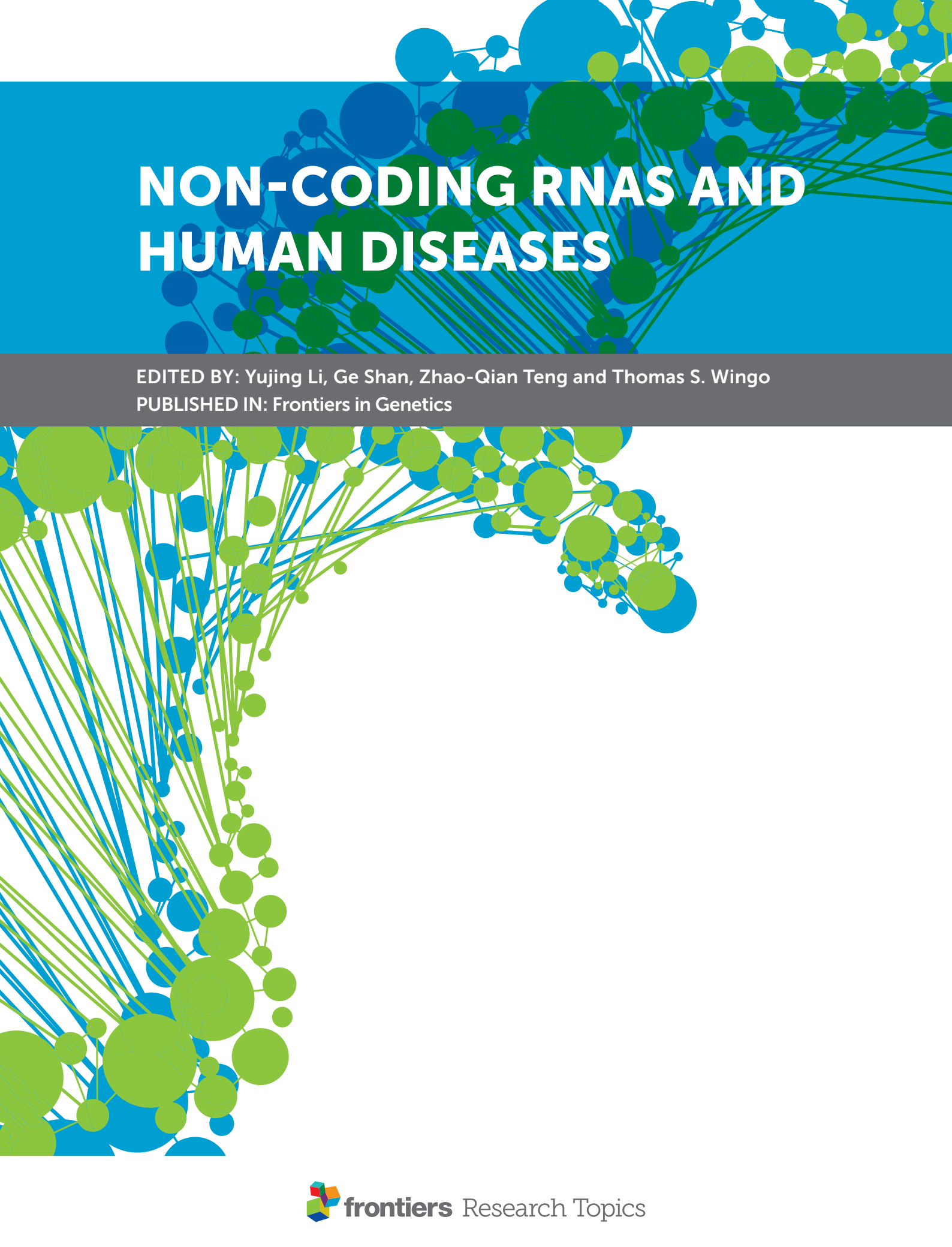




NON-CODING RNAS AND HUMAN DISEASES

EDITED BY: Yujing Li, Ge Shan, Zhao-Qian Teng and Thomas S. Wingo
PUBLISHED IN: Frontiers in Genetics



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ISSN 1664-8714

ISBN 978-2-88963-832-1

DOI 10.3389/978-2-88963-832-1

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NON-CODING RNAS AND HUMAN DISEASES

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Citation: Li, Y., Shan, G., Teng, Z.-Q., Wingo, T. S., eds. (2020). Non-Coding RNAs and Human Diseases. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-88963-832-1

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Editorial: Non-Coding RNAs and Human Diseases

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Keywords: miRNA, ncRNAs, lncRNA, circRNA, piRNA, cancer biology, neurological disorders

Editorial on the Research Topic

Non-Coding RNAs and Human Diseases

Non-coding RNA (ncRNA) are functional RNA molecules that are not translatable into proteins (Djebali et al., 2012; Lonsdale et al., 2013; Forrest et al., 2014). Initially, ncRNAs referred to tRNAs and rRNAs (Brown et al., 1992; St Laurent et al., 2015). Recent technical advances have led to the discovery and characterization of many new classes of ncRNAs (Hüttenhofer and Vogel, 2006). These new ncRNAs species include snRNAs, snoRNAs, miRNAs, siRNAs, piRNAs, exRNAs, long non-coding RNA (lncRNAs), scaRNAs, and circRNAs (He and Hannon, 2004; Gu et al., 2007; Esteller, 2011; Redzic et al., 2014; Wu and Yang, 2015). While not all of their functions are known, many of the ncRNA species appear to play essential roles regulating transcription and translation of genes and transcription of ncRNAs themselves. Thus, there is little surprise that ncRNAs are identified as playing important roles in normal physiologic processes, complex human traits, and human diseases (Diederichs et al., 2016; Li et al., 2018; Fernandes et al., 2019; Vijayan and Reddy, 2020). This special issue focused on the ncRNA, particularly circRNAs, lncRNAs, miRNAs, and their role in human disease. The aim of this issue is to provide a broad overview of current research on the diverse work being done to elucidate the role of ncRNAs in disease. A major theme that emerged was the potential role of miRNAs as prognostic markers or biomarkers of disease.

NcRNAs AND CANCER

ncRNAs play vital roles in tumorigenesis and tumor progression that is incompletely understood. Several investigators addressed the role of circRNA-miRNA-mRNA networks in different cancers, including hepatocellular carcinoma (HCC) (Sheng et al.), gastrointestinal stromal tumors (Jia et al.), and cervical cancer (Liu C. et al.). In a perspective, Molin et al. address the significance of circRNAs in MLL arranged acute leukemia (MLLre) recombinome. In lung adenocarcinoma, (Stewart et al.) performed a large-scale analysis of lncRNAs and find evidence for deregulated pseudogene-derived lncRNAs associated with cancer survival. microRNAs are among the better known ncRNAs. Here, Pereira et al., identify miRNAs associated with the development of gastric cancer to novel targets and potential early-stage indicators. The role of miRNAs to identify HCC progression was also explored using fecal-derived miRNAs by Wang et al. The role of miRNA and mRNA expression in endometrial cancer by Xu et al. using the The Cancer Genome Atlas (TCGA) identified mRNAs and miRNAs associated with patient survival suggesting a potential role for miRNAs in predicting clinical outcomes. The role of polymorphisms in miRNA and their contributions to cancer risk was explored by Choupani et al. by investigating the association of polymorphisms mir-196a-2 rs11614913 and mir-149 rs2292832 with multiple cancers (e.g., gynecological cancers, ovarian, breast, and HCC) in their updated meta-analysis. Finally, five review articles highlight the recent advances

OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 03 February 2020

Accepted: 29 April 2020

Published: 25 May 2020

Citation:

Li Y, Shan G, Teng Z-Q and Wingo TS
(2020) Editorial: Non-Coding RNAs
and Human Diseases.
Front. Genet. 11:523.
doi: 10.3389/fgene.2020.00523

in the understanding of lncRNAs, circRNAs, and miRNAs in cancer initiation and progression, provide insight into their potential as biomarkers and therapeutic targets (Dong et al.; Plousiou and Vannini; Bandini and Fanini; Khan et al.).

To date, ncRNAs, particularly lncRNAs and miRNAs, are implicated in resistance or sensitivity to chemotherapy. Work by Xiang et al. analyzed the Cancer Cell Line Encyclopedia database and identified 44 of ncRNAs differentially expressed and significantly related to resistance or sensitivity of therapy for the advanced or metastatic breast cancer using Lapatinib, a small molecule inhibitor of HER1 and HER2 receptors. Shi et al. assayed miRNAs to identify the cisplatin-resistance in C13K human ovarian cancer cell and its cisplatin-sensitive OV2008 parental cells, and they found miR-205-5p led to PTEN downregulation and the subsequent enhancement of its downstream target p-AKT significantly contributes to cisplatin resistance in C13K Cells. Finally, Wan et al. addressed the cardiotoxicity from compound doxorubicin (DOX), a broad-spectrum anti-tumor drug. Their study found that the severe heart failure incurred by DOX based chemotherapy attributed to the enhanced expression of p21.

ncRNAs AND LIVER AND CARDIOVASCULAR DISEASES

Non-alcoholic fatty liver disease (NAFLD) is a prevalent chronic liver condition that is associated with liver failure and HCC, Huang Z. et al. identified miRNAs that regulate the level of CYP3A4, an important drug-metabolizing enzyme associated with the progression of NAFLD, and they found that miR-200a-3p and miR-150-5p appear to directly regulate CYP3A4 and are involved in free fatty acid (FFA)-induced steatosis, implicating them in the NAFLD pathogenesis. The role of miRNAs was also examined in coronary artery disease (CAD), a prevalent human disease by Liu S. et al. who identified the miR-378a-5p-CDK1 axis as important in the proliferation and migration of vascular smooth muscle cells that can cause development of atherosclerosis and treatment failure for CAD.

REFERENCES

- Brown, C. J., Hendrich, B. D., Rupert, J. L., et al. (1992). The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* 71, 527–542. doi: 10.1016/0092-8674(92)90520-m
- Diederichs, S., Bartsch, L., Berkmann, J. C., et al. (2016). The dark matter of the cancer genome: aberrations in regulatory elements, untranslated regions, splice sites, non-coding RNA and synonymous mutations. *EMBO Mol. Med.* 8, 442–457. doi: 10.15252/emmm.201506055
- Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., et al. (2012). Landscape of transcription in human cells. *Nature* 489, 101–108. doi: 10.1038/nature11233
- Esteller, M. (2011). Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12, 861–874. doi: 10.1038/nrg3074

ncRNAs AND NEUROLOGIC DISEASE

The role of lncRNAs to potentially address the wide-range of clinical severity and age-at-onset for spinocerebellar ataxia type 3, a rare neurodegenerative disease identified six lncRNAs that were initially identified in blood and then tested in cerebellum Li T. et al. In multiple sclerosis (MS), the most common chronic neurologic disease in young adults, identified circRNAs MS-associated genes and showed evidence that top MS-GWAS results are enriched for blocks with circRNAs thereby suggesting a potential novel role for circRNAs in MS pathogenesis (Paraboschi et al.). Review articles focused on recent progress of ncRNAs in many neurologic diseases including Alzheimer's Disease (Liu X. et al.), fragile X syndrome (Huang G. et al.; Zhou et al.), and neurodevelopmental disorders (Li L. et al.; Zhang et al.).

As the research and review articles of this issue highlight, ncRNAs are a diverse group of RNA species that are likely important contributors to human disease. They provide a unique window into disease pathogenesis and ability to target networks of genes and cellular processes. Future work to understand ncRNAs and their role in human illness will undoubtedly be aided by incorporating ncRNAs with transcriptomic and proteomic data. Such studies will be able to provide a more complete model of disease pathogenesis. We hope that future work will also examine the potential role of ncRNAs in prospectively collected samples from clinical trials or population-based samples to evaluate their suitability as prognostic indicators and biomarkers of disease.

AUTHOR CONTRIBUTIONS

YL, Z-QT, GS, and TW drafted the editorial. YL and TW revised the editorial with contributions from all authors. All authors approved the final version.

FUNDING

This work was supported by NIH grants RF1 AG057470, U01 AG061357, P50 AG025688, R56 AG062256, R56 AG060757, and R01 AG056533 (to TW).

- Fernandes, J. C. R., Acuña, S. M., Aoki, J. I., Floeter-Winter, L. M., and Muxel, S. M. (2019). Non-coding RNAs in the regulation of gene expression: physiology and disease. *Noncoding RNA* 5:17. doi: 10.3390/ncrna5010017
- Forrest, A. R. R., Kawaji, H., Rehli, M., Baillie, J. K., de Hoon, M. J., Hablerle, V., et al. (2014). A promoter-level mammalian expression atlas. *Nature* 507, 462–470. doi: 10.1038/nature13182
- Gu, S. G., Pak, J., Barberan-Soler, S., Ali, M., Fire, A., and Zahler, A. M. (2007). Distinct ribonucleoprotein reservoirs for microRNA and siRNA populations in *C. elegans*. *RNA* 13, 1492–1504. doi: 10.1261/rna.581907
- He, L., and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 5, 522–531. doi: 10.1038/nrg1379
- Hüttenhofer, A., and Vogel, J. (2006). Experimental approaches to identify non-coding RNAs. *Nucleic Acids Res.* 34, 635–646. doi: 10.1093/nar/gkj469

- Li, X., Yang, L., and Chen, L. L. (2018). The biogenesis, functions, and challenges of circular RNAs. *Mol. Cell* 71, 428–442. doi: 10.1016/j.molcel.2018.06.034
- Lonsdale, J., Thomas, J., Salvatore, M., Phillips, R., Lo, E., Shad, S., et al. (2013). The Genotype-Tissue Expression (GTEx) project. *Nat. Genet.* 45, 580–585. doi: 10.1038/ng.2653
- Redzic, J. S., Balaj, L., van der Vos, K. E., and Breakefield, X. O. (2014). Extracellular RNA mediates and marks cancer progression. *Semin. Cancer Biol.* 28, 14–23. doi: 10.1016/j.semcancer.2014.04.01
- St Laurent, G., Wahlestedt, C., and Kapranov, P. (2015). The landscape of long noncoding RNA classification. *Trends Genet.* 31, 239–251. doi: 10.1016/j.tig.2015.03.007
- Vijayan, M., and Reddy, P. H. (2020). Non-coding RNAs based molecular links in type 2 diabetes, ischemic stroke, and vascular dementia. *J. Alzheimers Dis.* doi: 10.3233/JAD-200070
- Wu, N., and Yang, B. B. (2015). The biological functions of non-coding RNAs: from a line to a circle. *Discoveries* 3:e48. doi: 10.15190/d.2015.40
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Long Noncoding RNA Can Be a Probable Mechanism and a Novel Target for Diagnosis and Therapy in Fragile X Syndrome

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OPEN ACCESS

Edited by:

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Italian National Research Council
(CNR), Italy
Sandeep Kumar,
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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 14 December 2018

Accepted: 30 April 2019

Published: 22 May 2019

Citation:

Huang G, Zhu H, Wu S, Cui M and
Xu T (2019) Long Noncoding RNA
Can Be a Probable Mechanism and a
Novel Target for Diagnosis and
Therapy in Fragile X Syndrome.
Front. Genet. 10:446.
doi: 10.3389/fgene.2019.00446

Fragile X syndrome (FXS) is the most common congenital hereditary disease of low intelligence after Down syndrome. Its main pathogenic gene is fragile X mental retardation 1 (FMR1) gene associated with intellectual disability, autism, and fragile X-related primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS). FMR1 gene transcription leads to the absence of fragile X mental retardation protein (FMRP). How to relieve or cure disorders associated with FXS has also become a clinically disturbing problem. Previous studies have recently shown that long noncoding RNAs (lncRNAs) contribute to the pathogenesis. And it has been identified that several lncRNAs including FMR4, FMR5, and FMR6 contribute to developing FXPOI/FXTAS, originating from the FMR1 gene locus. FMR4 is a product of RNA polymerase II and can regulate the expression of relevant genes during differentiation of human neural precursor cells. FMR5 is a sense-oriented transcript while FMR6 is an antisense lncRNA produced by the 3' UTR of FMR1. FMR6 is likely to contribute to developing FXPOI, and it overlaps exons 15–17 of FMR1 as well as two microRNA binding sites. Additionally, BC1 can bind FMRP to form an inhibitory complex and lncRNA TUG1 also can control axonal development by directly interacting with FMRP through modulating SnoN–Ccd1 pathway. Therefore, these lncRNAs provide pharmaceutical targets and novel biomarkers. This review will: (1) describe the clinical manifestations and traditional pathogenesis of FXS and FXTAS/FXPOI; (2) summarize what is known about the role of lncRNAs in the pathogenesis of FXS and FXTAS/FXPOI; and (3) provide an outlook of potential effects and future directions of lncRNAs in FXS and FXTAS/FXPOI researches.

Keywords: long noncoding RNA, fragile X syndrome, fragile X-related primary ovarian insufficiency, fragile X-associated tremor/ataxia syndrome, FMR4, FMR6, BC1, TUG1

INTRODUCTION

Fragile X syndrome (FXS) is a congenital hereditary disease associated with low intelligence, and is second common only to Down syndrome. The main pathogenic gene of the fragile X syndrome (FXS) is the fragile X mental retardation 1 (FMR1) gene located at Xq27.3, and it was first cloned in 1991 by Verkerk et al. (1991). Between Xq27 and Xq28, the chromosomes

are abnormally concentrated during meiosis, forming an extremely fragile filamentous site. Therefore, this syndrome is regarded as a fragile X chromosome syndrome. As well, the 5' end untranslated region (UTR) of the gene has a highly conserved CpG island with a length of 250 bp, which includes a trinucleotide repeat expansion (CGG)_n whose sequence is abnormally amplified and methylated in individuals with fragile X chromosome syndrome (Bardoni and Mandel, 2002). Based on the CGG trinucleotide repeat numbers, the sequence is divided into full mutation, permutation, gray zone, and normality (Allingham-Hawkins et al., 1999; Bardoni and Mandel, 2002). In addition, full mutation, an expansion beyond 200 repeats, is associated with typical clinical symptoms of FXS which is responsible for intellectual disability, autism, and so on. Besides, permutation which corresponds to 55–200 repeats may contribute to development of fragile X-related primary ovarian insufficiency (FXPOI) and the fragile X-associated tremor/ataxia syndrome (FXTAS). A size of 45–54 is known as a gray zone, while normality corresponds to 5–44 repeats (Wittenberger et al., 2007; Ciaccio et al., 2017).

There is a lot of evidence that all proteins originate from only 1.2% of the genome, although 40% or higher of the human genome is transcribed into RNA (Carninci et al., 2005; Cheng et al., 2005; Consortium et al., 2007), known as non-protein-coding RNA (ncRNA) and enriched in the brain (Djebali et al., 2012). As well, ncRNAs are classified into different categories based on their functions and sizes. For instance, ncRNAs are differentiated into short and long noncoding RNAs (lncRNAs). Also, we introduced the long noncoding RNAs (lncRNAs) in this article, having a wide range of functions. They are also a kind of ncRNA with a length of more than 200 nucleotides while short noncoding RNAs certainly have less than 200 nucleotides. Short noncoding RNAs include microRNAs (miRNAs), small-interfering RNA (siRNA), and piwi-interacting RNA (piwi-RNA) and small nuclear RNAs (snRNA) (Zhou et al., 2019). For example, miRNAs are noncoding single-stranded RNAs consisting of 18–25 nucleotides in length (Akbari Kordkheylly et al., 2019). Previous studies have recently indicated that lncRNAs contribute to the pathogenesis of both the full mutation and premutation carriers, especially the nervous disorders. Still, there is no summary of function and mechanism of lncRNAs in FXS patients and premutation carriers, while the article sums up and explains the link between FXS and lncRNAs in detail.

FUNCTIONS AND MECHANISMS OF LONG NONCODING RNAs

ncRNAs play a significant role in human diseases. It is also demonstrable that ncRNAs have multiple functions. For example, pi-RNAs can repress translation (Fire et al., 1998; Carmell et al., 2007; Houwing et al., 2007), miRNAs can inhibit translation, while siRNAs can lead to silencing of a wide range of genetic targets and degrade mRNA (Fire et al., 1998; Dana et al., 2017). And a lot of evidence implicates that numerous protein-coding genes such as FMR1 have antisense transcripts. In some instances,

antisense transcription manipulation may result in sense transcription inhibition or make sense transcription more stable. These phenomena are known as discordant regulation and concordant regulation, respectively (Katayama et al., 2005). However, it is still not certain whether the exact regulation mechanisms of sense transcriptions are regulated by antisense partners, which may be various and complicated; thus, there is a need for more and further studies. Previous researches have revealed that the expression pattern of ncRNAs has changed in many patients suffering from human disorders, such as cardiovascular disease, neuronal dysfunction, and cancer (Haemmig and Feinberg, 2017; Nicolas, 2017; Cai et al., 2019; Shao et al., 2019; Zhang et al., 2019). They all indicate that ncRNAs can be functionally related to human diseases. Therefore, ncRNAs are probably taken for potential drug targets (Khalil et al., 2008; Nicolas, 2017). Short noncoding RNAs have been extensively explored and reviewed that they tend to affect the gene expression through the interference with translation or posttranscriptional mechanisms (Rother and Meister, 2011; Ojha et al., 2019). However, we only learned in recent years that lncRNAs can contribute to regulating cellular functions and/or physiology as regulatory factors. As the function of the lncRNAs gradually surfaces, lncRNAs have attracted more and more attention as potential biomarkers and/or drug targets (Kung et al., 2013; Tang et al., 2014; Zhu et al., 2018; Zou et al., 2018).

In particular, lncRNAs can originate from both sense and antisense chains of genes that can encode proteins. As well, they are likely to be a transcript of promoter, intron, and 3' end region. More specifically, lncRNAs are grouped into five categories according to the nearest protein-coding genes, which are sense, antisense, bidirectional, intronic, and intergenic lncRNAs. Besides, protein-coding genes are defined as the sense DNA. Transcriptions of sense lncRNAs incline to the occurrence of the sense DNA strand, which is different in the antisense lncRNAs. However, they all overlap one exon or more. Bidirectional lncRNAs are transcribed from the promoter in two directions, and their length is usually several hundred base pairs (bps) (Sarfi et al., 2019). Intronic lncRNAs do not overlap any exons and are the transcripts that originate from introns in any directions. Meanwhile, intergenic lncRNAs are stand-alone, meaning that they can exist in the sequence space without contain protein-coding genes, also known as large intergenic (or intervening) ncRNAs (Kung et al., 2013; Kumar and Goyal, 2017). At the same time, lncRNAs can interact with DNA, RNA, and protein, and other biological macromolecules and perform many biological functions such as regulation of the activity of transcription and the epigenetic landscape of their original locus (Wang and Chang, 2011; Huarte, 2015; Klinge, 2018). In addition, lncRNAs usually play a crucial role in cis- or trans-regulation of gene expression at their original sites or other locus in the genome. They also perform scaffolding function and remodeling chromatin through recruiting epigenetic complexes and ribonucleotide nucleotide proteins. The functions of other lncRNAs are executed *via* targeting mRNAs or regulating the post-transcriptional mechanism of genes. The subcellular locations of lncRNAs have a significant effect on their functional properties. The lncRNAs which are

located in the nucleus can regulate genetic transcription and perform epigenetic modification by binding DNA to generate RNA–DNA triplex complex. As well, cytoplasmic lncRNAs can affect the stability of mRNAs and act on posttranscriptional regulation (Mercer and Mattick, 2013). For example, lncRNAs which are located in the nucleus can contribute to both RNA processing and protein modifications *via* interacting with RNA-binding proteins (RBPs) (He et al., 2019). In addition, they may contribute to up-regulated expression of mRNAs by acting as miRNA sponges and reduce the regulatory effects of miRNAs (Kinney and Pradhan, 2013; Militello et al., 2017). Also, they may be involved in protein synthesis or interact with other proteins and RNAs to influence cellular signaling cascades (Kinney and Pradhan, 2013; Sanchez Calle et al., 2018). lncRNAs can also generate a secondary and/or tertiary structure that provides multiple binding sites for proteins and other regulatory RNAs (Liu et al., 2017). For example, lncRNAs may bind DNA-binding proteins and prevent DNA-binding proteins from attaching to related transcription factors. A typical example is that some lncRNAs can stop DNMT1 from binding its targeted DNA. Thus, the methylation of the targeted DNA has been affected to some extent. As a result, transcriptional activation of the gene is influenced (Hung et al., 2011). As well, lncRNAs can play an important role in DNA damage response and cellular division (Hajjari et al., 2014). No matter the biological mechanism of lncRNAs, there is enough evidence that lncRNAs are involved in the numerous normal and abnormal cell functions.

THE CLINICAL MANIFESTATIONS AND TRADITIONAL PATHOGENESIS OF FRAGILE X SYNDROME

In essence, men are more likely to get FXS than women. This is because the X chromosome is linked to recessive inheritance. In fact, about 80% of male patients have intellectual disabilities. According to IQ, the severity is classified as mild, moderate, and severe mental disability, with corresponding IQ values at 40–54, 50–70, and less than 20, respectively. Many patients with full mutation are disabled moderately (Greco et al., 2002). They tend to suffer from midface hypoplasia, language barrier, autism, and macro-orchidism (Verkerk et al., 1991) while women are considered to be the carriers of FXS. More specifically, about 70% of women have normal intelligence as carriers, and female FXS patients only tend to have mild mental retardation. Female premutation is likely to suffer from FXPOI, leading to a reproductive decline.

The full mutation is responsible for FXS associated with inherited intellectual and developmental disability. With full mutation, the promoters and CpG islands of FMR1 gene are highly methylated. Meanwhile, the associated histone proteins are hyperacetylated and chromatin aggregated. Then, the silencing of FMR1 gene transcription is attributed to the absence of fragile X mental retardation protein (FMRP), a protein product encoded by the causative gene (Fu et al., 1991; Ali et al., 2017). The expression of FMRP begins at the early stage of

development and lasts a lifetime. The expression of FMRP widely exists within all mammalian tissues. However, it is particularly abundant in the testis and brain. In the brain, FMRP exists mainly in the cytoplasm of neurons, including soma, dendrites, and synapses. FMRP, a kind of mRNA-binding protein, can associate with ribosomes and be involved in the aggregation of mRNA as well as regulation of the transcription efficiency of targeted genes. It also participates in protein synthesis of axons and dendrites. Therefore, the absence of FMRP might lead to the abnormal translation of mRNA and the abnormal structure and function of synapsis, which would affect the function of the nervous system (Pieretti et al., 1991; Sutcliffe et al., 1992; Darnell and Klann, 2013). The deduction supports that the deficient expression of FMRP is responsible for intellectual disability, thus patients exhibit a series of clinical manifestations (Weiler et al., 1997; Brown et al., 1998; Antar et al., 2005). Meanwhile, FMRP can potentially play an important role in the nucleus. Some existing researches have reported that FMRP may also be involved in mediating the DNA-damage response pathway through binding to methylated H3K79 chromatin (Liu et al., 2012).

THE CLINICAL MANIFESTATIONS AND TRADITIONAL PATHOGENESIS OF FRAGILE X-ASSOCIATED TREMOR/ ATAXIA SYNDROME AND FRAGILE X-RELATED PRIMARY OVARIAN INSUFFICIENCY

The Clinical Manifestations of Fragile X-Associated Tremor/Ataxia Syndrome and Fragile X-Related Primary Ovarian Insufficiency

Premature ovarian insufficiency (POI) is the cessation of ovarian function before 40 years of age. POI refers to the loss of germination and hormone function before normal physiological menopause as a result of the exhaustion of ovarian follicles (Hoek et al., 1997). As well, the risk of menstrual dysfunction, diminished ovarian reserve, and infertility is increased due to POI. In contrast, the age of menopause is decreased for POI patients. Laboratory tests have revealed that hypogonadism and elevated gonadotropin serum levels, which are characterized by low estradiol (E2) levels (<20 pg/ml), increased gonadotropin levels (follicle-stimulating hormone ([FSH] > 20 IU/l), low anti-Müllerian hormone (AMH) levels – <0.5 ng/ml (<1 ng/ml), and low inhibin B levels (Bellipanni et al., 2001). FSH levels may vary from cycle to cycle. Meanwhile, AMH is considered the best marker of ovarian reserve. As well, estrogen deficiency leads to the first symptoms: excessive sweating, tension, diminished libido, hot flushes, weakness, and mucous membrane dryness. In addition, substantial chronic hypogonadism may cause bone injuries and a higher risk of bone fracture. Even for younger women with POI, it is possible to have a consequent decrease in bone mineral density. Therefore, densitometry testing

is necessary. Deficient estrogen levels are associated with metabolic disorders, thus can lead to cardiovascular diseases such as hypercholesterolaemia, atherosclerosis, and urogenital atrophy – infections and vaginal dryness (Fink et al., 2018). However, lower fertility or even infertility is one of the most troubling POF-associated problems for young women. However, for a majority of the women suffering from premature ovarian insufficiency (POI), etiology is still completely unknown. The risk of POI for premutation carriers with CGG repeat on one allele has been found to be high (up to 35%), while only 1% of women in the general population suffer from POI. As well, noncarriers might experience menopause 5 years later than premutation carriers, since these carriers' ovarian function is obviously destroyed (Bretherick et al., 2005; Bodega et al., 2006; Mailick et al., 2014). Recent studies have demonstrated that overt premature ovarian insufficiency is correlated with premutation repeat lengths. Also, there is a linear relationship between the size of CGG trinucleotide repeats and the risk of POI/ovarian phenotype. With an increase in the size of repeats, the risk of POI elevates and reaches a plateau, but decreases beyond 80–100 repeats (Sullivan et al., 2005; Ennis et al., 2006; Tejada et al., 2008). Allen and colleagues realized that the average age of menopause tends to decline in all premutation carriers, which is apparently the medium-sized repeats with the lowest menopause age. It means that medium-sized repeats have lower odds ratio for fertility and an increased rate of dizygotic twinning compared with normal individuals while the rate of spontaneous abortion does not increase. This indicates that it puts no damage on the quality of oocyte. Both low and high repeats tended to suffer the same experience from insufficient ovarian reserve, although not as serious as medium-sized carriers (Allen et al., 2007).

FXTAS is a neurodegenerative disorder characterized by progressive intention tremor, gait ataxia, psychiatric symptoms, parkinsonism, cognitive decline, and autonomic disorders, which typically occurs after 50 years of age (Hagerman et al., 2001; Jacquemont et al., 2003; Kong et al., 2017). Main principal neuropathological features of FXTAS include Purkinje cell loss, brain atrophy, and ubiquitin-positive intranuclear inclusions which are mostly present in single, large (~2–5 μm of diameter), and spherical aggregates (Weiler et al., 1997; Greco et al., 2002, 2007; Jacquemont et al., 2003). These aggregates exist in different areas of the brain, especially in the hippocampus, and they are also found in the brain's Purkinje cells. Additionally, rare intranuclear inclusions, which are ubiquitin-positive and contain various chaperones, are also detected in the tissues outside of the central nervous system (Iwahashi et al., 2006). In particular, FXTAS is more common in males than in females (Rodriguez-Revena et al., 2009). Previous studies have found that FMR1 premutation can contribute to FXTAS development (Garcia-Arocena and Hagerman, 2010). As well, female premutation carriers do not suffer from FXTAS in most cases due to the defense mechanism that the pathogenic mutant, allele (located at X-chromosome) may become inactivated randomly. In addition, it affects an estimated 46% of males and 17% of females. Compared to carriers of the CGG premutation allele with control (<30) CGG repeat size, the FMR1 mRNA level of the

former has increased 2 to 8-folds *via* brain and blood analysis (Tassone et al., 2000, 2004). Tassone and colleagues attributed the higher FMR1 mRNA level to an increased transcriptional activity of the FMR1 gene (Tassone et al., 2007), which may be due to epigenetic modifications in the result of CGG repeat expansion itself (Todd et al., 2010; Usdin and Kumari, 2015).

The Traditional Pathogenesis of Fragile X-Associated Tremor/Ataxia Syndrome and Fragile X-Related Primary Ovarian Insufficiency

However, little is known about the mechanisms that contribute to the development of FXPOI and FXTAS. These individuals with an allele of an expansion beyond 200 CGC trinucleotide repeats lead to the incomplete absence of FMRP, but they still perform their function normally. Thus, insufficient FMRP does not submit to be the culprit of developing FXPOI/FXTAS (Sherman et al., 2014). Kenneson put forward that FMR1 RNAs' transcription of the permutation carriers was positively relevant with the size of CGG trinucleotide repeats while the FMRP translation was negatively correlated with the size of CGG trinucleotide repeats (Kenneson et al., 2001). The excessive FMR1 mRNAs in premutation carriers contribute to several proteins' dysregulation and deposition. Along with FMR1 mRNAs, these proteins are present in several parts of the body in the form of cell inclusions, which is located in CNS, peripheral nervous system (particularly autonomic ganglia), pituitary, and Leydig cells (Greco et al., 2006; Gokden et al., 2009). Therefore, partial FMRP deficiency and/or RNA toxicity may be involved in the pathogenic mechanism of FXPOI/FXTAS. In addition, abnormal translation of the CGG repeats can result in the production of polyalanine (FMRpolyA). The polyglycine (FMRpolyA) and other polypeptides containing proteins are neurotoxic (Hall and Berry-Kravis, 2018). FMRpolyG proteins can be detected in the brains and other tissues of individuals, but FMRpolyA could only be demonstrated in transfected cells with FXTAS. The phenomenon illustrated that FMRpolyA may contribute to the development of FXTAS (Hukema et al., 2015).

The theory suggests that dynamic intranuclear long rCGG RNA, translated by the gene with CGG trinucleotide repeats, can contribute to making normal cells dead and nonfunctional *via* tracking and binding to a wide range of RNA-binding proteins (RBP). The intranuclear long rCGG RNA is sequesters-specific and its sequestration can make the viability of normal cells decreased (Sellier et al., 2010). Also, the phenomenon is confirmed in another study, suggesting the fully mutated carriers or those with 4,199 methylated repeated alleles lead to silencing the expression of FMR1 gene, but did not suffer from FXPOI or FXTAS. That leads to the conclusion that the silence of disease-causing gene (FMR1) and protein products (FMRP) is not the main culprit. On the contrary, FMR1 transcript levels increase with premutation carriers. In addition, Elizur et al. have confirmed the fact that FMR1 mRNAs of both male and female premutation carriers are up-regulated, and FMR1 mRNAs play a significant role in FXTAS and FXPOI (Elizur et al., 2014).

CATEGORIES OF PATHOGENIC LONG NONCODING RNAs ORIGINATING FROM FMR1 GENE

Previous studies have indicated that lncRNAs contribute to the pathogenesis of both permutation and full mutation carriers, especially nervous disorders. In addition, it is reported that the expression of lncRNAs is different, and several relevant lncRNAs originate from the FMR1 gene locus in both FXS patients and premutation carriers. This suggests that they may be markers to diagnose or evaluate relevant disorders (Figure 1; Ladd et al., 2007; Khalil et al., 2008). And the discovered lncRNAs originating from FMR1, including FMR4, FMR5, and FMR6 as shown in Figure 1. Additionally, BC1 RNA and lncRNA TUG1 which originate from other genes also contribute to making permutation carriers sick.

FMR4 Can Affect Cell Proliferation or Differentiation

FMR4, an untranslated primate-specific lncRNA (2.4 kb), is transcribed upstream of FMR1 in the antisense direction. It is widely expressed in body development. In particular, FMR4 has been widely detected during our growing up years. Also, the expression of FMR4 exists in some adult tissues such as brain, small intestine, spleen, colon, liver, and placenta except in the ovaries, prostate, pancreas, or testes (Hinds et al., 1993). During embryonic and/or fetal development, FMR4 is likely to express in these organs and tissues (ovaries, prostate, pancreas, or testes). Similarly, the expression of FMR4 is detected highly in kidney and heart of fetus. Considering that a number of people suffering from FXS have cardiac dysfunctions, such as prolapse of mitral valve and aortic root dilation, FMR4 which expresses highly in heart may have a functional role to play in the relevant pathogenic mechanism (Sreeram et al., 1989).

FMR4, a product of RNA polymerase II, can be detected in normal people as well as in premutation carriers, but not in FXS with full mutation. It also has similar half-life to FMR1 mRNA. Similar to FMR1 mRNA level, the expression of FMR4 is up-regulated in premutation carriers and silenced in brain

tissue of full mutation carriers (FXS). Some previous researches have shown that overexpression and knockdown of FMR4 can alter the expression of these genes, which has an effect on cellular proliferation or differentiation. As a chromatographic transcript, FMR4 can induce the changes of transcriptional levels by directly aiming at mRNAs splicing, editing, or stability, and by binding histone-modifying enzymes to develop complexes. However, it is not certain whether these observed results are affected by epigenetic changes, RNA-protein interactions, or downstream effects. The study also confirmed the methyl-CpG-binding domain protein 4 (MBD4), which is a negatively responsive gene of FMR4. MBD4 is a member of MBD nucleoprotein family, and it has two domains: one is methyl-CpG-binding domain which can specifically bind methylated CpG, and the other one is a DNA glycosylase domain which is associated with the catalytic activity. Thus, MBD4 is crucial in DNA mismatch repair, inhibition of transcription, and the regulation of apoptosis (Yakovlev et al., 2017). In addition, the study revealed that FMR4 very likely shares a bidirectional promoter with FMR1. The expression of FMR4 is developmentally regulated and shows negative relation to the expression of both FMR1 and MBD4 in human neural precursor cells, which are in differentiation. Therefore, all evidence implicates that as a kind of lncRNAs and FMR4 can regulate the function of relevant genes which can regulate gene, and the transcript may act in cellular development (Peschansky et al., 2015). However, Peschansky and colleagues also suggested that the genes regulated by FMR4 enrich and are involved in cell proliferation and neural development. S-phase marker assays further exhibited that FMR4 may up-regulated cell proliferation, rather than differentiation of human neural precursor cells (hNPCs). They confirmed the theory by using transfection, qPCR, and subcellular fractionation, and other technologies. The experimental group is that HEK293T cells are transfected with pcDNA3.1-FMR4, while the control group is that cells are transfected with empty pcDNA3.1 control vector or silencer negative control siRNA aiming at FMR4. And FMR4 is highly expressed in the experimental group but not in the control group. They found that the silencing and overexpression of FMR4 can lead to the genome-wide change in histone methylation. The situation also

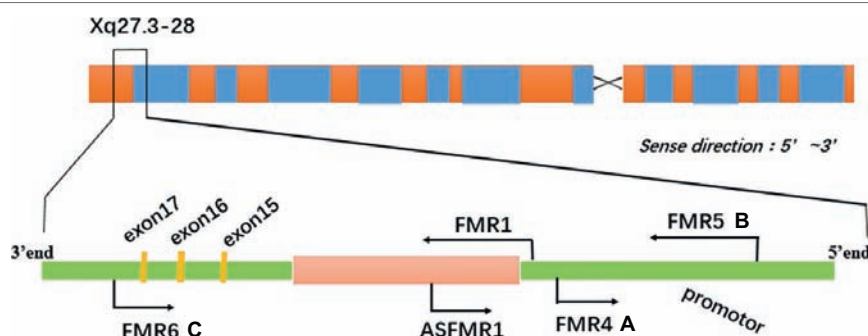


FIGURE 1 | The transcriptional landscape of the FMR1 gene locus is complicated (A) FMR4 is transcribed upstream of FMR1 in the antisense direction. (B) FMR5 is a sense-oriented transcript from the FMR1 promoter and its transcription start site (TSS) is situated 1 kb upstream from FMR1 TSS. (C) FMR6 is transcribed in the antisense direction from the 3' UTR of FMR1 overlapping exons 15–17.

exists in other mRNAs which have been confirmed to act in developmental or neurophysiological roles. FMR4 works mainly by forming scaffolds for the recruitment of histone-modifying complexes or other proteins to affect the stability, splicing, or editing of relevant mRNAs (Figure 2; Peschansky et al., 2016).

The phenomenon was also found in the research of Ahmad M and colleagues. As mentioned above, there are no overlaps between the FMR4 and FMR1 because FMR4 lies in the upstream of FMR1 with 2.4 kb in the antisense direction. Thus, they used relevant siRNAs to knockout FMR1 and found that there has been no change in the expression of FMR4. Thus, they reported that expression of FMR4 cannot be affected by FMR1. These observations have indicated that FMR4 is not directly derived from regulatory transcript for FMR1. But siRNA knockdown of FMR4 can cause an increase of cell apoptosis and the changes of cell cycle. In addition, they simultaneously found that the overexpression of FMR4 resulted in an increased proliferation *in vitro*. Thus, FMR4 has a significant effect on cell proliferation *in vitro*. The evidence implicated that FMR4 can act at anti-apoptosis in HeLa cells and HEK293T and suggests that studying genomic locus can make us discover unknown functions of the gene. They also speculate that changes in the expression of FMR4 may affect the clinical manifestations of FXS or associated disorders (Khalil et al., 2008).

Another transcript, ASFMR1, originates from the CGG expanded repeats in the 5' UTR of FMR1, and was reported by Ladd and colleagues. The expression of ASFMR1 is silenced in full carriers and increased in premutation carriers, with the changes being similar to FMR4. Ladd and colleagues also evaluated that FMR4 is likely to be nested in the 3' UTR of ASFMR1 due to its one splice variant overlapping that of FMR4. Besides, ASFMR1 is widely expressed in human tissues with relatively high expression in brain. The ASFMR1 transcript is transported to the cytoplasm and contains a potential proline-rich ORF, indicating that ASFMR1 has a conserved cellular function and can potentially be associated with FXS and FXTAS (Ladd et al., 2007).

FMR5 Is a Kind of Sense-Oriented Long Noncoding RNA

FMR5 is a sense-oriented lncRNA and its transcription begins around 1 kb upstream from the FMR1 transcription start site (TSS) which overlaps with the FMR1 promoter. Meanwhile, FMR5 and FMR6 are discovered by using a new technology called "Deep-RACE." This technology can combine next-generation sequencing with rapid amplification of cDNA ends (RACE). The expression of FMR5 reportedly appeared in some brain tissue with full mutation, permutation carriers, and normal individuals (Pastori et al., 2014). There are similar expression levels of FMR5 in brain tissue with normal people, full mutation, and permutation carriers, which shows that FMR5 transcription is not related to chromatin methylated modifications. Kumari and Usdin also indicated that the transcription of low-riched transcripts such as FMR5 may be repressed by the essence of negative histone marks in the FMR1 locus. This is consistent with the discovery that trimethylation of histone H4 at lysine 20 (H4K20me3) as a negative chromatin mark as well as trimethylation of histone H3 at lysine 9 (H3K9me3) are related to exon 1 of the FMR1 gene, such as CGG expanded repeats, but not connected with the promoter region (Kumari and Usdin, 2010). At the same time, lower levels of three positive chromatin marks, including H3K4 dimethylation (H3K4me2), H3 acetylation (H3Ac), and H4 acetylation (H4Ac) combine with the FMR1 promoter in full mutation carriers (Gheldof et al., 2006). Kumari and Usdin suggested negative histone modifications on silenced FMR1 may contribute to developing FXS because these modifications enrich FX alleles, and the intrinsic and local repeats may lead to the silence of FMR1 (Kumari and Usdin, 2010).

FMR6 Regulates Translational Efficiency and/or Stability of FMR1

The expression of FMR5 and that of FMR6 are dependent on completely different patterns. FMR6, a spliced lncRNA, is

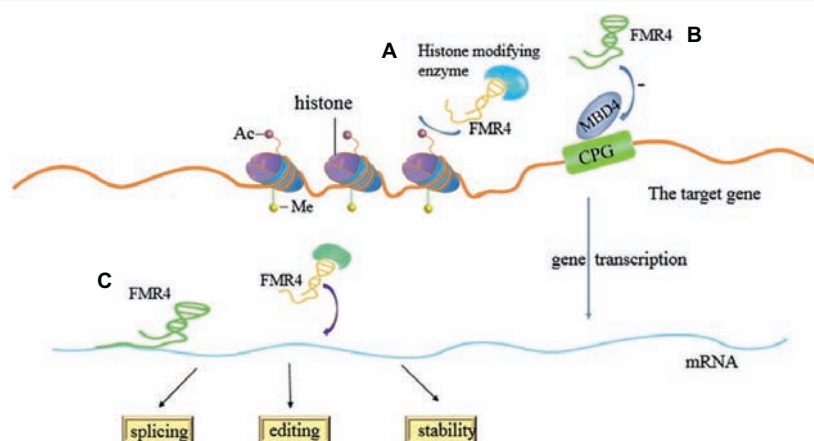


FIGURE 2 | Regulation of FMR4 in a variety of ways. **(A)** FMR4 binds histone-modifying enzymes to develop complexes which can regulate transcription of the target gene. **(B)** FMR4 can play role in DNA mismatch repair, transcriptional inhibition, and apoptosis regulation as a negatively regulating factor of MBD4. **(C)** FMR4 can induce the changes of transcriptional levels by directly aiming at mRNAs splicing, editing, or stability, or by forming scaffolds for the recruitment of functional protein.

transcribed by the 3' UTR of FMR1 overlapping exons 15–17 in the antisense direction, and FMR6 may combine with the FMR1 mRNA because it is complementary to the 3' region of FMR1. A study found that selective siRNA aiming to knockdown the nonoverlapping regions of the β -secretase-1 antisense transcript (BACE1-AS) made the expression of β -secretase-1 (BACE1) mRNA and protein decreased, which indicated that BACE1-AS can regulate BACE1 mRNA and BACE1 protein expression subsequently (Faghihi et al., 2008). And it follows that FMR6 may also regulate the splicing and stability of FMR1 mRNA and the expression level of FMRP. As well, FMR6 overlaps two microRNA binding sites, including miR-19a and miR-19b located in the 3' UTR of FMR1, and the lncRNA may regulate translational efficiency or stability of FMR1 mRNA by the another way of combining with microRNAs (Pastori et al., 2014).

The expression of FMR6 is down-regulated in brain tissue from premutation carriers and fragile X patients (Pastori et al., 2014). Meanwhile, Shai E. Elizur and colleagues carried out a study to evaluate whether the accumulation of lncRNAs contributes to developing FXPOI. Their research suggested that FMR6 is expressed in ovarian granular cells from both premutation carriers and fragile X patients similar to FMR1 mRNA, and there is a marked nonlinearity between the FMR6 level of ovarian granular cells and the size of CGG repeats. Females in the medium-range CGG repeats (80–120) obviously keep up with higher FMR6 levels of granulosa cells. In addition, the transcription level of FMR6 is negatively associated with the number of oocytes detected. These findings indicate that in ovary granulosa cells of females with FXPOI, the accumulation of both FMR6 and FMR1 except FMR4, may result in ovarian dysfunction. Although the FMR1 premutation can result in primary ovarian insufficiency, the relationship is nonlinear but still not exact between ovarian reserve and CGG repeat numbers. We can speculate FXPOI is the result of an increased accumulation of FMR1 and FMR6 lncRNAs in ovarian granular cells in the intermediate range (80–120 CGG repeat). Also, more well-studied observations are required to explore the exact mechanism by which FMR1 mRNA and FMR6 contribute to FXPOI, and estimate whether these research findings might also be extended to the normal-range CGG repeats (Elizur et al., 2016).

THE FMRP-BC1-mRNA INHIBITORY COMPLEX

Presynaptic localization of FMRP has been confirmed in the CNS. FMRP can regulate the development of both axon and dendrite. Meanwhile, methylation of FMR1 gene two-way promoter can regulate the expression of lncRNAs. In addition, FMRP, a kind of protein involved in regulating the efficiency of translation and transporting messenger ribonucleoprotein (mRNP), can combine with the dendritic brain cytoplasmic RNA 1 (ncRNA BC1) to form the FMRP-BC1 complex. This complex can inhibit the translation of a certain subset of FMRP-targeted mRNAs in neurons.

BC1 ncRNAs have been regarded as negative translation regulators. Knockdown of BC1 RNAs' expression can lead to remarkably increased neuronal excitability and epilepsy (Zhong et al., 2009). The BC1 RNA can also play a role as an adaptor molecule to connect several mRNAs with FMRP (Zalfa et al., 2005). BC200, BC1 analog in primates, can have more advantages compared with BC1 because its RNA distribution is also able to indicate the localization of dendrites and neuron-specific expression (Tiedge et al., 1993). Also, the FMRP have exhibited enough binding sites as a regulatory and transport factor, such as two evidently characterized KH domains which can bind RNAs, and the N and C termini which have affinity for RNA. However, only the RGG box of FMRP can only bind RNA with special sequence and/or structure. Similarly, G quartet is a kind of RNA with rich G. It can directly bind FMRP, recognize and connect with the above four domains (Darnell et al., 2001; Schaeffer et al., 2001). Also, a new RNA-binding motif originating from the N terminus (NT) of FMRP has been identified to be capable of binding to BC200 specifically and directly. In addition, the FMRP-BC1/BC200 complex can cover up the signal which can regulate the FMRP cycle in and out of the nucleus. Thus, the transportation of mRNP is affected and the relevant proteins cannot be transported into the nucleus, while the breakdown of the complex permits proteins to reenter the nucleus freely (Zalfa et al., 2005). Studies *in vitro* have indicated that BC1 can play its functional inhibition role by binding both PABA and eIF4A (the translational initiation factor) (Wang et al., 2002). Meanwhile, Lacoux C and colleagues proposed that the interaction between BC1 and FMRP may be regulated by 2'-O-methylation. They demonstrated that BC1 RNAs in neurons are 2'-O-methylated differentially and have an effect in binding FMRP to the complex (Figure 3; Lacoux et al., 2012).

LONG NONCODING RNA TUG1 REGULATE AXONAL DEVELOPMENT VIA MODULATING SnoN-CCD1 PATHWAY

Current evidence confirms that lncRNA TUG1 has an obvious and close relationship with FMRP for patients suffering from cancer. Guo and colleagues pointed out that the transcription of FMRP is successfully suppressed in neurons transfected by Fmr1shRNA. They found out that the length and complexity of the dendrites were all reduced in the neurons whose FMRP expression was deficient compared with the control group whose FMRP expression was normal. As such, the data can confirm that FMRP plays a crucial role in axonal development. However, there was no significant change in the length and complexity of the dendrites in TUG1-deficient neurons transfected by TUG1 shRNA. The researchers found that down-regulated TUG1 expression slightly led to developing axon better in neurons and up-regulated TUG1 expression results in significantly shortening the axonal length. Meanwhile, FMRP deficiency led to overexpression of TUG1 and knockdown of TUG1 expression can repair the defects of axonal development in FMRP-deficient neurons. It indicates that TUG1 may interact with FMRP to

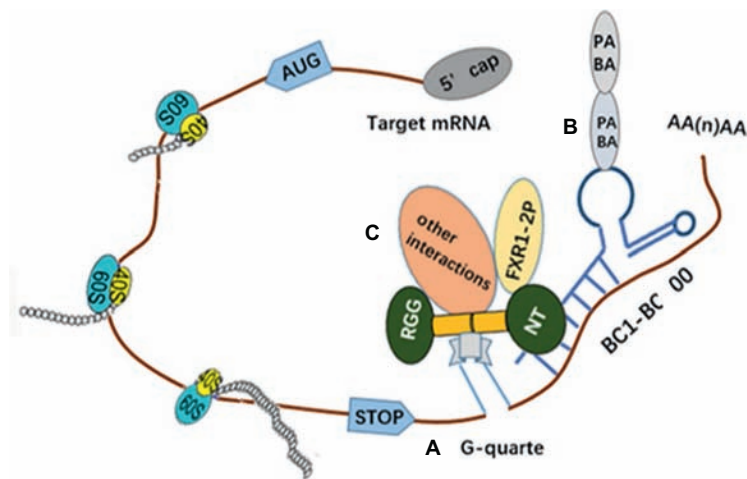


FIGURE 3 | It is an illustration of how the BC2/200-FMRP complex recognizes and inhibits the translation of mRNAs. **(A)** The NT of FMRP can bind to the targeted mRNAs simultaneously *via* five regions of the longer stem loop of BC1 RNAs and G-quarte, and it would repress steadily the translation of the targeted mRNAs. **(B)** Poly(A)-binding protein (PABP) can connect with BC1 that actually act on the poly(A) tail of targeted mRNAs. **(C)** Other interactions also play a role in preventing the targeted mRNAs being.

specifically regulate axonal development of neurons. At the same time, they found that TUG1 can regulate axonal development by interfering with the SnoN-Ccd1 pathway, which is known to be involved in the development of axons. In addition, the reduced length of axon due to TUG1 up-regulation and FMRP deficiency can be rescued by the overexpression of Ccd1. However, making FMR1 silenced and TUG1 overexpressed does not alter the whole protein expression level of SnoN. It demonstrates that the interaction between TUG1 and FMRP regulates SnoN activity that would not be dependent on the ubiquitin-proteasome system. The system has been proved to be capable of activating SnoN pathway because the ubiquitin ligase Cdh1/APC can accelerate SnoN ubiquitination and subsequent degradation, and can consequently inhibit axonal development (Konishi et al., 2004; Guo et al., 2018).

It has been known that lncRNAs can affect gene expression level *via* binding to specific transcriptional factors and inhibit or enhance the activity of these specific transcriptional factors. Similarly, TUG1 can also inhibit the transcriptional activity of SnoN by binding with SnoN, which would lead to a decreased expression of Ccd1, a well-known SnoN-targeted gene. Therefore, these evidence provide another potential mechanism of how lncRNA TUG1 can regulate the transcriptional activity of SnoN. In other words, lncRNAs can also combine with particular transcriptional factors to repress or promote the activities of these transcriptional factors, thus regulate the expression of relevant genes, just as TUG1 can bind with SnoN to inhibit its transcriptional activity. This will result in the down-regulated expression of Ccd1, known as SnoN-targeted gene (Geisler and Coller, 2013). A large number of previous studies have proved that lncRNA TUG1 is closely related to FMRP for patients suffering from cancer. However, whether the relationship between lncRNA TUG1 and FMRP for patients suffering from FXS and FXPOI/FXTAS is the same or not, there is a need for more in-depth studies to verify it. If the answer is yes,

then it could open new avenues of research and treatment for FXS and FXPOI/FXTAS.

CONCLUSION AND PERSPECTIVE

The expression of FMR6, FMR5, FMR4, and FMR1 is different in patients' brain tissues with FXS and FXTAS/FXPOI, which could probably be as a result of the differences in CGG trinucleotide repeat numbers, DNA methylation degree, or histone modification degree. FMR4, a product of RNA polymerase II, indicated increased expression in premutation carriers and silenced expression in full mutation carriers. It can also regulate the expression of relevant genes during differentiation of human neural precursor cells (Kumari and Usdin, 2010). Meanwhile, both FMR5 and FMR6 are new transcripts from the FMR1 gene locus. The expression of FMR5 appears in some human brain regions in both FXS patients and premutation carriers, while the expression of FMR6 is silenced in premutation and full mutation carriers. For FXS and FXTAS/FXPOI patients, it is feasible that the levels of these transcripts (FMR6, FMR5, FMR4 and FMR1) can correspond to different clinical manifestations and results. Therefore, these lncRNAs may be taken for markers to diagnose and evaluate FXS and FXTAS/FXPOI. And there is a need for additional studies to evaluate whether any undiscovered functional properties of each transcript may result in clinical phenotypes of FXS and FXTAS/FXPOI patients (Pastori et al., 2014). Most importantly, several studies have suggested that FMRP also has a direct interaction with BC1, which affects its functional regulation and transportation. As well, lncRNA TUG1 can bind it to decrease its stability. In addition, lncRNA TUG1 can regulate axonal development by combining with SnoN and mediating SnoN-Ccd1 pathway. Also, there is a need to deeply explore an alternative potential mechanism of modulating the transcriptional activity of SnoN by lncRNA TUG1 and the function of lncRNA TUG1 in

patients suffering from FXS and FXPOI/FXTAS (Fabian and Sonenberg, 2012). lncRNAs can be considered a new biomarker for human disease. It has been applied to new diagnostic or prognostic markers in bodily fluid samples, such as urine samples of patients suffering from cancer to detect the lncRNA prostate cancer antigen 3. The lncRNA can improve diagnosis of prostate cancer (Reis and Verjovski-Almeida, 2012). Similarly, to determine whether the lncRNAs' expression levels are related to clinical manifestations of fragile X Syndrome, it is necessary to detect these transcripts in a large number of patients. In particular, blood samples provide the most practical choice for both experimental and potential prognostic purposes. By understanding the mystery of these lncRNAs, the diagnosis and treatment of FXS, along with its associated disorders, may be more accurate and effective in the future.

REFERENCES

- Akbari Kordkheyl, V., Khonakdar Tarsi, A., Mishan, M. A., Tafazoli, A., Bardania, H., Zarpou, S., et al. (2019). Effects of quercetin on microRNAs: a mechanistic review. *J. Cell. Biochem.* 1–15. doi: 10.1002/jcb.28663
- Ali, E. Z., Yakob, Y., Md Desa, N., Ishak, T., Zakaria, Z., Ngu, L. K., et al. (2017). Molecular analysis of fragile X syndrome (FXS) among Malaysian patients with developmental disability. *Malays. J. Pathol.* 39, 99–106.
- Allen, E. G., Sullivan, A. K., Marcus, M., Small, C., Dominguez, C., Epstein, M. P., et al. (2007). Examination of reproductive aging milestones among women who carry the FMR1 premutation. *Hum. Reprod.* 22, 2142–2152. doi: 10.1093/humrep/dem148
- Allingham-Hawkins, D. J., Babul-Hirji, R., Chitayat, D., Holden, J. J., Yang, K. T., Lee, C., et al. (1999). Fragile X premutation is a significant risk factor for premature ovarian failure: the international collaborative POF in fragile X study – data. *Am. J. Med. Genet.* 83, 322–325. doi: 10.1002/(SICI)1096-8628(19990402)83:4<322::AID-AJMG17>3.0.CO;2-B
- Antar, L. N., Dictenberg, J. B., Plociniak, M., Afroz, R., and Bassell, G. J. (2005). Localization of FMRP-associated mRNA granules and requirement of microtubules for activity-dependent trafficking in hippocampal neurons. *Genes Brain Behav.* 4, 350–359. doi: 10.1111/j.1601-183X.2005.00128.x
- Bardoni, B., and Mandel, J. L. (2002). Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. *Curr. Opin. Genet. Dev.* 12, 284–293. doi: 10.1016/S0959-437X(02)00300-3
- Bellipanni, G., Bianchi, P., Pierpaoli, W., Bulian, D., and Ilyia, E. (2001). Effects of melatonin in perimenopausal and menopausal women: a randomized and placebo controlled study. *Exp. Gerontol.* 36, 297–310. doi: 10.1016/S0531-5565(00)00217-5
- Bodega, B., Bione, S., Dalpra, L., Toniolo, D., Ornaghi, F., Vegetti, W., et al. (2006). Influence of intermediate and uninterrupted FMR1 CGG expansions in premature ovarian failure manifestation. *Hum. Reprod.* 21, 952–957. doi: 10.1093/humrep/dei432
- Bretherick, K. L., Fluker, M. R., and Robinson, W. P. (2005). FMR1 repeat sizes in the gray zone and high end of the normal range are associated with premature ovarian failure. *Hum. Genet.* 117, 376–382. doi: 10.1007/s00439-005-1326-8
- Brown, V., Small, K., Lakkis, L., Feng, Y., Gunter, C., Wilkinson, K. D., et al. (1998). Purified recombinant Fmrp exhibits selective RNA binding as an intrinsic property of the fragile X mental retardation protein. *J. Biol. Chem.* 273, 15521–15527. doi: 10.1074/jbc.273.25.15521
- Cai, Q., Wang, S., Jin, L., Weng, M., Zhou, D., Wang, J., et al. (2019). Long non-coding RNA GBCDRLnc1 induces chemoresistance of gallbladder cancer cells by activating autophagy. *Mol. Cancer* 18, 82–99. doi: 10.1186/s12943-019-1016-0
- Carmell, M. A., Girard, A., van de Kant, H. J., Bourc'his, D., Bestor, T. H., de Rooij, D. G., et al. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev. Cell* 12, 503–514. doi: 10.1016/j.devcel.2007.03.001

AUTHOR CONTRIBUTIONS

GH and HZ wrote the manuscript, carried out image analysis, and contributed to this work equally. SW drew the figures. SW and MC co-supervised GH and HZ, participated in discussions, and commented on the manuscript. TX conceived the idea, directed, and critically reviewed the manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (81302242), Jilin Province Development and Reform Commission funds (20180201032YY), and Education Department of Jilin Province (JJKH20170840KJ).

- Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda, N., et al. (2005). The transcriptional landscape of the mammalian genome. *Science* 309, 1559–1563. doi: 10.1126/science.1112014
- Cheng, J., Kapranov, P., Drenkow, J., Dike, S., Brubaker, S., Patel, S., et al. (2005). Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science* 308, 1149–1154. doi: 10.1126/science.1108625
- Ciaccio, C., Fontana, L., Milani, D., Tabano, S., Miozzo, M., and Esposito, S. (2017). Fragile X syndrome: a review of clinical and molecular diagnoses. *Ital. J. Pediatr.* 43:39. doi: 10.1186/s13052-017-0355-y
- Consortium, E. P., Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigo, R., Gingeras, T. R., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799–816. doi: 10.1038/nature05874
- Dana, H., Chalbatani, G. M., Mahmoodzadeh, H., Karimloo, R., Rezaiean, O., Moradzadeh, A., et al. (2017). Molecular mechanisms and biological functions of siRNA. *Int. J. Biomed. Sci.* 13, 48–57.
- Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 107, 489–499. doi: 10.1016/S0092-8674(01)00566-9
- Darnell, J. C., and Klann, E. (2013). The translation of translational control by FMRP: therapeutic targets for FXS. *Nat. Neurosci.* 16, 1530–1536. doi: 10.1038/nn.3379
- Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., et al. (2012). Landscape of transcription in human cells. *Nature* 489, 101–108. doi: 10.1038/nature11233
- Elizur, S. E., Dratviman-Storobinsky, O., Derech-Haim, S., Lebovitz, O., Dor, J., Orvieto, R., et al. (2016). FMR6 may play a role in the pathogenesis of fragile X-associated premature ovarian insufficiency. *Gynecol. Endocrinol.* 32, 334–337. doi: 10.3109/09513590.2015.1116508
- Elizur, S. E., Lebovitz, O., Derech-Haim, S., Dratviman-Storobinsky, O., Feldman, B., Dor, J., et al. (2014). Elevated levels of FMR1 mRNA in granulosa cells are associated with low ovarian reserve in FMR1 premutation carriers. *PLoS One* 9:e105121. doi: 10.1371/journal.pone.0105121
- Ennis, S., Ward, D., and Murray, A. (2006). Nonlinear association between CGG repeat number and age of menopause in FMR1 premutation carriers. *Eur. J. Hum. Genet.* 14, 253–255. doi: 10.1038/sj.ejhg.5201510
- Fabian, M. R., and Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat. Struct. Mol. Biol.* 19, 586–593. doi: 10.1038/nsmb.2296
- Faghihi, M. A., Modarresi, F., Khalil, A. M., Wood, D. E., Sahagan, B. G., Morgan, T. E., et al. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat. Med.* 14, 723–730. doi: 10.1038/nm1784
- Fink, D. A., Nelson, L. M., Pyeritz, R., Johnson, J., Sherman, S. L., Cohen, Y., et al. (2018). Fragile X associated primary ovarian insufficiency (FXPOI): case report and literature review. *Front. Genet.* 9:529. doi: 10.3389/fgene.2018.00529

- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888
- Fu, Y. H., Kuhl, D. P., Pizzuti, A., Pieretti, M., Sutcliffe, J. S., Richards, S., et al. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 67, 1047–1058. doi: 10.1016/0092-8674(91)90283-5
- Garcia-Arocena, D., and Hagerman, P. J. (2010). Advances in understanding the molecular basis of FXTAS. *Hum. Mol. Genet.* 19, R83–R89. doi: 10.1093/hmg/ddq166
- Geisler, S., and Collier, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat. Rev. Mol. Cell Biol.* 14, 699–712. doi: 10.1038/nrm3679
- Gheldof, N., Tabuchi, T. M., and Dekker, J. (2006). The active FMR1 promoter is associated with a large domain of altered chromatin conformation with embedded local histone modifications. *Proc. Natl. Acad. Sci. USA* 103, 12463–12468. doi: 10.1073/pnas.0605343103
- Gokden, M., Al-Hinti, J. T., and Harik, S. I. (2009). Peripheral nervous system pathology in fragile X tremor/ataxia syndrome (FXTAS). *Neuropathology* 29, 280–284. doi: 10.1111/j.1440-1789.2008.00948.x
- Greco, C. M., Berman, R. F., Martin, R. M., Tassone, F., Schwartz, P. H., Chang, A., et al. (2006). Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain* 129, 243–255. doi: 10.1093/brain/awh683
- Greco, C. M., Hagerman, R. J., Tassone, F., Chudley, A. E., Del Bigio, M. R., Jacquemont, S., et al. (2002). Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain* 125, 1760–1771. doi: 10.1093/brain/awf184
- Greco, C. M., Soontrapornchai, K., Wirojanan, J., Gould, J. E., Hagerman, P. J., and Hagerman, R. J. (2007). Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. *J. Urol.* 177, 1434–1437. doi: 10.1016/j.juro.2006.11.097
- Guo, Y., Chen, X., Xing, R., Wang, M., Zhu, X., and Guo, W. (2018). Interplay between FMRP and lncRNA TUG1 regulates axonal development through mediating SnoN-Ccd1 pathway. *Hum. Mol. Genet.* 27, 475–485. doi: 10.1093/hmg/ddx417
- Haemmmig, S., and Feinberg, M. W. (2017). Targeting lncRNAs in cardiovascular disease: options and expeditions. *Circ. Res.* 120, 620–623. doi: 10.1161/CIRCRESAHA.116.310152
- Hagerman, R. J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., et al. (2001). Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology* 57, 127–130. doi: 10.1212/WNL.57.1.127
- Hajjari, M., Khoshnevisan, A., and Shin, Y. K. (2014). Molecular function and regulation of long non-coding RNAs: paradigms with potential roles in cancer. *Tumour Biol.* 35, 10645–10663. doi: 10.1007/s13277-014-2636-z
- Hall, D. A., and Berry-Kravis, E. (2018). Fragile X syndrome and fragile X-associated tremor ataxia syndrome. *Handb. Clin. Neurol.* 147, 377–391. doi: 10.1016/B978-0-444-63233-3.00025-7
- He, R. Z., Luo, D. X., and Mo, Y. Y. (2019). Emerging roles of lncRNAs in the post-transcriptional regulation in cancer. *Genes Dis.* 6, 6–15. doi: 10.1016/j.gendis.2019.01.003
- Hinds, H. L., Ashley, C. T., Sutcliffe, J. S., Nelson, D. L., Warren, S. T., Housman, D. E., et al. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nat. Genet.* 3, 36–43. doi: 10.1038/ng0193-36
- Hoek, A., Schoemaker, J., and Drexhage, H. A. (1997). Premature ovarian failure and ovarian autoimmunity. *Endocr. Rev.* 18, 107–134. doi: 10.1210/edrv.18.1.0291
- Houwing, S., Kamminga, L. M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., et al. (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* 129, 69–82. doi: 10.1016/j.cell.2007.03.026
- Huarte, M. (2015). The emerging role of lncRNAs in cancer. *Nat. Med.* 21, 1253–1261. doi: 10.1038/nm.3981
- Hukema, R. K., Buijsen, R. A., Schonewille, M., Raske, C., Severijnen, L. A., Nieuwenhuizen-Bakker, I., et al. (2015). Reversibility of neuropathology and motor deficits in an inducible mouse model for FXTAS. *Hum. Mol. Genet.* 24, 4948–4957. doi: 10.1093/hmg/ddv216
- Hung, T., Wang, Y., Lin, M. F., Koegel, A. K., Kotake, Y., Grant, G. D., et al. (2011). Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. *Nat. Genet.* 43, 621–629. doi: 10.1038/ng.848
- Iwahashi, C. K., Yasui, D. H., An, H. J., Greco, C. M., Tassone, F., Nannen, K., et al. (2006). Protein composition of the intranuclear inclusions of FXTAS. *Brain* 129, 256–271. doi: 10.1093/brain/awh650
- Jacquemont, S., Hagerman, R. J., Leehey, M., Grigsby, J., Zhang, L., Brunberg, J. A., et al. (2003). Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *Am. J. Hum. Genet.* 72, 869–878. doi: 10.1086/374321
- Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., et al. (2005). Antisense transcription in the mammalian transcriptome. *Science* 309, 1564–1566. doi: 10.1126/science.1112009
- Kenneson, A., Zhang, F., Hagedorn, C. H., and Warren, S. T. (2001). Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. *Hum. Mol. Genet.* 10, 1449–1454. doi: 10.1093/hmg/10.14.1449
- Khalil, A. M., Faghihi, M. A., Modarresi, F., Brothers, S. P., and Wahlestedt, C. (2008). A novel RNA transcript with antiapoptotic function is silenced in fragile X syndrome. *PLoS One* 3:e1486. doi: 10.1371/journal.pone.0001486
- Kinney, S. R., and Pradhan, S. (2013). Ten eleven translocation enzymes and 5-hydroxymethylation in mammalian development and cancer. *Adv. Exp. Med. Biol.* 754, 57–79. doi: 10.1007/978-1-4419-9967-2_3
- Klinge, C. M. (2018). Non-coding RNAs in breast cancer: intracellular and intercellular communication. *Noncoding RNA* 4, 40–73. doi: 10.3390/ncrna4040040
- Kong, H. E., Zhao, J., Xu, S., Jin, P., and Jin, Y. (2017). Fragile X-associated tremor/ataxia syndrome: from molecular pathogenesis to development of therapeutics. *Front. Cell. Neurosci.* 11:128. doi: 10.3389/fncel.2017.00128
- Konishi, Y., Stegmüller, J., Matsuda, T., Bonni, S., and Bonni, A. (2004). Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science* 303, 1026–1030. doi: 10.1126/science.1093712
- Kumar, M. M., and Goyal, R. (2017). lncRNA as a therapeutic target for angiogenesis. *Curr. Top. Med. Chem.* 17, 1750–1757. doi: 10.2174/1568026617666161116144744
- Kumari, D., and Usdin, K. (2010). The distribution of repressive histone modifications on silenced FMR1 alleles provides clues to the mechanism of gene silencing in fragile X syndrome. *Hum. Mol. Genet.* 19, 4634–4642. doi: 10.1093/hmg/ddq394
- Kung, J. T., Colognori, D., and Lee, J. T. (2013). Long noncoding RNAs: past, present, and future. *Genetics* 193, 651–669. doi: 10.1534/genetics.112.146704
- Lacoux, C., Di Marino, D., Boyl, P. P., Zalfa, F., Yan, B., Ciotti, M. T., et al. (2012). BC1-FMRP interaction is modulated by 2'-O-methylation: RNA-binding activity of the tudor domain and translational regulation at synapses. *Nucleic Acids Res.* 40, 4086–4096. doi: 10.1093/nar/gkr1254
- Ladd, P. D., Smith, L. E., Rabaia, N. A., Moore, J. M., Georges, S. A., Hansen, R. S., et al. (2007). An antisense transcript spanning the CGG repeat region of FMR1 is upregulated in premutation carriers but silenced in full mutation individuals. *Hum. Mol. Genet.* 16, 3174–3187. doi: 10.1093/hmg/ddm293
- Liu, Z., Chang, Q., Yang, F., Liu, B., Yao, H. W., Bai, Z. G., et al. (2017). Long non-coding RNA NEAT1 overexpression is associated with unfavorable prognosis in patients with hepatocellular carcinoma after hepatectomy: a Chinese population-based study. *Eur. J. Surg. Oncol.* 43, 1697–1703. doi: 10.1016/j.ejso.2017.06.013
- Liu, W., Jiang, F., Bi, X., and Zhang, Y. Q. (2012). Drosophila FMRP participates in the DNA damage response by regulating G2/M cell cycle checkpoint and apoptosis. *Hum. Mol. Genet.* 21, 4655–4668. doi: 10.1093/hmg/ddc307
- Mailick, M. R., Hong, J., Greenberg, J., Smith, L., and Sherman, S. (2014). Curvilinear association of CGG repeats and age at menopause in women with FMR1 premutation expansions. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 165B, 705–711. doi: 10.1002/ajmg.b.32277
- Mercer, T. R., and Mattick, J. S. (2013). Structure and function of long noncoding RNAs in epigenetic regulation. *Nat. Struct. Mol. Biol.* 20, 300–307. doi: 10.1038/nsmb.2480
- Militello, G., Weirick, T., John, D., Doring, C., Dimmeler, S., and Uchida, S. (2017). Screening and validation of lncRNAs and circRNAs as miRNA sponges. *Brief. Bioinform.* 18, 780–788. doi: 10.1093/bib/bbw053
- Nicolas, F. E. (2017). Role of ncRNAs in development, diagnosis and treatment of human cancer. *Recent Pat. Anticancer Drug Discov.* 12, 128–135. doi: 10.2174/1574892812666170105113415
- Ojha, R., Nandani, R., Pandey, R. K., Mishra, A., and Prajapati, V. K. (2019). Emerging role of circulating microRNA in the diagnosis of human infectious diseases. *J. Cell. Physiol.* 234, 1030–1043. doi: 10.1002/jcp.27127

- Pastori, C., Peschansky, V. J., Barbouth, D., Mehta, A., Silva, J. P., and Wahlestedt, C. (2014). Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in fragile X syndrome and fragile X-associated tremor/ataxia syndrome. *Hum. Genet.* 133, 59–67. doi: 10.1007/s00439-013-1356-6
- Peschansky, V. J., Pastori, C., Zeier, Z., Motti, D., Wentzel, K., Velmeshev, D., et al. (2015). Changes in expression of the long non-coding RNA FMR4 associate with altered gene expression during differentiation of human neural precursor cells. *Front. Genet.* 6:263. doi: 10.3389/fgene.2015.00263
- Peschansky, V. J., Pastori, C., Zeier, Z., Wentzel, K., Velmeshev, D., Magistri, M., et al. (2016). The long non-coding RNA FMR4 promotes proliferation of human neural precursor cells and epigenetic regulation of gene expression in trans. *Mol. Cell. Neurosci.* 74, 49–57. doi: 10.1016/j.mcn.2016.03.008
- Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T., et al. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* 66, 817–822.
- Reis, E. M., and Verjovski-Almeida, S. (2012). Perspectives of long non-coding RNAs in cancer diagnostics. *Front. Genet.* 3:32. doi: 10.3389/fgene.2012.00032
- Rodriguez-Revela, L., Madrigal, I., Pagonabarraga, J., Xuncla, M., Badenas, C., Kulisevsky, J., et al. (2009). Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *Eur. J. Hum. Genet.* 17, 1359–1362. doi: 10.1038/ejhg.2009.51
- Rother, S., and Meister, G. (2011). Small RNAs derived from longer non-coding RNAs. *Biochimie* 93, 1905–1915. doi: 10.1016/j.biochi.2011.07.032
- Sanchez Calle, A., Kawamura, Y., Yamamoto, Y., Takeshita, F., and Ochiya, T. (2018). Emerging roles of long non-coding RNA in cancer. *Cancer Sci.* 109, 2093–2100. doi: 10.1111/cas.13642
- Sarfi, M., Abbastabar, M., and Khalili, E. (2019). Long noncoding RNAs biomarker-based cancer assessment. *J. Cell. Physiol.* 1–16. doi: 10.1002/jcp.28417
- Schaeffer, C., Bardoni, B., Mandel, J. L., Ehresmann, B., Ehresmann, C., and Moine, H. (2001). The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. *EMBO J.* 20, 4803–4813. doi: 10.1093/emboj/20.17.4803
- Sellier, C., Rau, F., Liu, Y., Tassone, F., Hukema, R. K., Gattoni, R., et al. (2010). Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients. *EMBO J.* 29, 1248–1261. doi: 10.1038/emboj.2010.21
- Shao, L. L., Jiang, Y. H., Jiang, L. Y., Yang, C. H., and Qi, Y. Z. (2019). Long non-coding RNA and mRNA analysis of Ang II-induced neuronal dysfunction. *Mol. Biol. Rep.* 1–16. doi: 10.1007/s11033-019-04783-x
- Sherman, S. L., Curnow, E. C., Easley, C. A., Jin, P., Hukema, R. K., Tejada, M. I., et al. (2014). Use of model systems to understand the etiology of fragile X-associated primary ovarian insufficiency (FXPOI). *J. Neurodev. Disord.* 6, 26–37. doi: 10.1186/1866-1955-6-26
- Sreeram, N., Wren, C., Bhate, M., Robertson, P., and Hunter, S. (1989). Cardiac abnormalities in the fragile X syndrome. *Br. Heart J.* 61, 289–291. doi: 10.1136/hrt.61.3.289
- Sullivan, A. K., Marcus, M., Epstein, M. P., Allen, E. G., Anido, A. E., Paquin, J. J., et al. (2005). Association of FMR1 repeat size with ovarian dysfunction. *Hum. Reprod.* 20, 402–412. doi: 10.1093/humrep/deh635
- Sutcliffe, J. S., Nelson, D. L., Zhang, F., Pieretti, M., Caskey, C. T., Saxe, D., et al. (1992). DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum. Mol. Genet.* 1, 397–400. doi: 10.1093/hmg/1.6.397
- Tang, J., Zhuo, H., Zhang, X., Jiang, R., Ji, J., Deng, L., et al. (2014). A novel biomarker Linc00974 interacting with KRT19 promotes proliferation and metastasis in hepatocellular carcinoma. *Cell Death Dis.* 5:e1549. doi: 10.1038/cddis.2014.518
- Tassone, F., Beilina, A., Carosi, C., Albertosi, S., Bagni, C., Li, L., et al. (2007). Elevated FMR1 mRNA in premutation carriers is due to increased transcription. *RNA* 13, 555–562. doi: 10.1261/rna.280807
- Tassone, F., Hagerman, R. J., Garcia-Arocena, D., Khandjian, E. W., Greco, C. M., and Hagerman, P. J. (2004). Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. *J. Med. Genet.* 41:e43. doi: 10.1136/jmg.2003.012518
- Tassone, F., Hagerman, R. J., Loesch, D. Z., Lachiewicz, A., Taylor, A. K., and Hagerman, P. J. (2000). Fragile X males with unmethylated, full mutation trinucleotide repeat expansions have elevated levels of FMR1 messenger RNA. *Am. J. Med. Genet.* 94, 232–236. doi: 10.1002/1096-8628(20000918)94:3<232::AID-AJMG9>3.0.CO;2-H
- Tejada, M. I., Garcia-Alegria, E., Bilbao, A., Martinez-Bouzas, C., Beristain, E., Poch, M., et al. (2008). Analysis of the molecular parameters that could predict the risk of manifesting premature ovarian failure in female premutation carriers of fragile X syndrome. *Menopause* 15, 945–949. doi: 10.1097/gme.0b013e3181647762
- Tiedge, H., Chen, W., and Brosius, J. (1993). Primary structure, neural-specific expression, and dendritic location of human BC200 RNA. *J. Neurosci.* 13, 2382–2390. doi: 10.1523/JNEUROSCI.13-06-02382.1993
- Todd, P. K., Oh, S. Y., Krans, A., Pandey, U. B., Di Prospero, N. A., Min, K. T., et al. (2010). Histone deacetylases suppress CGG repeat-induced neurodegeneration via transcriptional silencing in models of fragile X tremor ataxia syndrome. *PLoS Genet.* 6:e1001240. doi: 10.1371/journal.pgen.1001240
- Usdin, K., and Kumari, D. (2015). Repeat-mediated epigenetic dysregulation of the FMR1 gene in the fragile X-related disorders. *Front. Genet.* 6:192. doi: 10.3389/fgene.2015.00192
- Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65, 905–914. doi: 10.1016/0092-8674(91)90397-H
- Wang, K. C., and Chang, H. Y. (2011). Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 43, 904–914. doi: 10.1016/j.molcel.2011.08.018
- Wang, H., Iacoangeli, A., Popp, S., Muslimov, I. A., Imataka, H., Sonenberg, N., et al. (2002). Dendritic BC1 RNA: functional role in regulation of translation initiation. *J. Neurosci.* 22, 10232–10241. doi: 10.1523/JNEUROSCI.22-23-10232.2002
- Weiler, I. J., Irwin, S. A., Klintsova, A. Y., Spencer, C. M., Brazelton, A. D., Miyashiro, K., et al. (1997). Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc. Natl. Acad. Sci. USA* 94, 5395–5400.
- Wittenberger, M. D., Hagerman, R. J., Sherman, S. L., McConkie-Rosell, A., Welt, C. K., Rebar, R. W., et al. (2007). The FMR1 premutation and reproduction. *Fertil. Steril.* 87, 456–465. doi: 10.1016/j.fertnstert.2006.09.004
- Yakovlev, D. A., Kuznetsova, A. A., Fedorova, O. S., and Kuznetsov, N. A. (2017). Search for modified DNA sites with the human methyl-CpG-binding enzyme MBD4. *Acta Nat.* 9, 88–98.
- Zalfa, F., Adinolfi, S., Napoli, I., Kuhn-Holsken, E., Urlaub, H., Achsel, T., et al. (2005). Fragile X mental retardation protein (FMRP) binds specifically to the brain cytoplasmic RNAs BC1/BC200 via a novel RNA-binding motif. *J. Biol. Chem.* 280, 33403–33410. doi: 10.1074/jbc.M504286200
- Zhang, Q., Zhang, Y., and Wang, Y. (2019). GHET1 acts as a prognostic indicator and functions an oncogenic lncRNA in cervical cancer. *Biosci. Rep.* 39, 1–21. doi: 10.1042/BSR20182506
- Zhong, J., Chuang, S. C., Bianchi, R., Zhao, W., Lee, H., Fenton, A. A., et al. (2009). BC1 regulation of metabotropic glutamate receptor-mediated neuronal excitability. *J. Neurosci.* 29, 9977–9986. doi: 10.1523/JNEUROSCI.3893-08.2009
- Zhou, Y., Hu, Y., Sun, Q., and Xie, N. (2019). Non-coding RNA in fragile X syndrome and converging mechanisms shared by related disorders. *Front. Genet.* 10:139. doi: 10.3389/fgene.2019.00139
- Zhu, C., Cheng, D., Qiu, X., Zhuang, M., and Liu, Z. (2018). Long noncoding RNA SNHG16 promotes cell proliferation by sponging microRNA-205 and upregulating ZEB1 expression in osteosarcoma. *Cell. Physiol. Biochem.* 51, 429–440. doi: 10.1159/000495239
- Zou, T., Wang, P. L., Gao, Y., and Liang, W. T. (2018). Long noncoding RNA HOTTIP is a significant indicator of ovarian cancer prognosis and enhances cell proliferation and invasion. *Cancer Biomark.* 1–7. doi: 10.3233/CBM-181727 [Epub ahead of print]

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CeRNA Expression Profiling Identifies KIT-Related circRNA-miRNA-mRNA Networks in Gastrointestinal Stromal Tumour

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to RNA,
a section of the journal
Frontiers in Genetics

Received: 13 December 2018

Accepted: 09 August 2019

Published: 10 September 2019

Citation:

Jia N, Tong H, Zhang Y, Katayama H,
Wang Y, Lu W, Zhang S and
Wang J (2019) CeRNA Expression
Profiling Identifies KIT-Related
circRNA-miRNA-mRNA Networks
in Gastrointestinal Stromal Tumour.
Front. Genet. 10:825.
doi: 10.3389/fgene.2019.00825

Gastrointestinal stromal tumours (GISTs) are the most common human sarcomas and are typically located in the stomach or small intestine. Although circular RNAs (circRNAs) reportedly play vital roles in tumour oncogenesis and progression, the molecular basis of the aggressive tumour biology of these circRNAs in GISTs remains unclear. In this study, we applied SBC ceRNA microarrays to screen for tumour-specific circRNA profiles in GISTs and identified that a total of 5,770 circRNAs and 1,815 mRNAs were differentially expressed in GISTs. Three significantly differential circRNAs (circ_0069765, circ_0084097, and circ_0079471) and their host genes (KIT, PLAT, and ETV1) were also verified in 68 pairs of GISTs and adjacent normal gastrointestinal tissues by qRT-PCR. A GIST-specific circRNA-miRNA-mRNA regulatory network analysis demonstrated that the specific KIT-related regulatory networks involved the three circRNAs, the circRNA host genes and three miRNAs (miR-142-5p, miR-144-3p and miR-485-3p), which may be key regulators of GISTs that could serve as molecular biomarkers and potential therapeutic targets for this malignant disease.

Keywords: circRNAs, KIT, PLAT, ETV1, regulatory networks analysis

INTRODUCTION

As one of the most common non-epithelial neoplasms, gastrointestinal stromal tumours (GISTs) are located in the stomach (55.6%), small intestine (31.8%), colon and rectum (6.0%), and oesophagus and abdominal cavity (6.2%) and have various clinical features ranging from asymptomatic to nonspecific abdominal discomfort, pain, haemorrhage and tumour abdominal (Joensuu et al., 2012); the incidence of GISTs is 10-15 cases per million per year in 19 countries (Soreide et al., 2016). It is not necessary for GIST patients to exhibit liver metastasis or intraperitoneal dissemination to perform an assessment of the tumour risk. However, clinicopathological factors, including the tumour size, mitotic count and anatomical location, were associated with cancer patient survival (Fletcher et al., 2002; Markku Miettinen and Jerzy Lasota, 2006; Joensuu, 2008), and complete surgical resection remains the primary treatment method for localized GISTs (Ho and Blanke, 2011). GISTs can be characterized by the expression of CD117 or PDGFRA protein in neoplastic cells, and a gain-of-function mutation may exist in

the type III receptor tyrosine kinase gene (c-KIT or PDGFR- α) at the genetic level (Hirota et al., 1998; Heinrich et al., 2003b). KIT is a receptor tyrosine kinase that is upregulated in the interstitial cells of Cajal, which are pacemakers responsible for digestive movement (Chi et al., 2010). KIT mutations frequently occur in exons 9, 11, 13 and 17 (Heinrich et al., 2003a; Corless et al., 2004) and play a vital role in GIST pathogenesis (Mazur and Clark, 1983; Hirota et al., 1998). In addition, a PDGFR- α mutation, which is present in 1/3 of KIT-negative GIST patients, mainly affects exons 12, 14 and 18 and can induce tyrosine kinase autophosphorylation, activate signalling molecules containing SH2 domains, and initiate various cancer-related pathways (Wozniak et al., 2012).

Additionally, deregulated circular RNAs (circRNAs) were investigated in acute myeloid leukaemia, breast cancer, gastric cancer, and prostate cancer (Patop and Kadener, 2018; Xia et al., 2018) and found to be involved in a variety of tumour-specific progression events, such as proliferation, invasion and metastasis (Li F et al., 2015; Li J et al., 2015; Wilusz, 2017; Yang et al., 2018; Patop and Kadener, 2018). These deregulated circRNAs exhibit cell- or tissue-specific expression, exist in a steady state on tissues, in the cellular nucleus and cytosol, on extracellular exosomes, and in body fluid and may serve as potential biomarkers of cancer (Gao and Zhao, 2018). Several deregulated circRNAs have been reported to contribute to promoting cell proliferation, such as circPVT1 in gastric tumours, circABCB10 in breast tumours and circBANP in colon tumours (Patop and Kadener, 2018). The downregulation of circITCH was also observed in bladder carcinoma, oesophageal squamous cell carcinoma, lung cancer, colon and rectal cancer and hepatocellular carcinoma (Patop and Kadener, 2018). circRNAs, which have a head-to-tail connected ring structure of exon or intron cyclization, are generated from pre-mRNAs (Wilusz, 2018) and play a sponge role by absorbing microRNAs for binding to the miRNAs of target genes, which could be indirectly influenced by circRNAs forming competing endogenous RNA (ceRNA) networks with circRNAs (Kim et al., 2009). The overexpression of circITCH passively modulated diverse tumour cellular processes by binding miR-17 via specific miRNA-binding sites, which had evident effects on the aggressive biological behaviours mediated by the circITCH/miR-17, miR-224/p21, and PTEN axis (Yang et al., 2018). We previously revealed that the differentially expressed circRNAs (circ_0062019 and circ_0057558) and the host gene SLC19A1 of circ_0062019 could be used as potential novel biomarkers of prostate cancer (Xia et al., 2018). However, to note, no altered circRNAs have been reported in GISTs, and we still lack adequate in-depth knowledge about the biological function of circRNAs in GISTs.

In this study, we first analysed the ceRNA expression profile in gastrointestinal stromal tumour using high-throughput circRNA gene microarray and verified the differential circRNAs in GISTs by qRT-PCR. Our exploration of the circRNA-miRNA-mRNA network could help by adding a new dimension to the study of the molecular mechanisms of GISTs and provide new directions for GIST diagnosis and treatment.

MATERIALS AND METHODS

Patients and Specimens

This study included patients with GIST who underwent partial or complete resection between Sept 2012 and Oct 2017 at Shanghai Public Health Clinical Center, Fudan University, China. The study was approved by the Medical Ethics Commission of Shanghai Public Health Clinical Center. All patients had understood all aspects of the informed consent and signed the informed consent forms before undergoing surgeries. During the operation, 68 pairs of GIST and adjacent normal gastrointestinal tissue samples were collected from obvious lesions and the corresponding gastric or intestinal tissues, which were 1–3 centimetre distant from the tumour edge and contained no obvious cancer cells. After removal from the body, the fresh samples were rapidly intensively chilled in liquid nitrogen within 5 min of excision to avoid degradation. Then, the frozen specimens were stored in a -80°C refrigerator. All enrolled patients were diagnosed for the first time through a pathological examination before undergoing surgical resection. The definitive diagnosis of all cases required tissue biopsy, which relied on endoscopic ultrasound-guided fine-needle aspiration. The tumour histological grading were based on malignancy risk stratification of the gastrointestinal stromal cell tumours by tumour size, mitotic count, and location (Markku Miettinen and Jerzy Lasota, 2006).

Cell Line, Plasmid and Cell Transfection

The human gastrointestinal stromal tumour cell lines GIST-T1 and GIST-882 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The GIST-T1 cells were cultured in McCoy5A's medium, and the GIST-882 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) (HyClone, Logan, UT, USA) under the culture conditions of 37°C and 5% CO_2 . A circ_0084097 and an NC control pLCDH-ciR empty vector were synthesized by Genesee Biotech Co. Ltd. (Guangzhou, China) and transfected into the GIST-T1 cells by using Lipofectamine 2000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. The transfection efficiency was assessed using qRT-PCR.

RNA Purification and SBC ceRNA Microarrays

The total RNA was isolated and purified with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and a TIANGEN total RNA Isolation Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. The isolated RNAs were stored at -80°C . The RNA was qualified, and the RNA integrity number was determined by an Agilent 2100 bioanalyser, while the RNA concentration was analysed using a NanoDrop-2000 spectrophotometer (NanoDrop, USA). For the ceRNA microarray, the included RNA samples were obtained from 3 pairs of GIST and adjacent normal gastrointestinal

tissue samples. cRNA was synthesized and amplified with an Agilent Low Input Quick Amp WT Labeling Kit (Santa Clara, CA, US) and can be labelled by cyanine 3-labelled CTP with T7 RNA polymerase. The labelled cRNA was purified by an RNeasy mini kit (Qiagen, USA) and loaded onto SBC Human (4*180K) ceRNA microarrays including 88,371 circRNAs and 18,853 mRNAs (Shanghai Biotechnology corporation, Shanghai, China). The signals were scanned by an Agilent G2565CA Microarray Scanner. The raw data were obtained by Agilent Feature Extraction (v10.7). After normalization of the raw data with R software, the differentially expressed mRNAs and circRNAs were filtrated according to the fold change and Student t-test. The normalized signal value is the value calculated by log2. All ceRNAs with a fold change (FC) $\geq \pm 2$, a p-value < 0.05 and intensity > 7.0 were included for further statistical analysis. The complete ceRNA array datasets were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE131481.

Regulatory Network and Pathway Analysis of the Differential mRNAs and the Host Genes of the Differential circRNAs in GISTs

To further investigate the functions of these differential mRNAs in GISTs, the functions of the differential genes were annotated with GO and KEGG pathway analyses (Xia et al., 2018). CircInteractome (<https://circinteractome.nia.nih.gov/>) was used to predict the putative miRNAs of the three circRNAs and the potential circRNA/miRNA interaction (Dudekula et al., 2016). TargetsCan7.2 (https://circinteractome.org/vert_72) was used to predict the targeted miRNAs of the three host genes. We overlapped the two predicted results. Finally, we selected the top miRNAs with the highest context scores (score > 85) to establish a circRNA-miRNA-host gene network, which was illustrated by Cytoscape3.5.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis of the Differentially Expressed circRNAs and Their Host Genes in GISTs

In total, 3 circRNAs were chosen for experimental validation by qRT-PCR. As an exonuclease, RNase R can only act on RNA from its 3' to 5' end but does not degrade circRNA (Suzuki et al., 2006). Therefore, to distinguish the expression between the linear mRNA and circRNA, total RNAs were incubated for 20 min at 37°C with or without RNase R (Epicentre Technologies, Madison, WI), and the resulting RNAs were purified using an RNeasy Total RNA Kit (Qiagen, Beijing, China) and transcribed into cDNA. The cDNAs were synthesized with reverse transcriptase using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The PCR comprised 50 ng cDNA, 10 μ l of 2 x PCR Master mix (SYBR Premix Ex Taq™ II kit) (TaKaRa), 0.8 μ l primer forward (10 μ M), 0.8 μ l primer reverse (10 μ M), and 0.4 μ l of ROX reference Dye and was performed on an ABI ViiA 7 (Applied Biosystems, DE, USA) as follows: denaturation at 95°C for 10

min, amplification at 95°C for 15 s over 40 cycles, followed by annealing and extension at 60°C for 1 min. The results of the relative expression levels were obtained by calculating the raw data using the $2^{-\Delta\Delta Ct}$ method. 18S rRNA served as an internal control for the normalization. The numbers of exons and exact sequences of circ_0084097 produced from PLAT, circ_0069765 from KIT, and circ_0079471 from ETV1 were validated by Sanger sequencing. All the primers for circ_0084097, circ_0069765, and circ_0079471 were designed by Shanghai Biotechnology corporation and shown in **Tables S1** and **S2**.

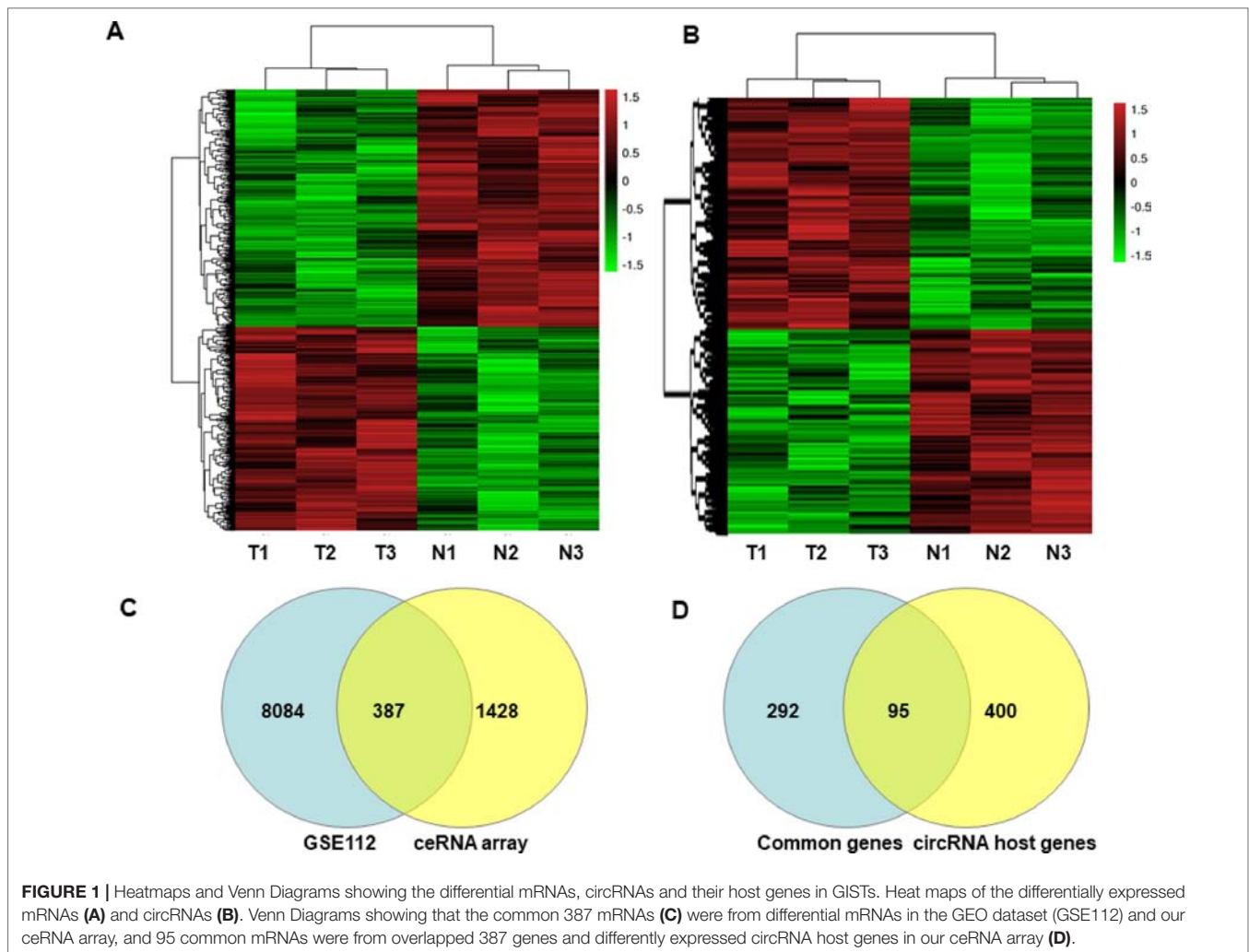
Statistical Analysis

To compare the GIST and adjacent normal gastrointestinal tissue samples, the significance of the relative quantification validation was conducted by Student t-test for the paired analysis. All tests were 2-sided, and $p < 0.05$ was regarded as statistical significance. The data were analysed with Statistical Program for Social Sciences (SPSS) 16.0 software (SPSS, Chicago, IL, USA).

RESULTS

Differentially Expressed mRNAs and circRNAs in GISTs

The characteristics of the GIST patient population and the clinical details of the three samples from the GIST patients chosen for the SBC ceRNA arrays are shown in **Table S3**. The ceRNA arrays were performed to investigate the differentially expressed mRNAs and circRNAs in GISTs. Volcano plots were used to present the significant differences in the extracted data between the GIST and adjacent normal gastrointestinal tissue samples and show the expressed difference in mRNAs (**Figure S1A**) and circRNAs (**Figure S1B**) between the GIST and adjacent tissues. Based on the differences in their expression levels, hierarchical clustering showed the differentially expressed mRNA (**Figure 1A**) and circRNA (**Figure 1B**) expression profile among 3 pairs of GIST and adjacent normal gastrointestinal tissue samples. In total, 1,815 mRNAs (839 upregulated mRNAs and 976 downregulated mRNAs) (**Table 1**) and 5,770 circRNAs (3,122 upregulated circRNAs and 2,648 downregulated circRNAs) (**Table 2**) were differentially expressed between the GIST and adjacent normal gastrointestinal tissue samples ($p < 0.05$ and $FC \geq \pm 2$). After screening the differentially expressed mRNAs by retrieving the GEO database (GSE112) and utilizing GEO2R in analysing the array data (**Table S4**), Venn diagrams were generated to show the 387 common differentially expressed genes (DEGs) selected in our array and GEO dataset GSE112 (**Figure 1C**). Finally, 95 DEGs were also identified as the host genes of DEcircRNAs in GISTs. In total, 54 circRNA host genes were upregulated, and 41 DEcircRNA host genes were downregulated in the GIST tumour tissues from these three GISTs patients, which was consistent with the expression level of the circRNAs ($p < 0.05$ and $FC \geq \pm 2$) (**Figure 1D** and **Table 3**).



Functional Pathway Analysis of Differential mRNAs and circRNA Host Genes in GISTs

Subsequently, a Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of these differentially expressed mRNAs and the host genes of the differential circRNAs in GISTs was performed to determine the top 5 pathways of the differential mRNAs, which included Biosynthesis of unsaturated fatty acids, Vitamin B6 metabolism, Notch signalling pathway, Dilated cardiomyopathy, ABC transporters and Hypertrophic cardiomyopathy (HCM) (Figure 2A); several circRNA host genes were enriched in the pathways of One carbon pool by folate, D-Glutamine and D-glutamate metabolism, ECM-receptor interaction, Adherens junction, and Nicotinate and nicotinamide metabolism (Figure 2B). Moreover, several common pathways involved the differentially expressed mRNAs and host genes of differential circRNAs in GISTs, including vascular smooth muscle contraction, Notch signalling pathway, nicotinate and nicotinamide metabolism, N-Glycan biosynthesis, hypertrophic cardiomyopathy (HCM), focal adhesion ECM-receptor interaction, Dilated

cardiomyopathy, axon guidance and Arrhythmogenic right ventricular cardiomyopathy (ARVC).

KIT-Related circRNA-miRNA-mRNA Regulatory Network Analysis in GISTs

Among ceRNA expression profiling in GISTs, we found three circRNAs (circ_0069765, circ_0084097, and circ_0079471) and their host genes (KIT, PLAT, and ETV1) were up-regulated in GISTs. The molecular analysis of KIT becomes one of the two gold standards of diagnosis in GISTs. Mutation in the KIT gene is the key oncogenic drivers in the majority of GISTs (Wu et al., 2019), which is also potentiated by a positive feedback circuit that involves the ETS transcription factor ETV1 (Duensing, 2015; Wu et al., 2019). Besides, PLAT (Tissue-Type Plasminogen Activator) as a node with VEGFC, PGF and CHD7 in the functional networks was also verified to be significantly enriched in blood vessel development involved in the tissue specificity of GISTs (Ma et al., 2018), which pushed us to analyze KIT related circRNA-miRNA-mRNA regulatory network in GISTs. Thus, three circRNAs derived from above

TABLE 1 | Partially differentially expressed mRNAs in GIST.

Gene Symbol	Gene bank Accession No	Fold change	p-values
MC4R	NM_005912	518.09	0.031
F2RL2	NM_004101	312.59	0.032
LY6H	NM_001130478	205.12	0.008
NPPC	NM_024409	141.15	0.001
FBN2	NM_001999	133.50	0.019
OBSCN	NM_001271223	132.13	0.000
PTPRH	NM_002842	124.36	0.000
ASTN1	NM_004319	112.92	0.027
SCG5	NM_001144757	102.57	0.010
ITGA10	NM_003637	94.89	0.006
TBX5	NM_000192	93.62	0.037
PRKCQ	NM_006257	92.00	0.001
ANO1	NM_018043	84.12	0.001
ABCA12	NM_173076	80.20	0.005
CIT	NM_001206999	63.97	0.000
SH3GL3	NM_001301109	63.28	0.004
KIT	NM_000222	61.06	0.011
DPP10	NM_001178036	57.54	0.018
TENM1	NM_001163278	56.20	0.001
ROBO2	NM_001290040	54.12	0.007
GYG2	NM_003918	-215.06	0.016
PKD1L2	NM_052892	-228.15	0.014
AZGP1	NM_001185	-242.01	0.004
MFAP5	NM_003480	-242.88	0.002
ABCA8	NM_001288985	-258.21	0.002
VIT	NM_053276	-279.41	0.001
CYP4B1	NM_001099772	-281.41	0.004
PI16	NM_153370	-306.57	0.004
HBA1	NM_000558	-315.48	0.008
ITLN1	NM_017625	-335.62	0.004
ALDH1L1	NM_012190	-375.76	0.024
PLIN4	NM_001080400	-392.80	0.009
CFD	NM_001928	-565.13	0.001
C14orf180	NM_001286400	-681.24	0.016
PPP1R1A	NM_006741	-709.72	0.015
HRASLS5	NM_054108	-740.50	0.017
ADH1C	NM_000669	-846.95	0.001
ADH1B	NM_001286650	-886.39	0.021
ADH1A	NM_000667	-969.78	0.003
TUSC5	NM_172367	-2204.07	0.025

TABLE 2 | Partially differentially expressed circRNAs in GIST.

circRNA_ID	Gene bank Accession No	Fold change	p-values	Host gene
hsa_circ_0065978	NM_001161581	598.50	0.020	POC1A
hsa_circ_0016772	NM_001098623	253.92	0.008	OBSCN
hsa_circ_0016774	NM_001098623	233.44	0.005	OBSCN
hsa_circ_0016775	NM_001098623	231.51	0.004	OBSCN
hsa_circ_0016776	NM_001098623	213.33	0.007	OBSCN
hsa_circ_0016773	NM_001098623	209.56	0.004	OBSCN
hsa_circ_0016778	NM_001098623	201.52	0.004	OBSCN
hsa_circ_0069236	NM_001145847	184.99	0.006	PROM1
hsa_circ_0016780	NM_001098623	180.51	0.001	OBSCN
hsa_circ_0016779	NM_001098623	169.85	0.005	OBSCN
hsa_circ_0023311	NM_018043	137.75	0.001	ANO1
hsa_circ_0028697	NM_001206999	125.79	0.003	CIT
hsa_circ_0028694	NM_001206999	124.24	0.000	CIT
hsa_circ_0028687	NM_001206999	113.21	0.000	CIT
hsa_circ_0045305	NM_138363	107.60	0.000	CEP95
hsa_circ_0073782	NM_001999	103.58	0.023	FBN2
hsa_circ_0023310	NM_018043	103.44	0.000	ANO1
hsa_circ_0003570	NM_014661	98.82	0.000	FAM53B
hsa_circ_0023309	NM_018043	98.25	0.000	ANO1
hsa_circ_0073792	NM_001999	94.47	0.019	FBN2
hsa_circ_0081375	NM_001185	-102.41	0.001	AZGP1
hsa_circ_0037139	NM_000517	-105.37	0.020	HBA2
hsa_circ_0025368	NM_003480	-106.22	0.000	MFAP5
hsa_circ_0035024	NM_001015001	-107.93	0.006	CKMT1A
hsa_circ_0037141	NM_000558	-113.19	0.021	HBA1
hsa_circ_0006751	NM_014241	-115.81	0.003	PTPLA
hsa_circ_0017695	NM_024693	-119.12	0.038	ECHDC3
hsa_circ_0001946	NM_004065	-122.17	0.021	CDR1
hsa_circ_0002091	NM_014241	-130.33	0.000	PTPLA
hsa_circ_0087206	NM_000689	-132.06	0.001	ALDH1A
hsa_circ_0005754	NM_001103184	-146.44	0.022	FMN1
hsa_circ_0080888	NM_006379	-159.28	0.001	SEMA3C
hsa_circ_0025367	NM_003480	-159.49	0.000	MFAP5
hsa_circ_0080897	NM_006379	-159.96	0.001	SEMA3C
hsa_circ_0048861	NM_000064	-181.48	0.004	C3
hsa_circ_0048858	NM_000064	-182.24	0.006	C3
hsa_circ_0048867	NM_000064	-182.38	0.007	C3
hsa_circ_0048892	NM_000064	-196.57	0.010	C3
hsa_circ_0048870	NM_000064	-218.98	0.005	C3
hsa_circ_0048871	NM_000064	-279.58	0.004	C3

parental genes were selected for further investigation although there were some top change circRNAs in **Table 2**. circ_0069765, which is located on chr4 q12 (chr4:55569889-55603446), is derived from a non-coding regulatory region of KIT (**Figure S2A**). circ_0079471, which is located on chr7 p21.2 (chr7:13949257-13975521), is a regulatory circRNA within a long non-coding region of ETV1 (**Figure S2B**). However, circ_0084097 stems from a non-coding regulatory region contained a promoter blank adjacent to the promoter region of PLAT, which is located on chr8 p12 (chr8:42046451-42050729) (**Figure S2C**). Based on the miRNA site prediction, we predicted the targeted miRNAs of the three differential circRNAs in circular RNA Interactome (<https://circinteractome.nia.nih.gov/>) (Dudekula et al., 2016). To obtain insight into reciprocal circRNA, miRNA and mRNA regulation, we constructed a regulatory circRNA-miRNA-mRNA network using Cytoscape software and clarified the interaction among the three circRNAs (circ_0069765, circ_0084097, and circ_0079471),

their host genes (KIT, PLAT, and ETV1) and seven predicted miRNAs (miR-144-3p, miR-1246, miR-485-3p, miR-142-3p, miR-142-5p, miR-326 and miR-324-5p), which is shown in **Figure 3**. In the figure, the upregulated circRNAs and their host genes are marked in red, and the downregulated miRNAs that had been reported in previous studies investigating cancer tissues are marked in green. Evidently, miR-144-3p, and miR-485-3p are common target miRNAs of all three host genes (KIT, PLAT, and ETV1), and miR-142-5p is a targeted miRNA of KIT and PLAT. We also found that miR-1246 was predicted as the common targets of both circ_0069765 and circ_0084097 and their host genes (KIT and PLAT), and miR-326 was predicted as the common targets of both circ_0069765 and circ_0079471. Thus, the specific regulatory networks including the three circRNAs (circ_0069765, circ_0084097, and circ_0079471), their host genes (KIT, PLAT, and ETV1) and the three miRNAs (miR-142-5p, miR-144-3p and miR-485-3p) may be key regulators in GISTs.

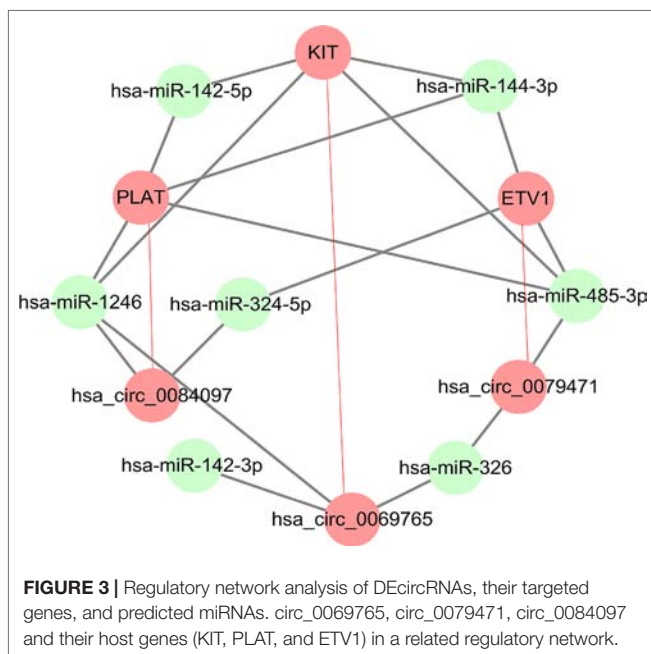
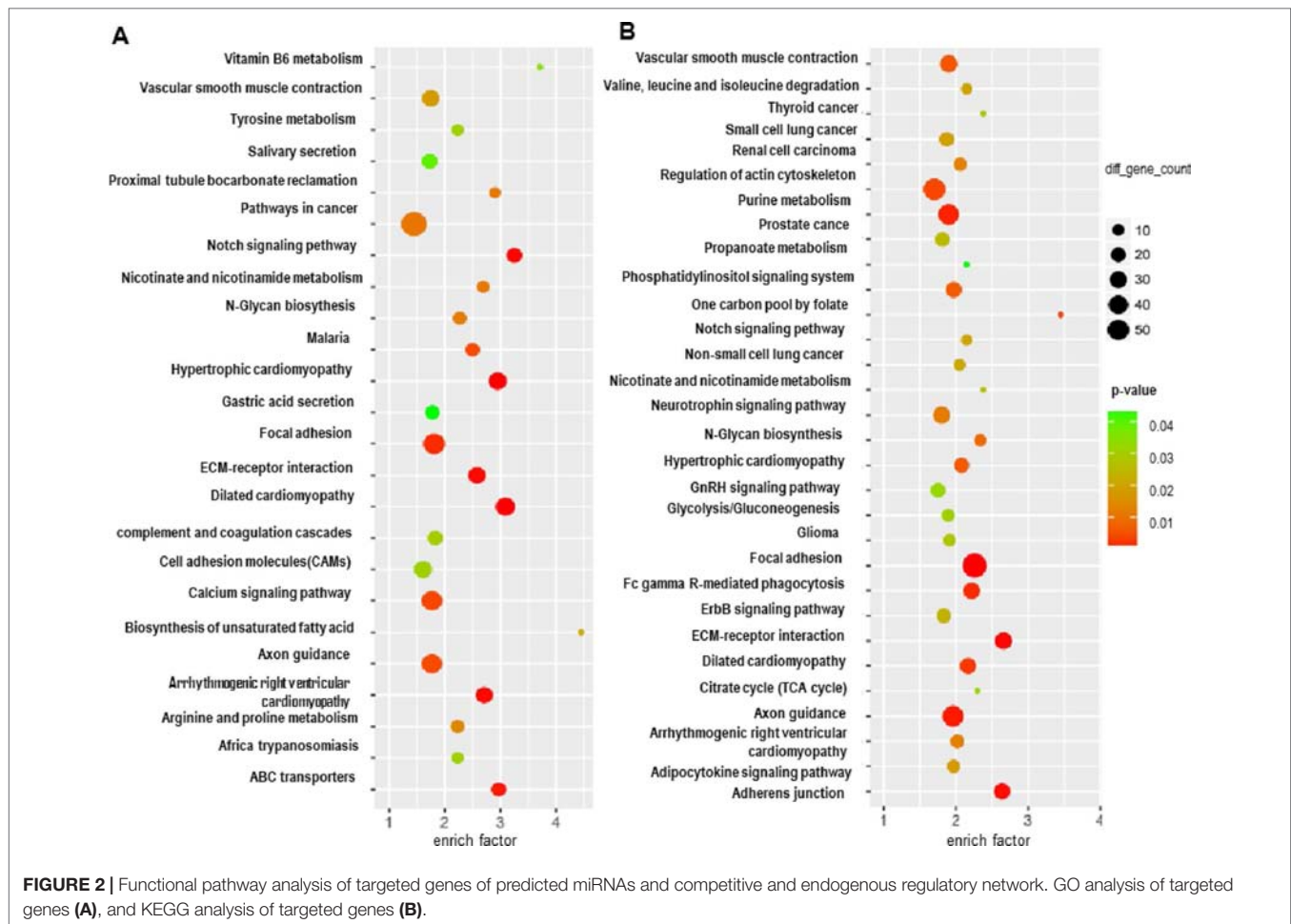
TABLE 3 | Partial DEcircRNAs and DEGs as host genes in GISTs.

circRNA_ID	Gene bank Accession No	Fold change	p-values	Host gene	Fold change	p-values
hsa_circ_0073782	NM_001999	103.58	0.023	FBN2	133.50	0.019
hsa_circ_0017609	NM_006257	39.41	0.014	PRKCCQ	92.00	0.001
hsa_circ_0028697	NM_001206999	125.79	0.003	CIT	63.97	0.000
hsa_circ_0069765	NM_000222	66.92	0.002	KIT	61.06	0.011
hsa_circ_0056201	NM_001178036	43.76	0.047	DPP10	57.54	0.018
hsa_circ_0091277	NM_198465	70.33	0.000	NRK	51.90	0.000
hsa_circ_0079471	NM_004956	36.48	0.005	ETV1	45.36	0.009
hsa_circ_0008714	NM_001025390	66.38	0.004	AMPD3	33.80	0.003
hsa_circ_0071585	NM_000892	5.39	0.023	KLKB1	24.89	0.026
hsa_circ_0086362	NM_002839	65.77	0.009	PTPRD	23.58	0.009
hsa_circ_0073242	NM_004385	20.36	0.000	VCAN	23.06	0.000
hsa_circ_0084097	NM_000930	19.09	0.013	PLAT	22.19	0.007
hsa_circ_0015753	NM_198503	14.62	0.001	KCNT2	19.19	0.007
hsa_circ_0027663	NM_001135805	29.87	0.001	SYT1	18.99	0.009
hsa_circ_0055922	NM_201555	24.80	0.008	FHL2	16.85	0.000
hsa_circ_0047919	NM_152721	6.70	0.041	DOK6	16.10	0.011
hsa_circ_0052028	NM_002975	16.82	0.003	CLEC11A	16.07	0.003
hsa_circ_0070911	NM_014278	12.28	0.001	HSPA4L	15.89	0.001
hsa_circ_0070487	NM_005723	14.36	0.013	TSPAN5	15.41	0.007
hsa_circ_0069722	NM_145263	29.12	0.002	SPATA18	14.52	0.001
hsa_circ_0080961	NM_000927	25.22	0.019	ABCB1	12.98	0.001
hsa_circ_0015816	NM_205860	-19.14	0.020	NR5A2	-9.17	0.024
hsa_circ_0014229	NM_019554	-41.00	0.008	S100A4	-10.00	0.003
hsa_circ_0033629	NM_001311	-13.51	0.007	CRIP1	-10.04	0.015
hsa_circ_0000895	NM_002229	-8.78	0.009	JUNB	-10.11	0.007
hsa_circ_0070442	NM_007351	-31.47	0.003	MMRN1	-11.11	0.004
hsa_circ_0013276	NM_001013660	-9.10	0.001	FRRS1	-11.70	0.005
hsa_circ_0087214	NM_000700	-38.15	0.033	ANXA1	-12.81	0.007
hsa_circ_0078299	NM_005100	-14.43	0.002	AKAP12	-14.88	0.003
hsa_circ_0060545	NM_002999	-7.21	0.034	SDC4	-16.55	0.016
hsa_circ_0032974	NM_006329	-23.91	0.029	FBLN5	-17.26	0.010
hsa_circ_0000074	NM_002228	-13.85	0.018	JUN	-17.69	0.006
hsa_circ_0055622	NM_207328	-2.59	0.039	GPT2	-18.34	0.002
hsa_circ_0046941	NM_002071	-5.80	0.043	GNAL	-23.67	0.002
hsa_circ_0008591	NM_053025	-18.81	0.025	MYLK	-26.71	0.016
hsa_circ_0049487	NM_001299	-38.06	0.048	CNN1	-28.60	0.041
hsa_circ_0070510	NM_016242	-23.03	0.038	EMCN	-31.55	0.029
hsa_circ_0039466	NM_175617	-11.67	0.001	MT1E	-35.88	0.002
hsa_circ_0056473	NM_032995	-12.44	0.007	ARHGEF4	-45.11	0.000
hsa_circ_0003625	NM_032784	-50.11	0.008	RSPO3	-60.82	0.009
hsa_circ_0025368	NM_003480	-196.57	0.000	MFAP5	-242.90	0.002

Differential circRNAs (circ_0069765, circ_0079471 and circ_0084097) and Their Host Genes Were Verified in GISTs by qRT-PCR

The genomic structure shows that circ_0069765 contains six exons from the KIT gene (**Figure S2A**), circ_0079471 contains four exons from ETV1 gene (**Figure S2B**), and circ_0084097 contains three exons from PLAT gene (**Figure S2C**). All the “head-to-tail” splicing sites of the three circRNAs are presented in **Figure S2**. The distinct products of these three circRNAs were amplified using outward-facing primers and confirmed by Sanger sequencing (**Figures S3A–C**). We found that circ_0069765, circ_0079471 and circ_0084097 were resistant to RNase R, compared to the linear mRNAs (Data not shown). Next, we detected the expression level

of circ_0069765, circ_0079471, circ_0084097 and their corresponding host genes by real-time PCR (qRT-PCR) analyses. The relative expression of the three circRNAs (circ_0069765, circ_0079471 and circ_0084097) was evidently upregulated in the GIST tissues compared with that in the adjacent noncancerous tissues ($p < 0.001$); in addition, the three host genes, i.e., KIT, PLAT and ETV1, were upregulated ($p < 0.001$) (**Figure 4**). The qRT-PCR analyses revealed that 44 of 66 (66.67%) tumours had increased circ_0069765 (4.68-fold); 60 of 65 (92.30%) tumours had increased host gene KIT mRNA (1404.20-fold) expression; 63 of 68 (92.65%) tumours had increased circ_0084097 expression (156.86-fold); 61 of 68 (89.71%) tumours had increased host gene PLAT mRNA (462.43-fold) expression; 59 of 68 (86.76%) tumours had increased circ_0079471 (118.10-fold) expression; and 62 of



66 (93.94%) tumours had increased host gene ETV1 mRNA (678.60-fold) expression. These findings were consistent with the tissue microarray data and showed the significant upregulation tendency of the three circRNAs and three host genes. Finally, we identified that markable positive correlations were present between PLAT and three verified circRNAs ($p < 0.05$) (Table S5). We also noted a non-negative correlation between two circRNAs and ETV1 (Table S5, $*p < 0.05$). Interestingly, an obvious correlation was observed not only between the genes ETV1 and PLAT ($p < 0.001$) but also between the circRNAs circ_0069765 and circ_0079471 and between circ_0079471 and circ_0084097 ($p < 0.05$) (Table S5). To clarify the characteristics of these differential circRNAs and their host genes in GIST cancer, a Pearson correlation analysis was applied to analyse the correlation between these circRNAs/their host genes and the corresponding clinical parameters. As shown in Table 4, circ_0084097 and its host gene PLAT are negatively correlated with metastasis of tumours significantly related to the stomach ($p < 0.05$). PLAT was also negatively correlated with the tumour diameter ($p < 0.05$) (Table 4), indicating that circ_0084097 and PLAT may be related to the early stage of stomach stromal tumour.

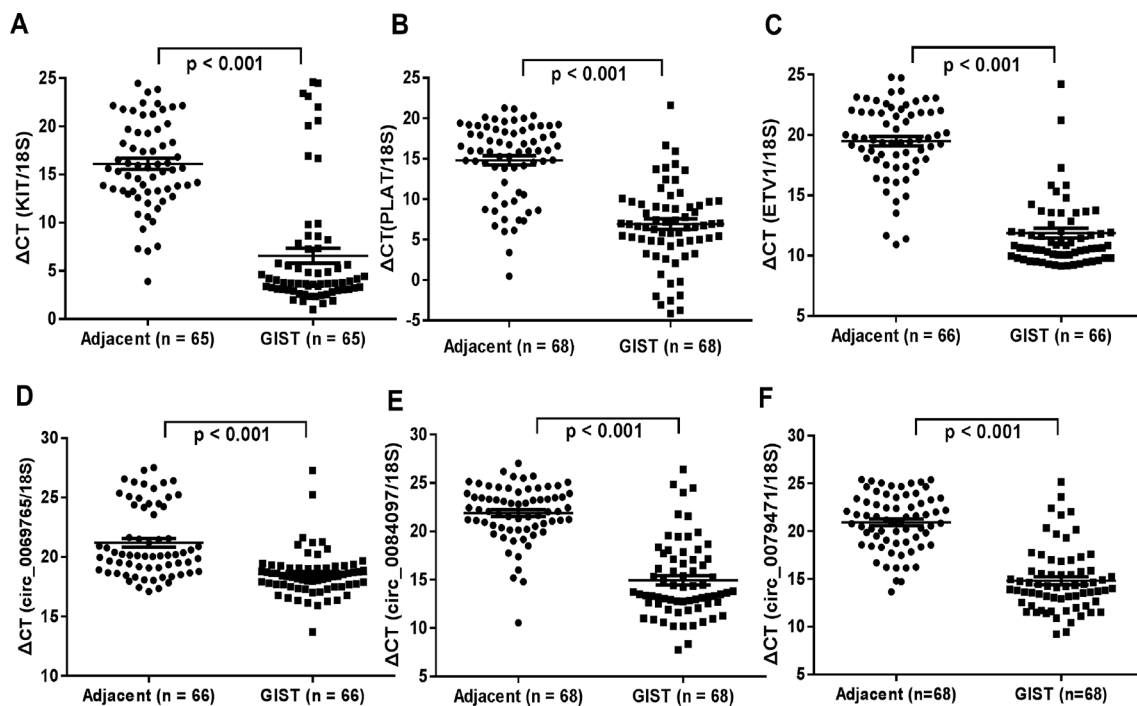


FIGURE 4 | qRT-PCR analysis of the gene expression levels of the three differentially expressed circRNAs and their host genes in GISTs. **(A)** KIT; **(B)** PLAT; **(C)** ETV1; **(D)** circ_0069765; **(E)** circ_0084097; and circ_0079471 **(F)**.

TABLE 4 | Correlation analysis of circ_0084097/PLAT expression in tumour tissue samples of GIST patients and their clinical factors.

Characteristic	circ_0084097		Person correlation	p-value	PLAT		Person correlation	p-value
	Low	High			Low	High		
Location			0.310	0.010			0.284	0.019
Stomach	15	24			13	26		
Other	19	10			21	8		
Metastasis			-0.246	0.045			-0.242	0.049
Yes	7	4			8	3		
No	26	30			25	31		
Vascular invasion			-0.218	0.074			-0.197	0.108
Yes	7	3			7	3		
No	27	31			27	31		
Adhesion			-0.1	0.419			-0.198	0.106
Yes	15	15			19	11		
No	19	19			15	23		
Maxium tumour diameter			-0.059	0.635			-0.298	0.013
<10 cm	25	28			22	31		
>10 cm	9	6			13	3		
NIH grade			0.106	0.388			-0.084	0.496
low risk	15	11			12	14		
intermediate and high risk	19	23			22	20		

Bold values denote statistical significance at the $p < 0.05$ level.

DISCUSSION

In this study, the ceRNA expression profile showed that the mRNA and circRNA expression profile in the gastric stromal tumour tissues was distinguished from that in matched tissues adjacent to the tumour and found that a total of 3,122 circRNAs were significantly

upregulated and 2,648 were significantly downregulated in the tumour tissues. More importantly, 95 differentially expressed genes had been filtered by overlapping circRNA host genes and significant mRNAs of GSE112. We found several common pathways involving the differential mRNAs and the host genes of differential circRNAs in GISTs, including vascular smooth muscle contraction, Notch

signalling pathway, nicotinate and nicotinamide metabolism, N-Glycan biosynthesis, Hypertrophic cardiomyopathy (HCM), Focal adhesion, ECM-receptor interaction, Dilated cardiomyopathy, Axon guidance, and Arrhythmogenic right ventricular cardiomyopathy (ARVC) (Figure 2). Three molecular inhibitors of the Wnt signalling pathway have been reported to be tumour suppressors in various in vitro and in vivo GIST models harbouring a KIT mutation. The Wnt antagonist DKK4 was apparently downregulated in advanced human GISTs (Zeng et al., 2017). The Notch signalling pathway has also been reported to be a tumour suppressor in GIST cells harbouring a KIT mutation. The downstream target of notch (dominant-negative Hes1) was apparently upregulated in GIST patients with longer relapse-free survival (Yang et al., 2018). In addition, the focal adhesion signalling pathway played a critical role in the proliferation of both imatinib-sensitive and resistant GIST cells (Zeng et al., 2017). We demonstrated that the Wnt, Notch and Focal adhesion signalling pathways are associated with GIST cell proliferation.

Notably, 95 genes were not only differentially expressed linear RNAs but also maternal genes that generated various differentially expressed circular RNAs in our study. circ_0069765, circ_0079471 and circ_0084097 were selected for the validation of the array results,

and we detected the expression of these circRNAs in 68 pairs of tissue samples and showed that the three circRNAs were significantly upregulated in tumour tissues, while their host genes KIT, PLAT and ETV1 had a similar rising trend in expression. Furthermore, the expression levels of these three circRNAs and their host genes were also checked in GIST cell lines. We only found that circ_0069765 was significantly upregulated in the GIST-T1 and GIST-882 cells and that circ_0079471 and its host gene ETV1 were overexpressed in the GIST-T1 cells compared to the normal stomach stromal tissue by a qRT-PCR analysis (all $p < 0.05$) (Figure S4).

Web tools for miRNA target-site prediction for circRNA that have a sequence-based recognition system come with the context scores which have the advantage of being predictive for all types of interactions. There is not standard score for selecting top miRNAs. We selected top miRNAs with the high context score (score > 85) for the three differential circRNAs to establish a circRNA-miRNA-host gene network in GIST (Table S6) and found that miR-142-3p, miR-142-5p, miR-149, miR-324-5p, miR-326, miR-485-3p and miR-1246 might interact with circ_0069765, circ_0079471 and circ_0084097. Interestingly, miR-1246, miR-142-5p, and miR-324-5p were downregulated in the GIST cells (GIST-882 and GIST-T1)

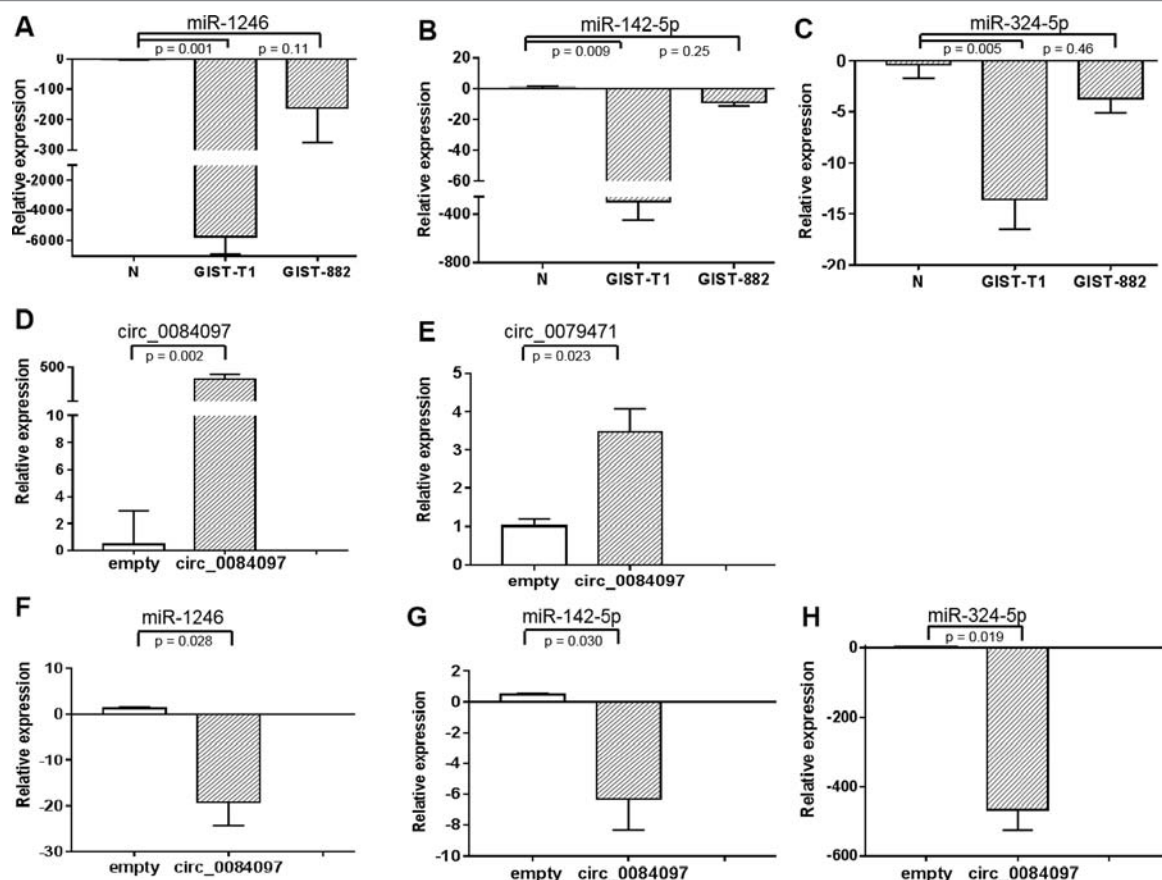


FIGURE 5 | Gene expression levels of miR-1246, miR-142-5p, and miR-324-5p in GIST-T1 and GIST-882 cells (A–C) (A) miR-1246; (B) miR-142-5p; (C) miR-324-5p and their expression in GIST-T1 with circ_0079471 by overexpression of circ_0084097 (D–H) (D) circ_0084097; (E) circ_0079471; (F) miR-1246; (G) miR-142-5p; (H) miR-324-5p were analysed by qRT-PCR.

compared to the normal stomach stromal tissue in the qRT-PCR analysis (Figures 5A–C). In the analysis of the function of the ceRNAs and their interaction, we confirmed that these three miRNAs were also repressed and that circ_0079471 was upregulated in GIST-T1 cells by overexpression of circ_0084097 (Figures 5D–H), which was consistent with our circRNA-miRNA regulatory network analysis in GISTs (Figure 4). Thus, these miRNAs may be linked to several host genes, including KIT, PLAT, and ETV1. In GISTs, a KIT proximal domain mutation, especially in exon 11, can induce ligand-independent kinase phosphorylation and activate downstream signal transduction pathway, including AKT, MAPK and STAT (Corless et al., 2011). The molecular targeted agent, Imatinib, blocks KIT / PDGFRA signalling by binding the ATP-binding pocket required for phosphorylation and activation of the receptor. The application of imatinib had changed from a single drug model to a combination with surgical treatment, which was essential to complete surgical resection, alleviate the disease, prolong survival and improve the quality of life, especially among postoperative patients (Huang et al., 2016). Unfortunately, initially sensitive tumours acquired imatinib resistance due to a KIT secondary mutation. Sunitinib and regorafenib are two additional multikinase inhibitors approved as second- and third-line therapies, respectively, and are available for the treatment of imatinib-resistance GIST (Demetri et al., 2006; Demetri et al., 2013). It has been found that non-small cell lung cancer tumourigenesis was suppressed by the overexpression of miR142-5p, which also regulated tumour cell PD-L1 expression and enhanced anti-tumour immunity in pancreatic cancer (Jia et al., 2017; Wang et al., 2017a). The downregulation of miR-142-5p was significantly associated with the recurrence and poor prognosis of gastric cancer (GC) and promoted tumour metastasis by regulating CYR61 expression (Yan et al., 2019). miR-144-3p was significantly downregulated in hepatocellular carcinoma, glioblastoma, multiple myeloma and pancreatic cancer and inhibited proliferation, migration and tumour metastasis by targeting SGK3 (Wu et al., 2017), FZD7 (Cheng et al., 2017), c-Met (Zhao et al., 2017) and FOSB (Liu et al., 2018). The repression of miR-485-3p was also found in breast cancer. The overexpression of miR-485-3p can inhibit mitochondrial respiration and breast cancer cell metastasis by inhibiting PGC-1 α expression (Lou et al., 2016). Low serum levels of miR-485-3p were related to poor survival in patients with glioblastoma (Wang et al., 2017b). The miR-324-5p-mediated suppression of NF- κ B activation was reported to be responsible for inhibition breast cancer cell invasion and migration (Song et al., 2015). The expression of miR-1246 was downregulated in lung cancer cell lines and cervical cancer tissue, was negatively correlated with the clinical stage and inhibited cell invasion and the EMT by targeting CXCR4 (Yang et al., 2015; Xu et al., 2018). miR-149 was downregulated in ovarian cancer, colorectal cancer and lung cancer. The overexpression of miR-149 increased the drug sensitivity of cancer cells and inhibited the EMT through the FOXM1/cyclin D1/MMP2 axis (Ke et al., 2013; Xu et al., 2015; Sun et al., 2018). Thus, the decreased expression and functional inhibition of these miRNAs in cancer further

support our hypothesis that circ_0069765, circ_0079471 and circ_0084097 function to regulate the more comprehensive circRNAs-miRNAs-genes network.

In summary, the present research revealed the ceRNA expression profiles in GISTs and identified that three circRNAs (circ_0069765, circ_0079471 and circ_0084097) and three host genes (KIT, ETV1 and PLAT) were upregulated in GISTs using qRT-PCR. We further demonstrated that the specific regulatory networks including three circRNAs (circ_0069765, circ_0084097, and circ_0079471), their host genes (KIT, PLAT, and ETV1) and three miRNAs (miR-142-5p, miR-144-3p and miR-485-3p) may be key regulators in GISTs and are likely involved in tumour oncogenesis and progression. In future investigations, it is worth considering the verification of the molecular mechanism of these specific circRNAs to regulate GIST occurrence and development. A greater understanding of the mechanisms of the involvement of specific circRNAs in GIST tumour malignancy is necessary for the identification of possible therapeutic targets.

DATA AVAILABILITY

The datasets generated for this study can be found at NCBI using accession number GSE131481 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131481>).

ETHICS STATEMENT

The study included patients with GIST who underwent partial or complete resection at Shanghai Public Health Clinical Center, Fudan University, China between Sept 2016 and Oct 2017. The study was approved by the Medical Ethics Commission of Shanghai Public Health Clinical Center.

AUTHOR CONTRIBUTIONS

JW and HT contributed to the conception; NJ, HT, YZ, HK, YW, WL, SZ, and JW analyzed the data; NJ and JW wrote the manuscript; and JW revised the manuscript.

FUNDING

This research was supported by a grant from the National Natural Science Foundation of China (81672383), the National Special Research Program of China for Important Infectious Diseases (2018ZX10302103-003). The grant (KY-GW-2017-09) (HT) was from Shanghai Public Health Clinical Center, Shanghai, China.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00825/full#supplementary-material>

REFERENCES

- Cheng, Z. X., Song, Y. X., Wang, Z. Y., Wang, Y., and Dong, Y. (2017). miR-144-3p serves as a tumor suppressor by targeting FZD7 and predicts the prognosis of human glioblastoma. *Eur. Rev. Med. Pharmacol. Sci.* 21, 4079–4086.
- Chi, P., Chen, Y., Zhang, L., Guo, X., Wongvipat, J., Shamu, T., et al. (2010). ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature* 467, 849–853. doi: 10.1038/nature09409
- Corless, C. L., Barnett, C. M., and Heinrich, M. C. (2011). Gastrointestinal stromal tumours: origin and molecular oncology. *Nat. Rev. Cancer* 11, 865–878. doi: 10.1038/nrc3143
- Corless, C. L., Fletcher, J. A., and Heinrich, M. C. (2004). Biology of gastrointestinal stromal tumors. *J. Clin. Oncol.* 22, 3813–3825. doi: 10.1200/JCO.2004.05.140
- Fletcher, C. D., Berman, J. J., Corless, C., Gorstein, F., Lasota, J., Longley, B. J., et al. (2002). Diagnosis of gastrointestinal stromal tumors: a consensus approach. *Hum. Pathol.* 33. doi: 10.1053/hupa.2002.124119
- Demetri, G. D., van Oosterom, A. T., Garrett, C. R., Blackstein, M. E., Shah, M. H., Verweij, J., et al. (2006). Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* 368, 1329–1338. doi: 10.1016/S0140-6736(06)69446-4
- Demetri, G. D., Reichardt, P., Kang, Y. K., Blay, J. Y., Rutkowski, P., Gelderblom, H., et al. (2013). Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 381, 295–302. doi: 10.1016/S0140-6736(12)61857-1
- Dudekula, D. B., Panda, A. C., Grammatikakis, I., De, S., Abdelmohsen, K., and Gorospe, M. (2016). CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. *RNA Biol.* 13, 34–42. doi: 10.1080/15476286.2015.1128065
- Duensing, A. (2015). Targeting ETV1 in gastrointestinal stromal tumors: tripping the circuit breaker in GIST? *Cancer Discov.* 5, 231–233. doi: 10.1158/2159-8290.CD-15-0116
- Gao, Y., and Zhao, F. (2018). Computational strategies for exploring circular RNAs. *Trends Genet.* 34, 389–400. doi: 10.1016/j.tig.2017.12.016
- Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C. J., Joseph, N., et al. (2003b). PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 299, 708–710. doi: 10.1126/science.1079666
- Heinrich, M. C., Corless, C. L., Demetri, G. D., Blanke, C. D., Von Mehren, M., Joensuu, H., et al. (2003a). Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J. Clin. Oncol.* 21, 4342–4349. doi: 10.1200/JCO.2003.04.190
- Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., et al. (1998). Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 279, 577–580. doi: 10.1126/science.279.5350.577
- Ho, M. Y., and Blanke, C. D. (2011). Gastrointestinal stromal tumors: disease and treatment update. *Gastroenterology* 140, 1372–1376, e1372. doi: 10.1053/j.gastro.2011.03.017
- Huang, K. K., McPherson, J. R., and Tay, S. T. (2016). SETD2 histone modifier loss in aggressive GI stromal tumours. *Gut* 65, 1960–1972. doi: 10.1136/gutjnl-2015-309482
- Jia, L., Xi, Q., Wang, H., Zhang, Z., Liu, H., Cheng, Y., et al. (2017). miR-142-5p regulates tumor cell PD-L1 expression and enhances anti-tumor immunity. *Biochem. Biophys. Res. Commun.* 488, 425–431. doi: 10.1016/j.bbrc.2017.05.074
- Joensuu, H. (2008). Risk stratification of patients diagnosed with gastrointestinal stromal tumor. *Hum. Pathol.* 39, 1411–1419. doi: 10.1016/j.humpath.2008.06.025
- Joensuu, H., Vehtari, A., Riihimäki, J., Nishida, T., Steigen, S. E., Brabec, P., et al. (2012). Risk of recurrence of gastrointestinal stromal tumour after surgery: an analysis of pooled population-based cohorts. *Lancet Oncol.* 13, 265–274. doi: 10.1016/S1470-2045(11)70299-6
- Ke, Y., Zhao, W., Xiong, J., and Cao, R. (2013). miR-149 inhibits non-small-cell lung cancer cells EMT by targeting FOXM1. *Biochem. Res. Int.* 2013, 506731. doi: 10.1155/2013/506731
- Kim, H. H., Kuwano, Y., Srikantan, S., Lee, E. K., Martindale, J. L., and Gorospe, M. (2009). HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev.* 23, 1743–1748. doi: 10.1101/gad.1812509
- Li, F., Zhang, L., Lu, J., Li, W., and Zhou, Y. (2015). Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/ β -catenin pathway. *Oncotarget* 6, 6001–6013. doi: 10.18632/oncotarget.3469
- Li, J., Yang, J., Zhou, P., Le, Y., Zhou, C., Wang, S., et al. (2015). Circular RNAs in cancer: novel insights into origins, properties, functions and implications. *Am. J. Cancer Res.* 5, 472–480.
- Liu, S., Luan, J., and Ding, Y. (2018). miR-144-3p Targets FosB Proto-oncogene, AP-1 Transcription Factor Subunit (FOSB) to suppress proliferation, migration, and invasion of PANC-1 pancreatic cancer cells. *Oncol. Res.* 26, 683–690. doi: 10.3727/096504017X14982585511252
- Lou, C., Xiao, M., Cheng, S., Lu, X., Jia, S., Ren, Y., et al. (2016). MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1 α expression. *Cell Death Dis.* 7, e2159. doi: 10.1038/cddis.2016.27
- Ma, N., Xu, H., Zhou, Y., Liu, M., Wei Zhou, J., and Jie Wang, C. (2018). Analyzing the molecular mechanism of the tissue specificity of gastrointestinal stromal tumors by using bioinformatics approaches. *J. Buon.* 23, 1149–1155.
- Markku Miettinen, M., and Jerzy Lasota, M. (2006). Gastrointestinal stromal tumors: Pathology and prognosis at different sites. *Semin. Diagn. Pathol.* 23, 70–83. doi: 10.1053/j.semmp.2006.09.001
- Mazur, M. T., and Clark, H. B. (1983). Gastric stromal tumors: reappraisal of histogenesis. *Am. J. Surg. Pathol.* 7, 507–519. doi: 10.1097/00000478-198309000-00001
- Patop, I. L., and Kadener, S. (2018). circRNAs in Cancer. *Curr. Opin. Genet. Dev.* 48, 121–127. doi: 10.1016/j.gde.2017.11.007
- Song, L., Liu, D., Zhao, Y., He, J., Kang, H., Dai, Z., et al. (2015). Sinomenine inhibits breast cancer cell invasion and migration by suppressing NF- κ B activation mediated by IL-4/miR-324-5p/CUEDC2 axis. *Biochem. Biophys. Res. Commun.* 464, 705–710. doi: 10.1016/j.bbrc.2015.07.004
- Soreide, K., Sandvik, O. M., Soreide, J. A., Giljaca, V., Jureckova, A., and Bulusu, V. R. (2016). Global epidemiology of gastrointestinal stromal tumours (GIST): a systematic review of population-based cohort studies. *Cancer Epidemiol.* 40, 39–46. doi: 10.1016/j.canep.2015.10.031
- Sun, L., Zhai, R., Zhang, L., and Zhao, S. (2018). MicroRNA-149 suppresses the proliferation and increases the sensitivity of ovarian cancer cells to cisplatin by targeting X-linked inhibitor of apoptosis. *Oncol. Lett.* 15, 7328–7334. doi: 10.3892/ol.2018.8240
- Suzuki, H., Zuo, Y., Wang, J., Zhang, M. Q., Malhotra, A., and Mayeda, A. (2006). Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing. *Nucleic Acids Res.* 34, e63. doi: 10.1093/nar/gkl151
- Wang, Z., Liu, Z., Fang, X., and Yang, H. (2017a). MiR-142-5p suppresses tumorigenesis by targeting PIK3CA in non-small cell lung cancer. *Cell Physiol. Biochem.* 43, 2505–2515. doi: 10.1159/000484459
- Wang, Z. Q., Zhang, M. Y., Deng, M. L., Weng, N. Q., Wang, H. Y., and Wu, S. X. (2017b). Low serum level of miR-485-3p predicts poor survival in patients with glioblastoma. *PLoS One* 12, e0184969. doi: 10.1371/journal.pone.0184969
- Wilusz, J. E. (2017). Circular RNAs: unexpected outputs of many protein-coding genes. *RNA Biol.* 14, 1007–1017. doi: 10.1080/15476286.2016.1227905
- Wilusz, J. E. (2018). A 360 degrees view of circular RNAs: from biogenesis to functions. *Wiley Interdiscip. Rev. RNA* 9, e1478. doi: 10.1002/wrna.1478
- Wozniak, A., Rutkowski, P., Piskorz, A., Ciwoniuk, M., Osuch, C., Bylina, E., et al. (2012). Prognostic value of KIT/PDGFR α mutations in gastrointestinal stromal tumours (GIST): Polish Clinical GIST Registry experience. *Ann. Oncol.* 23, 353–360. doi: 10.1093/annonc/mdr127
- Wu, C. E., Tzen, C. Y., Wang, S. Y., and Yeh, C. N. (2019). Clinical diagnosis of Gastrointestinal Stromal Tumor (GIST): from the molecular genetic point of view. *Cancers (Basel)* 11, pii: E679. doi: 10.3390/cancers11050679
- Wu, M., Huang, C., Huang, X., Liang, R., Feng, Y., and Luo, X. (2017). MicroRNA-144-3p suppresses tumor growth and angiogenesis by targeting SGK3 in hepatocellular carcinoma. *Oncol. Rep.* 38, 2173–2181. doi: 10.3892/or.2017.5900
- Xia, Q., Ding, T., Zhang, G., Li, Z., Zeng, L., Zhu, Y., et al. (2018). Circular RNA expression profiling identifies prostate cancer-specific circRNAs in prostate cancer. *Cell Physiol. Biochem.* 50, 1903–1915. doi: 10.1159/000494870
- Xu, K., Liu, X., Mao, X., Xue, L., Wang, R., Chen, L., et al. (2015). MicroRNA-149 suppresses colorectal cancer cell migration and invasion by directly targeting

- forkhead box transcription factor FOXM1. *Cell Physiol. Biochem.* 35, 499–515. doi: 10.1159/000369715
- Xu, X., Cao, L., Zhang, Y., Lian, H., Sun, Z., and Cui, Y. (2018). MicroRNA-1246 inhibits cell invasion and epithelial mesenchymal transition process by targeting CXCR4 in lung cancer cells. *Cancer Biomark* 21, 251–260. doi: 10.3233/CBM-170317
- Yan, J., Yang, B., Lin, S., Xing, R., and Lu, Y. (2019). Downregulation of miR-142-5p promotes tumor metastasis through directly regulating CYR61 expression in gastric cancer. *Gastric Cancer*. 22, 302–313. doi: 10.1007/s10120-018-0872-4
- Yang, C., Yuan, W., Yang, X., Li, P., Wang, J., Han, J., et al. (2018). Circular RNA circ-ITCH inhibits bladder cancer progression by sponging miR-17/miR-224 and regulating p21, PTEN expression. *Mol. Cancer*, 17, 19. doi: 10.1186/s12943-018-0771-7
- Yang, Y., Xie, Y. J., Xu, Q., Chen, J. X., Shan, N. C., and Zhang, Y. (2015). Down-regulation of miR-1246 in cervical cancer tissues and its clinical significance. *Gynecol. Oncol.* 138, 683–688. doi: 10.1016/j.ygyno.2015.06.015
- Zeng, S., Seifert, A. M., Zhang, J. Q., Cavnar, M. J., Kim, T. S., Balachandran, V. P., et al. (2017). Wnt/beta-catenin signaling contributes to tumor malignancy and is targetable in gastrointestinal stromal tumor. *Mol. Cancer Ther.* 16, 1954–1966. doi: 10.1158/1535-7163.MCT-17-0139
- Zhao, Y., Xie, Z., Lin, J., and Liu, P. (2017). MiR-144-3p inhibits cell proliferation and induces apoptosis in multiple myeloma by targeting c-Met. *Am. J. Transl. Res.* 9, 2437–2446.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Role of Non-Coding RNAs in Neurodevelopmental Disorders

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Non-coding RNAs, a group of ribonucleic acids that are ubiquitous in the body and do not encode proteins, emerge as important regulatory factors in almost all biological processes in the brain. Extensive studies have suggested the involvement of non-coding RNAs in brain development and neurodevelopmental disorders, and dysregulation of non-coding RNAs is associated with abnormal brain development and the etiology of neurodevelopmental disorders. Here we provide an overview of the roles and working mechanisms of non-coding RNAs, and discuss potential clinical applications of non-coding RNAs as diagnostic and prognostic markers and as therapeutic targets in neurodevelopmental disorders.

Keywords: non-coding RNA, neurodevelopmental disorder, miRNA, piRNA, snoRNA, lncRNA

INTRODUCTION

Non-coding RNAs are RNA molecules that are not translated into proteins. Recent advances in genomic sequencing technologies and functional assays enable a more in-depth understanding of their characteristics (Mattick, 2011; Djebali et al., 2012; Obiols-Guardia and Guil, 2017). The transcription process of non-coding RNAs is precisely orchestrated in time and space (Okazaki Y., et al., 2002; Djebali et al., 2012). Different developmental stages or tissue types have distinct transcriptional landscapes (Carninci et al., 2005; Kapranov et al., 2007). The central nervous system is a sophisticated and precise system which is responsible for guiding our daily activities such as sports, learning, emotion and language. Rapidly growing evidence indicated that non-coding RNAs play indispensable roles in brain development, function, and the etiology of neurodevelopmental diseases.

Here, we review the diversity and biogenesis processes of non-coding RNAs, and summarize their versatile roles in neurodevelopmental disorders. We also discuss potential clinical applications of non-coding RNAs as diagnostic and prognostic markers and as therapeutic targets in neurodevelopmental disorders.

CHARACTERISTICS OF NON-CODING RNAs

Abundant and functionally important types of non-coding RNAs include ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), as well as regulatory non-coding RNAs which mainly consist of microRNA (miRNA), PIWI-interacting RNA (piRNA), small nucleolar RNA (snoRNA), small interfering RNAs (siRNAs), long non-coding RNA (lncRNA), and Circular RNAs (CircRNAs).

OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
RNA, a section of the
journal *Frontiers in Genetics*

Received: 09 December 2018

Accepted: 25 September 2019

Published: 20 November 2019

Citation:

Zhang S-F, Gao J and Liu C-M
(2019) The Role of Non-Coding RNAs
in Neurodevelopmental Disorders.
Front. Genet. 10:1033.
doi: 10.3389/fgene.2019.01033

Non-coding RNAs play a critical role in epigenetics regulation of gene expression in addition to their roles at the transcriptional and post-transcriptional level (David and Bartel, 2004; Alexander et al., 2010).

miRNAs are small single-stranded molecules (20–24 nt) that have seed sequences complementary to sequences on target mRNAs transcripts through the 3'UTR, leading to silencing of the target gene. The miRNA gene is transcribed by RNA polymerases II and III to generate a primary microRNA precursor molecule (pri-miRNA). The pri-miRNA then undergoes nuclear cleavage by Drosha/DGCR8 to form a precursor microRNA (pre-miRNA). The pre-miRNA is transported from the nucleus into the cytoplasm by Exportin 5, and then processed by Dicer/TRBP into a miRNA duplex which is unwound by a helicase. The mature miRNA is incorporated into the RNA-induced silencing complex (Risch et al.) which mediates down-regulation of gene expression by either translational repression or mRNA degradation (David and Bartel, 2004; Esteller, 2011; Rajman and Schratt, 2017) (**Figure 1**).

piRNAs are small non-coding RNAs (24–31 nt) that can silence transposons and regulate gene expression by directing PIWI proteins of Argonaute family to specific genomic loci (Fabio Mohn and Brennecke, 2015) (**Figure 1**). piRNAs biogenesis is divided into primary and secondary pathways (Zamore et al., 2018). In primary pathway, primary piRNAs are transcribed from genomic loci called piRNA clusters (Brennecke et al., 2007; Li et al., 2013). Primary piRNAs are spliced by endonuclease into tail-to-head phased precursor piRNA that are catalyzed by the mitochondrial protein Zucchini/PLD6 (Ipsaro et al., 2012; Nishimasu et al., 2012; Fabio Mohn and Brennecke, 2015). Each pre-piRNA begins with a 5' monophosphate, a prerequisite for loading RNA into nearly all Argonaute proteins (Schirle et al., 2014; Wang et al., 2014). Once the PIWI protein captures pre-piRNA, the 3' terminal is trimmed by a single-stranded RNA exonuclease called Trimmer/PNLDC1 to the appropriate size (Kawaoka et al., 2011; Tang et al., 2016; Ding et al., 2017).

Finally, the small RNA methyltransferase Hen1/HENMT1 adds a 2-O-methyl moiety to the 3' ends of the mature piRNAs (Horwich et al., 2007; Lim et al., 2015). The secondary piRNA biogenesis pathway, also known as the “ping-pong” cycle, is a piRNA-directed piRNA synthesis pathway that produces a piRNA *via* interaction with PIWI proteins (Brennecke et al., 2007; Gunawardane et al., 2007; Wang et al., 2014) (**Figure 1**). So far, functions of piRNA are mainly concentrated in regulation of genomic stability *via* silencing transposon (**Figure 1**).

snoRNAs are 60–300 nt nucleotide long, metabolically stable RNAs, which are usually concentrated in Cajal bodies or nucleoli (Ding et al., 2008). snoRNAs are produced by the transcription of RDR2. Compared with miRNA, snoRNA transcription events occur only in the nucleus (Vaucheret, 2006). Initial, transcripts of snoRNA enter the cytoplasm for processing and modifying and subsequently return to the nucleus. snoRNA can catalyze sequence-specific 2'-O-methylation and pseudouridine acidification of ribosomal RNA (rRNA) by forming protein complexes with splicing function (Kiss-László et al., 1996; Jingwei Ni et al., 1997). A new study has found that snoRNAs are indispensable for processing and stability of lncRNA (Xing and Chen, 2018).

lncRNA are a family of long-chain non-coding RNA that are usually longer than 200 nt, which regulate various developmental and physiological processes (Hu et al., 2016; Zhou et al., 2016; Lekka and Hall, 2018). Almost all lncRNAs transcripts do not contain open reading frames (Wilhelm et al., 2014), which are produced by RNA polymerase II, followed by capping and forming 3' polyadenylate tails (**Figure 1**) (Lekka and Hall, 2018). lncRNAs can interact with other epigenetic regulators to direct histone-modified enzymes or DNA-methylated enzymes to the specific gene loci and modulate gene expression (Tripathi et al., 2010; Gong and Maquat, 2011). For example, BDNF-AS can recruit EZH2 and PRC2 complex to the promoter region of BDNF to down-regulate the expression of BDNF (Modarresi et al.). In addition, lncRNAs might act as enhancers to activate

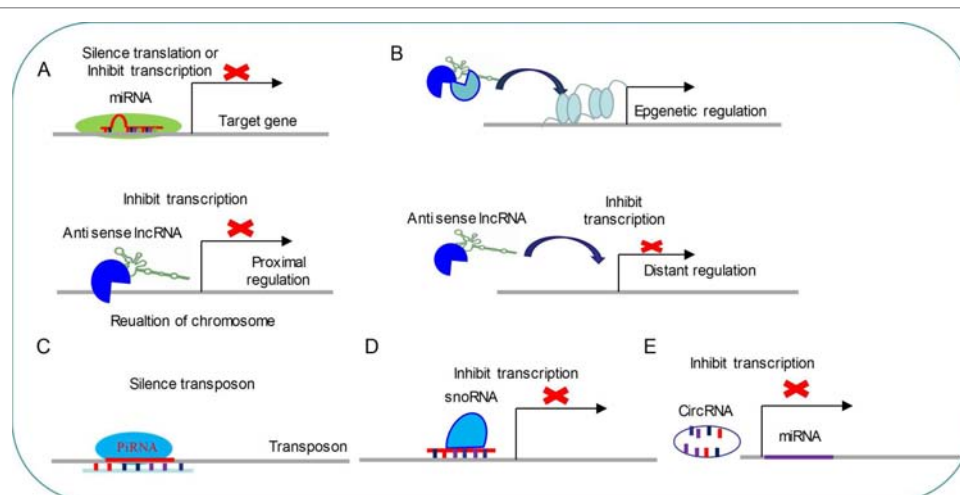


FIGURE 1 | Common regulatory mechanism of non-coding RNAs (A) miRNAs modulate target genes by silencing mRNA translation or inhibiting mRNA transcription. (B) lncRNAs regulate gene transcription by three distinct ways. (C) piRNAs target transposon for silencing its expression. (D) SnoRNAs inhibit transcription by targeting specific loci. (E) CircRNAs are able to repress miRNA expressions.

gene transcription (Orom et al., 2010), and target miRNAs to silence their inhibitory functions (Cesana et al., 2011; Lai et al., 2013). lncRNAs can also interact with proteins to modulate gene expression in different levels or direct their appropriate spatial subcellular localization (Chu et al., 2015; Lekka and Hall, 2018).

Circular RNAs (CircRNAs) are recently emerged as a new class of endogenous noncoding RNAs (ncRNAs) which might regulate gene expression (Memczak et al., 2013; You et al., 2015). These RNAs are usually processed into loops after transcription (Piwecka et al., 2017). Large-scale sequencing and analysis results have demonstrated that thousands of circular RNAs are present in mammalian and nematodes (Memczak et al., 2013; Liang Chen 2015). The published data also have indicated that CircRNAs are highly abundant in mammalian brain compared to other analyzed tissues (Memczak et al., 2013). What's more, the majority of detected mouse CircRNAs are also expressed as CircRNAs in human brain, which suggests that CircRNAs are very conserved between species (Liang Chen 2015). Identically, transcription of circular RNA is orchestrated by developmental stage and tissue specificity (Hansen et al., 2013; Liang Chen 2015; Piwecka et al., 2017), which indicates that CircRNAs might serve as regulatory RNAs, especially in brain (**Figure 1**). Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function (Hansen et al., 2013; Piwecka et al., 2017). For example, cerebellar degeneration-related protein 1 transcript (CDR1as)

contains more than 70 selectively conserved miRNA target sites. CDR1as strongly suppresses miR-7 activity and results in increased levels of miR-7 targets (Hansen et al., 2013; Piwecka et al., 2017). Functionally, CDR1as and its interaction with miRNAs are important for sensorimotor gating and synaptic transmission (Piwecka et al., 2017). Using high resolution *in situ* hybridization technology, the researchers found that visualized CircRNAs punctate into the dendrites of neurons, and many CircRNAs change their abundance abruptly at a time corresponding to synaptogenesis. Together, these data indicate that CircRNAs play important roles in regulating synaptic function (You et al., 2015).

NON-CODING RNA IN NEURODEVELOPMENTAL DISORDERS

Human brain displays the richest repertoire of ncRNA species, and where several different ncRNA molecules are known to be involved in crucial steps for neurodevelopment (Mehler and Mattick, 2006; Taft et al., 2010; Obiols-Guardia and Guil, 2017). Abnormal expression of non-coding RNA has been linked with pathologies of several neurodevelopmental diseases, including Autism spectrum disorders (ASD), Fragile X syndrome (FXS), Down syndrome (DS), Rett syndrome, and Prader-Willi Angelman syndrome (**Table 1**).

TABLE 1 | Non-coding RNAs are related to neurodevelopmental disorders.

Neurodevelopmental Disorders	Dysregulated Non-coding RNAs	Functional examples	References
Autism Spectrum Disorders	miR-197-5p, miR-328-3p, miR-424-5p, miR-500a-5p, miR-313-5a. Up: miR-619-5p, miR-365a-3p miR-664a-3p, miR-188, RAY1/ST7, ST7OT1-4, ST7AS1-4, ST7OT1-3, SHANK2-AS, MSNP1AS	MiR-188 serves to fine-tune synaptic plasticity by regulating autism susceptibility genes Nrp-2 expression. Overexpression of SHANK2-AS reduces the complexity of neurites, and inhibits the proliferation of neuronal stem cells and promotes their apoptosis	(Talebizadeh et al., 2008; Tewarit Sarachana 2010; Lee et al., 2012; Kerin et al., 2012; Trindade et al., 2013; van de et al., 2013; Mundalil Vasu et al., 2014; DeWitt et al., 2016; Constantin, 2017; Kichukova et al., 2017; Luo et al., 2018)
Fragile X Syndrome	miR-302, miR-125, miR-132, let-7c, miR-9, miR-100, miR-124, miR-125a, miR-125b, miR-127, miR-128, miR-132, miR-138, miR-143, miR-219, FMR4, FMR5, FMR6	MiR-125 and miR-132 interact with FMRP to regulate the signal transduction of metabolic glutamate receptors (mGluR1) and NMDAR. FMR4, FMR5 and FMR6 are detectable in the majority of patient leukocyte RNA samples.	(Ladd et al., 2007; Khalila 2009; Taft et al., 2010; Esteller, 2011; van de et al., 2013; Lin, 2015)
Down Syndrome	miR-99a, let-7c, miR-125b-2, miR-155, miR-802	DS dementia strongly correlates with overexpression of miR-155 on chromosome 21 with concomitant reduction of multiple CNS-functional targets, including BACH1, CoREST1, Cyclin D1, BCL6, BCL10, BIM, and SAPK4.	(Malinge et al., 2009; Fillat and Altafaj, 2012; Asim et al., 2015; Tili et al., 2018)
Rett Syndrome	miR-184, miR-30a, miR-381, miR-495, miR-130a, miR-132, miR-200a, miR-302c, DQ541777, AK081227, AK087060, BDNF-AS	MiR-184, miR-30a, miR-381, and miR-495 are aberrantly up-regulated in MeCP2 knockout mice. These miRNAs are known for repressing the expression of important modulators of neuronal development, such as Bdnf and Numbl. BDNF is known to be aberrantly diminished in RTT individuals, it can be speculated that the lncRNA BDNF-AS might be an important therapeutic target for treating RTT.	(Nomura et al., 2008; Urdinguio et al., 2010; Wu et al., 2010; Petazzi et al., 2013; Lin et al., 2014; Mo 2015; Katz et al., 2016; Rodrigues et al., 2016; Zhang et al., 2016)
Prader-Willi syndrome & Angelman syndrome	SNRPN, SNRD116, Ube3a-ATS	Mice with deletion of SNRD116 (MBII85) snoRNA clusters demonstrated obvious PWS phenotypes. In AS patients, the maternal Ube3a allele is inactive, the paternal allele is intact but epigenetically silenced through the Ube3a-ATS part of LNCAT at the Ube3a locus.	(Muscatelli et al., 2000; Landers et al., 2005; Skryabin et al., 2007; Sahoo et al., 2008; de Smith et al., 2009; Buiting, 2010; Meng et al., 2012)

Autism Spectrum Disorders

ASD is a developmental disorder that affects communication and behavior, which is characterized by repetitive patterns of behavior, interests, or activities, problems in social interactions, and psychological problems in children (Balachandar et al., 2016). Children with ASD have co-occurring language problems, intellectual disabilities, and epilepsy at higher rates than the general population. While the exact cause of ASD has remained somewhat of a mystery, dozens of genes have been identified to potentially contribute to disease susceptibility (Edward et al., 1985; Bailey et al., 1995; Risch et al., 1999; Klei et al., 2012; Kang, 2014). For example, polymorphisms in the *FMR1* gene have been reported to be associated with autism (Reddy, 2005), however, no consistent association between *FMR1* polymorphisms and autism has been demonstrated. Therefore, ASD is probably not caused by one single genetic factor. Recent studies suggest that epigenetic mechanisms, such as non-coding RNAs, may play a major role in the pathogenesis of ASD (Constantin, 2017).

Abnormal expression levels of miRNAs, including miR-132, miR-23a, miR-93, miR-106b, miR-146b and miRNA-148b, were observed in the serum, lymphoblastoid cells, or cerebellar cortex of autistic patients (Abu-Elneel et al., 2008; Talebizadeh et al., 2008; Tewarit Sarachana1 2010; Mundalil Vasu et al., 2014) (Figure 2). Many autism susceptibility genes are predictive targets of these differently expressed miRNAs, which further strengthens the causal relationship between miRNAs and autism (Abu-Elneel et al., 2008; Talebizadeh et al., 2008; Tewarit Sarachana1, 2010; Constantin, 2017).

A profiling study of circulating serum miRNAs in children with ASD reveals that differentially expressed miRNAs in serum may be involved in the molecular pathway of ASD (Kichukova et al., 2017), and serum miR-197-5p, miR-328-3p, miR-424-5p, miR-619-5p, miR-500a-5p, miR-313-5a, miR-365a-3p and miR-664a-3p may be served as potential biomarkers of ASD (Kichukova et al., 2017) (Table 1). Moreover, the interaction between miRNAs and autism risk genes in the cerebellum was observed in animal models of ASD. For example, miR-188 is down-regulated in autism patient (Trindade et al., 2013) and is up-regulated in response to long-term potentiation (Lee et al., 2012). MiR-188 promotes dendritic spine formation by blocking the expression of neuropilin-2, a well-known candidate gene of ASD (Lee et al., 2012) (Figure 2).

More than 200 lncRNAs are differentially expressed in the prefrontal cortex and cerebellum of ASD patients (Rennert, 2013). The autism loci containing *RAY1/ST7* (suppression of tumorigenicity 7) encode at least four non-coding genes (*ST7OT1-4*, *ST7AS1-4*) which are located in the sense or antisense chains that potentially regulate *RAY1/ST7*. Several rare mutations of *RAY1/ST7* or *ST7OT1-3* genes have been detected in autistic patients (Vincent et al., 2002; van de et al., 2013) (Figure 2).

Genome-wide differential expression analysis of blood samples from ASD patients (Carninci et al.) identified 2407 up-regulated and 1522 down-regulated lncRNAs in peripheral blood leukocytes of ASD. The pathway enrichment analysis of these differently

expressed lncRNAs revealed that they were mainly involved in synaptic vesicle circulation, long-term inhibition and long-term potentiation of neural pathways (van de et al., 2013). Differential expression of lncRNAs *SHANK2-AS* and *BDNF-AS* was also observed in ASD (van de et al., 2013; Wang et al., 2015; Tang et al., 2017) (Figure 2). In neurons, *SHANK2-AS* and *Shank2* can form double-stranded RNA that inhibit the expression of *Shank2*. Overexpression of *SHANK2-AS* reduces the complexity of neurites, and inhibits the proliferation of neuronal stem cells and promotes their apoptosis (Luo et al., 2018).

Moesin is a protein that in human is encoded by the *MSN* gene, which regulates neuronal structure and immune response. *MSN*-binding *MSNPI-AS* is highly expressed in postmortem cerebral cortex samples from individuals with ASD. Increased *MSNPI-AS* expression is also observed in individuals carrying the ASD-associated rs4307059 T allele (Kerin et al., 2012) (Figure 2). By targeting the *MSNPI-AS* gene promoter, *MSNPI-AS* knockdown disrupts the expression of 318 genes in neuroblastoma neural progenitor cells, many of which are involved in chromatin organization and immune response, indicating multiple transcriptional and translational functions of *MSNPIAS* in ASD-relevant biological processes (DeWitt et al., 2016) (Table 1).

Fragile X Syndrome

FXS is the most common inherited cause of mental disorder and ASD (Lin, 2015; Hecht et al., 2017). FXS is caused by *FMR1* (fragile X mental retardation 1) inactivation or dysfunction (Verkerk et al., 1991). *FMR1* is required for normal neuronal connectivity and plasticity. The *FMR1* gene contains a CGG-repeat present in the 5'UTR which can be unstable upon transmission to the next generation. FXS patients have a repeat length exceeding 200 CGGs that generally leads to methylation of the repeat and the promoter region, resulting in silencing of *FMR1* gene expression (Fu et al., 1991; Dominique Heitz et al., 1992; Tang et al., 2017).

Several miRNAs have already been proven to involve in the development of FXS (Lin, 2015). During the embryonic stage, miR-302 is specifically expressed by embryonic stem cells, and it blocks the translation of *FMR1*, which is required to repress differentiation. At blastocyst stage, down-regulation of miR-302 promotes FMRP synthesis and subsequent neuronal development. In the normal neuronal development, FMRP, as an RNA-binding protein, interacts with miR-125 and miR-132 to regulate the signal transduction of metabolic glutamate receptors (mGluR1) and N-methyl-D-aspartate receptors (NMDAR) (Lin, 2015) (Figure 2). In addition, let-7c, miR-9, miR-100, miR-124, miR-125a, miR-125b, miR-127, miR-128, miR-132, miR-138, miR-143 and miR-219 might also interact with fragile X mental retardation protein (FMRP) to regulate neuronal development (Lin, 2015) (Table 1).

lncRNAs have recently emerged to influence the pathogenesis of FXS (Esteller, 2011; Taft et al., 2010; van de et al., 2013). The *FMR1* bidirectional promoter is capable of translating lncRNA *FMR4* or *FMR1-AS1* which is an antisense transcription that overlaps the CGG repeat region (Khalila

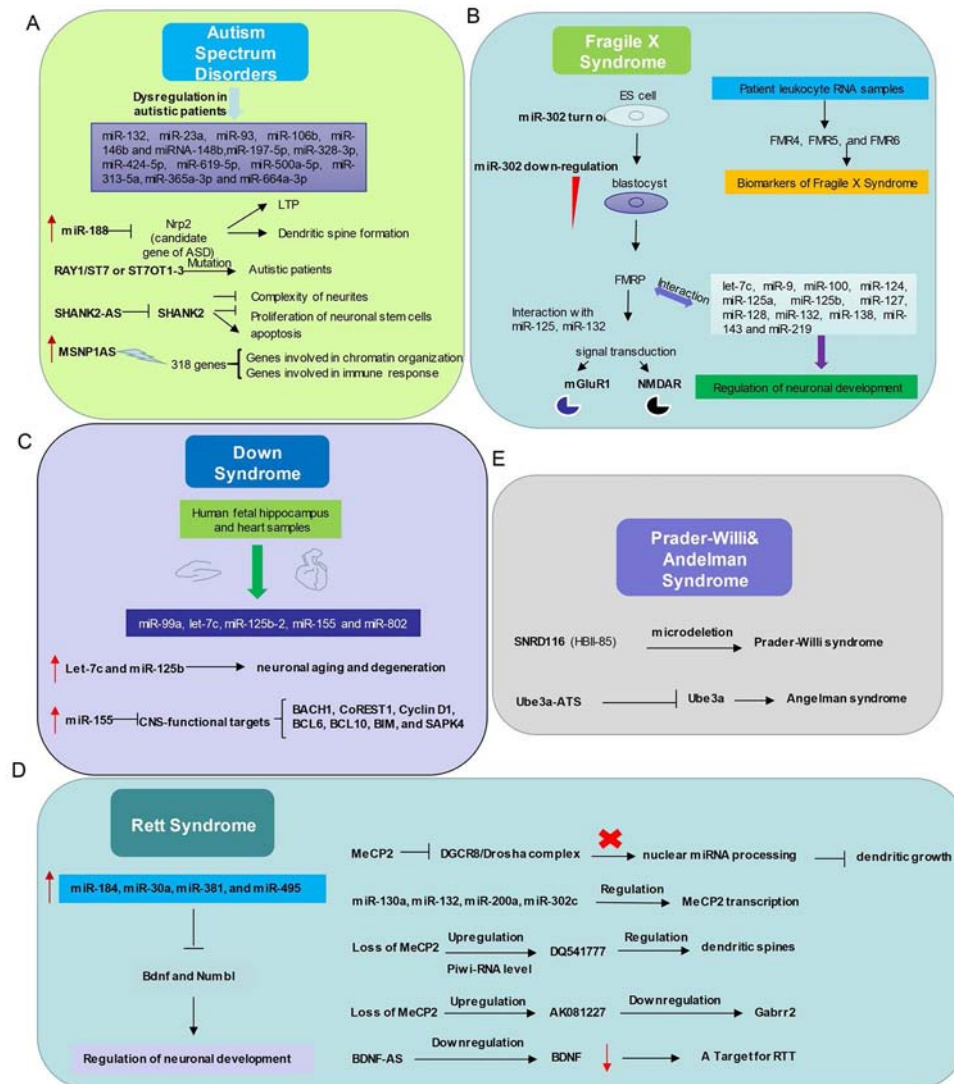


FIGURE 2 | Relationship between non-coding RNAs and neurodevelopmental disorders. **(A)** In Autism Spectrum Disorders, miR-132, miR-23a, miR-93, miR-106b, miR-146b and miRNA-148b, miR-197-5p, miR-328-3p, miR-424-5p, miR-619-5p, miR-500a-5p, miR-313-5a, miR-365a-3p and miR-664a-3p expression levels are abnormal. miR-188, RAY1/ST7 or ST7OT1-3, SHANK2-AS, MSNP1AS participate in ASD with distinct ways. **(B)** The interactions between non-coding RNAs and mechanism diagram of Fragile X Syndrome. microRNAs and long non-coding RNAs regulate Fragile X Syndrome process and might be a class of biomarkers of Fragile X Syndrome. **(C)** Dysregulation of microRNAs related to Down Syndrome in human fetal hippocampus and heart samples. **(D)** Left, the expression level of several microRNAs up-regulates in Rett Syndrome patients and these microRNAs play important roles in modulating neuronal development. Right, relationship between Mecp2 and Non-coding RNAs. **(E)** Microdeletion of SnoRNA SNRD (HBI-85) leads to Prader-Willi syndrome like phenotype. Long non-coding RNA Ube3a-ATS represses Ube3a, which gives rise to Angelman syndrome.

2009; Ladd et al., 2007) (Figure 2). *FMR4* plays a critical role in regulating cell cycle, proliferation, and apoptosis of human neural precursor cells (Khalila 2009).

LncRNAs *FMR5* and *FMR6* have recently been linked to FXS (Pastori et al., 2014) (Figure 2). *FMR5* is a sense lncRNA transcribed upstream of the *FMR1* promoter, while *FMR6* is an antisense transcript overlapping the 3'-UTR of *FMR1*. The expression of *FMR4*, *FMR5*, and *FMR6* is detectable in the majority of patient leukocyte RNA samples, suggesting that it may be reliable biomarkers for FXS (Wahlestedt, 2013).

Down Syndrome

DS is a neurodevelopmental disorder caused by the presence of all or part of a third copy of chromosome 21 (Asim et al., 2015). This disorder has been characterized with many clinical manifestations, including dementia, defects of the immunity system and congenital heart, and abnormalities of facial growth, gastrointestinal tract, and endocrine system (Malinge et al., 2009). Five miRNAs (miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) have been found to be overexpressed in human fetal hippocampus and heart samples from individuals with DS (Fillat

and Altafaj, 2012) (**Figure 2**). Let-7c and miR-125b have been shown to enhance neuronal aging and degeneration (Chawla et al., 2016). Recent evidence suggests that DS dementia strongly correlates with overexpression of miR-155 on chromosome 21 with concomitant reduction of multiple CNS-functional targets, including BACH1, CoREST1, Cyclin D1, BCL6, BCL10, BIM, and SAPK4 (Tili et al., 2018) (**Figure 2**).

Rett Syndrome

RTT is a neurodevelopmental disorder caused by the loss of function of methyl-CpG-binding protein 2 (MeCP2) (Chahrouh and Zoghbi, 2007; Obiols-Guardia and Guil, 2017). Because chromosome Y does not exist MeCP2, the disease occurs almost entirely in women (Weng et al., 2011; Obiols-Guardia and Guil, 2017). MeCP2 protein is highly expressed in neurons, acting as a transcriptional repressor and activator, depending on the context (Luikenhuis et al., 2004). Growing evidence suggests that various non-coding RNAs might play important roles in the development of RTT (Obiols-Guardia and Guil, 2017).

Due to direct or indirect deregulation following MeCP2 loss of function, disrupted miRNA expression has been reported in the disease progress of RTT (Urduingio et al., 2010; Lyu et al., 2016). For instance, miR-184, miR-30a, miR-381, and miR-495 are aberrantly up-regulated in MeCP2 knockout mice (Nomura et al., 2008; Wu et al., 2010) (**Figure 2**). These miRNAs are known for repressing the expression of important modulators of neuronal development, such as *Bdnf* and *Numbl* (Liu et al., 2010; Wu et al., 2010). MeCP2 also interacts with pri-miRNA processing machines and affects their activity. For example, DGCR8/Drosha complex can be inhibited by MeCP2, thus affecting nuclear miRNA processing and dendritic growth (Cheng et al., 2014). Interestingly, miRNAs can also regulate MeCP2 transcription, such as miR-130a (Zhang et al., 2016), miR-132 (Lyu et al., 2016), miR-200a, and miR-302c (Rodrigues et al., 2016) (**Figure 2**). Future studies on the link between MeCP2 and the miRNA population will broaden our knowledge of regulatory network affected in RTT and will help develop better therapeutic strategies.

PiRNA expression levels are altered globally in the absence of MeCP2 (Saxena et al., 2012). There are at least 12 hippocampus-abundant piRNAs up-regulated with a fold change of over 1.5 in the cerebellum of MeCP2 KO mice. Among them, DQ541777, which is implicated in regulating the size of dendritic spines, is the 5th most abundant piRNA in the cerebellum libraries (Lee et al., 2011; Saxena et al., 2012) (**Figure 2**). More specific functions of these dysregulated piRNAs in the pathophysiology of RTT are expected to be discovered in near future.

The aberrant lncRNA transcriptome is also present in the brain of RTT mice. For instance, the AK081227 and AK087060 transcripts are up-regulated in MeCP2-null brains (Petazzi et al., 2013). The overexpression of AK081227 mediated by the *Mecp2* loss is associated with the down-regulation of its host coding protein gene *Gabrr2*, a major inhibitory neurotransmitter in the mammalian brain where it acts at GABA receptors, which are ligand-gated chloride channels (Petazzi et al., 2013). The neurotrophic BDNF is known to be

aberrantly diminished in RTT individuals (Katz et al., 2016), it can be speculated that the lncRNA BDNF-AS might be an important therapeutic target for treating RTT. Although lncRNAs play an important role in neuronal development, their roles in the pathogenesis of RTT is largely unknown (Mo 2015; Lin et al., 2014; Ng et al., 2013).

Prader-Willi Syndrome and Angelman Syndrome

Chromosome 15q11-q13 is a region containing a lot of genomic imprinting genes (Kalsner and Chamberlain, 2015). Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS) are two different types of neurodevelopmental disorders which are caused by loss of function or overexpression of at least one imprinted gene at the 15q11-q13 locus (Kalsner and Chamberlain, 2015). PWS is characterized by intellectual disability, irritability, short stature, and low fertility/hypogonadism (Buiting, 2010). NECDIN and small ribonucleoprotein polypeptide N (SNRPN) are functionally related to the pathological features of the disease (Francoise Mustelli, 2000). SNRPN downstream introns contain SNRD116 (HBII-85) snoRNA clusters, and paternal genetic microdeletion of SNRPN clusters might lead to PWS (Sahoo et al., 2008; de Smith et al., 2009) (**Figure 2**). Actually, mice with deletion of MBII85 snoRNA clusters demonstrated obvious PWS phenotypes (Skryabin et al., 2007), indicating non-coding RNAs can be tightly regulated and may play critical roles in the pathology of PWS.

The pathological features of AS include delayed development, intellectual disability, severe speech impairment, and problems with movement and balance (Buiting, 2010). AS is caused by the deletion and/or mutation of *Ube3a* on the maternal chromosome. While in patients with AS the maternal *Ube3a* allele is inactive, the paternal allele is intact but epigenetically silenced through the *Ube3a*-ATS part of LNCAT (large non-coding antisense transcript) at the *Ube3a* locus (Runte et al., 2001). Elucidating the mechanisms of how *Ube3a*-ATS involves in silencing the paternal *Ube3a* may lead to new therapies for AS (Landers et al., 2005; Meng et al., 2012) (**Figure 2**).

Conclusions

Non-coding RNAs have emerged as important regulators in the brain development and function. Although the number and functional subclasses of non-coding RNAs has steadily increased, it still likely represents only a small fraction of the total RNA transcriptome underlying the ontogeny and functional complexity of mammalian brain functions in health and disease. Aberrant expression of non-coding RNAs has linked with various neurodevelopmental diseases. The regulatory network of non-coding RNAs in neurodevelopmental disorders is very complicated, and the molecular mechanisms of non-coding RNA causing neurodevelopmental diseases are still largely unknown. However, we still hope that this review raises awareness of the central roles that large non-coding RNAs and their complex regulatory networks in brain development and function.

With more defined molecular function and mechanism, Non-coding RNAs have the great potential to serve as disease

biomarkers or drug targets, especially in neurodevelopmental disorders. For example, miR-132, miR-23a, miR-93, miR-106b, miR-146b and miRNA-148b are abnormally expressed in autistic patients (Mahesh Mundalil Vasu1, 2014; Trindade et al., 2013). miR-99a, let-7c, miR-125b-2, miR-155 and miR-802 are found to be overexpressed in human samples from individuals with Down Syndrome (Fillat and Altafaj, 2012). In fragile X syndrome, the expression of FMR4, FMR5, and FMR6 is detectable in the majority of patient leukocyte RNA samples, suggesting that it may be reliable biomarkers for Fragile X Syndrome (Wahlestedt, 2013). The analysis and integration of this information with other datasets will get new clues of non-coding RNAs as reliable biomarkers.

A large number of studies have suggested that non-coding RNAs might be new promising targets for the treatment of neurodevelopmental disorders. However, several challenges remain to be investigated before non-coding RNAs can be routinely used in outbreak investigation and clinical practice. Firstly, more efficient non-coding RNAs delivery systems are needed to develop. Secondly, the biology of non-coding RNAs, such as their structural motifs, stability, degradation, and gene regulatory network, needs further investigations. Finally, preclinical research and clinical trials are required to determine the safe dose and therapeutic potentials of non-coding RNAs.

REFERENCES

- Abu-Elneel, K., Liu, T., Gazzaniga, F. S., Nishimura, Y., Wall, D. P., Geschwind, D. H., et al. (2008). *Heterogeneous dysregulation of microRNAs across the autism spectrum*. *Neurogenetics* 9 (3), 153–161. doi: 10.1007/s10048-008-0133-5
- Alexander, R. P., Fang, G., Rozowsky, J., Snyder, M., and Gerstein, M. B. (2010). Annotating non-coding regions of the genome. *Nat. Rev. Genet.* 11, 559–571. doi: 10.1038/nrg2814
- Asim, A., Kumar, A., Muthuswamy, S., Jain, S., and Agarwal, S. (2015). Down syndrome: an insight of the disease. *J. Biomed. Sci.* 22, 41. doi: 10.1186/s12929-015-0138-y
- Bailey, A., LeCouteur, A., Gottesman, I., Bolton, P., Simonoff, E., et al. (1995). *Autism as a strongly genetic disorder: evidence from a British twin study*. *Psychol. Med.* 25 (1), 63–77. doi: 10.1017/S0033291700028099
- Balachandar, V., Dhivya, V., Gomathi, M., Mohanadevi, S., Venkatesh, B., and Geetha, B. (2016). A review of Rett syndrome (RTT) with induced pluripotent stem cells. *Stem Cell Invest.* 3, 52. doi: 10.21037/sci.2016.09.05
- Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., et al. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128, 1089–1103. doi: 10.1016/j.cell.2007.01.043
- Brug, M. P., and Wahlestedt, C. (2012). Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat. Biotechnol.* 30, 453–459. doi: 10.1038/nbt.2158
- Buiting, K. (2010). Prader-Willi syndrome and Angelman syndrome. *Am. J. Med. Genet. Part. C Semin. Med. Genet.* 154C, 365–376. doi: 10.1002/ajmg.c.30273
- Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda, N., et al. (2005). The transcriptional landscape of the mammalian genome. *Science* 309, 1559–1563. doi: 10.1126/science.1112014
- Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., et al. (2011). A Long Noncoding RNA Controls Muscle Differentiation by Functioning as a Competing Endogenous RNA. *Cell* 147, 947. doi: 10.1016/j.cell.2011.09.028
- Chahrouh, M., and Zoghbi, H. Y. (2007). The story of Rett syndrome: from clinic to neurobiology. *Neuron* 56, 422–437. doi: 10.1016/j.neuron.2007.10.001
- Chawla, G., Deosthale, P., Childress, S., Wu, Y. C., and Sokol, N. S. (2016). A let-7-to-miR-125 MicroRNA Switch Regulates Neuronal Integrity and Lifespan in *Drosophila*. *PLoS Genet.* 12, e1006247. doi: 10.1371/journal.pgen.1006247
- Cheng, T. L., Wang, Z., Liao, Q., Zhu, Y., Zhou, W. H., Xu, W., et al. (2014). MeCP2 suppresses nuclear microRNA processing and dendritic growth by regulating the DGCR8/Drosha complex. *Dev. Cell* 28, 547–560. doi: 10.1016/j.devcel.2014.01.032
- Chu, C., Zhang, Q. C., da Rocha, S. T., Flynn, R. A., Bharadwaj, M., Calabrese, J. M., et al. (2015). Systematic discovery of Xist RNA binding proteins. *Cell* 161, 404–416. doi: 10.1016/j.cell.2015.03.025
- Constantin, L. (2017). The Role of MicroRNAs in Cerebellar Development and Autism Spectrum Disorder During Embryogenesis. *Mol. Neurobiol.* 54, 6944–6959. doi: 10.1007/s12035-016-0220-9
- David, P., and Bartel, (2004). MicroRNAs. *Genomics Rev. Biogenesis Mech. Funct.* 116, 281–297. doi: 10.1016/s0092-8674(04)00045-5
- de Smith, A. J., Purmann, C., Walters, R. G., Ellis, R. J., Holder, S. E., Van Haelst, M. M., et al. (2009). A deletion of the HBII-85 class of small nucleolar RNAs (snoRNAs) is associated with hyperphagia, obesity and hypogonadism. *Hum. Mol. Genet.* 18, 3257–3265. doi: 10.1093/hmg/ddp263
- DeWitt, J. J., Hecht, P. M., Grepo, N., Wilkinson, B., Evgrafov, O. V., Morris, K. V., et al. (2016). Transcriptional Gene Silencing of the Autism-Associated Long Noncoding RNA MSNP1AS in Human Neural Progenitor Cells. *Dev. Neurosci.* 38, 375–383. doi: 10.1159/000453258
- Ding, D., Liu, J., Dong, K., Midic, U., Hess, R. A., Xie, H., et al. (2017). PNLD1 is essential for piRNA 3' end trimming and transposon silencing during spermatogenesis in mice. *Nat. Commun.* 8, 819. doi: 10.1038/s41467-017-00854-4
- Ding, F., Li, H. H., Zhang, S., Solomon, N. M., Camper, S. A., Cohen, P., et al. (2008). SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice. *PLoS One* 3, e1709. doi: 10.1371/journal.pone.0001709
- Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., et al. (2012). Landscape of transcription in human cells. *Nature* 489, 101–108. doi: 10.1038/nature11233
- Dominique Heitz, D. D., Imbert, G., Kretz, C., and Mandel, J.-L., (1992). *Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant of the transition to full mutation*. *J. Med. Genet.* 29 (11), 794–801. doi: 10.1136/jmg.29.11.794

AUTHOR CONTRIBUTIONS

C-ML directed the manuscript preparation. S-FZ, JG, and C-ML wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the National Key Research and Development Program of China Project (Grant No. 2016YFA0101402), the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDA16010302), the National Science Foundation of China (No. 91753140, 81771224, 31571043).

- Edward, R., Ritvo, M. D., Anne Spence, M., Ph.D., B. J., Freeman, Mason-Brothers, Anne, et al. (1985). Evidence for Autosomal Recessive Inheritance in 46 Families With Multiple Incidences of Autism. *Am. J. Psychiatry* 142 (2), 187–92.
- Esteller, M. (2011). Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12, 861–874. doi: 10.1038/nrg3074
- Fabio Mohn, D. H., and Brennecke, J., (2015). Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. *Science* 348 (6236), 812–817. doi: 10.1126/science.aaa1039
- Fillat, C., and Altafaj, X. (2012). Gene therapy for Down syndrome. *Prog. Brain Res.* 197, 237–247. doi: 10.1016/B978-0-444-54299-1.00012-1
- Fu, Y. H., Kuhl, D. P., Pizzutti, A., Pieretti, M., Sutcliffe, J. S., Richards, S., et al. (1991) Variation of the CGG Repeat at the Fragile X Site Results in Genetic Instability_ Resolution of the Sherman Paradox. *Prog. Brain Res.* 197, 237–47. doi: 10.1016/B978-0-444-54299-1.00012-1
- Gong, C., and Maquat, L. E. (2011). lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. *Nature* 470, 284–288. doi: 10.1038/nature09701
- Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., et al. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. *Science* 315, 1587–1590. doi: 10.1126/science.1140494
- Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993
- Hecht, M., Tabib, A., Kahan, T., Orlanski, S., Gropp, M., Tabach, Y., et al. (2017). Epigenetic mechanism of FMR1 inactivation in Fragile X syndrome. *Int. J. Dev. Biol.* 61, 285–292. doi: 10.1387/ijdb.170022hc
- Horwich, M. D., Li, C., Matrangola, C., Vagin, V., Farley, G., Wang, P., et al. (2007). The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr. Biol.* 17, 1265–1272. doi: 10.1016/j.cub.2007.06.030
- Hu, S., Wang, X., and Shan, G. (2016). Insertion of an Alu element in a lncRNA leads to primate-specific modulation of alternative splicing. *Nat. Struct. Mol. Biol.* 23, 1011–1019. doi: 10.1038/nsmb.3302
- Ipsaro, J. J., Haase, A. D., Knott, S. R., Joshua-Tor, L., and Hannon, G. J. (2012). The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. *Nature* 491, 279–283. doi: 10.1038/nature11502
- Jingwei Ni, A. L. T., Maurille, J., and Fournier. (1997). Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* 89 (4), 565–73.
- Kalsner, L., and Chamberlain, S. J. (2015). Prader-Willi, Angelman, and 15q11-q13 Duplication Syndromes. *Pediatr. Clinics North Am.* 62, 587–606. doi: 10.1016/j.pcl.2015.03.004
- Kang, S. (2014). Research round-up. *Lancet Psychiatry* 1, 14. doi: 10.1016/S2215-0366(14)70266-4
- Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Duttagupta, R., Willingham, A. T., et al., (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* 316, 1484–1488. doi: 10.1126/science.1138341
- Katz, D. M., Bird, A., Coenraads, M., Gray, S. J., Menon, D. U., Philpot, B. D., et al. (2016). Rett Syndrome: Crossing the Threshold to Clinical Translation. *Trends Neurosci.* 39, 100–113. doi: 10.1016/j.tins.2015.12.008
- Kawaoka, S., Izumi, N., Katsuma, S., and Tomari, Y. (2011). 3' end formation of PIWI-interacting RNAs in vitro. *Mol. Cell* 43, 1015–1022. doi: 10.1016/j.molcel.2011.07.029
- Kerin, T., Ramanathan, A., Rivas, K., Grepo, N., Coetzee, G. A., and Campbell, D. B. (2012). A noncoding RNA antisense to moesin at 5p14.1 in autism. *Sci. Transl. Med.* 4, 128ra140. doi: 10.1126/scitranslmed.3003479
- Khalila, A. M. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc. Natl. Acad. Sci. U. S. A.* 106 (28), 11667–72.
- Kichukova, T. M., Popov, N. T., Ivanov, I. S., and Vachev, T. I. (2017). Profiling of Circulating Serum MicroRNAs in Children with Autism Spectrum Disorder using Stem-loop qRT-PCR Assay. *Folia Med.* 59 (1), 43–52. doi: 10.1515/folmed-2017-0009
- Kiss-László, Z., Henry, Y., Bachelier, J. P., Caizergues-Ferrer, M., and Kiss, T., (1996). "Site-Specific Ribose Methylation of Preribosomal RNA," A Novel Function for Small Nucleolar RNAs. *Cell* 85 (7), 1077–1088. doi: 10.1016/S0092-8674(00)81308-2
- Klei, L., Sander, S. J., Murtha, M. T., Hus, V., Lowe, J. K., Willsey, A. J., et al., (2012). Common genetic variants, acting additively, are a major source of risk for autism. *Folia Med. (Plovdiv)* 59 (1), 43–52. doi: 10.1515/folmed-2017-0009
- Ladd, P. D., Smith, L. E., Rabaia, N. A., Moore, J. M., Georges, S. A., Hansen, R. S., et al. (2007). An antisense transcript spanning the CGG repeat region of FMR1 is upregulated in premutation carriers but silenced in full mutation individuals. *Hum. Mol. Genet.* 16, 3174–3187. doi: 10.1093/hmg/ddm293
- Lai, F., Orom, U. A., Cesaroni, M., Beringer, M., Taatjes, D. J., Blobel, G. A., et al. (2013). Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature* 494, 497–501. doi: 10.1038/nature11884
- Landers, M., Calciano, M. A., Colosi, D., Glatt-Deeley, H., Wagstaff, J., and Lalande, M. (2005). Maternal disruption of Ube3a leads to increased expression of Ube3a-ATS in trans. *Nucleic Acids Res.* 33, 3976–3984. doi: 10.1093/nar/gki705
- Lee, E. J., Banerjee, S., Zhou, H., Jammalamadaka, A., Arcila, M., Manjunath, B. S., et al. (2011). Identification of piRNAs in the central nervous system. *RNA* 17, 1090–1099. doi: 10.1261/rna.2565011
- Lee, K., Kim, J. H., Kwon, O. B., An, K., Ryu, J., Cho, K., et al. (2012). An activity-regulated microRNA, miR-188, controls dendritic plasticity and synaptic transmission by downregulating neuropilin-2. *J. Neurosci.* 32, 5678–5687. doi: 10.1523/JNEUROSCI.6471-11.2012
- Lekka, E., and Hall, J. (2018). Noncoding RNAs in disease. *FEBS Lett.* 592, 2884–2900. doi: 10.1002/1873-3468.13182
- Li, X. Z., Roy, C. K., Dong, X., Bolcun-Filas, E., Wang, J., Han, B. W., et al., (2013). An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes. *Mol. Cell* 50, 67–81. doi: 10.1016/j.molcel.2013.02.016
- Liang Chen, C. H. (2015). Circular RNAs in Eukaryotic Cells. *Curr. Genomics* 16, 312–318. doi: 10.2174/1389202916666150707161554
- Lim, S. L., Qu, Z. P., Kortschak, R. D., Lawrence, D. M., Geoghegan, J., Hempfling, A. L., et al., (2015). HENMT1 and piRNA Stability Are Required for Adult Male Germ Cell Transposon Repression and to Define the Spermatogenic Program in the Mouse. *PLoS Genet.* 11, e1005620. doi: 10.1371/journal.pgen.1005620
- Lin, N., Chang, K. Y., Li, Z., Gates, K., Rana, Z. A., Dang, J., et al., (2014). An evolutionarily conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. *Mol. Cell* 53, 1005–1019. doi: 10.1016/j.molcel.2014.01.021
- Lin, S. L. (2015). microRNAs and Fragile X Syndrome. *Adv. Exp. Med. Biol.* 888, 107–121. doi: 10.1007/978-3-319-22671-2_7
- Liu, C., Teng, Z. Q., Santistevan, N. J., Szulwach, K. E., Guo, W., Jin, P., et al. (2010). Epigenetic regulation of miR-184 by MBD1 governs neural stem cell proliferation and differentiation. *Cell Stem Cell* 6, 433–444. doi: 10.1016/j.stem.2010.02.017
- Luikenhuis, S., Giacometti, E., Beard, C. F., and Jaenisch, R. (2004). Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice. *Proc. Natl. Acad. Sci. U.S.A.* 101, 6033–6038. doi: 10.1073/pnas.0401626101
- Luo, T., Liu, P., Wang, X. Y., Li, L. Z., Zhao, L. P., Huang, J., et al. (2018). Effect of the autism-associated lncRNA Shank2-AS on architecture and growth of neurons. *J. Cell. Biochem.* 1–9. doi: 10.1002/jcb.27471
- Lyu, J. W., Yuan, B., Cheng, T. L., Qiu, Z. L., and Zhou, W. H. (2016). Reciprocal regulation of autism-related genes MeCP2 and PTEN via microRNAs. *Sci. Rep.* 6, 20392. doi: 10.1038/srep20392
- Malinge, S., Izraeli, S., and Crispino, J. D. (2009). Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome. *Blood* 113, 2619–2628. doi: 10.1182/blood-2008-11-163501
- Mattick, J. S. (2011). Long noncoding RNAs in cell and developmental biology. *Semin. Cell Dev. Biol.* 22, 327. doi: 10.1016/j.semcdb.2011.05.002
- Mehler, M. F., and Mattick, J. S. (2006). Non-coding RNAs in the nervous system. *J. Physiol.* 575, 333–341. doi: 10.1113/jphysiol.2006.113191
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al., (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Meng, L., Person, R. E., and Beaudet, A. L. (2012). Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. *Hum. Mol. Genet.* 21, 3001–3012. doi: 10.1093/hmg/dds130
- Mo, C. F. (2015). Loss of non-coding RNA expression from the DLK1-DIO3 imprinted locus correlates with reduced neural differentiation potential in human embryonic stem cell lines. *Stem Cell Res. Ther.* 6, 1. doi: 10.1186/scrt535

- Modarresi, F., Faghihi, M. A., Lopez-Toledano, M. A., Fatemi, R. P., Magistri, M., Brothers, S. P., et al. (2012) Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation *Nat Biotechnol.* 30, 453–459.
- Mundalil Vasu, M., Anitha, A., Thanseem, I., Suzuki, K., Yamada, K., Takahashi, T., et al. (2014). *Serum microRNA profiles in children with autism. Mol. Autism* 5, 40.
- Muscattelli, F., Abrous, D. N., Massacrier, A., Boccaccio, I., Le Moal, M., Cau, P., et al. (2000). *Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human prader-Willi syndrome. Hum. Mol. Genet.* 9 (20), 3101–3110. doi: 10.1093/hmg/9.20.3101
- Ng, S. Y., Bogu, G. K., Soh, B. S., and Stanton, L. W. (2013). The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. *Mol. Cell* 51, 349–359. doi: 10.1016/j.molcel.2013.07.017
- Nishimasu, H., Ishizu, H., Saito, K., Fukuhara, S., Kamatani, M. K., Bonnefond, L., et al. (2012). Structure and function of Zucchini endoribonuclease in piRNA biogenesis. *Nature* 491, 284–287. doi: 10.1038/nature11509
- Nomura, T., Kimura, M., Horii, T., Morita, S., Soejima, H., Kudo, S., et al. (2008). MeCP2-dependent repression of an imprinted miR-184 released by depolarization. *Hum. Mol. Genet.* 17, 1192–1199. doi: 10.1093/hmg/ddn011
- Obiols-Guardia, A., and Guil, S. (2017). The Role of Noncoding RNAs in Neurodevelopmental Disorders: The Case of Rett Syndrome. *Adv. Exp. Med. Biol.* 978, 23–37. doi: 10.1007/978-3-319-53889-1_2
- Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., et al. (2002). *Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature* 420 (6915), 563–73.
- Orom, U. A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., et al. (2010). Long noncoding RNAs with enhancer-like function in human cells. *Cell* 143, 46–58. doi: 10.1016/j.cell.2010.09.001
- Pastori, C., Peschansky, V. J., Barbouth, D., Mehta, A., Silva, J. P., and Wahlestedt, C. (2014). Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. *Hum. Genet.* 133, 59–67. doi: 10.1007/s00439-013-1356-6
- Petazzi, P., Sandoval, J., Szczesna, K., Jorge, O. C., Roa, L., Sayols, S., et al. (2013). Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model. *RNA Biol.* 10, 1197–1203. doi: 10.4161/rna.24286
- Piwecka, M., Glazar, P., Hernandez-Miranda, L. R., Memczak, S., Wolf, S. A., Rybak-Wolf, A., et al. (2017). Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science* 357, 1460–1463. doi: 10.1126/science.aam8526
- Rajman, M., and Schratt, G. (2017). MicroRNAs in neural development: from master regulators to fine-tuners. *Development* 144, 2310–2322. doi: 10.1242/dev.144337
- Reddy, K. S. (2005). Cytogenetic abnormalities and fragile-X syndrome in Autism Spectrum Disorder. *BMC Med. Genet.* 6, 3. doi: 10.1186/1471-2350-6-3
- Rennert, M.N.Z.O.M. (2013). *Aberrant Expression of Long Noncoding RNAs in Autistic Brain. J. Mol. Neurosci.* 49 (3), 589–93. doi: 10.1007/s12031-012-9880-8
- Risch, N., Spiker, D., Lotspeich, L., Nouri, N., Hinds, D., Hallmayer, J., et al. (1999). A genomic screen of autism: evidence for a multilocus etiology. *Am. J. Hum. Genet.* 65, 493–507. doi: 10.1086/302497
- Rodrigues, D. C., Kim, D. S., Yang, G., Zaslavsky, K., Ha, K. C., Mok, R. S., et al. (2016). MECP2 Is Post-transcriptionally Regulated during Human Neurodevelopment by Combinatorial Action of RNA-Binding Proteins and miRNAs. *Cell Rep.* 17, 720–734. doi: 10.1016/j.celrep.2016.09.049
- Runte, M., Huttenhofer, A., Gross, S., Kieffmann, M., Horsthemke, B., and Buiting, K. (2001). The IC-SNURF-SNRPN transcript serves as a host for multiple small nuclear RNA species and as an antisense RNA for UBE3A. *Hum. Mol. Genet.* 10, 2687–2700. doi: 10.1093/hmg/10.23.2687
- Sahoo, T., del Gaudio, D., German, J. R., Shinawi, M., Peters, S. U., Person, R. E., et al. (2008). Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster. *Nat. Genet.* 40, 719–721. doi: 10.1038/ng.158
- Saxena, A., Tang, D., and Carninci, P. (2012). piRNAs warrant investigation in Rett Syndrome: an omics perspective. *Dis. Markers* 33, 261–275. doi: 10.1155/2012/396737
- Schirle, N. T., Sheu-Gruttadauria, J., and MacRae, I. J. (2014). Structural basis for microRNA targeting. *Science* 346, 608–613. doi: 10.1126/science.1258040
- Skryabin, B. V., Gubar, L. V., Seeger, B., Pfeiffer, J., Handel, S., Robeck, T., et al. (2007). Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation. *PLoS Genet.* 3, e235. doi: 10.1371/journal.pgen.0030235
- Taft, R. J., Pang, K. C., Mercer, T. R., Dinger, M., and Mattick, J. S. (2010). Non-coding RNAs: regulators of disease. *J. Pathol.* 220 (2), 126–39. doi: 10.1002/path.2638
- Talebizadeh, Z., Butler, M. G., and Theodoro, M. F. (2008). Feasibility and relevance of examining lymphoblastoid cell lines to study role of microRNAs in autism. *Autism Res. Official J. Int. Soc. Autism Res.* 1, 240–250. doi: 10.1002/aur.33
- Tang, J., Yu, Y., and Yang, W. (2017). Long noncoding RNA and its contribution to autism spectrum disorders. *CNS Neurosci. Ther.* 23, 645–656. doi: 10.1111/cns.12710
- Tang, W., Tu, S., Lee, H. C., Weng, Z., and Mello, C. C. (2016). The RNase PARN-1 Trims piRNA 3' Ends to Promote Transcriptome Surveillance in *C. elegans*. *Cell* 164, 974–984. doi: 10.1016/j.cell.2016.02.008
- Tewarit Sarachana, R. Z. (2010). *Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. Genome Med.* 2 (4), 23. doi: 10.1186/gm144
- Tili, E., Mezache, L., Michaille, J. J., Amann, V., Williams, J., Vandiver, P., et al. (2018). microRNA 155 up regulation in the CNS is strongly correlated to Down's syndrome dementia. *Ann. Diagn. Pathol.* 34, 103–109. doi: 10.1016/j.anndiagpath.2018.03.006
- Trindade, A. J., Medvetz, D. A., Neuman, N. A., Myachina, F., Yu, J., Priolo, C., et al. (2013). MicroRNA-21 is induced by rapamycin in a model of tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM). *PLoS One* 8, e60014. doi: 10.1371/journal.pone.0060014
- Tripathi, V., Ellis, J. D., Shen, Z., Song, D. Y., Pan, Q., Watt, A. T., et al. (2010). The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol. Cell* 39, 925–938. doi: 10.1016/j.molcel.2010.08.011
- Urdinguio, R. G., Fernandez, A. F., Lopez-Nieva, P., Rossi, S., Huertas, D., Kulis, M., et al. (2010). Disrupted microRNA expression caused by Mecp2 loss in a mouse model of Rett syndrome. *Epigenetics* 5, 656–663. doi: 10.4161/epi.5.7.13055
- van de, V., Gordebeke, P. M., Khoshab, N., Tiesinga, P. H., Buitelaar, J. K., Kozicz, T., et al. (2013). Long non-coding RNAs in neurodevelopmental disorders. *Front. Mol. Neurosci.* 6, 53. doi: 10.3389/fnmol.2013.00053
- Vaucheret, H. (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev.* 20, 759–771. doi: 10.1101/gad.1410506
- Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65, 905–914. doi: 10.1016/0092-8674(91)90397-H
- Vincent, J. B., Petek, E., Thevarkunnel, S., Kolozsvari, D., Cheung, J., Patel, M., et al. (2002). The RAY1/ST7 Tumor-Suppressor Locus on Chromosome 7q31 Represents a Complex Multi-transcript System. *Genomics* 80, 283–294. doi: 10.1006/geno.2002.6835
- Wahlestedt, C.P.V.J.P.D.B.A.M.J.P.S.C. (2013). Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. *Hum. Genet.* (1), 59–67. doi: 10.1007/s00439-013-1356-6
- Wang, W., Yoshikawa, M., Han, B. W., Izumi, N., Tomari, Y., Weng, Z., et al. (2014). The initial uridine of primary piRNAs does not create the tenth adenine that is the hallmark of secondary piRNAs. *Mol. Cell* 56, 708–716. doi: 10.1016/j.molcel.2014.10.016
- Wang, Y., Zhao, X., Ju, W., Flory, M., Zhong, J., Jiang, S., et al. (2015). Genome-wide differential expression of synaptic long noncoding RNAs in autism spectrum disorder. *Trans. Psychiatry* 5, e660. doi: 10.1038/tp.2015.144
- Weng, S. M., Bailey, M. E., and Cobb, S. R. (2011). Rett syndrome: from bed to bench. *Pediatr Neonatol* 52, 309–316. doi: 10.1016/j.pedneo.2011.08.002
- Wilhelm, M., Schlegl, J., Hahne, H., Gholami, A. M., Lieberenz, M., Savitski, M. M., et al. (2014). Mass-spectrometry-based draft of the human proteome. *Nature* 509, 582–587. doi: 10.1038/nature13319
- Wu, H., Tao, J., Chen, P. J., Shahab, A., Ge, W., Hart, R. P., et al. (2010). Genome-wide analysis reveals methyl-CpG-binding protein 2-dependent regulation of

- microRNAs in a mouse model of Rett syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18161–18166. doi: 10.1073/pnas.1005595107
- Xing, Y. H., and Chen, L. L. (2018). Processing and roles of snoRNA-ended long noncoding RNAs. *Crit. Rev. Biochem. Mol. Biol.*, 1–11. doi: 10.1080/10409238.2018.1508411
- You, X., Vlatkovic, I., Babic, A., Will, T., Epstein, I., Tushev, G., et al., (2015). Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat. Neurosci.* 18, 603–610. doi: 10.1038/nn.3975
- Zamore, P. G., Colpan, I., and Cansu Cecchini, K., (2018). “A Single Mechanism of Biogenesis,” in *Initiated and Directed by PIWI Proteins, Explains piRNA Production in Most Animals*. *Mol. Cell* 71 (5), 775–790.e5. doi: 10.1016/j.molcel.2018.08.007
- Zhang, Y., Chen, M., Qiu, Z., Hu, K., McGee, W., Chen, X., et al. (2016). MiR-130a regulates neurite outgrowth and dendritic spine density by targeting MeCP2. *Protein Cell* 7, 489–500. doi: 10.1007/s13238-016-0272-7
- Zhou, S., Ding, F., and Gu, X. (2016). Non-coding RNAs as Emerging Regulators of Neural Injury Responses and Regeneration. *Neurosci. Bull.* 32, 253–264. doi: 10.1007/s12264-016-0028-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aberrant Expression of Pseudogene-Derived lncRNAs as an Alternative Mechanism of Cancer Gene Regulation in Lung Adenocarcinoma

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OPEN ACCESS

Edited by:

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Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 16 November 2018

Accepted: 11 February 2019

Published: 06 March 2019

Citation:

Stewart GL, Enfield KSS, Sage AP, Martinez VD, Minatel BC, Pewarchuk ME, Marshall EA and Lam WL (2019) Aberrant Expression of Pseudogene-Derived lncRNAs as an Alternative Mechanism of Cancer Gene Regulation in Lung Adenocarcinoma. *Front. Genet.* 10:138. doi: 10.3389/fgene.2019.00138

Transcriptome sequencing has led to the widespread identification of long non-coding RNAs (lncRNAs). Subsequently, these genes have been shown to hold functional importance in human cellular biology, which can be exploited by tumors to drive the hallmarks of cancer. Due to the complex tertiary structure and unknown binding motifs of lncRNAs, there is a growing disparity between the number of lncRNAs identified and those that have been functionally characterized. As such, lncRNAs deregulated in cancer may represent critical components of cancer pathways that could serve as novel therapeutic intervention points. Pseudogenes are non-coding DNA sequences that are defunct relatives of their protein-coding parent genes but retain high sequence similarity. Interestingly, certain lncRNAs expressed from pseudogene loci have been shown to regulate the protein-coding parent genes of these pseudogenes in *trans* particularly because of this sequence complementarity. We hypothesize that this phenomenon occurs more broadly than previously realized, and that aberrant expression of lncRNAs overlapping pseudogene loci provides an alternative mechanism of cancer gene deregulation. Using RNA-sequencing data from two cohorts of lung adenocarcinoma, each paired with patient-matched non-malignant lung samples, we discovered 104 deregulated pseudogene-derived lncRNAs. Remarkably, many of these deregulated lncRNAs (i) were expressed from the loci of pseudogenes related to known cancer genes, (ii) had expression that significantly correlated with protein-coding parent gene expression, and (iii) had lncRNA protein-coding parent gene expression that was significantly associated with survival. Here, we uncover evidence to suggest the lncRNA-pseudogene-protein-coding gene axis as a prominent mechanism of cancer gene regulation in lung adenocarcinoma, and highlights the clinical utility of exploring the non-coding regions of the cancer transcriptome.

Keywords: long non-coding RNAs, pseudogenes, lung cancer, gene regulation, non-coding RNAs

INTRODUCTION

Lung cancer is an enormous health burden, representing the most common cause of cancer death worldwide. While advances in imaging technology have improved early detection, the outcome for lung cancer patients remains poor. This is largely attributed to limited therapeutic options and short-term response due to tumor heterogeneity. Despite extensive sequencing efforts to characterize exomes and protein-coding transcriptomes of lung tumors, less than half of lung adenocarcinoma (LUAD) patients harbor clinically targetable mutated driver genes, highlighting the need to explore alternative mechanisms of cancer gene deregulation (Chan and Hughes, 2015).

Long non-coding RNAs (lncRNAs, > 200 nt) have emerged as important players in cell biology. lncRNAs have been observed to function through a wide variety of regulatory mechanisms, targeting DNA, proteins, and other RNA species; specifically with defined roles in RNA degradation or stabilization, protein translocation and complex formation, and recruitment of complexes to transcriptional loci (Lee et al., 1999; Wang et al., 2011; Kung et al., 2013; Simon et al., 2013). This broad functional repertoire has been shown to be exploited by many cancer types, including LUAD, to drive various hallmarks of cancer (Bhan et al., 2017; Peng et al., 2017). One of the first cancer-associated lncRNA transcripts, *MALAT1* (Metastasis Associated Lung Adenocarcinoma Transcript 1) was discovered in lung cancer. *MALAT1* is overexpressed in metastatic lung tumors and functions *in trans* through transcriptional regulation of multiple genes involved in cell motility (Tano et al., 2010; Gutschner et al., 2013). Since the discovery of *MALAT1*, many other lncRNAs have been shown to play a direct role in nearly every major cancer type. Tumor suppressive lncRNA transcripts are commonly observed to be downregulated, for instance the lncRNA *TARID*, which promotes the expression of the tumor suppressor gene *TCF21* through active promoter demethylation (Arab et al., 2014). Conversely oncogenic lncRNAs can be overexpressed, such as *NEAT1*, an architectural lncRNA associated with metastasis in many cancer types (Hirose et al., 2014; Blume et al., 2015; Yamazaki et al., 2018; Zhang et al., 2018). As such, lncRNAs may represent critical regulators of oncogenic-driver pathways that could serve as undiscovered clinical intervention points in LUAD.

While lncRNAs have been observed to be important in cancer biology, functional prediction of newly-discovered lncRNAs remains a major challenge. However, many lncRNAs expressed from pseudogene loci have been shown to regulate the specific genes with which they have sequence homology. Pseudogenes are DNA sequences that are defunct relatives of functional protein-coding genes (herein referred to as parent genes) and arise during either gene duplication events, or the reverse transcription of an mRNA transcript into a new genomic location. Through evolution these duplicated genes have acquired mutations such as premature stop codons and frameshifts, which results in the loss of protein coding ability, while still retaining a high degree of sequence homology with the original parent gene (Khachane and Harrison, 2009). Recently, pseudogene-derived lncRNAs have been shown to regulate their parent genes and this novel mechanism has been observed in many tumor types, including

lung cancer (Sun et al., 2017; Hu et al., 2018). A prominent example is the tumor suppressor gene *PTEN* (chromosome 10), regulated both positively by *PTENP1* (chromosome 9), a lncRNA transcribed from the sense strand of the pseudogene locus, and negatively by the lncRNA *PTENP1-AS1*, which is transcribed from the strand antisense to the parent gene (Johnsson et al., 2013).

Pseudogenes have been continually omitted from large RNA-sequencing datasets due to the complexity of separating highly similar pseudogene sequences from parent genes. However, Milligan et al. (2016) recently generated an atlas of lncRNAs overlapping pseudogenes, which has provided a foundation for their analysis in RNA sequencing datasets. We hypothesize that the functions of pseudogene-derived lncRNAs are an under-explored mechanism of gene regulation that occurs more broadly than previously realized, and that these events contribute to the tumorigenesis of LUAD. We performed next generation RNA-sequencing on microdissected LUAD tumors and matched non-malignant tissue to identify deregulated lncRNAs expressed from pseudogene loci (herein referred to as Ψ -lncs). We then explored the relationships of these Ψ -lncs with their parent genes, and explored their significance in relation to patient clinical features in our discovery dataset as well as a validation dataset.

RESULTS

Ψ -lnc Expression Is Deregulated in Lung Adenocarcinoma

Pseudogenes vary widely in terms of length, gene fraction, and identity to parent genes, and can be expressed as lncRNAs that are sense, antisense, partial overlapping, or internal to the parent. In light of this variation, Ψ -lncs are observed to have vastly different regulatory effects on downstream target genes (Figure 1A). In our curation of Ψ -lncs in LUAD, we have included those that have exonic overlap with a pseudogene (partial or full length) and considered both sense and antisense transcripts (Supplementary Table S1). Ψ -lncs were analyzed in an in-house discovery (BCCA, $n = 72$) and external validation (TCGA, $n = 108$) cohort of LUAD and paired non-malignant lung tissues (Table 1). We identified aberrantly expressed Ψ -lncs that are significantly deregulated in both the discovery and validation datasets with the same direction of expression alteration (Ψ -lncs upregulated or downregulated in tumors compared to matched non-malignant tissue).

We found 104 lncRNAs expressed from 102 pseudogene loci to be significantly deregulated in LUAD (Supplementary Table S2). To our surprise, we found that the majority of these deregulated Ψ -lncs were downregulated in tumors (Figures 2A,B). Most of these were unannotated lncRNAs, such as *RP11-1007O24.3*, which was downregulated in tumors, with only 24 of the total deregulated Ψ -lncs having been previously described in scientific literature annotated in PubMed, albeit none in the field of pseudogene-mediated deregulation (Supplementary Table S3 and Figure 2B). Twenty of these 24 have been described in the context of cancer, with only four in lung cancer. This includes *DGCR5*, a lncRNA we found to be overexpressed in

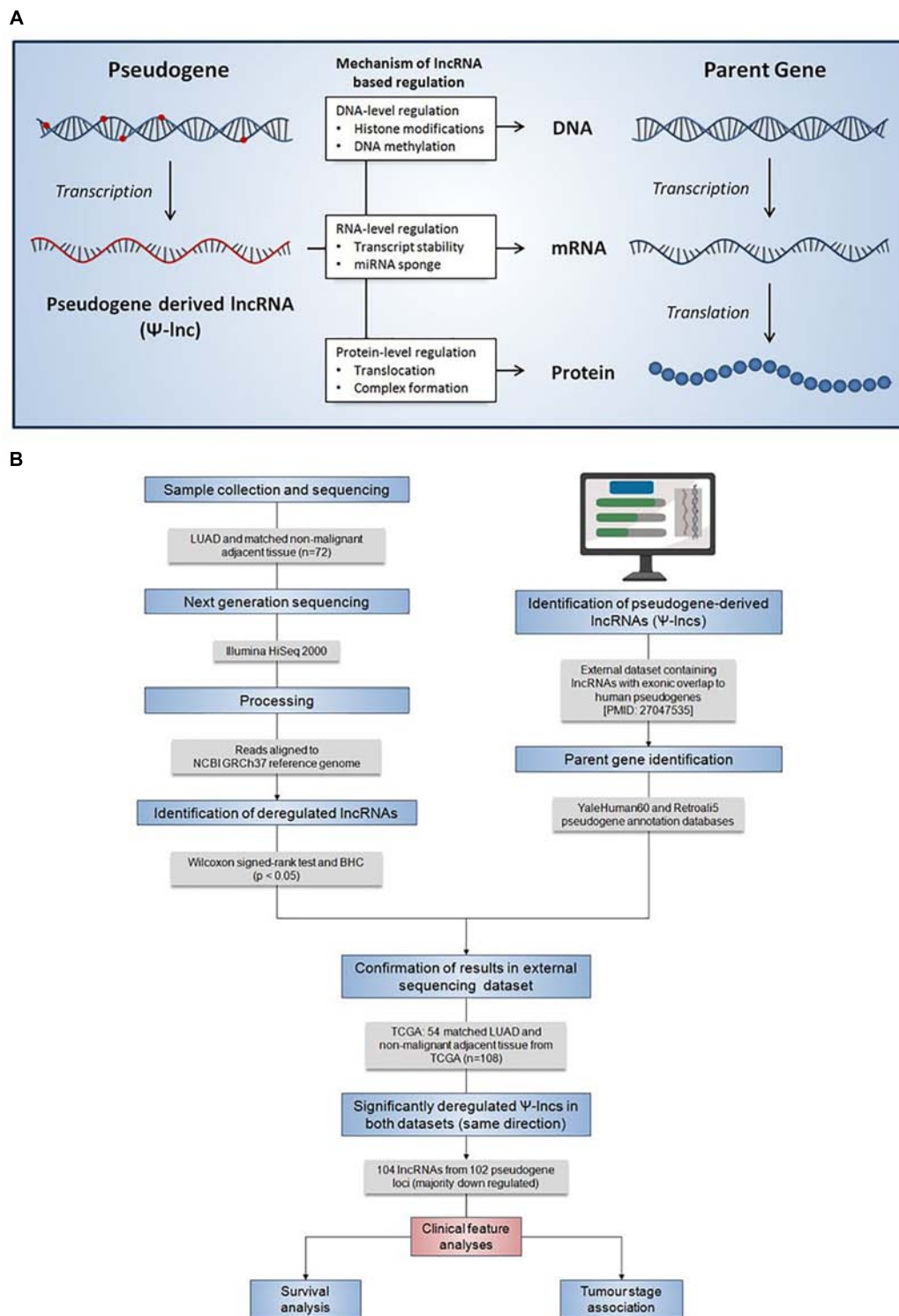


FIGURE 1 | Summary of the regulatory mechanisms of Ψ -lncs and the analysis pipeline for the identification of their deregulation in lung adenocarcinoma.

(A) Summary of the regulatory mechanisms of pseudogene-derived lncRNAs (Ψ -lncs) that retain sequence homology with the parent gene. Overall, lncRNAs have been shown to function through a variety of regulatory mechanisms, acting on the DNA, RNA, and protein levels. **(B)** Flow diagram description of the analysis pipeline applied for the identification of deregulated Ψ -lncs. Patient LUAD samples were collected and subjected to next generation sequencing to quantify RNA expression. Gene expression was then compared between tumors and matched non-malignant tissue to identify significantly deregulated transcripts. lncRNAs with exonic overlap to known pseudogenes were then identified and confirmed in a 2nd set of LUAD and matched non-malignant tissue. Deregulated Ψ -lncs were then assayed to determine associations with clinical features.

TABLE 1 | Patient clinical characteristics of the discovery (BCCA), validation (TCGA), and survival analysis (KmPlotter) datasets.

Characteristic	BCCA	TCGA	KmPlotter
Samples (pairs)	72 (36)	108 (54)	866 (n/a)
Sex			
Male	10	24	344
Female	26	30	318
Average age	70	67	–
Stage			
I	20 (56%)	28 (52%)	370 (69%)
II	11 (31%)	14 (30%)	136 (25%)
III	3 (8%)	10 (19%)	24 (4%)
IV	1 (3%)	2 (4%)	4 (1%)
Ethnicity			
Caucasian	11 (31%)	51 (94%)	–
Asian	14 (39%)	–	–
Unknown	11 (31%)	–	–
Black	–	3 (6%)	–
Smoking			
Current	5 (14%)	7 (13%)	246 (Ever) ^a
Former	6 (17%)	36 (67%)	
Never	25 (69%)	5 (9%)	246

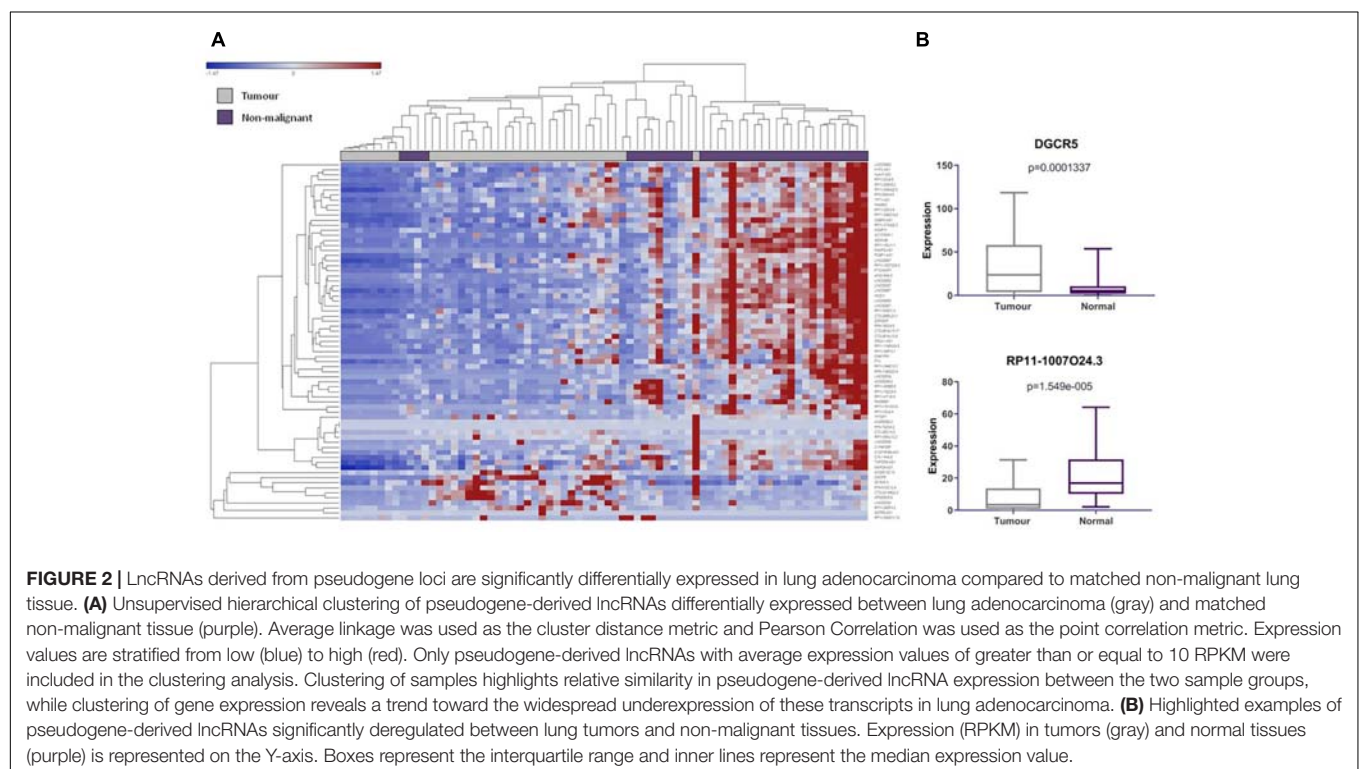
^a Ever smokers is a term that includes both current and former smokers.

tumors. *DGCR5* has been reported to promote LUAD progression by sequestering a variety of miRNAs involved in cell cycle regulation, although it has not been investigated with regard to its pseudogene-derived nature (Chen et al., 2017; Dong et al., 2018; Luo et al., 2018).

We were interested in examining the genetic events that could impact pseudogene loci, and thus affect Ψ -lnc expression. We mapped the chromosomal distribution of the deregulated Ψ -lncs, finding them to be distributed throughout the genome and detected on most chromosomes, except for chromosomes 4 and Y (**Figure 3**). The locations of each of the parent genes of deregulated Ψ -lncs are similarly distributed through the genome (**Supplementary Table S4**). We then determined the overlap of these genes with regions of recurrent chromosomal amplification and deletion as determined by The Cancer Genome Atlas (TCGA) for LUAD (Zack et al., 2013). While some Ψ -lncs overlap with regions of recurrent deletion, the majority do not, indicating that they may be regulated by mechanisms other than copy number alteration (**Figure 3**).

Global Patterns of Ψ -lnc and Parental Gene Expression

As a first step to identify deregulated Ψ -lncs that may function through regulation of their respective parent genes, we explored whether Ψ -lncs with significantly deregulated expression were associated with altered parent gene transcript levels (Poliseno et al., 2010; Johnsson et al., 2013; Feng et al., 2017; Huang et al., 2018). We obtained parent gene information for the 95 deregulated Ψ -lncs and determined that they shared 104 parent genes. Some pseudogenes contained multiple lncRNAs, and some lncRNAs overlapped multiple pseudogenes, constituting a total number of 116 Ψ -lnc-parent gene pairings. For each Ψ -lnc-parent pair we compared groups of tumors with high levels of Ψ -lnc expression to those with low levels of Ψ -lnc expression.



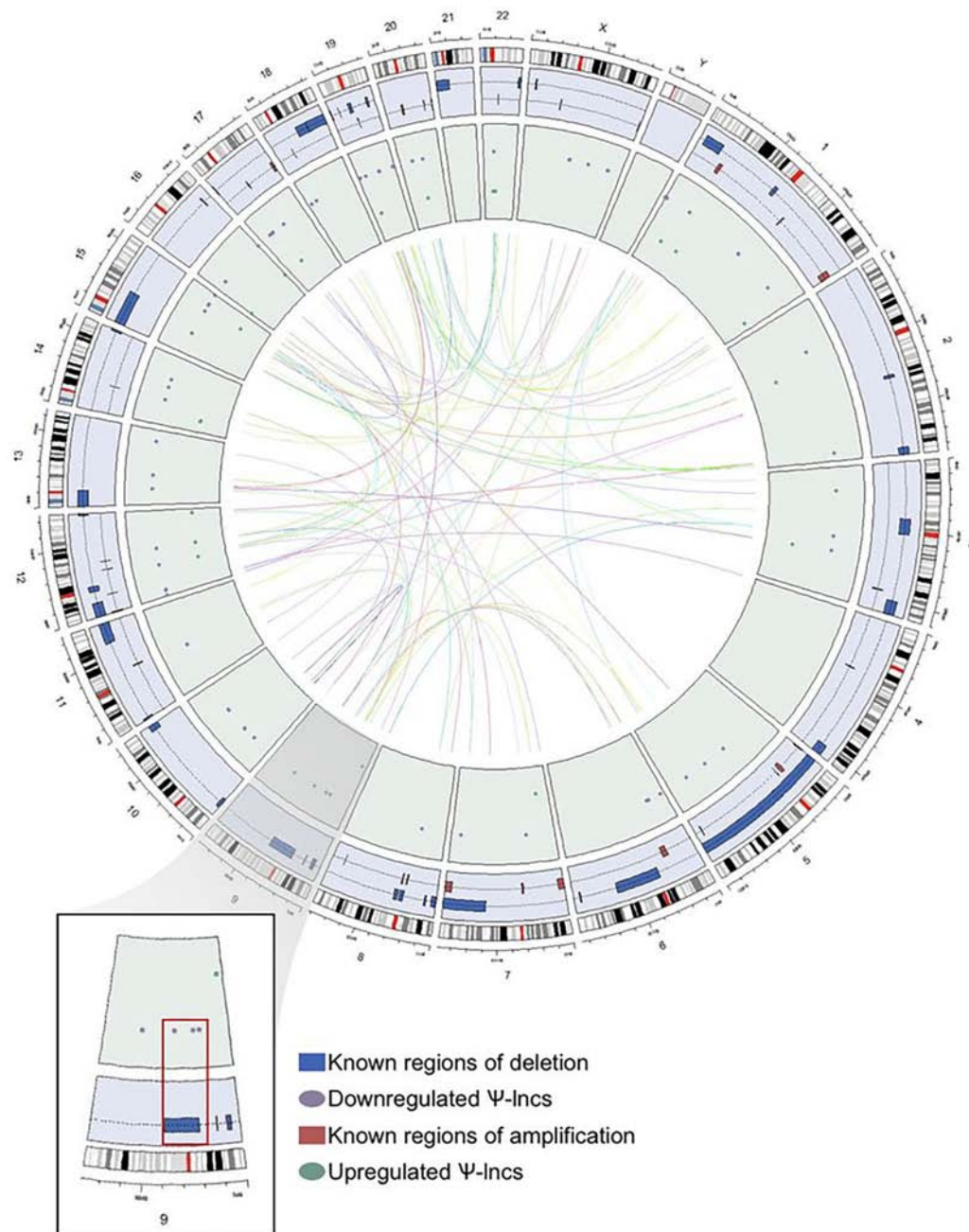


FIGURE 3 | Genome-wide distribution of deregulated pseudogene-derived lncRNAs in lung adenocarcinoma. Circular representation of the genomic distribution of the deregulated pseudogene-derived lncRNAs discovered in our study, as well as known regions of copy number alterations in lung adenocarcinoma as described by TCGA. The outer concentric circle represents the human karyotype from the genomic build hg19. The blue concentric circle contains known regions of copy number amplification (red boxes) and deletion (blue boxes) that have been previously published. The inner green circle represents the specific genomic location of our pseudogene-derived lncRNAs found to be either upregulated (green circles) or downregulated (purple circles) in lung adenocarcinoma. Finally, the inner connecting lines represent the interaction between the deregulated pseudogene-derived lncRNAs and the locations of their respective protein-coding parent genes. Chromosome 9 (magnified region) highlights that some of the downregulated pseudogene-derived lncRNAs overlap with genomic regions frequently deleted in lung adenocarcinoma.

We found that 33 Ψ -lncs have a significant expression relationship with their parent gene in at least 1 dataset (**Supplementary Table S5**). This included 21 sense Ψ -lnc-parent-gene pairs, and 13 antisense Ψ -lnc-parent gene pairs. Having identified

Ψ -lncs with expression associated with parent gene expression, we investigated whether parent genes had known oncogenic or tumor suppressive roles. We performed a literature search to determine if any had been previously described in the context

TABLE 2 | Parent genes of deregulated Ψ -lncs previously described in cancer literature.

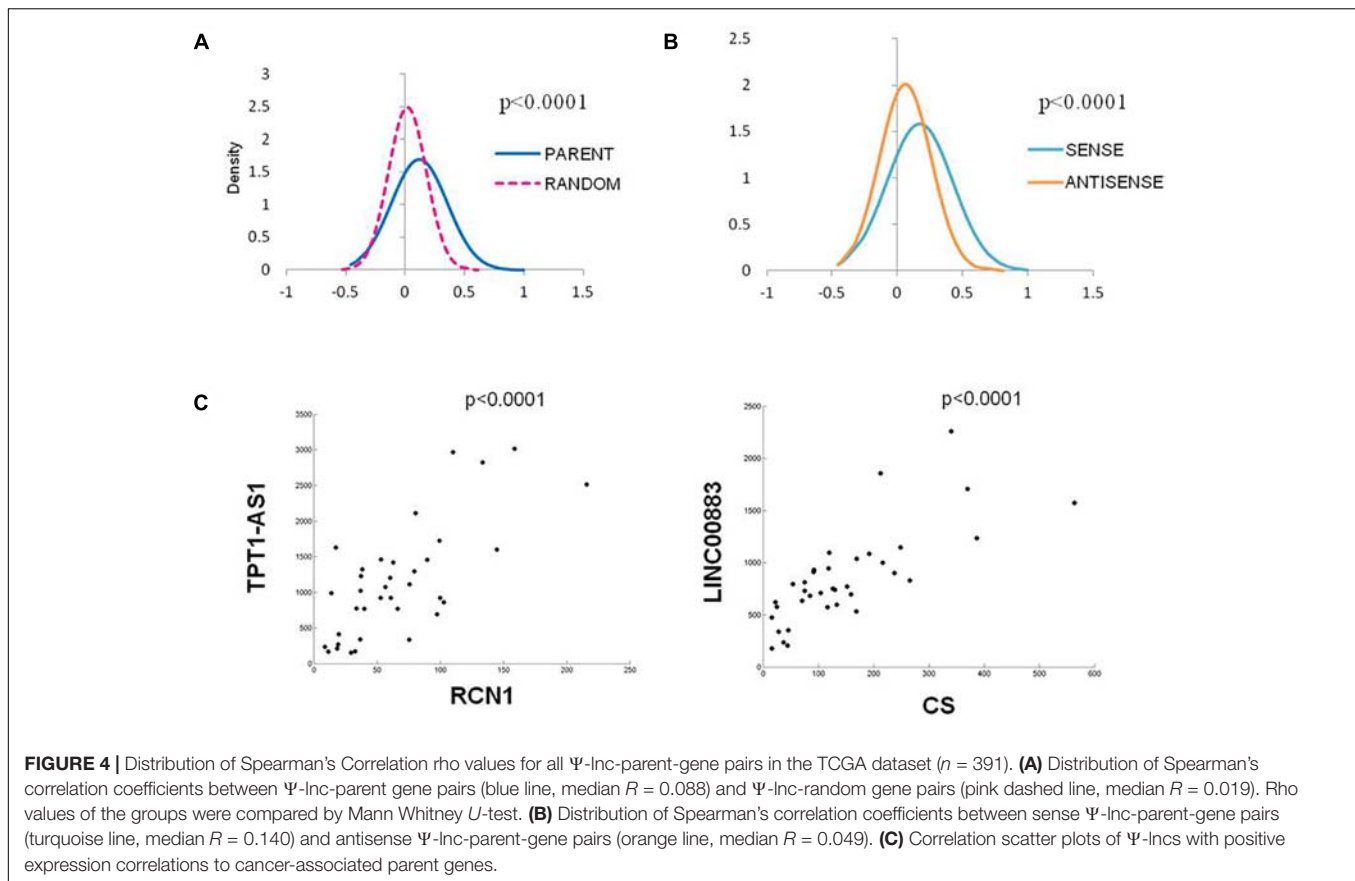
Parent Gene	Cancer ^a	Lung ^a Cancer	Ψ -lnc	Parent Gene	Cancer ^a	Lung ^a Cancer	Ψ -lncRNA
ADC	8	–	RP11-439L8.3	MT1	>1000	95	MMP24-AS1
AGAP1	12	–	AGAP11	NEBL	17	–	LINC00342
ARIH1	4	–	RP11-1007O24.3	NUP210	9	2	H1FX-AS1
ATXN7L3	5	–	RP11-56G10.2	PCBP2	26	–	PCBP1-AS1
BCR	>1000	>1000	AC008132.13	PKD1	238	5	RP11-1186N24.5
BMS1	4	–	AGAP11	POTEF	4	–	RP11-193H5.1
CDC42	1657	129	RP11-390F4.3	PPARGC1B	37	1	RP11-527N22.1
CECR7	3	–	AP000525.9;	PPY	91	5	CTD-2008P7.8
			CTD-2314B22.3	PRKX	12	2	RP11-526I2.1
CELSR1	11	2	DGCR5	PTCHD3	2	–	PTCHD3P1
CHRNA1	8	–	RP11-650L12.2	PTMA	34	–	LINC00987
CIC	–	96	RP11-34P13.7	PZP	20	–	LINC00987
CS	–	10	LINC00883;	RAB11FIP1	16	1	RP3-368A4.5
			RP11-446H18.5	RAB40B	5	–	LL0XNC01-250H12.3
CSPG4	62	5	DNM1P51	RCN1	17	1	TPT1-AS1
CTAGE1	49	–	RP1-122P22.2	RNASEH1	7	–	RP11-344E13.3
CYP4F2	29	–	CYP4F35P	RPL21	5	–	AC005062.2
CYP4F3	10	1	FAM95B1	RPL23A	8	–	HLA-F-AS1
CYP4F31P	10	1	CYP4F35P;	RPSA	51	3	FTX
			FAM95B1	RPSAP58	–	3	LINC00466
DFFB	33	1	TOPORS-AS1	SEMA3A	163	25	SEMA3B
DRD2	121	10	AP000438.2	SHQ1	10	1	RP11-50E11.3
EGLN1	158	5	RP11-182J1.1	SNAPC5	4	–	LY86-AS1
FAM103A1	1	–	RP11-324H6.5	SNX18	2	–	RP11-435B5.5
GPR39	8	–	RP11-399O19.9	SRSF9	5	–	RP11-752G15.3
HMBG1	>1000	111	RP11-349A22.5;	TACC3	122	21	LINC00667
			ZBED3-AS1	TOMM40	10	2	CTD-2314B22.3
HMG2P46	81	1	RAMP2-AS1	TPTE	69	7	TPTEP1
KRT8	94	7	RP5-1198O20.4	TUBB4B	5	–	RP11-386G11.10
LINC00657	2	–	CTA-14H9.5;	TULP3	5	–	LINC00359
			HCG11	VENTX	8	1	RP11-81H3.2
LMNB2	3	1	RP11-161H23.9	VWF	733	52	TPTEP1
MARK4	25	3	CTD-2201G3.1	ZNF14	2	–	CTD-2666L21.1;
MICE	1	–	HLA-F-AS1				ZNF833P
MIPEPP3	4	1	C1QTNF9B-AS1	ZNF44	1	–	CTD-2666L21.1
				ZNF584	1	–	CTD-2619J13.17

^a denotes number of entries in PubMed.

of cancer. Interestingly, we found that 65 of these parent genes had been previously described in cancer, and of those, 33 had been described in lung cancer (Table 2). Of the 34 significantly differentially expressed Ψ -lnc-parent gene pairs, 25 parent genes were described in cancer. This includes lung cancer associated genes like CS, which affects tumor drug response, as well as RCN1, which is associated with poor prognosis and tumor progression in lung cancer (Chen et al., 2014, 2018).

As the vast majority of the deregulated Ψ -lncs that were correlated with their parent gene had positive associations, we were interested whether this was a global phenomenon or exclusive to deregulated genes. We performed a Spearman's correlation analysis on every Ψ -lnc-parent-gene pair with expression data in our dataset irrespective of deregulation status ($n = 390$ gene pairs). We plotted the distribution of Spearman's rho (ρ) values for the Ψ -lnc-parent-gene pairs

and compared them to the rho values for Ψ -lncs paired to randomly selected genes. We found that the Ψ -lnc-parent-gene pairs have significantly more positive relationships than the random gene pairs in both the BCCA (Mann Whitney U -test, $p < 0.0001$) and TCGA datasets (Mann Whitney U -test, $p < 0.0001$; Figure 4A). Studies have shown that lncRNAs transcribed from opposite strands can have different regulatory effects on target genes (Johnsson et al., 2013; Balbin et al., 2015). To determine if transcriptional orientation has an effect on Ψ -lnc-parent relationships we compared the Spearman's rho values of sense Ψ -lnc-parent pairs to antisense Ψ -lnc-parent pairs. In both datasets we observed that the sense Ψ -lnc-parent pairs to have significantly more positive relationships than the antisense Ψ -lnc-parent pairs (Mann Whitney U -test, TCGA set ($p < 0.0001$), and BCCA set ($p < 0.0025$) (Figure 4B and Supplementary Table S6). Strongly positively



correlated Ψ -lnc-parent pairs include *TPT1-AS1/RCN1* and *LINC00887/CS* (Figure 4C).

Ψ -lncs and Their Parent Genes Are Associated With Patient Survival

If the aberrant expression of Ψ -lncs is biologically relevant, it follows that they may be relevant in tumor aggressiveness, stage, and patient survival. We performed a two-group analysis using a Mann Whitney U -test between Stage I tumors and Stage II-IV tumors, as the majority of our tumors fell into these categories (Table 1). Of the deregulated Ψ -lncs we found *CTC-250I14.3* to be associated with Stage 1 disease and downregulated in both the BCCA and TCGA LUAD cohorts (Supplementary Figure S1). Our discovery datasets were limited in sample size for survival association analysis; therefore, we examined a third cohort of 719 LUAD from the KM Plotter database (Kaplan–Meier Plotter)¹ (Table 1). This cohort was limited to genes with probe coverage on microarray platforms. A total of 19 of the deregulated Ψ -lncs were represented on this platform, yet, the majority of these Ψ -lncs (16 of 19) were significantly associated with poor overall survival (log-rank $p < 0.05$, Table 3).

While we were not able to investigate the survival associations of all deregulated Ψ -lncs as many were not covered by the microarray platforms, the majority (72 out of 103) of

their parent genes were represented. We discovered that 67 of these parent genes were associated with patient survival. Twenty-eight of these survival associated parent genes were also significantly associated with the expression of their paired deregulated Ψ -lnc. Furthermore, we found 11 pairs where both Ψ -lnc and parent gene are associated with patient survival. For example *RP11-1007O24.3*, a Ψ -lnc downregulated in tumors, is positively associated with expression of survival-associated parent gene *ARIH1* in both the BCCA and TCGA datasets (Table 3 and Figure 5A). Further examples also include Ψ -lnc-parent pairs such as *ZBED3-AS1* and *HMGB1*, which are positively correlated at the expression level, and both significantly associated with survival (Figure 5B). We also observe Ψ -lnc-parent pairs that are associated with survival, but do not share an expression relationship such as *LINC00667* and parent gene *TACC3* (Figure 5C). Collectively, our discovery of the broad deregulation of Ψ -lncs, many of which are survival-associated and associated with parent gene expression, may indicate that Ψ -lncs impact LUAD biology through *trans* regulation of their cancer-associated parent genes.

DISCUSSION

Here we expand upon the work done by Milligan et al. (2016), completing the first large-scale analysis of lncRNA expression from pseudogene loci in LUAD and paired non-malignant lung

¹<http://kmplot.com/analysis/>

TABLE 3 | Associations between Ψ -lncRNA, parent gene expression, and patient outcome.

Ψ -lncRNA	Survival p (KmPlotter)	Parent Gene	Survival p (KmPlotter) ^a	Association with Parent (p , BCCA)	Association with Parent (p , TCGA)
AC008132.13	—	BCR	0.00043 ^a	0.0173	—
AGAP11	0.0099 ^b	AGAP1	5.80E-10 ^b	0.0005	—
AGAP11	—	BMS1	0.00018 ^a	0.0121	—
AP000525.9	—	CECR7	3.20E-06 ^a	—	0.0371
CTA-14H9.5	—	LINC00657	6.00E-06 ^b	—	—
CTD-2314B22.3	—	CECR7	3.20E-06 ^a	—	0.0402
CTD-2619J13.17	—	ZNF584	1.70E-08 ^a	—	0.0205
CTD-2666L21.1	—	ZNF44	2.00E-15 ^b	—	0.0013
CTD-2666L21.1	—	ZNF14	1.90E-12 ^a	—	—
DGCR5	4.00E-04 ^a	CELSR1	2.70E-02 ^a	—	—
FAM66C	3.00E-04 ^b	DEFB130	—	—	—
FAM95B1	—	CYP4F3	2.00E-01 ^b	—	—
HCG11	1.40E-08 ^b	LINC00657	6.00E-06 ^b	—	0.0046
LINC00342	1.80E-06 ^b	NEBL	2.30E-05 ^b	—	—
LINC00466	4.60E-06 ^a	RPSAP58	—	—	—
LINC00639	0.0087 ^b	ZFP41	0.014 ^b	—	—
LINC00667	4.50E-08 ^b	TACC3	6.20E-09 ^a	—	—
LINC00883	2.90E-03 ^b	CS	0.00041 ^a	0.0449	—
LINC00957	0.042 ^b	RASA4B	0.035 ^a	—	—
LINC00982	1.30E-06 ^b	n/a ^c	—	—	—
LINC00987	—	PZP	0.02 ^a	—	<0.0001
LY86-AS1	0.00025 ^b	SNAPC5	5e-06 ^b	—	—
MMP24-AS1	0.023 ^a	MT1	1.20E-12 ^a	—	—
PCBP1-AS1	—	PCBP2	4.30E-13 ^b	—	—
RAMP2-AS1	0.014 ^b	HMG2P46	—	—	—
RP11-1007O24.3	—	ARIH1	0.00093 ^b	<0.0001	0.0030
RP11-1186N24.5	—	PKD1	0.0013 ^b	—	<0.0001
RP11-182J1.1	—	EGLN1	1.20E-08 ^b	0.0011	—
RP11-344E13.3	—	RNASEH1	1.00E-07 ^b	0.0007	—
RP11-349A22.5	—	HMG1	7.50E-10	—	—
RP11-34P13.7	—	CIC	1.20E-08 ^a	—	0.0205
RP11-390F4.3	—	CDC42	1.30E-05	0.0449	—
RP11-439L8.3	—	ADC	3.30E-09	0.0205	—
RP11-446H18.5	—	CS	0.00041 ^a	—	—
RP11-93K22.13	—	FAM86B3P	6.30E-05 ^b	—	0.0129
TOPORS-AS1	—	DFB	9.10E-08 ^b	—	0.0033
TPT1-AS1	0.00019 ^b	RCN1	0.016 ^a	0.0242	—
ZBED3-AS1	1.10E-10 ^b	HMG1	7.50E-10 ^b	0.0100	—
ZNF833P	—	ZNF491	0.016 ^b	—	—
ZNF833P	—	ZNF14	1.90E-12 ^a	—	0.0079

^a Denotes poor overall survival associated with high gene expression.

^b Denotes poor overall survival associated with low gene expression.

^c No information for the parent gene.

tissue. We discovered a broadly positive association between Ψ -lncs and parent-gene expression, suggestive of an alternative mechanism of cancer gene regulation. While there have been singular examples of deregulated pseudogene-derived lncRNAs in cancer, we show that this phenomenon is widespread in LUAD. In addition to being correlated with Ψ -lnc expression, we find that many of the parent genes of these deregulated lncRNAs are annotated cancer genes and are significantly associated with patient survival, highlighting how these previously unappreciated non-coding genes may affect LUAD biology.

While the identification of lncRNAs associated with cancer phenotypes is increasing, a great challenge in the field remains the accurate downstream prediction of lncRNA function. Unlike protein-coding genes or small non-coding RNAs, features like complex folding patterns and unknown binding motifs have

contributed to the challenging functional characterization of lncRNAs. We utilized the sequence similarity found between lncRNAs expressed from pseudogene loci, and their parent genes to predict the function of this subset of lncRNAs in LUAD. We identified a set of 104 Ψ -lncs deregulated in LUAD in two independent datasets. This greatly increases the number of deregulated lncRNAs known to be expressed from pseudogene loci in LUAD. Interestingly, the majority of these Ψ -lncs were under-expressed in tumors compared to non-malignant tissues, suggesting that they may have tumor-suppressive roles, and that their downregulation is advantageous to LUAD tumorigenesis.

Under-expression was not significantly associated with regions of recurrent copy number deletion in LUAD, although a subset of deregulated Ψ -lnc loci were localized to these regions (Figure 3). These observations suggest that they may be regulated by alternative molecular mechanisms, including broad chromosomal aberrations that affect whole chromosome arms, or epigenetic mechanisms. For example, endogenous retroviruses and repetitive elements often become aberrantly expressed in cancer due to deregulated methylation patterns (Kassiotis, 2014). We did not observe enrichment of Ψ -lncs or their parent genes on any chromosomes, despite the fact that pseudogenes are known to be overabundant on the human X chromosome (Drouin, 2006).

The direction of transcription often affects lncRNA function (Balbin et al., 2015). For example, the *PTEN* pseudogene (*PTENP1*) expressed in the sense direction can function as a decoy for inhibitory miRNAs that would otherwise cause translational inhibition of the *PTEN* parent mRNA. Conversely, when the antisense lncRNA is expressed from the *PTENP1* locus, the transcript is able to localize to the *PTEN* parent locus and recruit chromatin-remodeling machinery, which leads to the silencing of *PTEN* transcription (Poliseno et al., 2010; Johnsson et al., 2013). Both mechanisms have been coopted by cancer cells for their respective tumor suppressive and oncogenic roles (Hu et al., 2018). We found sense Ψ -lnc-parent pairs, (which account for 208 out of 391 Ψ -lncs examined) to be more positively correlated than antisense Ψ -lnc-parent pairs in both cohorts (Figure 4B). This may imply that Ψ -lncs are more likely to regulate their parent gene in a positive manner which may occur through mechanisms such as miRNA sponging or transcript stabilization when transcribed in the sense direction (Glenfield and McLysaght, 2018; Hu et al., 2018). The distribution of Spearman's ρ -values for antisense-parent gene pairs suggests a more even split between positive and negative regulation. A limitation of this study is that we cannot discount the possibility that sequencing reads for sense overlapping Ψ -lncs that have sequence homology with their parent gene are being mapped to the parent gene instead of the Ψ -lnc. This potential issue warrants further investigation considering both the large number of annotated pseudogenes in the genome ($n = 13,000$) and the possibility of false interpretation of sequencing data for both protein-coding and non-coding genes.² While these alignment errors could affect sense Ψ -lnc-parent gene pairs,

²<https://www.genenames.org/cgi-bin/statistics>

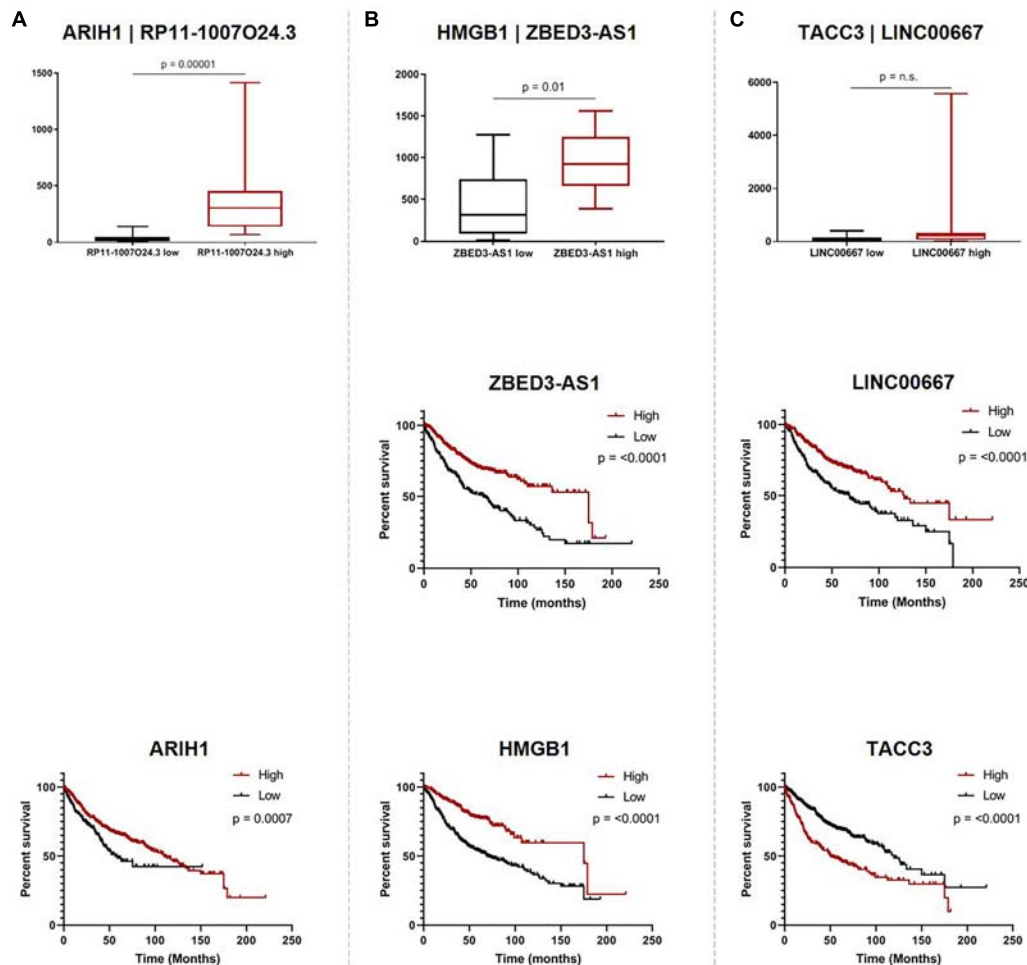


FIGURE 5 | Associations of pseudogene-derived lncRNAs (upper row), their respective parent gene expression levels and their potential impact on patient outcome (middle row and bottom row). Expression and survival associations as seen for: **(A)** *RP11-1007O24.3* and parent gene *ARIH1*, **(B)** *ZBED3-AS1* and parent gene *HMGB1*, and **(C)** *LINC00667* and parent gene *TACC3*. Expression associations (upper row) for each pseudogene-derived lncRNA and parent gene pair were found by stratifying samples into tertiles by high (red) and low (black) expression of the pseudogene-derived lncRNA, and plotting the expression of the parent-gene (RPKM) on the y-axis. Survival associations were found for pseudogene-derived lncRNAs (middle row) and their respective parent genes (bottom row). Samples were stratified into tertiles with high (red) and low (black) expression of the gene-of-interest, and the significance of the associations were assessed using the logRank method through GraphPad Prism 8 software on data obtained from kmPlot ($n = 673$). Survival information was not available for *RP11-1007O24.3* and it was thus omitted from this analysis.

antisense Ψ -lnc-parent gene pairs are not subjected to this same technical artifact. Recently, RNA-sequencing analysis strategies have emerged that begin to address this issue, and long read RNA-sequencing could be used to reduce errors in sequence alignment.

When looking at the parent genes of these deregulated Ψ -lncs we were interested to find that many had previously described roles in cancer (Table 2). This includes *EGLN1*, a well described cancer gene involved in regulation of tumor hypoxia, and *CDC42*, an oncogene involved in cell cycle control (Chan and Giaccia, 2010; Maldonado and Dharmawardhane, 2018). Many of these deregulated Ψ -lncs were also associated with clinical parameters such as patient survival and patient stage, in addition to the correlated expression between Ψ -lncs and their parent genes (Table 3). For example, *ZBED3-AS1*

and *HMGB1* were positively correlated at the expression level, and low expression of both genes was associated with poor patient survival (Figure 5B). We also observed Ψ -lnc-parent pairs where both genes are associated with survival, but do not share an expression relationship. *LINC00667* and parent gene *TACC3*, for example, are both survival-associated, but not correlated at the expression level. *TACC3* is a component of the *TACC3*/ch-TOG/clathrin protein complex, and roles in complex assembly have been previously observed for lncRNAs (Munschauer et al., 2018; Figure 5C). Thus, it is possible that *LINC00667* is involved in a form of regulation that would not affect transcript levels, including protein-complex assembly. While we were unable to assess survival associations for many of the deregulated Ψ -lncs, they may still impact patient survival

through regulation of their parent genes. We found 28 out of 34 expression-associated Ψ -lnc-parent gene pairs to have parent genes associated with patient survival. *RP11-1007O24.3*, for example, was positively correlated with survival-associated parent gene *ARIH1* expression in both cohorts. *ARIH1* has been previously described to be a mediator of DNA-damage response and mitophagy in cancer cells (Figure 5A; Perdomo et al., 1988; von Stechow et al., 2015). The potential regulatory impact of Ψ -lncs on their clinically relevant parent genes is considerable and may represent a novel avenue for targeted therapies. While this study focused on the broad effects of Ψ -lnc deregulation, future studies utilizing *in vitro* and *in vivo* experiments will be necessary to determine the specific mechanisms of parent gene regulation.

As Ψ -lncs may represent an unexplored area of cancer-associated parent gene regulation, their therapeutic relevance should be further explored. lncRNAs make ideal targets for therapies that target RNA products such as Antisense Oligonucleotide (ASO) therapies, since RNA is their final functional state, rather than the intermediate product for protein-coding genes (Crooke et al., 2018). In addition, ASOs are easier and less costly to develop than small molecule inhibitors, and are in development as aerosol sprays that may be ideal for lung cancer treatment (Kjems and Howard, 2012; Li and Chen, 2013). However, as ASOs target through complementary sequence pairing, they would have to be designed in such a way as to not interfere with the parent gene, especially in the case of Ψ -lncs expressed from the sense strand.

This strategy of identifying lncRNAs aberrantly expressed from pseudogene loci may be useful when applied to other cancer types. Indeed, we see that several of our deregulated Ψ -lncs have been described in other tumor types, such as *TPT1-AS1* in cervical cancer, and *HGC11* in both prostate cancer and hepatocellular carcinoma (Zhang et al., 2016; Xu et al., 2017; Jiang et al., 2018). Additionally, as lncRNA expression is highly tissue specific, the application of this approach to other cancer types may yield novel disease-specific Ψ -lnc-parent pairs, highlighting the clinical utility of examining these previously underappreciated transcripts. Overall, Ψ -lnc-cancer-parent-gene axes represent alternative mechanisms of cancer gene regulation, and their identification is a critical step toward the functional characterization of lncRNAs.

CONCLUSION

There is a growing need to functionally characterize lncRNAs. Pseudogene-derived lncRNAs have been shown to be involved in cancer and regulate the expression of their parent genes. We show here how pervasive this gene regulatory mechanism is in LUAD samples. We identify a large set of deregulated Ψ -lncs, with aberrant expression observed in RNA-sequencing data from two LUAD cohorts of paired tumor and non-malignant lung tissue samples. We show that these deregulated Ψ -lncs have clinical value and that the parent genes, many of which are correlated with Ψ -lnc expression, have been implicated in cancer phenotypes and are associated with clinical outcome. Together,

our results highlight the important roles of the non-coding transcriptome in cancer cellular biology.

MATERIALS AND METHODS

Next Generation Sequencing of Lung Adenocarcinoma Patient Samples Discovery Cohort

We performed Next Generation Illumina HiSeq RNA sequencing on a set of 36 micro-dissected LUAD tumors and matched adjacent non-malignant tissue ($n = 72$). Our British Columbia Cancer Agency cohort (BCCA) was composed of fresh-frozen LUAD tumors and matched non-malignant lung parenchymal tissue collected from 36 patients at the Vancouver General Hospital with approval from the University of British Columbia-BCCA Research Ethics Board. Consent obtained from the tissue donors of this study was both informed and written. To avoid field effects non-malignant samples were collected from areas > 2 cm away from the tumor. In order to reduce contaminating sequences derived from alternative cell types, tissue microdissection was guided by a pathologist. Samples used in this study contained $> 80\%$ tumor cell or $> 80\%$ non-malignant cell content. Total RNA was extracted using Trizol reagent and standard procedures.

Processing of RNA-Sequencing Data

Total RNA was used for library construction at the Genome Sciences Center (GSC, Vancouver, BC, Canada). Briefly, samples were first analyzed using Agilent Bioanalyzer RNA nanochip, and samples that passed quality check were arrayed into a 96-well plate. PolyA⁺ RNA was purified using the 96-well MultiMACS mRNA isolation kit on the MultiMACS 96 separator (Miltenyi Biotec, Germany) from 2 μ g total RNA with on-column DNase I-treatment as per the manufacturer's instructions. Double-stranded cDNA was synthesized from the purified polyA⁺-RNA using the Superscript Double-Stranded cDNA Synthesis kit (Life Technologies, United States) and random hexamer primers at a concentration of 5 μ M. The paired-end sequencing library was prepared following the GSC paired-end library preparation protocol, which is strand specific. Sequencing was performed using the Illumina HiSeq 2000 platform. Raw sequencing reads were subject to a quality control process. Reads with a length < 50 nt (under two thirds of maximum read length of 75 nt) and quality level (Phred) < 20 were discarded. High quality reads (.fastq files) were aligned to the NCBI GRCh37 reference human genome build using the STAR aligner (v 2.4.1d) under default parameters (Dobin et al., 2013). Aligned reads (.bam files) were quantified using Ensembl Transcripts (Release 75) reference annotations (Flicek et al., 2014). Raw RNA sequencing reads from each patient (tumor and corresponding non-malignant tissue) were deposited at BioProject.³ Quantification was performed using the Partek Flow platform as reads per kilobase per million (RPKM). RNA sequencing (.bam files) and clinical

³<http://www.ncbi.nlm.nih.gov/bioproject/516232>

data for a secondary set of LUAD tumors and matched non-malignant tissue ($n = 108$) were downloaded from The Cancer Genome Atlas (TCGA) Data Portal for validation purposes⁴ (Table 1). Expression profiles from TCGA were processed as described above.

Identification of lncRNAs Expressed From Pseudogene Loci and Corresponding Parent Genes

Ψ -lnc Annotation

Milligan et al. (2016) recently published a global atlas of lncRNAs that have exonic overlap with positionally non-redundant (unique) pseudogenes from 3 major pseudogene databases. Using this resource we obtained a list of lncRNAs overlapping pseudogene loci (Supplementary Table S1) that we used as a foundation for our expression analysis. As the degree of sequence overlap required for a pseudogene-derived lncRNA to regulate its parent gene is unknown, we did not restrict our analysis to full-length, expressed pseudogenes, and included lncRNAs with any exonic overlap, including sense and antisense transcripts in order to annotate the most comprehensive list of Ψ -lncs (lncRNAs overlapping pseudogene loci).

Parent Gene Annotation

The parent gene information was also extracted for all pseudogenes overlapping our list of Ψ -lncs that have parent-gene annotations in the YaleHuman60 and Retroali5 databases (Supplementary Table S7). Manual literature search was performed for parent genes of deregulated Ψ -lncs that were not contained in these databases.

Statistical Analysis

Identification of Significantly Deregulated Ψ -lncs in Paired LUAD and Non-Malignant Lung Tissue

Gene expression for protein-coding and non-coding genes was compared between tumors and non-malignant tissue and significantly deregulated genes were identified using a Wilcoxon signed-rank test ($p < 0.05$) and subjected to a Benjamini-Hochberg (BH) FDR correction. Ψ -lncs as identified previously were extracted, and those that were significantly deregulated between tumors and non-malignant tissue, in both our discovery (BCCA) and validation (TCGA) were selected for further analysis (Figure 1B).

Ψ -lncs and Parent Gene Expression

Tumors were sorted by Ψ -lnc expression for each Ψ -lnc-parent gene pair, and grouped into top and bottom Ψ -lnc expressing tertiles. Parent gene expression was then compared between the two groups using the Mann Whitney U -test (p -value ≤ 0.05). We performed a global expression analysis to determine whether Ψ -lnc-parent pairs were more positive or negatively correlated than random chance. For all Ψ -lncs with expression data in both datasets, Spearman's correlation rho

values were calculated for Ψ -lnc-parent gene pairs ($n = 390$) and compared to Ψ -lnc-random gene pairs. Random genes in our expression matrices were selected to pair with each Ψ -lnc. Each gene was assigned a number and pairs were chosen by using a random number generator.⁵ Spearman's rho values were then plotted, smoothed, and compared (Mann Whitney U -test, p -value ≤ 0.05). Rho value distribution was also compared between sense lnc-parent gene pairs ($n = 208$) and antisense lnc-parent pairs ($n = 182$).

Literature Searches

To determine if each gene of interest (Ψ -lnc or parent gene) had been previously described in the context of tumors we searched Pubmed using the terms "gene + cancer" or "gene + lung cancer."

Hierarchical Clustering and Data Visualization

Unsupervised hierarchical clustering was performed in order to visualize and examine the expression of the Ψ -lncs in individual samples (Figure 2A). Average Linkage was used as a cluster distance metric, while Pearson Correlation was used as a point distance metric. To visualize the expression patterns of the most highly expressed Ψ -lncs, those with an average expression value of ≥ 10 RPKM in either tumor or non-malignant samples were included in the analysis.

Distribution of Deregulated Ψ -lncs Across Genome

Locations of Ψ -lncs and parent genes were compared to identify their genomic position (Supplementary Table S4). Circular plot visualization was performed using the R-package Circlize (Figure 3; Gu et al., 2014). LUAD-specific regions of significant recurrent somatic copy number alterations had been previously identified by TCGA, and were used in this study to determine if the deregulated Ψ -lncs overlapped with frequently altered regions (Zack et al., 2013). All genomic coordinates correspond to the NCBI GRCh37 reference human genome build.

Clinical Features

Survival Analysis

A large public clinical database (Kaplan-Meier Plotter)⁶ comprised of 719 LUAD samples was used to determine the association between both protein-coding and non-coding gene expression with patient outcomes. Similar to the BCCA and TCGA cohorts, the patient samples in this 3rd dataset were mostly comprised of Stage I and Stage II tumors (Table 1). Of the 104 deregulated Ψ -lncs, 19 were represented in this database, while 70% of parent genes (72 out of 103) were present. Default settings were used and a log-rank (Mantel-Cox) test was applied to compare survival between groups of tumors with high and low expression of each gene tested, where $p < 0.05$ was considered statistically significant. The optimal expression cut-off was selected for each gene.

⁴<https://portal.gdc.cancer.gov/>

⁵<https://www.random.org/>

⁶<http://kmplot.com/analysis/>

Association With Tumor Stage

The majority of tumors for BCCA and TCGA fell into the categories of Stage I and Stage II (Table 1). We compared expression of deregulated Ψ -lncs between Stage I tumors and tumors classified as Stage II and above using a Mann Whitney U -test (p -value ≤ 0.05).

AUTHOR CONTRIBUTIONS

GS was responsible for the project design. GS, KE, AS, VM, BM, MP, EM, and WL contributed to data acquisition, data analysis, interpretation of results, and manuscript preparation. WL was the principal investigator of this project. All authors have read, edited, and approved the final manuscript.

FUNDING

This study was supported by grants from the Canadian Institutes for Health Research (CIHR FDN-143345) and Scholarships from CIHR. EM is a Vanier Canada Graduate Scholar, and is additionally supported by UBC Faculty of Medicine.

REFERENCES

- Arab, K., Park, Y. J., Lindroth, A. M., Schafer, A., Oakes, C., Weichenhan, D., et al. (2014). Long noncoding RNA TARID directs demethylation and activation of the tumor suppressor TCF21 via GADD45A. *Mol. Cell* 55, 604–614. doi: 10.1016/j.molcel.2014.06.031
- Balbin, O. A., Malik, R., Dhanasekaran, S. M., Prensner, J. R., Cao, X., Wu, Y. M., et al. (2015). The landscape of antisense gene expression in human cancers. *Genome Res.* 25, 1068–1079. doi: 10.1101/gr.180596.114
- Bhan, A., Soleimani, M., and Mandal, S. S. (2017). Long noncoding RNA and cancer: a new paradigm. *Cancer Res.* 77, 3965–3981. doi: 10.1158/0008-5472.CAN-16-2634
- Blume, C. J., Hotz-Wagenblatt, A., Hullein, J., Sellner, L., Jethwa, A., Stolz, T., et al. (2015). p53-dependent non-coding RNA networks in chronic lymphocytic leukemia. *Leukemia* 29, 2015–2023. doi: 10.1038/leu.2015.119
- Chan, B. A., and Hughes, B. G. (2015). Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. *Transl. Lung. Cancer Res.* 4, 36–54. doi: 10.3978/j.issn.2218-6751.2014.05.01
- Chan, D. A., and Giaccia, A. J. (2010). PHD2 in tumour angiogenesis. *Br. J. Cancer* 103, 1–5. doi: 10.1038/sj.bjc.6605682
- Chen, E. G., Zhang, J. S., Xu, S., Zhu, X. J., and Hu, H. H. (2017). Long non-coding RNA DGCR5 is involved in the regulation of proliferation, migration and invasion of lung cancer by targeting miR-1180. *Am. J. Cancer Res.* 7, 1463–1475.
- Chen, L., Liu, T., Zhou, J., Wang, Y., Wang, X., Di, W., et al. (2014). Citrate synthase expression affects tumor phenotype and drug resistance in human ovarian carcinoma. *PLoS One* 9:e115708. doi: 10.1371/journal.pone.0115708
- Chen, X., Shao, W., Huang, H., Feng, X., Yao, S., and Ke, H. (2018). Overexpression of RCN1 correlates with poor prognosis and progression in non-small cell lung cancer. *Hum. Pathol.* 83, 140–148. doi: 10.1016/j.humpath.2018.08.014
- Crooke, S. T., Witztum, J. L., Bennett, C. F., and Baker, B. F. (2018). RNA-targeted therapeutics. *Cell Metab.* 27, 714–739. doi: 10.1016/j.cmet.2018.03.004
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635
- Dong, H. X., Wang, R., Jin, X. Y., Zeng, J., and Pan, J. (2018). LncRNA DGCR5 promotes lung adenocarcinoma (LUAD) progression via inhibiting hsa-mir-22-3p. *J. Cell Physiol.* 233, 4126–4136. doi: 10.1002/jcp.26215

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00138/full#supplementary-material>

FIGURE S1 | Expression of lncRNA CTC-250I14.3 is significantly associated with Stage I disease in both the BCCA and TCGA LUAD cohorts (Mann Whitney U -test).

TABLE S1 | Detailed information of all pseudogene-derived lncRNAs.

TABLE S2 | Statistical analysis of the deregulated lncRNAs in the BCCA and TCGA cohorts (BHC-corrected p -values).

TABLE S3 | Number of PUBMED entries for lncRNA cancer-association.

TABLE S4 | Genomic locations of significant lncRNAs and their respective parent genes.

TABLE S5 | Expression correlations between the lncRNAs and their respective parent genes in the BCCA and TCGA cohorts.

TABLE S6 | Spearman's correlation coefficients for global expression analysis of Ψ -lnc-parent pairs.

TABLE S7 | Parent gene information for pseudogenes contained in Retrolali5 and Yale60 databases.

- Drouin, G. (2006). Processed pseudogenes are more abundant in human and mouse X chromosomes than in autosomes. *Mol. Biol. Evol.* 23, 1652–1655. doi: 10.1093/molbev/msl048
- Feng, F., Qiu, B., Zang, R., Song, P., and Gao, S. (2017). Pseudogene PHBP1 promotes esophageal squamous cell carcinoma proliferation by increasing its cognate gene PHB expression. *Oncotarget* 8, 29091–29100. doi: 10.18632/oncotarget.16196
- Flicek, P., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., et al. (2014). Ensembl 2014. *Nucleic Acids Res.* 42, D749–D755. doi: 10.1093/nar/gkt1196
- Glenfield, C., and McLysaght, A. (2018). Pseudogenes provide evolutionary evidence for the competitive endogenous RNA hypothesis. *Mol. Biol. Evol.* 35, 2886–2899. doi: 10.1093/molbev/msy183
- Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). circlize Implements and enhances circular visualization in R. *Bioinformatics* 30, 2811–2812. doi: 10.1093/bioinformatics/btu393
- Gutschner, T., Hammerle, M., Eissmann, M., Hsu, J., Kim, Y., Hung, G., et al. (2013). The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. *Cancer Res.* 73, 1180–1189. doi: 10.1158/0008-5472.CAN-12-2850
- Hirose, T., Virnicchi, G., Tanigawa, A., Naganuma, T., Li, R., Kimura, H., et al. (2014). NEAT1 long noncoding RNA regulates transcription via protein sequestration within subnuclear bodies. *Mol. Biol. Cell* 25, 169–183. doi: 10.1091/mbc.E13-09-0558
- Hu, X., Yang, L., and Mo, Y. Y. (2018). Role of pseudogenes in tumorigenesis. *Cancers* 10:256. doi: 10.3390/cancers10080256
- Huang, J. L., Cao, S. W., Ou, Q. S., Yang, B., Zheng, S. H., Tang, J., et al. (2018). The long non-coding RNA PTTG3P promotes cell growth and metastasis via up-regulating PTTG1 and activating PI3K/AKT signaling in hepatocellular carcinoma. *Mol. Cancer* 17:93. doi: 10.1186/s12943-018-0841-x
- Jiang, H., Huang, G., Zhao, N., Zhang, T., Jiang, M., He, Y., et al. (2018). Long non-coding RNA TPT1-AS1 promotes cell growth and metastasis in cervical cancer via acting AS a sponge for miR-324-5p. *J. Exp. Clin. Cancer Res.* 37:169. doi: 10.1186/s13046-018-0846-8
- Johnsson, P., Ackley, A., Vidarsdottir, L., Lui, W. O., Corcoran, M., Grander, D., et al. (2013). A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. *Nat. Struct. Mol. Biol.* 20, 440–446. doi: 10.1038/nsmb.2516
- Kassiotis, G. (2014). Endogenous retroviruses and the development of cancer. *J. Immunol.* 192, 1343–1349. doi: 10.4049/jimmunol.1302972

- Khachane, A. N., and Harrison, P. M. (2009). Assessing the genomic evidence for conserved transcribed pseudogenes under selection. *BMC Genomics* 10:435. doi: 10.1186/1471-2164-10-435
- Kjems, J., and Howard, K. A. (2012). Oligonucleotide delivery to the lung: waiting to inhale. *Mol. Ther. Nucleic Acids* 1:e1. doi: 10.1038/mtna.2011.1
- Kung, J. T., Colognori, D., and Lee, J. T. (2013). Long noncoding RNAs: past, present, and future. *Genetics* 193, 651–669. doi: 10.1534/genetics.112.146704
- Lee, J. T., Davidow, L. S., and Warshawsky, D. (1999). Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat. Genet.* 21, 400–404. doi: 10.1038/7734
- Li, C. H., and Chen, Y. (2013). Targeting long non-coding RNAs in cancers: progress and prospects. *Int. J. Biochem. Cell Biol.* 45, 1895–1910. doi: 10.1016/j.biocel.2013.05.030
- Luo, J., Zhu, H., Jiang, H., Cui, Y., Wang, M., Ni, X., et al. (2018). The effects of aberrant expression of lncRNA DGCR5/miR-873-5p/TUSC3 in lung cancer cell progression. *Cancer Med.* doi: 10.1002/cam4.1566 [Epub ahead of print].
- Maldonado, M. D. M., and Dharmawardhane, S. (2018). Targeting Rac and Cdc42 GTPases in cancer. *Cancer Res.* 78, 3101–3111. doi: 10.1158/0008-5472.CAN-18-0619
- Milligan, M. J., Harvey, E., Yu, A., Morgan, A. L., Smith, D. L., Zhang, E., et al. (2016). Global intersection of long non-coding RNAs with processed and unprocessed pseudogenes in the human genome. *Front. Genet.* 7:26. doi: 10.3389/fgene.2016.00026
- Munschauer, M., Nguyen, C. T., Sirokman, K., Hartigan, C. R., Hogstrom, L., Engreitz, J. M., et al. (2018). The NORAD lncRNA assembles a topoisomerase complex critical for genome stability. *Nature* 561, 132–136. doi: 10.1038/s41586-018-0453-z
- Peng, W. X., Koirala, P., and Mo, Y. Y. (2017). lncRNA-mediated regulation of cell signaling in cancer. *Oncogene* 36, 5661–5667. doi: 10.1038/onc.2017.184
- Perdomo, R., Alvarez, C., Geninazzi, H., Ferreira, C., Monti, J., Parada, R., et al. (1988). Early diagnosis of hydatidosis by ultrasonography. *Lancet* 1:244. doi: 10.1016/S0140-6736(88)91097-5
- Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J., and Pandolfi, P. P. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 465, 1033–1038. doi: 10.1038/nature09144
- Simon, M. D., Pinter, S. F., Fang, R., Sarma, K., Rutenberg-Schoenberg, M., Bowman, S. K., et al. (2013). High-resolution Xist binding maps reveal two-step spreading during X-chromosome inactivation. *Nature* 504, 465–469. doi: 10.1038/nature12719
- Sun, M., Nie, F. Q., Zang, C., Wang, Y., Hou, J., Wei, C., et al. (2017). The pseudogene DUXAP8 promotes non-small-cell lung cancer cell proliferation and invasion by epigenetically silencing EGR1 and RHOB. *Mol. Ther.* 25, 739–751. doi: 10.1016/j.ymthe.2016.12.018
- Tano, K., Mizuno, R., Okada, T., Rakwal, R., Shibato, J., Masuo, Y., et al. (2010). MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. *FEBS Lett.* 584, 4575–4580. doi: 10.1016/j.febslet.2010.10.008
- von Stechow, L., Typas, D., Carreras Puigvert, J., Oort, L., Siddappa, R., Pines, A., et al. (2015). The E3 ubiquitin ligase ARIH1 protects against genotoxic stress by initiating a 4EHP-mediated mRNA translation arrest. *Mol. Cell. Biol.* 35, 1254–1268. doi: 10.1128/MCB.01152-14
- Wang, K. C., Yang, Y. W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., et al. (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* 472, 120–124. doi: 10.1038/nature09819
- Xu, Y., Zheng, Y., Liu, H., and Li, T. (2017). Modulation of IGF2BP1 by long non-coding RNA HCG11 suppresses apoptosis of hepatocellular carcinoma cells via MAPK signaling transduction. *Int. J. Oncol.* 51, 791–800. doi: 10.3892/ijo.2017.4066
- Yamazaki, T., Fujikawa, C., Kubota, A., Takahashi, A., and Hirose, T. (2018). CRISPRa-mediated NEAT1 lncRNA upregulation induces formation of intact paraspeckles. *Biochem. Biophys. Res. Commun.* 504, 218–224. doi: 10.1016/j.bbrc.2018.08.158
- Zack, T. I., Schumacher, S. E., Carter, S. L., Cherniack, A. D., Saksena, G., Tabak, B., et al. (2013). Pan-cancer patterns of somatic copy number alteration. *Nat. Genet.* 45, 1134–1140. doi: 10.1038/ng.2760
- Zhang, M., Weng, W., Zhang, Q., Wu, Y., Ni, S., Tan, C., et al. (2018). The lncRNA NEAT1 activates Wnt/beta-catenin signaling and promotes colorectal cancer progression via interacting with DDX5. *J. Hematol. Oncol.* 11:113. doi: 10.1186/s13045-018-0656-7
- Zhang, Y., Zhang, P., Wan, X., Su, X., Kong, Z., Zhai, Q., et al. (2016). Downregulation of long non-coding RNA HCG11 predicts a poor prognosis in prostate cancer. *Biomed. Pharmacother.* 83, 936–941. doi: 10.1016/j.biopha.2016.08.013

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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LncRNAs GIHCG and SPINT1-AS1 Are Crucial Factors for Pan-Cancer Cells Sensitivity to Lapatinib

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OPEN ACCESS

Edited by:

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Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 15 November 2018

Accepted: 16 January 2019

Published: 19 February 2019

Citation:

Xiang Z, Song S, Zhu Z, Sun W,
Gifts JE, Sun S, Li QS, Yu Y and Li KK
(2019) LncRNAs GIHCG and
SPINT1-AS1 Are Crucial Factors for
Pan-Cancer Cells Sensitivity to
Lapatinib. *Front. Genet.* 10:25.
doi: 10.3389/fgene.2019.00025

Lapatinib is a small molecule inhibitor of EGFR (HER1) and ERBB2 (HER2) receptors, which is used for treatment of advanced or metastatic breast cancer. To find the drug resistance mechanisms of treatment for EGFR/ERBB2 positive tumors, we analyzed the possible effects of lncRNAs. In this study, using CCLE (Cancer Cell Line Encyclopedia) database, we explored the relationship between the lncRNAs and Lapatinib sensitivity/resistance, and then validated those findings through *in vitro* experiments. We found that the expression of EGFR/ERBB2 and activation of ERBB pathway was significantly related to Lapatinib sensitivity. GO (Gene Ontology) analysis of top 10 pathways showed that the sensitivity of Lapatinib was positively correlated with cell keratin, epithelial differentiation, and cell-cell junction, while negatively correlated with signatures of extracellular matrix. Forty-four differentially expressed lncRNAs were found between the Lapatinib sensitive and resistant groups (fold-change > 1.5, $P < 0.01$). Gene set variation analysis (GSVA) was performed based on 44 lncRNAs and genes in the top 10 pathways. Five lncRNAs were identified as hub molecules. Co-expression network was constructed by more than five lncRNAs and 199 genes in the top 10 pathways, and three lncRNAs (GIHCG, SPINT1-AS1, and MAGI2-AS3) and 47 genes were identified as close-related molecules. The three lncRNAs in epithelium-derived cancers were differentially expressed between sensitive and resistant groups, but no significance was found in non-epithelium-derived cancer cells. Correlation analysis showed that SPINT1-AS1 ($R = -0.715$, $P < 0.001$) and GIHCG ($R = 0.557$, $P = 0.013$) were correlated with the IC50 of epithelium-derived cancer cells. In further experiments, GIHCG knockdown enhanced cancer cell susceptibility to Lapatinib, while high level of SPINT1-AS1 was a sensitive biomarker of NCI-N87 and MCF7 cancer cells to Lapatinib. In conclusions, lncRNAs GIHCG and SPINT1-AS1 were involved in regulating Lapatinib sensitivity. Up-regulation of GIHCG was a drug-resistant biomarker, while up-regulation of SPINT1-AS1 was a sensitive indicator.

Keywords: pan-cancer, computational analysis, lncRNAs, lapatinib, targeted therapy

INTRODUCTION

Lapatinib is a small molecular drug that has been shown to be a dual tyrosine kinase inhibitor, which is involved in the EGFR/HER1 and ERBB2/HER2 pathways and suppresses the autophosphorylation of these receptors. Clinically, it has been used in combination therapy with capecitabine in patients with advanced or metastatic breast cancer that overexpressed ERBB2/HER2 in the cases of previous treatment with anthracyclines, taxanes, or trastuzumab (Herceptin) (Geyer et al., 2006). In addition, a satisfactory response rate has also been found with Lapatinib treatment for ERBB2-positive progressive gastric cancer (Cetin et al., 2014; Satoh et al., 2014). However, in patients with head and neck squamous cell carcinoma, Lapatinib combined with radiotherapy did not show therapeutic effects (Harrington et al., 2015). Similarly, in ERBB2/EGFR positive metastatic bladder cancer patients who underwent first-line chemotherapy didn't get benefit from Lapatinib maintenance treatment (Powles et al., 2017). Therefore, uncovering the drug-resistant mechanism of Lapatinib will help improve the therapeutic effects of Lapatinib targeted therapy and find new sensitive biomarkers.

Long non-coding RNAs (lncRNAs) are a large class of transcribed RNA molecules that are longer than 200 nucleotides but do not encode proteins. In addition to the regulation of diverse cellular processes, such as epigenetics, cell cycle, and cell differentiation, they have been found to play important roles in carcinogenesis, tumor development, and treatment resistance (Heery et al., 2017; Peng et al., 2017; Hahne and Valeri, 2018; Wang et al., 2018; Wu et al., 2018). For instance, Ma et al. found that lncRNAs CASC9 and EWAST1 were two crucial molecules associated to EGFR-TKIs resistant in non-small cell lung cancer (Ma et al., 2017).

The Cancer Cell Line Encyclopedia (CCLE) database (<https://portals.broadinstitute.org/ccle>) is an open access resource with the most completely integrated datasets of cancer cells genomes and drug effectiveness. It includes the experimental datasets of drug treatment of 24 kinds of chemical compounds in almost 1,000 cancer cell lines of various human cancers (Barretina et al., 2012). Kim et al. used CCLE database in their recent publication. They found that high levels of FGFR and integrin $\beta 3$ are resistant to crizotinib treatment, suggesting that FGFR, and integrin $\beta 3$ could be predictive markers for Met-targeted therapy (Kim et al., 2015). To date, there is a limited number of studies (Jiang et al., 2014; Niknafs et al., 2016; Bester et al., 2018; Li D. et al., 2018; Sun et al., 2018) to explore lncRNAs by CCLE database. In this study, we analyzed the lncRNAs of whole-genome datasets of CCLE after treatment with Lapatinib on pan-cancer cell lines, and proposed crucial lncRNAs GIHCG and SPINT1-AS1 involved in regulating Lapatinib sensitivity.

MATERIALS AND METHODS

Data Extraction From CCLE

There are 5,344 lncRNA probes and 49,331 non-lncRNA probes in the whole-genome gene expression profile chip used in CCLE (Barretina et al., 2012). There are 1,037 cell lines of various cancer

types in the database. Among those, 504 cell lines had been treated with Lapatinib and got IC50 (half maximal inhibitory concentration) data and 501 cell lines were examined by microarrays. Since the study focused on solid tumors, we deleted cell lines of hematopoietic and lymphoid cell lines. Finally, 420 solid tumor cell lines were enrolled in the study (Table 1).

Cancer Cell Lines and Cell Culture

Nineteen cancer cell lines were used for validating experiments *in vitro*. Four of those were gastric cancer cell lines (NCI-N87, SGC-7901, AGS, and MKN-45), three were melanoma cell lines (MuM-2C, MV3, and A-375), three were hepatocarcinoma cell lines (LM3, 97L, and Huh7), three were thyroid cancer cell lines (KHM-5M, CAL-62, and C643), two were breast cancer cell lines (MCF7 and SK-BR-3), two were pancreatic cancer cell lines (TCC-PAN2 and BxPC3), and two were colorectal cancer lines (DLD-1 and NCIH-747). Cell lines NCI-N87, MuM-2C, LM3, MV3, Huh7, SGC-7901, CAL-62, AGS, MCF7, C643, 97L, SK-BR-3, KHM-5M, A-375, TCC-PAN2, MKN-45, and BxPC3 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cell lines DLD-1 and NCIH-747 were purchased from The Global Bioresource Center ATCC (Maryland, USA). The cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with 95% air and 5% CO₂.

Transient Transfection of siRNAs

SPINT1-AS1 and GIHCG siRNAs were transfected into cancer cells by Lipofectamine 2000 (Invitrogen, Carlsbad, California,

TABLE 1 | The distribution of 420 cancer cell lines of solid tumor.

Cancer types	Count
Autonomic ganglia	10
Biliary tract	1
Bone	11
Breast	29
Central nervous system	29
Endometrium	20
Kidney	9
Large intestine	23
Liver	19
Lung	91
Esophagus	15
Ovary	28
Pancreas	28
Pleura	7
Prostate	3
Salivary gland	1
Skin	40
Soft tissue	12
Stomach	18
Thyroid	5
Upper aerodigestive tract	7
Urinary tract	14

TABLE 2 | Lapatinib IC50 of 420 cancer cell lines.

CCLE cell line names	Cell type	IC50 (μM)*
SNU1	Stomach	8
KMRC2	Kidney	8
HEYA8	Ovary	8
NCIH1915	Lung	8
SH10TC	Stomach	8
JMSU1	Urinary tract	8
UACC62	Skin	8
SKLU1	Lung	8
ES2	Ovary	8
SNU398	Liver	8
MSTO211H	Pleura	8
HMC18	Breast	8
HS229T	Lung	8
HS895T	Skin	8
NCIH1092	Lung	8
8505C	Thyroid	8
RKO	Large intestine	8
SW1573	Lung	8
NCIH2172	Lung	8
IGR37	Skin	8
T24	Urinary tract	8
NCIH1581	Lung	8
HLF	Liver	8
MG63	Bone	8
HS840T	Upper aerodigestive tract	8
DMS114	Lung	8
HS936T	Skin	8
FU97	Stomach	8
NCIH2052	Pleura	8
8305C	Thyroid	8
RERFLCAI	Lung	8
SW579	Thyroid	8
TOV112D	Ovary	8
HS729	Soft tissue	8
KMRC1	Kidney	8
SJSA1	Bone	8
HUH1	Liver	8
1321N1	Central nervous system	8
TC71	Bone	8
KELLY	Autonomic ganglia	8
NCIH520	Lung	8
IGR39	Skin	8
EN	Endometrium	8
U118MG	Central nervous system	8
639V	Urinary tract	8
HGC27	Stomach	8
UMUC3	Urinary tract	8
42MGBA	Central nervous system	8
SKNBE2	Autonomic ganglia	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μM)*
CALU1	Lung	8
NCIH211	Lung	8
HEC59	Endometrium	8
BFTC909	Kidney	8
RPMI7951	Skin	8
IPC298	Skin	8
NCIH1651	Lung	8
MDAMB436	Breast	8
SKNDZ	Autonomic ganglia	8
DKMG	Central nervous system	8
IALM	Lung	8
NCIH1792	Lung	8
JHH6	Liver	8
PSN1	Pancreas	8
HOS	Bone	8
CAL78	Bone	8
U87MG	Central nervous system	8
GI1	Central nervous system	8
NCIH1155	Lung	8
SBC5	Lung	8
IMR32	Autonomic ganglia	8
NCIH460	Lung	8
WM2664	Skin	8
MEWO	Skin	8
BT549	Breast	8
SKMEL30	Skin	8
NCIH1703	Lung	8
HEP3B217	Liver	8
TT2609C02	Thyroid	8
HEPG2	Liver	8
SKNAS	Autonomic ganglia	8
NCIH1944	Lung	8
SW1271	Lung	8
COLO679	Skin	8
DAOY	Central nervous system	8
SHP77	Lung	8
NCIH1299	Lung	8
VMRCRCZ	Kidney	8
LOXIMVI	Skin	8
NCIH1339	Lung	8
HS746T	Stomach	8
SKHEP1	Liver	8
NCIH1694	Lung	8
COV504	Ovary	8
NCIH1793	Lung	8
SNU423	Liver	8
JHUEM2	Endometrium	8
CALU6	Lung	8
J82	Urinary tract	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μ M)*
UACC257	Skin	8
G402	Soft tissue	8
MESSA	Soft tissue	8
HT1080	Soft tissue	8
MPP89	Pleura	8
OVTOKO	Ovary	8
SUIT2	Pancreas	8
SIMA	Autonomic ganglia	8
H4	Central nervous system	8
WM1799	Skin	8
A673	Bone	8
NCIH1975	Lung	8
MDAMB157	Breast	8
SKMEL5	Skin	8
SKES1	Bone	8
NCIH2452	Pleura	8
NCIH647	Lung	8
SAOS2	Bone	8
NCIH2023	Lung	8
NCIH226	Lung	8
SF295	Central nervous system	8
SW620	Large intestine	8
NCIH661	Lung	8
HS939T	Skin	8
HS578T	Breast	8
HCC44	Lung	8
EFO21	Ovary	8
KPNSI9S	Autonomic ganglia	8
SF126	Central nervous system	8
HS739T	Breast	8
NCIH1693	Lung	8
TOV21G	Ovary	8
KALS1	Central nervous system	8
A375	Skin	8
CHP212	Autonomic ganglia	8
SW1990	Pancreas	8
LOUNH91	Lung	8
OV90	Ovary	8
SKMEL2	Skin	8
NCIH23	Lung	8
YKG1	Central nervous system	8
WM88	Skin	8
ACHN	Kidney	8
SKNFI	Autonomic ganglia	8
DU145	Prostate	8
GAMG	Central nervous system	8
MDAMB435S	Skin	8
NCIH2087	Lung	8
NCIH1563	Lung	8
HEC6	Endometrium	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μ M)*
NCIH2228	Lung	8
SW1353	Bone	8
RD	Soft tissue	8
SNU387	Liver	8
OC316	Ovary	8
SKNSH	Autonomic ganglia	8
FUOV1	Ovary	8
LCLC103H	Lung	8
HCC15	Lung	8
KNS60	Central nervous system	8
PK45H	Pancreas	8
HT1197	Urinary tract	8
KP4	Pancreas	8
GB1	Central nervous system	8
HT144	Skin	8
U2OS	Bone	8
HLE	Liver	8
COLO741	Skin	8
TCCSUP	Urinary tract	8
LN18	Central nervous system	8
NCIH810	Lung	8
JHH2	Liver	8
T98G	Central nervous system	8
QGP1	Pancreas	8
IGROV1	Ovary	8
LN229	Central nervous system	8
OVCAR4	Ovary	8
JHH4	Liver	8
HS944T	Skin	8
BCPAP	Thyroid	8
HS683	Central nervous system	8
NCIH2009	Lung	8
GMS10	Central nervous system	8
G401	Soft tissue	8
A172	Central nervous system	8
HEC1B	Endometrium	8
HEC251	Endometrium	8
SW900	Lung	8
OC315	Ovary	8
JHOS2	Ovary	8
RERFLCMS	Lung	8
ISTMES1	Pleura	8
RVH421	Skin	8
MFE296	Endometrium	8
HS766T	Pancreas	8
HCC78	Lung	8
MKN7	Stomach	8
C32	Skin	8
HEC265	Endometrium	8
NCIH1184	Lung	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μ M)*
SW480	Large intestine	8
NCIH522	Lung	8
NCIH650	Lung	8
OC314	Ovary	8
COV318	Ovary	8
HS852T	Skin	8
NCIH727	Lung	8
EFO27	Ovary	8
SJRH30	Soft tissue	8
KNS81	Central nervous system	8
SNU449	Liver	8
A2058	Skin	8
HS294T	Skin	8
SNU182	Liver	8
COLO205	Large intestine	8
HUCCT1	Biliary tract	8
ISHIKAWAHERAKLIO02ER	Endometrium	8
LS411N	Large intestine	8
PATU8902	Pancreas	8
PC3	Prostate	8
SKMEL24	Skin	8
C3A	Liver	8
AN3CA	Endometrium	8
SNGM	Endometrium	8
TE1	Esophagus	8
NCIH1573	Lung	8
HCT116	Large intestine	8
NCIH1568	Lung	8
HPAC	Pancreas	8
HEC151	Endometrium	8
OVMANA	Ovary	8
HCC56	Large intestine	8
HEC1A	Endometrium	8
CAK12	Kidney	8
CAPAN2	Pancreas	8
NCIH1373	Lung	8
NCIH1048	Lung	8
CAS1	Central nervous system	8
HCC1569	Breast	8
SNU475	Liver	8
LS123	Large intestine	8
NCIH1341	Lung	8
PANC0403	Pancreas	8
MOGGCCM	Central nervous system	8
IM95	Stomach	8
ONCODG1	Ovary	8
NCIH747	Large intestine	8
WM115	Skin	8
DBTRG05MG	Central nervous system	8
EFE184	Endometrium	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μ M)*
HS695T	Skin	8
KYM1	Soft tissue	8
MORCPR	Lung	8
CORL105	Lung	8
PL45	Pancreas	8
SQ1	Lung	8
TEN	Endometrium	8
T84	Large intestine	8
HCC1395	Breast	8
ZR751	Breast	8
RERFGC1B	Stomach	8
DETROIT562	Upper aerodigestive tract	8
DV90	Lung	8
SW780	Urinary tract	8
KYSE510	Esophagus	8
SKMEL31	Skin	8
NCIH1869	Lung	8
NCIH441	Lung	8
NCIH2085	Lung	8
CORL23	Lung	8
OCUM1	Stomach	8
SNUC2A	Large intestine	8
TE5	Esophagus	8
MKN45	Stomach	8
KP3	Pancreas	8
KNS42	Central nervous system	8
KLE	Endometrium	8
SW1417	Large intestine	8
KMBC2	Urinary tract	8
LC1SQSF	Lung	8
OVSAHO	Ovary	8
VMRCLCD	Lung	8
KP2	Pancreas	8
BT20	Breast	8
RT4	Urinary tract	8
EFM19	Breast	8
KYSE70	Esophagus	8
A253	Salivary gland	8
COLO201	Large intestine	8
SW48	Large intestine	8
SU8686	Pancreas	8
MFE280	Endometrium	8
CAMA1	Breast	8
KURAMOCHI	Ovary	8
COLO678	Large intestine	8
HUPT3	Pancreas	8
HCC1187	Breast	8
T47D	Breast	8
MDAMB415	Breast	8
HSC2	Upper aerodigestive tract	8

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μM)*
KYSE150	Esophagus	8
UACC812	Breast	8
ONS76	Central nervous system	8
KNS62	Lung	8
PANC1005	Pancreas	7.987659
ISTMES2	Pleura	7.889611
NCIH1355	Lung	7.860697
KYSE30	Esophagus	7.858886
22RV1	Prostate	7.847305
MIAPACA2	Pancreas	7.469959
JHOS4	Ovary	7.408363
A204	Soft tissue	7.399833
HCC70	Breast	7.36332
NCIH2286	Lung	7.359588
MALME3M	Skin	7.325411
GCIY	Stomach	7.255416
PK1	Pancreas	7.236271
786O	Kidney	7.178035
T3M10	Lung	7.170651
A2780	Ovary	7.146677
SKLMS1	Soft tissue	7.136584
HT1376	Urinary tract	7.084046
HUPT4	Pancreas	7.0557
PANC0327	Pancreas	6.904092
SW1088	Central nervous system	6.737086
SNU16	Stomach	6.697771
PLCPRF5	Liver	6.669433
HARA	Lung	6.656741
MELHO	Skin	6.552444
RT112	Urinary tract	6.525924
K029AX	Skin	6.444433
EBC1	Lung	6.372372
MCAS	Ovary	6.3241
COLO320	Large intestine	6.295312
PK59	Pancreas	6.190494
HT29	Large intestine	5.884947
TE9	Esophagus	5.855279
WM983B	Skin	5.68912
KCIMOHI	Pancreas	5.619114
TYKNU	Ovary	5.343411
8MGBA	Central nervous system	5.22662
PANC0203	Pancreas	5.197284
NCIH1650	Lung	5.152449
NIHOVCAR3	Ovary	5.117735
OVCAR8	Ovary	5.095931
JHH7	Liver	4.92477
HMCB	Skin	4.767848
MKN74	Stomach	4.689733
HCT15	Large intestine	4.666833
WM793	Skin	4.641666

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μM)*
BXPC3	Pancreas	4.599786
HCC1806	Breast	4.378565
ESS1	Endometrium	4.373962
SCC9	Upper aerodigestive tract	4.287216
MHHES1	Bone	4.274786
A549	Lung	4.227246
HPAFII	Pancreas	4.222833
GCT	Soft tissue	4.213955
C2BBE1	Large intestine	4.099345
KE39	Stomach	4.05606
LU99	Lung	3.926637
VMRCRCW	Kidney	3.895097
KYSE410	Esophagus	3.808475
KYSE520	Esophagus	3.773011
NCIH2030	Lung	3.72418
OE33	Esophagus	3.538352
HDQP1	Breast	3.104604
G361	Skin	3.047757
RL952	Endometrium	3.012983
NCIH2122	Lung	2.934416
NCIH28	Pleura	2.911829
LS513	Large intestine	2.880553
MCF7	Breast	2.845194
NCIH358	Lung	2.83834
ASPC1	Pancreas	2.785628
KYSE450	Esophagus	2.574543
NUGC3	Stomach	2.410753
SCC25	Upper aerodigestive tract	2.398599
SW403	Large intestine	2.379555
LUDLU1	Lung	2.319642
MDAMB468	Breast	2.312559
5637	Urinary tract	2.307768
PC14	Lung	2.149659
L33	Pancreas	2.124577
CAL12T	Lung	1.951666
CAL851	Breast	1.899548
HCC4006	Lung	1.854881
NCIH2444	Lung	1.746528
AZ521	Stomach	1.659918
SCABER	Urinary tract	1.511766
SKMES1	Lung	1.476444
HCC1954	Breast	1.457828
MDAMB453	Breast	1.4379
NCIH322	Lung	1.362128
TE15	Esophagus	1.285878
HCC2935	Lung	1.239924
769P	Kidney	1.057461
MFE319	Endometrium	1.026923
SKOV3	Ovary	0.983712
KYSE180	Esophagus	0.876243

(Continued)

TABLE 2 | Continued

CCLE cell line names	Cell type	IC50 (μM)*
FADU	Upper aerodigestive tract	0.823073
SKCO1	Large intestine	0.71562
KYSE140	Esophagus	0.68893
CAL27	Upper aerodigestive tract	0.688771
CHL1	Skin	0.675993
TE11	Esophagus	0.63775
JHH5	Liver	0.569108
CALU3	Lung	0.494588
MDAMB175V1	Breast	0.468741
NCIH1666	Lung	0.386496
NCIH1648	Lung	0.373409
HCC827	Lung	0.372134
NCIH3255	Lung	0.333763
NCIH2170	Lung	0.300981
TE617T	Soft tissue	0.242928
CCK81	Large intestine	0.240195
SKBR3	Breast	0.196392
AU565	Breast	0.18321
NUGC4	Stomach	0.171543
ZR7530	Breast	0.166593
BT474	Breast	0.116183
NCIN87	Stomach	0.066107

*Extracted from CCLE database (<https://portals.broadinstitute.org/ccle>).

IC50 (μM) is half maximal inhibitory concentration (IC50), which is defined as a drug concentration producing absolute 50% inhibition of growth in cell proliferation assay. By definition, this metric relies on the assumption, that at a high concentration of the drug, 100% effect is achieved as all cells die in a proliferation assay.

USA) according to the manufacturer's instructions. The siRNA sequences are shown in Table S1.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated using the TRIzol solution (Invitrogen, California, USA). The cDNA was synthesized using Reverse Transcription kit (TOYOBO, Japan). Real-time PCR was performed in 10 μl reaction mixtures with the HT 7900 (Applied Biosystems, Foster City, USA) using SYBRTM Select Master Mix (Applied Biosystems, Foster City, USA). The sequences of primers were designed and synthesized by Sunny Biotech (Shanghai, China): The primer sequences are shown in Table S1.

Cell Viability Assay

Five thousand cells of different cancers were placed in each well of 96-well plates (100 μl /well). Different concentrations of Lapatinib (Selleck, Houston, USA) were incubated for 48 h. After adding 10 μl CCK-8 for 2 h, OD value was measured at 450 nm by spectrophotometry (BioTek, Vermont, USA).

Data Analysis

The “corrplot” and “pheatmap” package in R software were utilized for visualizing pearson correlation analysis and cluster analysis by “euclidean” method. The Benjamini and Hochberg method was used to calculate P. adjust value. By means of “clusterProfiler” package in R, GSEA (Gene Set Enrichment

Analysis) and GO (Gene Ontology) analyses were carried out to explore involved gene clusters. GSEA is a computational method based on previous publication by Subramanian et al. (2005). GO analysis is a kind of gene enrichment analysis to classify gene set on three aspects: molecular function, cellular component and biological process (Ashburner et al., 2000). Differentially expressed lncRNAs and genes with difference larger than 1.5-fold were obtained by “limma” package, which is often used to explore differentially expressed genes between two phenotypes (Ritchie et al., 2015). The top 10 gene clusters of all cancer cell lines were scored using “GSVA” package (Gene Set Variation Analysis,) in R language, which utilizes non-parametric unsupervised method for evaluating gene set enrichment (GSE) in transcriptomic data (Hanzelmann et al., 2013). Cytoscape software was applied to establish co-expression network and determine hub lncRNAs. The inhibiting ratio and Lapatinib IC50 were calculated according to OD value by GraphPad Prism 6.0 (Inc., La Jolla, CA, USA). The relative RNA levels were calculated by $2^{-\Delta\Delta\text{CT}}$ ($\Delta\text{CT} = \text{LncRNA}^{\text{CT value}} - \text{GAPDH}^{\text{CT value}}$, $\Delta\Delta\text{CT} = \Delta\text{CT} - \Delta\text{CT}^{\text{min}}$, $\Delta\text{CT}^{\text{min}}$: minimum ΔCT of expression levels of lncRNA GIHCG or SPINT1-AS1 in cell line). Student's *t*-tests were performed by GraphPad Prism 6.0. *P* < 0.05 was considered statistically significant.

RESULTS

Lapatinib IC50 From Pan-Cancer Cell Lines Analysis

The CCLE data of Lapatinib IC50 of the selected 420 cell lines was shown in Table 2. The upper limit of IC50 was originally determined as 8 μM for those cancer cell lines in the database. There were 302 cancer cell lines with IC50 higher than 8 μM , which were insensitive to Lapatinib drug. There were 118 cancer cell lines with IC50 lower than 8 μM , which were relatively sensitive to Lapatinib drug. Taking 8 μM of IC50 as a threshold, we categorized 420 cancer cell lines into two groups, high_IC50 (*n* = 302) and low_IC50 (*n* = 118). Since EGFR and ERBB2 are the targets of the Lapatinib drug, the expression levels of EGFR, and ERBB2 in high_IC50 and low_IC50 groups were analyzed. The expression levels of EGFR and ERBB2 were significantly higher in low-IC50 group than in high-IC50 (Figure 1A, *P* = 0.006 and *P* < 0.001, respectively). The distribution tendency of 22 types of solid cancer cell lines in high-IC50 (up to 8 μM) and low_IC50 (lower than 8 μM) groups is presented in Figure 1B. GSEA analysis showed that ERBB pathway-related genes were enriched in low_IC50 group (Figure 1C, ERBB signaling pathway NES = −1.81, *P* < 0.002, p. adjust = 0.064; regulation of ERBB signaling pathway NES = −1.69, *P* < 0.002, p. adjust = 0.064).

Pathway Analysis Involved in Lapatinib Sensitivity

To illustrate the mechanism of Lapatinib resistance, we selected genes with fold-change > 1.5 times to perform GO analysis (Table S2). In the top 10 involved pathways, Lapatinib sensitivity was positively associated with cell keratin, epithelial differentiation,

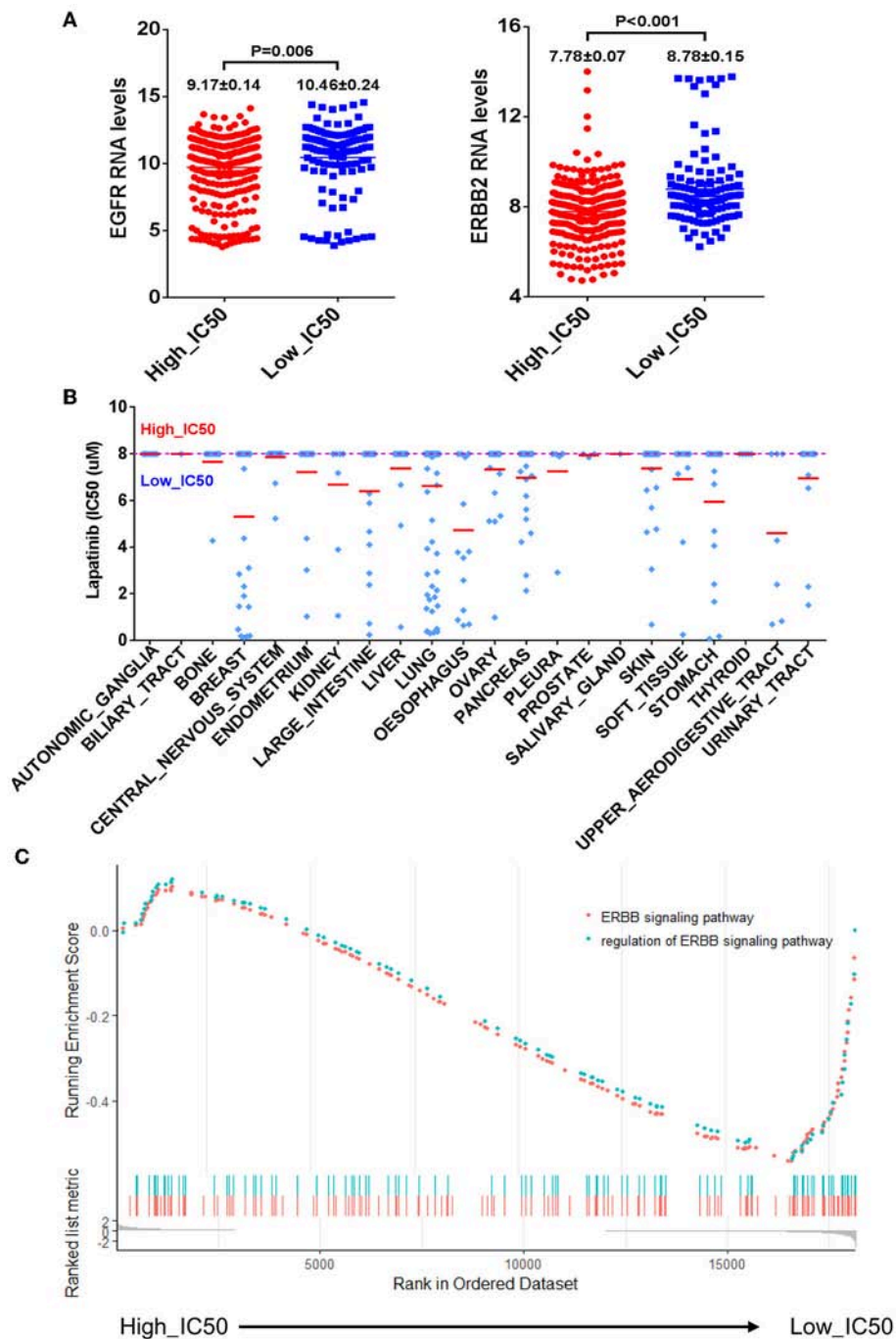
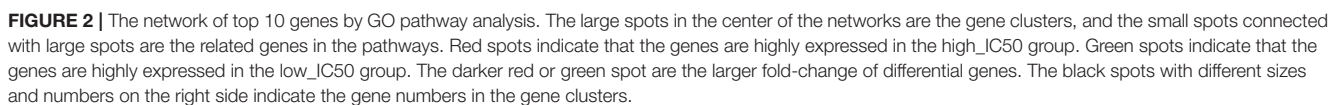
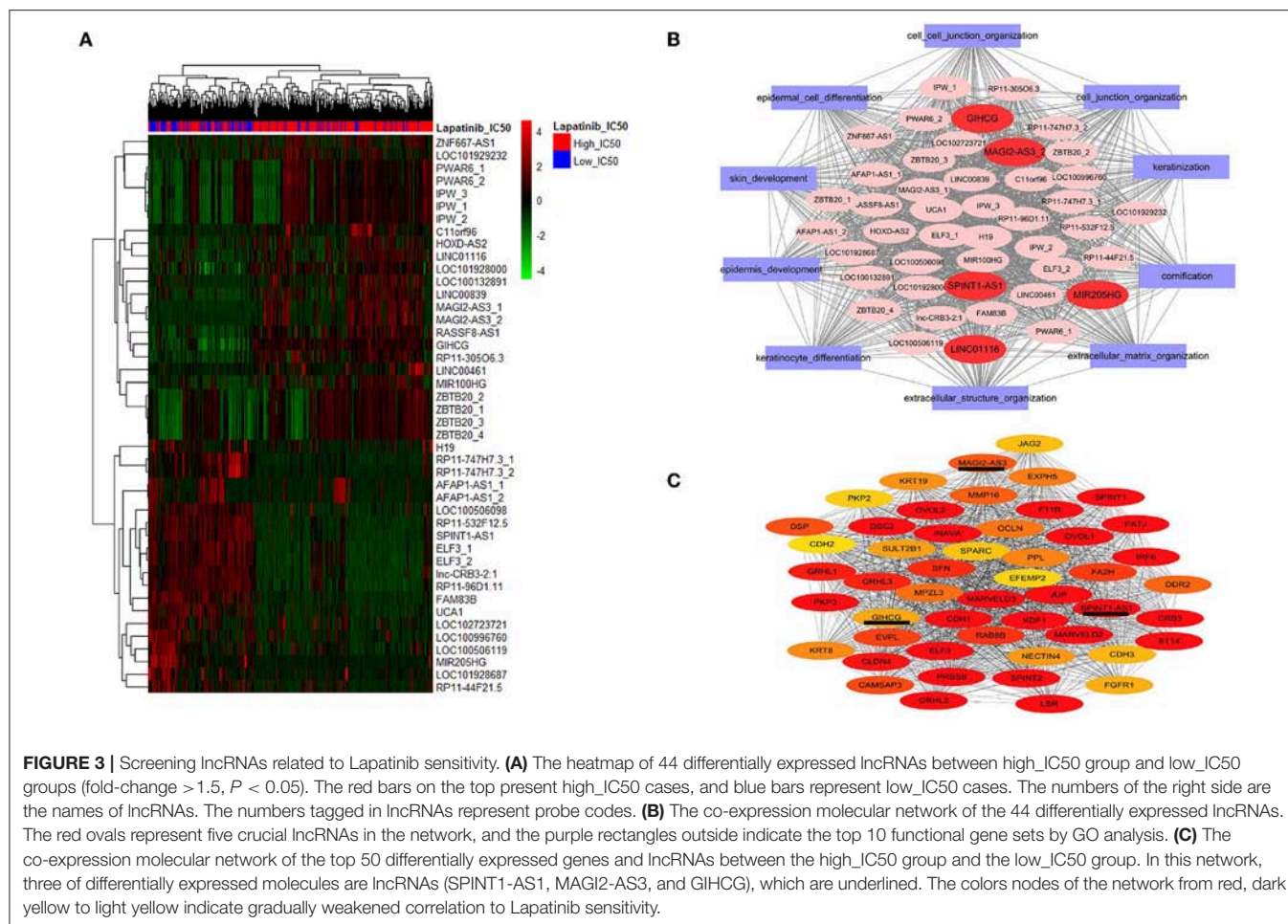


FIGURE 1 | The correlation of mRNA expression levels of EGFR and ERBB2 and Lapatinib IC50. **(A)** The bar charts of mRNA expression levels of EGFR (left) and ERBB2 (right) of cancer cell lines between the high_IC50 and low_IC50 groups of Lapatinib drug. The expression levels of EGFR and ERBB2 are significantly higher in the low_IC50 group than that in the high_IC50 group ($p < 0.01$). **(B)** The distribution tendency of 22 types of solid cancer cell lines in high-IC50 (up to $8 \mu\text{M}$) and low_IC50 (lower than $8 \mu\text{M}$). The red lines represent mean value of Lapatinib IC50. **(C)** The enrichment analysis of ERBB signaling pathway reveals that ERBB signaling pathway is significantly enriched in Lapatinib low_IC50 group. “Y” axis indicates the enrichment score (ES) value, and “X” axis indicates genes according to differential expression value between high_IC50 and low_IC50 groups. The blue and red dot curves represent ES value. The bottom barcodes represent the leading gene set that strongly contributed to ES value. The positive ES value represents positive correlation to Lapatinib IC50, and minus ES value represents negative correlation to Lapatinib IC50.



We further screened the differentially expressed lncRNAs, and 44 lncRNAs were identified between the high_IC50 group and low_IC50 group (**Figure 3A** and **Table 3**, fold-change >1.5, $P < 0.01$). Then, we selected genes in the top 10 pathways and 44 differential lncRNAs for the construction of the co-expression network. The enrichment scores of the top 10 pathway genes in every cancer cell lines were calculated and determined by GSEA analysis. Five lncRNAs were highlighted as the hub factors in the top 10 regulating pathways (**Figure 3B**). The

We divided the 420 cancer cell lines into epithelium derived group ($n = 278$) and non-epithelium derived group ($n = 142$; including nervous system, bone, cartilage, and pleura). The differential expression levels of the three lncRNAs between the



two groups are presented in **Figure 4A**. In the epithelium-derived group, the differential expression levels of the three lncRNAs between Lapatinib high_IC50 and low_IC50 groups were significantly different (**Figure 4B**, $P < 0.05$). In the non-epithelium groups, there was no significant difference of the three lncRNAs between Lapatinib high_IC50 and low_IC50 groups. Higher expressing level of SPINT1-AS1 was found in epithelium-derived cancer cells, and higher expressing levels of MAGI2-AS3 and GIHCG were observed in the non-epithelium group.

Differentially expressed genes (1.5-fold change) between the Lapatinib high_IC50 and low_IC50 groups in epithelial group (Table S3) were utilized to perform GO analysis. Enhanced signatures of cell keratin, epithelial differentiation, and cell-cell junction were observed in Lapatinib low_IC50 group, and decreased signature of extracellular matrix were observed in Lapatinib low_IC50 group (**Figure 5**, $P < 0.001$, $P_{\text{adjust}} < 0.001$).

Correlation of LncRNAs SPINT1-AS1, GIHCG, or MAGI2-AS3 and Lapatinib Sensitivity in Epithelial Group

Correlation analysis revealed that Lapatinib IC50 of the non-epithelial group was higher than that of the epithelial group

(**Figure 6A**). Of the three critical lncRNAs, SPINT1-AS1, and GIHCG were the lncRNAs most correlated to Lapatinib sensitivity (**Figure 6B**). SPINT1-AS1 and GIHCG were selected as key factors of affecting Lapatinib sensitivity of epithelial cancers. The up-regulation of SPINT1-AS1 was found in low_IC50 group and increased GIHCG was found in high_IC50 group (**Figure 6C**).

Validating Study of GIHCG and SPINT1-AS1 on Regulating Lapatinib Sensitivity *in vitro*

In validating experiments, we examined expression levels of GIHCG and SPINT1-AS1 in seven types of cancer cell lines (thyroid cancer, pancreatic cancer, liver cancer, melanoma, gastric cancer, breast cancer, and colorectal cancer) and Lapatinib IC50 of the same cancer cell lines. Correlation analysis showed that higher expression levels of SPINT1-AS1 were significantly associated with lower Lapatinib IC50 (**Figure 7A**, $R = -0.715$, $P < 0.001$), while higher expression levels of GIHCG were significantly related to higher Lapatinib IC50 (**Figure 7A**, $R = 0.557$, $P = 0.013$).

The sensitive cancer cell lines of NCI-N87 (gastric cancer) and MCF7 (breast cancer), as well as the resistant cancer cell lines of NCIH-747 (colon cancer) and BxPC3 (pancreatic cancer)

TABLE 3 | Differentially expressed lncRNAs between Lapatinib high_IC50 and low_IC50 groups of 420 cancer cell lines (fold-change > 1.5, $P < 0.01$).

Probes	Title	Symbol	Ensemble transcript id version	Log FC	P-value	Adj. P-value
225381_at	mir-100-let-7a-2 cluster host gene (non-protein coding)	MIR100HG	ENSG00000255248.7	1.339024	4.98E-08	1.48E-05
226546_at	uncharacterized LOC100506844	GIHCG	ENSG00000257698.1	1.19665	1.52E-15	8.13E-12
228564_at	Long intergenic non-protein coding RNA 1116	LINC01116	ENSG00000163364.9	1.122804	4.24E-06	0.000493
227554_at	MAGI2 antisense RNA 3	MAGI2-AS3	ENSG00000234456.7	1.096172	2.73E-07	5.84E-05
1566482_at	NA	RP11-305O6.3	ENSG00000250280.2	0.961776	3.96E-08	1.24E-05
213156_at	Zinc finger and BTB domain containing 20	ZBTB20	ENSG00000259976.3	0.942404	6.68E-06	0.000649
213158_at	Zinc finger and BTB domain containing 20	ZBTB20	ENSG00000259976.3	0.908785	1.6E-05	0.001179
244741_s_at	ZNF667 antisense RNA 1 (head to head)	ZNF667-AS1	ENSG00000166770.10	0.873077	0.000703	0.019471
229480_at	MAGI2 antisense RNA 3	MAGI2-AS3	ENSG00000234456.7	0.870971	4.07E-07	8.05E-05
229493_at	HOXD cluster antisense RNA 2	HOXD-AS2	ENSG00000237380.6	0.795366	2.89E-07	5.94E-05
227082_at	Zinc finger and BTB domain containing 20	ZBTB20	ENSG00000259976.3	0.780225	5.64E-05	0.003174
226587_at	Prader Willi/Angelman region RNA 6	PWAR6	ENSG00000257151.1	0.777959	0.0002	0.008638
242358_at	RASSF8 antisense RNA 1	RASSF8-AS1	ENSG00000246695.7	0.770905	9.02E-08	2.29E-05
236075_s_at	Uncharacterized LOC101928000	LOC101928000	ENSG00000234327.7	0.766575	6.6E-06	0.000649
221974_at	Imprinted in Prader-Willi syndrome (non-protein coding) /// uncharacterized LOC101930404 /// Prader Willi/Angelman region RNA, SNRPN neighbor /// small nucleolar RNA, C/D box 107 /// small nucleolar RNA, C/D box 115–13 /// small nucleolar RNA, C/D box 115–26 /// small nucleolar RNA, C/D box 115–7 /// small nucleolar RNA, C/D box 116–22 /// small nucleolar RNA, C/D box 116–28 /// small nucleolar RNA, C/D box 116–4 /// small nuclear ribonucleoprotein polypeptide N	IPW /// LOC101930404 /// PWARN /// SNORD107 /// SNORD115-13 /// SNORD115-26 /// SNORD115-7 /// SNORD116-22 /// SNORD116-28 /// SNORD116-4 /// SNRPN	ENSG00000224078.13	0.719911	0.000535	0.016616
227099_s_at	Chromosome 11 open reading frame 96	C11orf96	ENSG00000254409.2	0.686826	0.001963	0.037596
217520_x_at	Uncharacterized LOC101929232 /// PDCD6IP pseudogene 2	PDCD6IP2	ENSG00000274253.4	0.671638	1.03E-05	0.000862
226591_at	Prader Willi/Angelman region RNA 6	PWAR6	ENSG00000257151.1	0.665136	0.000597	0.018108
233562_at	Long intergenic non-protein coding RNA 839	LINC00839	ENSG00000185904.11	0.644287	0.000226	0.009558
228370_at	Imprinted in Prader-Willi syndrome (non-protein coding) /// uncharacterized LOC101930404 /// Prader Willi/Angelman region RNA, SNRPN neighbor /// small nucleolar RNA, C/D box 107 /// small nucleolar RNA, C/D box 115–13 /// small nucleolar RNA, C/D box 115–26 /// small nucleolar RNA, C/D box 115–7 /// small nucleolar RNA, C/D box 116–22 /// small nucleolar RNA, C/D box 116–28 /// small nucleolar RNA, C/D box 116–4	IPW /// LOC101930404 /// PWARN /// SNORD107 /// SNORD115-13 /// SNORD115-26 /// SNORD115-7 /// SNORD116-22 /// SNORD116-28 /// SNORD116-4	ENSG00000224078.13	0.63548	0.004004	0.056605
230272_at	Long intergenic non-protein coding RNA 461 /// microRNA 9-2	LINC00461 /// MIR9-2	ENSG00000245526.10	0.633241	0.000333	0.011874

(Continued)

TABLE 3 | Continued

Probes	Title	Symbol	Ensemble transcript id version	Log FC	P-value	Adj. P-value
227121_at	Zinc finger and BTB domain containing 20	ZBTB20	ENSG00000259976.3	0.622039	6.47E-05	0.003438
228438_at	Uncharacterized LOC100132891	LOC100132891	ENSG00000235531.9	0.610992	0.00111	0.026335
213447_at	Imprinted in Prader-Willi syndrome (non-protein coding) /// uncharacterized LOC101930404 /// Prader Willi/Angelman region RNA, SNRPN neighbor /// small nucleolar RNA, C/D box 107 /// small nucleolar RNA, C/D box 115–13 /// small nucleolar RNA, C/D box 115–26 /// small nucleolar RNA, C/D box 115–7 /// small nucleolar RNA, C/D box 116–22 /// small nucleolar RNA, C/D box 116–28 /// small nucleolar RNA, C/D box 116–4 /// small nuclear ribonucleoprotein polypeptide N	IPW /// LOC101930404 /// PWARN /// SNORD107 /// SNORD115-13 /// SNORD115-26 /// SNORD115-7 /// SNORD116-22 /// SNORD116-28 /// SNORD116-4 /// SNRPN	ENSG00000224078.13	0.603999	0.000792	0.021388
238632_at	NA	RP11-44F21.5	ENSG00000260265.1	−0.58771	0.000615	0.018108
224646_x_at	H19, imprinted maternally expressed transcript (non-protein coding) /// microRNA 675	H19 /// MIR675	ENSG00000130600.18	−0.66521	0.008633	0.089285
243729_at	NA	RP11-747H7.3	ENSG00000260711.2	−0.68534	2.63E-09	1.08E-06
1557779_at	Uncharacterized LOC101928687	LOC101928687	ENSG00000231131.6	−0.69525	0.000133	0.006282
229296_at	Uncharacterized LOC100506119	LOC100506119	ENSG00000233901.5	−0.74915	3.12E-05	0.001938
1557094_at	Uncharacterized LOC100996760	LOC100996760	ENSG00000276850.4	−0.80357	4.07E-05	0.002362
223779_at	AFAP1 antisense RNA 1	AFAP1-AS1	ENSG00000272620.1	−0.80513	6.36E-05	0.003434
235921_at	Uncharacterized LOC102723721	LOC102723721	ENSG00000223784.1	−0.81799	9.33E-06	0.000804
1558216_at	AFAP1 antisense RNA 1	AFAP1-AS1	ENSG00000272620.1	−0.84595	0.000286	0.010641
242874_at	NA	RP11-747H7.3	ENSG00000260711.2	−0.92003	9.63E-11	6.43E-08
227985_at	Uncharacterized LOC100506098	LOC100506098	ENSG00000233834.6	−1.04243	7.5E-08	2E-05
236279_at	NA	NA	ENSG00000275234.1	−1.04592	6.12E-10	2.97E-07
232202_at	Family with sequence similarity 83, member B	FAM83B	ENSG00000261116.1	−1.07231	2.29E-10	1.22E-07
238742_x_at	Uncharacterized LOC102724362	SPINT1-AS1	ENSG00000261183.5	−1.10252	6.38E-14	1.7E-10
226755_at	MIR205 host gene (non-protein coding)	MIR205HG	ENSG00000230937.11	−1.11922	1.11E-10	6.61E-08
242354_at	NA	RP11-532F12.5	ENSG00000261183.5	−1.19239	5.99E-13	8.01E-10
229223_at	NA	RP11-96D1.11	ENSG00000262160.1	−1.26926	1.78E-12	1.59E-09
201510_at	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	ELF3	ENSG00000249007.1	−1.54591	1.36E-13	2.42E-10
210827_s_at	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	ELF3	ENSG00000249007.1	−1.63868	8.23E-13	8.79E-10
227919_at	Urothelial cancer associated 1 (non-protein coding)	UCA1	ENSG00000214049.7	−1.65999	9.68E-08	2.35E-05

log FC, log2 of fold-change. Positive value indicates increased expression in high_IC50 group, and negative value indicates decreased expression in high_IC50 group. NA, Not available.

were selected for a subsequent validating study. After knocking-down expression levels of GIHCG and SPINT1-AS1 by small interfering RNAs, Lapatinib IC50, and inhibitory rate of cancer cells were detected. Among three small interference sequences of GIHCG and SPINT1-AS1 mRNAs, siRNA sequence 3 of GIHCG (Si3, **Figure 7B**), and siRNA sequence 1 of SPINT1-AS1 (Si1, **Figure 7C**) were identified as effective siRNAs for further experiments.

Knocking-down of GIHCG could significantly enhance the sensitivity to Lapatinib in MCF7 and BxPC3 cancer cell lines (**Figure 7D**), while down-regulation of SPINT1-AS1 could promote resistance to Lapatinib in NCI-N87 and MCF7 cancer cell lines (**Figure 7E**). To clarify whether there is a mutual regulatory relationship between GIHCG and SPINT1-AS1, we detected the expression level of SPINT1-AS1 after GIHCG knockdown and vice versa. As shown in **Figures 7F,G**,

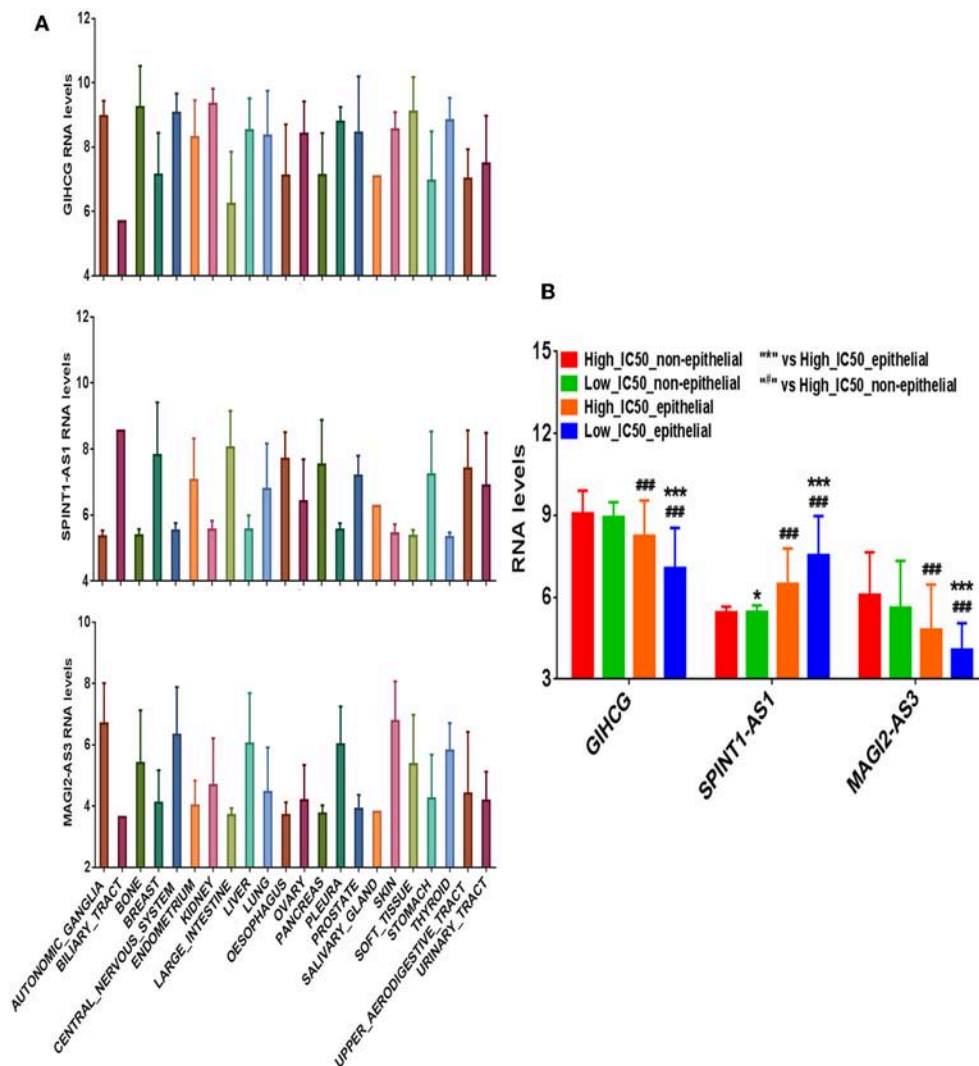


FIGURE 4 | The correlation of expression levels of three crucial lncRNAs and originated sites of cancer cell lines. **(A)** The expression levels of GIHCG, SPINT1-AS1, and MAGI2-AS3 on 22 types of cancer cell lines. **(B)** The bar charts of expression levels of GIHCG, SPINT1-AS1, and MAGI2-AS3 between Lapatinib high_IC50 and low_IC50 groups in epithelial cancer cell lines and non-epithelial cancer cell lines.

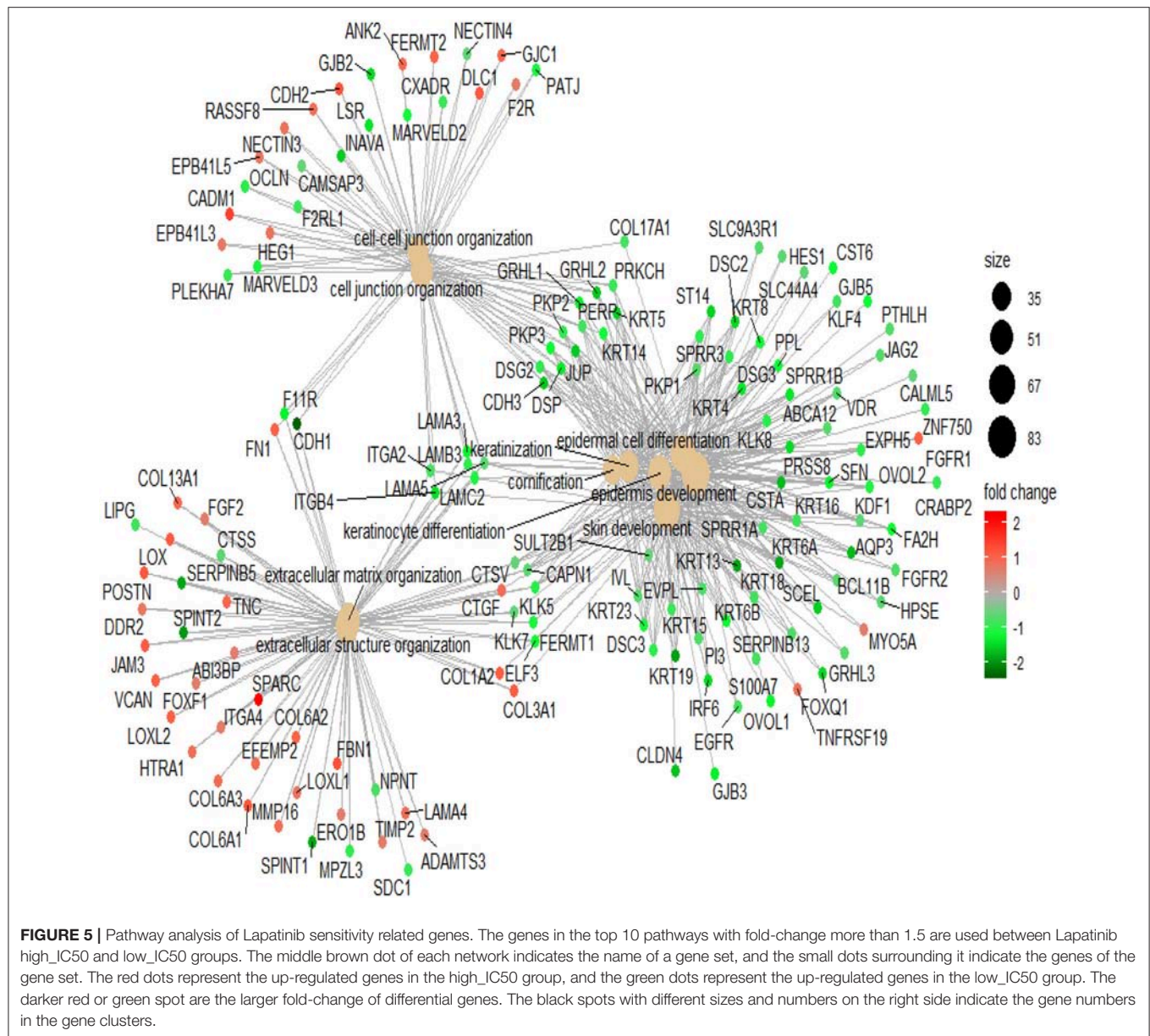
suppression of GIHCG in Lapatinib resistant cancer cell lines NCIH-747 and BxPC3 could induce up-regulation of SPINT1-AS1 ($P < 0.05$), while knockdown of SPINT1-AS1 did not change the expression level of GIHCG ($P > 0.05$).

DISCUSSION

lncRNA is an important regulatory molecule in drug resistance during chemotherapy or gene targeted therapy (Li et al., 2016; Dong et al., 2018; Wu et al., 2018; Zhou et al., 2018). In this study, we analyzed Lapatinib sensitivity to EGFR and ERBB2 targeted therapy pan-cancer cell line wide. We noticed that Lapatinib sensitivity was not only positively correlated to the activation of EGFR and ERBB2 signaling pathways, but also positively associated to cell keratin, epithelial differentiation, and cell-cell junction. The Lapatinib sensitivity of cancer cell lines

was negatively associated to extracellular matrix signature. By screening differentially expressed lncRNAs and establishing co-expression network between Lapatinib high_IC50 and low_IC50 groups, three key lncRNAs, SPINT1-AS1, GIHCG, and MAGI2-AS3, were found. Of those, GIHCG and SPINT1-AS1 were only differentially expressed in epithelial derived cancers. SPINT1-AS1 was negatively related to Lapatinib IC50, whereas GIHCG was positively associated to Lapatinib IC50. By siRNAs treatment, downregulation of SPINT1-AS1 could promote Lapatinib resistance, while downregulation of GIHCG promoted Lapatinib sensitivity. The combination of bioinformatical approach and experimental study confirmed that lncRNAs were involved in regulating sensitivity to Lapatinib targeted therapy.

PI3K/Akt, Ras/Raf/MEK/ERK1/2, and PLC γ pathways are downstream pathways of EGFR and ERBB2 and play important roles in cell proliferation and survival of multiple cancers



(Roskoski, 2014). The expression levels of EGFR and ERBB2 are positively correlated to Lapatinib sensitivity (Rusnak et al., 2007; Xiang et al., 2018). Trastuzumab (Herceptin) is a molecular targeted drug of ERBB2-positive metastatic/advanced breast cancer and gastric cancer (Bang et al., 2010; Loibl and Gianni, 2017). Lapatinib is a small molecule chemical, which proved effective for ERBB2-positive advanced or metastatic breast cancer when combined with capecitabine after previous treatment with anthracyclines, paclitaxel, or trastuzumab (Geyer et al., 2006). In gastric cancer, treatment with Lapatinib plus capecitabine and oxaliplatin also revealed anti-cancer effects on HER2-amplified gastroesophageal adenocarcinoma, especially in Asian and younger patients (Hecht et al., 2016). LncRNAs emerged as one of the new resistance mechanisms to chemotherapy or

molecule targeted therapy. By bioinformatics analysis, Lapatinib sensitive cancer cells exhibited enrichment of genes related to cell keratin, epithelial differentiation, and cell-cell junction. The ERBB family plays an important role in regulating cell differentiation (Pellat et al., 2017). We noticed that Lapatinib sensitivity is positively correlated to ERBB pathway activation. It means that cancer cells sensitive to Lapatinib drug often showed enrichment of cell differentiation-related genes, while Lapatinib-resistant cancer cells are often accompanied by enrichment of extracellular matrix pathway (D'Amato et al., 2015; Khan et al., 2016; Lin et al., 2017; Watson et al., 2018). Furthermore, increases of extracellular matrix could further induce epithelial-mesenchymal transition of cancer cells (Tzanakakis et al., 2018).

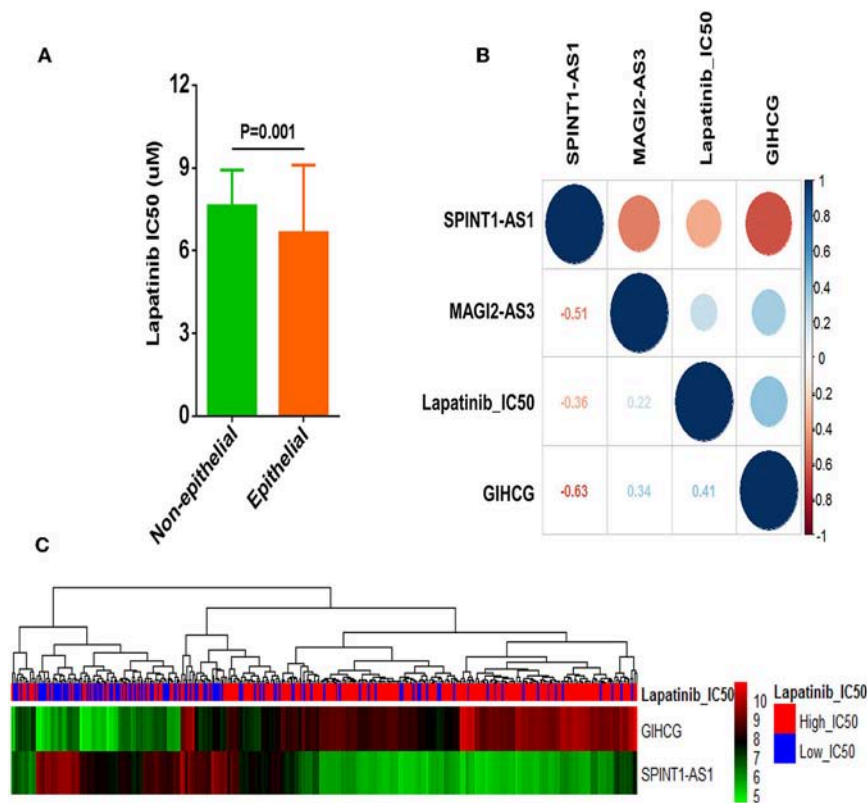


FIGURE 6 | Correlation analysis of three crucial lncRNAs GIHCG, SPINT1-AS1, MAGI2-AS3, and Lapatinib sensitivity in epithelial cancer cells. **(A)** Non-epithelial cancer cells showed higher Lapatinib_IC50 than epithelial cancer cells in the CCLE database. **(B)** Correlation between three lncRNAs and Lapatinib IC50 of epithelial cancer cells. Red circles represent negative correlation, and blue circles represent positive correlation. The number of the lower left grids indicates correlation coefficient between two factors (all P -values <0.001). **(C)** The heatmap presents expression levels of GIHCG and SPINT1-AS1 in Lapatinib high_IC50 and low_IC50 groups of epithelial cancer cell lines.

Although the role of lncRNAs in cancer progression and Lapatinib resistance have been reported in other studies (Russell et al., 2015; Li et al., 2016; Liang et al., 2018; Ma et al., 2018), this is the first study that proved that lncRNAs GIHCG and SPINT1-AS1 are involved in regulating therapeutic sensitivity to Lapatinib. Based on pan-cancer cell lines analysis, Lapatinib IC50 is significantly different between non-epithelial cancer cell lines, and epithelial cancer cell lines. As the inhibitor of miR-200b/200a/429, lncRNA GIHCG was shown effectively promoting the progression of liver cancer through inducing methylation of miR-200b/200a/429 promoter (Sui et al., 2016). GIHCG is also involved in promoting cancer proliferation and migration in tongue and renal cancers (D'Aniello et al., 2018; Ma et al., 2018). However, there is no study showing whether or not GIHCG could regulate Lapatinib drug sensitivity in cancers. lncRNA SPINT1-AS1 is a Kunitz type 1 antisense RNA1, belonging to serine peptidase inhibitor. An increased expression of SPINT1-AS1 has been observed in colorectal cancer (Li C. et al., 2018). It is also the first time that lncRNA SPINT1-AS1 has been found regulating Lapatinib drug sensitivity on multiple cancer cells. In validating experiments, the knockdown of SPINT1-AS1 did not result in the up-regulation of GIHCG. We speculated

that GIHCG may regulate SPINT1-AS1 expression through regulating promoter methylation or by manner of competitive endogenous RNA (ceRNA) (Zhang G. et al., 2018; Zhang L. et al., 2018). However, the mutual regulatory mechanisms of lncRNA GIHCG and SPINT1-AS1 remain to be studied in the future.

CONCLUSION

In conclusion, the current study proposed a group of lncRNAs related to Lapatinib sensitivity based on pan-cancer cell lines analysis. By subsequent experimental study, lncRNAs GIHCG and SPINT1-AS1 were firstly identified as crucial lncRNAs in regulating Lapatinib resistance or sensitivity in epithelium-derived cancer cell lines. SPINT1-AS1 is a Lapatinib sensitivity predictor, while GIHCG is a predictive molecule for Lapatinib resistance.

ETHICS STATEMENT

The protocols used in this study were approved by Rui Jin Hospital Ethics Review Boards. Written

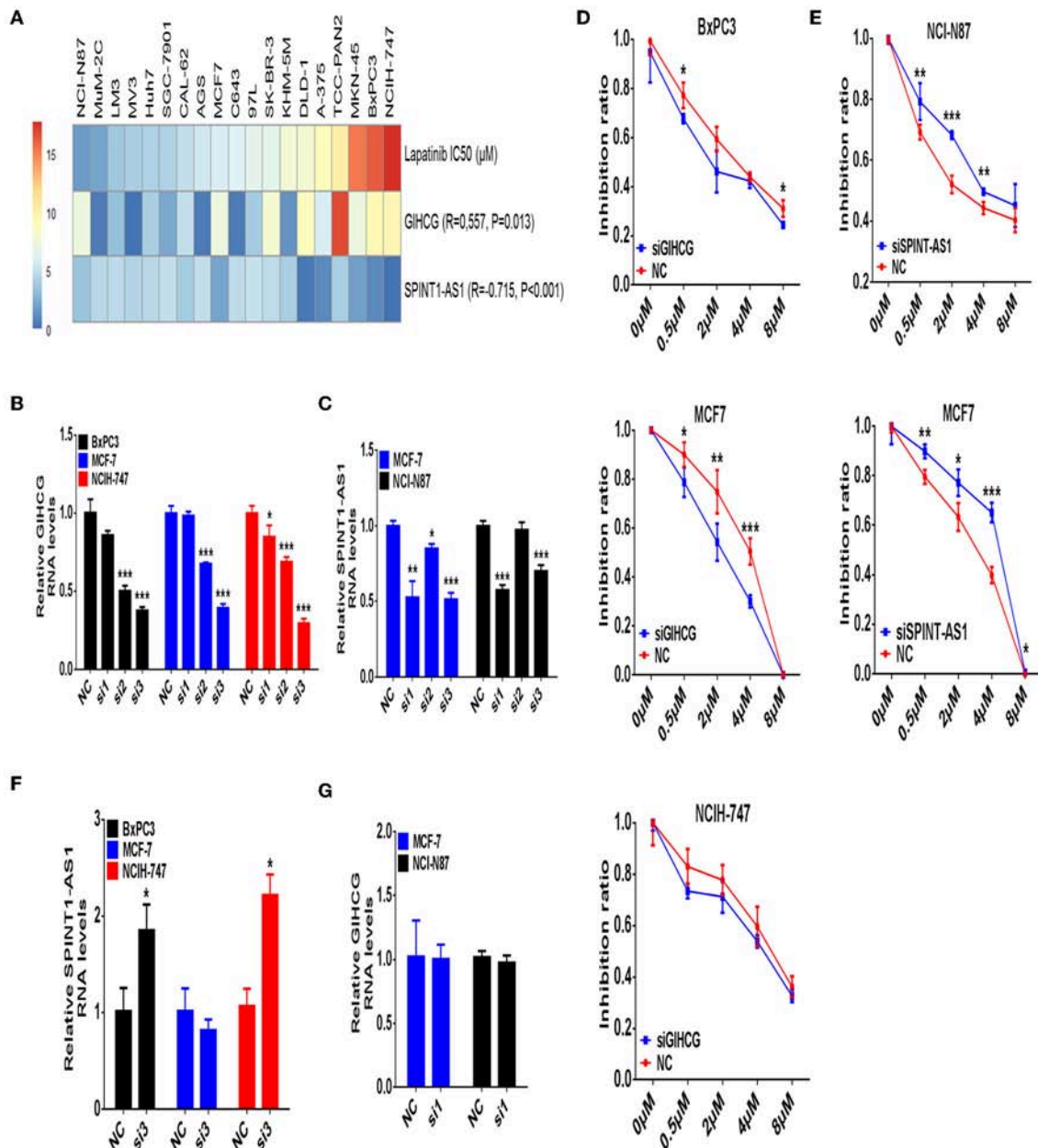


FIGURE 7 | Validating study of lncRNAs GIHCG and SPINT1-AS1 on regulating Lapatinib sensitivity. **(A)** The Lapatinib IC₅₀ and expression levels of GIHCG and SPINT1-AS1 are assayed on 19 cancer cell lines from different types of cancer origin. The expression level of GIHCG is positively related to Lapatinib IC₅₀ ($R = 0.557$, $P = 0.013$), while the expression level of SPINT1-AS1 is negatively related to Lapatinib IC₅₀ ($R = -0.715$, $P < 0.001$). **(B)** Knockdown of GIHCG is performed by siRNA in BxPC3, MCF7, and NCIH-747 cancer cells. **(C)** Knockdown of SPINT1-AS1 is performed by siRNA in NCI-N87 and MCF7 cancer cells. **(D)** Knockdown of GIHCG shows enhancing Lapatinib sensitivity in BxPC3, MCF7, and NCIH-747 cancer cells. **(E)** Knockdown of SPINT1-AS1 shows promoting Lapatinib resistance in NCI-N87 and MCF7 cancer cells. **(F)** Knockdown of GIHCG discloses increased SPINT1-AS1 expression in BxPC3 and NCIH-747 cancer cells. **(G)** Knockdown of SPINT1-AS1 does not increase GIHCG expression in NCI-N87 and MCF7 cancer cells. Experimental group vs. negative control (NC), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

informed consents were obtained from all human material donors in accordance with the Declaration of Helsinki. Animals were used according to the protocols approved by Rui Jin Hospital Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KL and YY conceived and designed the experiments. ZX, ShS, ZZ, JG, and QL performed the experiments. ZX, ZZ, SaS, WS, YY, and KL analyzed the data. ZX, ShS, ZZ, SaS, WS, YY, and KL

contributed reagents, materials, and analysis tools. ZX, YY, and KL wrote the paper.

FUNDING

This project was supported by the National Natural Science Foundation (NSF 81270622 and 81772505), Bagui Talent Foundations (T3120097921, T3120099202, A3120099201, and C31200992001), Innovation Foundation for Key Laboratory of Processing for Non-ferrous Metal and Featured Materials (AE3390003605), National Key R&D Program of China (2017YFC0908300, 2016YFC1303200), China 973 Program

REFERENCES

- Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. The gene ontology consortium. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556
- Bang, Y. J., Van Cutsem, E., Feyereislova, A., Chung, H. C., Shen, L., Sawaki, A., et al. (2010). Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 376, 687–697. doi: 10.1016/S0140-6736(10)61121-X
- Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., et al. (2012). The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483, 603–607. doi: 10.1038/nature11003
- Bester, A. C., Lee, J. D., Chavez, A., Lee, Y. R., Nachmani, D., Vora, S., et al. (2018). An integrated genome-wide CRISPRa approach to functionalize lncRNAs in drug resistance. *Cell* 173, 649–664 e620. doi: 10.1016/j.cell.2018.03.052
- Cetin, B., Benekli, M., Turker, I., Koral, L., Ulas, A., Dane, F., et al. (2014). Lapatinib plus capecitabine for HER2-positive advanced breast cancer: a multicentre study of anatolian society of medical oncology (ASMO). *J. Chemother.* 26, 300–305. doi: 10.1179/1973947813Y.0000000147
- D'Amato, V., Raimondo, L., Formisano, L., Giuliano, M., De Placido, S., Rosa, R., et al. (2015). Mechanisms of lapatinib resistance in HER2-driven breast cancer. *Cancer Treat. Rev.* 41, 877–883. doi: 10.1016/j.ctrv.2015.08.001
- D'Aniello, C., Pisconti, S., Facchini, S., Imbimbo, C., and Cavaliere, C. (2018). Long noncoding RNA GIHCG is a potential diagnostic and prognostic biomarker and therapeutic target for renal cell carcinoma. *Eur. Rev. Med. Pharmacol. Sci.* 22, 1169–1170. doi: 10.26355/eurrev_201803_14452
- Dong, H., Wang, W., Mo, S., Chen, R., Zou, K., Han, J., et al. (2018). SP1-induced lncRNA AGAP2-AS1 expression promotes chemoresistance of breast cancer by epigenetic regulation of MyD88. *J. Exp. Clin. Cancer Res.* 37:202. doi: 10.1186/s13046-018-0875-3
- Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., et al. (2006). Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med.* 355, 2733–2743. doi: 10.1056/NEJMoa064320
- Hahne, J. C., and Valeri, N. (2018). Non-coding RNAs and resistance to anticancer drugs in gastrointestinal tumors. *Front. Oncol.* 8:226. doi: 10.3389/fonc.2018.00226
- Hanzelmann, S., Castelo, R., and Guinney, J. (2013). GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 14:7. doi: 10.1186/1471-2105-14-7
- Harrington, K., Temam, S., Mehanna, H., D'Cruz, A., Jain, M., D'Onofrio, I., et al. (2015). Postoperative adjuvant lapatinib and concurrent chemoradiotherapy followed by maintenance lapatinib monotherapy in high-risk patients with resected squamous cell carcinoma of the head and neck: a phase III, randomized, double-blind, placebo-controlled study. *J. Clin. Oncol.* 33, 4202–4209. doi: 10.1200/JCO.2015.61.4370
- Hecht, J. R., Bang, Y. J., Qin, S. K., Chung, H. C., Xu, J. M., Park, J. O., et al. (2016). Lapatinib in combination with capecitabine plus oxaliplatin in human epidermal growth factor receptor 2-positive advanced (2013CB733700 and 2011CB510102), Shanghai Science and Technology Committee (18411953100), the Cross-Institute Research Fund of Shanghai Jiao Tong University (YG2017ZD01, YG2015MS62), Innovation Foundation of Translational Medicine of Shanghai Jiao Tong University School of Medicine (15ZH4001, TM201617, and TM 201702), and Technology Transfer Project of Science & Technology Dept. Shanghai Jiao Tong University School of Medicine.
- ACKNOWLEDGMENTS**
- We acknowledge open database of CCLE.
- or metastatic gastric, esophageal, or gastroesophageal adenocarcinoma: TRIO-013/LOGIC-a randomized phase III trial. *J. Clin. Oncol.* 34, 443–451. doi: 10.1200/JCO.2015.62.6598
- Heery, R., Finn, S. P., Cuffe, S., and Gray, S. G. (2017). Long non-coding RNAs: key regulators of epithelial-mesenchymal transition, tumour drug resistance and cancer stem cells. *Cancers* 9:E38. doi: 10.3390/cancers9040038
- Jiang, M., Huang, O., Xie, Z., Wu, S., Zhang, X., Shen, A., et al. (2014). A novel long non-coding RNA-ARA: adriamycin resistance-associated. *Biochem. Pharmacol.* 87, 254–283. doi: 10.1016/j.bcp.2013.10.020
- Khan, I. A., Yoo, B. H., Masson, O., Baron, S., Corkery, D., Delleire, G., et al. (2016). ErbB2-dependent downregulation of a pro-apoptotic protein Perp is required for oncogenic transformation of breast epithelial cells. *Oncogene* 35, 5759–5769. doi: 10.1038/onc.2016.109
- Kim, B., Wang, S., Lee, J. M., Jeong, Y., Ahn, T., Son, D. S., et al. (2015). Synthetic lethal screening reveals FGFR as one of the combinatorial targets to overcome resistance to Met-targeted therapy. *Oncogene* 34, 1083–1093. doi: 10.1038/onc.2014.51
- Li, C., Li, W., Zhang, Y., Zhang, X., Liu, T., Yang, Y., et al. (2018). Increased expression of antisense lncRNA SPINT1-AS1 predicts a poor prognosis in colorectal cancer and is negatively correlated with its sense transcript. *OncoTargets Ther.* 11, 3969–3978. doi: 10.2147/OTT.S163883
- Li, D., Zhang, J., Wang, M., Li, X., Gong, H., Tang, H., et al. (2018). Activity dependent LoNA regulates translation by coordinating rRNA transcription and methylation. *Nat. Commun.* 9:1726. doi: 10.1038/s41467-018-04072-4
- Li, W., Zhai, L., Wang, H., Liu, C., Zhang, J., Chen, W., et al. (2016). Downregulation of lncRNA GAS5 causes trastuzumab resistance in breast cancer. *Oncotarget* 7, 27778–27786. doi: 10.18632/oncotarget.8413
- Liang, Y., Chen, X., Wu, Y., Li, J., Zhang, S., Wang, K., et al. (2018). lncRNA CASC9 promotes esophageal squamous cell carcinoma metastasis through upregulating LAMC2 expression by interacting with the CREB-binding protein. *Cell Death Differ* 25, 1980–1995. doi: 10.1038/s41418-018-0084-9
- Lin, C. H., Jokela, T., Gray, J., and LaBarge, M. A. (2017). Combinatorial microenvironments impose a continuum of cellular responses to a single pathway-targeted anti-cancer compound. *Cell Rep.* 21, 533–545. doi: 10.1016/j.celrep.2017.09.058
- Loibl, S., and Gianni, L. (2017). HER2-positive breast cancer. *Lancet* 389, 2415–2429. doi: 10.1016/S0140-6736(16)32417-5
- Ma, L., Wang, Q., Gong, Z., Xue, L., and Zuo, Z. (2018). Long noncoding RNA GIHCG enhanced tongue squamous cell carcinoma progression through regulating miR-429. *J. Cell. Biochem.* 119, 9064–9071. doi: 10.1002/jcb.27164
- Ma, P., Zhang, M., Nie, F., Huang, Z., He, J., Li, W., et al. (2017). Transcriptome analysis of EGFR tyrosine kinase inhibitors resistance associated long noncoding RNA in non-small cell lung cancer. *Biomed. Pharmacother.* 87, 20–26. doi: 10.1016/j.biopha.2016.12.079
- Niknafs, Y. S., Han, S., Ma, T., Speers, C., Zhang, C., Wilder-Romans, K., et al. (2016). The lncRNA landscape of breast cancer reveals a role for DSCAM-AS1 in breast cancer progression. *Nat. Commun.* 7:12791. doi: 10.1038/ncomms12791

- Pellat, A., Vaquero, J., and Fouassier, L. (2017). Role of ErbB/HER family of receptor tyrosine kinases in cholangiocyte biology. *Hepatology* 67, 762–773. doi: 10.1002/hep.29350
- Peng, W. X., Koirala, P., and Mo, Y. Y. (2017). LncRNA-mediated regulation of cell signaling in cancer. *Oncogene* 36, 5661–5667. doi: 10.1038/onc.2017.184
- Powles, T., Huddart, R. A., Elliott, T., Sarker, S. J., Ackerman, C., Jones, R., et al. (2017). Phase III, double-blind, randomized trial that compared maintenance lapatinib versus placebo after first-line chemotherapy in patients with human epidermal growth factor receptor 1/2-positive metastatic bladder cancer. *J. Clin. Oncol.* 35, 48–55. doi: 10.1200/JCO.2015.66.3468
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., et al. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43:e47. doi: 10.1093/nar/gkv007
- Roskoski, R. Jr. (2014). The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacol. Res.* 79, 34–74. doi: 10.1016/j.phrs.2013.11.002
- Rusnak, D. W., Alligood, K. J., Mullin, R. J., Spehar, G. M., Arenas-Elliott, C., Martin, A. M., et al. (2007). Assessment of epidermal growth factor receptor (EGFR, ErbB1) and HER2 (ErbB2) protein expression levels and response to lapatinib (Tykerb, GW572016) in an expanded panel of human normal and tumour cell lines. *Cell Prolif.* 40, 580–594. doi: 10.1111/j.1365-2184.2007.00455.x
- Russell, M. R., Penikis, A., Oldridge, D. A., Alvarez-Dominguez, J. R., McDaniel, L., Diamond, M., et al. (2015). CASC15-S is a tumor suppressor lncRNA at the 6p22 neuroblastoma susceptibility locus. *Cancer Res.* 75, 3155–3166. doi: 10.1158/0008-5472.CAN-14-3613
- Satoh, T., Xu, R. H., Chung, H. C., Sun, G. P., Doi, T., Xu, J. M., et al. (2014). Lapatinib plus paclitaxel versus paclitaxel alone in the second-line treatment of HER2-amplified advanced gastric cancer in Asian populations: TyTAN—a randomized, phase III study. *J. Clin. Oncol.* 32, 2039–2049. doi: 10.1200/JCO.2013.53.6136
- Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15545–15550. doi: 10.1073/pnas.0506580102
- Sui, C. J., Zhou, Y. M., Shen, W. F., Dai, B. H., Lu, J. J., Zhang, M. F., et al. (2016). Long noncoding RNA GIHCG promotes hepatocellular carcinoma progression through epigenetically regulating miR-200b/a/429. *J. Mol. Med.* 94, 1281–1296. doi: 10.1007/s00109-016-1442-z
- Sun, Q., Tripathi, V., Yoon, J. H., Singh, D. K., Hao, Q., Min, K. W., et al. (2018). MIR100 host gene-encoded lncRNAs regulate cell cycle by modulating the interaction between HuR and its target mRNAs. *Nucleic Acids Res.* 46, 10405–10416. doi: 10.1093/nar/gky696
- Tzanakakis, G., Kavasi, R. M., Voudouri, K., Berdiaki, A., Spyridaki, I., Tsatsakis, A., et al. (2018). Role of the extracellular matrix in cancer-associated epithelial to mesenchymal transition phenomenon. *Dev. Dyn.* 247, 368–381. doi: 10.1002/dvdy.24557
- Wang, Q., Li, C., Tang, P., Ji, R., Chen, S., and Wen, J. (2018). A minimal lncRNA-mRNA signature predicts sensitivity to neoadjuvant chemotherapy in triple-negative breast cancer. *Cell. Physiol. Biochem.* 48, 2539–2548. doi: 10.1159/000492698
- Watson, S. S., Dane, M., Chin, K., Tatarova, Z., Liu, M., Liby, T., et al. (2018). Microenvironment-mediated mechanisms of resistance to HER2 inhibitors differ between HER2+ breast cancer subtypes. *Cell Syst.* 6, 329–342 e326. doi: 10.1016/j.cels.2018.02.001
- Wu, L., Pan, C., Wei, X., Shi, Y., Zheng, J., Lin, X., et al. (2018). lncRNA KRAL reverses 5-fluorouracil resistance in hepatocellular carcinoma cells by acting as a ceRNA against miR-141. *Cell Commun. Signal.* 16:47. doi: 10.1186/s12964-018-0260-z
- Xiang, Z., Huang, X., Wang, J., Zhang, J., Ji, J., Yan, R., et al. (2018). Cross-database analysis reveals sensitive biomarkers for combined therapy for ERBB2+ gastric cancer. *Front. Pharmacol.* 9:861. doi: 10.3389/fphar.2018.00861
- Zhang, G., Li, S., Lu, J., Ge, Y., Wang, Q., Ma, G., et al. (2018). lncRNA MT1JP functions as a ceRNA in regulating FBXW7 through competitively binding to miR-92a-3p in gastric cancer. *Mol. Cancer* 17:87. doi: 10.1186/s12943-018-0829-6
- Zhang, L., Kang, W., Lu, X., Ma, S., Dong, L., and Zou, B. (2018). lncRNA CASC11 promoted gastric cancer cell proliferation, migration and invasion *in vitro* by regulating cell cycle pathway. *Cell Cycle* 17, 1886–1900. doi: 10.1080/15384101.2018.1502574
- Zhou, Z., Lin, Z., He, Y., Pang, X., Wang, Y., Ponnusamy, M., et al. (2018). The long non-coding RNA D63785 regulates chemotherapy sensitivity in human gastric cancer by targeting miR-422a. *Mol. Ther. Nucleic Acids.* 12, 405–419. doi: 10.1016/j.omtn.2018.05.024

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular Mechanisms in Clear Cell Renal Cell Carcinoma: Role of miRNAs and Hypermethylated miRNA Genes in Crucial Oncogenic Pathways and Processes

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OPEN ACCESS

Edited by:

Ge Shan,
University of Science and Technology
of China, China

Reviewed by:

Alessio Paone,
Sapienza University of Rome, Italy
Graziella Curtale,
The Scripps Research Institute,
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equally to this work

Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 15 November 2018

Accepted: 22 March 2019

Published: 24 April 2019

Citation:

Braga EA, Fridman MV,
Loginov VI, Dmitriev AA and
Morozov SG (2019) Molecular
Mechanisms in Clear Cell Renal Cell
Carcinoma: Role of miRNAs
and Hypermethylated miRNA Genes
in Crucial Oncogenic Pathways
and Processes. *Front. Genet.* 10:320.
doi: 10.3389/fgene.2019.00320

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Clear cell renal cell carcinoma (ccRCC) is the third most common urological cancer, and it has the highest mortality rate. The increasing drug resistance of metastatic ccRCC has resulted in the search for new biomarkers. Epigenetic regulatory mechanisms, such as genome-wide DNA methylation and inhibition of protein translation by interaction of microRNA (miRNA) with its target messenger RNA (mRNA), are deeply involved in the pathogenesis of human cancers, including ccRCC, and may be used in its diagnosis and prognosis. Here, we review oncogenic and oncosuppressive miRNAs, their putative target genes, and the crucial pathways they are involved in. The contradictory behavior of a number of miRNAs, such as suppressive and anti-metastatic miRNAs with oncogenic potential (for example, miR-99a, miR-106a, miR-125b, miR-144, miR-203, miR-378), is examined. miRNAs that contribute mostly to important pathways and processes in ccRCC, for instance, PI3K/AKT/mTOR, Wnt- β , histone modification, and chromatin remodeling, are discussed in detail. We also separately consider their participation in crucial oncogenic processes, such as hypoxia and angiogenesis, metastasis, and epithelial-mesenchymal transition (EMT). The review also considers the interactions of long non-coding RNAs (lncRNAs) and miRNAs of significance in ccRCC. Recent advances in the understanding of the role of hypermethylated miRNA genes in ccRCC and their usefulness as biomarkers are reviewed based on our own data and those available in the literature. Finally, new data and perspectives concerning the clinical applications of miRNAs in the diagnosis, prognosis, and treatment of ccRCC are discussed.

Keywords: clear cell renal cell carcinoma, microRNA, target genes, angiogenesis, epithelial-mesenchymal transition, metastasis, hypermethylated miRNA genes, long non-coding RNA

INTRODUCTION

Renal cell carcinoma (RCC) is diagnosed in approximately 90% of patients with kidney cancer. RCC has the highest mortality rate among urogenital cancers (Vasudev et al., 2012). Approximately 270,000 new cases of RCC and 116,000 RCC-related deaths are reported globally every year (Randall et al., 2014). RCC is a heterogeneous group of epithelial tumors, among which clear cell RCC (ccRCC) is the most common and accounts for 70–80% of the reported cases of RCC (Cairns, 2010). The severity of ccRCC is higher than that of papillary kidney cancer and chromophobe tumors (Cairns, 2010). Lack of effective diagnostics in the early stages of the disease, increasing mortality rate, and resistance to therapies in patients with metastatic ccRCC emphasize the need to discover new biomarkers that are applicable for the early diagnosis of ccRCC and detection of metastasis.

Epigenetic regulatory mechanisms involving DNA methylation at the genomic transcriptional level and the interaction of non-coding RNAs, in particular microRNAs (miRNAs), with target messenger RNA (mRNA) at the post-transcriptional level are important in the regulation of genes and proteins (Baylin and Jones, 2016). miRNAs are involved in the regulation of cell proliferation, differentiation, and stress responses, and in the regulation of other fundamental biological processes and signaling pathways (Cora et al., 2017). Alterations in expression and regulatory functions of miRNA can be one of the key factors of various pathogenesises. miRNAs are involved in the development of more than 300 diseases, including oncological diseases. The number of publications aimed at identifying target genes and signaling pathways that involve miRNAs has increased (Reddy, 2015; Dragomir et al., 2018; Gattolliat et al., 2018). miRNAs are important positive or negative regulators of all processes characteristic of the pathogenesis of tumors, which include control of the cell cycle, apoptosis, neo-angiogenesis, tissue invasion, and metastasis (Exposito-Villen et al., 2018; Goradel et al., 2018; Kashyap et al., 2018; Mens and Ghanbari, 2018).

miRNAs are classified as being oncogenic or oncosuppressive (tumor suppressive) based on their stimulating or inhibiting effects, respectively, on tumor development. The targets of oncogenic miRNAs usually include the mRNAs of the tumor suppressor genes. In contrast, the targets of miRNAs that act to suppress tumor development are oncogenes and genes involved in tumor progression. The regulation of tumor suppressor miRNAs involves methylation of the promoter regions of their genes. The methylation suppresses their expression and subsequently inhibits their suppressor function. These processes occur more often in genes encoding tumor suppressor miRNAs than in genes encoding tumor suppressor proteins (Kunej et al., 2011; Piletic and Kunej, 2016). Methylation of tumor suppressor miRNA genes and the interaction of miRNAs with target mRNAs have a systemic effect on key processes and signaling pathways involved in carcinogenesis (Lopez-Serra and Esteller, 2012; Loginov et al., 2015; Baylin and Jones, 2016). Studies of methylation and profiling of miRNA expression have driven the design of minimally invasive diagnostics, and recent advances in the field of cancer epigenomics have highlighted the profound

possibilities of these approaches in clinical practice (Li et al., 2015; Xing and He, 2016; Morris and Latif, 2017).

Epigenetic mechanisms involved in ccRCC genesis have received less attention than other cancers, such as colorectal, lung, breast, and prostate carcinomas. Nevertheless, many genes susceptible to hypermethylation have been identified, including *VHL*, *RASSF1A*, *CDH1*, and *APAF1*, and reported as promising biomarkers of ccRCC (for example, Dmitriev et al., 2014; Braga et al., 2015; Xing and He, 2016; Morris and Latif, 2017). Data on the expression profiles of miRNAs, their target genes in ccRCC, and their potential use in the clinic are accumulating and have been covered in earlier reviews (for example, Li et al., 2015; He Y.H. et al., 2018). However, the contribution of methylation of miRNA genes to epigenetic regulatory mechanisms in ccRCC remains unclear, although interesting results have been reported.

This review examines the expression profiles, targets, and functions of oncogenic and tumor suppressor miRNAs, their role in molecular mechanisms and signaling pathways of ccRCC, as well as their clinical potential. In addition, based on data obtained in our studies and the published literature, we consider in detail the advances made in studies of hypermethylated miRNA genes in ccRCC and their usefulness as biomarkers.

Several long non-coding RNAs (lncRNAs) have been recently described in RCC, as has their involvement in regulatory interactions as competing endogenous RNA (ceRNA), whereby lncRNAs can act as miRNA sponges to modulate gene expression (Li et al., 2017; Chan and Tay, 2018). Moreover, integrative analyses have enabled construction of a ccRCC regulatory ceRNA network comprising 89 lncRNAs, 10 miRNAs, and 22 mRNAs (Yin et al., 2018). In general, however, very few publications have addressed lncRNAs in relation to ccRCC (60 papers on the topic were found indexed in PubMed as of January 2019). In this review, we also consider some data on lncRNAs and their interactions with miRNAs that are relevant to ccRCC.

ONCOGENIC AND SUPPRESSIVE MIRNAS IN MAIN SIGNALING PATHWAYS OF CCRCC

Expression microchips and quantitative PCR have been used to identify miRNAs with decreased expression levels in kidney tumors, presumably oncosuppressor miRNAs, and with increased expression levels in ccRCC, presumably oncogenic miRNAs (for example, Li et al., 2015). The expression of the miRNA genes is generally evaluated by determining the levels of mature miRNA, which is very stable and thus an effective biomarker (Andorfer et al., 2011).

Tumor suppressor miRNAs include miR-34a (Yamamura et al., 2012; Zhang C. et al., 2014; Toraih et al., 2017), miR-30c (Huang J. et al., 2013), miR-30d (Wu et al., 2013), miR-99a (Cui et al., 2012), miR-133a (Kawakami et al., 2012), miR-133b (Wu et al., 2014), miR-138 (Ding et al., 2018), miR-141 (Li W. et al., 2014), miR-143 (Yoshino et al., 2013), miR-182 (Kulkarni et al., 2018), miR-187 (Zhao et al., 2013), miR-199a (Qin et al., 2018), miR-200c (Ding et al., 2018), miR-205 (Xu et al., 2018), and others. These miRNAs target mRNAs of oncogenes

or genes encoding proteins mediating the progression of kidney tumors. For example, the targets of the crucially important miR-34a are *c-MET* (Toraih et al., 2017), *c-MYC* (Yamamura et al., 2012), and *NOTCH1* (Zhang C. et al., 2014) oncogenes. All are involved in the proliferation and activation of the cell cycle. Many targets of suppressor miRNAs are associated with invasion and migration. For example, matrix metalloproteinase 9 (MMP-9) is a validated target of miR-133b (Wu et al., 2014). *VIM*, *EZH2*, *ZEB2* (Ding et al., 2018), and *HIF1A* (Song et al., 2011) are regulated by miR-138.

Typical oncogenic miRNAs include miR-7 (He H. et al., 2018), miR-21 (Li X. et al., 2014; Chen J. et al., 2017; Cui et al., 2017), miR-155 (Ji et al., 2017), miR-590-5p (Xiao et al., 2013), and others. Their targets, on the contrary, are tumor suppressor genes, which include *PDCD4* (Li X. et al., 2014), *PTEN* (Cui et al., 2017), *TIMP3* (Chen J. et al., 2017), *FOXO3A* (Ji et al., 2017), *PBRM1* (Xiao et al., 2013), non-coding *MEG3* (He H. et al., 2018), and others.

Modern methodologies have provided a great deal of information concerning the miRNA expression profiles in ccRCC (He H. et al., 2015; Wotschovsky et al., 2016). Sixty-three differentially expressed miRNAs have been identified by analyzing the massive sequencing data published in The Cancer Genome Atlas (Liang et al., 2017).

To date, a substantial amount of data has been obtained on the role of miRNA in the regulation of target genes in ccRCC and its pathogenesis (Jung et al., 2009; Redova et al., 2011; Heinzelmann et al., 2014). A study of miRNA and mRNA gene networks constructed on the basis of their expression profiles in ccRCC reported a key role for miR-106a-5p. The loss of this miRNA led to the increased expression of 49 putative targets (Müller and Nowak, 2014). Other miRNAs implicated in this study were miR-135a-5p (32 targets), miR-206 (28 targets), miR-363-3p (22 targets), and miR-216b (13 targets) (Müller and Nowak, 2014). The targets included genes that affect apoptosis, metastasis, cellular mobility, and oncogenes (*c-MET*, *VEGFA*, *NRP2*, and *FLT1*). A similar study (Butz et al., 2015) pinpointed miR-124-3p, miR-30a-5p, and miR-200c-3p as the most significant miRNAs affecting protein expression in ccRCC; the expressions of these miRNAs were often reduced.

Notably, since the von Hippel Lindau (*VHL*) gene has an important role in both familial and sporadic kidney cancer, the genes associated with *VHL*-dependent regulation are important candidates in studying the spectrum of miRNAs that alter *VHL* expression (Neal et al., 2010). Loss of *VHL* function results in constitutive activation of the hypoxia-inducible factor (HIF) pathway, which leads to hypoxia and subsequent expression of angiogenic factors. Oncogenic miRNAs that enhance the development of hypoxia and angiogenesis and their targets have been identified. The next section of this review is devoted to a detailed look at the *VHL*/HIF pathways and the involved miRNAs.

The significance of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway (Joosten et al., 2018) associated with the escape from apoptosis, growth, and proliferation of cells in kidney cancer must be noted. The Cancer Genome Atlas Research

Network indicates frequent mutations in the genes of this pathway in ccRCC. The PI3K/AKT/mTOR pathway can also stimulate HIF through mTOR modulation (Cancer Genome Atlas Research, 2013).

miR-148a targets *AKT2*, and this interaction leads to the suppression of cell growth, colony formation, migration, and invasion and tumor growth in xenografts. The miR-148a level is decreased in RCC and is inversely correlated with tumor size, stage, and metastasis to lymph nodes (Cao et al., 2017). In cultured RCC cells, the inhibition of *FLOT1* by miR-182-5p reduces the phosphorylation and activation of *AKT2* and subsequent phosphorylation of *FOXO3a* (Xu et al., 2014). Oncosuppressor and transcription factor *FOXO3A* is translocated out of the nucleus upon phosphorylation by proteins such as Akt (protein kinase B). *FOXO3a* decreases proliferation and arrests cells in the G1 phase. The level of miR-182-5p is often reduced in RCC (Xu et al., 2014).

On the contrary, miR-122 mimic increases the level of phosphorylation of *AKT2* and mTOR. In addition to its enhanced expression in RCC, miR-122 reduces the expression of the Sprouty RTK signaling antagonist 2 (*SPRY2*) gene. The product of this gene is the inhibitor of Ras/MAPK signaling pathway (Wang Z. et al., 2017). Thus, while miR-148a and miR-182-5p act as tumor suppressors (Fan Y. et al., 2016; Cao et al., 2017), miR-122 acts as an oncogene (Lian et al., 2013).

The expression of miR-99a was first shown to be downregulated in RCC tumor tissues and correlated with poor survival (Cui et al., 2012). The authors also reported that the use of its mimic suppressed cell growth, migration, invasion *in vitro* and *in vivo*, and induced cell arrest at the G1 phase (Cui et al., 2012). However, in contrast to these results, which indicated tumor suppression activity of miR-99a, a recent study (Oliveira et al., 2017) described the upregulation of miR-99a and downregulation of its target gene mTOR in most of the ccRCC samples examined. These findings indicated the oncogenic activity of miR-99a. Both studies implicated mTOR as the direct target of miR-99a in renal cancer cells. Further studies are needed to definitively determine the suppressive or oncogenic activity of miR-99a in ccRCC.

miR-144 also targets *mTOR* in RCC. The reduced expression of miR-144 has been inversely correlated with the stage and size of the tumor, while increased miR-144 expression can suppress cell growth and arrest cells in the G1 phase (Xiang et al., 2016). However, another study (Xiao et al., 2017) identified miR-144-3p as an oncomiRNA. The authors described that the overexpression of miR-144-3p promoted proliferation, migration, invasion, and chemoresistance in ccRCC cells. They also identified AT-rich interactive domain 1A (*ARID1A*) as a direct target gene of miR-144-3p in ccRCC. *ARID1A* is a transcription regulator that is a part of the SNF/SWI remodeling complex. The data to date highlight the lack of clarity concerning the role of miR-144 in ccRCC. Further in-depth studies are required.

miR-137 expression is reduced in RCC and miR-137 suppresses the activation of the PI3K/AKT signaling pathway. This leads to reduced proliferation, migration, and invasiveness of cells, enhanced apoptosis, and suppressed tumor growth in

xenografts (Zhang and Li, 2016). The lncRNA *SNHG1* also negatively regulates this miRNA in RCC (Zhao S. et al., 2018).

The target of most oncogenic miRNAs in ccRCC is the tumor suppressor phosphatase and tensin homolog (*PTEN*) gene, which is the negative regulator of the PI3K/AKT/mTOR pathway. In particular, *PTEN* directly interacts with miR-23b (Zaman et al., 2012), miR-193-3p (Liu L. et al., 2017), miR-21 (Cui et al., 2017), and miR-22 (Fan W. et al., 2016), although miR-22 acts as a tumor suppressor. Appropriate interactions that affect the expression of *PTEN* may be significant in the development of the disease. Aggressive ccRCC specimens are characterized by a reduced level of *PTEN* (Cancer Genome Atlas Research, 2013). There are some indications that miR-193a-3p and miR-224 can affect the PI3K/AKT pathway by targeting the ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (*ST3GalIV*) gene in RCC (Pan et al., 2018a).

The influence of some miRNAs on the PI3K/AKT pathway is depicted in **Figure 1**.

It is believed that the effect of the reduced expression of miR-124-3p in ccRCC is due to an increase in the expression of its putative targets, *CAV1* and *FLOT1*, which are cytoplasmic membrane proteins associated with caveolae-mediated endocytosis (Butz et al., 2015). *CAV1* expression causes the AKT-dependent formation of lamellipodia, which increases cell migration and invasion. In addition, it activates the Ras-extracellular signal-regulated kinase (Ras-ERK) pathway and suppresses anoikis, a form of programmed cell death specific to non-malignant cells induced by the detachment of anchorage-dependent cells. *FLOT1* is also associated with endocytosis and activation of epidermal growth factor receptor, and subsequent activation of MAPK (Butz et al., 2015).

Studies have also shown the importance of the Wnt/ β -catenin signaling pathway in RCC and indicated that many WNT antagonist genes are inhibited by methylation of their promoters in ccRCC (Joosten et al., 2018). In ccRCC, miR-106b-5p can activate this pathway, having as its targets the following negative regulators of the Wnt/ β -catenin pathway: *LZTFL1*, *SFRP1*, and *DKK2* (Lu et al., 2017). Thus, being an oncogene, miR-106b-5p supports the aggressiveness and stemness phenotypes of ccRCC (Lu et al., 2017). miR-1260b also targets *DKK2* and stimulates the

proliferation and invasiveness of RCC cells, and its expression is enhanced in RCC (Hirata et al., 2013).

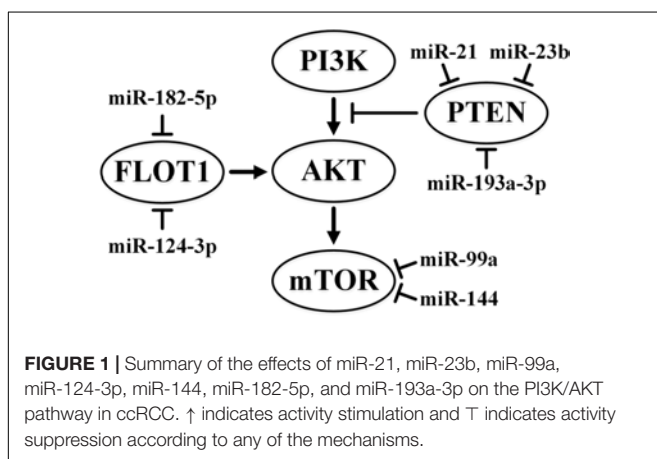
miR-203a targets the glycogen synthase kinase-3 β (*GSK3 β*) gene (Hu et al., 2014), whose product is involved in β -catenin degradation (Joosten et al., 2018). Thus, miR-203a also acts as an oncogene in RCC, being a predictor of poor prognosis (Hu et al., 2014). IGF-II mRNA binding protein 1 (IGF2BP1), which participates in the Wnt/ β -catenin pathway, is a target of miR-372 in the RCC cell lines, thus miR-372 acts as a tumor suppressor (Huang et al., 2015).

The roles of miRNAs in important processes in ccRCC, which include cell migration, invasion, and proliferation, were highlighted in a recent review (He Y.H. et al., 2018).

Notably, mutations in known oncogenes and tumor suppressors, such as *RAS*, *TP53*, *RB*, and *PTEN*, are not typical of RCC (Joosten et al., 2018). However, sporadic mutations occur with high frequency in the genes encoding proteins associated with histone modification and chromatin remodeling. These genes include *SETD2* (3–12%), *KDM5C* (3–8%), *KDM6A* (1%), *BAP1* (8–15%), and *PBRM1* (21–41%) (Cancer Genome Atlas Research, 2013). miRNA-mediated repression also contributes to the altered expression of these genes in RCC. For example, miR-106a-5p, which targets *SETD2*, functions as an oncogene in this case. *SETD2* is instrumental in suppressing and halting the cell cycle at the transition from G0 to G1, and also inhibits proliferation and stimulates apoptosis in RCC (Xiang et al., 2015). *SETD2* increases the binding of H3K36me3 to the p53 promoter that enhances the expression of p53. In ccRCC, miR-590-5p acts as an oncogene and its *PBRM1* target acts as a tumor suppressor (Xiao et al., 2013).

Immune checkpoint molecules play an important role in the control of carcinogenesis. In ccRCC, the B7 homolog 3 (*B7-H3*) immune checkpoint molecules are validated targets of miR-187. This miRNA exhibits the characteristics of a tumor suppressor, and its concentration is reduced in both tumor tissue and plasma that provides opportunities for use in diagnosis of patients with ccRCC. A low level of miR-187 is associated with advanced stages of the disease and a worse 5-year survival rate. *In vitro*, it reduces cell proliferation and mobility and suppresses tumor growth (Zhao et al., 2013). Non-classical human leukocyte antigen G (*HLA-G*) inhibits the cytotoxic activity of T-lymphocytes and natural killer cells. *HLA-G* is often expressed in RCC and is a target of miR-148a and miR-133a, and overexpression of these miRNAs suppresses its effect (Jasinski-Bergner et al., 2015).

It is interesting to note the dual functions of some miRNAs in ccRCC. For instance, one of the targets of miR-106a-5p is the P21 (RAC1) Activated Kinase 5 (*PAK5*) gene. *PAK5* is a serine/threonine kinase (activated by RAC) that regulates cytoskeleton dynamics, cell proliferation, and survival. miR-106a-5p is reportedly a tumor suppressor that reduces metastasis in xenografts and reduces migration and invasiveness of RCC cell lines (Pan et al., 2017). These data contradict the studies that reported oncogenic effects of miR-106a-5p, such as its ability to target *SETD2* and indirectly downregulate TP53 (Xiang et al., 2015) and VHL protein levels (Oliveira et al., 2017). The contradictory nature of the influence of miR-106a-5p on ccRCC is summarized in **Figure 2**.



Thus, some miRNAs (for example, miR-106a and miR-144) can show both tumor suppressor and oncogenic properties depending on the context, i.e., their effect is apparently dependent on the target gene and pathways involved. However, some contradictory data, for instance, for miR-99a (Cui et al., 2012; Oliveira et al., 2017), may be the result of inconvenient methodology or inadequate sampling of examined tumors.

The next few sections of this review will be devoted to discussing the regulation of angiogenesis and epithelial-mesenchymal transition (EMT), given the great importance of these processes in RCC.

MOLECULAR MECHANISMS IN HYPOXIA AND ANGIOGENESIS IN CCRCC: THE ROLE OF MIRNA

In most clear cell carcinomas of the kidney, the expression of the *VHL* gene is dysregulated in a similar manner (98% of cases feature gene deletion, with hypermethylation and point mutations in the remaining 2%). *VHL* binds HIF1A and degrades it only in the presence of oxygen. In the absence of oxygen, HIF1A dimerizes with HIF1B and leads to the activation of transcription of more than 40 genes, including the vascular endothelial growth factor (*VEGF*) family and erythropoietin (*EPO*) genes, genes encoding growth factors (e.g., *EGF*, *PDGF*) and glycolytic enzymes, and genes involved in glucose metabolism (e.g., *GLUT1/4*) (Roskoski, 2017). In the presence of *VHL* mutations downregulating its expression, the aforementioned regulatory influences of *VHL* on HIF1 are disrupted. Inactivation by oxygen is also a characteristic of HIF2A (Serocki et al., 2018). The VEGF-A protein of the VEGF family stimulates the dimerization and activation of the VEGF receptor 2 (VEGFR2). This interaction regulates, in particular, the angiogenesis process, ensuring the supply of oxygen and nutrients to the tumor and encouraging metastasis. The activation of VEGFR2, in turn, leads to the activations of the ERK1/2, AKT, Src, and p38

MAPK pathways, which are important for tumor development and metastasis. These activations occur through a cascade of phosphorylation events involving the tyrosine moiety of various proteins, which further recruit phosphotyrosine-binding proteins. These pathways are associated with key angiogenesis events that include cell proliferation, migration, survival, and vascular permeability (Roskoski, 2017). In patients with ccRCC being treated with VEGF and mTOR blockers, angiogenesis can be stimulated by angiopoietin 2, c-MET, or interleukin (Malouf et al., 2016).

While HIF1 responds to acute hypoxia, the adaptation to chronic hypoxia is mediated by the expression of HIF2 and HIF3 in endothelial tissues, while the expression of HIF1 is downregulated. miRNAs play a significant role in this adaptation. While there is a significant overlap of HIF1 and HIF2 targets, the role of HIF3 is much less clear. It has at least six isoforms. One suppresses the activity of HIF1 and HIF2, while others may activate the transcription of a number of genes (Serocki et al., 2018).

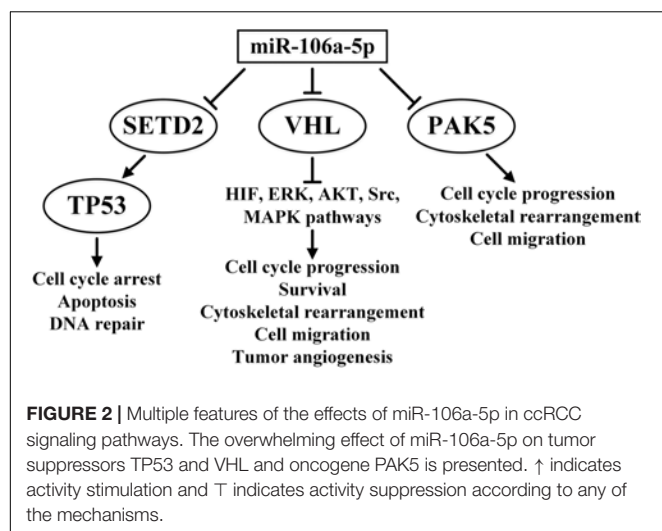
In response to prolonged hypoxia, 786-O, a *VHL*-defective cell line of ccRCC, displays upregulation of the expression of *LICAM* and *FBN1* genes. In addition to the downregulated expression of miR-100 and miR-378, 786-O hypoxic cells have downregulated expression levels of *AUTS2*, *MAPT*, *AGT*, and *USH1C* genes. Expression of miR-100 and miR-378 was also found to be significantly reduced in bone metastases of ccRCC (Chen S.C. et al., 2017).

In ccRCC, the response to hypoxia and metastasis is also modulated by the androgen receptor (AR). AR increases the expression of miR-185-5p, which targets *VEGF-C* (a gene encoding lymphangiogenic factor). At the same time, binding of miR-185-5p to the promoter region of *HIF2A* mRNA increases HIF2A expression and the subsequent expression of *VEGFA* (an angiogenic factor). This explains why AR-positive ccRCC metastasizes to the lung rather more often than to lymph nodes (Huang et al., 2017).

In RCC patients, especially those diagnosed with ccRCC and *VHL* disorders caused by familial mutations, disruption of signaling pathways inhibited by tyrosine kinase inhibitors and other related inhibitory drugs is most effective (Mickley et al., 2015). The possibility of using drugs based on miRNA mimics and anti-miR is also being considered for these purposes (Schanza et al., 2017; Serocki et al., 2018).

As mentioned before, miRNAs contribute significantly to regulation of the main hypoxia response pathways. At least 40 miRNAs affect the expression of HIF alone in various tissues, including miR-687 in embryonic renal tissue and miR-429 and miR-19a in endothelial cells (Serocki et al., 2018). Upregulation of miR-106a and miR-106b and downregulation of their target gene *VHL* has been described for ccRCC (Oliveira et al., 2017).

VEGFR2 is the putative target of miR-221 in metastatic RCC (Khella H.W.Z. et al., 2015). Accordingly, poor survival outcome in patients treated with the receptor tyrosine kinase inhibitor sunitinib correlates with a high level of miR-221 accompanied by low levels of *VEGFR2*. The effect of miR-221 is different for endothelial and tumor cells; it lowers proliferation and angiogenesis in human umbilical vein endothelial cells



(HUVECs) but increases the proliferation of ACHN kidney cancer cells (Khella H.W.Z. et al., 2015).

Responses to hypoxia are also influenced by lncRNA. Antisense transcripts of the 3' and 5' non-coding regions of *HIF1A* are 3'aHIF-1 α and 5'aHIF-1 α , respectively. In human lung epithelial cells, the expression of these lncRNAs is induced by hypoxia, and they can suppress the expression of *HIF1A* (Uchida et al., 2004). In addition, increased expression of the HOX transcript antisense intergenic RNA (HOTAIR) lncRNA is observed in RCC, and it correlates with the tumor progression. It has a direct inhibitory effect on miR-217, the target of which is *HIF1A*. Suppression of miR-217 reduces apoptosis, increases proliferation and migration of tumor cells, and stimulates EMT in RCC (Hong et al., 2017).

In RCC and ovarian cancer, miR-192 has anti-angiogenic properties due to the effect on its *EGR1* and *HOXB9* targets (Wu et al., 2016). In lung cancer, *EGR1* binds to the *VEGFA* proximal promoter, activating its expression (Shimoyamada et al., 2010). The effect of *HOXB9* on vascularization in breast cancer has been documented (Seki et al., 2012). Moreover, low levels of miR-192 in various malignant tumors are a predictor of poor prognosis (Wu et al., 2016).

The influence of some miRNAs on the VHL/HIF pathway is summarized in **Figure 3**.

In turn, the expression of certain miRNAs is affected by the proteins of the VHL-HIF-VEGF pathway (for example, Schanza et al., 2017). Thus, knockdown or genetic elimination of the *VHL* gene in RCC cells leads to an increase in miR-28-3p expression. The target of this miRNA is the gene for mitotic spindle checkpoint protein (Mad2), which blocks cells with unformed spindles from entering mitosis. Dysregulation of Mad2 expression leads to chromosomal instability (Hell et al., 2014). The expression of miR-204 in ccRCC cells is, on the contrary, induced by VHL. The target of miR-204 is *MAP1LC3B* (*LC3B*), which is associated with macro-autophagy (Mikhaylova et al., 2012). *LC3B*-mediated macro-autophagy is necessary for nutrient supply to the tumor during the period of metabolic

stress and is important for cell survival, tumor growth, and increased aggressiveness (Sato et al., 2007). At the same time, hypoxia in RCC suppresses miR-30c under the influence of HIF (Huang J. et al., 2013).

As a result of the interaction of different proteins of the VHL/HIF/VEGF pathway, along with the participation of miRNAs, positive and negative feedback loops may appear that prolongs the initial effect of hypoxia.

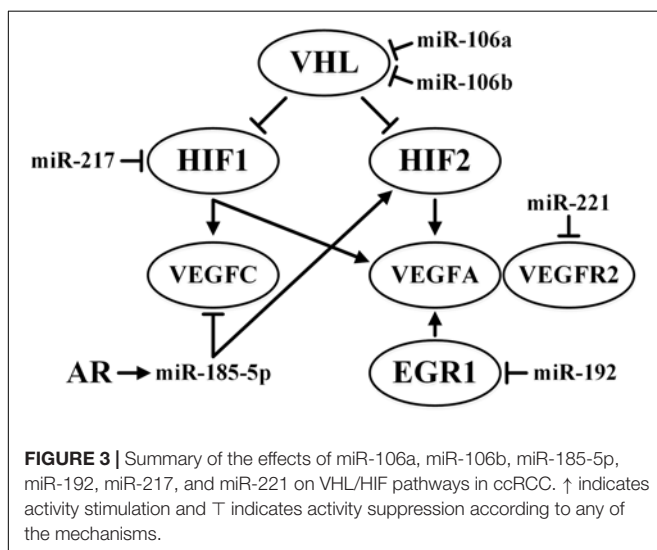
In mice, renal ischemia/reperfusion procedure was reported to reduce the concentration of 40 miRNAs by almost half, while the concentration of 36 other miRNAs more than doubled (Liu et al., 2012). Quantitative RT-PCR performed 4–12 h after the procedure confirmed an increase in miR-210 concentration. In HUVEC-12 cells, angiogenesis is stimulated by increases in the expression of VEGF and VEGFR2, which are triggered by an increased miR-210 concentration (Liu et al., 2012). Since the target of miR-210 in chondrocytes and hepatocellular carcinoma cells is *HIF3A*, it is highly likely that this miRNA may regulate the switch between acute hypoxia and chronic hypoxia responses in these cells (Serocki et al., 2018). HIF1A may regulate miR-210 and has been shown to enhance its expression in RCC (Nakada et al., 2011). HIF1A-mediated enhancement of miR-210 expression was also shown in breast cancer, while HIF2A showed no regulatory effect (Camps et al., 2008). However, in 786-O RCC cells, HIF2A also influenced miR-210 expression (Zhang Z. et al., 2009). Increased expression of miR-210 is most typical for ccRCC (Valera et al., 2011).

A feedback loop has been constructed for the cardiomyocytes. HIF1 increases transcription of the *MIR-21* gene, and miR-21, in turn, induces the expression of *HIF1A* via the regulation of PTEN/AKT signaling pathway (Liu et al., 2014). The expression of miR-21 is also increased in oral squamous cell carcinoma (OSCC). It is highly concentrated in the exosomes of OSCC cells and enhances the migratory and invasive properties of tumor cells in a mechanism that is dependent on HIF1A and HIF2A (Li et al., 2016).

During hypoxia, exosomes containing miR-135b are formed in multiple myeloma cells. Suppression of the *HIF1AN* by miR-135b in HUVEC cells leads to increased HIF1 activity and angiogenesis (Umezue et al., 2014). This mechanism remains turned on even after the restoration of the normal supply of oxygen, which prolongs the antihypoxic response.

Enhanced miR-18a expression after 24 h of hypoxia was observed in human choroidal endothelial cells (Han et al., 2015). Since *HIF1A* mRNA is the direct target of this miRNA, this interaction may act as the switch between the responses to acute hypoxia and chronic hypoxia (Han et al., 2015).

Hypoxia suppresses the expression of DICER, which is associated with miRNA processing. However, it is not understood how this does not lead to the total disruption of miRNA activity (Serocki et al., 2018). Some miRNAs, such as the tumor suppressor miR-182-5p, are more sensitive to hypoxic suppression of DICER expression than others. In addition to the aforementioned effect on the AKT pathway, miR-182-5p acts as an inhibitor of its target *HIF2A*, even though it is primarily manifest in VHL-deficient ccRCC cells (Fan Y. et al., 2016). The miR-200 family is another example of miRNAs that are sensitive



to disruption of DICER expression. DICER is the direct target of miR-122, a miRNA that is highly expressed in ccRCC, while the loss of DICER decreases the expression of the miR-200 family, leading to EMT (Fan Y. et al., 2018).

Most tumor cells respond to hypoxia by shifting their energy metabolism to glycolysis (the Warburg Effect). Many miRNAs participate in this shift, which is a characteristic of many solid tumors (Morais et al., 2017). In particular, in RCC, miR-1291 directly inhibits the expression of the *GLUT1* glucose transporter gene, and its levels in tumor tissue are reduced as compared to the levels in normal tissue (Yamasaki et al., 2013). In ccRCC, hypoxia-induced miR-101 expression can be a mechanism for activating glycolysis, as miR-101 inhibits the *TIGAR* (TP53-Induced Glycolysis and Apoptosis Regulator) gene, which is a member of the p53 pathway that is involved in mediating the exchange to the pentose phosphate pathway (Xu et al., 2017). However, *TIGAR*-mediated regulation of glycolysis has an anti-apoptotic effect, protecting against DNA damage.

lncRNAs also affect the transition to glycolysis (Serocki et al., 2018). In cell cultures obtained from solid tumors, HIF1A-inducible long intergenic non-coding (linc) RNA-p21 was shown to bind to HIF1A, disrupting its interaction with VHL and attenuating the ubiquitination of HIF1A. As a result, both HIF1A and lincRNA-p21 accumulate that causes a transition to glycolysis (Yang et al., 2014).

METASTASIS AND EMT IN KIDNEY CANCER: THE ROLE OF MIRNA

The incidence of metastasis is high in ccRCC (25–30%) and increases to over 50% after surgery (Rydzanicz et al., 2013). Metastatic ccRCC is extremely resistant to therapy, with responses to chemo-, radio-, and immunotherapy observed in no more than 10% of patients, with 5-year survival rates as low as 9% in the presence of distant metastases (Randall et al., 2014).

Metastasis of primary tumors occurs in several stages – from changes in biochemistry, morphology, and migration abilities of tumor cells to the appearance of surface receptors that provide directed migration to target organs, followed by the formation of specific microenvironment in the target organ, into which metastatic cells can enter and survive.

Epithelial-mesenchymal transition is an important stage in metastasis, during which changes occur in the properties of cancer cells. The changes contribute to metastasis. The process of EMT is characterized by a loss of cell polarity and intercellular bonds, as well as an increase in the migration activity of cells and their invasive properties. A critical event in EMT is the loss of E-cadherin and an increase in the activity of its transcriptional repressors, which include *ZEB1*, *ZEB2*, *TWIST*, *SNAIL*, and *SLUG* (Piva et al., 2016). EMT causes the development of sarcomatoid ccRCC (the most unfavorable form of cancer) due to the switched expression of E-cadherin to N-cadherin, dissociation of β -catenin from the membrane, and increased expression of *SNAIL* and *SPARC* (Conant et al., 2011).

An evaluation of the expression of 11 EMT markers in nephrectomized samples of RCC revealed that E-cadherin, clusterin, *TWIST*, and vimentin levels were significant predictors of recurrence (Harada et al., 2012).

Wilms' tumor, WT1, causes mesenchymal-to-epithelial transition (MET) in kidneys during their development. During development of other organs, such as the heart, this gene causes EMT. WT1 is absent in normal renal tissue but is expressed in ccRCC due to the decreased expression of VHL or expression of mutated VHL. WT1 expression in ccRCC in the presence of *SNAIL* and E-cadherin starts a "hybrid" process in which EMT and MET traits are simultaneously expressed (Sampson et al., 2014). The loss of VHL activity also stimulates EMT, thereby increasing the expression of HIF1A, which subsequently activates nuclear factor-kappa B (NF- κ B). The cytoplasmic expression of NF- κ B has been correlated with the invasiveness of ccRCC (Kankaya et al., 2015).

Chronic oxidative stress-stimulated malignant transformation of kidney cells is accompanied by their acquisition of stem characteristics and EMT (Mahalingaiah et al., 2015). This leads to a significant increase in the expression of genes such as *BCL2*, *CCND1* (CyclinD1), *BIRC5* (Survivin), and *PCNA*, as well as *VIM* (Vimentin), *ACTA2* (α -Smooth muscle actin, α -SMA) and *SNAIL1*, as well as a significant decrease in the expression of *CDH1* (E-cadherin), cytokeratin, and *CTNNB1* (β -catenin). The suppression of *OCT4* and *SNAIL* with small interfering RNA (siRNA) can partially reverse these changes (Mahalingaiah et al., 2015). The effect of hypoxia on EMT can also be partially achieved by reducing the expression of miR-30c and increasing the expression of its target, *SLUG*, which also leads to a decrease in E-cadherin expression and stimulation of cell migration in RCC (Huang J. et al., 2013).

Downregulation of *FOXO3a* in ccRCC also leads to an increase in expression of *SNAIL* that stimulates EMT (Ni et al., 2014). In ccRCC, *FOXO3a* is a target of oncogenic miR-155, which increases proliferation, colony formation, migration, and invasion (Ji et al., 2017). *SNAIL1* in ccRCC is the target of miR-30e-3p (minor variant), which inhibits invasion and migration of tumor cells (Wang D. et al., 2017).

The level of miR-720 is elevated in RCC, while its downregulation suppresses tumor growth (Bhat et al., 2017). miR-720 in RCC targets *CTNNA1* (α E-catenin) and *CDH1* (E-cadherin), thereby stimulating cell migration and invasiveness.

ZEB2 suppresses the expression of E-cadherin. In RCC, *ZEB2* enhances the migratory and invasive ability of cells and the expression of *ZEB2* correlates with more advanced forms of the disease and worse survival rates (see the review by Piva et al., 2016). One of the targets of miR-141 (a member of the miR-200 family) in RCC is *ZEB2* and thus it can suppress EMT (Li W. et al., 2014). Reduced expression of miR-141 has been correlated with a poor response to sunitinib (Berkers et al., 2013). In addition, in RCC, miR-141 binds to the lncRNA *HOTAIR* causing its cleavage by the Argonaute 2 (Ago2) complex (Chiyomaru et al., 2014). As already mentioned, *HOTAIR* is also capable of stimulating EMT (Hong et al., 2017). The high expression of miR-122 in ccRCC stimulates EMT as a result of the targeting of *DICER*

and *OCLN* (occludin), which plays an important role in tight junctions, which are characteristic of renal tissue. The loss of tight junctions leads to an increase in cell mobility (Jingushi et al., 2017). Downregulated expression of *DICER* results in the suppression of the maturation of the miR-200 family.

In RCC, three miRNAs – miR-192 (which has anti-angiogenic effects), miR-194, and miR-215 – target *ZEB2*, *MDM2*, and *TYMS* oncogenes (Khella H.W. et al., 2013). miR-30a is specifically downregulated in metastatic tumors compared to its expression in primary tumors (Butz et al., 2015). Other studies on ccRCC have implicated the *ZEB2* and glucose-regulated protein 78 (*GRP78*) genes as targets of miR-30a (Chen Z. et al., 2017; Wang C. et al., 2017).

Expression of the transcription factor *SOX4*, which is normally involved in embryogenesis, is limited to a small number of cell types in which the mature cells retain stem cell characteristics. Due to the influence of *SOX4* on the Wnt/ β -catenin, Notch1, and p53 pathways, as well as the components of the miRNA processing machinery (*DICER*, Argonaute 1, RNA helicase A), upregulation of the expression of *SOX4* can cause the development of different types of cancer and stimulate EMT. In contrast, the high expression of *SOX4* may be associated with a more favorable disease course in other types of cancer. In different cancer types, many miRNAs target *SOX4*. They usually act in an anti-oncogenic manner and are characterized by reduced expression in ccRCC (Geethadevi et al., 2018). In ccRCC, miR-138 has a tumor suppressor role via the targeting of *SOX4*. The effects include the inhibited proliferation, migration, and invasion of tumor cells, increased expression of E-cadherin, and decreased expression of vimentin (Liu F. et al., 2017).

In ccRCC, miR-210 targets *TWIST1*. When the expression of this miRNA is turned off, the cancer cells begin to display the EMT morphology and increased tumor growth is observed in xenografts. High *TWIST1* expression along with low expression of miR-210 is associated with a low survival rate in ccRCC. The aforementioned association of miR-210 with a response to hypoxia and its angiogenic behavior is important. It cannot be ruled out that miR-210 may show varied roles at different stages in the development of ccRCC (Yoshino et al., 2017).

The reduced expression of miR-203 in ccRCC primary tumors correlates with poor prognosis and metastasis, while increased expression of miR-203 suppresses RCC cell lines growth and ccRCC metastasis (Xu M. et al., 2015). The authors attributed this to the action of miR-203 on fibroblast growth factor 2 (*FGF2*) as a target. The *FGF2* protein is able to interact with the dimer of growth factor receptor-bound protein 2 (*GRB2*) involved in the PI3K/AKT and Ras/MAPK signaling pathways. Moreover, *FGF2* is involved in the fine regulation of the Ras/MAPK pathway via negative feedback. *FGF2* is overexpressed in many cancers (Ornitz and Itoh, 2015). The interaction between lncRNA *HOTAIR* and miR-203 leads to the suppression of miR-203 and stimulates EMT; when this interaction is eliminated, the expression of E-cadherin, claudin, *PTEN*, *p21*, and *p27* increase, while vimentin expression is reduced (Hong et al., 2017). Methylation of *MIR-203* is a specific characteristic of metastatic forms of ccRCC (see below and Pereyaslova et al., 2016).

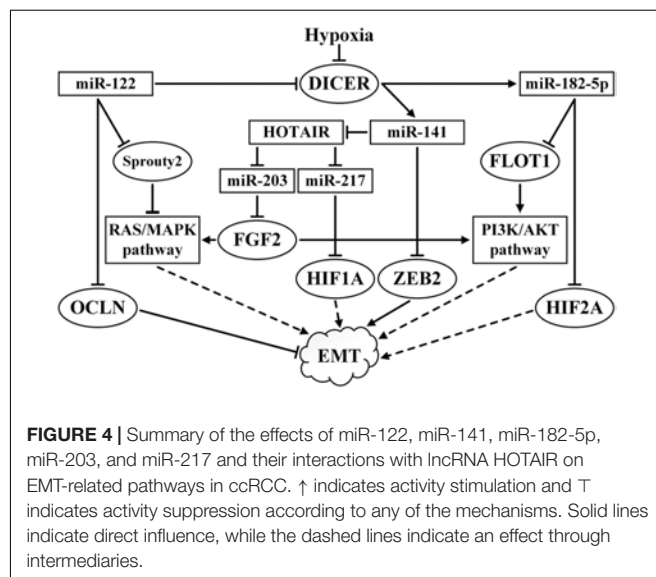
The influence of some miRNAs and lncRNA *HOTAIR* on EMT-related pathways is summarized in **Figure 4**.

The role of the cell environment, in particular the intercellular matrix, is important in metastasis. *CD44* is one of the important targets of miR-34a in the RCC lines. The *CD44* protein is a cell surface glycoprotein that is involved in the intercellular interaction, cell adhesion, and migration. *CD44* is a receptor for hyaluronic acid, osteopontin, collagen, and it can interact with matrix metalloproteinases. Ectopic expression of miR-34a stops cellular growth, migration, and invasion, and also significantly inhibits the growth of carcinoma in xenografts and metastasis in nude mice (Yu et al., 2014).

ADAM metalloproteinase domain 17 (*ADAM17*, also known as disintegrin and A metalloproteinase 17) is a target of miR-145 in RCC. The level of *ADAM17* is significantly increased in RCC, whereas the level of miR-145 is reduced. *ADAM17* stimulates cell proliferation and migration, and also negatively regulates miR-145 through tumor necrosis factor- α (*TNF α*). The result is the formation of a positive feedback loop. In healthy kidneys, this metalloproteinase is found in various types of cells capable of giving rise to RCC (Doberstein et al., 2013).

The expression of miR-186 is greatly reduced in RCC. This miRNA suppresses cell growth, colony formation, invasion, and arrests the cell cycle at the G0/G1 stage. It is believed that the effects of miR-186 are due to the action on *SENPI* (sentrin/SUMO-specific protease 1) as a target (Jiao et al., 2018). The *SENPI* protein is the regulator of the SUMO pathway, which triggers the detachment of SUMO from homeodomain interacting protein kinase 2 (*HIPK2*, a transcriptional regulator), histone deacetylase 1 (*HDAC1*), and metastasis associated 1 (*MTA1*). The influence of the mimic miR-186 on *SENPI* can suppress the pathway of NF- κ B, which reduces the expression of p-I κ B α and p-p65, as well as the underlying cyclin D1 and *MMP9*, and increases the expression of *p21* and *BCL-2* (Jiao et al., 2018).

Reduced expressions of miR-149-5p and miR-149-3p are correlated in ccRCC with increased tumor aggressiveness and



metastases (Okato et al., 2017). The authors attributed this to the fact that the target of both miRNAs is forkhead box protein M1 (*FOXM1*), a transcriptional activator that regulates the expression of cyclins, such as cyclin B1 and cyclin D1.

ABERRANTLY METHYLATED MIRNA GENES IN CCRCC

One of the critical means of regulating the expression of miRNA genes is the methylation of the CpG island that is adjacent to or overlaps with the miRNA gene (Han et al., 2007; Lopez-Serra and Esteller, 2012). It is assumed that the percentage of miRNA genes that are aberrantly methylated in tumors is several times higher than the genes encoding proteins, which increases their prospects as biomarkers (Kunej et al., 2011; Baylin and Jones, 2016; Piletic and Kunej, 2016). Methylation profiles of miRNA genes have been constructed for epithelial tumors that have different localizations, including the colon, lung, breast, prostate, and ovaries. These are being considered as new potential markers and marker systems for the diagnosis and prognosis of these malignant cancers (Pronina et al., 2017b; Torres-Ferreira et al., 2017; Heller et al., 2018; Loginov et al., 2018a,b; Shi et al., 2018; Zare et al., 2018).

In contrast to the most common and widely studied types of cancer, such as colon, lung, breast, and prostate cancer, less is known about the methylation of the miRNA genes in cCRCC. What is known mainly concerns genes of the miR-9 and miR-34 families (Hildebrandt et al., 2010; Vogt et al., 2011; Schiffgen et al., 2013). These studies have shown that the promoter of the gene coding for miR-34a is significantly methylated in RCC cell lines and that the expression of this miRNA is downregulated. Treatment with 5-aza-2'-deoxycytidine increases the expression of miR-34a and reduces the expression of its target (Yu et al., 2014).

The last 5 years have seen a significant increase in the understanding of the role of hypermethylation of miRNA genes in the pathogenesis of cCRCC. In cCRCC, miR-10b is characterized by a highly methylated promoter and significantly reduced expression (He C. et al., 2015). Transfection by lentivirus carrying this miRNA or treatment with demethylating agents inhibits cell proliferation, migration, and invasiveness in cell culture. However, the target genes that are significant in RCC are unknown (He C. et al., 2015). In stomach cancer, miR-10b acts as a tumor suppressor and targets microtubule-associated protein RP/EB family member 1 (*MAPRE1*) (Kim et al., 2011). In non-small-cell lung cancer and bladder cancer, it acts as an oncogene by enhancing cellular migration and invasion. The putative targets associated with this effect are *KLF4* and *HOXD10* (Xiao et al., 2014).

A high miR-21/miR-10b ratio in metastatic cCRCC correlates with a poor prognosis (Fritz et al., 2014). Interestingly, an increased level of MIR-21 expression that correlates with a poor prognosis is associated with hypomethylation of its promoter (Cancer Genome Atlas Research, 2013).

The aforementioned miR-182-5p tumor suppressor miRNA can also be regulated by methylation. An upstream CpG

island (8–10 kb) contains a putative transcription start site. Its methylation level is significantly increased in RCC cell cultures and is slightly elevated in ccRCC samples compared to the normal tissue, which may explain the decreased expression of this miRNA. One of the targets of miR-182-5p is *MALAT-1*. This gene produces a precursor transcript from which a ncRNA is derived by RNase P cleavage of a tRNA-like small ncRNA (known as mascRNA) from its 3' end. Its ribonucleoprotein complexes may act as a transcriptional regulator for numerous genes, including some genes involved in cancer metastasis and cell migration. Downregulation of *MALAT-1* leads to upregulation of p53 and downregulation of CDC20 and AURKA, which are the drivers of the cell cycle mitotic phase (Kulkarni et al., 2018).

The gene encoding miR-766-3p is highly methylated in RCC tissues compared to the normal tissues. This miRNA behaves like a suppressor. Its direct target is *SF2*, whose repression also reduces the expression of AKT and ERK (Chen C. et al., 2017). The promoter of miR-145, which targets the metalloproteinase gene *ADAM17*, is also strongly methylated in RCC. Treatment with 5-aza-2'-deoxycytidine increases the expression of miR-145 and reduces the expression of *ADAM17* (Doberstein et al., 2013). The promoter region of the MIR-200c gene is hypermethylated in RCC cell lines, which correlates with decreased expression, and it is not methylated in the normal renal cell line (Gao et al., 2014). The MIR-492 gene has a strongly methylated promoter in ccRCC and reduced expression. The use of 5-aza-2'-deoxycytidine or the histone deacetylase inhibitor 4-phenylbutyric acid increases the expression of miR-492 in ccRCC cell cultures, inhibits cell proliferation, and stimulates apoptosis and adhesion (Wu et al., 2015). The upstream promoter of MIR-106a gene is hypermethylated in RCC, which might be responsible for its downregulation (Pan et al., 2017).

Over the past 5 years, we have systematically analyzed the methylation of up to 20 miRNA genes in various cancers, including lung, breast, ovarian, and kidney cancer. These analyses have identified new markers and marker systems that are useful for the diagnosis and prognosis of these malignant diseases (Beresneva et al., 2013; Rykov et al., 2013; Pronina et al., 2017b; Braga et al., 2018; Loginov et al., 2018a,b; Varachev et al., 2018). Moreover, we have shown a correlation between the levels of expression and methylation, which has confirmed the functional role of methylation of a group of miRNA genes in the pathogenesis of breast, ovarian, and kidney cancer (Pronina et al., 2017b; Loginov et al., 2018a,b; Varachev et al., 2018).

We first found that the frequency of methylation of six miRNA genes (MIR-124-2, MIR-124-3, MIR-9-1, MIR-9-3, MIR-34b/c, and MIR-129-2) was significantly higher in malignant tumors of patients with cCRCC than in normal kidney tissues (Beresneva et al., 2013). Subsequent studies by our group established that 16 miRNA genes (MIR-124-1/-2/-3, -125b-1, MIR-129-2, MIR-132, MIR-137, MIR-193a, MIR-34b/c, MIR-375, MIR-203, MIR-9-1, MIR-9-3, MIR-107, MIR-130b, and MIR-1258) were hypermethylated and two miRNA genes (MIR-191, MIR-212) were hypomethylated in cCRCC (Pereyaslova et al., 2016; Loginov et al., 2017; Varachev et al., 2018). Moreover, seven miRNA genes (MIR-124-3, MIR-125b-1, MIR-129-2,

MIR-137, MIR-34b/c, MIR-375, MIR-9-3) were downregulated and correlated with altered methylation. The findings provided evidence of the functional significance of methylation in the deregulation of miRNA genes and in the pathogenesis of ccRCC (Varachev et al., 2018).

Besides hypermethylation, some miRNA genes studied (MIR-124-2/-3, -34b/c, MIR-129-2, MIR-107, MIR-148a, MIR-203) have been associated with aspects of ccRCC progression that include advanced pathologic stage, tumor size, and differentiation grade. In particular, hypermethylation of six miRNA genes (MIR-125b-1, MIR-129-2, MIR-203, MIR-375, MIR-107, and MIR-1258) significantly correlated with the presence of metastasis (Pereyaslova et al., 2016; Varachev et al., 2018), and most significantly for MIR-375 and MIR-1258 (p -value $\sim 10^{-7}$ – 10^{-8}).

On the basis of hypermethylated miRNA genes, novel marker systems have been suggested for ccRCC diagnosis (MIR-125b-1, MIR-375, MIR-137, MIR-193a) and prediction of metastasis (MIR-125b-1, MIR-375, MIR-107, MIR-1258, MIR-203) (Loginov et al., 2017; Varachev et al., 2018).

A Russian team of researchers demonstrated the correlation of the methylation of the MIR-129-2 gene with increased expression of genes, such as *RARB(2)*, *RHOA*, *NKIRAS1*, and *CHL1* (Pronina et al., 2017a). Preliminary data from another study indicated a negative correlation between the expression levels of miR-375 and the pro-apoptotic gene *APAF1* (Varachev et al., 2018). The targets of most hypermethylated miRNAs, identified by us recently remain unclear and further research is needed.

However, the roles of some of the novel hypermethylated miRNAs in the pathogenesis of kidney cancer have been studied. For instance, the reduced expression of miR-124 is considered a highly significant factor in ccRCC genesis and may be an essential predictor of survival (Butz et al., 2015). Moreover, the miRNA and gene expression data from 458 ccRCC and 254 normal kidney specimens were used to construct the integrated miRNA-target interaction network, which revealed miR-124 as a key miRNA contributing to the aggressive behavior of ccRCC (Butz et al., 2015).

Table 1 presents examples of miRNAs encoded by genes that are hypermethylated and downregulated in ccRCC. Data on target genes and functions in RCC are also provided. The information presented pertains mainly to those genes whose role of hypermethylation was first explored by our group and data from other authors concerning the functional roles, target genes, and in some cases the signaling pathways involved.

For five miRNAs we identified (miR-124-3p, miR-129-3p, miR-137, miR-203, and miR-375), functional studies by other authors confirmed suppressor properties and/or association with metastasis. The tumor suppressor and anti-metastatic functions of the hypermethylated MIR-129-2 gene we discovered (Beresneva et al., 2013), are consistent with the data that miR-129-3p downregulates multiple metastasis-related genes in RCC cells, including *SOX4*, and also decreased phosphorylation of focal adhesion kinase and expression of *MMP-2/9* (Chen et al., 2014). In addition, miR-129-3p is involved in miR-129-3p/TRPM7/AKT/FOXO1 signaling

pathway (Zhao Z. et al., 2018). The collective data confirm the ability of miR-129-3p to restrain metastasis in ccRCC.

In ccRCC, the hypermethylated MIR-137 gene (Varachev et al., 2018) abrogates the tumor-promoting effect of overexpressed ncRNA Small Nucleolar RNA Host Gene 1 (SNHG1), which is associated with metastasis and poor prognosis of ccRCC patients (Zhao S. et al., 2018).

Data on the nature of the role of miR-203 in RCC, either as a tumor suppressor or an oncogene, is inconsistent (Hu et al., 2014). However, in other cancer types (hepatocellular carcinoma, multiple myeloma, lung cancer, esophageal cancer, bladder cancer), miR-203 acts as a suppressor (Hu et al., 2014). It is possible that this miRNA has different roles at different stages of the development of ccRCC and/or in its different variants. Overall, we observed significantly more frequent hypermethylation of the MIR-203 gene in tumors of patients with ccRCC with metastases. Moreover, our data on the anti-metastatic activity of miR-203 are consistent with data from three other papers (see **Table 1**), including the results on the role of miRNA-203 in inhibition of EMT and metastatic genes (Dasgupta et al., 2018).

Our data concerning the correlation of miR-375 hypermethylation with metastasis strengthens the evidence that the suppression of the tumor aggressive phenotypes of ccRCC mediated by this miRNA regulates the oncogene *YWHAZ*, which is its direct target (Zhang X. et al., 2018). As discussed earlier, aberrantly methylated miR-148a was suggested to function as a tumor suppressor in RCC by targeting *AKT2* (Cao et al., 2017). A recent paper (Reustle et al., 2018) implicated the role of aberrantly methylated miR-212-3p and miR-132-3p in ccRCC in the post-transcriptional regulation of *BCRP/ABCG2* (breast cancer resistance protein), which contributes to the multi-drug resistance seen in cancer. Hypermethylated miR-193a-3p can affect the PI3K/AKT pathway in RCC (Pan et al., 2018a).

However, our data on the frequent hypermethylation of the MIR-125b-1 gene and the hypermethylation linkages with progression and metastasis (Varachev et al., 2018) do not agree with the increased level of this miRNA and its role in promoting cell (Jin et al., 2017). The properties of this miRNA are also ambiguous in other types of cancer, such as breast cancer (Loginov et al., 2018a). Additional studies are required to determine the role and mechanism of miR-125b in RCC.

Tumor suppressor or/and anti-metastatic properties were found for some miRNAs, encoded by 16 aberrantly methylated genes that discovered by us, including miR-124, miR-129, miR-132, miR-137, miR-148a, miR-203, and miR-375, as well as for miR-9 and miR-34 genes. However, further analyses should explore the expression and functional properties of some other miRNAs, which genes were also found as hypermethylated (miR-107, miR-125b, miR-1258, miR-130b, and miR-193a) or showed hypomethylation (miR-191, miR-212) in cancers of the kidney. Overall, our group has provided novel data on the contribution of methylation to the regulation of more than 10 miRNA genes in ccRCC (Beresneva et al., 2013; Loginov et al., 2017; Varachev et al., 2018). Further research and validation of target genes for these aberrantly methylated miRNAs are required. Moreover, hypermethylated

TABLE 1 | Examples of the most studied miRNAs encoded by hypermethylated genes in ccRCC, focusing on the role of aberrant methylation in miRNA deregulation, target genes, and functions.

miRNA	miRNA gene hypermethylation and downregulation	Target genes of miRNA and some pathways	Functions of miRNA in ccRCC	References
miR-124-3p	Hypermethylation of the <i>miR-124-2</i> gene correlates with decrease of miR-124-3p levels in ccRCC tissues and cell lines	<i>CAV1</i> , <i>FLOT</i> , <i>FZD5</i> (miR-124-3p/FZD5/PKC pathway), lncRNA HOTAIR, <i>ST8SIA4</i> (HOTAIR/miR-124/ST8SIA4 pathway)	Hypermethylation of <i>miR-124-2</i> is strongly associated with advanced pathologic stage and grade of differentiation. Restoration miR-124-3p reduces migration, invasion, proliferation, metastasis; decreased cell cycle S phase; increases patient survival, drug resistance. By sponging miR-124 HOTAIR as ceRNA upregulated ST8SIA4 and promoted the proliferation and metastasis in RCC	Butz et al., 2015; Long et al., 2015; Pan et al., 2018b; Varachev et al., 2018
miR-129-3p	Hypermethylation of the <i>miR-129-2</i> gene correlates with decrease of miR-129-3p levels in ccRCC tissues and cell lines	<i>SOX-4</i> , <i>MMP-2/9</i> , <i>TRPM7</i> (miR-129-3p/TRPM7/AKT/FOXO1 pathway)	Hypermethylation of <i>miR-129-2</i> is associated with advanced pathologic stage and metastasis in ccRCC; miR-129-3p inhibits migration, invasion, metastasis	Beresneva et al., 2013; Chen et al., 2014; Zhao Z. et al., 2018
miR-137	Downregulation of miR-137 in RCC tissues and cell lines correlates with hypermethylation of the <i>miR-137</i> gene	lncRNA SNHG1, oncogene <i>RLIP76</i>	Decrease proliferation, migration, invasion, metastasis; promotes apoptosis; inhibits PI3K/PKB pathway; tumor suppressor function confirmed <i>in vitro</i> and <i>in vivo</i>	Zhang and Li, 2016; Varachev et al., 2018; Wang M. et al., 2018; Zhao S. et al., 2018
miR-182-5p	Downregulation correlates with hypermethylation in RCC tissues and cell lines	HIF-2 α , lncRNA MALAT-1, <i>FLOT1</i>	Oncosuppressor; inhibited tumorigenicity <i>in vitro</i> and <i>in vivo</i> and induced mitotic cell cycle arrest and apoptosis	Xu et al., 2014; Fan Y. et al., 2016; Fang et al., 2018; Kulkarni et al., 2018
miR-203	Downregulation of miR-203 in RCC cell lines and ccRCC specimens; methylation of the <i>miR-203</i> gene is significantly increased only in metastatic tumors	<i>FGF2</i> , lncRNA SNHG14, N-WASP (SNHG14/miR-203/N-WASP pathway), lncRNA HOTAIR	<i>miR-203</i> methylation and low expression are associated with metastasis in ccRCC. SNHG14 sponging miR-203 and elevating N-WASP as ceRNA promotes ccRCC migration and invasion. Overexpression of miR-203 inhibited migration, invasion, and induced apoptosis and cell cycle arrest. miR-203-HOTAIR interaction inhibited EMT and metastatic genes	Xu M. et al., 2015; Liu G. et al., 2017; Dasgupta et al., 2018; Varachev et al., 2018
miR-375	Downregulation in RCC cell lines and ccRCC specimens; the <i>miR-375</i> gene is hypermethylated in ccRCC tissues	Oncogene <i>YWHAZ</i>	<i>miR-375</i> methylation and low expression are associated with metastasis ccRCC; according to functional studies miR-375 suppressed ccRCC cell proliferation, migration, and invasion	Varachev et al., 2018; Zhang X. et al., 2018
miR-766-3p	Downregulation caused by hypermethylation in RCC tissues	SF2 (miR-766-3p/SF2/P-AKT/P-ERK pathway)	Downregulation is associated with clinical stage and worse prognosis; upregulation attenuates cell cycle progression	Chen C. et al., 2017

miRNAs could someday be used as convenient markers for ccRCC in the clinic, as has been suggested for non-invasive diagnosis and prognosis of prostate cancer using miR-193b, miR-129-2, and miR-34b/c methylation in tissue and urine samples (Torres-Ferreira et al., 2017).

In Table 1, it is interesting to note that among the seven most studied hypermethylated miRNAs, four have lncRNAs as one or more targets. For example, miR-124-3p interacts with the lncRNA HOTAIR, miR-137 with lncRNA SNHG1, miR-182-5p with lncRNA MALAT1, and miR-203 with the HOTAIR and SNHG14 lncRNAs. These data reinforce the role of lncRNAs

in the regulatory functions of miRNA, apparently by acting primarily as ceRNA to reduce the content of specific miRNAs depending on other factors.

CLINICAL APPLICATION OF MIRNAS AS MARKERS FOR DIAGNOSIS AND PROGNOSIS

In this review, we included only the most convincing results obtained from studies featuring large sample sizes and detailed

statistical evaluations. Initially, we examined the features of miRNAs that are relevant concerning their diagnostic potential. These features are summarized in **Table 2**.

The diagnostic value of elevated miR-210 levels is well established. This may be due to chronic hypoxia observed in ccRCC (Zhang Z. et al., 2009). However, no correlation of miR-210 expression with tumor stage has been reported (Iwamoto et al., 2014). The expression of miR-1233 in RCC cell lines is also stimulated by hypoxia (Dias et al., 2017).

The target of miR-451 is an mRNA encoding the PSMB8 protein, which has a pro-inflammatory function, and which is presumably significant in RCC (Zhu et al., 2016). In RCC, miR-193a-3p affects the PI3K/AKT pathway (Liu L. et al., 2017; Pan et al., 2018a). miR-28-5p is associated with chromosomal instability (Hell et al., 2014). In addition, its target is Ras-related small GTP-binding oncoprotein RAP1B (Wang et al., 2016). miR-141 is involved in the suppression of EMT (Li W. et al., 2014).

Targets of miR-144 include *mTOR* (Xiang et al., 2016) and *MAP3K8* (Liu F. et al., 2016). Thus, miR-144 can suppress cellular proliferation, EMT, and metastasis. However, another study (Xiao et al., 2017) implicated miR-144 as having an oncogenic role, with involvements in the stimulation of proliferation, migration, invasion, and resistance to sunitinib. Furthermore, miR-144 acts as an upregulated marker in the plasma of ccRCC patients, particularly those with advanced pT stage, which is a characteristic of oncogenes (Lou et al., 2017). The inconsistent functional properties of miR-144 in ccRCC revealed to date highlight the need for further studies before its clinical application can be realized.

miR-378 stands apart from other miRNAs. In a study that used samples from patients at all stages of ccRCC (Redova et al., 2012),

the diagnostic value of the increased expression of miR-378 was found. However, when samples were used only from patients at the I-II stage, the decreased expression of miR-378 had diagnostic value (Wang et al., 2015). This dichotomy of miRNA – with an oncogenic role in some types of cancer or at certain stages of the same disease, while being a tumor suppressor in other cases – is quite common. In ccRCC, miR-378 could be a potential marker of the disease stage (Li H.C. et al., 2014; Fedorko et al., 2015). However, Hauser et al. (2012) rejected the correlation between its expression in serum and pathologic stage. The authors also did not find evidence of differential expression of miR-378 levels in diseased and healthy individuals. The expression of miR-378 is likely associated with angiogenesis (Li H.C. et al., 2014). Indeed, in the ccRCC cell line, the expression of miR-378 declines with prolonged hypoxia (Chen S.C. et al., 2017). However, the mechanisms of action of miR-378 in ccRCC have not yet been adequately studied.

The genes discussed above, including some with inconsistent features (miR-144 and miR-378), have been suggested for the diagnosis of ccRCC (**Table 2**). Thus, the methods of ccRCC diagnosis need further verification.

The level of some miRNAs can be valuable as prognostic markers of survival in ccRCC (**Table 3**). Reviews describing miRNA markers used to predict survival and metastasis in ccRCC, have been published (Fedorko et al., 2016; Ran et al., 2017; He Y.H. et al., 2018). In the present review, we present only the most recent studies that featured large sample sizes (**Table 3**). Unfortunately, the data obtained so far has not always been consistent. For example, an increase of miR-210, which serves as a diagnostic criterion for ccRCC, was associated with prolonged overall survival in one study (McCormick et al., 2013) and

TABLE 2 | Non-invasive markers suggested for use in ccRCC diagnosis: miRNA level and diagnostic value.

miRNA and level	Diagnostic value	Type and size of samples used	References
miR-378 ↑, miR-451 ↓	AUC 0.86, Sn 81%, Sp 83%	15 ccRCC + 90 RCC (73 ccRCC), 12+35 healthy controls	Redova et al., 2012
miR-210 ↑	AUC 0.81, Sn 74%, Sp 76%	Meta-analysis – 570 RCC patients	Chen et al., 2018
miR-378 ↑, miR-210 ↑	Sn 80%, Sp 78%	157 ccRCC; 12 chromophobe RCC; 26 papillary RCC	Fedorko et al., 2015
miR-193a-3p ↑, miR-362 ↑, miR-572 ↑, miR-28-5p ↓, miR-378 ↓	AUC 0.81 – for training sample set; AUC 0.80 – for the validating sample set	25+107 ccRCC patients	Wang et al., 2015
miR-210 ↑	Sn 70%, Sp 62%	82 ccRCC patients, serum exosomes	Zhang W. et al., 2018
miR-1233 ↑	Sn 81%, Sp 76%		
miR-34a ↓	Sn 81%, Sp 80%	30 ccRCC patients	Yadav et al., 2017
miR-141 ↓	Sn 75%, Sp 73%		
miR-1233 ↑	Sn 93%, Sp 100%		
miR-141 ↓, miR-1233 ↑	Sn 100%, Sp 73%.		
miR-144-3p ↑	AUC 0.91, Sn 87%, Sp 83% – ccRCC vs healthy donors; AUC 0.82, Sn 75%, Sp 72% – ccRCC vs renal angiomyolipomas	106 ccRCC, 28 renal angiomyolipomas, 123 healthy donors	Lou et al., 2017

Note: ↓ – decreased level of miRNA; ↑ – increased level of miRNA; AUC – area under the receiver operating characteristic curve, Sn – sensitivity, Sp – specificity. Only the most convincing results were included, with sample sizes ≥ 30 and detailed statistical evaluations: AUC, sensitivity, and specificity.

TABLE 3 | Prognostic markers for poor survival and metastasis in ccRCC: miRNA level and predictive value.

miRNA and level	Predictive value	References
miR-210 ↑	Lower stage and grade, miR-210 ↓ is associated with the worst OS	McCormick et al., 2013
miR-21 ↑, miR-126 ↓	Shorter CSS	Vergho et al., 2014
(miR-21/miR-10b)* ↑	Poor prognosis in M0	Fritz et al., 2014
miR-100 ↑	Worse OS and CSS	Wang et al., 2013
miR-155 ↓	Poor prognosis in stage III and IV (43 from 57 patients)	Shinmei et al., 2013
miR-217 ↓	Higher stage and grade, worse OS	Li H. et al., 2013
miR-497 ↓	Shorter OS	Zhao et al., 2015
miR-125b ↑	Worse CSS, early recurrence	Fu et al., 2014
miR-514 ↓	Primary M1 and recurrent	Wotschovsky et al., 2013
miR-204, miR-30c, miR-30a-3p, miR-30a-5p, miR-30e-3p, miR-30e-5p, miR-30c-2-3p ↓	M1 and distant metastasis vs M0	Heinzelmann et al., 2014
miR-30c, miR-126, miR-451 ↑	Shorter PFS and CSS	Heinzelmann et al., 2014
miR-215 ↓	Reduced DFS	Khella H.W. et al., 2013
miR-30a ↓	Hematogenous metastasis	Huang Q.B. et al., 2013
miR-630 ↑	Worst OS, metastasis	Zhao et al., 2014
miRNA-23b/27b cluster ↓	Worst OS, higher chance of disease recurrence	Ishihara et al., 2014
miR-29b ↑	Worst OS	Xu Y. et al., 2015
miR-126 ↓	Worst OS for large tumors (> 4 cm)	Khella H.W. et al., 2015
miR-203 ↓	Worst OS; FGF2 – target (mitogenic and angiogenic activities)	Xu M. et al., 2015
miR-21, miR-1260b, miR-210, miR-100, miR-125b, miR-221, miR-630, miR-497 ↑; miR-106b, miR-99a, miR-1826, miR-215, miR-217, miR-187, miR-129-3p, miR-23b, miR-27b, miR-126 ↓	miR-21 ↑ is associated with the worst OS, CSS, DFS; miR-126 ↓ is associated with the worst OS, CSS, DFS	Gu et al., 2015
miR-21 ↑, miR-210 ↑, miR-141 ↓, miR-200c ↓, miR-429 ↓	Using these 5 miRNAs, a classifier of the risk of recurrence was constructed	Tang and Xu, 2015
miR-210 ↑	Worst OS, higher chance of disease recurrence	Samaan et al., 2015
miR-194 ↓	Worst OS and DFS	Nofech-Mozes et al., 2016
miR-429 ↓	Association with metastases and shorter DFS and OS. Prevents a reduction in the level of E-cadherin in EMT, reduces motility	Machackova et al., 2016
miR-10a-5p ↓	Association with tumor progression. If in the prediction of survival by stage and degree data use miR-10a-5p, AUC increased from 0.942 up to 0.995	Kowalik et al., 2017
miR-10b ↓	Level of miR-10b is significantly lower in primary ccRCC vs normal tissues; it is lower in primary ccRCC vs oncocytoma; it is lower in metastatic ccRCC vs primary ccRCC; it is lower in stages III/IV vs I/II. Decrease in expression is associated with smaller DFS and OS	Khella et al., 2017
miR-224 ↑	Correlation with significantly worse survival	Fujii et al., 2017
miR-18a-5p ↑	Correlation with significantly worse survival	Zhou et al., 2018
miR-122-5p ↓ miR-206 ↓ in ccRCC and in benign renal tumors	miR-122-5p ↑ correlates with metastases and worse survival; miR-206 ↑ correlates with pT stage and metastases and worse survival	Heinemann et al., 2018
miR-21 ↑, miR-142-5p ↑, miR-194 ↓	Correlation with metastases is characterized by 87% sensitivity and 82% specificity	Lokeshwar et al., 2018

Note: ↓ – decreased level of miRNA; ↑ – increased level of miRNA; OS – overall survival; DFS – disease-free survival; CSS – cancer-specific survival; PFS – progression-free survival, * – ratio. Only the most recent (since 2013) works in which sets of >35 ccRCC samples were included.

decreased overall survival in another study (Samaan et al., 2015). For most miRNAs, the nature of interactions in ccRCC is not well understood.

Despite the relative simplicity of miRNA gene methylation studies, the approach has barely been used instead of the expression levels of miRNAs, to evaluate the prognosis of survival or predict metastasis in ccRCC. The only exception is a study (Gebauer et al., 2013) that described that the methylation of the gene encoding miR-124-3 was associated with a more advanced RCC stage, metastasis, and an increased risk of relapse in 111

RCC samples (80 ccRCC, with 77 paired with histologically normal tissue). Also, a recent review did not mention studies involving miRNA gene methylation in RCC (Ran et al., 2017). Studies we undertook identified novel biomarker systems by using hypermethylated miRNA genes as an indicator in ccRCC, both for the diagnosis (MIR-125b-1, MIR-375, MIR-137, and MIR-193a) and for the prediction of metastasis (MIR-125b-1, MIR-375, MIR-107, MIR-1258, and MIR-203) (Loginov et al., 2017; Varachev et al., 2018). The use of both biomarker systems in 70 patients was characterized by a high discrimination value

[area under the curve (AUC) 0.93], sensitivity (86%), and specificity (95%).

Few studies have explored the use of the miRNA level as an indicator to predict the response to ccRCC treatment. Most of the studies have focused on predicting the success of the response to tyrosine kinase inhibitors that are the first-line treatment for metastatic ccRCC. However, approximately 20% of the patients who are treated rapidly develop resistance to these drugs. For example, for 74 patients with metastatic ccRCC who were treated with tyrosine kinase inhibitors, early progression of the disease was observed in 16 (Garcia-Donas et al., 2016). Selection of increased miR-1307-3p and miR-425-5p levels as the biomarker predicted the risk of poor outcome to treatment with an AUC value of 0.75, which is better than the risks predicted using parameters based on clinicopathological factors. This result was revalidated in a group of 64 patients (Garcia-Donas et al., 2016). The predictors of the outcome of sunitinib treatment were studied in 123 patients with ccRCC (the dataset included metastatic and non-metastatic ccRCC patients). Of the 123 patients, 97 were characterized by prolonged (>22 months) progression-free survival (PFS) and the remaining 26 showed rapid progression of the disease in the first 3 months (Puente et al., 2017). It must be noted that, along with the other characteristic clinical indices, the former group was distinguished by the presence of high levels of the transcription factors HEYL, HEY, and HES, accompanied by elevated levels of miR-27b, miR-23b, and miR-628-5p, although miR-23b is considered as a typical oncogene (Zaman et al., 2012). In another study, 56 sunitinib-treated patients with metastatic ccRCC (24 responsive patients with PFS > 18 months and 32 non-responsive patients with PFS < 6 months) (Kovacova et al., 2018). A good response to the drug was positively associated with miR-942 and miR-133 levels, and the model based on using their expression levels as indicators gave predictions with an AUC value of 0.81.

Another aspect of the use of miRNA as biomarkers is the predictive capabilities of miRNA polymorphisms (variations or polymorphisms in sequences of miRNA, target mRNA, miRNA genes, and target miRNA genes and their pathways). The polymorphism in the MIR-34b/c promoter region has been amply studied. This polymorphism reduces the expression of this miRNA (allele C for rs4938723) in the homozygotic condition, increases the risk of RCC in the Chinese population, especially in elderly, men, smokers, and alcohol abusers (Zhang S. et al., 2014). Polymorphisms in the miRNA-binding sites of 102 genes belonging to the VHL-HIF1 α pathway and their effects on RCC risk have been analyzed (Wei et al., 2014). The most significant effect of two polymorphisms in the *MAPK1* gene, and the presence of four of the five adverse alleles in the *MAPK1*, *CDCP1*, *TFRC*, and *DEC1* gene set, was a more than twofold increase in the risk of RCC.

The genotype CC, a variant of the 3' untranslated region (UTR) of the SET domain containing lysine methyltransferase 8 (*SET8*) gene, significantly reduced the risk of ccRCC (the odds ratio = 0.318). This single nucleotide polymorphism, also designated rs16917496, is located in the miR-502 binding site within the 3'UTR of the *SET8* gene and is associated with

its downregulation in ccRCC. Knockdown of *SET8* inhibited proliferation, migration, and invasiveness of ccRCC cells in culture (Zhang et al., 2017). Many ccRCC-influencing genes are associated with remodeling, and *SET8* may be one of them (Joosten et al., 2018).

CONCLUSION

In recent years, the role of miRNA in the pathogenesis of ccRCC has been amply studied. In ccRCC, miRNAs involved in processes that include the response to hypoxia, EMT, and chromatin remodeling play significant roles. Nevertheless, the picture is far from complete. The search for new diagnostic and prognostic markers is still largely empirical and the associated pathways and processes are not clearly elucidated. For example, in a study involving prediction and correlation analyses, miRNA-mRNA pairs displayed a significant inverse relationship. miR-30b was one of these miRNAs, and its expression was negatively correlated with the highest number of genes in ccRCC (Liu et al., 2018). Among the latter, there are, for example, genes that are significant for tumor invasion, such as Integrin Subunit Alpha 5 (*ITGA5*). Nevertheless, almost nothing is known concerning the influence of miR-30b on ccRCC. The many potential biomarkers include some differentially expressed miRNAs or differentially methylated sites. However, only a few have been experimentally verified (Wang Z. et al., 2018).

For the diagnosis of ccRCC, the use of miRNAs like miR-144 has been proposed since their targets may be relevant for this cancer. However, this use is hampered by inconsistent findings, with both tumor suppressive and oncogenic activity reported in separate studies. Results of various studies for other miRNAs, including miR-106a, miR-125b, miR-203, and miR-378, have been contradictory. These discrepancies may be due to the varying roles of these miRNAs at different stages of cancer or in different forms of the disease or to their ability to interact with various targets depending on the cellular context.

Our recent contributions have revealed the potential for the use of the methylation profiles of miRNA genes in the search for reliable diagnostic and prognostic marker systems (i.e., high AUC). The results have suggested the need to expand the search for the selection of new miRNA genes regulated by methylation. Moreover, in-depth studies of the identified hypermethylated miRNA genes associated with metastasis with respect to their target genes and functions are necessary.

The recently discovered lncRNAs represent a novel class of potential prognostic biomarkers in ccRCC. Data on the function and clinical applications of lncRNA in ccRCC is accumulating, with 60 articles identified in PubMed as of January 2019. However, only a few of these studies (10%) explored the interactions of lncRNAs with miRNAs. Nevertheless, a detailed analysis of the molecular mechanisms of ccRCC in original articles allowed us to identify at least seven miRNAs (miR-122, miR-124-3p, miR-137, miR-141, miR-182-5p, miR-203, and miR-217) that interact with the lncRNAs (e.g., HOTAIR, MALAT-1, SNHG1, and SNHG14). Further research is needed

to elucidate these interactions in ccRCC and to assess their clinical potential.

AUTHOR CONTRIBUTIONS

EB, MF, VL, AD, and SM wrote the manuscript. All the authors revised the work critically for important intellectual content, approved the version to be published, and agreed to be

accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

The work was carried out within the framework of fundamental research for state academies for 2013-2020 (No. 0520-2019-0030).

REFERENCES

- Andorfer, C. A., Necela, B. M., Thompson, E. A., and Perez, E. A. (2011). MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer. *Trends Mol. Med.* 17, 313–319. doi: 10.1016/j.molmed.2011.01.006
- Baylin, S. B., and Jones, P. A. (2016). Epigenetic determinants of cancer. *Cold Spring Harb. Perspect. Biol.* 8:a019505. doi: 10.1101/cshperspect.a019505
- Beresneva, E. V., Rykov, S. V., Khodyrev, D. S., Pronina, I. V., Ermilova, V. D., Kazubskaya, T. P., et al. (2013). Methylation profile of group of miRNA genes in clear cell renal cell carcinoma and their involvement in cancer progression. *Russ. J. Genet.* 49, 320–328. doi: 10.1134/S1022795413030034
- Berkers, J., Govaere, O., Wolter, P., Beuselinck, B., Schoffski, P., van Kempen, L. C., et al. (2013). A possible role for microRNA-141 down-regulation in sunitinib resistant metastatic clear cell renal cell carcinoma through induction of epithelial-to-mesenchymal transition and hypoxia resistance. *J. Urol.* 189, 1930–1938. doi: 10.1016/j.juro.2012.11.133
- Bhat, N. S., Colden, M., Dar, A. A., Saini, S., Arora, P., Shahryari, V., et al. (2017). MicroRNA-720 regulates E-cadherin- α E-catenin complex and promotes renal cell carcinoma. *Mol. Cancer Ther.* 16, 2840–2848.
- Braga, E. A., Khodyrev, D. S., Loginov, V. I., Pronina, I. V., Senchenko, V. N., Dmitriev, A. A., et al. (2015). Methylation in the regulation of the expression of chromosome 3 and microRNA genes in clear-cell renal cell carcinomas. *Russ. J. Genet.* 51, 566–581. doi: 10.1134/S1022795415050026
- Braga, E. A., Loginov, V. I., Burdennyi, A. M., Filippova, E. A., Pronina, I. V., Kurevlev, S. V., et al. (2018). Five hypermethylated microRNA genes as potential markers of ovarian cancer. *Bull. Exp. Biol. Med.* 164, 351–355. doi: 10.1007/s10517-018-3988-y
- Butz, H., Szabo, P. M., Khella, H. W., Nofech-Mozes, R., Patocs, A., and Yousef, G. M. (2015). miRNA-target network reveals miR-124a as a key miRNA contributing to clear cell renal cell carcinoma aggressive behaviour by targeting CAV1 and FLOT1. *Oncotarget* 6, 12543–12557. doi: 10.18632/oncotarget.3815
- Cairns, P. (2010). Renal cell carcinoma. *Cancer Biomark.* 9, 461–473. doi: 10.3233/CBM-2011-0176
- Camps, C., Buffa, F. M., Colella, S., Moore, J., Sotiriou, C., Sheldon, H., et al. (2008). hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer. *Clin. Cancer Res.* 14, 1340–1348. doi: 10.1158/1078-0432.CCR-07-1755
- Cancer Genome Atlas Research, N. (2013). Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 499, 43–49. doi: 10.1038/nature12222
- Cao, H., Liu, Z., Wang, R., Zhang, X., Yi, W., Nie, G., et al. (2017). miR-148a suppresses human renal cell carcinoma malignancy by targeting AKT2. *Oncol. Rep.* 37, 147–154. doi: 10.3892/or.2016.5257
- Chan, J. J., and Tay, Y. (2018). Noncoding RNA:RNA regulatory networks in cancer. *Int. J. Mol. Sci.* 19:E1310. doi: 10.3390/ijms19051310
- Chen, C., Xue, S., Zhang, J., Chen, W., Gong, D., Zheng, J., et al. (2017). DNA-methylation-mediated repression of miR-766-3p promotes cell proliferation via targeting SF2 expression in renal cell carcinoma. *Int. J. Cancer* 141, 1867–1878. doi: 10.1002/ijc.30853
- Chen, J., Gu, Y., and Shen, W. (2017). MicroRNA-21 functions as an oncogene and promotes cell proliferation and invasion via TIMP3 in renal cancer. *Eur. Rev. Med. Pharmacol. Sci.* 21, 4566–4576.
- Chen, S. C., Chen, F. W., Hsu, Y. L., and Kuo, P. L. (2017). Systematic analysis of transcriptomic profile of renal cell carcinoma under long-term hypoxia using next-generation sequencing and bioinformatics. *Int. J. Mol. Sci.* 18:E2657. doi: 10.3390/ijms18122657
- Chen, X., Ruan, A., Wang, X., Han, W., Wang, R., Lou, N., et al. (2014). miR-129-3p, as a diagnostic and prognostic biomarker for renal cell carcinoma, attenuates cell migration and invasion via downregulating multiple metastasis-related genes. *J. Cancer Res. Clin. Oncol.* 140, 1295–1304. doi: 10.1007/s00432-014-1690-7
- Chen, Y., Wang, X., Zhu, X., and Shao, S. (2018). Detection performance of circulating microRNA-210 for renal cell carcinoma: a meta-analysis. *Clin. Lab.* 64, 569–576. doi: 10.7754/Clin.Lab.2017.171103
- Chen, Z., Zhang, J., Zhang, Z., Feng, Z., Wei, J., Lu, J., et al. (2017). The putative tumor suppressor microRNA-30a-5p modulates clear cell renal cell carcinoma aggressiveness through repression of ZEB2. *Cell Death Dis.* 8:e2859. doi: 10.1038/cddis.2017.252
- Chiyomaru, T., Fukuhara, S., Saini, S., Majid, S., Deng, G., Shahryari, V., et al. (2014). Long non-coding RNA HOTAIR is targeted and regulated by miR-141 in human cancer cells. *J. Biol. Chem.* 289, 12550–12565. doi: 10.1074/jbc.M113.488593
- Conant, J. L., Peng, Z., Evans, M. F., Naud, S., and Cooper, K. (2011). Sarcomatoid renal cell carcinoma is an example of epithelial–mesenchymal transition. *J. Clin. Pathol.* 64, 1088–1092. doi: 10.1136/jclinpath-2011-200216
- Cora, D., Re, A., Caselle, M., and Bussolino, F. (2017). MicroRNA-mediated regulatory circuits: outlook and perspectives. *Phys. Biol.* 14:045001. doi: 10.1088/1478-3975/aa6f21
- Cui, L., Zhou, H., Zhao, H., Zhou, Y., Xu, R., Xu, X., et al. (2012). MicroRNA-99a induces G1-phase cell cycle arrest and suppresses tumorigenicity in renal cell carcinoma. *BMC Cancer* 12:546. doi: 10.1186/1471-2407-12-546
- Cui, M., Liu, W., Zhang, L., Guo, F., Liu, Y., Chen, F., et al. (2017). Over-Expression of miR-21 and Lower PTEN Levels in Wilms' Tumor with aggressive behavior. *Tohoku J. Exp. Med.* 242, 43–52. doi: 10.1620/tjem.242.43
- Dasgupta, P., Kulkarni, P., Majid, S., Shahryari, V., Hashimoto, Y., Bhat, N. S., et al. (2018). MicroRNA-203 inhibits long noncoding RNA HOTAIR and regulates tumorigenesis through epithelial-to-mesenchymal transition pathway in renal cell carcinoma. *Mol. Cancer Ther.* 17, 1061–1069. doi: 10.1158/1535-7163.MCT-17-0925
- Dias, F., Teixeira, A. L., Ferreira, M., Adem, B., Bastos, N., Vieira, J., et al. (2017). Plasmatic miR-210, miR-221 and miR-1233 profile: potential liquid biopsies candidates for renal cell carcinoma. *Oncotarget* 8, 103315–103326. doi: 10.18632/oncotarget.21733
- Ding, J., Yeh, C. R., Sun, Y., Lin, C., Chou, J., Ou, Z., et al. (2018). Estrogen receptor beta promotes renal cell carcinoma progression via regulating LncRNA HOTAIR-miR-138/200c/204/217 associated CeRNA network. *Oncogene* 37, 5037–5053. doi: 10.1038/s41388-018-0175-6
- Dmitriev, A. A., Rudenko, E. E., Kudryavtseva, A. V., Krasnov, G. S., Gordiyuk, V. V., Melnikova, N. V., et al. (2014). Epigenetic alterations of chromosome 3 revealed by NotI-microarrays in clear cell renal cell carcinoma. *Biomed. Res. Int.* 2014:735292. doi: 10.1155/2014/735292
- Doberstein, K., Steinmeyer, N., Hartmetz, A. K., Eberhardt, W., Mittelbronn, M., Harter, P. N., et al. (2013). MicroRNA-145 targets the metalloprotease ADAM17 and is suppressed in renal cell carcinoma patients. *Neoplasia* 15, 218–230.
- Dragomir, M., Mafra, A. C. P., Dias, S. M. G., Vasilescu, C., and Calin, G. A. (2018). Using microRNA Networks to Understand Cancer. *Int. J. Mol. Sci.* 19:E1871. doi: 10.3390/ijms19071871

- Exposito-Villen, A., E Aránega, A., and Franco, D. (2018). Functional role of non-coding RNAs during epithelial-to-mesenchymal transition. *Noncoding RNA* 4:E14. doi: 10.3390/ncrna4020014
- Fan, W., Huang, J., Xiao, H., and Liang, Z. (2016). MicroRNA-22 is downregulated in clear cell renal cell carcinoma, and inhibits cell growth, migration and invasion by targeting PTEN. *Mol. Med. Rep.* 13, 4800–4806. doi: 10.3892/mmr.2016.5101
- Fan, Y., Li, H., Ma, X., Gao, Y., Bao, X., Du, Q., et al. (2016). Dicer suppresses the malignant phenotype in VHL-deficient clear cell renal cell carcinoma by inhibiting HIF-2alpha. *Oncotarget* 7, 18280–18294. doi: 10.18632/oncotarget.7807
- Fan, Y., Ma, X., Li, H., Gao, Y., Huang, Q., Zhang, Y., et al. (2018). miR-122 promotes metastasis of clear-cell renal cell carcinoma by downregulating Dicer. *Int. J. Cancer* 142, 547–560. doi: 10.1002/ijc.31050
- Fang, X., Sun, R., Hu, Y., Wang, H., Guo, Y., Yang, B., et al. (2018). miRNA-182-5p, via HIF2alpha, contributes to arsenic carcinogenesis: evidence from human renal epithelial cells. *Metalomics* 10, 1607–1617. doi: 10.1039/c8mt00251g
- Fedorko, M., Pacik, D., Wasserbauer, R., Juracek, J., Varga, G., Ghazal, M., et al. (2016). MicroRNAs in the pathogenesis of renal cell carcinoma and their diagnostic and prognostic utility as cancer biomarkers. *Int. J. Biol. Markers* 31, e26–e37. doi: 10.5301/ijbm.5000174
- Fedorko, M., Stanik, M., Iliev, R., Redova-Lojova, M., Machackova, T., Svoboda, M., et al. (2015). Combination of MiR-378 and MiR-210 serum levels enables sensitive detection of renal cell carcinoma. *Int. J. Mol. Sci.* 16, 23382–23389. doi: 10.3390/ijms161023382
- Fritz, H. K., Lindgren, D., Ljungberg, B., Axelson, H., and Dahlback, B. (2014). The miR(21/10b) ratio as a prognostic marker in clear cell renal cell carcinoma. *Eur. J. Cancer* 50, 1758–1765. doi: 10.1016/j.ejca.2014.03.281
- Fu, Q., Liu, Z., Pan, D., Zhang, W., Xu, L., Zhu, Y., et al. (2014). Tumor miR-125b predicts recurrence and survival of patients with clear-cell renal cell carcinoma after surgical resection. *Cancer Sci.* 105, 1427–1434. doi: 10.1111/cas.12507
- Fujii, N., Hirata, H., Ueno, K., Mori, J., Oka, S., Shimizu, K., et al. (2017). Extracellular miR-224 as a prognostic marker for clear cell renal cell carcinoma. *Oncotarget* 8, 109877–109888. doi: 10.18632/oncotarget.22436
- Gao, C., Peng, F. H., and Peng, L. K. (2014). MiR-200c sensitizes clear-cell renal cell carcinoma cells to sorafenib and imatinib by targeting heme oxygenase-1. *Neoplasia* 61, 680–689. doi: 10.4149/neo_2014_083
- Garcia-Donas, J., Beuselinck, B., Inglada-Perez, L., Grana, O., Schoffski, P., Wozniak, A., et al. (2016). Deep sequencing reveals microRNAs predictive of antiangiogenic drug response. *JCI Insight* 1:e86051. doi: 10.1172/jci.insight.86051
- Gattoliat, C. H., Couve, S., Meurice, G., Orear, C., Droin, N., Chiquet, M., et al. (2018). Integrative analysis of dysregulated microRNAs and mRNAs in multiple recurrent synchronized renal tumors from patients with von Hippel-Lindau disease. *Int. J. Oncol.* 53, 1455–1468. doi: 10.3892/ijo.2018.4490
- Gebauer, K., Peters, I., Dubrowskaja, N., Hennenlotter, J., Abbas, M., Scherer, R., et al. (2013). Hsa-mir-124-3 CpG island methylation is associated with advanced tumours and disease recurrence of patients with clear cell renal cell carcinoma. *Br. J. Cancer* 108, 131–138. doi: 10.1038/bjc.2012.537
- Geethadevi, A., Sharma, A., Sharma, M. K., and Parashar, D. (2018). An interplay between microRNA and SOX4 in the regulation of epithelial-mesenchymal transition and cancer progression. *Cancer Transl. Med.* 4, 17–27. doi: 10.4103/ctm.ctm_4_18
- Goradel, N. H., Mohammadi, N., Haghi-Aminjan, H., Farhood, B., Negahdari, B., and Sahebkar, A. (2018). Regulation of tumor angiogenesis by microRNAs: State of the art. *J. Cell. Physiol.* 234, 1099–1110. doi: 10.1002/jcp.27051
- Gu, L., Li, H., Chen, L., Ma, X., Gao, Y., Li, X., et al. (2015). MicroRNAs as prognostic molecular signatures in renal cell carcinoma: a systematic review and meta-analysis. *Oncotarget* 6, 32545–32560. doi: 10.18632/oncotarget.5324
- Han, F., Wu, Y., and Jiang, W. (2015). MicroRNA-18a decreases choroidal endothelial cell proliferation and migration by inhibiting HIF1A expression. *Med. Sci. Monit.* 21, 1642–1647. doi: 10.12659/MSM.893068
- Han, L., Witmer, P. D., Casey, E., Valle, D., and Sukumar, S. (2007). DNA methylation regulates MicroRNA expression. *Cancer Biol. Ther.* 6, 1284–1288.
- Harada, K., Miyake, H., Kusuda, Y., and Fujisawa, M. (2012). Expression of epithelial-mesenchymal transition markers in renal cell carcinoma: impact on prognostic outcomes in patients undergoing radical nephrectomy. *BJU Int.* 110(11 Pt C), E1131–E1137. doi: 10.1111/j.1464-410X.2012.11297.x
- Hauser, S., Wulfken, L. M., Holdenrieder, S., Moritz, R., Ohlmann, C. H., Jung, V., et al. (2012). Analysis of serum microRNAs (miR-26a-2*, miR-191, miR-337-3p and miR-378) as potential biomarkers in renal cell carcinoma. *Cancer Epidemiol.* 36, 391–394. doi: 10.1016/j.canep.2012.04.001
- He, C., Zhao, X., Jiang, H., Zhong, Z., and Xu, R. (2015). Demethylation of miR-10b plays a suppressive role in ccRCC cells. *Int. J. Clin. Exp. Pathol.* 8, 10595–10604.
- He, H., Dai, J., Zhuo, R., Zhao, J., Wang, H., Sun, F., et al. (2018). Study on the mechanism behind lncRNA MEG3 affecting clear cell renal cell carcinoma by regulating miR-7/RASL11B signaling. *J. Cell. Physiol.* 233, 9503–9515. doi: 10.1002/jcp.26849
- He, H., Wang, L., Zhou, W., Zhang, Z., Wang, L., Xu, S., et al. (2015). MicroRNA expression profiling in clear cell renal cell carcinoma: identification and functional validation of key miRNAs. *PLoS One* 10:e0125672. doi: 10.1371/journal.pone.0125672
- He, Y. H., Chen, C., and Shi, Z. (2018). The biological roles and clinical implications of microRNAs in clear cell renal cell carcinoma. *J. Cell. Physiol.* 233, 4458–4465. doi: 10.1002/jcp.26347
- Heinemann, F. G., Tolkach, Y., Deng, M., Schmidt, D., Perner, S., Kristiansen, G., et al. (2018). Serum miR-122-5p and miR-206 expression: non-invasive prognostic biomarkers for renal cell carcinoma. *Clin. Epigenetics* 10:11. doi: 10.1186/s13148-018-0444-9
- Heinzelmann, J., Unrein, A., Wickmann, U., Baumgart, S., Stapf, M., Szendroi, A., et al. (2014). MicroRNAs with prognostic potential for metastasis in clear cell renal cell carcinoma: a comparison of primary tumors and distant metastases. *Ann. Surg. Oncol.* 21, 1046–1054. doi: 10.1245/s10434-013-3361-3
- Hell, M. P., Thoma, C. R., Fankhauser, N., Christinat, Y., Weber, T. C., and Krek, W. (2014). miR-28-5p promotes chromosomal instability in VHL-associated cancers by inhibiting Mad2 translation. *Cancer Res.* 74, 2432–2443. doi: 10.1158/0008-5472.CAN-13-2041
- Heller, G., Altenberger, C., Steiner, I., Topkian, T., Ziegler, B., Tomasich, E., et al. (2018). DNA methylation of microRNA-coding genes in non-small-cell lung cancer patients. *J. Pathol.* 245, 387–398. doi: 10.1002/path.5079
- Hildebrandt, M. A., Gu, J., Lin, J., Ye, Y., Tan, W., Tamboli, P., et al. (2010). Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma. *Oncogene* 29, 5724–5728. doi: 10.1038/onc.2010.305
- Hirata, H., Ueno, K., Nakajima, K., Tabatabai, Z. L., Hinoda, Y., Ishii, N., et al. (2013). Genistein downregulates onco-miR-1260b and inhibits Wnt-signalling in renal cancer cells. *Br. J. Cancer* 108, 2070–2078. doi: 10.1038/bjc.2013.173
- Hong, Q., Li, O., Zheng, W., Xiao, W. Z., Zhang, L., Wu, D., et al. (2017). LncRNA HOTAIR regulates HIF-1alpha/AXL signaling through inhibition of miR-217 in renal cell carcinoma. *Cell Death Dis.* 8:e2772. doi: 10.1038/cddis.2017.181
- Hu, G., Lai, P., Liu, M., Xu, L., Guo, Z., Liu, H., et al. (2014). miR-203a regulates proliferation, migration, and apoptosis by targeting glycogen synthase kinase-3beta in human renal cell carcinoma. *Tumour Biol.* 35, 11443–11453. doi: 10.1007/s13277-014-2476-x
- Huang, J., Yao, X., Zhang, J., Dong, B., Chen, Q., Xue, W., et al. (2013). Hypoxia-induced downregulation of miR-30c promotes epithelial-mesenchymal transition in human renal cell carcinoma. *Cancer Sci.* 104, 1609–1617. doi: 10.1111/cas.12291
- Huang, Q., Sun, Y., Ma, X., Gao, Y., Li, X., Niu, Y., et al. (2017). Androgen receptor increases hematogenous metastasis yet decreases lymphatic metastasis of renal cell carcinoma. *Nat. Commun.* 8:918.
- Huang, Q. B., Ma, X., Zhang, X., Liu, S. W., Ai, Q., Shi, T. P., et al. (2013). Down-regulated miR-30a in clear cell renal cell carcinoma correlated with tumor hematogenous metastasis by targeting angiogenesis-specific DLL4. *PLoS One* 8:e67294. doi: 10.1371/journal.pone.0067294
- Huang, X., Huang, M., Kong, L., and Li, Y. (2015). miR-372 suppresses tumour proliferation and invasion by targeting IGF2BP1 in renal cell carcinoma. *Cell Prolif.* 48, 593–599. doi: 10.1111/cpr.12207
- Ishihara, T., Seki, N., Inoguchi, S., Yoshino, H., Tatarano, S., Yamada, Y., et al. (2014). Expression of the tumor suppressive miRNA-23b/27b cluster is a good prognostic marker in clear cell renal cell carcinoma. *J. Urol.* 192, 1822–1830. doi: 10.1016/j.juro.2014.07.001

- Iwamoto, H., Kanda, Y., Sejima, T., Osaki, M., Okada, F., and Takenaka, A. (2014). Serum miR-210 as a potential biomarker of early clear cell renal cell carcinoma. *Int. J. Oncol.* 44, 53–58. doi: 10.3892/ijo.2013.2169
- Jasinski-Bergner, S., Stoeck, C., Bukur, J., Massa, C., Braun, J., Huttelmaier, S., et al. (2015). Clinical relevance of miR-mediated HLA-G regulation and the associated immune cell infiltration in renal cell carcinoma. *Oncoimmunology* 4:e1008805. doi: 10.1080/2162402X.2015.1008805
- Ji, H., Tian, D., Zhang, B., Zhang, Y., Yan, D., and Wu, S. (2017). Overexpression of miR-155 in clear-cell renal cell carcinoma and its oncogenic effect through targeting FOXO3a. *Exp. Ther. Med.* 13, 2286–2292. doi: 10.3892/etm.2017.4263
- Jiao, D., Wu, M., Ji, L., Liu, F., and Liu, Y. (2018). MicroRNA-186 suppresses cell proliferation and metastasis through targeting sentrin-specific protease 1 in renal cell carcinoma. *Oncol. Res.* 26, 249–259. doi: 10.3727/096504017X14953948675430
- Jin, L., Zhang, Z., Li, Y., He, T., Hu, J., Liu, J., et al. (2017). miR-125b is associated with renal cell carcinoma cell migration, invasion and apoptosis. *Oncol. Lett.* 13, 4512–4520. doi: 10.3892/ol.2017.5985
- Jingushi, K., Kashiwagi, Y., Ueda, Y., Kitae, K., Hase, H., Nakata, W., et al. (2017). High miR-122 expression promotes malignant phenotypes in ccRCC by targeting occludin. *Int. J. Oncol.* 51, 289–297. doi: 10.3892/ijo.2017.4016
- Joosten, S. C., Smits, K. M., Aarts, M. J., Melotte, V., Koch, A., Tjan-Heijnen, V. C., et al. (2018). Epigenetics in renal cell cancer: mechanisms and clinical applications. *Nat. Rev. Urol.* 15, 430–451. doi: 10.1038/s41585-018-0023-z
- Jung, M., Mollenkopf, H. J., Grimm, C., Wagner, I., Albrecht, M., Waller, T., et al. (2009). MicroRNA profiling of clear cell renal cell cancer identifies a robust signature to define renal malignancy. *J. Cell. Mol. Med.* 13, 3918–3928. doi: 10.1111/j.1582-4934.2009.00705.x
- Kankaya, D., Kiremitci, S., Tulunay, O., and Baltaci, S. (2015). Gelsolin, NF-kappaB, and p53 expression in clear cell renal cell carcinoma: impact on outcome. *Pathol. Res. Pract.* 211, 505–512. doi: 10.1016/j.prp.2015.03.002
- Kashyap, D., Tuli, H. S., Garg, V. K., Goel, N., and Bishayee, A. (2018). Oncogenic and tumor-suppressive roles of MicroRNAs with special reference to apoptosis: molecular mechanisms and therapeutic potential. *Mol. Diagn. Ther.* 22, 179–201. doi: 10.1007/s40291-018-0316-1
- Kawakami, K., Enokida, H., Chiyomaru, T., Tatarano, S., Yoshino, H., Kagata, I., et al. (2012). The functional significance of miR-1 and miR-133a in renal cell carcinoma. *Eur. J. Cancer* 48, 827–836. doi: 10.1016/j.ejca.2011.06.030
- Khella, H. W., Bakhet, M., Allo, G., Jewett, M. A., Girgis, A. H., Latif, A., et al. (2013). miR-192, miR-194 and miR-215: a convergent microRNA network suppressing tumor progression in renal cell carcinoma. *Carcinogenesis* 34, 2231–2239. doi: 10.1093/carcin/bgt184
- Khella, H. W., Scorilas, A., Mozes, R., Mirham, L., Lianidou, E., Krylov, S. N., et al. (2015). Low expression of miR-126 is a prognostic marker for metastatic clear cell renal cell carcinoma. *Am. J. Pathol.* 185, 693–703. doi: 10.1016/j.ajpath.2014.11.017
- Khella, H. W. Z., Butz, H., Ding, Q., Rotondo, F., Evans, K. R., Kupchak, P., et al. (2015). miR-221/222 are involved in response to sunitinib treatment in metastatic renal cell carcinoma. *Mol. Ther.* 23, 1748–1758. doi: 10.1038/mt.2015.129
- Khella, H. W. Z., Daniel, N., Youssef, L., Scorilas, A., Nofech-Mozes, R., Mirham, L., et al. (2017). miR-10b is a prognostic marker in clear cell renal cell carcinoma. *J. Clin. Pathol.* 70, 854–859. doi: 10.1136/jclinpath-2017-204341
- Kim, K., Lee, H. C., Park, J. L., Kim, M., Kim, S. Y., Noh, S. M., et al. (2011). Epigenetic regulation of microRNA-10b and targeting of oncogenic MAPRE1 in gastric cancer. *Epigenetics* 6, 740–751.
- Kovacova, J., Juracek, J., Poprach, A., Buchler, T., Kopecky, J., Fiala, O., et al. (2018). Candidate MicroRNA biomarkers of therapeutic response to sunitinib in metastatic renal cell carcinoma: a validation study in patients with extremely good and poor response. *Anticancer Res.* 38, 2961–2965. doi: 10.21873/anticancer.12546
- Kowalik, C. G., Palmer, D. A., Sullivan, T. B., Teebagay, P. A., Dugan, J. M., Libertino, J. A., et al. (2017). Profiling microRNA from nephrectomy and biopsy specimens: predictors of progression and survival in clear cell renal cell carcinoma. *BJU Int.* 120, 428–440. doi: 10.1111/bju.13886
- Kulkarni, P., Dasgupta, P., Bhat, N. S., Shahryari, V., Shiina, M., Hashimoto, Y., et al. (2018). Elevated miR-182-5p associates with renal cancer cell mitotic arrest through diminished MALAT-1 expression. *Mol. Cancer Res.* 16, 1750–1760. doi: 10.1158/1541-7786.MCR-17-0762
- Kunej, T., Godnic, I., Ferdin, J., Horvat, S., Dovc, P., and Calin, G. A. (2011). Epigenetic regulation of microRNAs in cancer: an integrated review of literature. *Mutat. Res.* 717, 77–84. doi: 10.1016/j.mrfmmm.2011.03.008
- Li, H., Zhao, J., Zhang, J. W., Huang, Q. Y., Huang, J. Z., Chi, L. S., et al. (2013). MicroRNA-217, down-regulated in clear cell renal cell carcinoma and associated with lower survival, suppresses cell proliferation and migration. *Neoplasia* 60, 511–515. doi: 10.4149/neo_2013_066
- Li, H. C., Li, J. P., Wang, Z. M., Fu, D. L., Li, Z. L., Zhang, D., et al. (2014). Identification of angiogenesis-related miRNAs in a population of patients with renal clear cell carcinoma. *Oncol. Rep.* 32, 2061–2069. doi: 10.3892/or.2014.3403
- Li, L., Li, C., Wang, S., Wang, Z., Jiang, J., Wang, W., et al. (2016). Exosomes derived from hypoxic oral squamous cell carcinoma cells deliver miR-21 to normoxic cells to elicit a prometastatic phenotype. *Cancer Res.* 76, 1770–1780. doi: 10.1158/0008-5472.CAN-15-1625
- Li, M., Wang, Y., Cheng, L., Niu, W., Zhao, G., Raju, J. K., et al. (2017). Long non-coding RNAs in renal cell carcinoma: a systematic review and clinical implications. *Oncotarget* 8, 48424–48435. doi: 10.18632/oncotarget.17053
- Li, M., Wang, Y., Song, Y., Bu, R., Yin, B., Fei, X., et al. (2015). MicroRNAs in renal cell carcinoma: a systematic review of clinical implications (Review). *Oncol. Rep.* 33, 1571–1578. doi: 10.3892/or.2015.3799
- Li, W., Wang, Q., Su, Q., Ma, D., An, C., Ma, L., et al. (2014). Honokiol suppresses renal cancer cells' metastasis via dual-blocking epithelial-mesenchymal transition and cancer stem cell properties through modulating miR-141/ZEB2 signaling. *Mol. Cells* 37, 383–388. doi: 10.14348/molcells.2014.0009
- Li, X., Xin, S., He, Z., Che, X., Wang, J., Xiao, X., et al. (2014). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor PDCD4 and promotes cell transformation, proliferation, and metastasis in renal cell carcinoma. *Cell Physiol. Biochem.* 33, 1631–1642. doi: 10.1159/000362946
- Lian, J. H., Wang, W. H., Wang, J. Q., Zhang, Y. H., and Li, Y. (2013). MicroRNA-122 promotes proliferation, invasion and migration of renal cell carcinoma cells through the PI3K/Akt signaling pathway. *Asian Pac. J. Cancer Prev.* 14, 5017–5021.
- Liang, B., Zhao, J., and Wang, X. (2017). A three-microRNA signature as a diagnostic and prognostic marker in clear cell renal cancer: an *in silico* analysis. *PLoS One* 12:e0180660. doi: 10.1371/journal.pone.0180660
- Liu, F., Chen, N., Xiao, R., Wang, W., and Pan, Z. (2016). miR-144-3p serves as a tumor suppressor for renal cell carcinoma and inhibits its invasion and metastasis by targeting MAP3K8. *Biochem. Biophys. Res. Commun.* 480, 87–93. doi: 10.1016/j.bbrc.2016.10.004
- Liu, F., Lou, Y. L., Wu, J., Ruan, Q. F., Xie, A., Guo, F., et al. (2012). Upregulation of microRNA-210 regulates renal angiogenesis mediated by activation of VEGF signaling pathway under ischemia/perfusion injury in vivo and in vitro. *Kidney Blood Press Res.* 35, 182–191. doi: 10.1159/000331054
- Liu, F., Wu, L., Wang, A., Xu, Y., Luo, X., Liu, X., et al. (2017). MicroRNA-138 attenuates epithelial-to-mesenchymal transition by targeting SOX4 in clear cell renal cell carcinoma. *Am. J. Transl. Res.* 9, 3611–3622.
- Liu, G., Ye, Z., Zhao, X., and Ji, Z. (2017). SP1-induced up-regulation of lncRNA SNHG14 as a ceRNA promotes migration and invasion of clear cell renal cell carcinoma by regulating N-WASP. *Am. J. Cancer Res.* 7, 2515–2525.
- Liu, J., Liu, B., Guo, Y., Chen, Z., Sun, W., Gao, W., et al. (2018). Key miRNAs and target genes played roles in the development of clear cell renal cell carcinoma. *Cancer Biomark.* 23, 279–290. doi: 10.3233/CBM-181558
- Liu, L., Li, Y., Liu, S., Duan, Q., Chen, L., Wu, T., et al. (2017). Downregulation of miR-193a-3p inhibits cell growth and migration in renal cell carcinoma by targeting PTEN. *Tumour Biol.* 39:1010428317711951. doi: 10.1177/1010428317711951
- Liu, Y., Nie, H., Zhang, K., Ma, D., Yang, G., Zheng, Z., et al. (2014). A feedback regulatory loop between HIF-1alpha and miR-21 in response to hypoxia in cardiomyocytes. *FEBS Lett.* 588, 3137–3146. doi: 10.1016/j.febslet.2014.05.067
- Loginov, V. I., Beresneva, E. V., Kazubskaya, T. P., Braga, E. A., and Karpukhin, A. V. (2017). Methylation of 10 miRNA genes in clear cell renal cell carcinoma and their diagnostic value. *Cancer Urol.* 13, 27–33. doi: 10.17650/1726-9776-2017-13-3-27-33

- Loginov, V. I., Filippova, E. A., Kurevlev, S. V., Fridman, M. V., Burdennyy, A. M., and Braga, E. A. (2018a). Suppressive and Hypermethylated MicroRNAs in the Pathogenesis of Breast Cancer. *Russ. J. Genet.* 54, 770–787. doi: 10.1134/S1022795418070086
- Loginov, V. I., Pronina, I. V., Burdennyy, A. M., Filippova, E. A., Kazubskaya, T. P., Kushlinsky, D. N., et al. (2018b). Novel miRNA genes deregulated by aberrant methylation in ovarian carcinoma are involved in metastasis. *Gene* 662, 28–36. doi: 10.1016/j.gene.2018.04.005
- Loginov, V. I., Rykov, S. V., Fridman, M. V., and Braga, E. A. (2015). Methylation of miRNA genes and oncogenesis. *Biochemistry* 80, 145–162. doi: 10.1134/S0006297915020029
- Lokeshwar, S. D., Talukder, A., Yates, T. J., Hennig, M. J. P., Garcia-Roig, M., Lahorewala, S. S., et al. (2018). Molecular characterization of renal cell carcinoma: a potential three-MicroRNA prognostic signature. *Cancer Epidemiol. Biomarkers Prev.* 27, 464–472. doi: 10.1158/1055-9965.EPI-17-0700
- Long, Q. Z., Du, Y. F., Liu, X. G., Li, X., and He, D. L. (2015). miR-124 represses FZD5 to attenuate P-glycoprotein-mediated chemo-resistance in renal cell carcinoma. *Tumour Biol.* 36, 7017–7026. doi: 10.1007/s13277-015-3369-3
- Lopez-Serra, P., and Esteller, M. (2012). DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. *Oncogene* 31, 1609–1622. doi: 10.1038/onc.2011.354
- Lou, N., Ruan, A. M., Qiu, B., Bao, L., Xu, Y. C., Zhao, Y., et al. (2017). miR-144-3p as a novel plasma diagnostic biomarker for clear cell renal cell carcinoma. *Urol. Oncol.* 35, 36.e–36.e14. doi: 10.1016/j.urolonc.2016.07.012
- Lu, J., Wei, J. H., Feng, Z. H., Chen, Z. H., Wang, Y. Q., Huang, Y., et al. (2017). miR-106b-5p promotes renal cell carcinoma aggressiveness and stem-cell-like phenotype by activating Wnt/beta-catenin signalling. *Oncotarget* 8, 21461–21471. doi: 10.18632/oncotarget.15591
- Machackova, T., Mlcochova, H., Stanik, M., Dolezel, J., Fedorko, M., Pacik, D., et al. (2016). MiR-429 is linked to metastasis and poor prognosis in renal cell carcinoma by affecting epithelial-mesenchymal transition. *Tumour Biol.* 37, 14653–14658. doi: 10.1007/s13277-016-5310-9
- Mahalingaiah, P. K., Ponnusamy, L., and Singh, K. P. (2015). Chronic oxidative stress leads to malignant transformation along with acquisition of stem cell characteristics, and epithelial to mesenchymal transition in human renal epithelial cells. *J. Cell. Physiol.* 230, 1916–1928. doi: 10.1002/jcp.24922
- Malouf, G. G., Flippot, R., and Khayat, D. (2016). Therapeutic strategies for patients with metastatic renal cell carcinoma in whom first-line vascular endothelial growth factor receptor-directed therapies fail. *J. Oncol. Pract.* 12, 412–420. doi: 10.1200/JOP.2016.011809
- McCormick, R. I., Blick, C., Ragoussis, J., Schoedel, J., Mole, D. R., Young, A. C., et al. (2013). miR-210 is a target of hypoxia-inducible factors 1 and 2 in renal cancer, regulates ISC and correlates with good prognosis. *Br. J. Cancer* 108, 1133–1142. doi: 10.1038/bjc.2013.56
- Mens, M. M. J., and Ghanbari, M. (2018). Cell Cycle Regulation of Stem Cells by MicroRNAs. *Stem Cell Rev.* 14, 309–322. doi: 10.1007/s12015-018-9808-y
- Mickley, A., Kovaleva, O., Kzyshkowska, J., and Gratchev, A. (2015). Molecular and immunologic markers of kidney cancer-potential applications in predictive, preventive and personalized medicine. *EPMA J.* 6:20. doi: 10.1186/s13167-015-0042-2
- Mikhaylova, O., Stratton, Y., Hall, D., Kellner, E., Ehmer, B., Drew, A. F., et al. (2012). VHL-regulated MiR-204 suppresses tumor growth through inhibition of LC3B-mediated autophagy in renal clear cell carcinoma. *Cancer Cell* 21, 532–546. doi: 10.1016/j.ccr.2012.02.019
- Morais, M., Dias, F., Teixeira, A. L., and Medeiros, R. (2017). MicroRNAs and altered metabolism of clear cell renal cell carcinoma: potential role as aerobic glycolysis biomarkers. *Biochim. Biophys. Acta Gen. Subj.* 1861, 2175–2185. doi: 10.1016/j.bbagen.2017.05.028
- Morris, M. R., and Latif, F. (2017). The epigenetic landscape of renal cancer. *Nat. Rev. Nephrol.* 13, 47–60. doi: 10.1038/nrneph.2016.168
- Müller, S., and Nowak, K. (2014). Exploring the miRNA-mRNA regulatory network in clear cell renal cell carcinomas by next-generation sequencing expression profiles. *Biomed. Res. Int.* 2014:948408. doi: 10.1155/2014/948408
- Nakada, C., Tsukamoto, Y., Matsuura, K., Nguyen, T. L., Hijiya, N., Uchida, T., et al. (2011). Overexpression of miR-210, a downstream target of HIF1alpha, causes centrosome amplification in renal carcinoma cells. *J. Pathol.* 224, 280–288. doi: 10.1002/path.2860
- Neal, C. S., Michael, M. Z., Rawlings, L. H., Van der Hoek, M. B., and Gleadle, J. M. (2010). The VHL-dependent regulation of microRNAs in renal cancer. *BMC Med.* 8:64. doi: 10.1186/1741-7015-8-64
- Ni, D., Ma, X., Li, H. Z., Gao, Y., Li, X. T., Zhang, Y., et al. (2014). Downregulation of FOXO3a promotes tumor metastasis and is associated with metastasis-free survival of patients with clear cell renal cell carcinoma. *Clin. Cancer Res.* 20, 1779–1790. doi: 10.1158/1078-0432.CCR-13-1687
- Nofech-Mozes, R., Khella, H. W., Scorilas, A., Youssef, L., Krylov, S. N., Lianidou, E., et al. (2016). MicroRNA-194 is a marker for good prognosis in clear cell renal cell carcinoma. *Cancer Med.* 5, 656–664. doi: 10.1002/cam4.631
- Okato, A., Arai, T., Yamada, Y., Sugawara, S., Koshizuka, K., Fujimura, L., et al. (2017). Dual strands of Pre-miR-149 inhibit cancer cell migration and invasion through targeting FOXM1 in renal cell carcinoma. *Int. J. Mol. Sci.* 18:E1969. doi: 10.3390/ijms18091969
- Oliveira, R. C., Ivanovic, R. F., Leite, K. R. M., Viana, N. I., Pimenta, R. C. A., Junior, J. P., et al. (2017). Expression of micro-RNAs and genes related to angiogenesis in ccRCC and associations with tumor characteristics. *BMC Urol.* 17:113. doi: 10.1186/s12894-017-0306-3
- Ornitz, D. M., and Itoh, N. (2015). The Fibroblast Growth Factor signaling pathway. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 215–266. doi: 10.1002/wdev.176
- Pan, Y., Hu, J., Ma, J., Qi, X., Zhou, H., Miao, X., et al. (2018a). MiR-193a-3p and miR-224 mediate renal cell carcinoma progression by targeting alpha-2,3-sialyltransferase IV and the phosphatidylinositol 3 kinase/Akt pathway. *Mol. Carcinog.* 57, 1067–1077. doi: 10.1002/mc.22826
- Pan, Y., Wu, Y., Hu, J., Shan, Y., Ma, J., Ma, H., et al. (2018b). Long noncoding RNA HOTAIR promotes renal cell carcinoma malignancy through alpha-2, 8-sialyltransferase 4 by sponging microRNA-124. *Cell Prolif.* 51:e12507. doi: 10.1111/cpr.12507
- Pan, Y. J., Wei, L. L., Wu, X. J., Huo, F. C., Mou, J., and Pei, D. S. (2017). MiR-106a-5p inhibits the cell migration and invasion of renal cell carcinoma through targeting PAK5. *Cell Death Dis.* 8:e3155. doi: 10.1038/cddis.2017.561
- Pereyaslova, E., Loginov, V., Pronina, I., Dmitriev, A., Burdennyy, A., Kazubskaya, T. P., et al. (2016). Hypermethylation of MIR-129-2, MIR-203, and MIR-107 microRNA genes is associated with metastasis of clear cell renal cell cancer. *FEBS J.* 283, 251–251.
- Piletic, K., and Kunaj, T. (2016). MicroRNA epigenetic signatures in human disease. *Arch. Toxicol.* 90, 2405–2419.
- Piva, F., Giulietti, M., Santoni, M., Occhipinti, G., Scarpelli, M., Lopez-Beltran, A., et al. (2016). Epithelial to mesenchymal transition in renal cell carcinoma: implications for cancer therapy. *Mol. Diagn. Ther.* 20, 111–117. doi: 10.1007/s40291-016-0192-5
- Pronina, I. V., Klimov, E. A., Burdennyy, A. M., Beresneva, E. V., Fridman, M. V., Ermilova, V. D., et al. (2017a). Methylation of the genes for the MicroRNAs miR-129-2 and miR-9-1, changes in their expression, and activation of their potential target genes in clear cell renal cell carcinoma. *Mol. Biol.* 51, 61–71. doi: 10.1134/S0026893316060169
- Pronina, I. V., Loginov, V. I., Burdennyy, A. M., Fridman, M. V., Senchenko, V. N., Kazubskaya, T. P., et al. (2017b). DNA methylation contributes to deregulation of 12 cancer-associated microRNAs and breast cancer progression. *Gene* 604, 1–8. doi: 10.1016/j.gene.2016.12.018
- Puente, J., Lainez, N., Duenas, M., Mendez-Vidal, M. J., Esteban, E., Castellano, D., et al. (2017). Novel potential predictive markers of sunitinib outcomes in long-term responders versus primary refractory patients with metastatic clear-cell renal cell carcinoma. *Oncotarget* 8, 30410–30421. doi: 10.18632/oncotarget.16494
- Qin, Z., Wei, X., Jin, N., Wang, Y., Zhao, R., Hu, Y., et al. (2018). MiR-199a targeting ROCK1 to affect kidney cell proliferation, invasion and apoptosis. *Artif. Cells Nanomed. Biotechnol.* 46, 1920–1925. doi: 10.1080/21691401.2017.1396224
- Ran, L., Liang, J., Deng, X., and Wu, J. (2017). miRNAs in Prediction of Prognosis in Clear Cell Renal Cell Carcinoma. *Biomed. Res. Int.* 2017:4832931. doi: 10.1155/2017/4832931
- Randall, J. M., Millard, F., and Kurzrock, R. (2014). Molecular aberrations, targeted therapy, and renal cell carcinoma: current state-of-the-art. *Cancer Metastasis Rev.* 33, 1109–1124.
- Reddy, K. B. (2015). MicroRNA (miRNA) in cancer. *Cancer Cell Int.* 15:38. doi: 10.1186/s12935-015-0185-1

- Redova, M., Poprach, A., Nekvindova, J., Iliev, R., Radova, L., Lakomy, R., et al. (2012). Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma. *J. Transl. Med.* 10:55. doi: 10.1186/1479-5876-10-55
- Redova, M., Svoboda, M., and Slaby, O. (2011). MicroRNAs and their target gene networks in renal cell carcinoma. *Biochem. Biophys. Res. Commun.* 405, 153–156. doi: 10.1016/j.bbrc.2011.01.019
- Reustle, A., Fisel, P., Renner, O., Buttner, F., Winter, S., Rausch, S., et al. (2018). Characterization of the breast cancer resistance protein (BCRP/ABCG2) in clear cell renal cell carcinoma. *Int. J. Cancer.* 143, 3181–3193. doi: 10.1002/ijc.31741
- Roskoski, R. Jr. (2017). Vascular endothelial growth factor (VEGF) and VEGF receptor inhibitors in the treatment of renal cell carcinomas. *Pharmacol. Res.* 120, 116–132. doi: 10.1016/j.phrs.2017.03.010
- Rydzanicz, M., Wrzesinski, T., Bluyssen, H. A., and Wesoly, J. (2013). Genomics and epigenomics of clear cell renal cell carcinoma: recent developments and potential applications. *Cancer Lett.* 341, 111–126. doi: 10.1016/j.canlet.2013.08.006
- Rykov, S. V., Khodyrev, D. S., Pronina, I. V., Kazubskaya, T. P., Loginov, V. I., and Braga, E. A. (2013). Novel miRNA genes methylated in lung tumors. *Russ. J. Genet.* 49, 782–786. doi: 10.1134/S1022795413070119
- Samaan, S., Khella, H. W., Girgis, A., Scorilas, A., Lianidou, E., Gabril, M., et al. (2015). miR-210 is a prognostic marker in clear cell renal cell carcinoma. *J. Mol. Diagn.* 17, 136–144. doi: 10.1016/j.jmoldx.2014.10.005
- Sampson, V. B., David, J. M., Puig, I., Patil, P. U., de Herreros, A. G., Thomas, G. V., et al. (2014). Wilms' tumor protein induces an epithelial-mesenchymal hybrid differentiation state in clear cell renal cell carcinoma. *PLoS One* 9:e102041. doi: 10.1371/journal.pone.0102041
- Sato, K., Tsuchihara, K., Fujii, S., Sugiyama, M., Goya, T., Atomi, Y., et al. (2007). Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res.* 67, 9677–9684. doi: 10.1158/0008-5472.CAN-07-1462
- Schanza, L. M., Seles, M., Stotz, M., Fosselteder, J., Hutterer, G. C., Pichler, M., et al. (2017). MicroRNAs associated with von hippel-lindau pathway in renal cell carcinoma: a comprehensive review. *Int. J. Mol. Sci.* 18:E2495. doi: 10.3390/ijms18112495
- Schiffgen, M., Schmidt, D. H., von Rucker, A., Muller, S. C., and Ellinger, J. (2013). Epigenetic regulation of microRNA expression in renal cell carcinoma. *Biochem. Biophys. Res. Commun.* 436, 79–84. doi: 10.1016/j.bbrc.2013.05.061
- Seki, H., Hayashida, T., Jinno, H., Hirose, S., Sakata, M., Takahashi, M., et al. (2012). HOXB9 expression promoting tumor cell proliferation and angiogenesis is associated with clinical outcomes in breast cancer patients. *Ann. Surg. Oncol.* 19, 1831–1840. doi: 10.1245/s10434-012-2295-5
- Serocki, M., Bartoszewska, S., Janaszak-Jasiecka, A., Ochocka, R. J., Collawn, J. F., and Bartoszewski, R. (2018). miRNAs regulate the HIF switch during hypoxia: a novel therapeutic target. *Angiogenesis* 21, 183–202. doi: 10.1007/s10456-018-9600-2
- Shi, L., Li, X., Wu, Z., Li, X., Nie, J., Guo, M., et al. (2018). DNA methylation-mediated repression of miR-181a/135a/302c expression promotes the microsatellite-unstable colorectal cancer development and 5-FU resistance via targeting PLAG1. *J. Genet. Genomics* 45, 205–214. doi: 10.1016/j.jgg.2018.04.003
- Shimoyamada, H., Yazawa, T., Sato, H., Okudela, K., Ishii, J., Sakaeda, M., et al. (2010). Early growth response-1 induces and enhances vascular endothelial growth factor-A expression in lung cancer cells. *Am. J. Pathol.* 177, 70–83. doi: 10.2353/ajpath.2010.091164
- Shinmei, S., Sakamoto, N., Goto, K., Sentani, K., Anami, K., Hayashi, T., et al. (2013). MicroRNA-155 is a predictive marker for survival in patients with clear cell renal cell carcinoma. *Int. J. Urol.* 20, 468–477. doi: 10.1111/ij.1442-2042.2012.03182.x
- Song, T., Zhang, X., Wang, C., Wu, Y., Cai, W., Gao, J., et al. (2011). MiR-138 suppresses expression of hypoxia-inducible factor 1alpha (HIF-1alpha) in clear cell renal cell carcinoma 786-O cells. *Asian Pac. J. Cancer Prev.* 12, 1307–1311.
- Tang, K., and Xu, H. (2015). Prognostic value of meta-signature miRNAs in renal cell carcinoma: an integrated miRNA expression profiling analysis. *Sci. Rep.* 5:10272. doi: 10.1038/srep10272
- Toraih, E. A., Ibrahim, A. T., Fawzy, M. S., Hussein, M. H., Al-Qahtani, S. A. M., and Shaalan, A. A. M. (2017). MicroRNA-34a: a key regulator in the hallmarks of renal cell carcinoma. *Oxid. Med. Cell. Longev.* 2017:3269379. doi: 10.1155/2017/3269379
- Torres-Ferreira, J., Ramalho-Carvalho, J., Gomez, A., Menezes, F. D., Freitas, R., Oliveira, J., et al. (2017). MiR-193b promoter methylation accurately detects prostate cancer in urine sediments and miR-34b/c or miR-129-2 promoter methylation define subsets of clinically aggressive tumors. *Mol. Cancer* 16:26. doi: 10.1186/s12943-017-0604-0
- Uchida, T., Rossignol, F., Matthay, M. A., Mounier, R., Couette, S., Clottes, E., et al. (2004). Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. *J. Biol. Chem.* 279, 14871–14878. doi: 10.1074/jbc.M400461200
- Umez, T., Tadokoro, H., Azuma, K., Yoshizawa, S., Ohyashiki, K., and Ohyashiki, J. H. (2014). Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. *Blood* 124, 3748–3757. doi: 10.1182/blood-2014-05-576116
- Valera, V. A., Walter, B. A., Linehan, W. M., and Merino, M. J. (2011). Regulatory effects of microRNA-92 (miR-92) on VHL gene expression and the hypoxic activation of miR-210 in clear cell renal cell carcinoma. *J. Cancer* 2, 515–526.
- Varachev, V., Loginov, V., Pronina, I., Khodyrev, D., Kazubskaya, T., Morozov, A., et al. (2018). Hypermethylated tumor suppressor microRNAs as novel markers of clear cell renal cell carcinoma. *FEBS Open Bio* 8, 304–305.
- Vasudev, N. S., Selby, P. J., and Banks, R. E. (2012). Renal cancer biomarkers: the promise of personalized care. *BMC Med.* 10:112. doi: 10.1186/1741-7015-10-112
- Vergho, D., Kneitz, S., Rosenwald, A., Scherer, C., Spahn, M., Burger, M., et al. (2014). Combination of expression levels of miR-21 and miR-126 is associated with cancer-specific survival in clear-cell renal cell carcinoma. *BMC Cancer* 14:25. doi: 10.1186/1471-2407-14-25
- Vogt, M., Munding, J., Gruner, M., Liffers, S. T., Verdoodt, B., Hauk, J., et al. (2011). Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. *Virchows Arch.* 458, 313–322. doi: 10.1007/s00428-010-1030-5
- Wang, C., Cai, L., Liu, J., Wang, G., Li, H., Wang, X., et al. (2017). MicroRNA-30a-5p inhibits the growth of renal cell carcinoma by modulating GRP78 expression. *Cell Physiol. Biochem.* 43, 2405–2419. doi: 10.1159/000484394
- Wang, C., Hu, J., Lu, M., Gu, H., Zhou, X., Chen, X., et al. (2015). A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma. *Sci. Rep.* 5:7610. doi: 10.1038/srep07610
- Wang, C., Wu, C., Yang, Q., Ding, M., Zhong, J., Zhang, C. Y., et al. (2016). miR-28-5p acts as a tumor suppressor in renal cell carcinoma for multiple antitumor effects by targeting RAP1B. *Oncotarget* 7, 73888–73902. doi: 10.18632/oncotarget.12516
- Wang, D., Zhu, C., Zhang, Y., Zheng, Y., Ma, F., Su, L., et al. (2017). MicroRNA-30e-3p inhibits cell invasion and migration in clear cell renal cell carcinoma by targeting Snail1. *Oncol. Lett.* 13, 2053–2058. doi: 10.3892/ol.2017.5690
- Wang, G., Chen, L., Meng, J., Chen, M., Zhuang, L., and Zhang, L. (2013). Overexpression of microRNA-100 predicts an unfavorable prognosis in renal cell carcinoma. *Int. Urol. Nephrol.* 45, 373–379. doi: 10.1007/s11255-012-0374-y
- Wang, M., Gao, H., Qu, H., Li, J., Liu, K., and Han, Z. (2018). MiR-137 suppresses tumor growth and metastasis in clear cell renal cell carcinoma. *Pharmacol. Rep.* 70, 963–971. doi: 10.1016/j.pharep.2018.04.006
- Wang, Z., Qin, C., Zhang, J., Han, Z., Tao, J., Cao, Q., et al. (2017). MiR-122 promotes renal cancer cell proliferation by targeting Sprouty2. *Tumour Biol.* 39:1010428317691184. doi: 10.1177/1010428317691184
- Wang, Z., Zhang, Z., Zhang, C., and Xu, Y. (2018). Identification of potential pathogenic biomarkers in clear cell renal cell carcinoma. *Oncol. Lett.* 15, 8491–8499. doi: 10.3892/ol.2018.8398
- Wei, H., Ke, H. L., Lin, J., Shete, S., Wood, C. G., and Hildebrandt, M. A. (2014). MicroRNA target site polymorphisms in the VHL-HIF1alpha pathway predict renal cell carcinoma risk. *Mol. Carcinog.* 53, 1–7. doi: 10.1002/mc.21917
- Wotschovsky, Z., Busch, J., Jung, M., Kempkensteffen, C., Weikert, S., Schaser, K. D., et al. (2013). Diagnostic and prognostic potential of differentially expressed miRNAs between metastatic and non-metastatic renal cell carcinoma

- at the time of nephrectomy. *Clin. Chim. Acta* 416, 5–10. doi: 10.1016/j.cca.2012.11.010
- Wotschofsky, Z., Gummlich, L., Liep, J., Stephan, C., Kilic, E., Jung, K., et al. (2016). Integrated microRNA and mRNA signature associated with the transition from the locally confined to the metastasized clear cell renal cell carcinoma exemplified by miR-146-5p. *PLoS One* 11:e0148746. doi: 10.1371/journal.pone.0148746
- Wu, A., Wu, K., Li, M., Bao, L., Shen, X., Li, S., et al. (2015). Upregulation of microRNA-492 induced by epigenetic drug treatment inhibits the malignant phenotype of clear cell renal cell carcinoma in vitro. *Mol. Med. Rep.* 12, 1413–1420. doi: 10.3892/mmr.2015.3550
- Wu, C., Jin, B., Chen, L., Zhuo, D., Zhang, Z., Gong, K., et al. (2013). MiR-30d induces apoptosis and is regulated by the Akt/FOXO pathway in renal cell carcinoma. *Cell Signal.* 25, 1212–1221. doi: 10.1016/j.cellsig.2013.01.028
- Wu, D., Pan, H., Zhou, Y., Zhou, J., Fan, Y., and Qu, P. (2014). microRNA-133b downregulation and inhibition of cell proliferation, migration and invasion by targeting matrix metalloproteinase-9 in renal cell carcinoma. *Mol. Med. Rep.* 9, 2491–2498. doi: 10.3892/mmr.2014.2116
- Wu, S. Y., Rupaimoole, R., Shen, F., Pradeep, S., Pecot, C. V., Ivan, C., et al. (2016). A miR-192-EGR1-HOXB9 regulatory network controls the angiogenic switch in cancer. *Nat. Commun.* 7:11169. doi: 10.1038/ncomms11169
- Xiang, C., Cui, S. P., and Ke, Y. (2016). MiR-144 inhibits cell proliferation of renal cell carcinoma by targeting MTOR. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 36, 186–192. doi: 10.1007/s11596-016-1564-0
- Xiang, W., He, J., Huang, C., Chen, L., Tao, D., Wu, X., et al. (2015). miR-106b-5p targets tumor suppressor gene SETD2 to inactivate its function in clear cell renal cell carcinoma. *Oncotarget* 6, 4066–4079. doi: 10.18632/oncotarget.2926
- Xiao, H., Li, H., Yu, G., Xiao, W., Hu, J., Tang, K., et al. (2014). MicroRNA-10b promotes migration and invasion through KLF4 and HOXD10 in human bladder cancer. *Oncol. Rep.* 31, 1832–1838. doi: 10.3892/or.2014.3048
- Xiao, W., Lou, N., Ruan, H., Bao, L., Xiong, Z., Yuan, C., et al. (2017). Mir-144-3p promotes cell proliferation, metastasis, sunitinib resistance in clear cell renal cell carcinoma by downregulating ARID1A. *Cell. Physiol. Biochem.* 43, 2420–2433. doi: 10.1159/000484395
- Xiao, X., Tang, C., Xiao, S., Fu, C., and Yu, P. (2013). Enhancement of proliferation and invasion by MicroRNA-590-5p via targeting PBRM1 in clear cell renal carcinoma cells. *Oncol. Res.* 20, 537–544. doi: 10.3727/096504013X13775486749335
- Xing, T., and He, H. (2016). Epigenomics of clear cell renal cell carcinoma: mechanisms and potential use in molecular pathology. *Chin. J. Cancer Res.* 28, 80–91. doi: 10.3978/j.issn.1000-9604.2016.02.09
- Xu, M., Gu, M., Zhang, K., Zhou, J., Wang, Z., and Da, J. (2015). miR-203 inhibition of renal cancer cell proliferation, migration and invasion by targeting of FGF2. *Diagn. Pathol.* 10:24. doi: 10.1186/s13000-015-0255-7
- Xu, X., Liu, C., and Bao, J. (2017). Hypoxia-induced hsa-miR-101 promotes glycolysis by targeting TIGAR mRNA in clear cell renal cell carcinoma. *Mol. Med. Rep.* 15, 1373–1378. doi: 10.3892/mmr.2017.6139
- Xu, X., Wu, J., Li, S., Hu, Z., Xu, X., Zhu, Y., et al. (2014). Downregulation of microRNA-182-5p contributes to renal cell carcinoma proliferation via activating the AKT/FOXO3a signaling pathway. *Mol. Cancer* 13:109. doi: 10.1186/1476-4598-13-109
- Xu, X. W., Li, S., Yin, F., and Qin, L. L. (2018). Expression of miR-205 in renal cell carcinoma and its association with clinicopathological features and prognosis. *Eur. Rev. Med. Pharmacol. Sci.* 22, 662–670. doi: 10.26355/eurrev_201802_14291
- Xu, Y., Zhu, J., Lei, Z., Wan, L., Zhu, X., Ye, F., et al. (2015). Expression and functional role of miR-29b in renal cell carcinoma. *Int. J. Clin. Exp. Pathol.* 8, 14161–14170.
- Yadav, S., Khandelwal, M., Seth, A., Saini, A. K., Dogra, P. N., and Sharma, A. (2017). Serum microRNA expression profiling: potential diagnostic implications of a panel of serum microRNAs for clear cell renal cell cancer. *Urology* 104, 64–69. doi: 10.1016/j.urology.2017.03.013
- Yamamura, S., Saini, S., Majid, S., Hirata, H., Ueno, K., Chang, I., et al. (2012). MicroRNA-34a suppresses malignant transformation by targeting c-Myc transcriptional complexes in human renal cell carcinoma. *Carcinogenesis* 33, 294–300. doi: 10.1093/carcin/bgr286
- Yamasaki, T., Seki, N., Yoshino, H., Itesako, T., Yamada, Y., Tatarano, S., et al. (2013). Tumor-suppressive microRNA-1291 directly regulates glucose transporter 1 in renal cell carcinoma. *Cancer Sci.* 104, 1411–1419. doi: 10.1111/cas.12240
- Yang, F., Zhang, H., Mei, Y., and Wu, M. (2014). Reciprocal regulation of HIF-1 α and lincRNA-p21 modulates the Warburg effect. *Mol. Cell* 53, 88–100. doi: 10.1016/j.molcel.2013.11.004
- Yin, H., Wang, X., Zhang, X., Wang, Y., Zeng, Y., Xiong, Y., et al. (2018). Integrated analysis of long noncoding RNA associated-competing endogenous RNA as prognostic biomarkers in clear cell renal carcinoma. *Cancer Sci.* 109, 3336–3349. doi: 10.1111/cas.13778
- Yoshino, H., Enokida, H., Itesako, T., Kojima, S., Kinoshita, T., Tatarano, S., et al. (2013). Tumor-suppressive microRNA-143/145 cluster targets hexokinase-2 in renal cell carcinoma. *Cancer Sci.* 104, 1567–1574. doi: 10.1111/cas.12280
- Yoshino, H., Yonemori, M., Miyamoto, K., Tatarano, S., Kofuji, S., Nohata, N., et al. (2017). microRNA-210-3p depletion by CRISPR/Cas9 promoted tumorigenesis through revival of TWIST1 in renal cell carcinoma. *Oncotarget* 8, 20881–20894. doi: 10.18632/oncotarget.14930
- Yu, G., Li, H., Wang, J., Gumireddy, K., Li, A., Yao, W., et al. (2014). miRNA-34a suppresses cell proliferation and metastasis by targeting CD44 in human renal carcinoma cells. *J. Urol.* 192, 1229–1237. doi: 10.1016/j.juro.2014.05.094
- Zaman, M. S., Thamminana, S., Shahryari, V., Chiyomaru, T., Deng, G., Saini, S., et al. (2012). Inhibition of PTEN gene expression by oncogenic miR-23b-3p in renal cancer. *PLoS One* 7:e50203. doi: 10.1371/journal.pone.0050203
- Zare, M., Bastami, M., Solali, S., and Alivand, M. R. (2018). Aberrant miRNA promoter methylation and EMT-involving miRNAs in breast cancer metastasis: Diagnosis and therapeutic implications. *J. Cell. Physiol.* 233, 3729–3744. doi: 10.1002/jcp.26116
- Zhang, C., Mo, R., Yin, B., Zhou, L., Liu, Y., and Fan, J. (2014). Tumor suppressor microRNA-34a inhibits cell proliferation by targeting Notch1 in renal cell carcinoma. *Oncol. Lett.* 7, 1689–1694. doi: 10.3892/ol.2014.1931
- Zhang, H., and Li, H. (2016). miR-137 inhibits renal cell carcinoma growth in vitro and in vivo. *Oncol. Lett.* 12, 715–720. doi: 10.3892/ol.2016.4616
- Zhang, S., Guo, Z., Xu, J., Wang, J., Zhang, J., Cui, L., et al. (2017). miR-502-mediated histone methyltransferase SET8 expression is associated with clear cell renal cell carcinoma risk. *Oncol. Lett.* 14, 7131–7138. doi: 10.3892/ol.2017.7115
- Zhang, S., Qian, J., Cao, Q., Li, P., Wang, M., Wang, J., et al. (2014). A potentially functional polymorphism in the promoter region of miR-34b/c is associated with renal cell cancer risk in a Chinese population. *Mutagenesis* 29, 149–154. doi: 10.1093/mutage/geu001
- Zhang, W., Ni, M., Su, Y., Wang, H., Zhu, S., Zhao, A., et al. (2018). MicroRNAs in serum exosomes as potential biomarkers in clear-cell renal cell carcinoma. *Eur. Urol. Focus* 4, 412–419. doi: 10.1016/j.euf.2016.09.007
- Zhang, X., Xing, N. D., Lai, C. J., Liu, R., Jiao, W., Wang, J., et al. (2018). MicroRNA-375 suppresses the tumor aggressive phenotypes of clear cell renal cell carcinomas through regulating YWHAZ. *Chin. Med. J.* 131, 1944–1950. doi: 10.4103/0366-6999.238153
- Zhang, Z., Sun, H., Dai, H., Walsh, R. M., Imakura, M., Schelter, J., et al. (2009). MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT. *Cell Cycle* 8, 2756–2768. doi: 10.4161/cc.8.17.9387
- Zhao, J., Lei, T., Xu, C., Li, H., Ma, W., Yang, Y., et al. (2013). MicroRNA-187, down-regulated in clear cell renal cell carcinoma and associated with lower survival, inhibits cell growth and migration through targeting B7-H3. *Biochem. Biophys. Res. Commun.* 438, 439–444. doi: 10.1016/j.bbrc.2013.07.095
- Zhao, J. J., Chen, P. J., Duan, R. Q., Li, K. J., Wang, Y. Z., and Li, Y. (2014). Up-regulation of miR-630 in clear cell renal cell carcinoma is associated with lower overall survival. *Int. J. Clin. Exp. Pathol.* 7, 3318–3323.
- Zhao, S., Wang, Y., Luo, M., Cui, W., Zhou, X., and Miao, L. (2018). Long noncoding RNA small nucleolar RNA host gene 1 (SNHG1) promotes renal cell carcinoma progression and metastasis by negatively regulating miR-137. *Med. Sci. Monit.* 24, 3824–3831. doi: 10.12659/MSM.910866
- Zhao, X., Zhao, Z., Xu, W., Hou, J., and Du, X. (2015). Down-regulation of miR-497 is associated with poor prognosis in renal cancer. *Int. J. Clin. Exp. Pathol.* 8, 758–764.

- Zhao, Z., Zhang, M., Duan, X., Chen, Y., Li, E., Luo, L., et al. (2018). TRPM7 regulates AKT/FOXO1-dependent tumor growth and is an independent prognostic indicator in renal cell carcinoma. *Mol. Cancer Res.* 16, 1013–1023. doi: 10.1158/1541-7786.MCR-17-0767
- Zhou, L., Li, Z., Pan, X., Lai, Y., Quan, J., Zhao, L., et al. (2018). Identification of miR-18a-5p as an oncogene and prognostic biomarker in RCC. *Am. J. Transl. Res.* 10, 1874–1886.
- Zhu, S., Huang, Y., and Su, X. (2016). Mir-451 correlates with prognosis of renal cell carcinoma patients and inhibits cellular proliferation of renal cell carcinoma. *Med. Sci. Monit.* 22, 183–190.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptomics Analysis of Circular RNAs Differentially Expressed in Apoptotic HeLa Cells

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OPEN ACCESS

Edited by:

Yujing Li,
Emory University, United States

Reviewed by:

Yulin Jin,
Emory University, United States
Argyris Papantonis,
Universität zu Köln, Germany

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 14 November 2018

Accepted: 18 February 2019

Published: 13 March 2019

Citation:

Yaylak B, Erdogan I and Akgul B
(2019) Transcriptomics Analysis
of Circular RNAs Differentially
Expressed in Apoptotic HeLa Cells.
Front. Genet. 10:176.
doi: 10.3389/fgene.2019.00176

Apoptosis is a form of regulated cell death that plays a critical role in survival and developmental homeostasis. There are numerous reports on regulation of apoptosis by protein-coding genes as well as small non-coding RNAs, such as microRNAs. However, there is no comprehensive investigation of circular RNAs (circRNA) that are differentially expressed under apoptotic conditions. We have performed a transcriptomics study in which we first triggered apoptosis in HeLa cells through treatment with four different agents, namely cisplatin, doxorubicin, TNF- α and anti-Fas mAb. Total RNAs isolated from control as well as treated cells were treated with RNase R to eliminate the linear RNAs. The remaining RNAs were then subjected to deep-sequencing to identify differentially expressed circRNAs. Interestingly, some of the dys-regulated circRNAs were found to originate from protein-coding genes well-documented to regulate apoptosis. A number of candidate circRNAs were validated with qPCR with or without RNase R treatment as well. We then took advantage of bioinformatics tools to investigate the coding potential of differentially expressed RNAs. Additionally, we examined the candidate circRNAs for the putative miRNA-binding sites and their putative target mRNAs. Our analyses point to a potential for circRNA-mediated sponging of miRNAs known to regulate apoptosis. In conclusion, this is the first transcriptomics study that provides a complete circRNA profile of apoptotic cells that might shed light onto the potential role of circRNAs in apoptosis.

Keywords: apoptosis, circular RNA, RNA-seq, transcriptomics, HeLa

INTRODUCTION

Apoptosis is a tightly regulated mechanism of type 1 programmed cell death that mediates balance between survival and cell death. Apoptosis mainly proceeds with the extrinsic death receptor pathway or the intrinsic mitochondrial dysfunction pathway (Vince and Silke, 2009; Fuchs and Steller, 2011). Functional apoptosis pathway is vital for tissue homeostasis and organ development. Accordingly, dysregulation of apoptosis is associated with a wide range of diseases such as cancer, autoimmune diseases, neurodegenerative diseases, and acute pathologies (Favaloro et al., 2012). Therefore, much effort has been made to unravel molecular pathways that trigger and regulate apoptosis.

During past decades, a plethora of studies revealed that considerable amount of DNA called “dark matter of genome is transcribed but not translated” (Bertone et al., 2004). MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are well-known examples of non-coding RNAs

(ncRNAs) (Lewis et al., 2005; Lander, 2011). Recent studies revealed yet another type of ncRNAs, circRNAs, as a novel type of endogenous ncRNAs that has the potential to regulate not only the protein-coding genes but also ncRNAs as well (Salzman et al., 2013; Jeck et al., 2013; Memczak et al., 2013; Huang S. et al., 2017).

Circular RNAs are typically generated by back-splicing of exons from pre-mRNAs with a 3'-to-5' phosphodiester bond at the junction site (Chen, 2016). Since they do not possess a 5' cap or a poly(A) tail, they are frequently missed in poly(A)⁺ RNA profiling studies. Also, the unusual back-splicing generates a read that has been historically filtered out during conventional RNA-seq studies. The recent development of non-polyadenylated sequencing technology followed by exonuclease-mediated degradation of linear RNAs by RNase R resulted in the discovery of thousands of circular RNAs widely expressed in human (Suzuki et al., 2006). The majority of circular RNAs are conserved across species, and generally reveal tissue/developmental-stage-specific expression (Qu et al., 2015). Searching for out-of-order arrangement of exons known as a "backsplice" in combination with computational pipelines facilitated the genomewide identification of circRNAs (Jeck and Sharpless, 2014). Nonetheless, identification of backsplicing reads can be challenging because of high false positive rates of computational tools. These apparent backsplicing sequences can originate from different mechanisms such as tandem duplication, RNA-trans splicing and reverse transcriptase template switching rather than exonic circular RNAs (Cocquet et al., 2006; McManus et al., 2010; Jeck and Sharpless, 2014).

Circular RNAs can regulate gene expression through various mechanisms. Perhaps, one of the most prominent functions of circRNAs is to modulate endogenous miRNA activities by acting as miRNA sponges (Hansen et al., 2013; Memczak et al., 2013). CircRNAs might also regulate transcription by interacting with RNA polymerase II and U1 snRNP or various RNA binding proteins such as Mbl (Ashwal-Fluss et al., 2014). Moreover, the canonical pre-mRNA splicing and circularization may compete with each other, resulting in a decrease in the amount of the linear mRNA (Ashwal-Fluss et al., 2014). Besides, if the translation start site of an mRNA is included in the circularized exon, the remaining portion of the mRNA will miss a proper translation initiation site, negatively affecting its translation (Huang G. et al., 2017). Although circular RNAs were initially categorized as non-coding RNA, it was obvious that a portion of circRNAs held the potential for translation, especially those that retain an intact open reading frame. Spectacularly, cap-independent translation of a subset of circular RNAs has been reported recently, paving the way for truncated translation products from circRNAs (Pamudurti et al., 2017).

There are only a few studies that report the involvement of circRNAs in apoptosis. Circ_016423 has been reported to have a high possibility of regulating apoptosis in ischemic prenumbral cortex, potentially through harboring binding sites for various miRNAs (Mehta et al., 2017). Hsa_circ_0010729 was reported to regulate proliferation and migration as well as apoptosis in hypoxia-induced human umbilical vein endothelial

cells (HUVECs) by targeting miR-186-HIF- α axis (Dang et al., 2017). MFACR possesses a binding site for miR-652-3p, which in turn regulates the MTP18 mRNA, which codes for a nuclear-encoded mitochondrial membrane protein associated with apoptosis in cardiomyocytes (Wang et al., 2017).

Holdt et al. (2016) reported that overexpression of circANRIL, the circular form of lncRNA ANRIL, induces apoptosis in HEK-293 cells. Further, circular Rar1, circRar1, was shown to regulate apoptosis in N2a mouse neuroblastoma cells (Nan et al., 2016). Pro-apoptotic lncRpa and apoptosis-related circRar1 were shown to be regulated by miR-671. However, a complete genomewide profile of dysregulated circular RNAs under apoptotic conditions remains unknown. Here we report the first genome-wide profile of differentially expressed circular RNAs in apoptotic HeLa cells and provide bioinformatics data that suggest the potential translatability of certain candidate circRNAs.

MATERIALS AND METHODS

Cell Culture, Drug Treatment and Measurement of Apoptosis

HeLa cells were obtained from DKFZ GmbH (Germany). Cells were cultured in RPMI 1640 (with L-Glutamine, Gibco, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, United States) and 1% penicillin-streptomycin (Gibco) in an atmosphere of 5% CO₂ at 37°C. Based on the initial dose and time kinetics, the subsequent treatments were carried out in the following conditions (1) cisplatin (Santa Cruz, United States) at a concentration of 80 μ M for 16 h; (2) doxorubicin (Cell Signaling, United States) at a concentration of 0.5 μ M for 4 h; (3) anti-Fas mAb (Millipore, United States) at a concentration of 0.5 μ g/ml for 16 h; and (4) TNF- α (Millipore, United States) at a final concentration of 125 ng/ml TNF- α with 10 μ g/ml cycloheximide (Applchem, Germany) for 8 h. Untreated and dimethylsulfoxide (DMSO)-treated (0.1%) cells were used as negative control.

Following three biological replicates of drug treatments, 0.5×10^6 cells were trypsinized by 1 $\times$ Trypsin-EDTA (Gibco, United States) and washed in 1X cold PBS (Gibco, United States), followed by resuspension in 1 $\times$ Annexin binding buffer (Becton Dickinson, United States). The resuspended cells were stained with Annexin V-PE (Becton Dickinson, United States) and 7AAD (Becton Dickinson, United States) for 15 min in dark at room temperature and analyzed by FACS Canto (Becton Dickinson, United States).

Control and apoptotic cells were also stained with NucRedTM Dead 647 ReadyProbesTM Reagent (Invitrogen) to visualize apoptotic cells under fluorescence microscopy. HeLa cells (0.4×10^6 cells/well) were seeded in a six-well plate and treated with 4 drugs as explained above. DMSO (5%) (v/v) was used as positive control. At the end of treatment period, 2 drops/ml of medium was applied on the cells and cells were monitored by a fluorescent microscope (Zeiss Observer Z1) after 30 min of incubation in dark.

CircRNA Deep-Sequencing and Bioinformatics Analyses

Control and treated cells were sent to NOVAGENE (Hong Kong) in RNeasy for RNA isolation and deep sequencing using a previously published method optimized for circular RNA identification (Szabo and Salzman, 2016). Three biological replicates of total RNAs from control and treated cells were then subjected to poly(A) tail elimination, rRNA depletion and RNase-R treatment for circRNA enrichment prior to deep sequencing (Szabo and Salzman, 2016). The original raw data obtained from high throughput sequencing (Illumina HiSeq™2500) were first transformed to Sequenced Reads containing read sequence and corresponding base quality (in FASTQ format) through Base Calling. Novel circRNAs were identified and annotated using CIRCexplorer (Zhang et al., 2014), which remapped the unmapped reads to the genome by tophat's fusion-search to find back-spliced reads. The normalized expression of circRNAs in each sample was calculated based on the transcript per million (TPM) method (Zhou et al., 2010) using DESeq2 version 1.6.3 (Love et al., 2014) where the threshold was set as $\text{padj} < 0.05$.

Log2 ratios were clustered by K-means and self-organizing maps (SOM) algorithms as well as TPM. CircRNA and miRNA pairing were annotated by miRSystem (Lu et al., 2012) for further analysis. All differentially expressed circular RNAs, origin mRNAs and potential miRNA binding sites were classified based on their fold change, pathway-drug specificity and source genes. Translation potential of circRNAs was examined by CircInteractome¹, Ensembl², NCBI ORF Finder³, IRESite⁴ (http://iressite.org/), and SRAMP: prediction of mammalian N6-methyladenosine (m⁶A) sites⁵.

Initially, we focused on cisplatin-treated samples due to the widespread use of cisplatin in cancer treatment. Thus, 109 differentially expressed circular RNAs in cisplatin-treated samples were subjected to further bioinformatics analyses. StarBase⁶ was used to investigate potential miRNA-circRNA interactions. miRNAs with potential binding sites were then fed into miRSystem⁶ to identify putative target mRNAs as well as pathways that might be affected by the potential miRNA-circRNA interactions. To examine the translation potential of circRNA candidates, exon sources of circular candidates were downloaded from Ensembl (see text footnote 2). Those that originate from the first exons were searched for the presence of a potential internal ribosome entry site using IRESite (see text footnote 4). SRAMP (see text footnote 5) was used to analyze the presence of m⁶A sites on hsa_circ_0029693.

Validation of circRNAs by Cloning

Total RNA was isolated from cisplatin (80 μM) and DMSO (0.1%) (Applchem, Germany) treated cells using TRIzol (Thermo Fisher Scientific, United States) according to the

manufacturer's instructions. For all candidates, divergent primers were designed by using the CircInteractome web tool (see text footnote 1). These primers were specific to amplify backsplice junctions, excluding the potential for the amplification of linear mRNAs (Table 1). Total RNAs were then treated with RNase R (Epicenter) according to manufacturer's instructions to eliminate the linear RNAs and to enrich the circRNAs. Briefly, 2 μg of total RNAs, 1 μl buffer and 5 units of RNase R were mixed in a final volume of 10 μl and incubated for 30 min at 37°C. Subsequently, RNA clean-up was performed with Nucleospin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions.

cDNAs were prepared from RNase R⁺ and RNase R⁻ RNA samples with ProtoScript[®] first strand cDNA synthesis kit (NEB, United States) with random primers in a total volume of 50 μl according to the manufacturer's instructions. The cDNA reactions contained 200 ng of total RNAs or equivalent of RNase-treated samples. The candidate circRNAs were amplified by PCR using the following conditions: Initial denaturation at 95°C for 30 s, 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, extension at 68°C for 1 min, 1 cycle of final extension at 68°C for 5 min. PCR products were run on 1% agarose gel to examine the size and purity of the amplified products. Bands of the correct size were extracted from the gel using the gel extraction kit (Macherey Nagel, Germany). The purified fragments were cloned into the pCR[®]II TA vector using the TA cloning kit (Thermo Fisher Scientific, United States) and sequenced at the BIOMER center (IZTECH, Turkey) to validate the backsplice junction of target circRNAs.

qPCR Analyses

cDNAs prepared from RNase R⁺ and RNase R⁻ RNA samples were used as templates for qPCR with GoTaq q-PCR Master Mix (Promega, United States). Two step PCR amplification conditions were as follows: initial denaturation at 95 °C for 2 min, (40 cycles) denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

TABLE 1 | List of divergent primers.

	Forward primer	Reverse primer
hsa_circ_0012992 ^b	AGGCTTGGATCTTC AGCCATT	AGGTGGATGCACA AAATCTCAG
circ-HIPK3 ^b	TATGTTGGTGGATCC TGTTCCGCA	TGGTGGGTAGACCA AGACTTGTGA
Beta-actin ^a	CTGGGACGACATGGAGAAAA	AAGGAAGGCTGGAA GAGTGC
hsa_circ_0014824 ^b	ATCACAGGACCCA AACCTCTG	CTTGCCTCTGGTG CAGAAT
hsa_circ_0029693 ^b	TGCTAATGAATCG GGCACCT	TCTTTCCTTCCAT TTTTGTAGTTCC
hsa_circ_0029693 ^c	AAGCCTTGAGGG AAATCAGA	TGGCCTCATTG TTAGTCCAG
GAPDH ^a	ACTCCTCCACC TTTGACGC	GCTGTAGCCAAAT CGTTGTC

^aConvergent primer, ^bfor qPCR amplification, ^cfor cloning.

¹ circinteractome.nia.nih.gov

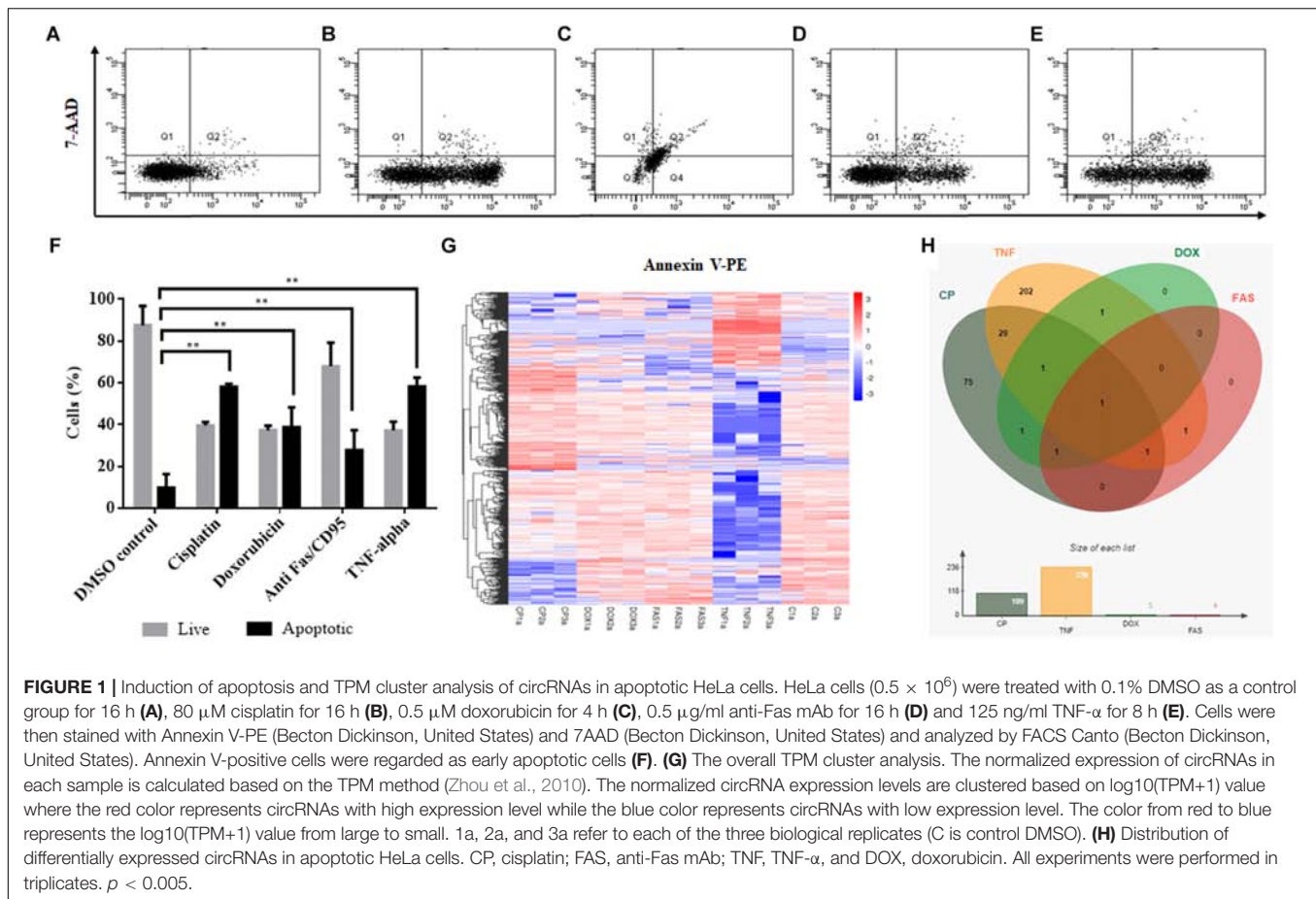
² ensembl.org/index.html

³ ncbi.nlm.nih.gov/orffinder/

⁴ cuilab.cn/sramp

⁵ starbase.sysu.edu.cn/

⁶ mirsystem.cgm.ntu.edu.tw



Statistical Analyses

All experiments were carried out at least in three biological replicates. Student's *t* test was used to analyze the data. A *P*-value of 0.05 was considered statistically significant unless indicated otherwise.

RESULTS AND DISCUSSION

Induction of Apoptosis and Deep Sequencing Analyses

Cisplatin and doxorubicin treatment were performed to induce apoptosis by intrinsic pathway while anti-Fas mAb and TNF- α treatments were carried out to trigger the extrinsic pathway (Boatright and Salvesen, 2003; Morgan et al., 2013; Huang et al., 2018). In order to attain an apoptosis rate of approximately 50%, drug /ligand concentrations and incubation periods were optimized through dose and time kinetics (data not shown). Figures 1A–E shows the dot blot analyses of cell populations after treatment at optimum doses and times. All drug and ligand treatments caused cells to shift to the Annexin V+/7AAD⁺ region (Figures 1B,D,E) except doxorubicin (Figure 1C), which caused cells to be included in Annexin V+/7AAD⁺ population. Apoptosis rates ranged between 27 and 58% (Figure 1F), depending on the treatment agent. Cisplatin and TNF- α

caused the highest apoptotic rate (58%) while anti-Fas-treated population displayed a lower rate of apoptosis (27%) due to the desensitization of HeLa cells to FasR stimulation (Holmström et al., 1999). Control and apoptotic cells were also stained with NucRedTM Dead 647 as a qualitative visualization of cell death (Supplementary Figure S1). The Annexin V+/7AAD⁺ state of the cells were interpreted as cells being caught at the early apoptotic phase and thus they were deemed suitable for downstream steps.

To identify differentially expressed circRNAs, total RNAs were subjected to circRNA enrichment as explained in “Materials and Methods.” The number of raw reads ranged from 81,360,972 to 106,246,444 whereas clean reads ranged from 79,147,032 to 103,277,920 with an error rate of 0.02 to 0.03%. Q30 score was between 91 and 94.43%, indicating fairly a low error rate. The lowest percentage of total mapped reads was 68.77% whereas the highest percentage of total mapped reads was 94.3%. For all samples, the primary source of circRNAs was introns while getting reads from exons and intergenic sequences as well. TPM cluster analysis was conducted to obtain normalized circRNA expression data. Read count value from expression level analysis of circRNA was used as input data for K-means and SOM clustering (Supplementary Figure S2). This clustering revealed that circRNAs within the same cluster exhibited the same changing trend of expression levels under

different conditions. Based on the TPM cluster analysis, it is apparent that circRNA expression differs among control, drug- and ligand-treated groups (**Figure 1G**). TNF- α and cisplatin treatment groups exhibited the highest number of dys-regulated circRNAs. However, the number of circRNAs differentially expressed in doxorubicin and anti-Fas mAb-treated HeLa cells were relatively low (**Figure 1H**). Venn diagram and size graph show that 109 circular RNA were differentially expressed in cisplatin-treated HeLa cells. Likewise a total of 236 circular RNAs showed differential expression in the TNF- α treated HeLa cells. Five and four circular RNA were differentially expressed in doxorubicin and anti-Fas mAb-treated HeLa cells, respectively. Consistent raw read numbers in three biological replicates of doxorubicin and anti-Fas mAb-treated samples suggest a drug-specific response rather than a technical problem, which begs for further investigation.

Candidate Selection

Because the drugs and ligands used in our studies are potent inducers of not only apoptosis but also other cellular phenotypes such as stress, proliferation or cell cycle, we applied a number of filtering criteria to select candidate circRNAs for further analyses. Basically we selected the dys-regulated circRNAs that (1) had the highest log change difference between control and drug treated cells, (2) had a statistical significance of differential expression, and (3) originate from mRNAs that were reported or have the potential to regulate apoptosis. Based on these criteria, we selected 10 candidates that met our criteria the most. Among these candidates, hsa_circ_0099768, hsa_circ_0083543, hsa_circ_0043795, hsa_circ_0051666, and hsa_circ_0014824 were upregulated under apoptotic conditions whereas the rest of them were downregulated (**Table 2**). All candidates exhibited differential expression under cisplatin treatment whereas hsa_circ_0099768 was differentially expressed under all treatment conditions. hsa_circ_0083543 was the other candidate differentially expressed in cisplatin-, doxorubicin-, and anti-Fas-treated HeLa cells.

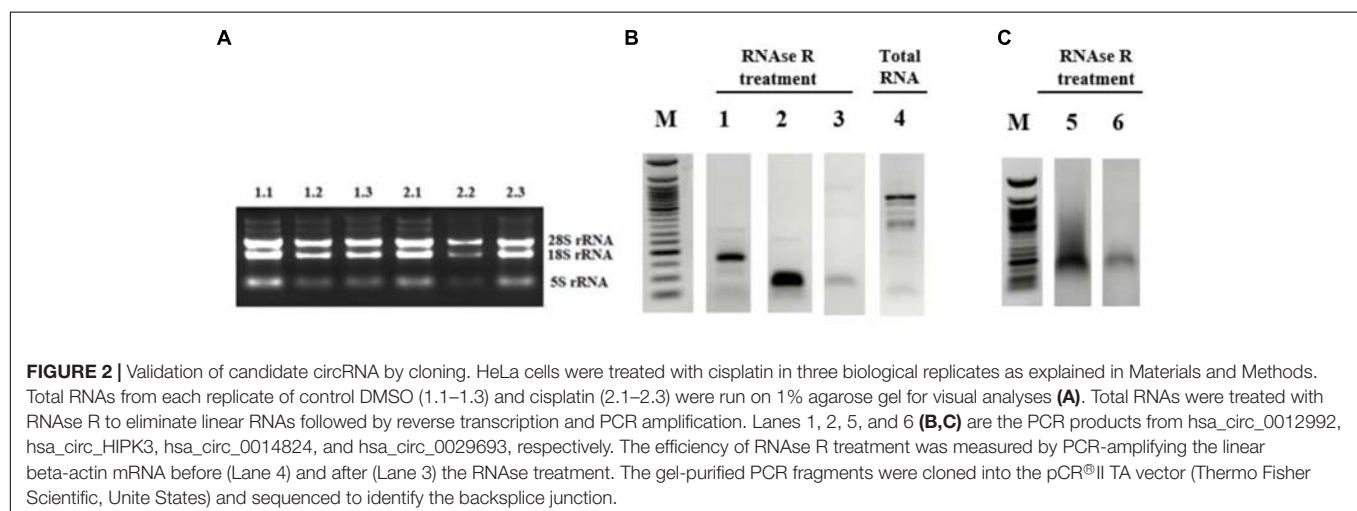
RNAse R Treatment and qPCR Validation

Although the bioinformatics algorithms eliminate artifacts from the circRNAs, additional experimental analyses are required to call the RNA-seq reads as circRNAs. Typically, RNAse R treatment is used to eliminate linear RNAs to prevent the potential amplification of tandem duplications (Panda et al., 2017). Additionally, the amplified products are sequenced to conclusively state the presence of a circRNA. To this extent, we used the total RNAs from control and cisplatin-treated cells (**Figure 2A**) and chose circ-HIPK3 as a positive control to test circRNA validation. Li et al. (2017) reported that circ-HIPK3 is indeed expressed in HeLa cells. We designed divergent primers using the Circinteractome tool (Dudekula et al., 2016) to amplify the positive control circ-HIPK3. qPCR was then performed with cDNAs that were reverse transcribed from both RNAse R⁺ and RNAse R⁻ samples.

TABLE 2 | Circular RNA candidates differentially expressed in apoptotic HeLa cells.

Candidates	P-Value	Log change	Parental mRNA
hsa_circ_0099768*	2,29E-04	71.045	FGF14
hsa_circ_0083543**	0.00053703	50.376	XPO7
hsa_circ_0043795	0.00068894	49.662	STAT5A
hsa_circ_0051666	0.0004384	50.975	BBC3
hsa_circ_0014824	9,18E-01	55.021	ARHGEF11
hsa_circ_0029693	0.00038011	-36.338	LATS2
hsa_circ_0081300	0.00039188	-36.291	TRRAP
hsa_circ_0012992	0.00014162	-53.499	USP33
hsa_circ_0080853	0.00011961	-53.925	PTPN12
hsa_circ_0097866	0.0001054	-54.228	TMEM132C

Circular RNAs that were significantly down- and up-regulated upon treatment with all agents is listed below with their parental coding mRNAs. The majority of top 5 upregulated and downregulated circular RNAs originated from protein coding genes well-documented to regulate apoptosis. P-value, log change and apoptotic regulator function of the origin linear mRNA were considered as criteria in candidate selection. * denotes the candidate common in all drug/ligand groups, ** denotes the candidate common in cisplatin, doxorubicin and anti-Fas group.



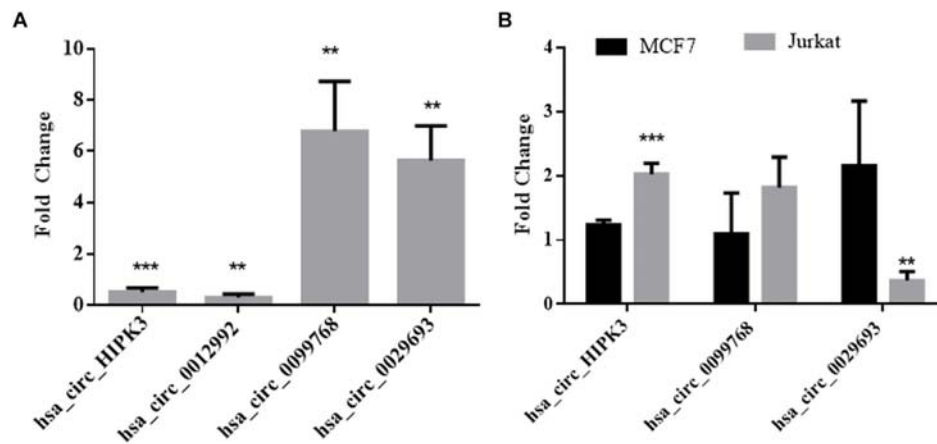


FIGURE 3 | qPCR analyses of candidate circRNA expression. Cells were treated with DMSO (control) and cisplatin as explained in Materials and Methods. RNase R-treated RNA samples were used to prepare cDNAs using ProtoScript® first strand cDNA synthesis kit (NEB, United States) with random primers. GoTaq q-PCR Master Mix (Promega, United States) was used for qPCR analyses. Data collected in three biological replicates were normalized against DMSO control and GAPDH. ** and *** denote $p \leq 0.01$ and $p \leq 0.001$, respectively. (A) HeLa cells; (B) MCF and Jurkat cells.

First, we examined the efficiency of RNase R treatment to eliminate linear RNAs using DMSO-treated control RNAs. As shown in **Figure 2B**, RNase R treatment resulted in the loss of the linear beta actin mRNA (Lane 3 vs. 4). However, the positive control circ-HIPK3 was resistant to the treatment with RNase R (**Figure 2B**, Lane 2). We also validated the resistance of our candidates, hsa_circ_0012992, hsa_circ_0014824, and hsa_circ_0029693 to RNase R (**Figure 2B**, Lanes 1 and 2C). All these analyses showed that we were able to eliminate the linear RNAs and enrich the circular forms under our experimental conditions.

We then performed qPCR analyses with total RNAs to quantitatively measure the circRNA transcripts in cisplatin-treated cells to validate circRNA-seq data. To this extent, we

subjected three candidates, hsa_circ_0012992, hsa_circ_0099768, and hsa_circ_0029693 to qPCR analyses, which were shown to exist in the circular form (**Figure 3**). As shown in **Figure 3**, hsa_circ_0012992 and hsa_circ_HIPK3 were down-regulated 3.3- and 2-fold, respectively, while hsa_circ_0099768 and hsa_circ_0029693 were upregulated 6.7- and 5.6-fold, respectively. We then carried out cisplatin treatments in two other cell lines, Jurkat and MCF-7, to examine whether this expression is cell-specific (**Figure 3**). Hsa_circ_HIPK3 expression was 2-fold downregulated in HeLa cells whereas its expression was upregulated in MCF7 and Jurkat cells 1.2 and 2-fold respectively. Expression of hsa_circ_0099768 was upregulated in HeLa and Jurkat cells. HeLa cells possessed the highest differential expression with a 6.7-fold change. Hsa_circ_0029693

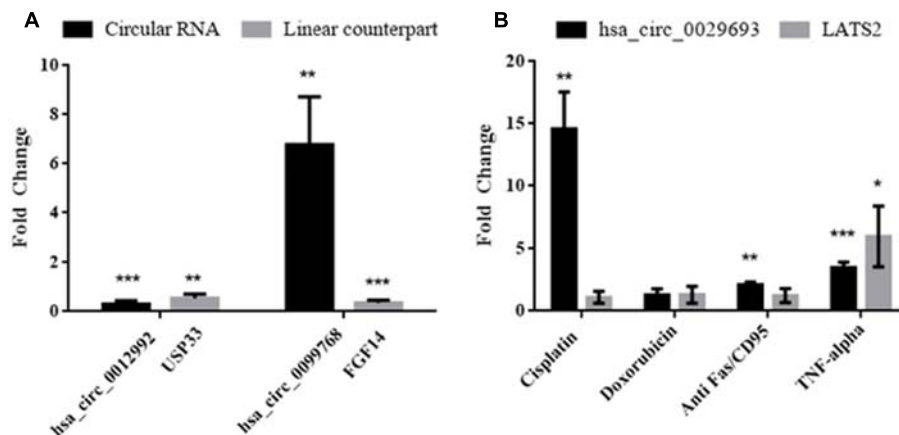


FIGURE 4 | Analyses of expression levels of circular and their counterpart linear mRNAs. For circRNA analyses, experiments were carried out as explained in **Figure 3**. For mRNA analyses, RNase R treatment was omitted before cDNA synthesis to amplify the linear RNAs. (A) HeLa cells treated with cisplatin only; (B) HeLa cells treated with cisplatin, doxorubicin, anti Fas, and TNF-alpha. Data, collected in three biological replicates, were normalized against DMSO control and GAPDH. *, **, and *** denote $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

was upregulated 5.6- and 2.1-fold in HeLa and MCF7 cells, respectively, while its expression was downregulated by 2.7-fold in Jurkat cells. All these results show that the induction of apoptosis results in differential expression of a number of circRNAs and their expression appears to be cell-specific at least for the candidate that we have analyzed in this study. Although our data provide an indirect relationship between the differential expression of certain circRNAs and apoptosis, further experiments are required to establish a direct link between an individual circRNA and apoptosis. For example, these candidates should be silenced or over-expressed to demonstrate the direct genotype-phenotype relationship.

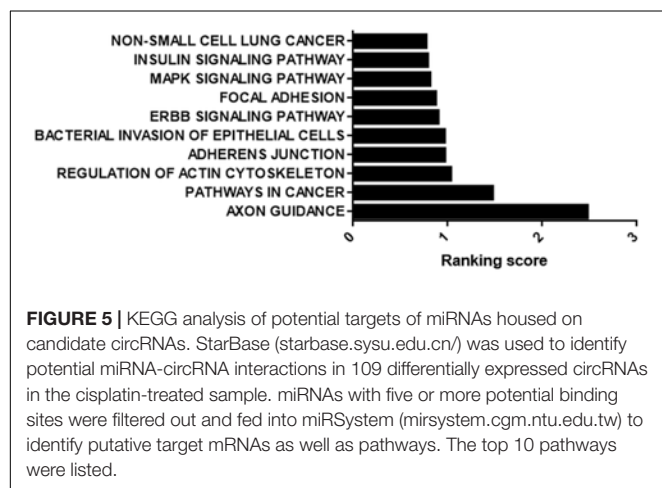
Molecular details of circRNA biogenesis are still in progress. It needs to be resolved whether canonical splicing precedes back-splicing or vice versa (exon skipping vs. direct back-splicing) (Chen and Yang, 2015). Recent evidence points to the use of constitutive exons in circRNA biogenesis at the expense of their linear counterpart (Aufiero et al., 2018). Thus, we subjected linear counterparts of the candidate circRNAs to qPCR analyses to examine whether circRNA expression is correlated with the template mRNA expression (Figure 4). LATS2, USP33, and FGF14 were the linear counterparts of hsa_circ_0029693, hsa_circ_0012992, and hsa_circ_0099768, respectively. In cisplatin-treated HeLa cells, USP33, the gene encoding a ubiquitin specific peptidase, was downregulated 2-fold in parallel with its circular counterpart hsa_circ_0012992, while hsa_circ_0099768 was upregulated 6.7-fold in apoptotic samples in contrary to its linear counterpart FGF14, the gene encoding fibroblast growth factor (Figure 4). The other candidate, has_circ_0029693 was also subjected to qPCR analysis with LATS2 in four drug/ligand-treated apoptotic samples. Both circular and linear counterparts were upregulated in TNF-alpha-induced apoptotic samples in 3.5- and 5.9-fold, respectively (Figure 4). These results suggest that has_circ_0099768 biogenesis is probably independent from its template mRNA biogenesis while the biogenesis of the other two candidates is correlated with the expression level of their template mRNAs. However, further analyses are required to examine the correlation between alternative splicing and circRNA biogenesis.

CircRNA-miRNA Interactions and Coding Potential

One potential function of differentially expressed circRNAs is to serve as miRNA sponges to regulate the functions of miRNAs and their target mRNAs (Chen, 2016). To examine whether our circRNAs might regulate apoptosis through miRNA-circRNA interactions, we checked the potential miRNA binding sites on the candidate circRNAs. Origin mRNAs of candidate circular RNAs are obtained from CircInteractome tool by entering circular RNA ID. Potential miRNA binding sites were obtained by using starBase v3.0 which is an open-source platform for studying the miRNA-circRNA interactions from CLIP-seq (Li et al., 2014). To qualify as a miRNA sponge, a circRNA is expected to harbor multiple binding sites for the same miRNA as illustrated in the case of cdr1as-miR-7 interaction (Li et al., 2014; Chen, 2016).

We prioritized the cisplatin-treated samples due to the cancer therapeutic potential of cisplatin. Interestingly, 71 out of 109 circRNAs possessed miRNA binding sites. Those 71 circRNAs were then classified based on the number of binding sites. miRNAs that were likely to bind to the same circRNA at least five times were analyzed by miRSystem. The target mRNAs of potentially sponged miRNAs were subjected to KEGG functional annotation analysis and the resulting top 10 pathways were presented in Figure 5. This analysis showed that the potential miRNA-circRNA interaction under our experimental setting is likely to modulate cellular processes such as pathways in cancer, cell adherence function and MAPK signaling pathway (Figure 5).

Perhaps, one of the most interesting functions of circRNAs is their translatability (Pamudurti et al., 2017). Potential translation of circRNAs could generate a truncated protein that could have a diverse array of functions in the cell. Thus, we also examined the translation potential of our candidates. Hsa_circ_0029693 originates from the 2nd exon that includes half of the 5'UTR and the first translated exon of the linear source gene LATS2. The resulting circRNA is 444 nt that harbors a 147-aa open reading frame and an in-frame stop codon (Figure 6A). The 3D structure of putative truncated protein product of has_circ_0029693 was created by SWISS-MODEL⁷. It might be a homodimer protein that contains caspase-3 and caspase-7 cleavage site motif but loses its protein kinase domain (Figure 6A). We used SRAMP (see text footnote 5) to check for potential m⁶A sites as these sites are potential indicator of translatability (Zhou et al., 2016). When we subjected has_circ_0029693 to such an analysis, a very high confident m⁶A site motif was detected in a site close proximity to translation start codon. Additionally, an IRES site was detected nearby the start codon (Figure 6B). All these analyses suggest that some of our candidates hold the potential for translation that could generate truncated products. Further experimental evidence would be required to conclusively demonstrate the translatability of circRNAs. For example, the polysome association of circRNAs would be a good support for their translatability. Perhaps, the most direct evident would be to detect these products (either the



⁷swissmodel.expasy.org

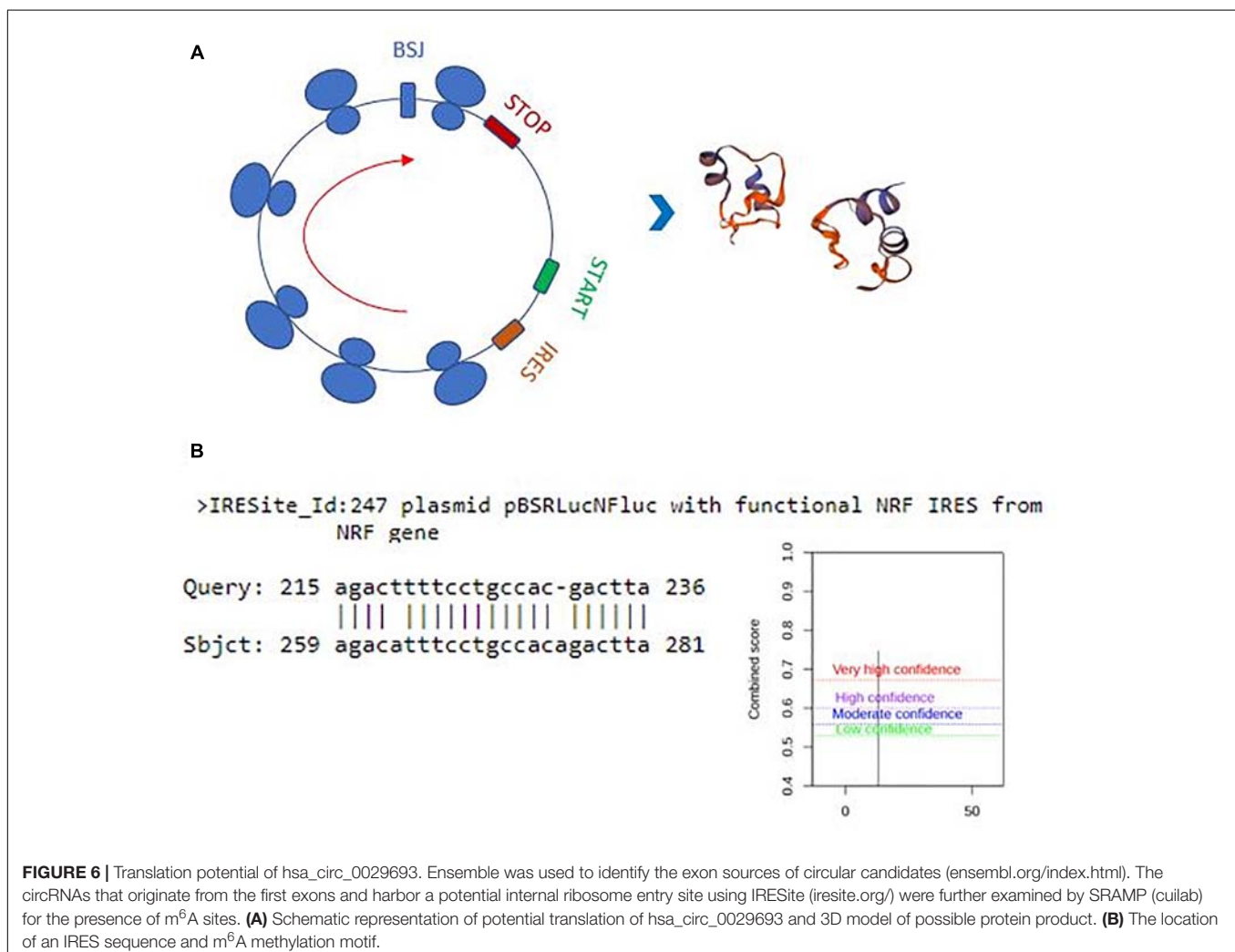
endogenous product or the over-expressed product) through mass spectroscopy. It will be quite interesting to investigate the potential role of these truncated products as they might potentially compete with the full-length proteins or have an entirely different function.

CONCLUSION

Apoptosis is a highly regulated cellular process that is important for cell survival and cell death and it is involved in various physiological as well as pathological conditions. In this study, we provide a comprehensive profile of circRNAs in HeLa cells to another (Figure 1G). At least in HeLa cells, TNF- α and cisplatin are more potent in circRNA induction. However, doxorubicin and anti-Fas mAb treatment caused a moderate level of circRNA differential expression. It is difficult to account for this difference in the number of differentially expressed circRNAs. The consistent read number in all three biological replicates potentially point to a drug-specific response rather than a technical problem but this point requires further investigation.

We then validated the existence of at least three circRNA candidates by PCR-amplification (Figure 2) and sequencing of the amplified fragments. qPCR analyses showed cell specific expression of circ-HIPK3 and hsa_circ_0029693 in HeLa, MCF-7 and Jurkat cells (Figure 3).

Interestingly, circRNAs, differentially expressed under apoptotic conditions, house binding sites for miRNAs reported to regulate apoptosis, suggesting a circRNA-miRNA-mRNA regulatory loop (Figure 5). Although we do not provide any experimental evidence at this point, our bioinformatics analyses provide an indirect link for a potential interaction between candidate circRNAs and miRNAs known to regulate apoptosis. However, more direct evidence would involve co-immunoprecipitation of circRNAs and miRNAs followed by PCR-amplification of the putative miRNAs. Also, silencing and/or over-expression would be helpful in demonstrating such direct interactions and their effect on the apoptotic phenotype. Our bioinformatics analyses also yielded interesting data at least with respect to the translatability of one candidate circRNA, hsa_circ_0029693. However, more solid evidence is required to claim the translation of a protein product from this circRNA.



In conclusion, although more experimental evidence is needed to demonstrate a direct link between differentially expressed candidate circRNAs and apoptosis, our data provide the first transcriptomics profile of circRNAs in HeLa cells. These data can be used in future studies to establish a direct link between candidates and apoptosis and also to study the effects of miRNA-circRNA interactions and putative truncated proteins on apoptosis.

DATA AVAILABILITY

Raw sequence reads have been deposited in the GEO database (GSE125249).

AUTHOR CONTRIBUTIONS

BA obtained the funding and designed the experiments. BY and IE performed the experiments. BY performed bioinformatics

analyses. BA, BY, and IE analyzed the data and wrote and approved the manuscript.

FUNDING

This study was funded by TUBITAK (Project No: 215Z081 to BA).

ACKNOWLEDGMENTS

The authors would like to thank BIOMER (Iztech, Turkey) and the specialists Ozgur Okur and Dane Ruscuklu for flow cytometry analyses and sequencing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00176/full#supplementary-material>

REFERENCES

- Ashwal-Fluss, R., Meyer, M., Pamudurti, N. R., Ivanov, A., Bartok, O., Hanan, M., et al. (2014). circRNA biogenesis competes with pre-mRNA splicing. *Mol. Cell.* 56, 55–66. doi: 10.1016/j.molcel.2014.08.019
- Aufiero, S., van den Hoogenhof, M. M. G., Rechman, Y. J., Beggali, A., van der Made, I., Kluin, J., et al. (2018). Carciac circRNAs arise mainly from constitutive exons rather than alternatively spliced exons. *RNA* 24, 815–827. doi: 10.1261/rna.064394.117
- Bertone, P., Stolc, V., Royce, T. E., Rozowsky, J. S., Urban, A. E., Zhu, X., et al. (2004). Global identification of human transcribed sequences with genome tiling arrays. *Science* 306, 2242–2246. doi: 10.1126/science.1103388
- Boatright, K. M., and Salvesen, G. S. (2003). Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* 15, 725–731. doi: 10.1016/j.ceb.2003.10.009
- Chen, L. L. (2016). The biogenesis and emerging roles of circular RNAs. *Nat. Rev. Mol. Cell. Biol.* 17, 205–211. doi: 10.1038/nrm.2015.32
- Chen, L. L., and Yang, L. (2015). Regulation of circRNA biogenesis. *RNA Biol.* 12, 381–388. doi: 10.1080/15476286.2015.1020271
- Cocquet, J., Chong, A., Zhang, G., and Veitia, R. A. (2006). Reverse transcriptase template switching and false alternative transcripts. *Genomics* 88, 127–131. doi: 10.1016/j.ygeno.2005.12.013
- Dang, Y., Quyang, X., Zhang, F., Wang, K., Lin, Y., Sun, B., et al. (2017). Circular RNAs expression profiles in human gastric cancer. *Sci. Rep.* 7:9060. doi: 10.1038/s41598-017-09076-6
- Dudekula, D. B., Panda, A. C., Grammatikakis, I., De, S., Abdelmohsen, K., and Gorospe, M. (2016). CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. *RNA Biol.* 13, 34–42. doi: 10.1080/15476286.2015.1128065
- Favaloro, B., Allocati, N., Graziano, V., Di Ilio, C., and De Laurenzi, V. (2012). Role of apoptosis in disease. *Aging* 4, 330–349. doi: 10.18632/aging.100459
- Fuchs, Y., and Steller, H. (2011). Programmed cell death in animal development and disease. *Cell* 147, 742–758. doi: 10.1016/j.cell.2011.10.033
- Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993
- Holdt, L. M., Stahringer, A., Sass, K., Pichler, G., Kulak, N. A., Wilfert, W., et al. (2016). Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. *Nat. Commun.* 7:12429. doi: 10.1038/ncomms12429
- Holmström, T. H., Tran, S. E., Johnson, V. L., Ahn, N. G., Chow, S. C., and Eriksson, J. E. (1999). Inhibition of mitogen-activated kinase signaling sensitizes HeLa cells to fas receptor-mediated apoptosis. *Mol. Cell. Biol.* 19, 5991–6002. doi: 10.1128/MCB.19.9.5991
- Huang, G., Li, S., Yang, N., Zou, Y., Zheng, D., and Xiao, T. (2017). Recent progress in circular RNAs in human cancers. *Cancer Lett.* 404, 8–18. doi: 10.1016/j.canlet.2017.07.002
- Huang, S., Yang, B., Chen, B. J., Bliim, N., Ueberham, U., Arendt, T., et al. (2017). The emerging role of circular RNAs in transcriptome regulation. *Genomics* 109, 401–407. doi: 10.1016/j.ygeno.2017.06.005
- Huang, Z., Lin, S., Long, C., Zhou, X., Fan, Y., Kuang, X., et al. (2018). Notch signaling pathway mediates Doxorubicin-driven apoptosis in cancers. *Cancer Manag. Res.* 10, 1439–1448. doi: 10.2147/CMARS160315
- Jeck, W. R., and Sharpless, N. E. (2014). Detecting and characterizing circular RNAs. *Nat. Biotechnol.* 32, 453–461. doi: 10.1038/nbt.2890
- Jeck, W. R., Sorrentino, J. A., Wang, K., Slevin, M. K., Burd, C. E., and Liu, J. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 19, 141–157. doi: 10.1261/rna.035667.112
- Lander, E. S. (2011). Initial impact of the sequencing of the human genome. *Nature* 470, 187–197. doi: 10.1038/nature09792
- Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035
- Li, J.-H., Liu, S., Zhou, H., Qu, L.-H., and Yang, J.-H. (2014). starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res.* 42, D92–D97. doi: 10.1093/nar/gkt1248
- Li, Y., Zheng, F., Xiao, X., Xie, F., Tao, D., Huang, C., et al. (2017). CircHIPK3 sponges miR-558 to suppress heparanase expression in bladder cancer cells. *EMBO Rep.* 18, 1646–1659. doi: 10.15252/embr.201643581
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8
- Lu, T.-P., Lee, C.-Y., Tsai, M.-H., Chiu, Y.-C., Hsiao, C. K., Lai, L.-C., et al. (2012). miRSystem: an integrated system for characterizing enriched functions and pathways of MicroRNA targets. *PLoS One* 7:e42390. doi: 10.1371/journal.pone.0042390
- McManus, C. J., Duff, M. O., Eipper-Mains, J., and Graveley, B. R. (2010). Global analysis of trans-splicing in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12975–12979. doi: 10.1073/pnas.1007586107
- Mehta, S. L., Pandi, G., and Vemuganti, R. (2017). Circular RNA expression profiles alter significantly in mouse brain after transient

- focal ischemia. *Stroke* 48, 2451–2548. doi: 10.1161/STROKEAHA.117.017469
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., and Rybak, A. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Morgan, S., Lopes, F., Gourley, C., Anderson, R. A., and Spears, N. (2013). Cisplatin and doxorubicin induce distinct mechanisms of ovarian follicle loss; imatinib provides selective protection only against cisplatin. *PLoS One* 8:e70117. doi: 10.1371/journal.pone.0070117
- Nan, A., Chen, L., Zhang, N., Liu, Z., Yang, T., Wang, Z., et al. (2016). A novel regulatory network among LncRpa, CircRar1, MiR-671 and apoptotic genes promotes lead-induced neuronal cell apoptosis. *Arch. Toxicol.* 91, 1671–1684. doi: 10.1007/s00204-016-1837-1
- Pamudurti, N. R., Bartok, O., Jens, M., Ashwal-Fluss, R., Stottmeister, C., Ruhe, L., et al. (2017). Translation of CircRNAs. *Mol. Cell* 66, 9–21. doi: 10.1016/j.molcel.2017.02.021
- Panda, A. C., De, S., Grammatikakis, I., Munk, R., Yang, X., Piao, Y., et al. (2017). High-purity circular RNA isolation method (RPAD) reveals vast collection of intronic circRNAs. *Nucleic Acid Res.* 45:e116. doi: 10.1093/nar/gkx297
- Qu, S., Yang, X., Li, X., Wang, J., Gao, Y., Shang, R., et al. (2015). Circular RNA: a new star of noncoding RNAs. *Cancer Lett.* 365, 141–148. doi: 10.1016/j.canlet.2015.06.003
- Salzman, J., Chen, R. E., Olsen, M. N., Wang, P. L., and Brown, P. O. (2013). Cell-type specific features of circular RNA expression. *PLoS Genet.* 9:e1003777. doi: 10.1371/journal.pgen.1003777
- Suzuki, H., Zou, Y., Wang, J., Zhang, M. Q., Malhotra, A., and Mayeda, A. (2006). Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing. *Nucleic Acids Res.* 34:e63. doi: 10.1093/nar/gkl151
- Szabo, L., and Salzman, J. (2016). Detecting circular RNAs: bioinformatic and experimental challenges. *Nat. Rev. Genet.* 17, 679–692. doi: 10.1038/nrg.2016.114
- Vince, J. E., and Silke, J. (2009). *Apoptosis: Regulatory Genes and Disease*. Hoboken, NJ: Wiley.
- Wang, K., Gan, T. Y., Li, N., Liu, C. Y., Zhou, L. Y., and Gao, J. N. (2017). Circular RNA mediates cardiomyocyte death via miRNA-dependent upregulation of MTP18 expression. *Cell Death Differ.* 24, 1111–1120. doi: 10.1038/cdd.2017.61
- Zhang, X. O., Wang, H. B., Zhang, Y., Lu, X., Chen, L. L., and Yang, L. (2014). Complementary sequence-mediated exon circularization. *Cell* 159, 134–147. doi: 10.1016/j.cell.2014.09.001
- Zhou, L., Chen, J., Li, Z., Li, X., Hu, X., Huang, Y., et al. (2010). Integrated profiling of microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell renal cell carcinoma. *PLoS One* 5:e15224. doi: 10.1371/journal.pone.0015224
- Zhou, Y., Zeng, P., Li, Y. H., Zhang, Z., and Cui, Q. (2016). SRAMP: prediction of mammalian N6-methyladenosine (m6A) sites based on sequence-derived features. *Nucleic Acids Res.* 44:e91. doi: 10.1093/nar/gkw104

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Analyses of a Panel of Transcripts Identified From a Small Sample Size and Construction of RNA Networks in Hepatocellular Carcinoma

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OPEN ACCESS

Edited by:

Subbaya Subramanian,
University of Minnesota Twin Cities,
United States

Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 14 November 2018

Accepted: 23 April 2019

Published: 08 May 2019

Citation:

Sheng Z, Wang X, Xu G, Shan G
and Chen L (2019) Analyses of a
Panel of Transcripts Identified From
a Small Sample Size and Construction
of RNA Networks in Hepatocellular
Carcinoma. *Front. Genet.* 10:431.
doi: 10.3389/fgene.2019.00431

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Dysregulation of mRNAs and non-coding RNAs (ncRNAs) plays critical roles in the progression of HCC. Here, we investigated HCC samples by RNA-seq and identified a series of dysregulated RNAs in HCC. Various bioinformatics analyses established long non-coding RNA (lncRNA)-mRNA co-expression and competing endogenous RNA (ceRNA) networks in circRNA-miRNA-mRNA axis, indicating the potential *cis* and/or *trans* regulatory roles of lncRNAs and circRNAs. Moreover, GO pathway analysis showed that these identified RNAs were associated with many biological processes that were related to tumorigenesis and tumor progression. In conclusion, we systematically established functional networks of lncRNA-mRNA, circRNA-miRNA-mRNA to further unveil the potential interactions and biological processes in HCC. These results provide further insights into gene expression network of HCC and may assist future diagnosis of HCC.

Keywords: hepatocellular carcinoma, lncRNA, miRNA, circRNA, network

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide, and the highest incidence rates in the world are reported in Asia and Africa, with China alone accounting for half of the cases in Asia and Africa (McGlynn and London, 2011). Clinical investigation shows that HCC is the sixth most common cancer and third main cause of cancer mortality in the world (Forner et al., 2018). The overall 5 years survival rate is less than 20% due to lack of early and timely detection and treatment (Lee et al., 2012). So far, the available treatment approaches for HCC mainly include resection, liver transplantation, image-guided tumor ablation, and systemic therapy. The 5 years survival rates of 60–70% can be achieved in early-stage HCC patients (Llovet and Bruix, 2003; Llovet et al., 2003). However, systematic therapies cannot improve the survival rates of the patients with advanced stage of HCC due to limited specific and effective biomarkers or targets for clinical treatments (Llovet and Bruix, 2003; Llovet et al., 2003; Marquardt et al., 2012).

Dysregulated gene expression is a common theme underneath human disease (Adams and Cory, 2007; Aguirre-Ghiso, 2007; Amaravadi and Thompson, 2007; Azam et al., 2010; Hanahan and Weinberg, 2011). The resulted abnormal mRNAs and non-coding RNAs (ncRNAs) play roles in the occurrence and progress of diseases such as cancers (Prensner et al., 2011; Kung et al., 2013; Iyer et al., 2015; Rusk, 2015; Klingenberg et al., 2017). Many mRNAs are encoded by tumor genes or tumor suppressor genes, and ncRNAs such as microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs) are also known to be associated with tumorigenesis and tumor progression (Panzitt et al., 2007; Wang et al., 2010; Wei et al., 2012; Zhang et al., 2012; Li G. et al., 2015; Palazzo and Lee, 2015; Zhou et al., 2016; Cui et al., 2017; Shen et al., 2018; Zhang and Wu, 2018).

In HCC, tumor genes such as procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), and splicing factor 3b subunit 4 (SF3B4) are overexpressed in HCC tumor tissues compared with adjacent tissues, and knocking down their expression levels can effectively suppress tumor invasion (Shen et al., 2018). Conversely, tumor suppressor genes such as Alcohol dehydrogenase 4 (ADH4) is significantly downregulated in tumor tissues, and patients with lower ADH4 expression levels have worse prognosis and lower overall survival rate (Wei et al., 2012).

In HCC, many miRNAs are found to play oncogenic or tumor suppressive roles (Zhang et al., 2012; Li G. et al., 2015; Zhou et al., 2016). For example, miR-421 can promote proliferation and migration in HCC cell by downregulating farnesoid X receptor (FXR), which is usually highly expressed in normal liver (Zhang et al., 2012). On the other hand, miR-708 is markedly downregulated in HCC tumor tissues compared with adjacent tissues. Low miR-708 level is related to tumor node metastasis (TNM) in advanced stage patients, and over expression of miR-708 can suppress the invasion and migration of in HCC cell lines *in vitro* (Li G. et al., 2015).

lncRNAs are also correlated to HCC (Chen et al., 2018). The lncRNA HULC is upregulated in HCC with high specificity (Panzitt et al., 2007; Wang et al., 2010). It can be detected both in tumor tissues and blood of HCC patients. Aberrantly elevated HULC promotes HCC invasion and progression by increasing expression of the oncogene HMGA2. The lncRNA PVT1 enhances the tumorigenesis and metastasis of HCC by acting as a competing endogenous RNA (ceRNA) for miR-186-5p, and the knockdown of PVT1 can effectively impede HCC invasion (Karreth et al., 2015; Cui et al., 2017).

CircRNAs are a large class of transcripts in multicellular organisms with emerging importance (Chen and Shan, 2015; Chen et al., 2015; Haque and Harries, 2017). One of functional mechanisms of circRNAs is acting as ceRNA (Hansen et al., 2013; Memczak et al., 2013). For example, the circRNA CDR1as functions as sponge of miR-7 in neuronal tissues (Memczak et al., 2013; Piwecka et al., 2017). Alterations in circRNA expression are found to be related to aberrant physiology and human diseases (Hansen et al., 2013; Memczak et al., 2013; Chen and Shan, 2015; Chen et al., 2015; Holdt et al., 2016; Piwecka et al., 2017).

In this study, we have identified differentially expressed mRNAs, miRNAs, lncRNAs, and circRNAs in fresh HCC

tissues through high-throughput RNA sequencing. Functional networks of lncRNA-mRNA and circRNA-miRNA-mRNA have been established to provide new insights for biomarkers and treatments in HCC.

MATERIALS AND METHODS

Clinical Samples

All fresh HCC patient tumor samples and adjacent tissues were collected from The First Affiliated Hospital of University of Science and Technology of China, which was approved by the Human Research Ethics Committee of University of Science and Technology of China (USTCEC201700007). Written informed consent was obtained from each patient for this study. All samples were rinsed with DEPC water and then kept in RNAhold (TransGen) within 30 min after removing from the operation. HCC patient tumor sample and adjacent tissue pairs were collected from 21 patients (12 males and 9 females with advanced stage HCC, all of them were HBsAg positive, and did not have anti-tumor therapy before surgery).

Total RNA Extraction

The clinical samples were cut into small pieces and homogenized in TRIzol reagent (Life Technologies) by homogenizer. Total RNA was extracted by using TRIzol reagent according to the manufacturer's instructions.

Transcriptome Data Analysis

Total RNAs from four pairs of HCC patient tumor tissues were extracted for high-throughput sequencing. Whole transcriptome libraries were constructed by the TruSeq Ribo Profile Library Prep Kit (Illumina, United States), according to the manufacturer's instructions. In brief, 10 µg total RNA was depleted rRNA with an Illumina Ribo-Zero Gold kit and purified for end repair and 5'-adaptor ligation. Then, reverse transcription was performed with random primers containing 3' adaptor sequences and randomized hexamers. Finally, the cDNAs were purified and amplified with thermo cycler. The PCR products of 300–500 bp were purified, quantified and stored at -80°C before sequencing. The libraries were subjected to 151 nt paired-end sequencing with an Illumina Nextseq 500 system (Novogene, China). Each library was generated a depth of 50–100 million read pairs and then adapters were removed with cutadapt to obtain clean reads. For mRNA and lncRNA analyses, the expression levels were calculated using TopHat2 and Cufflinks followed by the annotation references of Refseq and Ensemble transcript databases with the genome release Homo sapiens, hg19. The differentially expressed mRNA and lncRNA were determined by DESeq2 with the corresponding cutoff ($P < 0.001$, $\text{RPKM} \geq 10$, $|\log_2(\text{fold change})| \geq 1$ for mRNA and $P < 0.05$, $\text{RPKM} \geq 1$, $|\log_2(\text{fold change})| \geq 1$ for lncRNA). For circular RNA (circRNA) prediction, we identified the candidates with find_circ (Memczak et al., 2013) and the junction reads were calculated as Transcripts Per Kilobase Million (TPM). A criterion of $P \leq 0.05$, $\text{TPM} \geq 0.1$ and $|\log_2(\text{fold change})| \geq 1$ among four pairs of samples was used to identify differentially expressed

TABLE 1 | Primers for RT-qPCR validation.

Name	Primer sequence 5'–3'	Product size (bp)
C12orf75	F: GCAGGAGCAGCCAAAGATGT R: CTGGACACCATATTGACAGC	113
ZIC5	F: AGCCCTTCAAGGCCAAATAC R: GCTTGTGGATCTTGAGGTTT	129
PZP	F: TCAAGCTAGAAGCTGGCATC R: AAGCACAAATTCCTCCACGG	137
FAM65C	F: TGACATCGCCGACTTCTTCA R: TACAAGGAGCCCTTCCTGCT	177
C1QL1	F: CACCTACCATGTCTCATGC R: GCGTAGTCGTAGTTCTGGTC	120
TMEM74	F: GGAGCTCGCCATCAGCAAAA R: TTCCTGAAGACGTGGCTTC	142
GNAZ	F: CATCGCCGAGCTGACTATA R: CCACGTCCACCATCTTGAAG	132
LNC-TOB2P1	F: GTGACTACAGGAAGGTTAGC R: GAGAAGAGGCTGCATGAGAG	161
LOC100499489	F: TAGAGATGAGACGATGCAGG R: CAGCTCCACCCACGTGGTTT	110
LINC01093	F: AAGAGACTGGAGCCCTGAGT R: GCTGTAACCCCAAAACTCC	92
LOC100130899	F: GAGGTTTGTGCAATCAGG R: ACATACTCACGGCTGTGCCT	101
LOC200772	F: GAGGTTTGTGCAATCAGG R: ACATACTCACGGCTGTGCCT	135
LNC-FENDRR	F: AGGACCATTGAGCTCTAGAG R: ATGGGGTGAGCAAAAGCGCAT	141
LNC-NBPF22P	F: CCATGTGACCAACCAAGAACA R: CCATGTGACCAACCATCAATG	152
LNC-NMRAL2p	F: TGCAACACCGAAGGATTTCC R: CAAGTTCGAGGCTTCTCAT	148
ACTB	F: CCAACACAGTGCTGTCTGG R: GAGTACTTGCGCTCAGGAG	130
PLOD3	F: GTTTGTGGATAGCTACGACG R: CCACCTCTGGACGAACCTCTT	72
SF3B4	F: GGACCCTGAGATTGATGAGA R: GTCAGGGTCCCGCATAATTT	91
SNHG7	F: TGCTCACTGGAGATGACACG R: CACTGGAAGTCCATCACAGG	92
HULC	F: AGGAAGAGTCGTCACGAGAA R: TATTCGGCCTTTACTTCAG	124
ADH4	F: ATCAACAATGCCAAGGTCAC R: CCTATGATTCTGGAAGCTCC	113
COLEC10	F: GGCAATATTGGCAAGACTGG R: TCTCCAGGTATTCCAAGCAA	77
circAKR1B10	F: CCTTGTGAGGAAAGCCTTTG R: CAGCACACTTAGAGGAAGCT	199
circAKR1C3	F: AGGATTGGCCAAGTCCATCG R: CCATCGTTTGTCTCGTTGAG	211
circHMGCS1	F: CTCGGATGTTGCTGAATGAC R: CGGTCTAATGCACTGAGGTA	197
circC3P1	F: GGTGCAAGAACAGGAATCG R: TCACATGCTGTTGCTGGAG	137
miR-10b-3p	SL: GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACATTCCC F: CACGCAACAGATTCGATT R: CCAGTGCAGGGTCCGAGGTA	
miR-421	SL GTCGTATCCAGTGCAGGGTCCGAGGT	

(Continued)

TABLE 1 | Continued

Name	Primer sequence 5'–3'	Product size (bp)
	ATTCGCACTGGATACGACGCGCCC F: CACGCAATCAACAGACATTA R: CCAGTGCAGGGTCCGAGGTA	
miR-761	SL GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTGTGTC F: CACGCAGCAGCAGGGTGAAA R: CCAGTGCAGGGTCCGAGGTA	
miR-200a-3p	SL: GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACACATCG F: CACGCATAACACTGTCTGGT R: CCAGTGCAGGGTCCGAGGTA	
miR-200b-3p	SL: GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACTCATCA F: CACGCATAACTACTGCCTGGT R: CCAGTGCAGGGTCCGAGGTA	
miR-139-5p	SL: GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACACTGGA F: CACGCATCTACAGTGCACGT R: CCAGTGCAGGGTCCGAGGTA	

SL, Stem loop RT primer.

circRNAs. RNA-seq data were deposited in NCBI with the GEO accession code GSE128274.

Small RNA Data Analysis

For small RNA (sRNA) sequencing, eight sRNA libraries were generated with TruSeq small RNA (Illumina, United States) according to the manufacturer's instructions. Then the prepared libraries were sequenced with an Illumina Nextseq 500 system (Novogene, China). After filtering out the reads shorter than 15 nt, the remaining reads were mapped to the human genome (hg19) and the miRNA database in miRBase with bowtie (-v 1). The differentially expressed miRNAs were determined by DEseq2 with the cutoff of $P < 0.05$, $TPM \geq 1$, $|\log_2(\text{fold change})| \geq 1$.

Construction of Co-expression and CeRNA Network

For the co-expression network of significantly dysregulated lncRNAs and mRNAs, Pearson's correlations were calculated the co-expression analysis according to the expression levels in eight samples. A criterion of the coefficient parameter R-squared more than 0.99 was used for the remaining RNAs to further construct the network. For the competing endogenous RNAs (ceRNA) network of significantly dysregulated circRNAs and mRNAs, the miRNA/mRNA and miRNA/circRNA interaction were predicted with TargetScanHuman7.2. The above networks were both performed with Cytoscape¹.

GO Analysis

The significantly dysregulated mRNAs in co-expression and ceRNA network were both analyzed using GOrilla web-server with default parameters (Eden et al., 2009).

¹www.cytoscape.org

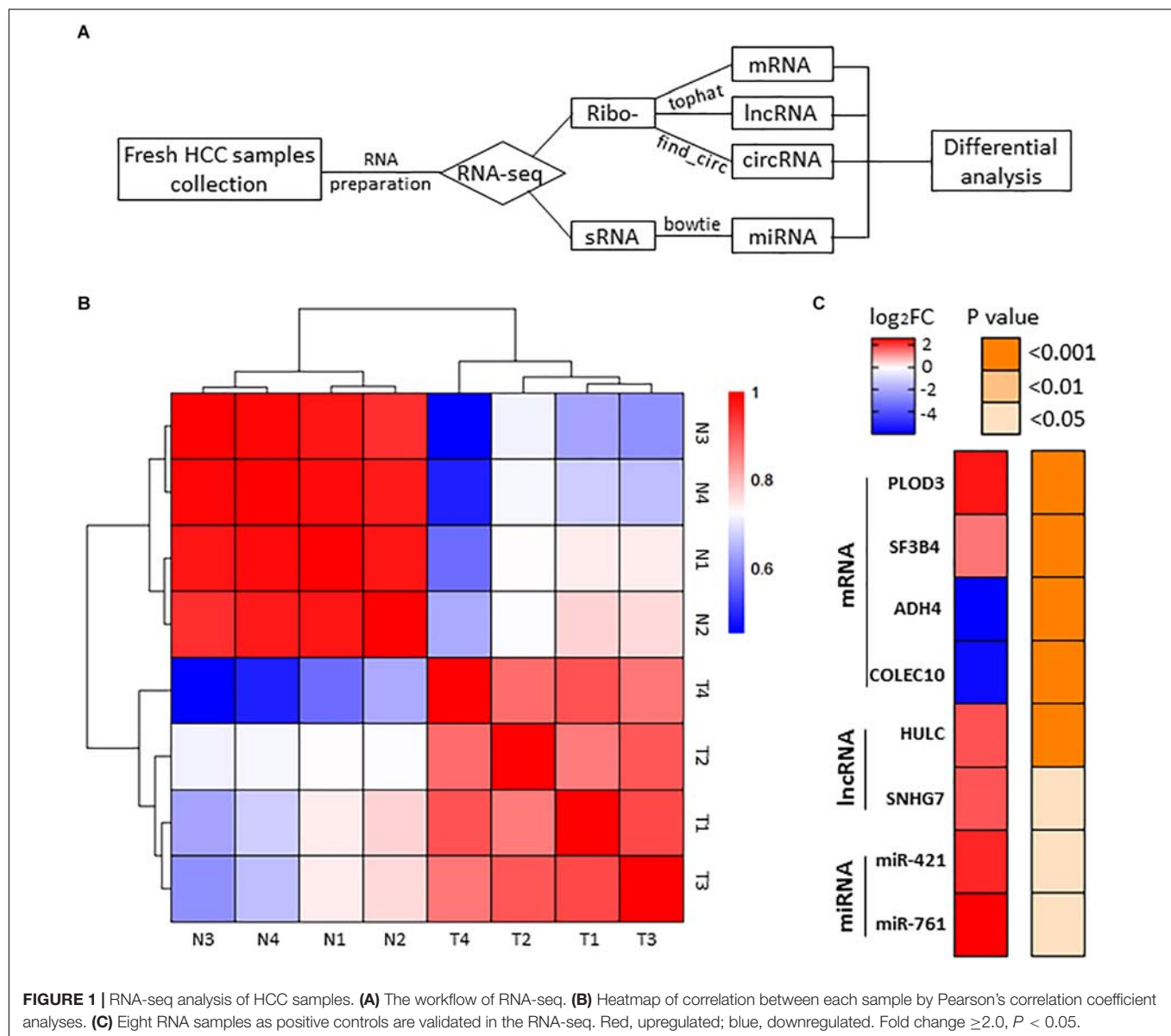


FIGURE 1 | RNA-seq analysis of HCC samples. **(A)** The workflow of RNA-seq. **(B)** Heatmap of correlation between each sample by Pearson's correlation coefficient analyses. **(C)** Eight RNA samples as positive controls are validated in the RNA-seq. Red, upregulated; blue, downregulated. Fold change ≥ 2.0 , $P < 0.05$.

Statistical Analysis

In all experiments, Student's *t*-tests were used to calculate *P*-values, as indicated in the figure legends. The values reported in the graphs represent averages of actual number of independent experiments, with error bars showing SD. After analysis of variance with *F*-tests, the statistical significance and *P*-values were evaluated with Student's *t*-tests.

Reverse Transcription and Real-Time Quantitative PCR

cDNA was prepared using GoScript Reverse Transcription System (Promega) according to the manufacturer's protocol. Notably, for the first strand cDNA synthesis of miRNA, stem-loop method was used (Kramer, 2011). Quantitative real-time PCR was performed with GoTaq SYBR Green qPCR

Master Mix (Promega) on a PikoReal 96 real-time PCR system followed by 40 amplification cycles (Thermo Fisher Scientific) according to standard procedures. Actually, all amplification curves already reached stationary stage before 35 amplification cycles, and the readings of Ct value were obtained at the exponential stage. Relative RNA expression was normalized to ACTB expression level. All primers are shown in Table 1.

RESULTS

Identification of Differentially Expressed RNAs in HCC Samples

To identify HCC-related RNAs, we used four HCC patients' fresh tumor tissues and paired adjacent non-tumor tissues for RNA sequencing. Differential expression of mRNAs, lncRNAs,

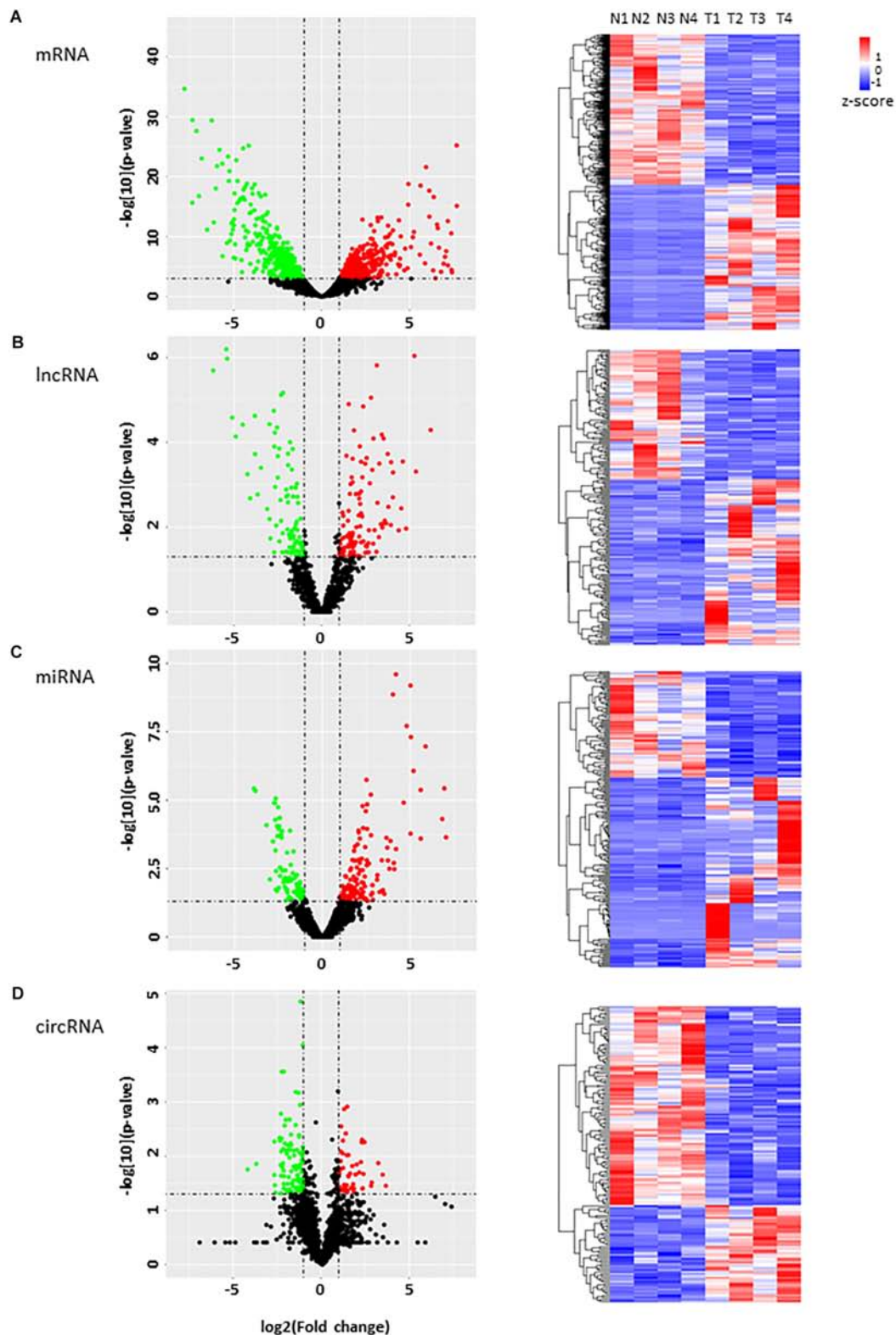


FIGURE 2 | Analysis of the RNA expression profile in HCC. **(A–D)** volcano plots (left) display differentially expressed transcripts, and the hierarchical cluster analyses on the right of each panel display differential expression of RNAs in four HCC tumor tissues and their controls. **(A)** mRNAs, **(B)** lncRNAs, **(C)** miRNAs, and **(D)** circRNA. Green and red dots represent downregulated and upregulated RNAs respectively in tumor tissues compared with adjacent tissues. Black dots indicate no significant difference.

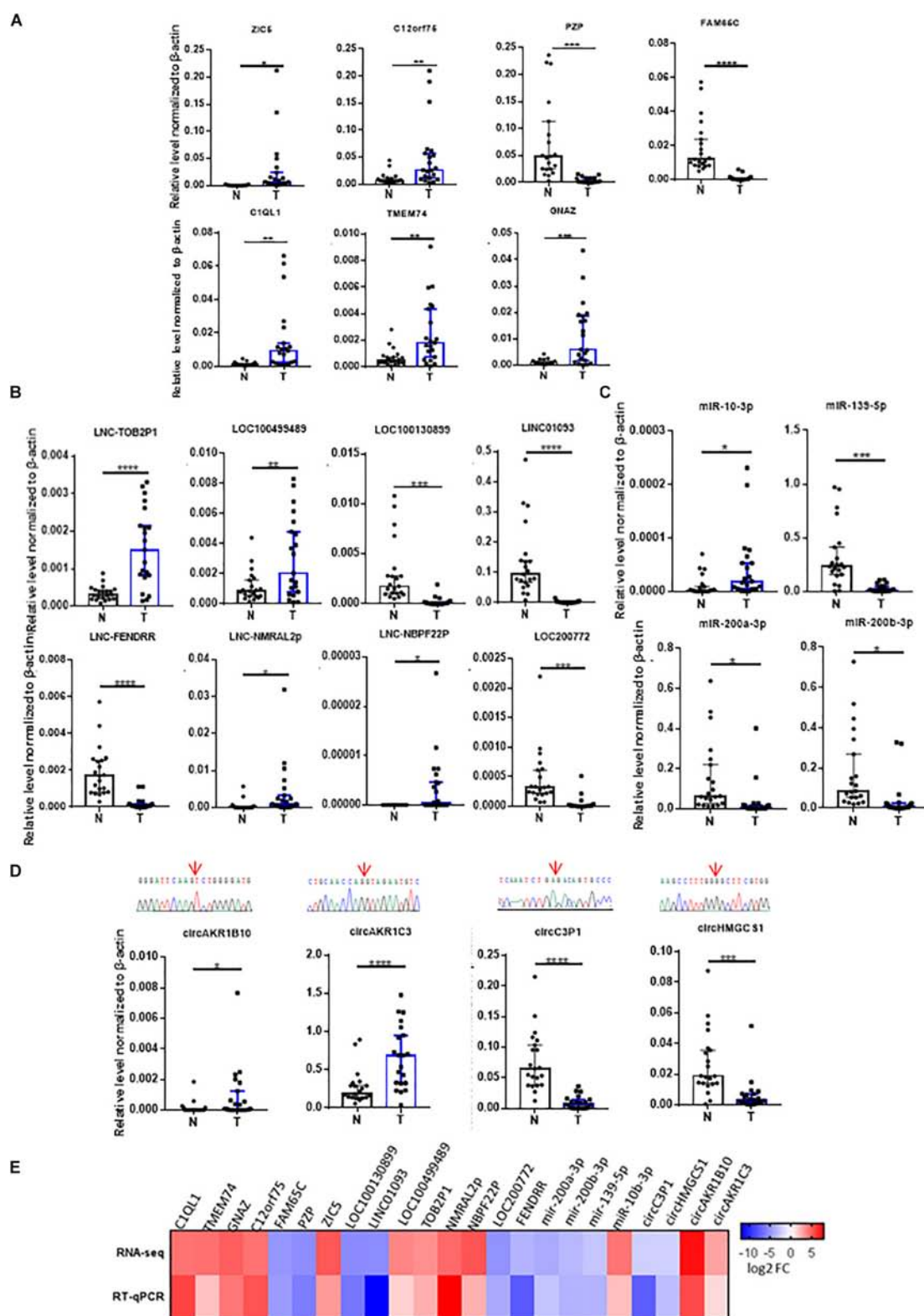


FIGURE 3 | Validation of RNAs identified from RNA-seq in 21 HCC tumor tissues by RT-qPCR. The validation results of **(A)** 7 dysregulated mRNAs, **(B)** 8 dysregulated lncRNAs, **(C)** 4 dysregulated microRNAs, and **(D)** 4 dysregulated circRNAs, red arrows above indicate junctions of circRNAs from the sequencing results. **(E)** The heatmap for the comparison of the RT-qPCR and RNA-seq. N, adjacent non-tumor tissues. T, tumor tissues. Data are median $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ are based on the Student's t -test.

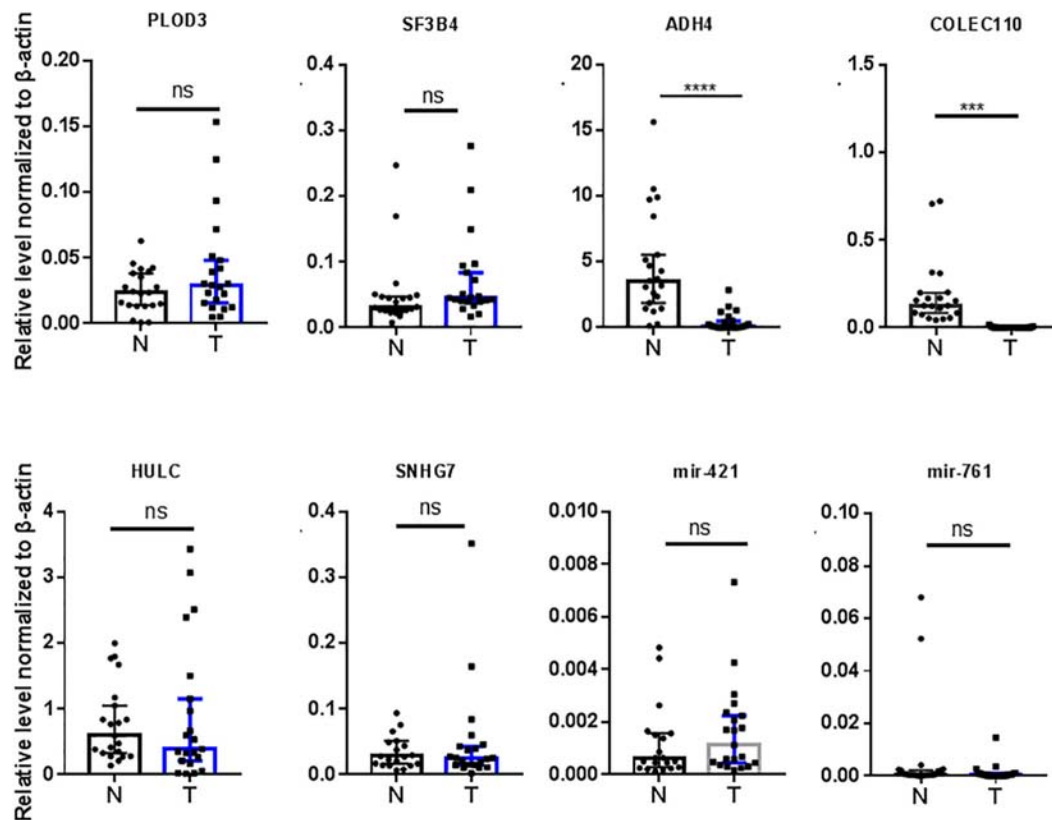


FIGURE 4 | Validation of RNAs previously reported by other studies in 21 HCC tumor tissues by RT-qPCR. N, adjacent non-tumor tissues. T, tumor tissues. Data are median $\pm$ SD. ns, not significant, *** $p < 0.001$, **** $p < 0.0001$ are based on the Student's t -test.

circRNAs, and miRNAs were then analyzed (Figure 1A). Pearson's correlation coefficient analysis showed that tumor tissues were positively correlated with each other, and control tissues also showed strong positive correlation (Figure 1B). It seems evident that the 4 tumor samples differ more from normal samples than among themselves, maybe due to the tumor heterogeneity. We noticed that previously reported transcripts with aberrant levels in HCC such as mRNAs (PLOD3, SF3B4, ADH4, and COLEC10), lncRNAs (HULC and SNHG7), and miRNAs (miR-421 and miR-761) were also identified in our RNA-seq (Figure 1C; Panzitt et al., 2007; Wang et al., 2010; Wei et al., 2012; Zhang et al., 2012; Li G. et al., 2015; Zhou et al., 2016; Shen et al., 2018; Zhang and Wu, 2018).

Next, we performed volcano plots for mRNAs, lncRNAs, miRNAs and circRNAs in HCC paired tissues (fold change ≥ 2.0 , $P < 0.05$) (Figures 2A–D). 919 differentially expressed mRNAs, 207 lncRNAs, 216 miRNAs, and 152 circRNAs were identified (Figures 2A–D). Among them, 452 mRNAs (49.18%), 116 lncRNAs (50.04%), 138 miRNAs (63.89%), and 50 circRNAs (32.89%) were upregulated, while 467 mRNAs (50.82%), 91 lncRNAs (43.96%), 78 miRNAs (36.11%), and 102 circRNAs (67.11%) were downregulated (Figures 2A–D). We also conducted a hierarchical cluster analysis to display the differential expression of four types of RNAs across eight samples (Figures 2A–D). Tumor and adjacent

non-tumor samples respectively were classified into different branch (Figures 2A–D).

RT-qPCR Verification of the Dysregulated RNAs in Clinical Samples

To validate the results of the RNA-seq, we selected 23 dysregulated RNA candidates including mRNAs, lncRNAs, miRNAs and circRNAs for RT-qPCR validation with 21 HCC patient sample pairs (Figures 3A–D). Housekeeping gene ACTB was used as the endogenous control. In the mRNA group, ZIC5, C12orf75, C1QL1, TMEM74, and GNAZ were significantly upregulated in tumor tissues compared to the adjacent control; PZP and FAM65C were significantly downregulated compared to the adjacent control (Figure 3A). In the lncRNA group, lncRNA-TOB2P1, LOC100499489, lnc-NMRAL2p, and lnc-NBPF22P were markedly upregulated in tumor tissues, while LINC01093, LOC100130899, LOC200772, and lnc-FENDRR were significantly downregulated in tumor tissues, compared to the adjacent controls (Figure 3B). In the miRNA group, miR-10-3p was significantly upregulated in tumor tissues, while miR-200a-3p, miR-200b-3p, and miR-139-5p were significantly downregulated in tumor tissues (Figure 3C). In the circRNA group, circAKR1B10 and circAKR1C3 were significantly upregulated in tumor tissues, while circHMGCS1 and circC3P1

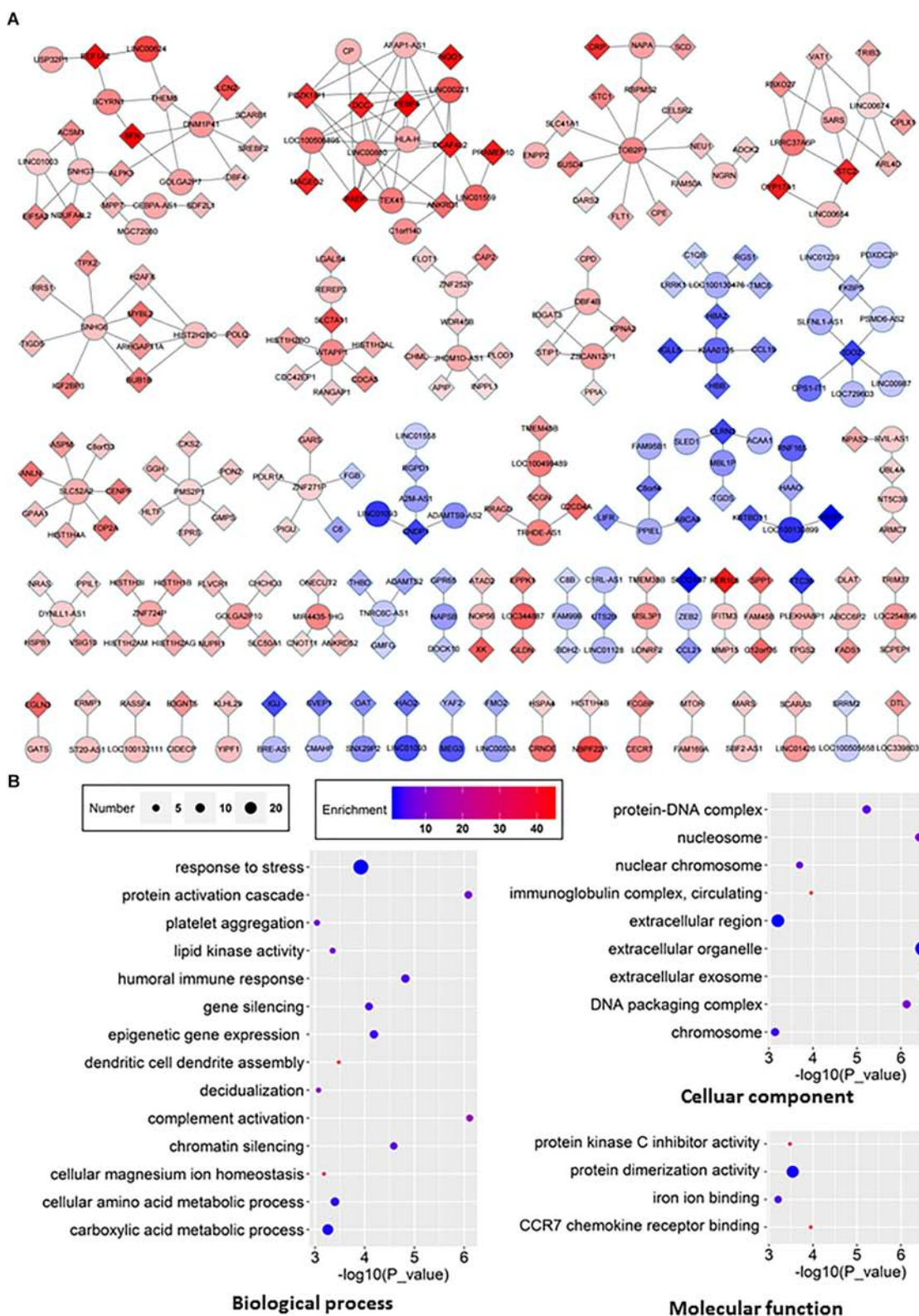
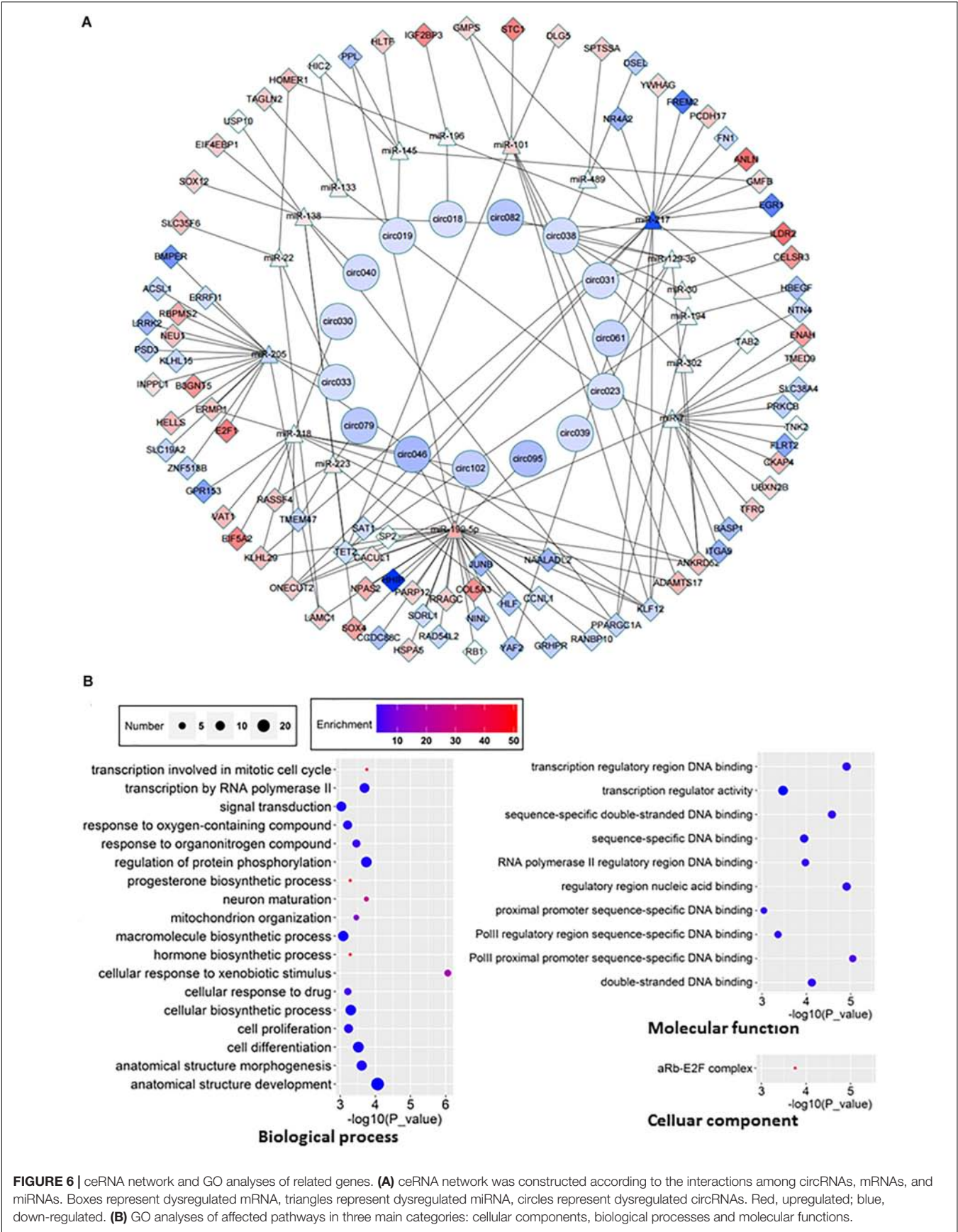


FIGURE 5 | The lncRNA-mRNA network in HCC and GO analyses of dysregulated genes. **(A)** The co-expression network between differentially expressed lncRNA and mRNA (correlation coefficient absolute value ≥ 0.99). The boxes represent mRNA, the circles represent lncRNA. Red, upregulated; blue, downregulated. **(B)** GO analyses and annotation of dysregulated genes in three main categories: biological processes, cellular components and molecular functions.



were markedly downregulated, compared to the adjacent controls (**Figure 3D**). For these 23 RNAs examined with 21 pairs of patient samples, the RT-qPCR verification was in accordance with RNA-seq results with high confidence (**Figure 3E**).

We also validated those transcripts with aberrant levels in HCC such as mRNAs previously identified by other studies (**Figure 1C**), with the same 21 HCC patient sample pairs with RT-qPCR (**Figure 4**). Among the eight transcripts (PLOC3, SF3B4, ADH4, COLEC10, HULC, SNHG7, miR-421, and miR-761), only two (ADH4 and COLEC10) transcripts were in accordance with RNA-seq results and previous reports (**Figure 4**; Wei et al., 2012; Zhang and Wu, 2018).

DISCUSSION

Co-expression Network of lncRNAs/mRNAs and GO Analysis

We then set out to investigate the identified lncRNAs associated with HCC. lncRNA functions can be predicted based on the functions of their co-expressed protein-coding genes, and alterations in the associations between these genes in clinical samples can be used to identify key lncRNAs in HCC (Cabili et al., 2011; Liao et al., 2011; Guo et al., 2013). We constructed a co-expression network of lncRNAs and co-expressed mRNAs based on the RNA-seq data to investigate their interactions (**Figure 5A**). Our analysis demonstrated that 98 lncRNAs interacted with 175 mRNAs (**Figure 5A**). The results of GO pathway analyses of the differentially RNAs showed that most co-expressed lncRNAs were closely related to several important pathways, including biological processes such as gene silencing, chromatin silencing, and response to stress, cellular components such as extracellular region, and molecular functions such as protein dimerization activity (**Figure 5B**).

Construction of CeRNA Network

One of molecular functions of circRNAs is ceRNA (Hansen et al., 2013; Memczak et al., 2013; Piwecka et al., 2017). In order to investigate the potential circRNAs acting as ceRNAs in HCC through regulating miRNAs and consequently modulating mRNAs, we constructed a ceRNA network among differentially expressed circRNAs, miRNAs, and mRNAs in HCC (**Figure 6A**). 15 circRNAs (**Table 2**), 17 miRNAs, and 89 mRNAs were found to be correlated in this ceRNA network. The results of GO pathway analyses showed that mRNAs in this network were correlated to regulations of biological processes such as protein phosphorylation, signal transduction, and cell proliferation, molecular functions such as transcription regulator activity and double-stranded DNA binding, and cellular components such as Rb-E2F complex (**Figure 6B**).

Survival Curves of Identified Genes

To explore the relationship between our identified targets and clinical observations, we then examined with survival curves in online database, UALCAN analysis². Total of 9 mRNAs, 7 of them

TABLE 2 | Information of circRNAs in **Figure 5A**.

circRNA	Genomic position	Gene symbol	circRNA ID
circ018	chr2 120885263 120932580	GLI2	Novel
circ019	chr13 33109905 33111164	N4BP2L2	hsa_circ_0100273
circ023	chr8 18725183 18730243	PSD3	hsa_circ_0136098
circ030	chr16 18852886 18856973	SMG1	hsa_circ_0006434
circ031	chr10 13322975 13337606	PHYH	hsa_circ_0093118
circ033	chr5 139819703 139828890	ANKHD1	hsa_circ_0001541
circ038	chr21 16386664 16415895	NRIP1	hsa_circ_0004771
circ039	chr12 12672795 12674397	DUSP16	hsa_circ_0003855
circ040	chr7 70227855 70236630	AUTS2	hsa_circ_0080420
circ046	chr6 161006077 161012133	LPA	hsa_circ_0131238
circ061	chr19 41382431 41414558	CYP2A7	Novel
circ079	chr5 78324255 78329231	DMGDH	hsa_circ_0129713
circ082	chr9 136302868 136303486	ADAMTS13	hsa_circ_0089372
circ095	chr6 161010584 161016567	LPA	Novel
circ0102	chr6 161006077 161016567	LPA	Novel

identified in our RNA-seq and qRT-PCR verification and 2 of them previously identified and further verified by this study, were analyzed with survival curves. 6 (ZIC5, C12orf75, PZP, FAM65C, ADH4, and COLEC110) out of the 9 mRNAs were correlated to survival curves with significance in HCC (**Figure 7**). Those genes we found upregulated in HCC (ZIC5, C12orf75) were positively correlated with survival curves (patients with higher expression levels in HCC survive shorter). Genes downregulated in HCC (PZP, FAM65C, ADH4, and COLEC110) were negatively correlated with survival curves (patients with higher expression levels in HCC survive longer). For other identified genes including significantly upregulated/downregulated mRNAs, lncRNAs, miRNAs, and circular RNAs, the UALCAN database does not have HCC-relevant information about them.

The incidence of HCC has been increasing, and the consequent mortality is also rising for the past decades (Forner et al., 2018). For the early-stage patients, it is amenable to potentially curative treatments such as resection, liver transplantation, image-guided tumor ablation and systemic therapy, which can increase the 5 years survival rates to 60–70% (Llovet and Bruix, 2008; Yu, 2016). However, patients who are diagnosed at the advanced stage of HCC are treated poorly due to lack of effective treatment options for potential liver disease. Early diagnosis and effective surveillance are required for the treatment of HCC patients to reduce the disease-related mortality. Future diagnosis and treatments call for novel HCC biomarkers and potential targets.

RNA biomarkers, as measurable clinical indicators, can be used to predict and detect some diseases state and symptoms outside the body of patient with unique advances. To provide effective treatment for HCC patients and insights for future diagnosis, several potential RNA biomarkers for HCC have also been investigated (Klingenberg et al., 2017). Accumulating evidences have shown that lncRNA and miRNAs are suitable potential markers for HCC (Xie et al., 2016; Birgani et al., 2018). It has been reported that more than half of the miRNAs genes are located in cancer-associated genomic regions or in fragile sites

²<http://ualcan.path.uab.edu/analysis.html>

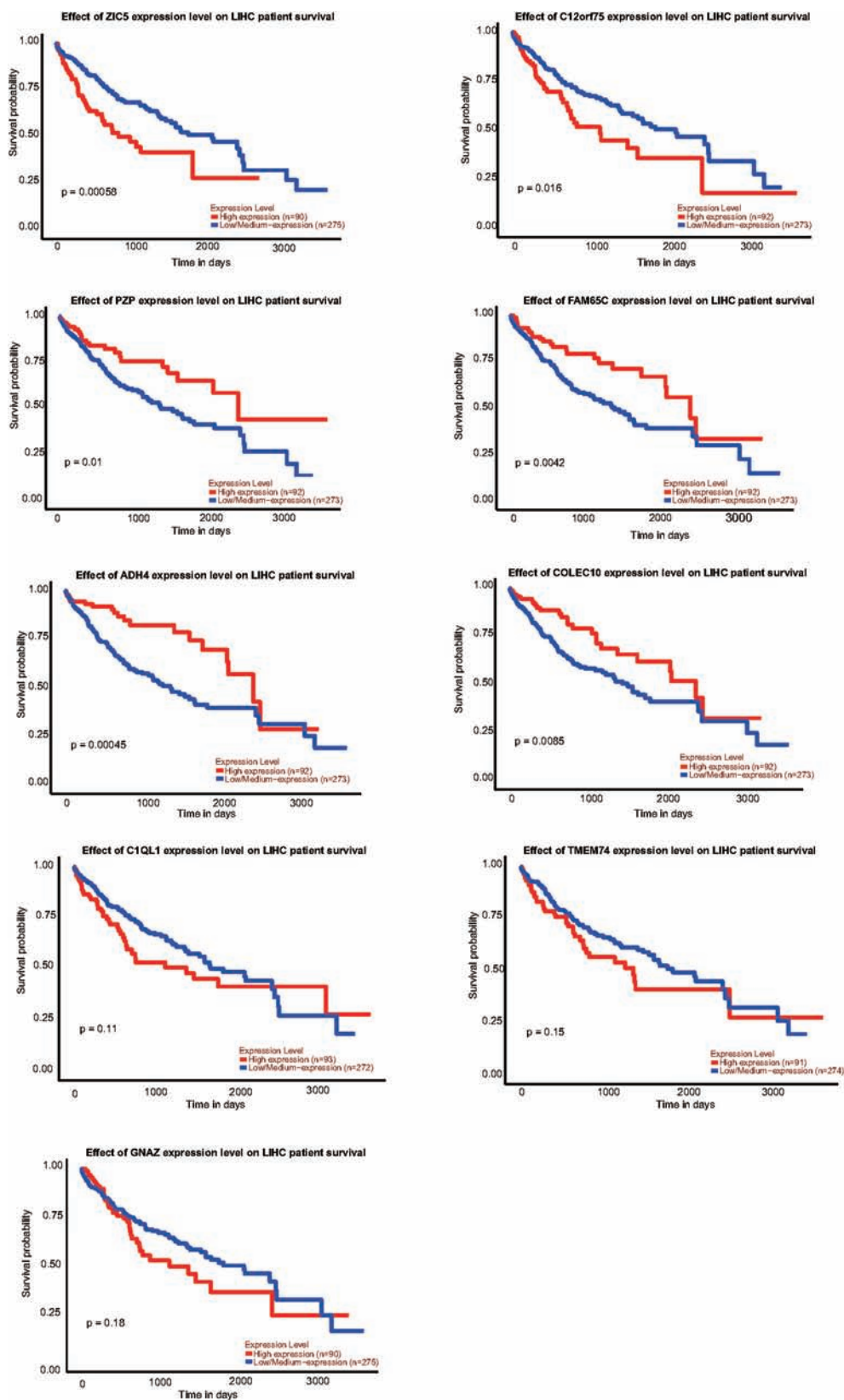


FIGURE 7 | Survival curves of identified genes analyzed by UALCAN analysis; p -value was calculated by log-rank test.

(Greene et al., 2017). Aberrant expression of lncRNAs, miRNAs, and circRNAs along with mRNAs may directly or indirectly lead to the progression of cancers due to their massive involvement (Ryan et al., 2010; Cheetham et al., 2013; Guarnerio et al., 2016).

RNA-seq is a powerful tool to study and detect the global transcriptome in tissues and cells (Sharma et al., 2010; Adey et al., 2013). In this study, we have identified 919 differentially expressed mRNA, 207 lncRNAs, 216 miRNAs, and 152 circRNAs in HCC through RNA-seq, these dysregulated RNAs, especially those validated with 21 patient HCC samples can be highlighted as potential biomarkers or therapeutic targets for HCC (Figure 3).

Although increasing pieces of evidence have demonstrated the role of aberrant expression of mRNA, miRNA, lncRNA, and circRNA in HCC, not many studies have systematically investigated the crosstalk among transcripts in this context. The co-expression network between lncRNA and mRNA (Figure 5A) and the ceRNA network of differential expression circRNA-miRNA-mRNA (Figure 6A) in our study provide insights for further investigation. Of course, both networks have their own limitation. For example, circRNAs can function not only as ceRNAs but also as transcriptional regulators (Li Z. et al., 2015; Hu and Zhou, 2018). Another interesting point is that circRNAs seem to be more often downregulated in tumor tissues as shown in this study as well as several other studies (Figure 2D; Zheng et al., 2016; Greene et al., 2017).

We examined the 8 RNAs reported in other studies, however only two were found consistent with our RT-qPCR results from 21 patients (Figure 4). The inconsistency may be due to the fact that all patients in this study were HBsAg positive with advanced stage HCC, and our patient cohort may be distinct from previous studies. We did not have an opportunity to investigate the potential exposure of the main HCC carcinogen aflatoxin of these patients, which may be a weakness of this study. Another limitation of the present study is that, just like most Chinese HCC patients, all patients in this study are already in the advanced stage upon their first diagnosis, due to limited coverage of preclinic screening. We also explored the correlation of the RNAs identified in our study with patient survival curves. 6 out of the 9 mRNAs were correlated to survival curves of HCC, indicating multiple transcripts identified in this study may play critical roles in the tumorigenesis and advance of HCC (Figure 7).

CONCLUSION

In conclusion, we have provided a comprehensive identification and analyses of the differentially expressed mRNAs,

miRNAs, lncRNAs, and circRNAs using RNA-seq, and some of these transcripts have been verified with clinic HCC samples. Functional network of lncRNA-mRNA and circRNA-miRNA-mRNA ceRNA network have been systematically established to further indicate potential interactions in HCC. GO pathway analyses also facilitate future studies on the specific mechanisms of HCC. We expect this work will serve as a valuable resource in future clinical diagnosis and therapy of HCC.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Human Research Ethics Committee of University of Science and Technology of China (USTCEC201700007) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Human Research Ethics Committee of University of Science and Technology of China (USTCEC201700007).

AUTHOR CONTRIBUTIONS

GX, GS, and LC designed and initiated this project. GS provided the major funding. GS and LC supervised the experiments. ZS and XW performed all the experiments. ZS, XW, LC, and GS analyzed the data and wrote the manuscript. All authors have discussed the results and made comments on the experiment.

FUNDING

This work was supported by the National Basic Research Program of China (2015CB943000), the National Key R&D Program of China (2018YFC1004500), the National Natural Science Foundation of China (31725016 and 31600657), and the Strategic Priority Research Program (Pilot study) “Biological basis of aging and therapeutic strategies” of the Chinese Academy of Sciences (XDPB10).

ACKNOWLEDGMENTS

We thank the Bioinformatics Center of the USTC, School of Life Sciences, for providing supercomputing resources.

REFERENCES

- Adams, J. M., and Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26, 1324–1337. doi: 10.1038/sj.onc.1210220
- Adey, A., Burton, J. N., Kitzman, J. O., Hiatt, J. B., Lewis, A. P., Martin, B. K., et al. (2013). The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. *Nature* 500, 207–211. doi: 10.1038/nature12064
- Aguirre-Ghiso, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. *Nat. Rev. Cancer* 7, 834–846. doi: 10.1038/nrc2256
- Amaravadi, R. K., and Thompson, C. B. (2007). The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin. Cancer Res.* 13, 7271–7279. doi: 10.1158/1078-0432.CCR-07-1595

- Azam, F., Mehta, S., and Harris, A. L. (2010). Mechanisms of resistance to antiangiogenesis therapy. *Eur. J. Cancer* 46, 1323–1332. doi: 10.1016/j.ejca.2010.02.020
- Birgani, M. T., Hajjari, M., Shahrisa, A., Khoshnevisan, A., Shoja, Z., Motahari, P., et al. (2018). Long non-coding rna snhg6 as a potential biomarker for hepatocellular carcinoma. *Pathol. Oncol. Res.* 24, 329–337. doi: 10.1007/s12253-017-0241-3
- Cabili, M. N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., et al. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* 25, 1915–1927. doi: 10.1101/gad.17446611
- Cheetham, S. W., Gruhl, F., Mattick, J. S., and Dinger, M. E. (2013). Long noncoding RNAs and the genetics of cancer. *Br. J. Cancer* 108, 2419–2425. doi: 10.1038/bjc.2013.233
- Chen, L., Dzakah, E. E., and Shan, G. (2018). Targetable long non-coding RNAs in cancer treatments. *Cancer Lett.* 418, 119–124. doi: 10.1016/j.canlet.2018.01.042
- Chen, L., Huang, C., Wang, X., and Shan, G. (2015). Circular RNAs in eukaryotic cells. *Curr. Genomics* 16, 312–318. doi: 10.2174/1389202916666150707161554
- Chen, L., and Shan, G. (2015). Circular RNAs remain peculiarly unclear in biogenesis and function. *Sci. China Life Sci.* 58, 616–618. doi: 10.1007/s11427-015-4855-y
- Cui, H., Zhang, Y., Zhang, Q., Chen, W., Zhao, H., and Liang, J. A. (2017). comprehensive genome-wide analysis of long noncoding RNA expression profile in hepatocellular carcinoma. *Cancer Med.* 6, 2932–2941. doi: 10.1002/cam4.1180
- Eden, E., Navon, R., Steinfeld, I., Lipson, D., and Yakhini, Z. (2009). GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10:48. doi: 10.1186/1471-2105-10-48
- Forner, A., Reig, M., and Bruix, J. (2018). Hepatocellular carcinoma. *Lancet* 391, 1301–1314. doi: 10.1016/S0140-6736(18)30010-2
- Greene, J., Baird, A. M., Brady, L., Lim, M., Gray, S. G., McDermott, R., et al. (2017). Circular RNAs: biogenesis, function and role in human diseases. *Front. Mol. Biosci.* 4:38. doi: 10.3389/fmolb.2017.00038
- Guarnerio, J., Bezzi, M., Jeong, J. C., Paffenholz, S. V., Berry, K., Naldini, M. M., et al. (2016). Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. *Cell* 165, 289–302. doi: 10.1016/j.cell.2016.07.035
- Guo, X., Gao, L., Liao, Q., Xiao, H., Ma, X., Yang, X., et al. (2013). Long non-coding RNAs function annotation: a global prediction method based on bi-colored networks. *Nucleic Acids Res.* 41:e35. doi: 10.1093/nar/gks967
- Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013
- Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993
- Haque, S., and Harries, L. W. (2017). Circular RNAs (circRNAs) in health and disease. *Genes* 8:E353. doi: 10.3390/genes8120353
- Holdt, L. M., Stahringer, A., Sass, K., Pichler, G., Kulak, N. A., Wilfert, W., et al. (2016). Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. *Nat. Commun.* 7:12429. doi: 10.1038/ncomms12429
- Hu, Q., and Zhou, T. (2018). ElciRNA-mediated gene expression: tunability and bimodality. *FEBS Lett.* 592, 3460–3471. doi: 10.1002/1873-3468.13253
- Iyer, M. K., Niknafs, Y. S., Malik, R., Singhal, U., Sahu, A., Hosono, Y., et al. (2015). The landscape of long non coding RNAs in the human transcriptome. *Nat. Genet.* 47, 199–208. doi: 10.1038/ng.3192
- Karreth, F. A., Reschke, M., Ruocco, A., Ng, C., Chapuy, B., Léopold, V., et al. (2015). The BRAF pseudogene functions as a competitive endogenous RNA and induces lymphoma in vivo. *Cell* 161, 319–332. doi: 10.1016/j.cell.2015.02.043
- Klingenberg, M., Matsuda, A., Diederichs, S., and Patel, T. (2017). Non-coding RNA in hepatocellular carcinoma: mechanisms, biomarkers and therapeutic targets. *J. Hepatol.* 67, 603–618. doi: 10.1016/j.jhep.2017.04.009
- Kramer, M. F. (2011). Stem-loop RT-qPCR for miRNAs. *Curr. Protoc. Mol. Biol.* 95:15. doi: 10.1002/0471142727.mb1510s95
- Kung, J. T., Colognori, D., and Lee, J. T. (2013). Long noncoding RNAs: past, present, and future. *Genetics* 193, 651–669. doi: 10.1534/genetics.112.146704
- Lee, S. S., Shin, H. S., Kim, H. J., Lee, S. J., Lee, H. S., Hyun, K. H., et al. (2012). Analysis of prognostic factors and 5-year survival rate in patients with hepatocellular carcinoma: a single-center experience. *Korean J. Hepatol.* 18, 48–55. doi: 10.3350/kjhep.2012.18.1.48
- Li, G., Yang, F., Xu, H., Yue, Z., Fang, X., and Liu, J. (2015). MicroRNA-708 is downregulated in hepatocellular carcinoma and suppresses tumor invasion and migration. *Biomed. Pharmacother.* 73, 154–159. doi: 10.1016/j.biopha.2015.05.010
- Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., et al. (2015). Exon-intron circular RNAs regulate transcription in the nucleus. *Nat. Struct. Mol. Biol.* 22, 256–264. doi: 10.1038/nsmb.2959
- Liao, Q., Liu, C., Yuan, X., Kang, S., Miao, R., Xiao, H., et al. (2011). Large-scale prediction of long non-coding RNA functions in a coding–non-coding gene co-expression network. *Nucleic Acids Res.* 39, 3864–3878. doi: 10.1093/nar/gkq1348
- Llovet, J. M., and Bruix, J. (2003). Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. *Hepatology* 37, 429–442. doi: 10.1053/jhep.2003.50047
- Llovet, J. M., and Bruix, J. (2008). Novel advancements in the management of hepatocellular carcinoma in 2008. *J. Hepatol.* 48, S20–S37. doi: 10.1016/j.jhep.2008.01.022
- Llovet, J. M., Burroughs, A., and Bruix, J. (2003). Hepatocellular carcinoma. *Lancet* 362, 1907–1917. doi: 10.1016/S0140-6736(03)14964-1
- Marquardt, J. U., Galle, P. R., and Teufel, A. (2012). Molecular diagnosis and therapy of hepatocellular carcinoma (HCC): an emerging field for advanced technologies. *J. Hepatol.* 56, 267–275. doi: 10.1016/j.jhep.2011.07.007
- McGlynn, K. A., and London, W. T. (2011). The global epidemiology of hepatocellular carcinoma: present and future. *Clin. Liver Dis.* 15, 223–243. doi: 10.1016/j.cld.2011.03.006
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495:333. doi: 10.1038/nature11928
- Palazzo, A. F., and Lee, E. S. (2015). Non-coding RNA: what is functional and what is junk? *Front Genet.* 6:2. doi: 10.3389/fgenet.2015.00002
- Panzitt, K., Tschernatsch, M. M., Guelly, C., Moustafa, T., Stradner, M., Strohmaier, H. M., et al. (2007). Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA. *Gastroenterology* 132, 330–342. doi: 10.1053/j.gastro.2006.08.026
- Piwecka, M., Glazar, P., Hernandez-Miranda, L. R., Memczak, S., Wolf, S. A., Rybak-Wolf, A., et al. (2017). Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science* 357:eaam8526. doi: 10.1126/science.aam8526
- Prensner, J. R., Iyer, M. K., Balbin, O. A., Dhanasekaran, S. M., Cao, Q., Brenner, J. C., et al. (2011). Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat. Biotech.* 29, 742–749. doi: 10.1038/nbt.1914
- Rusk, N. (2015). Understanding noncoding RNAs. *Nat. Methods* 12:35. doi: 10.1038/nmeth.3235
- Ryan, B. M., Robles, A. I., and Harris, C. C. (2010). Genetic variation in microRNA networks: the implications for cancer research. *Nat. Rev. Cancer* 10, 389–402. doi: 10.1038/nrc2867
- Sharma, C. M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiß, S., Sittka, A., et al. (2010). The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* 464:250. doi: 10.1038/nature08756
- Shen, Q., Eun, J. W., Lee, K., Kim, H. S., Yang, H. D., Kim, S. Y., et al. (2018). Barrier to autointegration factor 1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3, and splicing factor 3b subunit 4 as early-stage cancer decision markers and drivers of hepatocellular carcinoma. *Hepatology* 67, 1360–1377. doi: 10.1002/hep.29606
- Wang, J., Liu, X., Wu, H., Ni, P., Gu, Z., Qiao, Y., et al. (2010). CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. *Nucleic Acids Res.* 38, 5366–5383. doi: 10.1093/nar/gkq285
- Wei, R. R., Zhang, M. Y., Rao, H. L., Pu, H. Y., Zhang, H. Z., and Wang, H. Y. (2012). Identification of ADH4 as a novel and potential prognostic marker in hepatocellular carcinoma. *Med. Oncol.* 29, 2737–2743. doi: 10.1007/s12032-011-0126-3
- Xie, Q. Y., Almudevar, A., Whitney-Miller, C. L., Barry, C. T., and McCall, M. N. (2016). A microRNA biomarker of hepatocellular carcinoma recurrence

- following liver transplantation accounting for within-patient heterogeneity. *BMC Med. Genomics* 9:18. doi: 10.1186/s12920-016-0179-4
- Yu, S. J. (2016). A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010–2016. *Clin. Mol. Hepatol.* 22, 7–17. doi: 10.3350/cmh.2016.22.1.7
- Zhang, B., and Wu, H. (2018). Decreased expression of COLEC10 predicts poor overall survival in patients with hepatocellular carcinoma. *Cancer Manag. Res.* 10, 2369–2375. doi: 10.2147/CMAR.S161210
- Zhang, Y., Gong, W., Dai, S., Huang, G., Shen, X., Gao, M., et al. (2012). Downregulation of human farnesoid X receptor by miR-421 promotes proliferation and migration of hepatocellular carcinoma cells. *Mol. Cancer Res.* 10, 516–522. doi: 10.1158/1541-7786.MCR-11-0473
- Zheng, Q., Bao, C., Guo, W., Li, S., Chen, J., Chen, B., et al. (2016). Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat. Commun.* 7:11215. doi: 10.1038/ncomms11215
- Zhou, X., Zhang, L., Zheng, B., Yan, Y., Zhang, Y., Xie, H., et al. (2016). Micro RNA-761 is upregulated in hepatocellular carcinoma and regulates tumorigenesis by targeting Mitofusin-2. *Cancer Sci.* 107, 424–432. doi: 10.1111/cas.12904

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Long Non-coding RNA in Neuronal Development and Neurological Disorders

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OPEN ACCESS

Edited by:

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Reviewed by:

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Guangzhou Women and Children
Medical Center, China
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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 14 November 2018

Accepted: 27 December 2018

Published: 23 January 2019

Citation:

Li L, Zhuang Y, Zhao X and Li X
(2019) Long Non-coding RNA
in Neuronal Development
and Neurological Disorders.
Front. Genet. 9:744.
doi: 10.3389/fgene.2018.00744

Long non-coding RNAs (lncRNAs) are transcripts which are usually more than 200 nt in length, and which do not have the protein-coding capacity. LncRNAs can be categorized based on their generation from distinct DNA elements, or derived from specific RNA processing pathways. During the past several decades, dramatic progress has been made in understanding the regulatory functions of lncRNAs in diverse biological processes, including RNA processing and editing, cell fate determination, dosage compensation, genomic imprinting and development etc. Dysregulation of lncRNAs is involved in multiple human diseases, especially neurological disorders. In this review, we summarize the recent progress made with regards to the function of lncRNAs and associated molecular mechanisms, focusing on neuronal development and neurological disorders.

Keywords: long non-coding RNA, gene expression, neuronal development, neurological disorders, mechanism

INTRODUCTION

Over the past several decades, advances in genomic sequencing technology and findings from large-scale consortia have facilitated our understanding of the complexity and flexibility of mammalian genomes. The majority of mammalian genomes are transcribed, whereas only a few transcripts encode proteins, the majority of transcripts are non-coding RNAs (ncRNAs) (Roberts et al., 2014). Based on the length of transcripts, ncRNAs are usually classified into two categories: small non-coding RNAs and long non-coding RNAs (lncRNAs). Small ncRNAs are usually <200 nucleotides, including microRNAs, Piwi-interacting RNAs and small nuclear RNAs (snoRNAs). lncRNAs are >200 nucleotides and frequently transcribed by polymerase II, and share some features, e.g., 5'-capping, 3'-polyadenylation, alternative splicing and sequence conservation with mRNA (Ponting et al., 2009; Nagano and Fraser, 2011).

Although lncRNAs generally lack protein coding capacity, spatiotemporal-specific expression patterns have highlighted the diverse functions and complicated mechanisms of lncRNAs (Cao et al., 2018). Currently, it is widely accepted that lncRNAs play an important function in a variety of biological processes, including regulating gene expression, both at the transcriptional and the post-transcriptional level, shaping the chromatin conformation and imprinting the genomic loci (Lee and Bartolomei, 2013; Chen, 2016; Cao et al., 2018), and multiple diseases such as neurological disorders, cancer, and immunological diseases (Bian and Sun, 2011; Huarte, 2015; Wan et al., 2017).

In this review, we summarize the recent progress made regarding the functions of lncRNAs, especially the functions and associated mechanisms related to neurological disorders.

CHARACTERIZATION OF LncRNA

LncRNAs are generally transcribed from various genomic contexts and tend to have fewer exons than protein-coding transcripts (Iyer et al., 2015). Although there are still many challenges in annotation and interpretation of lncRNAs, because of the lack of an unambiguous classification framework, the existing lncRNAs can be subdivided into several categories based on their positional relation to protein coding genes, DNA elements or diverse mechanisms of processing (St Laurent et al., 2015; Kopp and Mendell, 2018) (Figure 1).

Sense lncRNAs are transcribed from the sense DNA strand, and have overlapping regions with protein-coding genes, including un-spliced sense partially intronic RNAs (PINs) and spliced transcripts resembling mRNAs (St Laurent et al., 2015). Further, natural antisense transcripts (NATs) of protein-coding genes have also been identified and many NATs share some opposite strand DNA sequences with the sense transcripts (Katayama et al., 2005). Some studies also indicate that NATs have either positive or negative effects on the corresponding sense transcripts or nearby protein-coding transcripts (Faghihi et al., 2010; Modarresi et al., 2012). For example, human brain-derived neurotrophic factor antisense RNA (*BDNF-AS*) was originally identified as natural antisense transcripts of neuronal transcriptional factor *BDNF*, shares 225 complementary nucleotides with *BDNF* mRNA and regulates the expression of *BDNF* both *in vivo* and *in vitro* (Modarresi et al., 2012; Fatemi et al., 2015).

Other studies indicate that intronic regions of coding genes produce a lot of lncRNAs. These intronic lncRNAs form the largest class of lncRNAs and are expressed independently from the pre-mRNA of protein coding genes. Many intronic lncRNAs fail to be debranched after splicing and form a covalent circle without 3' linear appendages, these circular intronic ncRNAs (ciRNAs) were found to play a regulatory role on their host genes (Zhang et al., 2013). In addition, circRNAs derived from the internal exons of pre-mRNAs through back-splicing, have also been found in various cell lines and tissues (Wu H. et al., 2017). These circular ncRNAs usually present tissue- and developmental stage-specific expression, such as the intensively studied cerebellar degeneration-related protein 1 (CDRIas) (Memczak et al., 2013).

A relatively well-characterized subclass of lncRNAs is large/long intergenic or intervening non-coding RNAs (lincRNAs), and transcribed from the intergenic regions. LincRNAs have no overlapping sequences with transcripts of either protein-coding genes or other types of genes (Clark and Blackshaw, 2014). At the molecular level, most annotated lincRNAs have mRNA-like features including 5'-cap structures, 3'-poly(A) tails, exon-exon splice junctions and association with ribosomes (Cabili et al., 2011). Compared with mRNA counterparts, lincRNAs exhibit a more tissue-specific expression,

a greater nuclear localization and less evolutionary conservation (Djebali et al., 2012).

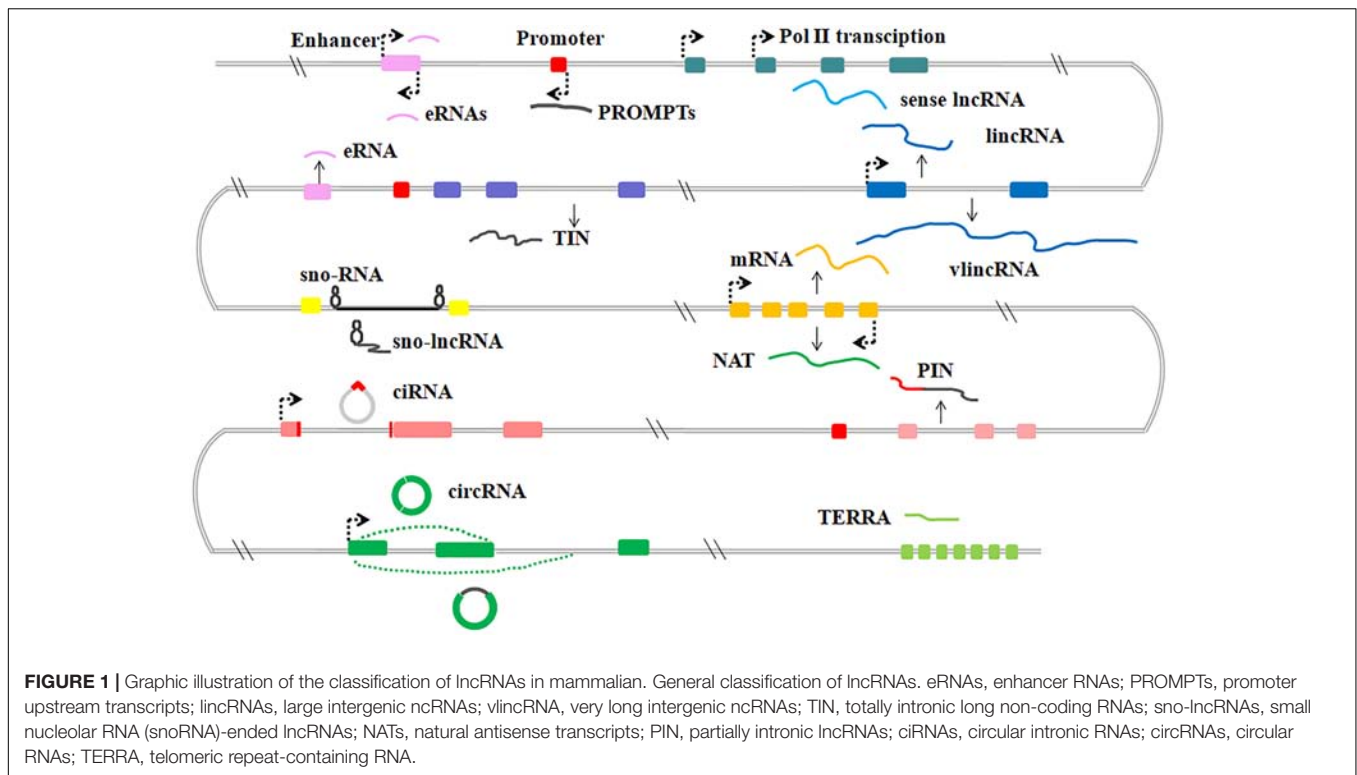
Promoter upstream transcripts (PROMPTs) localize in a fairly narrow region between ~500 and ~2500 nucleotides upstream of transcription start sites of nearby active protein-coding genes (Preker et al., 2011; Lloret-Llinares et al., 2016). It was reported that the expression levels of certain PROMPTs are altered in stress conditions, such as DNA damage responses and osmotic responses (Lloret-Llinares et al., 2016; Song et al., 2018). Enhancer-related lncRNAs (eRNAs) are bidirectional transcripts of enhancers and have enhancer-like functions. Increased binding of DNA hydroxylase Tet1 and histone methyltransferases Mll3/Mll4 and DNA hypomethylation and H3K27ac modifications at enhancers, may activate eRNAs transcription. Both PROMPTs and eRNAs are targets of the RNA exosome and display similarities during processing (Andersson et al., 2014; Wu H. et al., 2017).

Emerging evidence indicates that telomeric repeat-containing RNA (TERRA) is a heterogeneous lncRNA consisting of a combination of subtelomeric and telomeric sequences. These sequences are mostly transcribed from intrachromosomal telomeric repeats by pol II and polyadenylated at 3' region (Luke and Lingner, 2009). The length and expression level of human TERRA is influenced by the telomere length. The vast majority of mouse TERRA-binding sites were found in distal intergenic and intronic regions, where TERRA may regulate expression of target genes (Chu et al., 2017; Diman and Decottignies, 2018).

SnoRNA-ended lncRNAs (sno-lncRNAs) are transcripts of one intron flanked by two snoRNA genes that can be further processed to form snoRNA. sno-lncRNAs can be stabilized by snoRNPs formed by snoRNAs and specific protein components. *SLERT* is a representative Box H/ACA snoRNA-ended lncRNA and has been reported to be translocated to the nucleus by snoRNAs to function in pre-rRNA biogenesis (Wu H. et al., 2017).

PHYSIOLOGICAL FUNCTIONS OF LncRNA

Loss- and gain-of-function studies revealed that many lncRNAs are involved in various biological processes during development. Many lncRNAs have been found to regulate transcription via chromatin modulation, by working as molecular scaffolds for protein-protein interactions or interacting with chromatin modifying complexes and recruiting chromatin modifying complexes to specific loci, to activate or repress target gene expression. Some lncRNAs could affect transcription by modulating the binding of the general transcription machinery and regulatory factors (Wang and Chang, 2011; Fang and Fullwood, 2016; Wan et al., 2017; Lekka and Hall, 2018). Aside from modulating chromatin states, nuclear lncRNAs are involved in the RNA processing (Tripathi et al., 2010), turnover, silencing, translation and decay of mRNAs (Gong and Maquat, 2011; Carrieri et al., 2012; Geisler and Collier, 2013), or act as



miRNA decoys to neutralize miRNA-mediated mRNAs silencing and interact with signaling molecules, to modulate signaling pathways (Faghihi et al., 2010; Liu et al., 2015). In addition, some lncRNAs are determined to be precursors of certain miRNAs at particular stages of development (Dykes and Emanueli, 2017) (Table 1).

LncRNAs and Stem Cell Pluripotency and Differentiation

Accumulating evidence suggests that lncRNAs exert critical functions in pluripotency maintenance, reprogramming and lineage differentiation of stem cells (Wang and Chang, 2011; Ghosal et al., 2013). The long intergenic non-protein coding RNA regulator of reprogramming (*lincRNA-ROR*), increases the reprogramming efficiency of human induced pluripotent stem cells (iPSCs) and promotes the maintenance of embryonic stem cells (ESCs) pluripotency (Loewer et al., 2010). Similar to a miRNA sponge, *lincRNA-ROR* forms a regulatory feedback loop with miR-145 and OCT4, SOX2, and NANOG, and regulates ESC pluripotency (Wang et al., 2013). *MIAT* (myocardial infarction associated transcript) is a co-activator of Oct4 and participates in OCT4 and NANOG regulatory networks in mouse ESCs. Loss of *MIAT* reduces the expression of *Oct4*, *Sox2*, and *Klf4*, and inhibits ESCs proliferation (Sheik Mohamed et al., 2010).

LncRNAs and Development

Genomic imprinting is an important epigenetic mechanism and is crucial for normal development in mammals. It restricts gene expression on one of the two parental chromosomes in diploid

cells and affects both male and female descendants (Barlow and Bartolomei, 2014). *H19*, a maternally expressed 2.3 kb lncRNA, is generated from the highly conserved and imprinted vertebrate gene cluster insulin-like growth factor 2 (*Igf2*)/*H19*. *H19* transcripts are the precursors of miR-675-3p and miR-675-5p (Cai and Cullen, 2007). Before parturition, *H19* slows the growth of the placenta down partially, by down-regulating the RNA binding protein HuR. The decreased HuR cannot block the processing of miR-675, which further decreases the growth regulator *Igf1r* with *Igf2* as its main ligand (Keniry et al., 2012). Another two well-characterized lncRNAs that have been found to regulate genomic imprinting are *Kcnq1ot1* (*KCNQ1* opposite strand transcript 1) and *Airn* (antisense of *IGF2R* non-protein coding RNA), both are paternally expressed and regulate transcriptional silencing through a multilayered silencing pathway (Perry and Ulitsky, 2016).

Besides genomic imprinting, dosage compensation plays a vital role in equalizing the dosage of X-linked genes between males and females in heterogametic species. *Xist* is a 17 ~ 20 kb lncRNA and transcribed from the X inactivation center. During female development, *Xist* initiates X-chromosome inactivation (XCI), by progressively coating the future inactive X chromosome (Xi) and then utilizing its conserved A-repeat domain to bind PRC2, to form a transcriptionally silent nuclear compartment. The compartment is enriched by H3K27me3 and responsible for the chromosome-wide gene repression in the Xi (Chery and Larschan, 2014). *TSIX*, transcribed from the active chromosome (Xa), represses *Xist* at the early steps of X inactivation (Gendrel and Heard, 2014). Another lncRNA *JPX/ENOX*, which is transcribed from *Jpx/Enox* gene, that resides 10 kb upstream of

TABLE 1 | Diverse mechanisms of lncRNAs playing function.

Mechanism	lncRNA	Function	Relationship with target (Cis/Trans)	Reference
Chromatin remodeling	ANRIL	Repression at the CDKN2A/B locus	<i>Cis/trans</i>	Congrains et al., 2013
	BCAR4	SNIP1 and PNUTS recruitment	<i>Trans</i>	Xing Z. et al., 2014
	Braveheart	Activation of <i>MesP1</i>	<i>Trans</i>	Klattenhoff et al., 2013
	cga eRNA	Formation of looping between enhancer and promoter	<i>Cis</i>	Pnueli et al., 2015
	COOLAIR	Repression at the FLC locus	<i>Cis</i>	Marquardt et al., 2014
	FENDRR	PRC2 and TrxG/MLL complexes recruitment	<i>Trans</i>	Grote et al., 2013
	H19	Genomic imprinting	<i>Cis/trans</i>	Cai and Cullen, 2007
	HAUNT	Repression at the HOXA locus	<i>Cis</i>	Yin et al., 2015
	HOTAIR	Repression at the HOXD locus	<i>Trans</i>	Cai et al., 2014
	HOTTIP	Activation at the HOXA locus	<i>Cis</i>	Lian et al., 2016
	HOXD-AS1	Recruitment of WDR5 to target genes	<i>Trans</i>	Gu et al., 2017
	Kcnq1ot1	Imprinting at the KCNQ1 cluster	<i>Cis</i>	Chiesa et al., 2012
	lncTCF7	Recruitment of SWI/SNF complex to TCF7	<i>Cis</i>	Wu B. et al., 2018
	MEG3	Accumulation of p53 protein	<i>Cis/trans</i>	Zhou et al., 2007
	Oct4P4	Repression at the Oct4 locus	<i>Trans</i>	Scarola et al., 2015
	PAPAS	rRNA synthesis	<i>Cis</i>	Zhao Z.L. et al., 2018
	PARTICLE	Repression of methionine adenosyltransferase 2A	<i>Cis/trans</i>	O'leary et al., 2017
	TERRA	Felomeric heterochromatin formation	<i>Cis/trans</i>	Luke and Lingner, 2009
	TSIX	X inactivation	<i>Cis</i>	Sado et al., 2005
	XIST	X inactivation	<i>Cis</i>	Cerase et al., 2015
	Six3OS	Recruit histone modification enzymes to Six3 target genes	<i>Trans</i>	Rapicavoli et al., 2011
DNA methylation	APTR	Recruitment of PRC2 to CDKN1A/p21	<i>Trans</i>	Negishi et al., 2014
	DALI	DNA methylation on promoter regions of target genes	<i>Trans</i>	Chalei et al., 2014
	DUM	DNA methylation on Dppa2	<i>Cis</i>	Wang et al., 2015a
	Evf2/Dlx6as	Transcriptional repression of Evf2 and Dlx5	<i>Cis/trans</i>	Berghoff et al., 2013
	FIRRE	H3K27me3 methylation maintenance	<i>Trans</i>	Yang et al., 2015
miRNA binding	CDR1as	miR-7 decoy	<i>Trans</i>	Yu et al., 2016
	lincRNA-ROR	miR-145 binding	<i>Trans</i>	Wang et al., 2013
	lincRNA-ATB	miR-200s binding	<i>Trans</i>	Li et al., 2017
	UCA1	miR-184 sponge	<i>NA</i>	Li and Hu, 2015
	TUG1	miR-144/145 binding	<i>Trans</i>	Li et al., 2016
Transcriptional regulation	Uph	Repression at the <i>Hand2</i> locus	<i>Cis</i>	Kopp and Mendell, 2018
	Paupar	Negative regulation Pax6 expression	<i>Cis/trans</i>	Vance et al., 2014
	PANDA	Repression of NF-YA-mediated transcription	<i>NA</i>	Puvvula et al., 2014
	Airn	Imprinting at the IGF2R cluster	<i>Cis</i>	Kopp and Mendell, 2018
Post transcriptional regulation	RMST	Transcriptional coregulator of SOX2	<i>Trans</i>	Ng et al., 2013a.
	MALAT1	Ser/Arg splicing factor regulation	<i>Trans</i>	Wu Y.T. et al., 2015
	PCA3	PRUNE2 editing and stability	<i>Cis</i>	Salameh et al., 2015
	CCAT2	Alternative splicing of Glutaminase (GLS)	<i>Trans</i>	Xin Y. et al., 2017
	MIAT	Alternative splicing of DISC1, ERBB4	<i>Trans</i>	Sun et al., 2018
	Sirt1 AS	Promotion of <i>Sirt1</i> mRNA stability	<i>Cis</i>	Wang et al., 2014
	TINCR	Stability of multiple mRNAs	<i>Trans</i>	Kretz, 2013
	1/2-sbsRNA	Activation of STAU1-mediated decay	<i>Trans</i>	Gong and Maquat, 2011
	BACE1-AS	Positive regulation of BACE1	<i>Cis</i>	Modarresi et al., 2011
	aHIF	Nuclear membrane trafficking	<i>Cis</i>	Cayre et al., 2003
	AS UCHL1	UCHL1 mRNA translation	<i>Cis</i>	Carrieri et al., 2015
	lincRNA-p21	Translational suppression	<i>Cis</i>	Dimitrova et al., 2014
	ZEB2 NAT	Activation of ZEB2 translation	<i>Cis</i>	Bernardes De Jesus et al., 2018
	NORAD	Inhibition of PUM protein activity	<i>NA</i>	Lee et al., 2016

(Continued)

TABLE 1 | Continued

Mechanism	lncRNA	Function	Relationship with target (Cis/Trans)	Reference
	SNHG4/5/6	Localization of MTA2 protein in the nuclear	NA	Chang et al., 2016; Zhao et al., 2016
	LINK-A	Recruitment of BRK to GPNMB	NA	Wu D. et al., 2017
	NEAT1	Formation of nuclear paraspeckles	Trans	Clemson et al., 2009
	AOC4P	Degradation of vimentin	Trans	Wang et al., 2015b
	ASBEL	Localization of ANA/BTG3 mRNA in the nuclear	Cis	Zhao J. et al., 2018
	GAS5	Repression of glucocorticoid receptor-mediated transcription	NA	Kino et al., 2010

Xist, also involves in XCI through repressing the *TSIX* expression from the Xi and evicting nuclear protein CCCTC-binding factor (CTCF) away from promoter of *XIST* to activate the *XIST* expression from Xi (Sun et al., 2013).

LncRNAs in Neurodevelopment

A great number of lncRNAs are expressed during neural development and in the brain. Using high-throughput technologies, *in situ* hybridization, microarray analysis and RNA sequencing (RNA-seq), researchers have found that most of the lncRNAs examined (849 out of 1328) are expressed in specific cell types, subcellular compartments and different regions of the brain (Ng et al., 2013b; Shi et al., 2017). LncRNAs display differential expressions across the cortical layers, and region-specific expressions in the subventricular zone, dentate gyrus and olfactory bulb of mice (Belgard et al., 2011; Ramos et al., 2013). Based on a unique custom microarray platform, 8 lncRNAs were identified to be expressed in an age-dependent manner, from 36 surgically resected human neocortical samples, ranging from infancy to adulthood (Lipovich et al., 2014). However, the function of those lncRNAs need to be validated, through loss-of-function assays, RNA-protein association assays or assessments of RNA-chromatin association.

Some lncRNAs are also found to participate in neural cell fate determination, neuronal-glia fate switching and oligodendrocyte elaboration. An antisense transcript of the distal-less homeobox 1 (*Dlx1*), *Dlx1AS*, was discovered to be up-regulated during GABAergic differentiation and down-regulated during oligodendrocyte differentiation (Mercer et al., 2010). A subsequent study found that *Dlx1AS* participated in neurogenesis, implying its function in neuronal differentiation by regulating expression of its homeobox gene neighbors (Ramos et al., 2013). *Evf2*, a cloud-forming *Dlx5/6* ultra-conserved eRNA, influences the formation of GABAergic interneurons in both a mouse and human forebrain. In the developing ventral forebrain, *Evf2* regulates *Dlx5*, *Dlx6* and glutamate decarboxylase 1 (*Gad1*) expression by recruiting DLX1 and/or DLX2 and methyl CpG binding protein 2 (MeCP2) to specific DNA regulatory elements. *GAD1* is an enzyme responsible for catalyzing glutamate to form GABA. *Evf2* mouse mutants reduced GABAergic interneurons in the early postnatal dentate gyrus and hippocampus (Bond et al., 2009; Berghoff et al., 2013; Cajigas et al., 2018).

The vertebrate retina is comprised of three well-organized cell type-specific neuron layers, interconnected by synapses (Ng et al.,

2013b). *Six3OS* is the long non-coding opposite strand transcript (lncOST) of the homeodomain factor *Six3*. During mammalian eye development, *Six3* regulates both early eye formation and postnatal retinal cell specification. Knockdown of *Six3OS* leads to a decrease of bipolar cells and an increase of Müller glia, similar to the results in the knockdown of *Six3*. In contrast, overexpression of *Six3OS* decreased syntaxin positive cells. Gene perturbation studies revealed that *Six3OS* participates in retinal cell specification as a molecular scaffold, to regulate *Six3* activity rather than expression (Rapicavoli et al., 2011). *TUG1* (taurine upregulated gene 1), a spliced and polyadenylated lncRNA, is highly conserved in humans and mice. *TUG1* may participate in rod-photoreceptor genesis and inhibits cone-photoreceptor gene expression globally, by altering the chromatin configurations of photoreceptor-specific transcription factors *Crx* and *Nrl* (Young et al., 2005).

LncRNAs IN NEUROLOGIC DISORDERS

Emerging evidence has shown that the dysregulation of lncRNAs is related to multiple neurological disorders, such as schizophrenia (Scholz et al., 2010), autism spectrum disorder (ASD) (Wang et al., 2015c), Parkinson's (Ni et al., 2017), Huntington's (Sunwoo et al., 2017) and Alzheimer's diseases (Faghihi et al., 2008).

Schizophrenia (SCZ) is a debilitating mental disorder with a broad spectrum of neurocognitive impairments. Abundant data suggests that both genetic and environmental factors contribute to the pathophysiology of SCZ (Seidman and Mirsky, 2017). Several lncRNAs have been used as biomarkers and therapeutic targets for SCZ (Chen et al., 2016). lncRNA *MIAT*, also known as Gomafu or RNCR2, is down-regulated in SCZ upon neuronal activation (Sun et al., 2018). Previous studies found *MIAT* either acts as a competitive endogenous RNA (ceRNA) for miR-150-5p, miR-24, miR-22-3p or miR-150, to influence cell proliferation, apoptosis and migration, or participates in various signaling pathways by enhancing Nrf2 (nuclear factor erythroid 2-related factor 2) and Oct4 expression. Subsequent studies revealed that *MIAT* can directly bind to the splicing regulator quaking homolog (QKI) and splicing factor 1 (SF1), to modulate several gene expressions in the neuron. In SCZ patient brains, *DISC1* (disrupted in schizophrenia 1), *ERBB4* (v-erb-a erythroblastic leukemia viral oncogene homolog 4) and their alternatively

spliced variants were all down-regulated due to *MIAT* up-regulation. *MIAT* could act as a scaffold to affect alternative splicing of those SCZ-associated genes (Roberts et al., 2014; Liu et al., 2018; Sun et al., 2018).

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders characterized by impaired reciprocal social interactions, communication, and repetitive stereotyped behaviors (Tang et al., 2017). 222 differentially expressed lncRNAs have been identified from autistic brain tissues. 90% of these lncRNAs are oriented in or around known genes related to neurodevelopmental and psychiatric diseases, such as *UBE3A* (ubiquitin protein ligase E3A), which is associated with Angelman syndrome, that shares common features with ASD. At the same time, it has been found that the number of lncRNAs differentially expressed within a control sample, was much greater than that within an autistic sample (1375 lncRNAs vs. 236 lncRNAs, respectively) (Ziats and Rennert, 2013). A genome-wide association study (GWAS) of ASD identified a 3.9 kb lncRNA designated *MSNP1AS*, which is encoded by the opposite strand of the moesin pseudogene 1 (*MSNP1*). The sense transcript *MSN* encodes the moesin protein that regulates neuronal architecture and immune responses. *MSNP1AS* was found to be significantly upregulated in a postmortem ASD temporal cortex, and overexpression of *MSNP1AS* led to significant decreases in *MSN*, moesin, neurite number and length in cultured neurons. Thus, *MSNP1AS* contributes to ASD risk, by possibly influencing the sense transcript *MSN* expression negatively (Wilkinson and Campbell, 2013).

BACE1-AS is a conserved non-coding antisense transcript of β -secretase 1 (*BACE1*) and has been shown to be closely associated with Alzheimer's disease (AD). *BACE1* is responsible for the generation of β -amyloid and the amyloid plaques in the brain, which are the primary pathophysiology of AD. *BACE1-AS* is markedly up-regulated in AD brains and promotes the stability of *BACE1* through stabilizing *BACE1* mRNA, thereby increasing the *BACE1* protein and A β 1–42 levels (Faghihi et al., 2008). Knock down of *BACE1-AS* *in vivo* resulted in the down-regulation of both *BACE1* and *BACE1-AS*, along with reduced β -amyloid in the brain. In addition, the brain cytoplasmic RNA BC200 (*BCYRN1*), GDNF gene antisense transcript (*GDNF-AS*) and *Sox2* overlapping transcript (*Sox2OT*), all participate in progress and development in AD brains (Wan et al., 2017).

Huntington disease (HD) is a hereditary neurodegenerative disease with symptoms including dementia, chorea, and psychiatric disturbances. HD is caused by a CAG trinucleotide abnormal expansion in the first exon of the huntingtin gene and its probability of occurrence is 1/10000. Microarray data found that the expression of four lncRNAs significantly changed in HD brains: *NEAT1* (nuclear paraspeckle assembly transcript 1) and *TUG1* are upregulated, and *DGCR5* (DiGeorge syndrome critical region gene 5) and *MEG3* (maternally expressed 3) are downregulated. The up-regulation of *NEAT1* in HD might contribute to the pathogenic alteration of the transcriptional status, by sequestering various paraspeckle proteins (Sunwoo et al., 2017), whereas *TUG1* is possibly activated by p53 and then interacts with PRC2, to affect downstream HD-associated

genes. *DGCR5* and *MEG3*, are both direct targets of REST. Their down-regulation may result from the aberrant accumulation of REST in the nuclei of striatal neurons in HD (Johnson, 2012; Hwang and Zukin, 2018). Using the whole genome chromatin immunoprecipitation sequencing (ChIP-Seq) method, *HAR1*, a deeply conserved genomic region that is directly bound by REST was confirmed. This region encodes a pair of structured lncRNAs as well, *HAR1F* and *HAR1R*. Both *HAR1F* and *HAR1R* were downregulated in the striatum of HD patients (Johnson et al., 2010).

Parkinson's disease (PD) is one of most prevalent neurodegenerative disorders, characterized by progressive impairments of motor abilities caused by the loss of dopamine-producing cells in the brain. Antisense ubiquitin carboxy-terminal hydrolase L1 (*AS-Uchl1*) was discovered to up-regulate the translation of *Uchl1* protein at a post-transcriptional level depending on a 5' overlapping sequence and an embedded inverted SINEB2 sequence (Carrieri et al., 2012). *AS-Uchl1* is strongly down-regulated in neurochemical models of PD as a component of the Nurr1-dependent gene network and the subsequent reduced translation of *UCHL1* protein, lead to the perturbation of the ubiquitin-proteasome system (Carrieri et al., 2015). *H19* upstream conserved 1 and 2 (*Huc1* and *Huc2*), *lincRNA-p21*, *MALAT1*, *SNHG1*, and *TncRNA* were differentially expressed in PD patients (Kraus et al., 2017). As these lncRNAs are associated with synaptogenesis, proliferation and apoptosis, the expression of these lncRNAs precede the course of PD, suggesting they may be biomarkers of PD (Kraus et al., 2017).

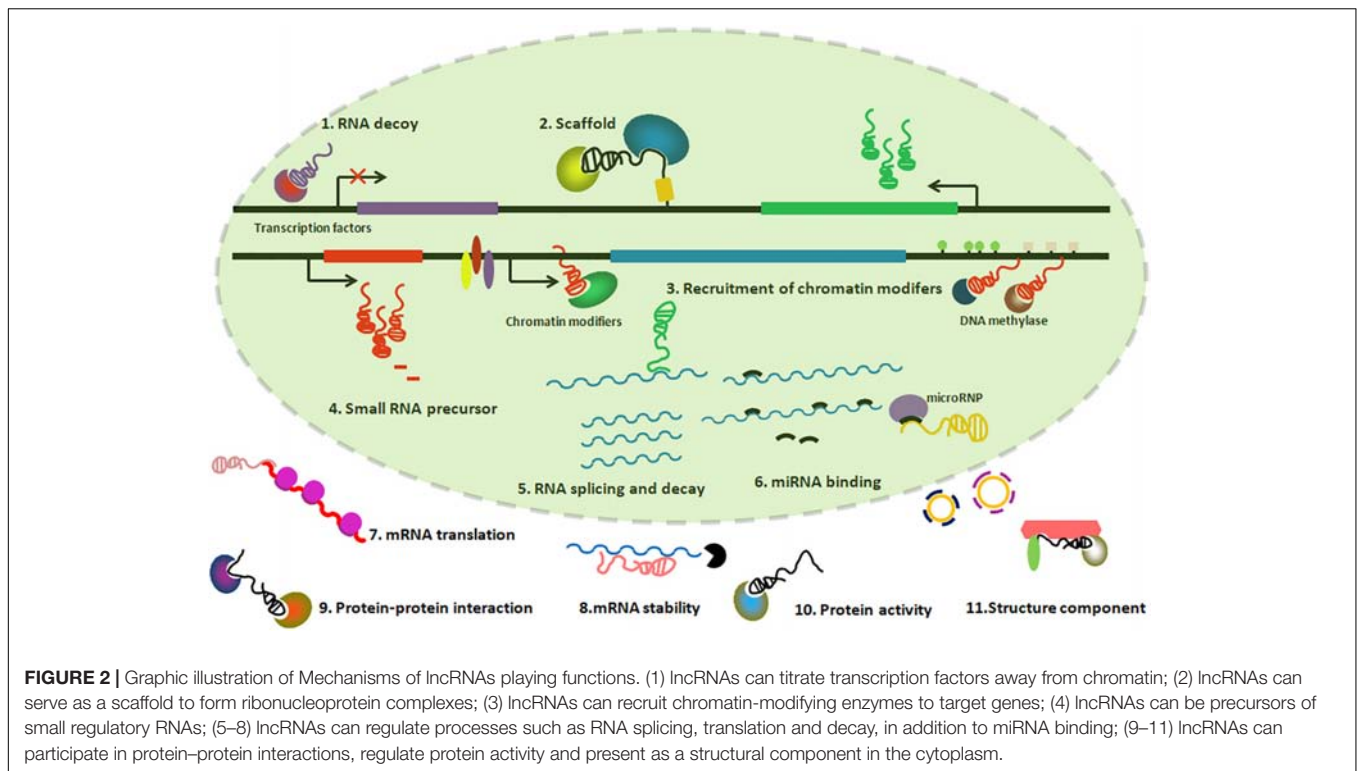
MECHANISMS OF LncRNAs IN BIOLOGICAL PROCESSES

lncRNAs could provide functions through differential mechanisms, including serving as molecular scaffolds, molecular signals, guiding chromatin modifiers, and miRNA sponges, etc. (Figure 2).

Molecular Scaffolds

Xist, a 17 ~ 20 kb lncRNA transcribed from the X inactivation center, initiates X-chromosome inactivation (XCI) by progressively coating the future inactive X chromosome (Xi). *Xist* can bind to chromatin-modifying complexes PRC2 through the conserved A-repeat domain and form a transcriptionally silent nuclear compartment. This compartment is responsible for the chromosome-wide gene repression in the Xi (Chery and Larschan, 2014). Another example of the lncRNA acting as a molecular scaffold is the *HOTAIR*, which are transcripts of the antisense strand of *HOXC* gene cluster, which can modulate nearby gene expression by interacting with PRC2 and lysine specific demethylase 1 (LSD1) (Tsai et al., 2010).

lncRNA *tsRMST*, an isoform of *RMST* (rhabdomyosarcoma 2 associated transcript) was highly expressed in human iPSCs and ESCs. Further studies revealed that *tsRMST* down-regulation leads to *NANOG* and the PRC2 complex component *SUZ12* fail to bind to the promoters of several inactive genes. These



genes are thereby activated and promote ectoderm and endoderm differentiation (Ng et al., 2012; Yu and Kuo, 2016).

Molecular Signals

Genomic imprinting restricts gene expression on one of the two parental chromosomes and the parental-specific gene expression in diploid cells, and affects both male and female descendants (Barlow and Bartolomei, 2014). *H19* transcripts are the precursors of miR-675-3p and miR-675-5p (Cai and Cullen, 2007). Before parturition, *H19* slows the growth of the placenta down partially, through down-regulating the RNA binding protein HuR. The decreased HuR fails to block the processing of miR-675, which further decreases the growth regulator *Igf1r* (Keniry et al., 2012).

MALAT1 (metastasis-associated lung adenocarcinoma transcript (1) is initially found as an abundant lncRNA in nuclear speckles to regulate processes of mRNA alternative splicing by modulating the levels of serine/arginine splicing factors (Tripathi et al., 2010). Recent studies confirmed that *MALAT1* is a sensitive prognostic marker for lung cancer metastasis and linked to several other human cancers (Gutschner et al., 2013).

Guiding Chromatin Modifiers

Genome regulation via DNA methylation and post-translational histone modifications by the activity of chromatin modifiers, is a well-documented function of lncRNA in eukaryotes (Bohmdorfer and Wierzbicki, 2015; Nanda et al., 2016). Altered DNA methylation patterns at CpG islands and mutations in chromatin modifiers, may result in oncogenesis (Haladyna et al., 2015; Nanda et al., 2016).

The first lncRNA identified to interact with both maintenance and *de novo* methylases, *Dum*, is tightly associated with myogenesis and transcriptionally induced by MyoD upon myoblast differentiation. *Dum* can recruit DNA methyltransferase1/3a/3b complex to the *Dppa2* promoter, through intra-chromosomal looping, mediated by RAD21 and NIPBL, resulting in two CpG loci hypermethylation and *Dppa2* silencing (Wang et al., 2015a). Recent studies of *HOTAIR* suggest that under hypoxia, *HOTAIR* expression is up-regulated in several cancer cells induced by the hypoxia-inducible factors (HIFs), recruiting hypoxia-response elements (HRE) to bind on the *HOTAIR* promoter. Along with HIFs, histone acetyltransferase CREB-binding protein (CBP/p300) and histone H3K4 specific methyltransferases, mixed lineage leukemia (MLL) family, are enriched in the HRE region of the *HOTAIR* promoter (Bhan et al., 2017).

Additionally, N-Myc can directly bind to the JMJD1A promoter to upregulate JMJD1A expression in neuroblastoma cells. The upregulated JMJD1A then directly binds to the *MALAT1* promoter to demethylate H3K9, to activate *MALAT1* expression (Tee et al., 2014; Peng et al., 2018). Furthermore, *H19* and mir-675 were found to participate in the adipogenesis through mir-675, targeting the histone deacetylase (HDAC) 4–6 3' untranslated regions and inducing HDACs 4–6 down-regulation. The reduced HDAC 4–6 then reduced *H19* expression, possibly by reducing the levels of CTCF occupancy in the *H19* imprinting control region. *H19* inhibition then facilitates the bone marrow mesenchymal stem cells differentiating into adipocytes (Huang et al., 2016).

miRNA Sponges

A handful of microRNAs have been reported to influence the mRNA stability of protein-coding genes on post-transcriptional level. Recent studies on lncRNAs discovered several miRNA-lncRNA interactions based on *in silico* and experimental analyses (Paraskevopoulou and Hatzigeorgiou, 2016). One of the well-studied lncRNAs which act as miRNA sponges, is *lincRNA-ROR*, which decoys miR-145 in self-renewing human ESCs. A regulatory feedback loop formed by *lincRNA-ROR*, miR-145 and the core transcription factors OCT4, SOX2, and NANOG is closely related to the ESCs pluripotency (Wang et al., 2013). *MALAT1* not only interacts with several splicing factors, but also binds to miRNAs including miR-101, miR-9, miR-125b, and miR217 to regulate the interactions between miRNA and mRNAs (Paraskevopoulou and Hatzigeorgiou, 2016).

Other Mechanisms

Some lncRNAs exert their function by maintaining DNA looping between enhancer and promoter regions, or by recruiting chromatin regulatory proteins to establish high affinity interactions between different regions of the DNA, resulting in closely positioned promoters and enhancers (Yang et al., 2013). For example, *linRNA-p21* regulates the expression of its neighboring gene *p21*, by *cis*-regulatory enhancer-like DNA elements, which embed within the *p21* gene body (Dimitrova et al., 2014). lncRNAs can also interact with DNA directly through nucleic-acid hybridization, and regulate nearby gene expression, such as *Airn* (antisense of IGF2R non-protein coding RNA), which produces transcription interference on paternal allele, by spanning the *Igf2* gene promoter (Latos et al., 2012). Additionally, lncRNAs may influence the three-dimensional organization of the mammalian nucleus. *FIRRE* (firre intergenic repeating RNA element) is transcribed from the X chromosome, and interacts with hnRNPU in the process of nuclear organization (Yang et al., 2015). *Trans*-acting lncRNAs also modulate the protein activity and RNA stability by directly binding in the nucleus or cytoplasm. lncRNA NORAD functions as a negative regulator of the RNA binding protein PUMILIO1

and 2 (PUM1 and 2) in the cytoplasm. The knockdown of NORAD leads to the degradation of PUM1/2 targeted mRNAs (Lee et al., 2016).

PERSPECTIVES

Previous studies have revealed that lncRNAs play an important role in neuronal development and function through differential mechanisms. The dysregulation of lncRNAs could result in neurological diseases. Advances in sequencing technologies and their applications will contribute substantially to uncovering and investigating novel lncRNAs and their functions.

One challenge in the lncRNA field is whether lncRNAs can be used as diagnostic biomarkers or therapeutic targets for diseases. Considering that the down-stream targets of lncRNAs could be broad, it is hard to use it as a specific “key” to one “lock.” Although the validation of their functions could be performed *in vitro* and *in vivo*, it is difficult to claim a specific target. Another challenge is to better understand the mechanisms of lncRNAs functions. It is highly necessary to develop proper genetic tools and to establish animal models to dissect the regulatory networks of lncRNAs, and their interaction with other epigenetic modifications.

AUTHOR CONTRIBUTIONS

LL, YZ, XZ, and XL wrote the manuscript. All authors commented on the manuscript.

FUNDING

LL was supported by the Natural Science Foundation of Zhejiang Province (Grant No. LQ18C060001). XL was supported in part by the National Key Research and Development Program of China (Grant No. 2016YFC0900400) and the National Natural Science Foundation of China (Grant Nos. 31771395 and 31571518).

REFERENCES

- Andersson, R., Gebhard, C., Miguel-Escalada, I., Hoof, I., Bornholdt, J., Boyd, M., et al. (2014). An atlas of active enhancers across human cell types and tissues. *Nature* 507, 455–461. doi: 10.1038/nature12787
- Barlow, D. P., and Bartolomei, M. S. (2014). Genomic imprinting in mammals. *Cold Spring Harb. Perspect. Biol.* 6:a018382. doi: 10.1101/cshperspect.a018382
- Belgard, T. G., Marques, A. C., Oliver, P. L., Abaan, H. O., Sirey, T. M., Hoerder-Suabedissen, A., et al. (2011). A transcriptomic atlas of mouse neocortical layers. *Neuron* 71, 605–616. doi: 10.1016/j.neuron.2011.06.039
- Berghoff, E. G., Clark, M. F., Chen, S., Cajigas, I., Leib, D. E., and Kohtz, J. D. (2013). Evf2 (Dlx6as) lncRNA regulates ultraconserved enhancer methylation and the differential transcriptional control of adjacent genes. *Development* 140, 4407–4416. doi: 10.1242/dev.099390
- Bernardes De Jesus, B., Marinho, S. P., Barros, S., Sousa-Franco, A., Alves-Vale, C., et al. (2018). Silencing of the lncRNA Zeb2-NAT facilitates reprogramming of aged fibroblasts and safeguards stem cell pluripotency. *Nat. Commun.* 9:94. doi: 10.1038/s41467-017-01921-6
- Bhan, A., Deb, P., Shihabeddin, N., Ansari, K. I., Brotto, M., and Mandal, S. S. (2017). Histone methylase MLL1 coordinates with HIF and regulate lncRNA HOTAIR expression under hypoxia. *Gene* 629, 16–28. doi: 10.1016/j.gene.2017.07.069
- Bian, S., and Sun, T. (2011). Functions of noncoding RNAs in neural development and neurological diseases. *Mol. Neurobiol.* 44, 359–373. doi: 10.1007/s12035-011-8211-3
- Bohmdorfer, G., and Wierzbicki, A. T. (2015). Control of chromatin structure by long noncoding RNA. *Trends Cell Biol.* 25, 623–632. doi: 10.1016/j.tcb.2015.07.002
- Bond, A. M., Vangompel, M. J. W., Sametsky, E. A., Clark, M. F., Savage, J. C., Disterhoft, J. F., et al. (2009). Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat. Neurosci.* 12:1020. doi: 10.1038/nn.2371
- Cabili, M. N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., et al. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* 25, 1915–1927. doi: 10.1101/gad.17446611

- Cai, B., Song, X. Q., Cai, J. P., and Zhang, S. (2014). HOTAIR: a cancer-related long non-coding RNA. *Neoplasma* 61, 379–391. doi: 10.4149/neo_2014_075
- Cai, X. Z., and Cullen, B. R. (2007). The imprinted H19 noncoding RNA is a primary microRNA precursor. *RNA* 13, 313–316. doi: 10.1261/rna.351707
- Cajigas, I., Chakraborty, A., Swyter, K. R., Luo, H., Bastidas, M., Nigro, M., et al. (2018). The Evf2 ultraconserved enhancer lncRNA functionally and spatially organizes megabase distant genes in the developing forebrain. *Mol. Cell* 71:e959. doi: 10.1016/j.molcel.2018.07.024
- Cao, H., Wahlestedt, C., and Kapranov, P. (2018). Strategies to annotate and characterize long noncoding RNAs: advantages and pitfalls. *Trends Genet.* 34, 704–721. doi: 10.1016/j.tig.2018.06.002
- Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S., et al. (2012). Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 491, 454–457. doi: 10.1038/nature11508
- Carrieri, C., Forrest, A. R. R., Santoro, C., Persichetti, F., Carninci, P., Zucchelli, S., et al. (2015). Expression analysis of the long non-coding RNA antisense to Uchl1 (AS Uchl1) during dopaminergic cells' differentiation in vitro and in neurochemical models of Parkinson's disease. *Front. Cell. Neurosci.* 9:114. doi: 10.3389/fncel.2015.00114
- Cayre, A., Rossignol, F., Clottes, E., and Penault-Llorca, F. (2003). aHIF but not HIF-1 α transcript is a poor prognostic marker in human breast cancer. *Breast Cancer Res.* 5, R223–R230. doi: 10.1186/bcr652
- Cerese, A., Pintacuda, G., Tattermusch, A., and Avner, P. (2015). Xist localization and function: new insights from multiple levels. *Genome Biol.* 16:166. doi: 10.1186/s13059-015-0733-y
- Chalei, V., Sansom, S. N., Kong, L. S., Lee, S., Montiel, J., Vance, K. W., et al. (2014). The long non-coding RNA dali is an epigenetic regulator of neural differentiation. *Elife* 3:e04530. doi: 10.7554/eLife.04530
- Chang, L., Yuan, Y., Li, C., Guo, T., Qi, H., Xiao, Y., et al. (2016). Upregulation of SNHG6 regulates ZEB1 expression by competitively binding miR-101-3p and interacting with UPF1 in hepatocellular carcinoma. *Cancer Lett.* 383, 183–194. doi: 10.1016/j.canlet.2016.09.034
- Chen, L. L. (2016). Linking long noncoding RNA localization and function. *Trends Biochem. Sci.* 41, 761–772. doi: 10.1016/j.tibs.2016.07.003
- Chen, S. D., Sun, X. Y., Niu, W., Kong, L. M., He, M. J., Li, W. S., et al. (2016). Aberrant expression of long non-coding RNAs in schizophrenia patients. *Med. Sci. Monitor* 22, 3340–3351. doi: 10.12659/MSm.896927
- Chery, J., and Larschan, E. (2014). X-marks the spot: X-chromosome identification during dosage compensation. *Biochim. Biophys. Acta* 1839, 234–240. doi: 10.1016/j.bbagr.2013.12.007
- Chiesa, N., De Crescenzo, A., Mishra, K., Perone, L., Carella, M., Palumbo, O., et al. (2012). The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases. *Hum. Mol. Genet.* 21, 10–25. doi: 10.1093/hmg/ddr419
- Chu, H. P., Cifuentes-Rojas, C., Kesner, B., Aeby, E., Lee, H. G., Wei, C., et al. (2017). TERRA RNA antagonizes ATRX and protects telomeres. *Cell* 170:e116. doi: 10.1016/j.cell.2017.06.017
- Clark, B. S., and Blackshaw, S. (2014). Long non-coding RNA-dependent transcriptional regulation in neuronal development and disease. *Front. Genet.* 5:164. doi: 10.3389/fgene.2014.00164
- Clemson, C. M., Hutchinson, J. N., Sara, S. A., Ensminger, A. W., Fox, A. H., Chess, A., et al. (2009). An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol. Cell* 33, 717–726. doi: 10.1016/j.molcel.2009.01.026
- Congrains, A., Kamide, K., Ohishi, M., and Rakugi, H. (2013). ANRIL: molecular mechanisms and implications in human health. *Int. J. Mol. Sci.* 14, 1278–1292. doi: 10.3390/ijms14011278
- Diman, A., and Decottignies, A. (2018). Genomic origin and nuclear localization of TERRA telomeric repeat-containing RNA: from Darkness to Dawn. *FEBS J.* 285, 1389–1398. doi: 10.1111/febs.14363
- Dimitrova, N., Zamudio, J. R., Jong, R. M., Soukup, D., Resnick, R., Sarma, K., et al. (2014). LincRNA-p21 activates p21 in cis to promote Polycomb target gene expression and to enforce the G1/S checkpoint. *Mol. Cell* 54, 777–790. doi: 10.1016/j.molcel.2014.04.025
- Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., et al. (2012). Landscape of transcription in human cells. *Nature* 489, 101–108. doi: 10.1038/nature11233
- Dykes, I. M., and Emanuelli, C. (2017). Transcriptional and post-transcriptional gene regulation by long non-coding RNA. *Genom. Proteom. Bioinform.* 15, 177–186. doi: 10.1016/j.gpb.2016.12.005
- Faghihi, M. A., Modarresi, F., Khalil, A. M., Wood, D. E., Sahagan, B. G., Morgan, T. E., et al. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat. Med.* 14, 723–730. doi: 10.1038/nm1784
- Faghihi, M. A., Zhang, M., Huang, J., Modarresi, F., Van Der Brug, M. P., Nalls, M. A., et al. (2010). Evidence for natural antisense transcript-mediated inhibition of microRNA function. *Genome Biol.* 11:R56. doi: 10.1186/gb-2010-11-5-r56
- Fang, Y. W., and Fullwood, M. J. (2016). Roles, functions, and mechanisms of long non-coding RNAs in cancer. *Genomics Proteomics Bioinformatics* 14, 42–54. doi: 10.1016/j.gpb.2015.09.006
- Fatemi, R. P., Salah-Uddin, S., Modarresi, F., Khoury, N., Wahlestedt, C., and Faghihi, M. A. (2015). Screening for small-molecule modulators of long noncoding RNA-protein interactions using Alphascreen. *J. Biomol. Screen.* 20, 1132–1141. doi: 10.1177/1087057115594187
- Geisler, S., and Coller, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. *Nat. Rev. Mol. Cell Biol.* 14, 699–712. doi: 10.1038/nrm3679
- Gendrel, A. V., and Heard, E. (2014). Noncoding RNAs and epigenetic mechanisms during X-chromosome inactivation. *Annu. Rev. Cell Dev. Biol.* 30, 561–580. doi: 10.1146/annurev-cellbio-101512-122415
- Ghosal, S., Das, S., and Chakrabarti, J. (2013). Long noncoding RNAs: new players in the molecular mechanism for maintenance and differentiation of pluripotent stem cells. *Stem Cells Dev.* 22, 2240–2253. doi: 10.1089/scd.2013.0014
- Gong, C. G., and Maquat, L. E. (2011). lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. *Nature* 470, 284–288. doi: 10.1038/nature09701
- Grote, P., Wittler, L., Hendrix, D., Koch, F., Wahrlich, S., Beisaw, A., et al. (2013). The tissue-specific lncRNA fendrr is an essential regulator of heart and body wall development in the mouse. *Dev. Cell* 24, 206–214. doi: 10.1016/j.devcel.2012.12.012
- Gu, P., Chen, X., Xie, R., Han, J., Xie, W., Wang, B., et al. (2017). lncRNA HOXD-AS1 regulates proliferation and chemo-resistance of castration-resistant prostate cancer via recruiting WDR5. *Mol. Ther. J. Am. Soc. Gene Ther.* 25, 1959–1973. doi: 10.1016/j.ymthe.2017.04.016
- Gutschner, T., Hammerle, M., and Diederichs, S. (2013). MALAT1 – a paradigm for long noncoding RNA function in cancer. *J. Mol. Med.* 91, 791–801. doi: 10.1007/s00109-013-1028-y
- Haladyna, J. N., Yamauchi, T., Neff, T., and Bernt, K. M. (2015). Epigenetic modifiers in normal and malignant hematopoiesis. *Epigenomics-UK* 7, 301–320. doi: 10.2217/Epi.14.88
- Huang, Y., Zheng, Y., Jin, C., Li, X., Jia, L., and Li, W. (2016). Long Non-coding RNA H19 inhibits adipocyte differentiation of bone marrow mesenchymal stem cells through epigenetic modulation of histone deacetylases. *Sci. Rep.* 6:28897. doi: 10.1038/srep28897
- Huarte, M. (2015). The emerging role of lncRNAs in cancer. *Nat. Med.* 21, 1253–1261. doi: 10.1038/nm.3981
- Hwang, J. Y., and Zukin, R. S. (2018). REST, a master transcriptional regulator in neurodegenerative disease. *Curr. Opin. Neurobiol.* 48, 193–200. doi: 10.1016/j.conb.2017.12.008
- Iyer, M. K., Niknafs, Y. S., Malik, R., Singhal, U., Sahu, A., Hosono, Y., et al. (2015). The landscape of long noncoding RNAs in the human transcriptome. *Nat. Genet.* 47, 199–208. doi: 10.1038/ng.3192
- Johnson, R. (2012). Long non-coding RNAs in huntington's disease neurodegeneration. *Neurobiol. Dis.* 46, 245–254. doi: 10.1016/j.nbd.2011.12.006
- Johnson, R., Richter, N., Jauch, R., Gaughwin, P. M., Zuccato, C., Cattaneo, E., et al. (2010). Human accelerated region 1 noncoding RNA is repressed by REST in Huntington's disease. *Physiol. Genomics* 41, 269–274. doi: 10.1152/physiolgenomics.00019.2010
- Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., et al. (2005). Antisense transcription in the mammalian transcriptome. *Science* 309, 1564–1566. doi: 10.1126/science.1112009
- Keniry, A., Oxley, D., Monnier, P., Kyba, M., Dandolo, L., Smits, G., et al. (2012). The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. *Nat. Cell Biol.* 14, 659–665. doi: 10.1038/ncb2521

- Kino, T., Hurt, D. E., Ichijo, T., Nader, N., and Chrousos, G. P. (2010). Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci. Signal.* 3:ra8. doi: 10.1126/scisignal.2000568
- Klattenhoff, C. A., Scheuermann, J. C., Surface, L. E., Bradley, R. K., Fields, P. A., Steinhauser, M. L., et al. (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. *Cell* 152, 570–583. doi: 10.1016/j.cell.2013.01.003
- Kopp, F., and Mendell, J. T. (2018). Functional classification and experimental dissection of long noncoding RNAs. *Cell* 172, 393–407. doi: 10.1016/j.cell.2018.01.011
- Kraus, T. F. J., Haider, M., Spanner, J., Steinmaurer, M., Dietinger, V., and Kretzschmar, H. A. (2017). Altered long noncoding RNA expression precedes the course of parkinson's disease—a preliminary report. *Mol. Neurobiol.* 54, 2869–2877. doi: 10.1007/s12035-016-9854-x
- Kretz, M. (2013). TINCR, stau1n1, and cellular differentiation. *RNA Biol.* 10, 1597–1601. doi: 10.4161/rna.26249
- Latos, P. A., Pauler, F. M., Koerner, M. V., Seneguin, H. B., Hudson, Q. J., Stocsits, R. R., et al. (2012). Airn transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. *Science* 338, 1469–1472. doi: 10.1126/science.1228110
- Lee, J. T., and Bartolomei, M. S. (2013). X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell* 152, 1308–1323. doi: 10.1016/j.cell.2013.02.016
- Lee, S., Kopp, F., Chang, T. C., Sataluri, A., Chen, B. B., Sivakumar, S., et al. (2016). Noncoding RNA NORAD regulates genomic stability by sequestering PUMILIO proteins. *Cell* 164, 69–80. doi: 10.1016/j.cell.2015.12.017
- Lekka, E., and Hall, J. (2018). Noncoding RNAs in disease. *FEBS Lett.* 592, 2884–2900. doi: 10.1002/1873-3468.13182
- Li, F., and Hu, C. P. (2015). Long non-coding RNA urothelial carcinoma associated 1 (UCA1): insight into its role in human diseases. *Crit. Rev. Eukaryot. Gene Expr.* 25, 191–197. doi: 10.1615/CritRevEukaryotGeneExpr.2015013770
- Li, J., Li, Z., Zheng, W., Li, X., Wang, Z., Cui, Y., et al. (2017). LncRNA-ATB: an indispensable cancer-related long noncoding RNA. *Cell Prolif.* 50:e12381. doi: 10.1111/cpr.12381
- Li, Z., Shen, J., Chan, M. T., and Wu, W. K. (2016). TUG1: a pivotal oncogenic long non-coding RNA of human cancers. *Cell Prolif.* 49, 471–475. doi: 10.1111/cpr.12269
- Lian, Y. F., Cai, Z. L., Gong, H. B., Xue, S. L., Wu, D. D., and Wang, K. M. (2016). HOTTIP: a critical oncogenic long non-coding RNA in human cancers. *Mol. Biosyst.* 12, 3247–3253. doi: 10.1039/c6mb00475j
- Lipovich, L., Tarca, A. L., Cai, J., Jia, H., Chugani, H. T., Sterner, K. N., et al. (2014). Developmental changes in the transcriptome of human cerebral cortex tissue: long noncoding RNA transcripts. *Cereb. Cortex* 24, 1451–1459. doi: 10.1093/cercor/bhs414
- Liu, B., Sun, L., Liu, Q., Gong, C., Yao, Y., Lv, X., et al. (2015). A cytoplasmic NF-kappaB interacting long noncoding RNA blocks IkappaB phosphorylation and suppresses breast cancer metastasis. *Cancer Cell* 27, 370–381. doi: 10.1016/j.ccr.2015.02.004
- Liu, Y. S., Rao, S. Q., Xu, Y., Zhang, F. Q., Wang, Z. Q., and Zhao, X. D. (2018). Changes in the level of long non-coding RNA gomafu gene expression in schizophrenia patients before and after antipsychotic medication. *Schizophr. Res.* 195, 318–319. doi: 10.1016/j.schres.2017.08.025
- Lloret-Llinares, M., Mapendano, C. K., Martlev, L. H., Lykke-Andersen, S., and Jensen, T. H. (2016). Relationships between PROMPT and gene expression. *RNA Biol.* 13, 6–14. doi: 10.1080/15476286.2015.1109769
- Loewer, S., Cabili, M. N., Guttman, M., Loh, Y. H., Thomas, K., Park, I. H., et al. (2010). Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. *Nat. Genet.* 42, 1113–1117. doi: 10.1038/ng.710
- Luke, B., and Lingner, J. (2009). TERRA: telomeric repeat-containing RNA. *EMBO J.* 28, 2503–2510. doi: 10.1038/emboj.2009.166
- Marquardt, S., Raittskin, O., Wu, Z., Liu, F. Q., Sun, Q. W., and Dean, C. (2014). Functional consequences of splicing of the antisense transcript COOLAIR on FLC transcription. *Mol. Cell* 54, 156–165. doi: 10.1016/j.molcel.2014.03.026
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Mercer, T. R., Qureshi, I. A., Gokhan, S., Dinger, M. E., Li, G., Mattick, J. S., et al. (2010). Long noncoding RNAs in neuronal-glial fate specification and oligodendrocyte lineage maturation. *BMC Neurosci.* 11:14. doi: 10.1186/1471-2202-11-14
- Modarresi, F., Faghihi, M. A., Lopez-Toledano, M. A., Fatemi, R. P., Magistri, M., Brothers, S. P., et al. (2012). Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat. Biotechnol.* 30, 453–459. doi: 10.1038/nbt.2158
- Modarresi, F., Faghihi, M. A., Patel, N. S., Sahagan, B. G., Wahlestedt, C., and Lopez-Toledano, M. A. (2011). Knockdown of BACE1-AS nonprotein-coding transcript modulates beta-amyloid-related hippocampal neurogenesis. *Int. J. Alzheimer's Dis.* 2011:929042. doi: 10.4061/2011/929042
- Nagano, T., and Fraser, P. (2011). No-nonsense functions for long noncoding RNAs. *Cell* 145, 178–181. doi: 10.1016/j.cell.2011.03.014
- Nanda, J. S., Kumar, R., and Raghava, G. P. S. (2016). dbEM: a database of epigenetic modifiers curated from cancerous and normal genomes. *Sci. Rep. UK* 6:19340. doi: 10.1038/Srep19340
- Negishi, M., Wongpalee, S. P., Sarkar, S., Park, J., Lee, K. Y., Shibata, Y., et al. (2014). A new lncRNA, APTTR, associates with and represses the CDKN1A/p21 promoter by recruiting polycomb proteins. *PLoS One* 9:e95216. doi: 10.1371/journal.pone.0095216
- Ng, S. Y., Bogu, G. K., Soh, B. S., and Stanton, L. W. (2013a). The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. *Mol. Cell.* 51, 349–359. doi: 10.1016/j.molcel.2013.07.017
- Ng, S. Y., Lin, L., Soh, B. S., and Stanton, L. W. (2013b). Long noncoding RNAs in development and disease of the central nervous system. *Trends Genet.* 29, 461–468. doi: 10.1016/j.tig.2013.03.002
- Ng, S. Y., Johnson, R., and Stanton, L. W. (2012). Human long non-coding RNAs promote pluripotency and neuronal differentiation by association with chromatin modifiers and transcription factors. *EMBO J.* 31, 522–533. doi: 10.1038/emboj.2011.459
- Ni, Y. H., Huang, H., Chen, Y. Q., Cao, M. H., Zhou, H. Z., and Zhang, Y. Y. (2017). Investigation of long non-coding RNA expression profiles in the substantia nigra of Parkinson's disease. *Cell Mol. Neurobiol.* 37, 329–338. doi: 10.1007/s10571-016-0373-0
- O'leary, V. B., Hain, S., Maugg, D., Smida, J., Azimzadeh, O., Tapio, S., et al. (2017). Long non-coding RNA PARTICLE bridges histone and DNA methylation. *Sci. Rep. UK* 7:1790. doi: 10.1038/s41598-017-01875-1
- Paraskevopoulou, M. D., and Hatzigeorgiou, A. G. (2016). Analyzing MiRNA-LncRNA interactions. *Methods Mol. Biol.* 1402, 271–286. doi: 10.1007/978-1-4939-3378-5_21
- Peng, K., Su, G., Ji, J., Yang, X., Miao, M., Mo, P., et al. (2018). Histone demethylase JMJD1A promotes colorectal cancer growth and metastasis by enhancing Wnt/beta-catenin signaling. *J. Biol. Chem.* 293, 10606–10619. doi: 10.1074/jbc.RA118.001730
- Perry, R. B., and Ulitsky, I. (2016). The functions of long noncoding RNAs in development and stem cells. *Development* 143, 3882–3894. doi: 10.1242/dev.140962
- Pnueli, L., Rudnizky, S., Yosefzon, Y., and Melamed, P. (2015). RNA transcribed from a distal enhancer is required for activating the chromatin at the promoter of the gonadotropin alpha-subunit gene. *Proc. Natl. Acad. Sci. U.S.A.* 112, 4369–4374. doi: 10.1073/pnas.1414841112
- Ponting, C. P., Oliver, P. L., and Reik, W. (2009). Evolution and functions of long noncoding RNAs. *Cell* 136, 629–641. doi: 10.1016/j.cell.2009.02.006
- Preker, P., Almvig, K., Christensen, M. S., Valen, E., Mapendano, C. K., Sandelin, A., et al. (2011). PROMoter uPstream transcripts share characteristics with mRNAs and are produced upstream of all three major types of mammalian promoters. *Nucleic Acids Res.* 39, 7179–7193. doi: 10.1093/nar/gkr370
- Puvvula, P. K., Desetty, R. D., Pineau, P., Marchio, A., Moon, A., Dejean, A., et al. (2014). Long noncoding RNA PANDA and scaffold-attachment-factor SAFA control senescence entry and exit. *Nat. Commun.* 5:5323. doi: 10.1038/ncomms6323
- Ramos, A. D., Diaz, A., Nellore, A., Delgado, R. N., Park, K. Y., Gonzales-Roybal, G., et al. (2013). Integration of genome-wide approaches identifies lncRNAs of adult neural stem cells and their progeny in vivo. *Cell Stem. Cell* 12, 616–628. doi: 10.1016/j.stem.2013.03.003

- Rapicavoli, N. A., Poth, E. M., Zhu, H., and Blackshaw, S. (2011). The long noncoding RNA Six3OS acts in trans to regulate retinal development by modulating Six3 activity. *Neural Dev.* 6:32. doi: 10.1186/1749-8104-6-32
- Roberts, T. C., Morris, K. V., and Wood, M. J. (2014). The role of long non-coding RNAs in neurodevelopment, brain function and neurological disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130507. doi: 10.1098/rstb.2013.0507
- Sado, T., Hoki, Y., and Sasaki, H. (2005). Tsix silences Xist through modification of chromatin structure. *Dev. Cell* 9, 159–165. doi: 10.1016/j.devcel.2005.05.015
- Salameh, A., Lee, A. K., Cardo-Vila, M., Nunes, D. N., Efstathiou, E., Staquicini, F. I., et al. (2015). PRUNE2 is a human prostate cancer suppressor regulated by the intronic long noncoding RNA PCA3. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8403–8408. doi: 10.1073/pnas.1507882112
- Scarola, M., Comisso, E., Pascolo, R., Chiaradia, R., Marion, R. M., Schneider, C., et al. (2015). Epigenetic silencing of Oct4 by a complex containing SUV39H1 and Oct4 pseudogene lncRNA. *Nat. Commun.* 6:7631. doi: 10.1038/ncomms8631
- Scholz, C. J., Jacob, C. P., Buttenschon, H. N., Kittel-Schneider, S., Boreatti-Hummer, A., Zimmer, M., et al. (2010). Functional variants of TSPAN8 are associated with bipolar disorder and schizophrenia. *Am. J. Med. Genet.* 153B, 967–972. doi: 10.1002/ajmg.b.31057
- Seidman, L. J., and Mirsky, A. F. (2017). Evolving notions of schizophrenia as a developmental neurocognitive disorder. *J. Int. Neuropsychol. Soc.* 23, 881–892. doi: 10.1017/S1355617717001114
- Sheik Mohamed, J., Gaughwin, P. M., Lim, B., Robson, P., and Lipovich, L. (2010). Conserved long noncoding RNAs transcriptionally regulated by Oct4 and nanog modulate pluripotency in mouse embryonic stem cells. *RNA* 16, 324–337. doi: 10.1261/rna.1441510
- Shi, C. H., Zhang, L., and Qin, C. (2017). Long non-coding RNAs in brain development, synaptic biology, and Alzheimer's disease. *Brain Res. Bull.* 132, 160–169. doi: 10.1016/j.brainresbull.2017.03.010
- Song, Y., Xuan, A., Bu, C., Ci, D., Tian, M., and Zhang, D. (2018). Osmotic stress-responsive promoter upstream transcripts (PROMPTs) act as carriers of MYB transcription factors to induce the expression of target genes in *Populus simonii*. *Plant Biotechnol. J.* doi: 10.1111/pbi.12955 [Epub ahead of print].
- St Laurent, G., Wahlestedt, C., and Kapranov, P. (2015). The landscape of long noncoding RNA classification. *Trends Genet.* 31, 239–251. doi: 10.1016/j.tig.2015.03.007
- Sun, C., Huang, L., Li, Z., Leng, K., Xu, Y., Jiang, X., et al. (2018). Long non-coding RNA MIAT in development and disease: a new player in an old game. *J. Biomed. Sci.* 25:23. doi: 10.1186/s12929-018-0427-3
- Sun, S., Del Rosario, B. C., Szanto, A., Ogawa, Y., Jeon, Y., and Lee, J. T. (2013). Jpx RNA activates xist by evicting CTCF. *Cell* 153, 1537–1551. doi: 10.1016/j.cell.2013.05.028
- Sunwoo, J. S., Lee, S. T., Im, W., Lee, M., Byun, J. I., Jung, K. H., et al. (2017). Altered expression of the long noncoding RNA NEAT1 in Huntington's disease. *Mol. Neurobiol.* 54, 1577–1586. doi: 10.1007/s12035-016-9928-9
- Tang, J., Yu, Y. Z., and Yang, W. (2017). Long noncoding RNA and its contribution to autism spectrum disorders. *CNS Neurosci. Ther.* 23, 645–656. doi: 10.1111/cns.12710
- Tee, A. E., Ling, D., Nelson, C., Atmadibrata, B., Dinger, M. E., Xu, N., et al. (2014). The histone demethylase JMJD1A induces cell migration and invasion by up-regulating the expression of the long noncoding RNA MALAT1. *Oncotarget* 5, 1793–1804. doi: 10.18632/oncotarget.1785
- Tripathi, V., Ellis, J. D., Shen, Z., Song, D. Y., Pan, Q., Watt, A. T., et al. (2010). The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol. Cell* 39, 925–938. doi: 10.1016/j.molcel.2010.08.011
- Tsai, M. C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J. K., Lan, F., et al. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329, 689–693. doi: 10.1126/science.1192002
- Vance, K. W., Sansom, S. N., Lee, S., Chalei, V., Kong, L. S., Cooper, S. E., et al. (2014). The long non-coding RNA Paupar regulates the expression of both local and distal genes. *EMBO J.* 33, 296–311. doi: 10.1002/embj.201386225
- Wan, P. X., Su, W. R., and Zhuo, Y. H. (2017). The role of long noncoding RNAs in neurodegenerative diseases. *Mol. Neurobiol.* 54, 2012–2021. doi: 10.1007/s12035-016-9793-6
- Wang, K. C., and Chang, H. Y. (2011). Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 43, 904–914. doi: 10.1016/j.molcel.2011.08.018
- Wang, L., Zhao, Y., Bao, X., Zhu, X., Kwok, Y. K., Sun, K., et al. (2015a). LncRNA dum interacts with dnmts to regulate Dppa2 expression during myogenic differentiation and muscle regeneration. *Cell Res.* 25, 335–350. doi: 10.1038/cr.2015.21
- Wang, T. H., Lin, Y. S., Chen, Y., Yeh, C. T., Huang, Y. L., Hsieh, T. H., et al. (2015b). Long non-coding RNA AOC4P suppresses hepatocellular carcinoma metastasis by enhancing vimentin degradation and inhibiting epithelial-mesenchymal transition. *Oncotarget* 6, 23342–23357. doi: 10.18632/oncotarget.4344
- Wang, Y., Zhao, X., Ju, W., Flory, M., Zhong, J., Jiang, S., et al. (2015c). Genome-wide differential expression of synaptic long noncoding RNAs in autism spectrum disorder. *Trans. Psychiatry* 5:e660. doi: 10.1038/tp.2015.144
- Wang, Y., Pang, W. J., Wei, N., Xiong, Y., Wu, W. J., Zhao, C. Z., et al. (2014). Identification, stability and expression of Sirt1 antisense long non-coding RNA. *Gene* 539, 117–124. doi: 10.1016/j.gene.2014.01.037
- Wang, Y., Xu, Z., Jiang, J., Xu, C., Kang, J., Xiao, L., et al. (2013). Endogenous miRNA sponge lincRNA-RoR regulates Oct4, Nanog, and Sox2 in human embryonic stem cell self-renewal. *Dev. Cell* 25, 69–80. doi: 10.1016/j.devcel.2013.03.002
- Wilkinson, B., and Campbell, D. B. (2013). Contribution of long noncoding RNAs to autism spectrum disorder risk. *Int. Rev. Neurobiol.* 113, 35–59. doi: 10.1016/B978-0-12-418700-9.00002-2
- Wu, B., Chen, M., Gao, M., Cong, Y., Jiang, L., Wei, J., et al. (2018). Down-regulation of lncTCF7 inhibits cell migration and invasion in colorectal cancer via inhibiting TCF7 expression. *Hum. Cell* 32, 31–40. doi: 10.1007/s13577-018-0217-y
- Wu, D., Zhao, B., Cao, X., and Wan, J. (2017). Long non-coding RNA LINK-A promotes glioma cell growth and invasion via lactate dehydrogenase A. *Oncol. Rep.* 38, 1525–1532. doi: 10.3892/or.2017.5806
- Wu, H., Yang, L., and Chen, L. L. (2017). The diversity of long noncoding RNAs and their generation. *Trends Genet.* 33, 540–552. doi: 10.1016/j.tig.2017.05.004
- Wu, Y. T., Huang, C., Meng, X. M., and Li, J. (2015). Long noncoding RNA MALAT1: insights into its biogenesis and implications in human disease. *Curr. Pharm. Design* 21, 5017–5028. doi: 10.2174/1381612821666150724115625
- Xin, Y., Li, Z., Zheng, H. Y., Chan, M. T. V., and Wu, W. K. K. (2017). CCAT2: a novel oncogenic long non-coding RNA in human cancers. *Cell Prolif.* 50:e12342. doi: 10.1111/cpr.12342
- Xing, Z., Lin, A., Li, C., Liang, K., Wang, S., Liu, Y., et al. (2014). LncRNA directs cooperative epigenetic regulation downstream of chemokine signals. *Cell* 159, 1110–1125. doi: 10.1016/j.cell.2014.10.013
- Yang, F., Deng, X., Ma, W., Berletch, J. B., Rabaia, N., Wei, G., et al. (2015). The lncRNA Firre anchors the inactive X chromosome to the nucleolus by binding CTCF and maintains H3K27me3 methylation. *Genome Biol.* 16:52. doi: 10.1186/s13059-015-0618-0
- Yang, L., Lin, C., Jin, C., Yang, J. C., Tanasa, B., Li, W., et al. (2013). lncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. *Nature* 500, 598–602. doi: 10.1038/nature12451
- Yin, Y. F., Yan, P. X., Lu, J. L., Song, G., Zhu, Y. Y., Li, Z. H., et al. (2015). Opposing roles for the lncRNA haunt and its genomic locus in regulating HOXA gene activation during embryonic stem cell differentiation. *Cell Stem Cell* 16, 504–516. doi: 10.1016/j.stem.2015.03.007
- Young, T. L., Matsuda, T., and Cepko, C. L. (2005). The noncoding RNA Taurine upregulated gene 1 is required for differentiation of the murine retina. *Curr. Biol.* 15, 501–512. doi: 10.1016/j.cub.2005.02.027
- Yu, C. Y., and Kuo, H. C. (2016). The trans-spliced long noncoding RNA tsRMST impedes human embryonic stem cell differentiation through WNT5A-mediated inhibition of the epithelial-to-mesenchymal transition. *Stem Cells* 34, 2052–2062. doi: 10.1002/stem.2386
- Yu, L., Gong, X., Sun, L., Zhou, Q., Lu, B., and Zhu, L. (2016). The circular RNA Cdr1as act as an oncogene in hepatocellular carcinoma through targeting miR-7 expression. *PLoS One* 11:e0158347. doi: 10.1371/journal.pone.0158347
- Zhang, Y., Zhang, X. O., Chen, T., Xiang, J. F., Yin, Q. F., Xing, Y. H., et al. (2013). Circular intronic long noncoding RNAs. *Mol. Cell* 51, 792–806. doi: 10.1016/j.molcel.2013.08.017

- Zhao, J., Zhang, C., Gao, Z., Wu, H., Gu, R., and Jiang, R. (2018). Long non-coding RNA ASBEL promotes osteosarcoma cell proliferation, migration, and invasion by regulating microRNA-21. *J. Cell. Biochem.* 119, 6461–6469. doi: 10.1002/jcb.26671
- Zhao, Z. L., Senturk, N., Song, C. L., and Grummt, I. (2018). lncRNA PAPAS tethered to the rDNA enhancer recruits hypophosphorylated CHD4/NuRD to repress rRNA synthesis at elevated temperatures. *Gene Dev.* 32, 836–848. doi: 10.1101/gad.311688.118
- Zhao, L., Guo, H., Zhou, B., Feng, J., Li, Y., Han, T., et al. (2016). Long non-coding RNA SNHG5 suppresses gastric cancer progression by trapping MTA2 in the cytosol. *Oncogene* 35, 5770–5780. doi: 10.1038/onc.2016.110
- Zhou, Y., Zhong, Y., Wang, Y., Zhang, X., Batista, D. L., Gejman, R., et al. (2007). Activation of p53 by MEG3 non-coding RNA. *J. Biol. Chem.* 282, 24731–24742. doi: 10.1074/jbc.M702029200
- Ziats, M. N., and Rennert, O. M. (2013). Aberrant expression of long noncoding RNAs in autistic brain. *J. Mol. Neurosci.* 49, 589–593. doi: 10.1007/s12031-012-9880-8
- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MicroRNAs and Androgen Receptor: Emerging Players in Breast Cancer

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Breast cancer (BC) is the most common cause of cancer among women, with a high incidence rate occurrence every year worldwide despite advances in its management. BC is characterized by a spectrum of subtypes which respond differently to treatments due to their biological features, representing the main issue in the control of this type of malignancy. Androgen receptor (AR) is emerging as a target to investigate among hormone receptors, since it seems to play a role at various stages of development of specific BC subsets. For this reason, in recent years AR has become very important in the clinical practice, although its role remains controversial. A number of studies have proposed a correlation between microRNAs (miRNAs), a class of gene expression modulators, and AR in prostate cancer (PC), but there are still few evidences about the relationship between miRNAs and AR in BC. The purpose of this review is to present a state of the art scenario with consideration to the most recent discoveries about miRNAs involved in the AR associated pathogenesis of BC, in order to provide new insights into the role of miRNAs as key drivers in the modulation of AR, and possible actors in the development and progression of BC. Moreover, we consider findings about involvement of AR signaling in all stages of BC, highlighting its association with different subsets of breast carcinomas and with pre- and postmenopausal state of patients.

Keywords: microRNAs, androgens, receptor, breast, cancer

OPEN ACCESS

Edited by:

Yujing Li,
Emory University, United States

Reviewed by:

Peter Igaz,
Semmelweis University, Hungary
Roberto Gherzi,
University of California, San Diego,
United States

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 18 October 2018

Accepted: 26 February 2019

Published: 19 March 2019

Citation:

Bandini E and Fanini F (2019)
MicroRNAs and Androgen Receptor:
Emerging Players in Breast Cancer.
Front. Genet. 10:203.
doi: 10.3389/fgene.2019.00203

INTRODUCTION

Breast cancer (BC) is the most common cause of cancer among women and in 2018 in the United States 266,120 new cases and 40,920 deaths have been estimated (Siegel et al., 2018). Despite advances in the management of the disease, a high incidence rate occurs every year worldwide as a consequence of several factors such as socioeconomic differences in the population (Lukong et al., 2017; Dean et al., 2018), ethnicity (DeSantis et al., 2017; Foy et al., 2018), dietary habits (Tabung et al., 2016; Nattenmüller et al., 2018), and disparities in screening programs (Miglioretti et al., 2015). The study of BC has highlighted a substantial tissue heterogeneity, showing several molecular profiles each with distinct clinical and biological features (Perou et al., 2000) which make this tumor differently responsive to treatments, and adverse in its management. In the last years, molecular profiling by gene expression and transcriptional studies has provided an important tool to classify BCs into four well-established subtypes: Luminal A, Luminal B, Basal-like, and

Abbreviations: AR, androgen receptor; BC, breast cancer; DFS, disease-free survival; DHT, dihydrotestosterone; E2, β -estradiol; ER, estrogen receptor; miRNAs, microRNAs; MA, molecular apocrine; OS, overall survival; PC, prostate cancer; PGR, progesterone receptor; RFS, relapse-free survival; TNBC, triple negative breast cancer.

human epidermal growth factor receptor 2 (Her2)-enriched (Parker et al., 2009). Among Basal like group, TNBC represents a heterogeneous category of cancer whose immunohistochemical classification lacks of ER, PGR, and HER2 protein expression. Up to day, several studies have been conducted in order to better identify molecular-based therapies. Integrated molecular analyses have significantly enhanced the knowledge about genomic drivers of the most common BC subtypes, giving prominence to the discovery of novel subtype-specific targets that can be exploited in the future especially for the treatment of TNBCs (Koboldt et al., 2012; Burstein et al., 2015). Lehmann et al. analyzed 587 TNBC cases and identified 6 TNBC subtypes displaying unique gene expression profile: two basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL) and a Luminal Androgen Receptor subtype (LAR), composed of AR driven tumors, and that has been suggested to be correlated to the previously identified by Farmer et al. “Molecular Apocrine” subtype (MA) (Farmer et al., 2005; Lehmann et al., 2011; Lehmann-Che et al., 2013). Among these subtypes, LAR type was found to be associated with older patient age, apocrine histologic features, low density of stromal tumor-infiltrating lymphocytes (TIL), and low Ki-67 labeling index (Kim et al., 2018). Sex steroid hormone receptors, ER and PGR, have always played a leading role in the development and progression of BC; however, in the last years, AR has emerged as a prominent player to focus attention on. In terms of therapeutic options, AR may provide a further strategy to counteract breast malignancy, especially in patients with ER negative (ER-) tumors that do not benefit from endocrine or Her2 targeted treatments. In the last decades several small molecules have been identified as critical regulators of transcription and translation of proteins involved in tumorigenesis, and among them miRNAs are the most studied. They belong to a broad family of small non-coding RNAs, and they deserve great attention since they can modify the expression of tumor suppressor genes or oncogenes, affecting signaling pathways of cancer cells. The function of miRNAs in BC has been deeply explored, with the first miRNA signature reported by Iorio et al., and followed by a plethora of studies that have determined a functional role of miRNAs in the disease (Iorio et al., 2005). Anyway, little is known about the emerging dysregulation mechanisms of miRNAs in the context of hormonal signaling, especially for androgens. In this review, we present a state of the art scenario about the role of AR in BC, highlighting the main issues about this “player” that is very debating especially for what concerns its function in different subsets of breast carcinomas. Moreover, we focus on the most recent discoveries about miRNAs involved in the AR associated pathogenesis of BC, since so far this topic has been considered almost exclusively in PC.

THE ROLE OF ANDROGEN RECEPTOR IN BREAST CANCER

Androgen receptor is a member of the family of steroid nuclear receptors which mediates the biological effects of androgens. It is well established that AR is considered an oncogenic driver at

all stages of PC, but its role in BC remains controversial. In fact, expression of AR splice variants (ARVs) has been elucidated in PC, but only recently the presence of multiple known and novel ARV transcripts has been demonstrated in a panel of BC cell lines and human tissues: AR-V1, -V3, -V4, -V7, and -V9 (Hu et al., 2014). In particular, AR-V7 was observed to be constitutively active and involved in androgen deprivation resistance in more than 50% of BC cases (Hickey et al., 2015). Whereas AR and ER- α have a quite similar structure and are co-expressed by many BCs, the role of AR may be different depending on the levels of both hormone receptors in the tumor environment. This becomes important in the evaluation of clinical practice, as in pre-clinical models of BC AR was able either to stimulate or inhibit cell proliferation (Macedo et al., 2006; Lin et al., 2009).

In the last years several studies have focused on the role of AR in ER- α positive (ER+) BCs, since there would seem to be a correlation between its expression grade and some clinical advantages. In fact high levels of AR are associated to reduced lymph node involvement, better DFS, RFS, and OS, response to endocrine therapies and chemotherapy, lower tumor grade, Ki67 expression, smaller tumor size and less necrosis, suggesting for AR a possible role as a tumor-suppressor in malignant breast epithelial cells (Peters et al., 2009; Castellano et al., 2010; Vera-Badillo et al., 2014). Rangel et al. investigated the prognostic impact of AR/ER ratio in 402 ER+ BC patients, showing its inverse relation with aggressiveness of biological features and worse prognosis (Rangel et al., 2018b). Similarly, Basile et al. reported that a high AR/ER ratio seems to be detrimental in BC treated with endocrine therapy (Basile et al., 2017), while in 2 validated BC cohorts, ER+ patients with AR positivity $\geq 78\%$ had the best survival, and among them those with a ratio of AR:ER $\alpha > 0.87$ exhibited the best outcomes (Ricciardelli et al., 2018). In a study involving 479 BC women, it has been evidenced that in ER+ patients the expression of forkhead box A1 (FOXA1), a pioneer factor which helps the recruitment of ER and AR to their response elements on the genome, was directly correlated to the presence of AR and to better outcome, providing additional knowledge about recurrence (Rangel et al., 2018a). Also Park et al. confirmed that ER+ patients with low expressed AR and FOXA1 tumors were significantly correlated to worse RFS (Park et al., 2017). Moreover, recent data showed that over 90% of metastasis from luminal tumors preserved FOXA1 expression (Ross-Innes et al., 2012), and the concomitant expression of AR and FOXA1 in metastatic lesions may promote the luminal to MA transition. In TNBCs, AR is expressed in 10–43% of cases but its prognostic value remains still unclear. Actually, larger cohort numbers should be needed to determine a role for AR in this peculiar subtype. In some studies involving TNBC cases, the presence of AR appeared correlated to an increase in overall mortality, lymph node metastasis and higher tumor stage (Hu et al., 2011; McGhan et al., 2014). Conversely, another group demonstrated that androgen pathways are associated with reduced aggression TNBC, and that AR loss may have a role in the progression of the tumor (McNamara et al., 2014). A meta-analysis involving 13 studies with 2826 TNBC cases, suggested a potential role of AR in a lower risk of recurrence highlighting that AR positive women showed prolonged DFS (Wang et al., 2016). After analysis of 135

invasive TNBC cases, AR and epidermal growth factor receptor (EGFR) expression was evaluated in order to stratify TNBCs into three risk groups: low risk (AR+ EGFR−) characterized by better prognosis and beneficial from anti-androgen therapies; high risk (AR− EGFR+) with worst prognosis, but better responsiveness to chemotherapy; and intermediate-risk (AR+ EGFR+, AR−, EGFR−) (Astvatsaturyan et al., 2018).

Molecular apocrine subtype has been studied *in vitro* using BC cell lines whose growth was promoted by AR expression. Robinson et al. demonstrated that in the absence of ER- α more than a half of AR binding events showed an analogous pattern to that of ER- α in ER+ cells, promoting the expression of ER target genes, and suggesting a role of AR as a ER- α mimic (Robinson et al., 2011). Anyway, the biological interaction between ER- α and AR still needs to be clarified. Curiously, in a transcriptomic study involving male BC, chromatin binding landscape of ER in relation to steroid hormone receptors including AR, was determined. Results showed that AR pathway was the only hormonal signaling more associated with the ER- α binding genes, confirming that genomic functions of ER- α and AR in BC are largely overlapping (Severson et al., 2018). For what concerns HER2-enriched BC subtype, it has been found strongly related to MA and studies have suggested a strong evidence of the proliferative role of AR (Ni et al., 2011; Chia et al., 2015). Lehmann-Che et al. tried to characterize MA tumors and found that they were all defined ER−, AR+, FOXA1+, with an overexpression of HER2 or prolactin induced protein (GCDFP15), useful for discriminating MA from basal-like (BL) in the context of ER− tumors. This distinction can be useful to include MA patients in specific “AR pathway” trials, being this subtype rather aggressive (Lehmann-Che et al., 2013). There are evidences that AR can promote ERK activation up-regulating HER2 gene transcription, therefore contributing to the growth of Her2+ BC (Naderi and Hughes-Davies, 2008; Chia et al., 2011). More recently, the functional role of AR was investigated by silencing assays and a reduction in the growth of Her2+ BC cells HCC1954 and SKBr3 was observed, also after treatment with

the androgen antagonist Enzalutamide, highlighting a function of AR in promoting the growth of Her2+ BC cells (He et al., 2017). Daemen and Manning explored HER2 amplification in 3155 breast tumors and found that the HER2-enriched (HER2E) subtype had a distinct transcriptional landscape independent of HER2-amplified (HER2A) that reflected and confirmed how AR signaling can replace ER-driven tumorigenesis (Daemen and Manning, 2018). In a study involving 1297 primary tumors and 336 paired axillary lymph node metastases, Kraby et al. found a highest proportion of AR positivity in the Luminal B subtype while the lowest was observed in the basal phenotype. Interestingly, in 60/72 cases a changeover from AR− primary tumor to AR+ lymph node metastasis occurred. Moreover, in primary tumors AR expression was an independent and favorable prognostic marker, particularly in the Luminal A subtype, and in grade 3 tumors (Kraby et al., 2018). All these observations underline the need for a more detailed classification of tumor samples aimed at a more targeted and personalized treatment of patients. The role of AR in BC subtypes is resumed in Table 1.

AR is expressed in all stages of BC (*in situ*, primary and metastatic). In fact, it is estimated that up to 90% of primary BC and up to 75% of metastatic lesions expressed AR (Hickey et al., 2012), as well as in the 50–80% of invasive BCs and in the 85% of ductal carcinoma *in situ* (DCIS) (Lim et al., 2014), although among the BC subtypes the frequency appears variable. Nevertheless, its role in breast carcinogenesis remains a debated topic as its contribution to the different tumor stages development and progression still needs to be clarified. Feng et al. reported the involvement of DHT in the initiation of epithelial-to-mesenchymal transition (EMT) of BC cells in an AR-dependent but ER-independent manner, indicating the role of androgens in cancer invasion and metastasis (Feng et al., 2017). Schrijver et al. investigated receptor conversion in 91 effusion metastasis, pleural and peritoneal, of 69 patients by immunohistochemistry and *in situ* hybridization. AR receptor status changed from positive in the primary tumor to negative in the effusion metastases or *vice versa* in 46–51% of cases, and

TABLE 1 | The role of AR in BC subtypes.

Tumor subtype	AR role	Reference
ER+	Tumor-suppressor: associated with low aggressiveness and better outcome	Peters et al., 2009; Castellano et al., 2010; Vera-Badillo et al., 2014; Basile et al., 2017; Kim et al., 2017; Rangel et al., 2018a,b; Ricciardelli et al., 2018
TNBC	Tumor-suppressor: associated with low aggressiveness and progression, and better outcome Oncogenic: associated with aggressiveness and worse outcome	McNamara et al., 2014; Wang et al., 2016 Hu et al., 2011; McGhan et al., 2014
TNBC AR+/EGFR− TNBC AR+/EGFR+ TNBC AR-/EGFR− TNBC AR-/EGFR+	Associated with better prognosis Group with intermediate risk Associated with worse prognosis	Astvatsaturyan et al., 2018
MA ER− HER2-enriched	ER mimic Proliferative	Robinson et al., 2011; Severson et al., 2018 Naderi and Hughes-Davies, 2008; Ni et al., 2011; Chia et al., 2015; He et al., 2017; Daemen and Manning, 2018
Luminal A primary tumors	Favorable prognostic marker	Kraby et al., 2018

this was more often associated in patients previously treated with ET (Schrijver et al., 2017). This new finding could be relevant for investigating AR-targeted therapies in ER- and endocrine resistant BC. RNA sequencing was performed to investigate CTCs isolated from blood samples of patients with metastatic ER+ BC, and a comparison between cases with progression in bone vs. visceral organs was made. Results showed that the most activated pathway in CTCs from bone was that of AR, especially involving splice variant AR-V7. Curiously, AR expression within CTCs was associated with the duration of treatment with aromatase inhibitors (AIs), proposing a possible mechanism in the contribution of acquired resistance to ET, and underlying the role of AR in BC bone metastasis together with the therapeutic option of its targeting in patients with metastatic setting (Aceto et al., 2018). Usually, the maintenance of the balance between DHT, the most potent endogenous AR ligand derived from testosterone (Labrie et al., 2003; Gao et al., 2005), and E2 ensures the physiological response of the breast tissue, including BC tissue, depending on the hormonal needs and the menopausal status. In fact, the circulating androgens concentration varies in woman in relation to pre and postmenopause state (Giovannelli et al., 2018). Whereas after menopause circulating level of E2 decrease dramatically up to 10-fold, androgens begin to acquire an important function (Rothman et al., 2011). Several studies have tried to analyze the correlation between circulating androgens and BC growth since this relationship remains unclear, although up to now a high serum testosterone level has been associated with an increased risk in postmenopausal women. It follows that an additional complication in understanding the role of AR is to be attributed to the menopausal state of patients, which seems to be a more significant variable than age. It would be important to distinguish between the intratumoral estrogen or androgen production, and to take into consideration the balance between these different sex hormones. The most of breast tumors are estrogen-dependent and are characterized by a high expression of ER that could interfere with the activity of AR and *vice versa*. Premenopausal patients BC tissues are characterized by higher production of estrogen, and in these individuals ovary is the main source of E2. Otherwise, in postmenopausal state estrogens derived from circulating adrenal androgens, such as androstenedione, and in these patients BC tissues presents lower levels of E2 and higher androgen levels (Takagi et al., 2018). How hormonal changes influence cancer development is still a discussed issue. Curiously, data showed that in recent decades incidence rates of advanced BC have increased for premenopausal women (Fahlén et al., 2018).

THE INTERACTION BETWEEN miRNAs AND ANDROGEN RECEPTOR IN BREAST CANCER

MicroRNAs are the most explored non-coding RNAs, and give rise to a large family of short (19–24 nucleotides) single-strand RNAs which take part in a variety of biological processes, such as cell proliferation, death, differentiation, and stress response (Bartel, 2004; Kozomara and Griffiths-Jones, 2014). They operate

recognizing a 2–7 nucleotides “seed-region” in the target mRNA, which can be localized in the 3′-UTR (Lewis et al., 2005), in the 5′-UTR (Lytle et al., 2007), or in the coding region (Forman et al., 2008). Their regulatory function on gene expression is performed through the control of translation of the mRNA target, which can result in downregulation but also in upregulation of the encoded protein (Ambros, 2004; Vasudevan et al., 2007). A decisive turning point was given by Fabbri et al., who highlighted for the first time the ability of miRNAs secreted by tumor-derived exosomes (TEX) to act as paracrine agonists of a specific receptor family suggesting an involvement in the tumor microenvironment interaction and a new possible target for cancer treatment (Fabbri et al., 2012). On this trail, other groups started to analyze the implication of miRs in tumor communication, growth and spread, and recently it has been demonstrated how breast-cancer TEX are able to carry precursor miRNAs (pre-miRNAs) complexed with Dicer, TRBP and AGO2 proteins displaying a cell-independent capacity to process pre-miRNAs into mature form, contributing to the comprehension of a cell-autonomous process occurring in exosomes when secreted into the extracellular space (Melo et al., 2014).

A number of studies have proposed a correlation between miRNAs and AR in PC (Shi et al., 2007; Epis et al., 2009; Ribas et al., 2009; Cao et al., 2010; Nadiminty et al., 2012), but there are still few evidences about the possible role of miRNAs in regulating AR expression in BC. For the first time, Nakano et al., through miRNAs Polymerase Chain reaction (PCR) Arrays, identified miR-363 as an androgen-inducible miRNA. In MCF-7 BC cells they highlighted a possible androgens-related feedback loop involving the gene *IQWD1* (IQ motif and WD repeats-1) and miR-363: under low androgens levels *IQWD1* was downregulated by miR-363, but this negative modulation did not occurred after DHT administration (**Figure 1A**). Interestingly, *IQWD1* has a role in protecting AR proteins from degradation *via* proteasome (Nakano et al., 2013). In AR+/ER- models, androgens seemed to mediate a negative correlation between miR-let-7a and the expression of its target oncogenes CMYC and KRAS. In particular, in the MA MDA-MB 453 and in the TNBC MDA-MB 231 cell lines treated with DHT a significant increase in let-7a expression was observed together with a decrease of CMYC and KRAS (**Figure 1B**). Similarly, in BC tissues the negative correlation was confirmed by IHC, highlighting a new androgen-induced AR activating signal pathway that directly upregulates let-7a and negatively regulates CMYC and KRAS, inhibiting proliferation of AR+/ER- cells (Lyu et al., 2014). Results about the tumor suppressive role of let-7a were confirmed also in AR+/ER+ BC cells, where DHT stimulation led to an AR translocation to the nucleus with transcriptional upregulation of let-7a, decreased cell proliferation, self-renewal capacities, invasion and migration (Zhang et al., 2018). Moreover, in order to deepen the effects of let-7a/AR pathway on breast tumor-initiating cells, Zhang et al. examined the expression of AR, let-7a and CD44⁺/CD24^{-/low} in invasive BC tissues. AR was significantly correlated to let-7a and CD44⁺/CD24^{-/low}, highlighting that patients expressing AR and let-7a could have a better outcome, unlike those with a CD44⁺/CD24^{-/low} phenotype which showed a worse prognosis. These findings put

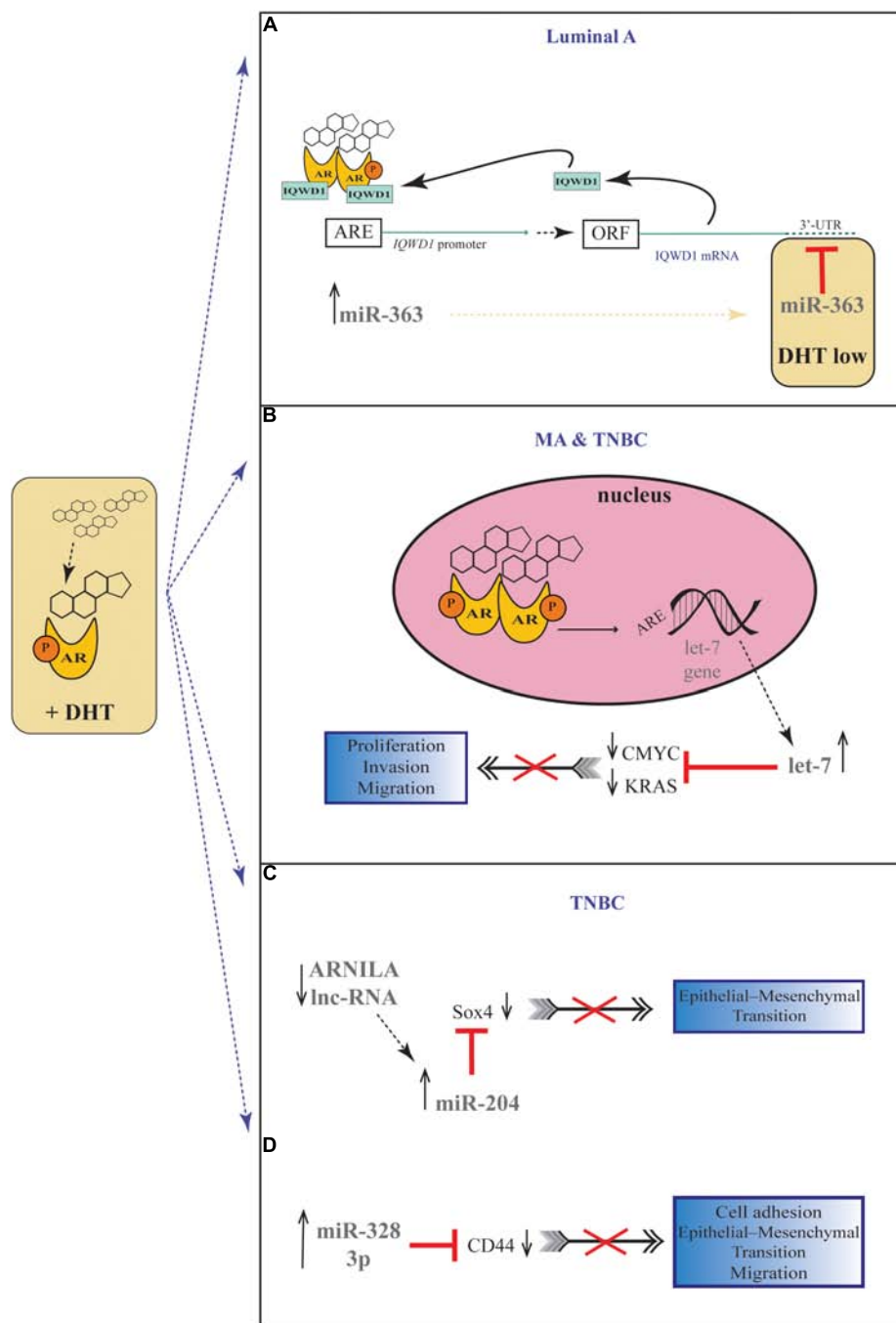
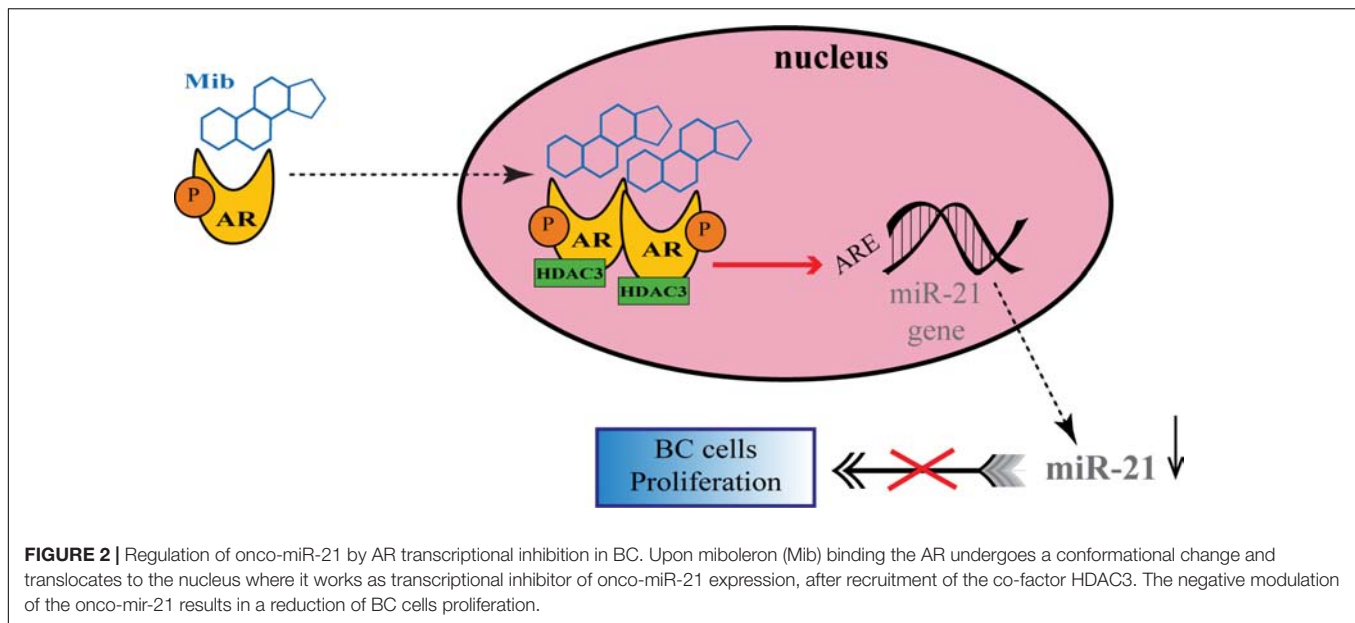


FIGURE 1 | Androgenic-inducible miRNAs involved in the process of BC progression. **(A)** In Luminal A BC cells, DHT treatment induces an androgen-related miRNA-mRNA pathway, involving miR-363 and its possible target gene *IQWD1*. In the presence of high levels of androgens, a *IQWD1* feed-forward regulation activates its own AR-mediated expression (Chen et al., 2007), and miR-363 significantly increased. Under relatively low level of DHT, *IQWD1* is negatively regulated by miR-363. **(B)** In MA and TNBC cells, the DHT administration results in an androgen-induced AR activating signal pathway which upregulates let-7 expression and negatively regulates *CMYC* and *KRAS* that are targets of let-7. **(C)** In TNBC cells, the lncRNA *ARNILA* is negatively regulated by AR after DHT treatment, causing a decreased adsorption of miR-204 which in turn inhibits Sox4 expression, a gene known to promote EMT. **(D)** In TNBC cells, DHT induces upregulation of miR-328-3p with concomitantly decrease of its target CD44, diminishing EMT, migration and adhesion.

in evidence that DHT-induced AR activation plays a critical role in BC, and that AR/let-7a signaling could be exploited as a new optional therapeutic target (Zhang et al., 2018). Another

study evidenced the interaction between AR and miRNAs in controlling BC cells behavior. Three BC cell lines (Luminals and MA subtypes) were screened for 84 miRNAs showing each of



them a distinct basal miRNAs expression profile. High level of let-7a and -7b found in MA-MDA-453 appeared to be distinctive for MA subtype, whereas miR-205 seemed to represent a marker in the luminal T47D and MCF-7 cells. Furthermore, treatment with the AR agonist CI-4AS-1 led to alterations in the expression profile of other micro-RNAs, such as miR-100 and miR-125 which were found significantly downregulated simultaneously with the increase and extracellular release of metalloprotease-13 (MMP13). Interestingly, the transfection of miR-100 and -125b abrogated the induction of MMP13, suggesting a correlation between these micro-RNAs and AR in the control of BC growth (Ahram et al., 2017). In TNBC, the gene SRY-box 4 (*Sox4*) is known to promote EMT, thereby progression, invasion and metastasis, and is found abnormally overexpressed. Yang et al. identified an AR negatively induced long non-coding RNA (lncRNA) ARNILA that correlated to poor PFS, tightly connected to AR and able to sequester miR-204, in turn facilitating the expression of its target *Sox4*. Particularly in AR+ carcinomas, ARNILA is suppressed by the action of DHT and AR, resulting in the decreased adsorption of miR-204 thus favoring *Sox4* expression inhibition. On the other hand, in AR- tumors, the action of ARNILA leads to *Sox4* expression by increasing the sequestration of miR-204, leading to the induction of EMT and metastatic propagation (Figure 1C). All together these findings spread light to the discovery of new lncRNA/miRNA/AR mechanisms correlated with poor clinical outcome by regulating EMT, migration, and invasion in TNBC (Yang et al., 2018). Again in TNBC, investigating the modulation of miRNAs after treatment with DHT a group reported miR-328-3p as the most upregulated. Concomitantly, CD44 target of miR-328-3p, decreased, diminishing cell adhesion, migration and EMT, and this result was confirmed also after miR-328-3p mimic transfection (Figure 1D) (Al-Othman et al., 2018). In another study, a total of 153 miRNAs were found to be differentially expressed in AR+ vs. AR- cell lines. The authors identified

miR-143, -145, -31, -30c, -30b-3p, 199a, and -181 as significantly downregulated in AR+ cells, while miR-933 and -5793 appeared as the most upregulated, suggesting a role for these miRNAs in the regulation of AR in BC (Shi et al., 2017). Again, the interaction between miR-30a and AR was explored, and miR-30a role was investigated in ER-, PR-, AR+, MDA-MB-453 BC cells. After DHT treatment, which activates Androgen-induced AR signal, a miRNAs profile was identified by miRNAs array, showing a downregulated expression of miR-30a, b and c (among which the most downregulated was miR-30a), and an upregulated expression of AR. Interestingly, in the AR mRNA 3'-untranslated region resides a bioinformatic putative miR-30a, b and c binding site confirming AR as a direct target of miR-30a. Nevertheless, AR does not bind miR-30a promoter region which could be downregulated through other AR-induced cell signaling pathways. This study identified a positive feedback mechanism of regulation which could be explained by two effects. First, the activation of AR expression and AR-induced signal downregulates miR-30a expression that in turn promotes AR availability. Second, the downregulation of miR-30a expression has a negative effect on the inhibition of cell growth induced by itself, being miR-30a a cancer suppressor gene (Lyu et al., 2017). Interestingly, Casaburi et al. reported that androgens can reduce BC cells proliferation by negative modulation of the onco-miR-21. By treatment with synthetic androgen miboleron (Mib) and Chromatin immune-precipitation (ChIP) analysis, they provided evidence that activated AR works as a transcriptional inhibitor of miR-21 expression. In particular, AR was able to bind the proximal promoter of miR-21 in a specific ARE sequence, involving the recruitment of HDAC3 as co-factor in the AR-mediated transcriptional repression (Figure 2). This hypothesis was also supported by a significant reduction of PolII binding in Mib treated extracts, providing further evidence about the protective role of androgens in BC cells (Casaburi et al., 2016).

DISCUSSION AND FUTURE HORIZONS

Many studies support the idea that BC is a heterogeneous pathology and this consideration is mainly motivated by the existence of different subtypes classified on the basis of hormone receptor expression. About 70–80% of BC express considerable level of ER and are estrogen-dependent (Takagi et al., 2018), but, as well, approximately 70–90% of them express AR, and close to 75% are considered to express both AR and ER (Vera-Badillo et al., 2014) suggesting a role of androgen hormones in the pathogenesis of BC. Although up today the association between levels of circulating androgens and BC risk is still under discussion, several studies identified AR as a marker of favorable prognosis and have demonstrated the anti-proliferative effects of androgens in BC cells (Andò et al., 2002; Lanzino et al., 2010; Takagi et al., 2010). Definitely, in this moment the scientific community can only state that the AR positivity makes more intricate the BC molecular outlook. It has been suggested that circulating androgens may have a role both as independent molecules and as substrate for estrogen synthesis, but limited to AR+/ER+ BC since in AR+/ER– BC they may act in a more homogenous way (Giovannelli et al., 2018). On the basis of different androgens effects on BC, several approaches having AR as a target have been evaluated, including AR agonists and antagonists.

Since the findings that miRNAs play a role in carcinogenesis and are deregulated in several types of tumor, they have obtained a lot of interest about their potential use as therapeutic agents in chemotherapy. Furthermore, the supposing of the existence of

a specific uptake can help the purpose to customize miRNAs as therapeutics alone or, more likely, in combination with today's anti-cancer therapies. Surely more stress should be placed on understanding the balance between AR and ER in relation to the different subtypes, which gives rise to main questions regarding a different response to endocrine therapies in BC. For instance, the interesting positive feedback mechanism identified by Lyu et al. between AR and miR-30a could be a starting point for further studies about the role of miRNAs as a therapeutic predictive markers, besides the identification of other miRs that are able to target AR, or molecules involved in the AR pathway, can certainly help to find more answers about this interaction (Lyu et al., 2017). Also, not to be underestimated is the recent intriguing branch of miRceptor which fits very well in the context of BC as a hormone-dependent tumor, and which is linked to broader themes such as the study of the tumor microenvironment. Consequently, it is reasonable to foresee how the interaction between miRNAs and AR in BC can become in the future an extensively investigated field, in order to increase the treatment chances and try to get much closer to BC personalized therapies.

AUTHOR CONTRIBUTIONS

EB wrote the first draft of the manuscript. FF contributed to the writing of the manuscript. FF and EB drew figures and tables. Both authors critically reviewed the manuscript and approved the final version of the manuscript.

REFERENCES

- Aceto, N., Bardia, A., Wittner, B. S., Donaldson, M. C., O'Keefe, R., Engstrom, A., et al. (2018). AR expression in breast cancer CTCs associates with bone metastases. *Mol. Cancer Res.* 16, 720–727. doi: 10.1158/1541-7786.MCR-17-0480
- Ahram, M., Mustafa, E., Zaza, R., Abu Hammad, S., Alhudhud, M., Bawadi, R., et al. (2017). Differential expression and androgen regulation of microRNAs and metalloprotease 13 in breast cancer cells. *Cell Biol. Int.* 41, 1345–1355. doi: 10.1002/cbin.10841
- Al-Othman, N., Hammad, H., and Ahram, M. (2018). Dihydrotestosterone regulates expression of CD44 via miR-328-3p in triple-negative breast cancer cells. *Gene* 675, 128–135. doi: 10.1016/j.gene.2018.06.094
- Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431, 350–355. doi: 10.1038/nature02871
- Andò, S., De Amicis, F., Rago, V., Carpino, A., Maggiolini, M., Panno, M. L., et al. (2002). Breast cancer: from estrogen to androgen receptor. *Mol. Cell. Endocrinol.* 193, 121–128. doi: 10.1016/S0303-7207(02)00105-3
- Astvatsaturyan, K., Yue, Y., Walts, A. E., and Bose, S. (2018). Androgen receptor positive triple negative breast cancer: clinicopathologic, prognostic, and predictive features. *PLoS One* 13:e0197827. doi: 10.1371/journal.pone.0197827
- Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. doi: 10.1016/S0092-8674(04)00045-5
- Basile, D., Cinausero, M., Iacono, D., Pelizzari, G., Bonotto, M., Vitale, M. G., et al. (2017). Androgen receptor in estrogen receptor positive breast cancer: beyond expression. *Cancer Treat. Rev.* 61, 15–22. doi: 10.1016/j.ctrv.2017.09.006
- Burstein, M. D., Tsimelzon, A., Poage, G. M., Covington, K. R., Contreras, A., Fuqua, S. A. W., et al. (2015). Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. *Clin. Cancer Res.* 21, 1688–1698. doi: 10.1158/1078-0432.CCR-14-0432
- Cao, P., Deng, Z., Wan, M., Huang, W., Cramer, S. D., Xu, J., et al. (2010). MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1 α /HIF-1 β . *Mol. Cancer* 9:108. doi: 10.1186/1476-4598-9-108
- Casaburi, I., Cesario, G. M., Donà, A., Rizza, P., Aquila, S., Avena, P., et al. (2016). Androgens downregulate miR-21 expression in breast cancer cells underlining the protective role of androgen receptor. *Oncotarget* 7, 12651–12661. doi: 10.18632/oncotarget.7207
- Castellano, I., Allia, E., Accortanzo, V., Vandone, A. M., Chiusa, L., Arisio, R., et al. (2010). Androgen receptor expression is a significant prognostic factor in estrogen receptor positive breast cancers. *Breast Cancer Res. Treat.* 124, 607–617. doi: 10.1007/s10549-010-0761-y
- Chen, P.-H., Tsao, Y.-P., Wang, C.-C., and Chen, S.-L. (2007). Nuclear receptor interaction protein, a coactivator of androgen receptors (AR), is regulated by AR and Sp1 to feed forward and activate its own gene expression through AR protein stability. *Nucleic Acids Res.* 36, 51–66. doi: 10.1093/nar/gkm942
- Chia, K., O'Brien, M., Brown, M., and Lim, E. (2015). Targeting the androgen receptor in breast cancer. *Curr. Oncol. Rep.* 17:4. doi: 10.1007/s11912-014-0427-8
- Chia, K. M., Liu, J., Francis, G. D., and Naderi, A. (2011). A feedback loop between androgen receptor and ERK signaling in estrogen receptor-negative breast cancer. *Neoplasia* 13, 154–166. doi: 10.1593/neo.101324
- Daemen, A., and Manning, G. (2018). HER2 is not a cancer subtype but rather a pan-cancer event and is highly enriched in AR-driven breast tumors. *Breast Cancer Res.* 20:8. doi: 10.1186/s13058-018-0933-y
- Dean, L. T., Gehlert, S., Neuhaus, M. L., Oh, A., Zanetti, K., Goodman, M., et al. (2018). Social factors matter in cancer risk and survivorship. *Cancer Causes Control* 29, 611–618. doi: 10.1007/s10552-018-1043-y

- DeSantis, C. E., Ma, J., Goding Sauer, A., Newman, L. A., and Jemal, A. (2017). Breast cancer statistics, 2017, racial disparity in mortality by state. *CA Cancer J. Clin.* 67, 439–448. doi: 10.3322/caac.21412
- Epis, M. R., Giles, K. M., Barker, A., Kendrick, T. S., and Leedman, P. J. (2009). miR-331-3p regulates ERBB-2 expression and androgen receptor signaling in prostate cancer. *J. Biol. Chem.* 284, 24696–24704. doi: 10.1074/jbc.M109.030098
- Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., et al. (2012). MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2110–E2116. doi: 10.1073/pnas.1209414109
- Fahlén, M., Zhang, H., Löfgren, L., Masironi, B., Von Schoultz, E., Von Schoultz, B. O., et al. (2018). Expression of progesterone and androgen receptors in the breast of premenopausal women, considering menstrual phase. *Anticancer Res.* 38, 1499–1510. doi: 10.21873/anticancer.12377
- Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., et al. (2005). Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene* 24, 4660–4671. doi: 10.1038/sj.onc.1208561
- Feng, J., Li, L., Zhang, N., Liu, J., Zhang, L., Gao, H., et al. (2017). Androgen and AR contribute to breast cancer development and metastasis: an insight of mechanisms. *Oncogene* 36, 2775–2790. doi: 10.1038/onc.2016.432
- Forman, J. J., Legesse-Miller, A., and Collier, H. A. (2008). A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14879–14884. doi: 10.1073/pnas.0803230105
- Foy, K. C., Fisher, J. L., Lustberg, M. B., Gray, D. M., DeGraffinreid, C. R., and Paskett, E. D. (2018). Disparities in breast cancer tumor characteristics, treatment, time to treatment, and survival probability among African American and white women. *NPJ Breast Cancer* 4:7. doi: 10.1038/s41523-018-0059-5
- Gao, W., Bohl, C. E., and Dalton, J. T. (2005). Chemistry and structural biology of androgen receptor. *Chem. Rev.* 105, 3352–3370. doi: 10.1021/cr020456u
- Giovannelli, P., Di Donato, M., Galasso, G., Di Zazzo, E., Bilancio, A., and Migliaccio, A. (2018). The androgen receptor in breast cancer. *Front. Endocrinol.* 9:492. doi: 10.3389/fendo.2018.00492
- He, L., Du, Z., Xiong, X., Ma, H., Zhu, Z., Gao, H., et al. (2017). Targeting androgen receptor in treating HER2 positive breast cancer. *Sci. Rep.* 7:14584. doi: 10.1038/s41598-017-14607-2
- Hickey, T. E., Irvine, C. M., Dvinge, H., Tarulli, G. A., Hanson, A. R., Ryan, N. K., et al. (2015). Expression of androgen receptor splice variants in clinical breast cancers. *Oncotarget* 6, 44728–44744. doi: 10.18632/oncotarget.6296
- Hickey, T. E., Robinson, J. L. L., Carroll, J. S., and Tilley, W. D. (2012). Minireview: the androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene? *Mol. Endocrinol.* 26, 1252–1267. doi: 10.1210/me.2012-1107
- Hu, D. G., Hickey, T. E., Irvine, C., Wijayakumara, D. D., Lu, L., Tilley, W. D., et al. (2014). Identification of androgen receptor splice variant transcripts in breast cancer cell lines and human tissues. *Horm. Cancer* 5, 61–71. doi: 10.1007/s12672-014-0171-4
- Hu, R., Dawood, S., Holmes, M. D., Collins, L. C., Schnitt, S. J., Cole, K., et al. (2011). Androgen receptor expression and breast cancer survival in postmenopausal women. *Clin. Cancer Res.* 17, 1867–1874. doi: 10.1158/1078-0432.CCR-10-2021
- Iorio, M. V., Ferracin, M., Liu, C.-G., Veronese, A., Spizzo, R., Sabbioni, S., et al. (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* 65, 7065–7070. doi: 10.1158/0008-5472.CAN-05-1783
- Kim, J.-Y., Park, K., Lee, E., Jung, H. H., Ahn, J. S., Im, Y.-H., et al. (2017). The effect of androgen receptor expression on clinical characterization of metastatic breast cancer. *Oncotarget* 8, 8693–8706. doi: 10.18632/oncotarget.14414
- Kim, S., Moon, B.-I., Lim, W., Park, S., Cho, M. S., and Sung, S. H. (2018). Feasibility of classification of triple negative breast cancer by immunohistochemical surrogate markers. *Clin. Breast Cancer* 18, e1123–e1132. doi: 10.1016/j.clbc.2018.03.012
- Koboldt, D. C., Fulton, R. S., McLellan, M. D., Schmidt, H., Kalicki-Veizer, J., McMichael, J. F., et al. (2012). Comprehensive molecular portraits of human breast tumours. *Nature* 490, 61–70. doi: 10.1038/nature11412
- Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42, D68–D73. doi: 10.1093/nar/gkt1181
- Kraby, M. R., Valla, M., Opdahl, S., Haugen, O. A., Sawicka, J. E., Engström, M. J., et al. (2018). The prognostic value of androgen receptors in breast cancer subtypes. *Breast Cancer Res. Treat.* 172, 283–296. doi: 10.1007/s10549-018-4904-x
- Labrie, F., Luu-The, V., Labrie, C., Bélanger, A., Simard, J., Lin, S.-X., et al. (2003). Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr. Rev.* 24, 152–182. doi: 10.1210/er.2001-0031
- Lanzino, M., Sisci, D., Morelli, C., Garofalo, C., Catalano, S., Casaburi, I., et al. (2010). Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells—identification of a novel androgen response element. *Nucleic Acids Res.* 38, 5351–5365. doi: 10.1093/nar/gkq278
- Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., et al. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Invest.* 121, 2750–2767. doi: 10.1172/JCI45014
- Lehmann-Che, J., Hamy, A.-S., Porcher, R., Barritault, M., Bouhidel, F., Habuelleh, H., et al. (2013). Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either HER2 or GCDP15. *Breast Cancer Res.* 15:R37. doi: 10.1186/bcr3421
- Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035
- Lim, E., Ni, M., Cao, S., Hazra, A., Tamimi, R. M., and Brown, M. (2014). Importance of breast cancer subtype in the development of androgen-receptor-directed therapy. *Curr. Breast Cancer Rep.* 6, 71–78. doi: 10.1007/s12609-014-0140-5
- Lin, H.-Y., Sun, M., Lin, C., Tang, H.-Y., London, D., Shih, A., et al. (2009). Androgen-induced human breast cancer cell proliferation is mediated by discrete mechanisms in estrogen receptor- α -positive and -negative breast cancer cells. *J. Steroid Biochem. Mol. Biol.* 113, 182–188. doi: 10.1016/j.jsmb.2008.12.010
- Lukong, K. E., Ogunbolude, Y., and Kamdem, J. P. (2017). Breast cancer in Africa: prevalence, treatment options, herbal medicines, and socioeconomic determinants. *Breast Cancer Res. Treat.* 166, 351–365. doi: 10.1007/s10549-017-4408-0
- Lytle, J. R., Yario, T. A., and Steitz, J. A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9667–9672. doi: 10.1073/pnas.0703820104
- Lyu, S., Liu, H., Liu, X., Liu, S., Wang, Y., Yu, Q., et al. (2017). Interrelation of androgen receptor and miR-30a and miR-30a function in ER-, PR-, AR+ MDA-MB-453 breast cancer cells. *Oncol. Lett.* 14, 4930–4936. doi: 10.3892/ol.2017.6781
- Lyu, S., Yu, Q., Ying, G., Wang, S., Wang, Y., Zhang, J., et al. (2014). Androgen receptor decreases CMYC and KRAS expression by upregulating let-7a expression in ER-, PR-, AR+ breast cancer. *Int. J. Oncol.* 44, 229–237. doi: 10.3892/ijo.2013.2151
- Macedo, L. F., Guo, Z., Tilghman, S. L., Sabnis, G. J., Qiu, Y., and Brodie, A. (2006). Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. *Cancer Res.* 66, 7775–7782. doi: 10.1158/0008-5472.CAN-05-3984
- McGhan, L. J., McCullough, A. E., Protheroe, C. A., Dueck, A. C., Lee, J. J., Nunez-Nateras, R., et al. (2014). Androgen receptor-positive triple negative breast cancer: a unique breast cancer subtype. *Ann. Surg. Oncol.* 21, 361–367. doi: 10.1245/s10434-013-3260-7
- McNamara, K. M., Yoda, T., Nurani, A. M., Shibahara, Y., Miki, Y., Wang, L., et al. (2014). Androgenic pathways in the progression of triple-negative breast carcinoma: a comparison between aggressive and non-aggressive subtypes. *Breast Cancer Res. Treat.* 145, 281–293. doi: 10.1007/s10549-014-2942-6
- Melo, S. A., Sugimoto, H., O'Connell, J. T., Kato, N., Villanueva, A., Vidal, A., et al. (2014). Cancer exosomes perform cell-independent MicroRNA biogenesis and promote tumorigenesis. *Cancer Cell* 26, 707–721. doi: 10.1016/j.ccell.2014.09.005
- Miglioretti, D. L., Zhu, W., Kerlikowske, K., Sprague, B. L., Onega, T., Buist, D. S. M., et al. (2015). Breast tumor prognostic characteristics and biennial vs annual mammography, age, and menopausal status. *JAMA Oncol.* 1, 1069–1077. doi: 10.1001/jamaoncol.2015.3084
- Naderi, A., and Hughes-Davies, L. (2008). A functionally significant cross-talk between androgen receptor and ErbB2 pathways in estrogen receptor negative breast cancer. *Neoplasia* 10, 542–548. doi: 10.1593/neo.08274

- Nadiminty, N., Tummala, R., Lou, W., Zhu, Y., Zhang, J., Chen, X., et al. (2012). MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells. *J. Biol. Chem.* 287, 1527–1537. doi: 10.1074/jbc.M111.278705
- Nakano, K., Miki, Y., Hata, S., Ebata, A., Takagi, K., McNamara, K. M., et al. (2013). Identification of androgen-responsive microRNAs and androgen-related genes in breast cancer. *Anticancer Res.* 33, 4811–4819.
- Nattenmüller, C. J., Kriegsmann, M., Sookthai, D., Fortner, R. T., Steffen, A., Walter, B., et al. (2018). Obesity as risk factor for subtypes of breast cancer: results from a prospective cohort study. *BMC Cancer* 18:616. doi: 10.1186/s12885-018-4548-6
- Ni, M., Chen, Y., Lim, E., Wimberly, H., Bailey, S. T., Imai, Y., et al. (2011). Targeting androgen receptor in estrogen receptor-negative breast cancer. *Cancer Cell* 20, 119–131. doi: 10.1016/j.ccr.2011.05.026
- Park, S., Koh, E., Koo, J. S., Kim, S. I., Park, B.-W., and Kim, K.-S. (2017). Lack of both androgen receptor and forkhead box A1 (FOXA1) expression is a poor prognostic factor in estrogen receptor-positive breast cancers. *Oncotarget* 8, 82940–82955. doi: 10.18632/oncotarget.20937
- Parker, J. S., Mullins, M., Cheang, M. C. U., Leung, S., Voduc, D., Vickery, T., et al. (2009). Supervised risk predictor of breast cancer based on intrinsic subtypes. *J. Clin. Oncol.* 27, 1160–1167. doi: 10.1200/JCO.2008.18.1370
- Perou, C. M., Sørli, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., et al. (2000). Molecular portraits of human breast tumours. *Nature* 406, 747–752. doi: 10.1038/35021093
- Peters, A. A., Buchanan, G., Ricciardelli, C., Bianco-Miotto, T., Centenera, M. M., Harris, J. M., et al. (2009). Androgen receptor inhibits estrogen receptor-activity and is prognostic in breast cancer. *Cancer Res.* 69, 6131–6140. doi: 10.1158/0008-5472.CAN-09-0452
- Rangel, N., Fortunati, N., Osella-Abate, S., Annaratone, L., Isella, C., Catalano, M. G., et al. (2018a). FOXA1 and AR in invasive breast cancer: new findings on their co-expression and impact on prognosis in ER-positive patients. *BMC Cancer* 18:703. doi: 10.1186/s12885-018-4624-y
- Rangel, N., Rondon-Lagos, M., Annaratone, L., Osella-Abate, S., Metovic, J., Mano, M. P., et al. (2018b). The role of the AR/ER ratio in ER-positive breast cancer patients. *Endocr. Relat. Cancer* 25, 163–172. doi: 10.1530/ERC-17-0417
- Ribas, J., Ni, X., Haffner, M., Wentzel, E. A., Salmasi, A. H., Chowdhury, W. H., et al. (2009). miR-21: an androgen receptor-regulated MicroRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Res.* 69, 7165–7169. doi: 10.1158/0008-5472.CAN-09-1448
- Ricciardelli, C., Bianco-Miotto, T., Jindal, S., Butler, L. M., Leung, S., McNeil, C. M., et al. (2018). The magnitude of androgen receptor positivity in breast cancer is critical for reliable prediction of disease outcome. *Clin. Cancer Res.* 24, 2328–2341. doi: 10.1158/1078-0432.CCR-17-1199
- Robinson, J. L. L., MacArthur, S., Ross-Innes, C. S., Tilley, W. D., Neal, D. E., Mills, I. G., et al. (2011). Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1. *EMBO J.* 30, 3019–3027. doi: 10.1038/emboj.2011.216
- Ross-Innes, C. S., Stark, R., Teschendorff, A. E., Holmes, K. A., Ali, H. R., Dunning, M. J., et al. (2012). Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature* 481, 389–393. doi: 10.1038/nature10730
- Rothman, M. S., Carlson, N. E., Xu, M., Wang, C., Swerdloff, R., Lee, P., et al. (2011). Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography–tandem mass spectrometry. *Steroids* 76, 177–182. doi: 10.1016/j.steroids.2010.10.010
- Schrijver, W. A. M. E., Schuurman, K., van Rossum, A., Consortium, D. D. B. C. M., Peeters, T., Ter Hoeve, N., et al. (2017). Loss of steroid hormone receptors is common in malignant pleural and peritoneal effusions of breast cancer patients treated with endocrine therapy. *Oncotarget* 8, 55550–55561. doi: 10.18632/oncotarget.15548
- Severson, T. M., Kim, Y., Joosten, S. E. P., Schuurman, K., van der Groep, P., Moelans, C. B., et al. (2018). Characterizing steroid hormone receptor chromatin binding landscapes in male and female breast cancer. *Nat. Commun.* 9:482. doi: 10.1038/s41467-018-02856-2
- Shi, X.-B., Xue, L., Yang, J., Ma, A.-H., Zhao, J., Xu, M., et al. (2007). An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19983–19988. doi: 10.1073/pnas.0706641104
- Shi, Y., Yang, F., Sun, Z., Zhang, W., Gu, J., and Guan, X. (2017). Differential microRNA expression is associated with androgen receptor expression in breast cancer. *Mol. Med. Rep.* 15, 29–36. doi: 10.3892/mmr.2016.6019
- Siegel, R. L., Miller, K. D., and Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J. Clin.* 68, 7–30. doi: 10.3322/caac.21442
- Tabung, F. K., Steck, S. E., Liese, A. D., Zhang, J., Ma, Y., Caan, B., et al. (2016). Association between dietary inflammatory potential and breast cancer incidence and death: results from the Women's Health Initiative. *Br. J. Cancer* 114, 1277–1285. doi: 10.1038/bjc.2016.98
- Takagi, K., Miki, Y., Ishida, T., Sasano, H., and Suzuki, T. (2018). The interplay of endocrine therapy, steroid pathways and therapeutic resistance: importance of androgen in breast carcinoma. *Mol. Cell. Endocrinol.* 466, 31–37. doi: 10.1016/j.mce.2017.09.011
- Takagi, K., Miki, Y., Nagasaki, S., Hirakawa, H., Onodera, Y., Akahira, J., et al. (2010). Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment. *Endocr. Relat. Cancer* 17, 415–430. doi: 10.1677/ERC-09-0257
- Vasudevan, S., Tong, Y., and Steitz, J. A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. *Science* 318, 1931–1934. doi: 10.1126/science.1149460
- Vera-Badillo, F. E., Templeton, A. J., de Gouveia, P., Diaz-Padilla, I., Bedard, P. L., Al-Mubarak, M., et al. (2014). Androgen receptor expression and outcomes in early breast cancer: a systematic review and meta-analysis. *JNCI J. Natl. Cancer Inst.* 106, djt319–djt319. doi: 10.1093/jnci/djt319
- Wang, C., Pan, B., Zhu, H., Zhou, Y., Mao, F., Lin, Y., et al. (2016). Prognostic value of androgen receptor in triple negative breast cancer: a meta-analysis. *Oncotarget* 7, 46482–46491. doi: 10.18632/oncotarget.10208
- Yang, F., Shen, Y., Zhang, W., Jin, J., Huang, D., Fang, H., et al. (2018). An androgen receptor negatively induced long non-coding RNA ARNILA binding to miR-204 promotes the invasion and metastasis of triple-negative breast cancer. *Cell Death Differ.* 25, 2209–2220. doi: 10.1038/s41418-018-0123-6
- Zhang, W., Liu, X., Liu, S., Qin, Y., Tian, X., Niu, F., et al. (2018). Androgen receptor/let-7a signaling regulates breast tumor-initiating cells. *Oncotarget* 9, 3690–3703. doi: 10.18632/oncotarget.23196

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-coding RNA in Fragile X Syndrome and Converging Mechanisms Shared by Related Disorders

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 14 October 2018

Accepted: 11 February 2019

Published: 01 March 2019

Citation:

Zhou Y, Hu Y, Sun Q and Xie N
(2019) Non-coding RNA in Fragile X
Syndrome and Converging
Mechanisms Shared by Related
Disorders. *Front. Genet.* 10:139.
doi: 10.3389/fgene.2019.00139

Fragile X syndrome (FXS) is one of the most common forms of hereditary intellectual disability. It is also a well-known monogenic cause of autism spectrum disorders (ASD). Repetitive trinucleotide expansion of CGG repeats in the 5'-UTR of *FMR1* is the pathological mutation. Full mutation CGG repeats epigenetically silence *FMR1* and thus lead to the absence of its product, fragile mental retardation protein (FMRP), which is an indispensable translational regulator at synapsis. Loss of FMRP causes abnormal neural morphology, dysregulated protein translation, and distorted synaptic plasticity, giving rise to FXS phenotypes. Non-coding RNAs, including siRNA, miRNA, and lncRNA, are transcribed from DNA but not meant for protein translation. They are not junk sequence but play indispensable roles in diverse cellular processes. FXS is the first neurological disorder being linked to miRNA pathway dysfunction. Since then, insightful knowledge has been gained in this field. In this review, we mainly focus on how non-coding RNAs, especially the siRNAs, miRNAs, and lncRNAs, are involved in FXS pathogenesis. We would also like to discuss several potential mechanisms mediated by non-coding RNAs that may be shared by FXS and other related disorders.

Keywords: non-coding RNA, Fragile X syndrome, RNAi mediated epigenetic silencing, microRNA mediated translational regulation, potential converging mechanisms

INTRODUCTION

Non-coding RNA is a kind of transcript from DNA but not meant for protein translation. Accumulating evidence has shown that non-coding RNA molecules play indispensable roles in diverse cellular processes. Understanding mechanisms mediated by these non-coding RNAs are of great importance for understanding the pathogenic process of relevant diseases. Functional molecules classified into this category have included micro-RNA (miRNA), long non-coding RNA (lncRNA), small-interfering RNA (siRNA), transfer RNA (tRNA), ribonucleoprotein RNA (rRNA), and piwi-interacting RNA (piwi-RNA), etc. (Cao et al., 2006; Beermann et al., 2016; Treiber et al., 2018).

Fragile X syndrome (FXS) is one of the most common forms of hereditary intellectual disability. It is also a well-known monogenic cause of autism spectrum disorders (ASD) (Wang et al., 2012). *FMR1* is the responsible gene. The repetitive trinucleotide expansion of CGG repeats in the 5'-untranslated region (5'-UTR) of *FMR1* is the pathological mutation. Normal individuals

usually bear CGG repeat expansion ranging from 6 to 55, while in FXS patients, this expansion often reaches beyond 200, known as the full mutation (Santoro et al., 2012). The *FMR1* gene was first cloned in 1991, rendering FXS the first discovered disease caused by trinucleotide expansion mutation (Verkerk et al., 1991). In addition to this mutation, conventional mutations including gross deletions, small indels, and missense or nonsense mutations have also been reported (Luo et al., 2014, 2015; Myrick et al., 2014). Over the last decade, FXS caused by the full mutation CGG repeat expansion receives the most intense attention. The full mutation CGG repeats lead to epigenetic silencing of *FMR1* and absence of its product, fragile mental retardation protein (FMRP) (Xie et al., 2016). FMRP is a complex RNA binding protein which plays indispensable roles in synaptic plasticity. It has four important RNA binding motifs including one arginine-glycine-glycine (RGG box) and three K homology domains (KH0, KH1, and KH2), recognizing special RNA secondary structures, such as the kissing complex and G quadruplex (Darnell et al., 2001, 2005; Myrick et al., 2015). By binding with target mRNA, FMRP mainly functions as a translation repressor at synapsis, regulating local translation spatially and temporarily to shape synaptic structure and plasticity (Nakamoto et al., 2007; Bassell and Warren, 2008; Huang et al., 2014). Most of FXS patients' phenotypes could be attributed to the loss of FMRP. Clinically, the most common symptom is intellectual retardation. Other neurological symptoms include ASD, attention deficit hyperactivity disorder (ADHD), and epilepsy. Non-neurological symptoms include macroorchidism, distinct facial features (elongated faces, protruded ears, and big forehead), and connective tissue abnormalities (mitral valve prolapse, flat fee, joint hyperextensibility, and high arched palate) (Jacquemont et al., 2007).

FXS is the first neurological disorder found to be linked to the miRNA pathway (Jin et al., 2004a). In this review, we mainly focus on how non-coding RNAs, especially the siRNAs, miRNAs, and lncRNAs, are involved in FXS pathogenesis. We would also like to discuss several potential mechanisms mediated by non-coding RNAs that may be shared by FXS and other related disorders.

NON-CODING RNA MEDIATED MECHANISMS IN FXS PATHOPHYSIOLOGY

How RNAi Is Involved in the Epigenetic Silencing of *FMR1*?

RNA interference (RNAi) refers to the process of mRNA degradation or translation inhibition mediated by small RNAs. It is a mechanism regulating gene expression at the post-transcriptional level in eukaryotic cells. siRNA and miRNA are the two most important types of small RNAs involved. Although their origins are different, they share similar downstream machinery when encountered with Dicer, an RNase III-like enzyme initiating RNAi. Precursors of siRNA or miRNA are cleaved by Dicer to be short double-stranded RNAs (dsRNAs).

In these duplexes, only one functional strand is kept, while the other one is degraded. The functional strand is the mature siRNA or miRNA that finally assembles with Argonaute proteins (AGO) to form the RNA-induced silencing complex (RISC). A major difference lies in that siRNA requires perfect base-pairing with the target sequence to guide AGO to the targeted locus while miRNA could tolerate several mismatches. AGO associated with siRNA usually induces mRNA degradation, in contrast, AGO loaded with miRNA tends to cause translation inhibition (Ha and Kim, 2014; Holoch and Moazed, 2015; Hu et al., 2017).

Full mutation CGG expansion triggers extensive DNA methylation, repressive histone modification, and chromatin condensation in the 5'-UTR of *FMR1*, transcriptionally silencing the gene and leading to loss of FMRP (Coffee et al., 1999; Biacsi et al., 2008; Alisch et al., 2013). How are the abnormal epigenetic markers triggered and maintained? Although this epigenetic silencing process has been the focus of study over the last two decades, detailed mechanisms are still mysterious. The consensus is that full mutation CGG repeat is the prerequisite trigger to initiate and maintain the repressive epigenetic changes of *FMR1*. Several models have been indicated to date, albeit it is still difficult to form an integrated one. The first model is DNA based. Secondary structures formed by the CGG repeats serve as substrates for DNA methyltransferase to initiate *de novo* DNA methylation, or as targets bound by repeat binding proteins to recruit repressor complexes (Smith et al., 1994; Bulut-Karslioglu et al., 2012). The second model is RNA based, where hairpin structures in mRNA formed by CGG repeats exceeding a certain threshold trigger the RNAi pathway to deposit repressive epigenetic markers (Kim et al., 2006; Usdin et al., 2014). The third model is a blended one, where the DNA:RNA hybrid is at play. During transcription, hybridization of the nascent RNA to its unzipped DNA template forms a special R-loop, which may act as a structural block or nucleosome analogy to induce epigenetic silencing (Colak et al., 2014; Groh et al., 2014). Our discussion below is focused on the RNA based model. What may be the role of RNAi in the epigenetic silencing of *FMR1*?

RNAi has been suggested as a conservative mechanism participating in the formation of heterochromatin in fission yeast. In this scenario, the siRNA serves as a localizer for the RISC complex to achieve site-specific epigenetic modulation. RISC is associated with Ago1 (the yeast argonaute homolog), Chp1 (a protein required for methylation of H3K9), and Tas3 (a protein required for localization of the chromatin). This RISC-like heterochromatin-targeting complex is termed RITS (Reinhart and Bartel, 2002; Volpe et al., 2002; Verdel et al., 2004).

In the context of FXS, it was observed that stable hairpin-structured RNAs containing pre-mutation CGG repeats could be processed by Dicer to generate small RNAs. Based on this observation and the role siRNA has in heterochromatin formation. A model for the RNAi mediated methylation of full mutation CGG repeats was proposed in 2004. In early embryo development, *FMR1* gene containing the full mutation CGG repeats is being transcribed actively. Bi-directional transcription of the DNA template generates dsRNAs bearing full mutation CGG repeats. Dicer further cleaves these dsRNAs to produce small RNAs. After being incorporated into the RITS complex,

similar to siRNAs, these small RNAs guide RITS to the CGG expansion region, recruiting epi-effectors, such as histone methyltransferase and DNA methyltransferase, to induce *FMR1* silencing epigenetically (Jin et al., 2004a; **Figure 1**). Thus, RNAi may play a critical role in the epigenetic silencing process of *FMR1*.

How miRNA Is Involved in FMRP Mediated Translational Regulation?

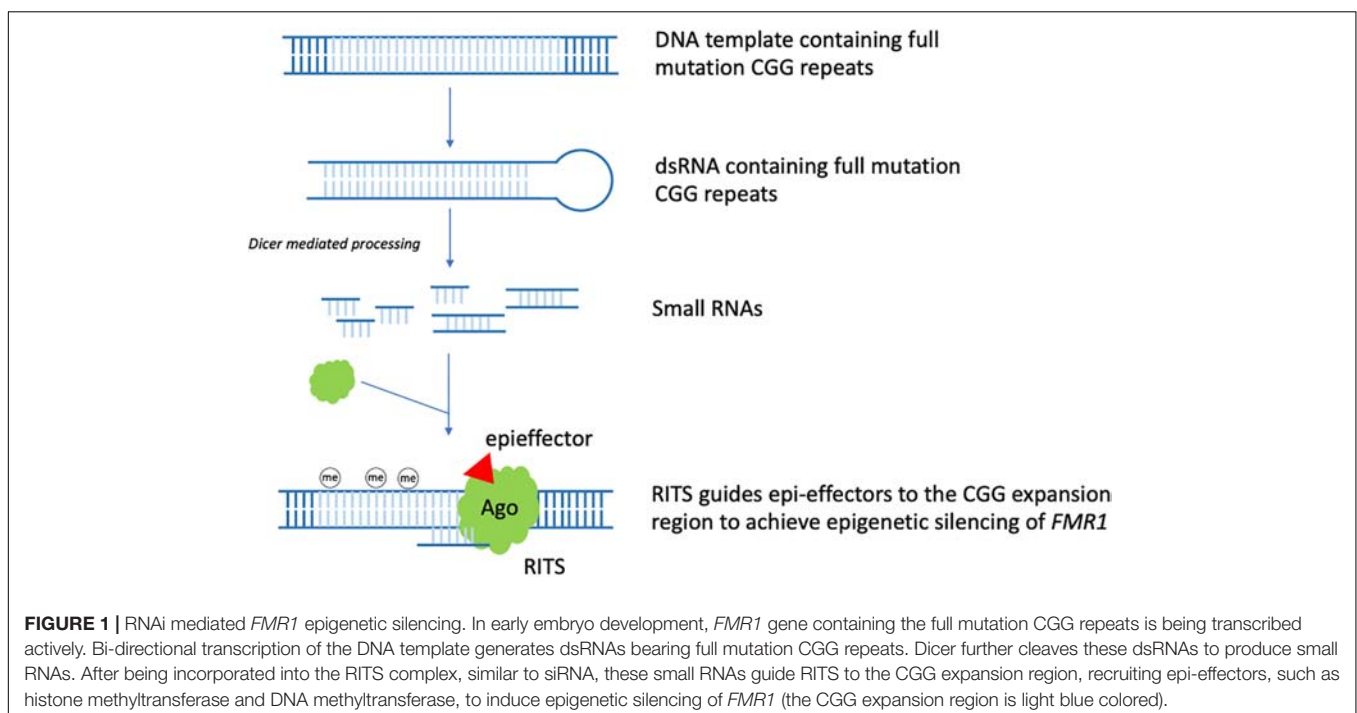
miRNA is a short non-coding single-stranded RNA molecule consisting of 20–25 nucleotides. Its biogenesis experiences a series of events. The Primary microRNA (pri-miRNA) was first converted to the precursor microRNA (pre-miRNA) by a microprocessor comprising of DGCR8 and DROSHA proteins. Next, Dicer cleaves the pre-miRNA to generate short dsRNAs, the functional strand of which further assembles with AGO proteins (also known as PIWI/PAZ-domain proteins) to form the RISC complex. This functional strand is the mature miRNA. It functions as a localizer as well as a translation repressor by binding to the 3'-untranslated region (3'-UTR) of the mRNA targets (Carthew and Sontheimer, 2009; Holoch and Moazed, 2015).

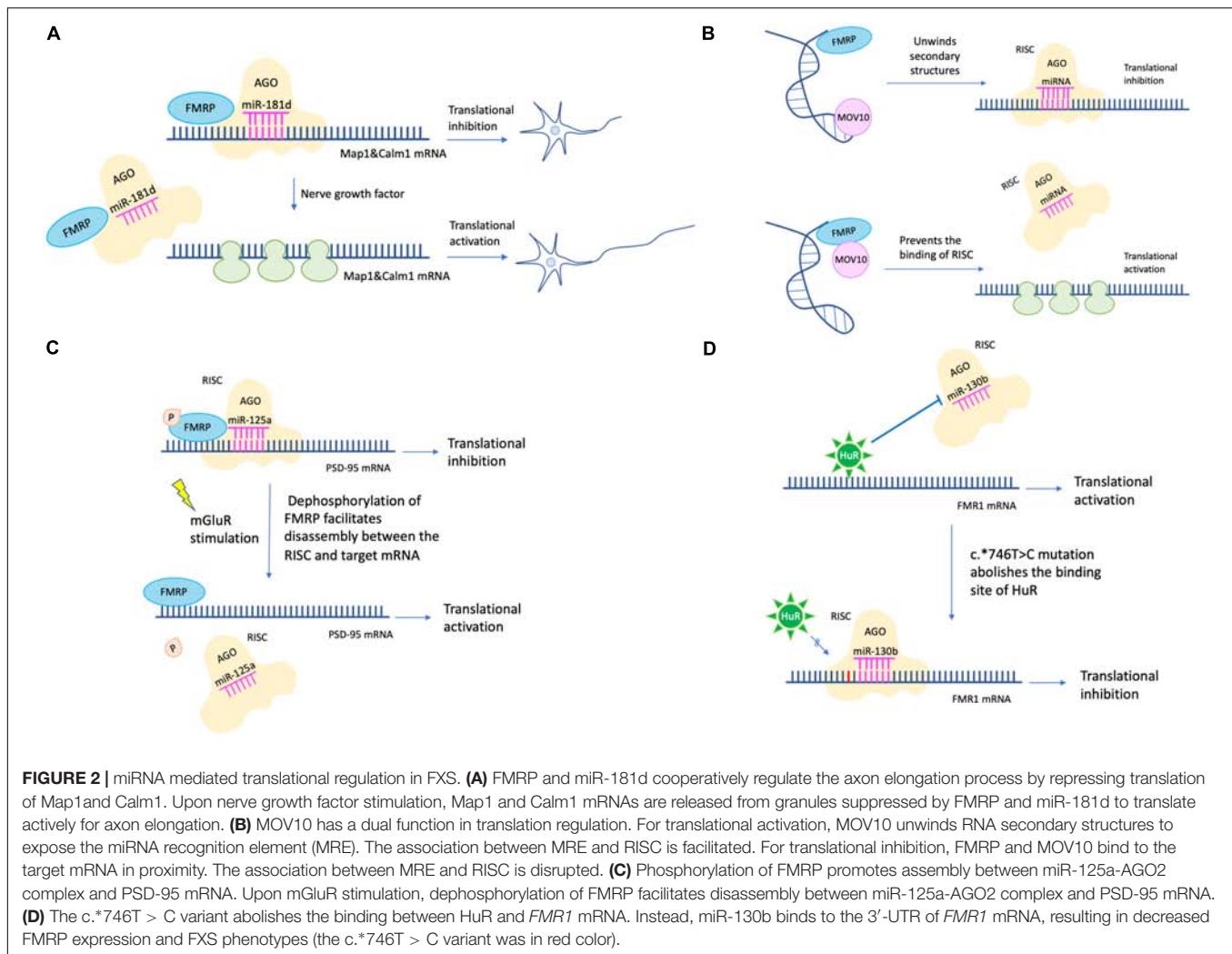
FXS is the first neurological disease being linked to miRNA pathway dysfunction (Jin et al., 2004a). FMRP is a RNA binding protein which mainly functions as a translational repressor. Studies from several groups suggested that FMRP is associated with critical components of the miRNA pathway. Loss of FMRP may cause aberrant miRNA profiling (Caudy et al., 2002; Ishizuka et al., 2002). The hippocampus of post-natal day 7 *FMR1* knockout mice showed significantly different miRNA expression profiles compared to the WT (Liu et al., 2015). In drosophila,

dfmr1 is associated with Ago1. Absence or partial loss of Ago1 impairs FMRP-mediated regulation of neural development and synaptogenesis (Jin et al., 2004b). Recently, in a FXS mice model, FMRP was shown to participate in pri-miRNA processing by upregulating DROSHA expression at the translational level. Loss of FMRP was associated with accumulation of specific pri-miRNAs and reduction of pre-miRNAs (Wan et al., 2017).

Efforts have been made to clarify detailed mechanisms of how FMRP regulates protein translation via the miRNA pathway. For example, FMRP could stabilize the binding of miRNA to its target mRNA via the KH domain (Plante et al., 2006). Besides, partly dependent on miR-125b, FMRP negatively regulates the expression of NR2A, a NMDA receptor subunit affecting synaptic plasticity (Edbauer et al., 2010). Moreover, FMRP regulates axon guidance gene via the miRNA pathway by repressing the RE-1 silencing transcription factor (REST) (Halevy et al., 2015). FMRP and miR-181d cooperatively regulate the axon elongation process by repressing translation of Map1 (a microtubule-associated protein) and Calm1 (a calcium signaling regulator). Upon nerve growth factor stimulation, Map1 and Calm1 mRNAs are released from granules suppressed by FMRP and miR-181d to translate actively for axon elongation (Wang et al., 2015; **Figure 2A**).

Later studies in succession provided further insight into how FMRP represses target mRNA translation via RNAi. A possible mechanism is FMRP interacts with MOV10, a RNA helicase implicated in miRNA pathway. MOV10 has a dual function in translational control. It assists the miRNA-mediated translational repression for specific RNAs but inhibits others. In the former case, MOV10 binds to the 3'-UTR of target mRNA, unwinds specific RNA secondary structures, and exposes the miRNA recognition elements (MRE) to facilitate the interaction between MRE and RISC. In the latter scenario, both FMRP





and MOV10 bind to the target mRNA in proximity. The association between MRE and RISC is disrupted (Kenny et al., 2014; **Figure 2B**). Another mechanism is that FMRP utilizes miRNA to control translation at synapses temporally and spatially. For example, phosphorylation of FMRP promotes assembly between miR-125a-AGO2 complex and PSD-95 mRNA. Upon mGluR stimulation, dephosphorylation of FMRP facilitates disassembly between miR-125a-AGO2 complex and PSD-95 mRNA (Muddashetty et al., 2011; **Figure 2C**).

Endogenous FMRP expression level is also regulated by the miRNA pathway. For example, the expression level of miR-130b was negatively correlated with the FMRP level in mice embryonic neural precursor cells (NPC) (Gong et al., 2013). In humans, miR-130b also seems to be a negative regulator of FMRP. Utilizing a patient-derived cell line, researchers identified a RNA binding protein HuR which may function as a RISC antagonist, as its binding site overlaps with that of miR-130b. This patient has no full mutation, however, the c.*746T > C variant in 3'-UTR renders him with significantly decreased FMRP level and FXS phenotypes. A likely mechanism is that the c.*746T > C variant abolishes the binding between HuR and

FMR1 mRNA. Instead, miR-130b binds to the 3'-UTR of *FMR1* mRNA, resulting in decreased FMRP expression (Suhl et al., 2015; **Figure 2D**). Additionally, in a zebrafish FXS model, researchers transgenically overexpressed rCGG (CGG trinucleotide repeats) motif via high titer retroviral delivery. As the expression level of rCGG increased, the level of *FMR1*-miRNA was increased, oppositely, the level of *FMR1* transcription was decreased, indicating that rCGG derived-miRNA participated in *FMR1* transcription suppression (Lin, 2015).

In sum, miRNA facilitates the FMRP mediated translational regulation via diverse mechanisms to shape synaptic plasticity and morphology, to achieve translational control temporally and spatially, and to regulate endogenous FMRP level (**Figure 2**).

lncRNA Participates in FXS Pathophysiology

Long non-coding RNA (lncRNA) is a genome transcript longer than 200nt. As a major category of non-coding RNA, lncRNA participates in formation of RNA-protein complexes, gene regulation, modulation of protein localization, and X

chromosome inactivation (Wu R. et al., 2016). In FXS, lncRNA may affect the cell proliferation process and may serve as novel clinical biomarkers. Similar to miRNA and siRNA, its role in FXS pathogenesis should not be ignored.

FMR4 is a 2.4 kb lncRNA, which is primate-specific and plays an anti-apoptotic role in human cells. It is transcribed from *FMR4*, which resides upstream *FMR1*. *FMR4* and *FMR1* a bi-directional promoter. In FXS, both of them are silenced due to the CGG repeat expansion, which may contribute to FXS pathogenesis (Khalil et al., 2008). FMR4 is also a trans-acting element which regulates hundreds of genes involved in neural development. By altering chromatin state epigenetically, FMR4 selectively modulates the cellular proliferation of human NPC (Peschansky et al., 2016). Later on, combining the technology of rapid amplification of cDNA ends (RACE) and next generation sequencing, researchers investigated the *FMR1* gene locus systematically and found two novel lncRNAs, FMR5, and FMR6. FMR5 is transcribed in the sense direction, the transcription start site (TSS) of which resides upstream the *FMR1* TSS. FMR6 is transcribed in the antisense direction, beginning from the 3'-UTR of *FMR1*. FMR5 and FMR6 may be novel clinical biomarkers due to their distinct expression patterns between full mutation and premutation patients (Pastori et al., 2014). A recent study provides new clues in how lncRNA is involved in FXS pathophysiology. TUG1 is a lncRNA which prevents axonal growth by negatively regulating the SnoN-Ccd1 pathway. In WT mice, FMRP directly binds with TUG1 to decrease its stability. However, in FXS mice, absence of FMRP leads to TUG1 overexpression. TUG1 interacts with SnoN, a crucial transcriptional regulator which controls axonal growth via regulating the actin-binding protein Ccd1. By affecting the transcriptional activity of SnoN, overexpression of TUG1 decreases the level of Ccd1 mRNA and protein, leading to abnormal axonal growth (Guo Y. et al., 2018). Albeit reference regarding this section is scarce, the importance of lncRNA is non-negligible, as evidenced by the research findings described above.

miRNA Links FMRP to Glia Function

Glutamate is a major excitatory neurotransmitter in brains. Dysregulation of glutamate in neurons participates in various neuropsychiatric disorders by affecting synaptic plasticity. Interestingly, abnormal regulation of glutamate in cells surrounding neurons may also be an important pathogenic process. For the encircled neurons may become more exciting. For example, in the astrocytes isolated from the cortex of *FMR1*-deficient mice, the GLT1 expression, a major glutamate transporter of glia, is significantly reduced compared to the WT, so is the uptake of glutamate. Additionally, treating the cortical slices with GLT1 inhibitors results in significantly enhanced neuronal excitability in *FMR1*-deficient mice but not in controls. Moreover, based on an astroglia-specific conditional FMRP knockout and restoration mice model, researchers found that selective re-expression of FMRP in astrocytes of *FMR1*-deficient mice rescues phenotypes of the decreased GLT1 expression in cortical astrocytes and the abnormal spine morphology in cortical pyramidal neurons. Upregulation of GLT1 expression

alleviates enhanced neuronal excitability and corrects spine abnormality in *FMR1*-deficient mice. These results together indicate that absence of FMRP may impair the ability of astrocytes, via dysregulated GLT1, to remove excessive glutamate from neurons and thus lead to neuronal hyperexcitability in FXS (Higashimori et al., 2013, 2016).

Notably, the regulation of GLT1 may be mediated by exosomes. Exosomes are membrane vesicles secreted by cells. They contain various signaling molecules including miRNAs. After being secreted into extracellular space, exosomes could be fused into surrounding cells to assist intercellular communication (Xiao et al., 2017). miR-124a has a positive effect on the expression level of GLT1 both *in vivo* and *in vitro*. Transfer of miR-124a from neurons to astrocytes via exosomes selectively increases the astroglial GLT1 protein level without affecting the mRNA level, suggesting the exosome-derived miR-124a is a regulator of the GLT1 expression (Morel et al., 2013).

It is likely that absence of FMRP may cause aberrant miRNA function in the neuron/astroglia interaction and thus affect GLT1 expression and glutamate uptake ability of astrocytes. More studies are needed to shed light on this topic.

NON-CODING RNA MEDIATED MECHANISMS SHARED BY FXS AND RELATED DISORDERS

miRNA of FXTAS May Also Be Pivotal to FXS

Fragile X tremor ataxia syndrome (FXTAS) is an aging-related neurodegeneration disorder, featured by progressive tremor, ataxia, and cognition decline. In FXTAS, *FMR1* containing the premutation CGG repeat expansion (60–200 repeats) is not silenced. Instead, it is transcribed actively, resulting in significantly higher mRNA level than normal but less FMRP protein. It is thought that the gain-of-function RNA toxicity is to blame, of which the miRNA pathway is at play. For instance, in neuronal cells and brain tissues from FXTAS patients, RNA aggregates containing pre-mutation CGG repeats decrease mature miRNA levels by sequestering RNA-binding proteins which are crucial to miRNA biogenesis, such as DGCR8 and DROSHA (Sellier et al., 2013). In olfactory neurons of *C. elegans*, expression of the expanded CGG repeats weakens the olfactory plasticity formation by interacting with the *C. elegans* specific argonaute ALG-2 (Juang et al., 2014). Additionally, miRNA-277, 424, 101, 129-5p, and 221 have also been indicated in the pathogenesis of FXTAS (Tan et al., 2012; Alvarez-Mora et al., 2013; Zongaro et al., 2013).

FXTAS is the neurodegenerative disorder most closely related to FXS. FXTAS and FXS may have overlapping mechanisms in the earlier neurodevelopmental stage or the later neurodegenerative stage, as either the premutation or the full mutation *FMR1* is transcribed actively at the very beginning, and both of them could lead to cognition impairment in late-life. miRNAs that are critical to FMRP function in

FXTAS may also be pivotal to FXS. Discovery of such miRNAs is necessary.

miRNA Regulating mTOR Activity Might Play a Role in FXS

Synaptic plasticity refers to the adaptability of synaptic transmission in an activity-dependent manner. Long-term potentiation (LTP) and long-term depression (LTD) are the two most common forms of synaptic plasticity. When the transmission is being strengthened for more than 1 h, it is termed LTP. When the transmission is being weakened for more than 1 h, it is termed LTD. LTP and LTD is the basis for learning and memory, the mGluR-LTD of which plays a central role in FXS pathogenesis (Heller et al., 2014).

Normally, the mGluR-LTD consists of two phases. In the early phase, when mGluR is stimulated, PP2A is instantly activated to dephosphorylate FMRP, derepressing mRNAs targeted by FMRP to cause a rapid synaptic translation burst. In the late phase, the mammalian target of rapamycin (mTOR) is activated. mTOR inhibits PP2A but activates S6K1, which phosphorylates FMRP again to counterbalance the previous translation burst. The overall effect of mGluR stimulation on the surface AMPA receptor is internalization (Nakamoto et al., 2007; Narayanan et al., 2007, 2008; Bassell and Warren, 2008).

In FXS, on the one hand, the signaling cascade from mGluR stimulation to mTOR activation comprises of homer, PIKE, PI3K, PDK1/2, AND Akt, etc. (Santoro et al., 2012). Since FMRP represses the activity of PIKE and PI3K, loss of FMRP results in increased mTOR signaling activity (Sharma et al., 2010). On the other hand, the translation repression at basal state mediated by phosphorylated FMRP is abolished due to FMRP deficiency. As a result, no matter whether there is a stimulus or not, mRNAs targeted by FMRP are always being transcribed actively. The temporarily released translation burst described above is lost, resulting in persistent and increased AMPA receptor internalization. Collectively, the mGluR/mTOR/LTD signaling is exaggerated in FXS (Bassell and Warren, 2008; Santoro et al., 2012).

Theoretically, any miRNA regulating components along the mGluR/mTOR/LTD pathway might play a role in FXS pathogenesis. As described below, accumulating evidence has suggested a bunch of miRNAs could either increase or decrease the activity of mTOR signaling. Although most of them are not directly linked to the pathophysiology of FXS, it is still worth noting that some of them might help unveil novel clues for studying overlapping mechanisms between FXS and other disorders.

Firstly, some miRNAs increase mTOR activity. For example, miR-125b is an oncogenic miRNA inhibiting the tumor suppressor p53. An important downstream target of p53 is *PIK3CA*, which encodes the p110 α subunit of PI3K. As the negative regulation of *PIK3CA* via p53 is weakened by miR-125b, the mTOR activity is increased (Astanehe et al., 2008; Zeng et al., 2012). miR-451 is also an oncogenic miRNA, which has been implicated in glioma. It may activate the mTOR pathway by inhibiting AMPK signaling in glioma and colorectal cancer

cells (Godlewski et al., 2010; Chen et al., 2014). Additionally, both miR-21 and miR-93 are oncogenic and manifest significantly differential expression profiling in ASD. They may increase the mTOR activity by inhibiting the putative target PTEN which is a negative regulator of PI3K (Sarachana et al., 2010; Kawano et al., 2015; Wu Y.E. et al., 2016; Guo X. et al., 2018).

On the contrary, miR-7 and miR-155 are miRNAs downregulating mTOR activity. miR-7 is a tumor suppressor targeting PIK3CD, Akt, and mTOR. PIK3CD is an integral catalytic subunit of PI3K. By inhibiting PI3K, Akt, and mTOR itself, the overall impact of miR-7 on mTOR pathway is suppression (Kefas et al., 2008; Fang et al., 2012). miR-155 is a negative regulator of mTOR via targeting multiple signals including RHEB. It has been implicated both in cancer and autism (Wan et al., 2014; Wu Y.E. et al., 2016).

Moreover, miR-199 seems to play a dual role in mTOR signaling. In cancer cells, it usually acts as a tumor suppressor by downregulating the mTOR pathway activity. In Rett syndrome, miR-199 is a positive regulator of the mTOR pathway activity. However, the MeCP2 deficiency hinders generation of the precursor-miR-199a. As a result, the positive regulation of mTOR signaling via miR-199 is weakened by MeCP2 deficiency, leading to decreased mTOR pathway activity and Rett syndrome phenotypes (Tsujimura et al., 2015).

Last but not least, in turn, the mTOR signaling pathway also regulates the biogenesis and activity of miRNAs. For instance, miR-21 is positively regulated by mTOR, while miR-125b is negatively regulated by mTOR (Ge et al., 2011; Bornachea et al., 2012; Ye et al., 2015).

miRNAs described here are mainly linked to cancer, ASD, and Rett syndrome. Although there is no evidence supporting a direct relationship between them and FXS pathophysiology, it is still possible that several of them may have aberrant functions in FXS. By increasing or decreasing mTOR activity, they might participate in the regulation of mGluR/mTOR/LTD signaling, and further contribute to the shaping of synaptic plasticity (Figure 3). More research on this topic is needed to clarify the linkage between miRNAs and mTOR pathway activity in FXS pathogenesis.

miRNA May Function as a Broad-Spectrum Therapeutic Agent

The increased mTOR pathway activity is a central pathogenic mechanism shared by FXS, aging, and several other neurological disorders such as autism and tuberous sclerosis, evidenced by reproducible results from yeast to mammalian animal models (Fabrizio et al., 2001; Jia et al., 2004; Johnson et al., 2013; Ebrahimi-Fakhari and Sahin, 2015; Kilincaslan et al., 2017). It is hopeful that the progress of mTOR activity regulation in one disorder may shed light on others.

The mTOR inhibitor has the potential to be a broad-spectrum therapeutic agent. For example, in *FMR1* KO mice, pharmacological inhibition of mTOR pathway rescues multiple FXS phenotypes, including excessive synaptic protein synthesis, persistent AMPA receptor internalization, and increased spine density (Gross et al., 2010). Optimistically, mTOR inhibitors have

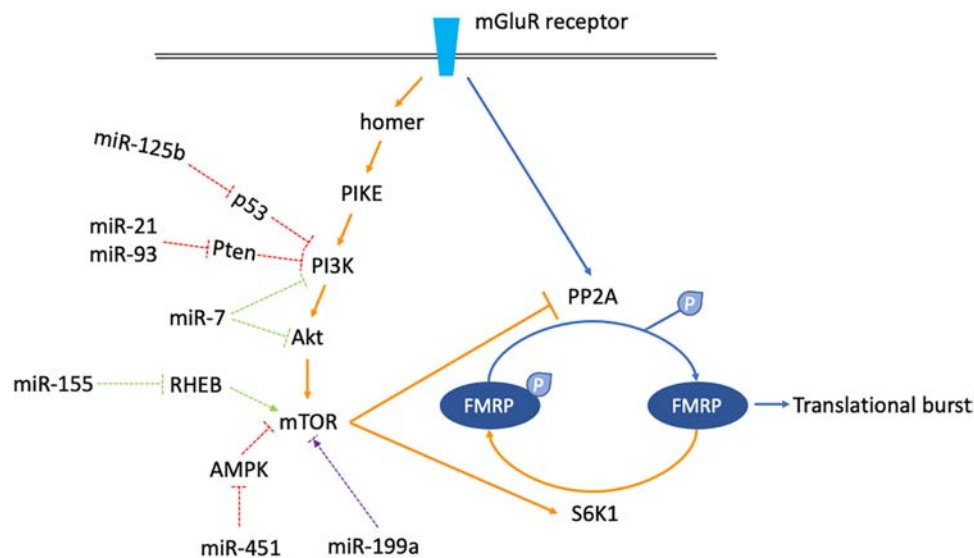


FIGURE 3 | Schematics shown miRNAs which might regulate mGluR/mTOR/LTD signaling. The mGluR/mTOR/LTD consists of two phases. In the early phase, when mGluR receptor is stimulated, the downstream phosphatase PP2A is activated instantly, dephosphorylating FMRP to allow translational burst (arrows are blue colored). In the late phase, activation of the mTOR signaling cascade activates the S6K1 kinase but inhibits the PP2A. FMRP is phosphorylated again, counterbalancing the previous translational burst (arrows are orange colored). miRNAs which might regulate components along the mGluR/mTOR/LTD pathway are classified into three categories. miR-125b, 21, 93, and 451 increase mTOR activity (dashed arrows are red colored). miR-7 and miR-155 decrease mTOR activity (dashed arrows are green colored). miR-199a has a dual function, increasing or decreasing mTOR activity in certain situations (dashed arrows are purple colored).

spawned several clinical trials for FXS (Luo et al., 2016). In aging mice, rapamycin, a classic mTOR inhibitor, was able to slow the aging process in multiple organs (Wilkinson et al., 2012). In tuberous sclerosis patients, everolimus was capable of controlling refractory epilepsy and alleviating autistic symptoms by inhibiting the mTOR pathway activity (Kilincaslan et al., 2017).

Theoretically, miRNAs that inhibit mTOR activity may have therapeutic effects similar to the chemicals described above. In this sense, understanding miRNA mediated mTOR regulation in FXS may help develop new therapeutic agents not only for FXS but also for other disorders where the mTOR dysregulation is an essential pathogenic mechanism.

miRNA Links FMRP to Alzheimer's Disease

Alzheimer's disease (AD) is pathologically featured by the A β plaques and neurofibrillary tangles containing hyperphosphorylated tau protein (Zhang et al., 2016). miR-132 and miR-125b are well established FMRP-associated miRNAs regulating synaptic structures and functions (Edbauer et al., 2010). Intriguingly, they are also involved in AD pathogenesis. miR-132 has protective effects on neurons both *in vivo* and *in vitro*, the mechanisms of which include reducing A β production and glutamate toxicity, targeting tau modifiers to decrease hyperphosphorylated tau proteins, inhibiting cell apoptosis, and strengthening hippocampal LTP (Smith et al., 2015; Hernandez-Rapp et al., 2016; Salta et al., 2016; Wang et al., 2017; El Fatimy et al., 2018). The expression level of miR-132 in cholinergic nucleus basalis Meynert is stable at early stages but decreases significantly as the disease course develops,

suggesting miR-132 may be a facilitator of neurodegeneration (Zhu et al., 2016). In contrast, miR-125b was shown to be a deleterious factor in AD pathogenesis. miRNA profiling of AD brains showed that miR-125b was significantly increased in the hippocampus (Lukiw, 2007). *In vitro* overexpression of miR-125b was shown to induce cell apoptosis, enhance oxidative stress, and promote tau hyperphosphorylation in the cell and animal models of AD (Banzhaf-Strathmann et al., 2014; Jin et al., 2018). As aforementioned, miRNAs assist FMRP mediated translational repression in FXS. Suboptimal ratio and assembly between FMRP, miRNA, and mRNA results in

TABLE 1 | Non-coding RNA involved in FXS pathogenesis.

Type	Mechanism	Reference
miR-125b	Facilitate FMRP to regulate NMDA receptor subunit expression Increase mTOR activity	Edbauer et al., 2010 Astanehe et al., 2008; Zeng et al., 2012
miR-181d	Regulate axon elongation	Wang et al., 2015
miR-130b	Regulate FMRP expression	Gong et al., 2013; Suhl et al., 2015
FMR4	lncRNA with anti-apoptotic function Modulate NPC proliferation	Khalil et al., 2008 Peschansky et al., 2016
FMR5,6	lncRNA may be used as biomarkers	Pastori et al., 2014
TUG1	lncRNA prevents axonal growth	Guo Y. et al., 2018
miR-124a	Regulate astroglial glutamate transportation	Morel et al., 2013
BC1	lncRNA may serve as an adaptor molecule of FMRP	Zhang et al., 2018

translation dysregulation. Meanwhile, mRNAs of $A\beta$ precursors and $A\beta$ receptors, including NMDARs, mGluR5, and PSD-95, are classic targets negatively regulated by FMRP. Therefore, establishing AD animal models lacking or overexpressing FMRP may help elucidate how FMRP interplays with miR-132 or miR-125b to regulate $A\beta$ related proteins at different disease stages in specific brain regions.

FMRP is also implicated in AD pathogenesis by interacting with lncRNA. It has been controversial whether the BC1 RNA, a lncRNA, functions as an adapter molecule for FMRP in FXS. Some researchers support the view that BC1 directly binds with FMRP to increase its affinity to target mRNAs (Zalfa et al., 2003, 2005; Napoli et al., 2008). Others argue that FMRP directly binds to the target mRNA. The interaction between FMRP and BC1 is non-specific. These two molecules act independently (Iacoangeli et al., 2008; Zhong et al., 2010). Notably, in the context of AD, this controversial issue seems to be reconciled to some extent. In the Tg2576 AD mice model, when FMRP binds to *APP* mRNA, *APP* translation is suppressed, subsequently producing less $A\beta$ protein. However, when BC1 binds to FMRP, the interaction between FMRP and *APP* mRNA is disrupted, resulting in active *APP* translation and increased $A\beta$ production, which further causes the spatial learning and memory deficits (Zhang et al., 2018). This study is a good example where FMRP indeed binds directly with either BC1 RNA or target mRNA. However, this binding does not necessarily include BC1 RNA and target mRNA simultaneously. Therefore, investigating how BC1 and other lncRNAs, *APP*, and FMRP interact with each other at synapsis may help understand the pathogenesis of both AD and FXS.

FMRP Regulates Stem Cell Fate via miRNA

The first evidence indicating FMRP may regulate germline stem cell fate via the miRNA pathway came in 2007. In *Drosophila*, the interaction between FMRP and Ago1 may facilitate maintenance but suppress differentiation of the germline stem cells (Yang et al., 2007). FMRP may also function via the bantam miRNA to regulate the germline stem cell fate (Yang et al., 2009). In testicle cells, FMRP was found to be associated with a bunch of miRNAs including miR-383, which is mainly expressed in spermatogonia. miR-383 is negatively regulated by FMRP during spermatogenesis, failure of which may contribute to male infertility (Tian et al., 2013). In the brain, miR-510, located near the fragile site on X chromosome, was associated with CGG expansion into full mutation in the neurons derived from mesenchymal stem cells. Bioinformatics analysis indicated that enhanced miR-510 expression might facilitate CGG expansion via regulating target genes such as VHL and PPP2R5E (Fazeli

et al., 2018). In mice embryonic NPC, the upregulation of miR-130b associates with decreased FMRP level and increased NPC proliferation tendency (Gong et al., 2013). The expression level of miR-181a, which translationally represses the expression of GluA2, a subunit of AMPA receptors, was increased in human NPCs derived from induced pluripotent stem cells. Since cells lacking the GluA2 subunit were more prone to differentiation, miR-181a may affect cell fate by interacting with the AMPA signaling pathway (Achuta et al., 2018). According to these data, it is fair to say that the FXS stem cell model provides an excellent monogenic platform for studying how miRNAs are involved in stem cell fate regulation, especially in testis and brain.

CLOSING REMARKS AND OUTLOOK

In summary, Non-coding RNA participates in FXS pathophysiology via multiple aspects. The RNAi mechanism provides a RNA-based model for explaining the epigenetic silencing of *FMR1*. The miRNA mainly facilitates FMRP's role as a translational repressor, controlling axon growth, shaping synaptic plasticity, and regulating endogenous FMRP level. The lncRNA also plays non-negligible roles (Table 1). Besides, understanding how non-coding RNA functions in FXS may help uncover converging mechanisms shared by FXS and related disorders. This list may well include FXTAS, ASD, Rett syndrome, AD, tuberous sclerosis, infertility, cancer, and aging, etc. In the future, studies dissecting the non-coding RNA profiling temporally and spatially are needed to identify the non-coding RNA regulation pattern in specific neurodevelopmental or neurodegenerative stages. The non-coding RNA that may function as a nexus between FXS and related disorders should be given more attention. As such research findings will help unveil converging mechanisms shared by different diseases and further contribute to the development of a broad-spectrum non-coding RNA therapeutic agent.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript writing, revision, and figure design.

ACKNOWLEDGMENTS

We thank all the kind support from Department of Geriatric Neurology, Xiangya Hospital, Central South University.

REFERENCES

- Achuta, V. S., Moykkynen, T., Peteri, U. K., Turconi, G., Rivera, C., Keinanen, K., et al. (2018). Functional changes of AMPA responses in human induced pluripotent stem cell-derived neural progenitors in fragile X syndrome. *Sci. Signal.* 11:eaan8784. doi: 10.1126/scisignal.aan8784
- Alisch, R. S., Wang, T., Chopra, P., Visootsak, J., Conneely, K. N., and Warren, S. T. (2013). Genome-wide analysis validates aberrant methylation in fragile X syndrome is specific to the *FMR1* locus. *BMC Med. Genet.* 14:18. doi: 10.1186/1471-2350-14-18
- Alvarez-Mora, M. I., Rodriguez-Revenga, L., Madrigal, I., Torres-Silva, F., Mateu-Huertas, E., Lizano, E., et al. (2013). MicroRNA expression profiling in blood from fragile X-associated tremor/ataxia syndrome patients. *Genes Brain Behav.* 12, 595–603. doi: 10.1111/gbb.12061
- Astanehe, A., Arenillas, D., Wasserman, W. W., Leung, P. C., Dunn, S. E., Davies, B. R., et al. (2008). Mechanisms underlying p53 regulation of PIK3CA

- transcription in ovarian surface epithelium and in ovarian cancer. *J. Cell Sci.* 121(Pt 5), 664–674. doi: 10.1242/jcs.013029
- Banzhaf-Strathmann, J., Benito, E., May, S., Arzberger, T., Tahirovic, S., Kretzschmar, H., et al. (2014). MicroRNA-125b induces tau hyperphosphorylation and cognitive deficits in Alzheimer's disease. *EMBO J.* 33, 1667–1680. doi: 10.15252/embj.201387576
- Bassell, G. J., and Warren, S. T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. *Neuron* 60, 201–214. doi: 10.1016/j.neuron.2008.10.004
- Beermann, J., Piccoli, M. T., Viereck, J., and Thum, T. (2016). Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol. Rev.* 96, 1297–1325. doi: 10.1152/physrev.00041.2015
- Biacsi, R., Kumari, D., and Usdin, K. (2008). SIRT1 inhibition alleviates gene silencing in Fragile X mental retardation syndrome. *PLoS Genet.* 4:e1000017. doi: 10.1371/journal.pgen.1000017
- Bornachea, O., Santos, M., Martinez-Cruz, A. B., Garcia-Escudero, R., Duenas, M., Costa, C., et al. (2012). EMT and induction of miR-21 mediate metastasis development in *Trp53*-deficient tumours. *Sci. Rep.* 2:434. doi: 10.1038/srep00434
- Bulut-Karslioglu, A., Perrera, V., Scaranaro, M., de la Rosa-Velazquez, I. A., van de Nobelen, S., Shukeir, N., et al. (2012). A transcription factor-based mechanism for mouse heterochromatin formation. *Nat. Struct. Mol. Biol.* 19, 1023–1030. doi: 10.1038/nsmb.2382
- Cao, X., Yeo, G., Muotri, A. R., Kuwabara, T., and Gage, F. H. (2006). Noncoding RNAs in the mammalian central nervous system. *Annu. Rev. Neurosci.* 29, 77–103. doi: 10.1146/annurev.neuro.29.051605.112839
- Carthew, R. W., and Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell* 136, 642–655. doi: 10.1016/j.cell.2009.01.035
- Caudy, A. A., Myers, M., Hannon, G. J., and Hammond, S. M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* 16, 2491–2496. doi: 10.1101/gad.1025202
- Chen, M. B., Wei, M. X., Han, J. Y., Wu, X. Y., Li, C., Wang, J., et al. (2014). MicroRNA-451 regulates AMPK/mTORC1 signaling and fascin1 expression in HT-29 colorectal cancer. *Cell. Signal.* 26, 102–109. doi: 10.1016/j.cellsig.2013.07.017
- Coffee, B., Zhang, F., Warren, S. T., and Reines, D. (1999). Acetylated histones are associated with FMR1 in normal but not fragile X-syndrome cells. *Nat. Genet.* 22, 98–101. doi: 10.1038/8807
- Colak, D., Zaninovic, N., Cohen, M. S., Rosenwaks, Z., Yang, W. Y., Gerhardt, J., et al. (2014). Promoter-bound trinucleotide repeat mRNA drives epigenetic silencing in fragile X syndrome. *Science* 343, 1002–1005. doi: 10.1126/science.1245831
- Darnell, J. C., Fraser, C. E., Mostovetsky, O., Stefani, G., Jones, T. A., Eddy, S. R., et al. (2005). Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes. *Genes Dev.* 19, 903–918. doi: 10.1101/gad.1276805
- Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 107, 489–499. doi: 10.1016/S0092-8674(01)00566-9
- Ebrahimi-Fakhari, D., and Sahin, M. (2015). Autism and the synapse: emerging mechanisms and mechanism-based therapies. *Curr. Opin. Neurol.* 28, 91–102. doi: 10.1097/WCO.0000000000000186
- Edbauer, D., Neilson, J. R., Foster, K. A., Wang, C. F., Seeburg, D. P., Batterton, M. N., et al. (2010). Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. *Neuron* 65, 373–384. doi: 10.1016/j.neuron.2010.01.005
- El Fatimy, R., Li, S., Chen, Z., Mushannen, T., Gongala, S., Wei, Z., et al. (2018). MicroRNA-132 provides neuroprotection for tauopathies via multiple signaling pathways. *Acta Neuropathol.* 136, 537–555. doi: 10.1007/s00401-018-1880-5
- Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M., and Longo, V. D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. *Science* 292, 288–290. doi: 10.1126/science.1059497
- Fang, Y., Xue, J. L., Shen, Q., Chen, J., and Tian, L. (2012). MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. *Hepatology* 55, 1852–1862. doi: 10.1002/hep.25576
- Fazeli, Z., Ghaderian, S. M. H., Najmabadi, H., and Omrani, M. D. (2018). High expression of miR-510 was associated with CGG expansion located at upstream of FMR1 into full mutation. *J. Cell. Biochem.* doi: 10.1002/jcb.27505 [Epub ahead of print].
- Ge, Y., Sun, Y., and Chen, J. (2011). IGF-II is regulated by microRNA-125b in skeletal myogenesis. *J. Cell Biol.* 192, 69–81. doi: 10.1083/jcb.201007165
- Godlewski, J., Nowicki, M. O., Bronisz, A., Nuovo, G., Palatini, J., De Lay, M., et al. (2010). MicroRNA-451 regulates LKB1/AMPK signaling and allows adaptation to metabolic stress in glioma cells. *Mol. Cell* 37, 620–632. doi: 10.1016/j.molcel.2010.02.018
- Gong, X., Zhang, K., Wang, Y., Wang, J., Cui, Y., Li, S., et al. (2013). MicroRNA-130b targets Fmr1 and regulates embryonic neural progenitor cell proliferation and differentiation. *Biochem. Biophys. Res. Commun.* 439, 493–500. doi: 10.1016/j.bbrc.2013.08.096
- Groh, M., Lufino, M. M., Wade-Martins, R., and Gromak, N. (2014). R-loops associated with triplet repeat expansions promote gene silencing in Friedreich ataxia and fragile X syndrome. *PLoS Genet.* 10:e1004318. doi: 10.1371/journal.pgen.1004318
- Gross, C., Nakamoto, M., Yao, X., Chan, C. B., Yim, S. Y., Ye, K., et al. (2010). Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome. *J. Neurosci.* 30, 10624–10638. doi: 10.1523/JNEUROSCI.0402-10.2010
- Guo, X., Qiu, W., Liu, Q., Qian, M., Wang, S., Zhang, Z., et al. (2018). Immunosuppressive effects of hypoxia-induced glioma exosomes through myeloid-derived suppressor cells via the miR-10a/Rora and miR-21/Pten pathways. *Oncogene* 37, 4239–4259. doi: 10.1038/s41388-018-0261-9
- Guo, Y., Chen, X., Xing, R., Wang, M., Zhu, X., and Guo, W. (2018). Interplay between FMRP and lncRNA TUG1 regulates axonal development through mediating SnoN-Ccd1 pathway. *Hum. Mol. Genet.* 27, 475–485. doi: 10.1093/hmg/ddx417
- Ha, M., and Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* 15, 509–524. doi: 10.1038/nrm3838
- Halevy, T., Czech, C., and Benvenisty, N. (2015). Molecular mechanisms regulating the defects in fragile X syndrome neurons derived from human pluripotent stem cells. *Stem Cell Rep.* 4, 37–46. doi: 10.1016/j.stemcr.2014.10.015
- Heller, E. A., Cates, H. M., Pena, C. J., Sun, H., Shao, N., Feng, J., et al. (2014). Locus-specific epigenetic remodeling controls addiction- and depression-related behaviors. *Nat. Neurosci.* 17, 1720–1727. doi: 10.1038/nn.3871
- Hernandez-Rapp, J., Rainone, S., Goupil, C., Dorval, V., Smith, P. Y., Saint-Pierre, M., et al. (2016). microRNA-132/212 deficiency enhances Abeta production and senile plaque deposition in Alzheimer's disease triple transgenic mice. *Sci. Rep.* 6:30953. doi: 10.1038/srep30953
- Higashimori, H., Morel, L., Huth, J., Lindemann, L., Dulla, C., Taylor, A., et al. (2013). Astroglial FMRP-dependent translational down-regulation of mGluR5 underlies glutamate transporter GLT1 dysregulation in the fragile X mouse. *Hum. Mol. Genet.* 22, 2041–2054. doi: 10.1093/hmg/ddt055
- Higashimori, H., Schin, C. S., Chiang, M. S., Morel, L., Shoneye, T. A., Nelson, D. L., et al. (2016). Selective deletion of Astroglial FMRP dysregulates glutamate transporter GLT1 and contributes to fragile X syndrome phenotypes in vivo. *J. Neurosci.* 36, 7079–7094. doi: 10.1523/JNEUROSCI.1069-16.2016
- Holoch, D., and Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. *Nat. Rev. Genet.* 16, 71–84. doi: 10.1038/nrg3863
- Hu, Y., Ehli, E. A., and Boomsma, D. I. (2017). MicroRNAs as biomarkers for psychiatric disorders with a focus on autism spectrum disorder: current progress in genetic association studies, expression profiling, and translational research. *Autism Res.* 10, 1184–1203. doi: 10.1002/aur.1789
- Huang, W., Luo, S., Ou, J., Zhu, F., Xia, Y., Xue, J., et al. (2014). Correlation between FMR1 expression and clinical phenotype in discordant dichorionic-diamniotic monozygotic twin sisters with the fragile X mutation. *J. Med. Genet.* 51, 159–164. doi: 10.1136/jmedgenet-2013-101978
- Iacoangeli, A., Rozhdestvensky, T. S., Dolzhanskaya, N., Tournier, B., Schutt, J., Brosius, J., et al. (2008). On BCL RNA and the fragile X mental retardation protein. *Proc. Natl. Acad. Sci. U.S.A.* 105, 734–739. doi: 10.1073/pnas.0710991105
- Ishizuka, A., Siomi, M. C., and Siomi, H. (2002). A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev.* 16, 2497–2508. doi: 10.1101/gad.1022002

- Jacquemont, S., Hagerman, R. J., Hagerman, P. J., and Leehey, M. A. (2007). Fragile-X syndrome and fragile X-associated tremor/ataxia syndrome: two faces of FMR1. *Lancet Neurol.* 6, 45–55. doi: 10.1016/S1474-4422(06)70676-7
- Jia, K., Chen, D., and Riddle, D. L. (2004). The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development* 131, 3897–3906. doi: 10.1242/dev.01255
- Jin, P., Alisch, R. S., and Warren, S. T. (2004a). RNA and microRNAs in fragile X mental retardation. *Nat. Cell Biol.* 6, 1048–1053. doi: 10.1038/ncb1104-1048
- Jin, P., Zarnescu, D. C., Ceman, S., Nakamoto, M., Mowrey, J., Jongens, T. A., et al. (2004b). Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat. Neurosci.* 7, 113–117. doi: 10.1038/nn1174
- Jin, Y., Tu, Q., and Liu, M. (2018). MicroRNA-125b regulates Alzheimer's disease through SphK1 regulation. *Mol. Med. Rep.* 18, 2373–2380. doi: 10.3892/mmr.2018.9156
- Johnson, S. C., Rabinovitch, P. S., and Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature* 493, 338–345. doi: 10.1038/nature11861
- Juang, B. T., Ludwig, A. L., Benedetti, K. L., Gu, C., Collins, K., Morales, C., et al. (2014). Expression of an expanded CGG-repeat RNA in a single pair of primary sensory neurons impairs olfactory adaptation in *Caenorhabditis elegans*. *Hum. Mol. Genet.* 23, 4945–4959. doi: 10.1093/hmg/ddu210
- Kawano, M., Tanaka, K., Itonaga, I., Ikeda, S., Iwasaki, T., and Tsumura, H. (2015). microRNA-93 promotes cell proliferation via targeting of PTEN in Osteosarcoma cells. *J. Exp. Clin. Cancer Res.* 34:76. doi: 10.1186/s13046-015-0192-z
- Kefas, B., Godlewski, J., Comeau, L., Li, Y., Abounader, R., Hawkinson, M., et al. (2008). microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res.* 68, 3566–3572. doi: 10.1158/0008-5472.CAN-07-6639
- Kenny, P. J., Zhou, H., Kim, M., Skariah, G., Khetani, R. S., Drnevich, J., et al. (2014). MOV10 and FMRP regulate AGO2 association with microRNA recognition elements. *Cell Rep.* 9, 1729–1741. doi: 10.1016/j.celrep.2014.10.054
- Khalil, A. M., Faghihi, M. A., Modarresi, F., Brothers, S. P., and Wahlestedt, C. (2008). A novel RNA transcript with antiapoptotic function is silenced in fragile X syndrome. *PLoS One* 3:e1486. doi: 10.1371/journal.pone.0001486
- Kilincaslán, A., Kok, B. E., Tekturk, P., Yalcinkaya, C., Ozkara, C., and Yapici, Z. (2017). Beneficial effects of everolimus on autism and attention-deficit/hyperactivity disorder symptoms in a group of patients with tuberous sclerosis complex. *J. Child Adolesc. Psychopharmacol.* 27, 383–388. doi: 10.1089/cap.2016.0100
- Kim, D. H., Villeneuve, L. M., Morris, K. V., and Rossi, J. J. (2006). Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat. Struct. Mol. Biol.* 13, 793–797. doi: 10.1038/nsmb1142
- Lin, S. L. (2015). microRNAs and Fragile X syndrome. *Adv. Exp. Med. Biol.* 888, 107–121. doi: 10.1007/978-3-319-22671-2_7
- Liu, T., Wan, R. P., Tang, L. J., Liu, S. J., Li, H. J., Zhao, Q. H., et al. (2015). A MicroRNA profile in Fmr1 knockout mice reveals MicroRNA expression alterations with possible roles in fragile X syndrome. *Mol. Neurobiol.* 51, 1053–1063. doi: 10.1007/s12035-014-8770-1
- Lukiw, W. J. (2007). Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport* 18, 297–300. doi: 10.1097/WNR.0b013e3280148e8b
- Luo, S., Huang, W., Xia, Q., Du, Q., Wu, L., and Duan, R. (2015). Mutational analyses of the FMR1 gene in Chinese pediatric population of fragile x suspects: low tolerance for point mutation. *J. Child Neurol.* 30, 803–806. doi: 10.1177/0883073814538508
- Luo, S., Huang, W., Xia, Q., Xia, Y., Du, Q., Wu, L., et al. (2014). Cryptic FMR1 mosaic deletion in a phenotypically normal mother of a boy with fragile X syndrome: case report. *BMC Med. Genet.* 15:125. doi: 10.1186/s12881-014-0125-2
- Luo, S. Y., Wu, L. Q., and Duan, R. H. (2016). Molecular medicine of fragile X syndrome: based on known molecular mechanisms. *World J. Pediatr.* 12, 19–27. doi: 10.1007/s12519-015-0052-0
- Morel, L., Regan, M., Higashimori, H., Ng, S. K., Esau, C., Vidensky, S., et al. (2013). Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. *J. Biol. Chem.* 288, 7105–7116. doi: 10.1074/jbc.M112.410944
- Muddashetty, R. S., Nalavadi, V. C., Gross, C., Yao, X., Xing, L., Laur, O., et al. (2011). Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. *Mol. Cell* 42, 673–688. doi: 10.1016/j.molcel.2011.05.006
- Myrick, L. K., Hashimoto, H., Cheng, X., and Warren, S. T. (2015). Human FMRP contains an integral tandem Agenet (Tudor) and KH motif in the amino terminal domain. *Hum. Mol. Genet.* 24, 1733–1740. doi: 10.1093/hmg/ddu586
- Myrick, L. K., Nakamoto-Kinoshita, M., Lindor, N. M., Kirmani, S., Cheng, X., and Warren, S. T. (2014). Fragile X syndrome due to a missense mutation. *Eur. J. Hum. Genet.* 22, 1185–1189. doi: 10.1038/ejhg.2013.311
- Nakamoto, M., Nalavadi, V., Epstein, M. P., Narayanan, U., Bassell, G. J., and Warren, S. T. (2007). Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15537–15542. doi: 10.1073/pnas.0707484104
- Napoli, I., Mercaldo, V., Boyl, P. P., Eleuteri, B., Zalfa, F., De Rubeis, S., et al. (2008). The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP. *Cell* 134, 1042–1054. doi: 10.1016/j.cell.2008.07.031
- Narayanan, U., Nalavadi, V., Nakamoto, M., Pallas, D. C., Ceman, S., Bassell, G. J., et al. (2007). FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A. *J. Neurosci.* 27, 14349–14357. doi: 10.1523/JNEUROSCI.2969-07.2007
- Narayanan, U., Nalavadi, V., Nakamoto, M., Thomas, G., Ceman, S., Bassell, G. J., et al. (2008). S6K1 phosphorylates and regulates fragile X mental retardation protein (FMRP) with the neuronal protein synthesis-dependent mammalian target of rapamycin (mTOR) signaling cascade. *J. Biol. Chem.* 283, 18478–18482. doi: 10.1074/jbc.C800055200
- Pastori, C., Peschansky, V. J., Barbouth, D., Mehta, A., Silva, J. P., and Wahlestedt, C. (2014). Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. *Hum. Genet.* 133, 59–67. doi: 10.1007/s00439-013-1356-6
- Peschansky, V. J., Pastori, C., Zeier, Z., Wentzel, K., Velmeshev, D., Magistri, M., et al. (2016). The long non-coding RNA FMR4 promotes proliferation of human neural precursor cells and epigenetic regulation of gene expression in trans. *Mol. Cell. Neurosci.* 74, 49–57. doi: 10.1016/j.mcn.2016.03.008
- Plante, I., Davidovic, L., Ouellet, D. L., Gobeil, L. A., Tremblay, S., Khandjian, E. W., et al. (2006). Dicer-derived microRNAs are utilized by the fragile X mental retardation protein for assembly on target RNAs. *J. Biomed. Biotechnol.* 2006:64347. doi: 10.1155/JBB/2006/64347
- Reinhart, B. J., and Bartel, D. P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science* 297:1831. doi: 10.1126/science.1077183
- Salta, E., Sierksma, A., Vanden Eynden, E., and De Strooper, B. (2016). miR-132 loss de-represses ITPKB and aggravates amyloid and TAU pathology in Alzheimer's brain. *EMBO Mol. Med.* 8, 1005–1018. doi: 10.15252/emmm.201606520
- Santoro, M. R., Bray, S. M., and Warren, S. T. (2012). Molecular mechanisms of fragile X syndrome: a twenty-year perspective. *Annu. Rev. Pathol.* 7, 219–245. doi: 10.1146/annurev-pathol-011811-132457
- Sarachana, T., Zhou, R., Chen, G., Manji, H. K., and Hu, V. W. (2010). Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. *Genome Med.* 2:23. doi: 10.1186/gm1144
- Sellier, C., Freyermuth, F., Tabet, R., Tran, T., He, F., Ruffenach, F., et al. (2013). Sequestration of DROSHA and DGCR8 by expanded CGG RNA repeats alters microRNA processing in fragile X-associated tremor/ataxia syndrome. *Cell Rep.* 3, 869–880. doi: 10.1016/j.celrep.2013.02.004
- Sharma, A., Hoeffer, C. A., Takayasu, Y., Miyawaki, T., McBride, S. M., Klann, E., et al. (2010). Dysregulation of mTOR signaling in fragile X syndrome. *J. Neurosci.* 30, 694–702. doi: 10.1523/JNEUROSCI.3696-09.2010
- Smith, P. Y., Hernandez-Rapp, J., Jolivet, F., Lecours, C., Bisht, K., Goupil, C., et al. (2015). miR-132/212 deficiency impairs tau metabolism and promotes pathological aggregation in vivo. *Hum. Mol. Genet.* 24, 6721–6735. doi: 10.1093/hmg/ddv377
- Smith, S. S., Laayoun, A., Lingeman, R. G., Baker, D. J., and Riley, J. (1994). Hypermethylation of telomere-like foldbacks at codon 12 of the human c-Ha-ras gene and the trinucleotide repeat of the FMR-1 gene of fragile X. *J. Mol. Biol.* 243, 143–151. doi: 10.1006/jmbi.1994.1640

- Suhl, J. A., Muddashetty, R. S., Anderson, B. R., Ifrim, M. F., Visootsak, J., Bassell, G. J., et al. (2015). A 3' untranslated region variant in FMR1 eliminates neuronal activity-dependent translation of FMRP by disrupting binding of the RNA-binding protein HuR. *Proc. Natl. Acad. Sci. U.S.A.* 112, E6553–E6561. doi: 10.1073/pnas.1514260112
- Tan, H., Poidevin, M., Li, H., Chen, D., and Jin, P. (2012). MicroRNA-277 modulates the neurodegeneration caused by Fragile X premutation rCGG repeats. *PLoS Genet.* 8:e1002681. doi: 10.1371/journal.pgen.1002681
- Tian, H., Cao, Y. X., Zhang, X. S., Liao, W. P., Yi, Y. H., Lian, J., et al. (2013). The targeting and functions of miRNA-383 are mediated by FMRP during spermatogenesis. *Cell Death Dis.* 4:e617. doi: 10.1038/cddis.2013.138
- Treiber, T., Treiber, N., and Meister, G. (2018). Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. *Nat. Rev. Mol. Cell Biol.* 20, 5–20. doi: 10.1038/s41580-018-0059-1
- Tsujimura, K., Irie, K., Nakashima, H., Egashira, Y., Fukao, Y., Fujiwara, M., et al. (2015). miR-199a Links MeCP2 with mTOR Signaling and Its dysregulation leads to rett syndrome phenotypes. *Cell Rep.* 12, 1887–1901. doi: 10.1016/j.celrep.2015.08.028
- Usdin, K., Hayward, B. E., Kumari, D., Lokanga, R. A., Sciascia, N., and Zhao, X. N. (2014). Repeat-mediated genetic and epigenetic changes at the FMR1 locus in the Fragile X-related disorders. *Front. Genet.* 5:226. doi: 10.3389/fgene.2014.00226
- Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I., et al. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 303, 672–676. doi: 10.1126/science.1093686
- Verkerk, A. J., Pieretti, M., Sutcliffe, J. S., Fu, Y. H., Kuhl, D. P., Pizzuti, A., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65, 905–914. doi: 10.1016/0092-8674(91)90397-H
- Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., and Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297, 1833–1837. doi: 10.1126/science.1074973
- Wan, G., Xie, W., Liu, Z., Xu, W., Lao, Y., Huang, N., et al. (2014). Hypoxia-induced MIR155 is a potent autophagy inducer by targeting multiple players in the MTOR pathway. *Autophagy* 10, 70–79. doi: 10.4161/auto.26534
- Wan, R. P., Zhou, L. T., Yang, H. X., Zhou, Y. T., Ye, S. H., Zhao, Q. H., et al. (2017). Involvement of FMRP in primary MicroRNA processing via enhancing *Drosha* translation. *Mol. Neurobiol.* 54, 2585–2594. doi: 10.1007/s12035-016-9855-9
- Wang, B., Pan, L., Wei, M., Wang, Q., Liu, W. W., Wang, N., et al. (2015). FMRP-mediated axonal delivery of miR-181d regulates axon elongation by locally targeting Map1b and Calm1. *Cell Rep.* 13, 2794–2807. doi: 10.1016/j.celrep.2015.11.057
- Wang, T., Bray, S. M., and Warren, S. T. (2012). New perspectives on the biology of fragile X syndrome. *Curr. Opin. Genet. Dev.* 22, 256–263. doi: 10.1016/j.gde.2012.02.002
- Wang, Y., Veremeyko, T., Wong, A. H., El Fatimy, R., Wei, Z., Cai, W., et al. (2017). Downregulation of miR-132/212 impairs S-nitrosylation balance and induces tau phosphorylation in Alzheimer's disease. *Neurobiol. Aging* 51, 156–166. doi: 10.1016/j.neurobiolaging.2016.12.015
- Wilkinson, J. E., Burmeister, L., Brooks, S. V., Chan, C. C., Friedline, S., Harrison, D. E., et al. (2012). Rapamycin slows aging in mice. *Aging Cell* 11, 675–682. doi: 10.1111/j.1474-9726.2012.00832.x
- Wu, R., Su, Y., Wu, H., Dai, Y., Zhao, M., and Lu, Q. (2016). Characters, functions and clinical perspectives of long non-coding RNAs. *Mol. Genet. Genomics* 291, 1013–1033. doi: 10.1007/s00438-016-1179-y
- Wu, Y. E., Parikshak, N. N., Belgard, T. G., and Geschwind, D. H. (2016). Genome-wide, integrative analysis implicates microRNA dysregulation in autism spectrum disorder. *Nat. Neurosci.* 19, 1463–1476. doi: 10.1038/nn.4373
- Xiao, T., Zhang, W., Jiao, B., Pan, C. Z., Liu, X., and Shen, L. (2017). The role of exosomes in the pathogenesis of Alzheimer' disease. *Transl. Neurodegener.* 6:3. doi: 10.1186/s40035-017-0072-x
- Xie, N., Gong, H., Suhl, J. A., Chopra, P., Wang, T., and Warren, S. T. (2016). Reactivation of FMR1 by CRISPR/Cas9-mediated deletion of the expanded CGG-repeat of the fragile X chromosome. *PLoS One* 11:e0165499. doi: 10.1371/journal.pone.0165499
- Yang, L., Duan, R., Chen, D., Wang, J., Chen, D., and Jin, P. (2007). Fragile X mental retardation protein modulates the fate of germline stem cells in *Drosophila*. *Hum. Mol. Genet.* 16, 1814–1820. doi: 10.1093/hmg/ddm129
- Yang, Y., Xu, S., Xia, L., Wang, J., Wen, S., Jin, P., et al. (2009). The bantam microRNA is associated with drosophila fragile X mental retardation protein and regulates the fate of germline stem cells. *PLoS Genet.* 5:e1000444. doi: 10.1371/journal.pgen.1000444
- Ye, P., Liu, Y., Chen, C., Tang, F., Wu, Q., Wang, X., et al. (2015). An mTORC1-Mdm2-Drosha axis for miRNA biogenesis in response to glucose- and amino acid-deprivation. *Mol. Cell* 57, 708–720. doi: 10.1016/j.molcel.2014.12.034
- Zalfa, F., Adinolfi, S., Napoli, I., Kuhn-Holsken, E., Urlaub, H., Achsel, T., et al. (2005). Fragile X mental retardation protein (FMRP) binds specifically to the brain cytoplasmic RNAs BC1/BC200 via a novel RNA-binding motif. *J. Biol. Chem.* 280, 33403–33410. doi: 10.1074/jbc.M504286200
- Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., et al. (2003). The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell* 112, 317–327. doi: 10.1016/S0092-8674(03)00079-5
- Zeng, C. W., Zhang, X. J., Lin, K. Y., Ye, H., Feng, S. Y., Zhang, H., et al. (2012). Camptothecin induces apoptosis in cancer cells via microRNA-125b-mediated mitochondrial pathways. *Mol. Pharmacol.* 81, 578–586. doi: 10.1124/mol.111.076794
- Zhang, T., Pang, P., Fang, Z., Guo, Y., Li, H., Li, X., et al. (2018). Expression of BC1 impairs spatial learning and memory in Alzheimer's disease via APP translation. *Mol. Neurobiol.* 55, 6007–6020. doi: 10.1007/s12035-017-0820-z
- Zhang, W., Jiao, B., Zhou, M., Zhou, T., and Shen, L. (2016). Modeling Alzheimer's disease with induced pluripotent stem cells: current challenges and future concerns. *Stem Cells Int.* 2016:7828049. doi: 10.1155/2016/7828049
- Zhong, J., Chuang, S. C., Bianchi, R., Zhao, W., Paul, G., Thakkar, P., et al. (2010). Regulatory BC1 RNA and the fragile X mental retardation protein: convergent functionality in brain. *PLoS One* 5:e15509. doi: 10.1371/journal.pone.0015509
- Zhu, Q. B., Unmehopa, U., Bossers, K., Hu, Y. T., Verwer, R., Balesar, R., et al. (2016). MicroRNA-132 and early growth response-1 in nucleus basalis of Meynert during the course of Alzheimer's disease. *Brain* 139(Pt 3), 908–921. doi: 10.1093/brain/awv383
- Zongaro, S., Hukema, R., D'Antoni, S., Davidovic, L., Barbry, P., Catania, M. V., et al. (2013). The 3' UTR of FMR1 mRNA is a target of miR-101, miR-129-5p and miR-221: implications for the molecular pathology of FXTAS at the synapse. *Hum. Mol. Genet.* 22, 1971–1982. doi: 10.1093/hmg/ddt044

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MiR-378a-5p Regulates Proliferation and Migration in Vascular Smooth Muscle Cell by Targeting CDK1

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Objective: Abnormal proliferation or migration of vascular smooth muscle cells (VSMCs) can lead to vessel lesions, resulting in atherosclerosis and in stent-restenosis (IRS). The purpose of our study was to establish the role of miR-378a-5p and its targets in regulating VSMCs function and IRS.

Methods: EdU assays and Cell Counting Kit-8 (CCK-8) assays were applied to evaluate VSMCs proliferation, wound healing assays and transwell assays were applied to assess cells migration. Furthermore, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to investigate the expression level of miR-378a-5p IRS patients and healthy individuals. Target genes were predicted using Target Scan and miRanda software, and biological functions of candidate genes were explored through bioinformatics analysis. Moreover, RNA-binding protein immunoprecipitation (RIP) was carried out to analyze the miRNAs interactions with proteins. We also used Immunofluorescence (IF) and fluorescence microscopy to determine the binding properties, localization and expression of miR-378a-5p with downstream target CDK1.

Results: The expression of miR-378a-5p was increased in the group with stent restenosis compared with healthy people, as well as in the group which VSMCs stimulated by platelet-derived growth factor-BB (PDGF-BB) compared with NCs. MiR-378a-5p over-expression had significantly promoted proliferative and migratory effects, while miR-378a-5p inhibitor suppressed VSMC proliferation and migration. CDK1 was proved to be the functional target of miR-378a-5p in VSMCs. Encouragingly, the expression of miR-378a-5p was increased in patients with stent restenosis compared with healthy people, as well as in PDGF-BB-stimulated VSMCs compared with control cells. Furthermore, co-transfection experiments demonstrated that miR-378a-5p over-expression promoted proliferation and migration of VSMCs specifically by reducing CDK1 gene expression levels.

Conclusion: In this investigatory, we concluded that miR-378a-5p is a critical mediator in regulating VSMC proliferation and migration by targeting CDK1/p21 signaling pathway. Thereby, interventions aimed at miR-378a-5p may be of therapeutic application in the prevention and treatment of stent restenosis.

Keywords: miR-378a-5p, vascular smooth muscle cell, stent-restenosis, proliferation, migration, atherosclerosis, CDK1

OPEN ACCESS

Edited by:

Zhao-Qian Teng,
Chinese Academy of Sciences, China

Reviewed by:

Chi-Ming Wong,
The University of Hong Kong,
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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 10 October 2018

Accepted: 15 January 2019

Published: 19 February 2019

Citation:

Liu S, Yang Y, Jiang S, Xu H,
Tang N, Lobo A, Zhang R, Liu S, Yu T
and Xin H (2019) MiR-378a-5p
Regulates Proliferation and Migration
in Vascular Smooth Muscle Cell by
Targeting CDK1. *Front. Genet.* 10:22.
doi: 10.3389/fgene.2019.00022

INTRODUCTION

Coronary artery disease (CAD) is a serious disease threatening human health with its high mortality rate. Percutaneous Coronary Intervention (PCI) and stent implantation are commonly applied in the treatment of these obstructive diseases (Feinberg, 2014; Buccheri et al., 2016). However, there will be new atherosclerosis around stent implantation, and this will further increase the rate of restenosis after stent implantation (Wang H. et al., 2015; Liu et al., 2018). As a foreign substance, a scaffold can cause damage to the tunica intima, followed by inflammation and platelet aggregation in the damaged areas; resulting in the formation of plaque and thrombosis in the long term (Liu et al., 2018; Tang et al., 2018). And after stent implantation, endothelialization occurs gradually which cannot be removed from the blood vessels again; so, if in-stent restenosis occurs, stent implantation must be repeated (Alfonso et al., 2006; Finn et al., 2007). Aberrant proliferation and migration of Vascular smooth muscle cells (VSMCs) were identified as the main causes of these adverse events (Krist et al., 2015; Afzal et al., 2016; Huang et al., 2017). When VSMCs are stimulated, they promote the transfer of VSMCs from tunica media to tunica intima, from the contractile to the secretory, while stimulating free VSMCs and fibroblasts to secrete a large amount of extracellular matrix; the extracellular matrix is continuously deposited in the blood vessels, causing the intima to gradually thicken, resulting in stenosis (Braun-Dullaues et al., 1998). Consequently, investigating key regulators and understanding the molecular mechanisms of VSMC biology has become a major method of treating atherosclerosis and stent restenosis.

MicroRNA (miRNA) is a type of small non-coding RNA that negatively modulates gene expression through mRNA translation repression or the induction of target mRNA instability (Gareri et al., 2016). Mounting shreds of evidence suggested that several miRNAs play critical roles in regulating VSMC proliferation and migration, such as miR-133, miR-221, miR-222, miR-663, miR-143, and miR-145 (Liu et al., 2009; Xin et al., 2009; Li et al., 2013; McDonald et al., 2015). Altering the expression of miRNA may have therapeutic potential in the prevention and treatment of stent restenosis.

It has been reported that the expression levels of miR-378a-5p in cardiac myocytes increased under some external stimuli (Wang et al., 2017; Rui et al., 2018). MiR-378a-5p is involved in the biological functions of some tumor cells (Luo et al., 2012), promoting proliferation in some tumor cells, and inhibiting proliferation in some others (Wang Z. et al., 2015). Considering that there may be tissue specificity associated with miR-378a-5p. However, the effect of miR-378a-5p in the regulation of VSMC biology remains unknown. The objective of the study is to investigate the potential roles of miR-378a-5p, as well as the molecular mechanisms of VSMCs proliferation and migration. Firstly, we screened and identified differential expression of miR-378 in patients with stent restenosis, then we studied the effect of up-regulation and down-regulation miR-378a-5p on the biological function of VSMCs. We also found that CDK1 was a potential gene target for the miR-378a-5p. Meanwhile, p21 may be the downstream target of CDK1. Consequently,

miR-378a-5p is a key modulator to regulate proliferation and migration of VSMC partly by modulating the level of CDK1 gene expression. In this way, we have enough reason to believe that miR-378a-5p could be used as a diagnostic marker for early diagnosis, monitoring, and treatment of molecular targets for stent restenosis.

MATERIALS AND METHODS

Blood Samples Acquisition and Baseline Clinical Characteristics Collection

Thirty-two persons were collected at the affiliated hospital of Qingdao University in Qingdao, China from June 2017 through February 2018. According to whether ISR was detected, they were classified into two groups: (1) The ISR group ($n = 14$): ISR is defined as a diameter stenosis greater than 50% in coronary angiography that occurs within the stent or 5 mm at the proximal or distal end of the stent; (2) The normal group ($n = 18$): 18 healthy persons without coronary heart disease as the control group. Basic information of all individuals collected, including age, gender, history of diabetes, drinking, hypertension, and smoking was noted. The research was supported by the Institutional Review Boards of Qingdao University Health Science Center. Paper version of informed consent was acquired from all subjects and the regional ethics committee in Qingdao, China approved the study protocol. The information of all clinical people is displayed in **Supplementary Table 3**.

Test Animals

All experimental laboratory animals were approved by the Animal Care and Use Committee. C57BL/6 and ApoE^{-/-} mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. There were 3 mice in each group. The control group was given a normal diet, the experimental group was given a western diet (conventional mouse feed+0.15% cholesterol+21% fat) for 12 weeks, then cardiac blood was collected from mice weighing 25–30 g for further experiments.

Cell Culture

The VSMC was purchased from the Chinese Type Culture Collection (Chinese Academy of Sciences, Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, United States) containing 10% fetal bovine serum (ExCell Bio.) in a 5% CO₂ humidified incubator at 37°C. MiR-378a-5p mimics, miR-378a-5p inhibitor and negative control oligonucleotide (NC) (GenePharma, Shanghai, China) were transfected into the VSMCs using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, United States).

Western Blot Analysis

Cells lysates were prepared in buffer mixture containing 1 ml RIPA (Solarbio, Beijing, China), 0.1 mM PMSF reagent and a protease inhibitor cocktail (Roche, Basel, Switzerland) for 10 min on ice, after which protein samples were separated by 10% SDS-PAGE, then transferred into 0.45 μ m polyvinylidene

difluoride (PVDF) membrane, membranes were blocked with 5% not-fat milk in Tris-buffered saline-Tween 20 (TBS-T) for 1 h. And incubated with a rabbit anti-CDK1 monoclonal antibody (1:10000 dilution; Abcam, MA, United States) or anti- β -actin (1:2500 dilution; Cell Signaling Technology, United States). Then being washed three times with TBS-Tween 20, the secondary antibodies were added. Finally, the signals were visualized with Supersensitive ECL Chemiluminescent Kit, according to the directions of the manufacturer. The quantification of the protein bands was performed using ImageJ 1.8.0.

RNA Extraction and qRT-PCR

Total RNA was extracted from the collected blood samples using TRIzol (Invitrogen, Grand Island, NY, United States), then treatment with DNase I (Takara, Otsu, Japan), then reverse RNA with reverse transcriptase kit (Takara) and mature miRNA levels were assessed using SYBR Green Real-time PCR Master Mix (Takara) according to the manufacturer's guidance. The following primers which used in the experiment showed in **Supplementary Tables 1, 2**. U6 and GAPDH are based on different detection genes as reference genes, respectively. Analysis of qRT-PCR results using the $2^{-\Delta\Delta Ct}$ method.

RNA Binding Protein Immunoprecipitation (RIP)

RNA-binding protein immunoprecipitation assays are performed to identify regions of the genome with RNA-binding proteins. In RIP assays, VSMCs were lysed in RIPA buffer containing 0.1 mM PMSF and 1% protease inhibitor cocktail on ice. After 10 min, the collecting cells were centrifuged at 12000 rpm for 20 min, the next step is to take 500 μ g cell lysates incubated with the CDK1 antibody at 4°C overnight. Then add protein A/G-agarose beads and incubate for 4 h at 4°C with shaking. Immunoprecipitation separates RNA-binding proteins and their bound RNA. Furthermore, the combination of RIP

and quantitative qRT-PCR can present experimental results more intuitively.

Cell Proliferation

Cell Counting Kit-8 (CCK-8) assay was performed to assess VSMC proliferation. Cells were incubated in DMEM at a density of 5×10^3 cells per well in 96-well plates for 24 h after transfection. And then the cells were maintained in 10 μ l /well CCK-8 solution (7Sea-Cell Counting Kit, Shanghai, China) for an additional 1 h. Finally, the value was measured at 450 nm absorbance.

Another way to test cell proliferation is to use the EdU assay, VSMCs were cultured with EdU solution (50 nmol/L) (RiboBio, Guangzhou, China) for 2 h, then VSMCs were stained according to the product instructions. And finally, the pictures were obtained by a fluorescence microscope (Zeiss, LSM510, META). Image J 1.8.0 was used for analysis of the data.

Cell Migration

Transwell assay and wound healing assay were performed to assess the migratory ability of VSMC.

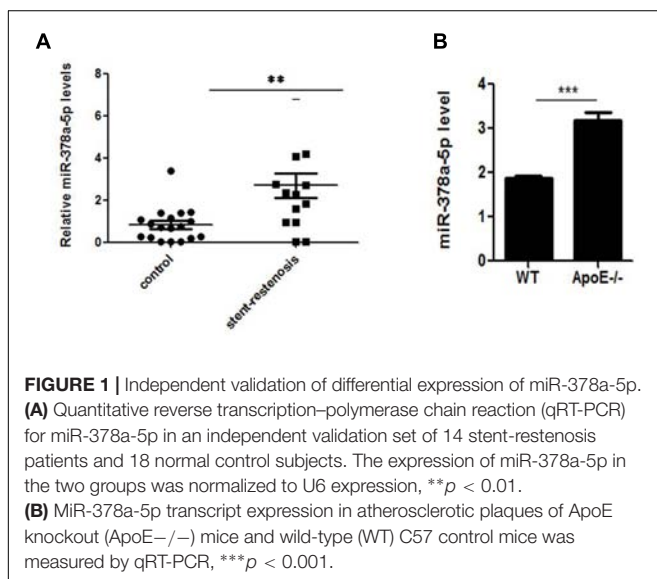
Vascular smooth muscle cells were maintained in 6-well plates 12 h before transfection. After transfected, cells were cultured for 24 h in normal medium and then an additional 24 h in serum-free DMEM. After resuspending VSMCs in serum-free DMEM (5×10^5 cells/ml), a mixture of 200 μ l was added to the upper chamber of a transwell insert (Corning, Tewksbury, NY, United States) in a 24-well plate. Meanwhile, the lower chamber added 500 μ l DMEM supplemented with 10% FBS. After 24 h incubation, PBS-rinsed cotton was used to wipe off the cells remaining on the upper side of the membrane. Then the cells were fixed with 4% paraformaldehyde for 1 h and dyed with 0.1% crystal violet for 30 min. After three washes, migrated VSMCs were recorded with a Zeiss LSM510 META microscope (20 $\times$ magnification), the 5 randomly fields were selected to count the cells.

Wound Healing Assay

Vascular smooth muscle cells were grown up to 60–70% in six-well plates. Then, cells were transfected with miR-378a-5p mimics, miR-378a-5p inhibitor, and NC. After 24 h, the wounds were made by a 1000- μ l disposable pipette tip, which had reached almost 100% confluence. Distance on both sides of the scratch was visualized and photographed immediately and at different time points after wounding using a Zeiss LSM510 META microscope.

Statistical Analysis

All data presented in this paper were the mean $\pm$ SD of at least three independent experiments, and an experiment performed with three samples for *in vitro* experiments. Data analyses were carried out using the GraphPad Prism 5 software. The quantitative data were presented as means $\pm$ SEM. Statistical analysis of the two groups by *t*-test, the different *P*-values indicate the different statistical significance: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.



RESULTS

MiR-378a-5p Expression in Stent-Restenosis Patients and ApoE $-/-$ Mice

MiR-378a-5p expression were detected between the patients with stent-restenosis and control group, respectively, by qRT-PCR, in which we found that miR-378a-5p expression levels were upregulated in stent-restenosis patients compared with control group (Figure 1A); MiR-378a-5p expression levels were higher in atherosclerotic plaques of ApoE knockout (ApoE $-/-$) mice than in control subjects as measured by qRT-PCR (Figure 1B).

MiR-378a-5p Promoted VSMCs Proliferation and Migration

MiR-378a-5p regulates biological function of VSMC. VSMCs were transfected with miR-378a-5p mimics (25 nM) and miR-378a-5p inhibitor (100 nM), both of them had visible transfection efficiency (Figure 2A). MiR-378a-5p mimics-transfected cells have stronger proliferative capacity compared with NC (25 nmol/L) (Figure 2B). Meanwhile, VSMC proliferation was assessed using the EdU assay; representative staining of the nucleus of proliferating VSMCs was shown in Figure 2C. EdU incorporation measured by confocal laser microscopy. In addition, we observed that miR-378a-5p significantly promoted cell migration in miR-378a-5p mimics-transfected

cells compared with NC, the miR-378a-5p inhibitor has the opposite effect (Figures 2D,E). Sequence of RNAs used in this study showed in Supplementary Table 4.

MiR-378a-5p Promoted Proliferation and Migration in PDGF-BB-Stimulated VSMCs

In order to detect the effects of miR-378a-5p in PDGF-BB-stimulated VSMC, we transfect miR-378a-5p mimics, miR-378a-5p inhibitor and NC into VSMCs. MiR-378a-5p was upregulated after PDGF-BB treatment in a time-dependent manner (Figure 3A). MiR-378a-5p mimic-induce miR-378a-5p up-regulation significantly increased VSMC migration compared with NC, the miR-378a-5p inhibitors have the opposite effect, as demonstrated by wound closure assay (Figures 3B,C) and transwell assay (Figures 3D,E). To detect the effect of PDGF-BB on VSMC phenotype switching genes, VSMCs were treated with PDGF-BB (50 ng/ml) for 12 h, the expression levels of sm-MHC2 and α -SMA protein decreased after being transfected with miR-378a-5p mimics (Figures 3F-I). These results demonstrate that miR-378a-5p promoted proliferation and migration in PDGF-BB-stimulated VSMCs.

Identification of CDK1 as a Direct Target of MiR-378a-5p in VSMCs

Target Scan algorithms found that CDK1 was a potential miR-378a-5p target. We found that the putative seed sequences

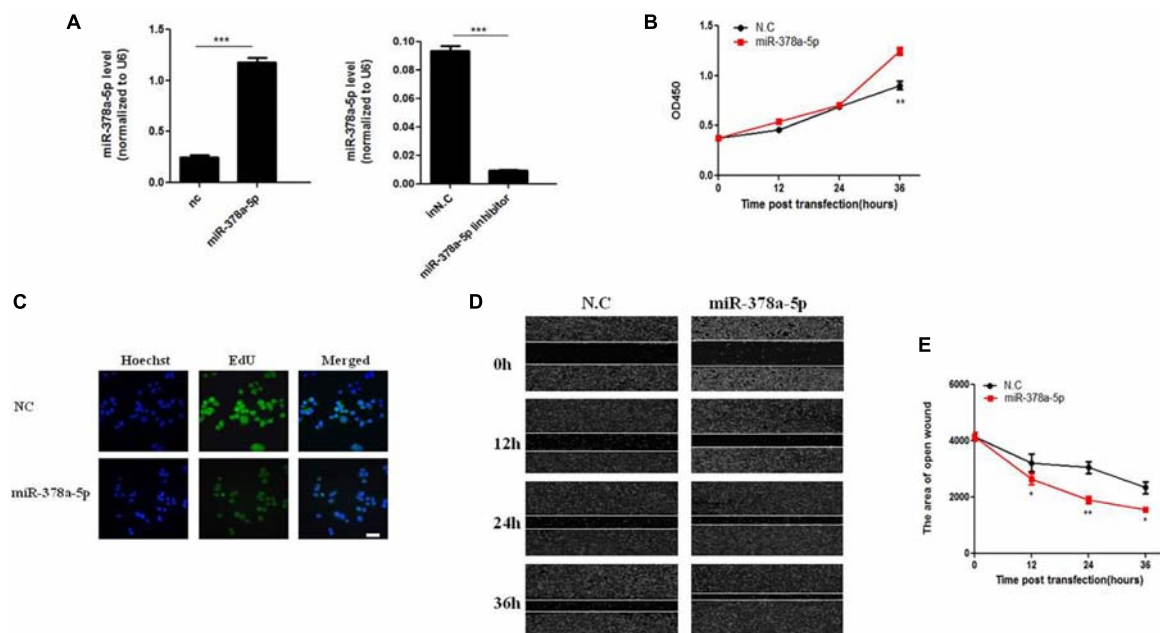


FIGURE 2 | Effects of miR-378a-5p on the proliferation and migration in vascular smooth muscle cell (VSMC). **(A)** Expression levels of miR-378a-5p were examined by qRT-PCR after transfection of miR-378a-5p mimics, miR-378a-5p inhibitor and negative control oligonucleotide (NC), *** $p < 0.001$. **(B)** The CCK-8 assay was performed to investigate the proliferation of VSMCs with miR-378a-5p mimics, miR-378a-5p inhibitor transfection, at 0, 12, 24, and 36 h, respectively. **(C)** Representative micrographs of EdU staining of VSMCs, with control or miR-378a-5p transfection, scale bar = 20 μ m. **(D,E)** miR-378a-5p effects on VSMC migration ability as determined via classic scratch assay and its quantification analysis, all images were taken under the same magnification, data are presented as mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$.

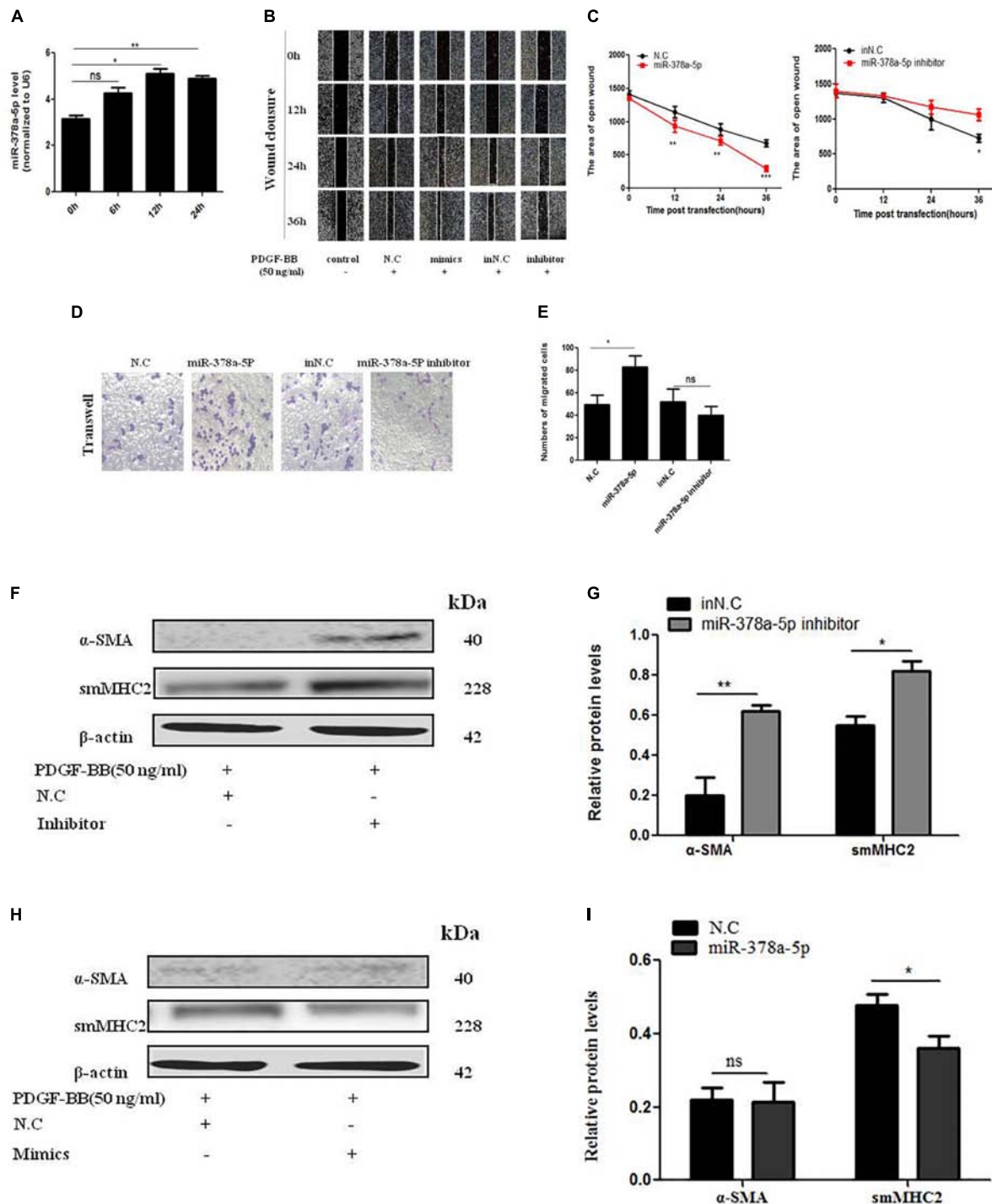


FIGURE 3 | MiR-378a-5p promotes platelet-derived growth factor-BB (PDGF-BB)-induced VSMC proliferation and migration. **(A)** miR-378a-5p expression levels were increased in VSMCs under the time gradient of PDGF-BB-induced (50 ng/ml), compared with quiescent cells, as demonstrated using qRT-PCR, data are presented as mean $\pm$ SEM. * p < 0.05 and ** p < 0.01. **(B,C)** miR-378a-5p abrogated PDGF-BB-mediated effects on VSMC migration ability as determined via classic scratch assay and its quantification analysis, all images were taken under the same magnification. **(D,E)** The miR-378a-5p mimics significantly increased PDGF-BB-induced (50 ng/ml) VSMC migration, as determined by transwell assay (original magnification: $\times$ 200) and its quantification analysis, data are presented as mean $\pm$ SEM. * p < 0.05. **(F)** Representative western blots of VSMC phenotype marker genes transfected with miR-378a-5p inhibitor compared with inN.C. under the condition with PDGF-BB (50 ng/ml) stimulated for 12 h. **(G)** Quantitative analysis of differentiation marker gene expression in the two groups, data are presented as mean $\pm$ SEM. * p < 0.05 and ** p < 0.01. **(H)** Representative western blots of VSMC phenotype marker genes transfected with miR-378a-5p mimics compared with NC under the condition with PDGF-BB (50 ng/ml) stimulated for 12 h. **(I)** Quantitative analysis of differentiation marker gene expression in the two groups, data are presented as mean $\pm$ SEM. * p < 0.05.

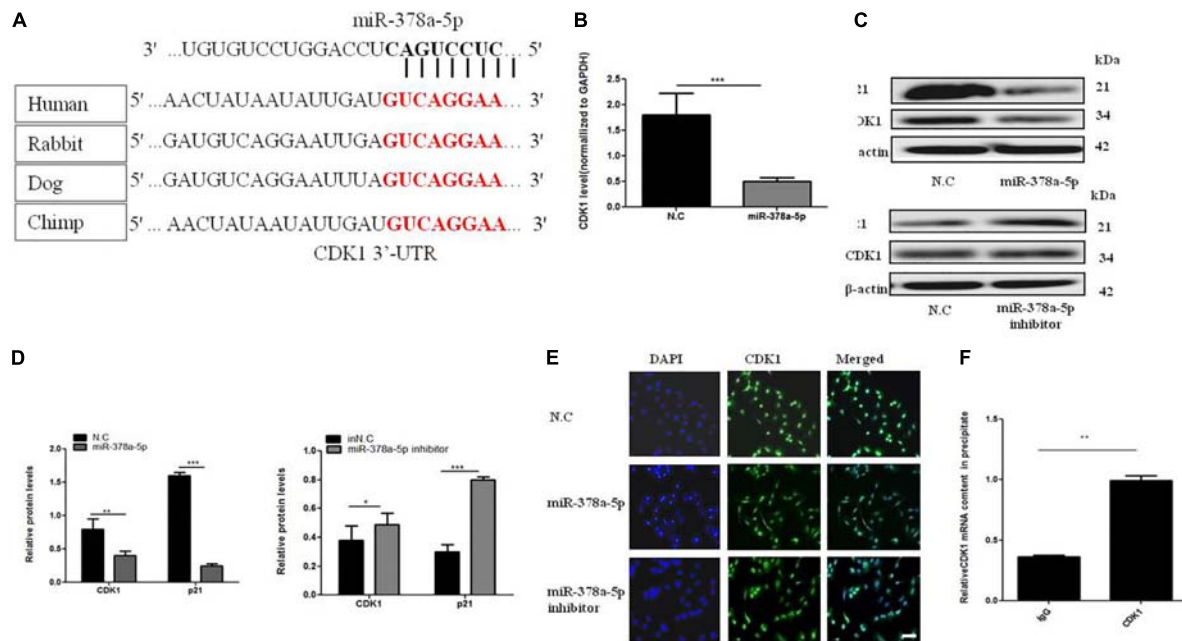


FIGURE 4 | MiR-378a-5p participates in the regulation of *CDK1* expression. **(A)** There is a potential seed sequence of miR-378a-5p in the 3'-UTR of *CDK1*, the putative seed sequences for the 3'-UTR of *CDK1* were highly conserved in different species. **(B)** qRT-PCR, **(C,D)** western blot were used to determine *CDK1* mRNA and protein expression levels and p21 protein levels in VSMCs transfected with miR-378a-5p mimics (25 nmol/L), inhibitor (100 nmol/L), NC (25 nmol/L) and inNC (25 nmol/L) after PDGF-BB stimulation, data are presented as mean $\pm$ SEM, * p < 0.05, ** p < 0.01, and *** p < 0.001. **(E)** Typical fluorescence photomicrograph by laser scan confocal microscopy, scale bar = 20 μ m. The photographs of nuclei (blue) and *CDK1* (green) fluorescence were taken under a same field and then were merged. **(F)** Real-time PCR validation of *CDK1*-associated mRNAs identified by RIP, data are presented as mean $\pm$ SEM. ** p < 0.01.

for miR-378a-5p within the 3'-UTR of *CDK1* were highly conserved and there has a potential seed sequence of miR-378a-5p in the 3'-UTR of *CDK1* (Figure 4A). To illustrate the relationship between *CDK1* and miR-378a-5p, we transfected VSMCs with miR-378a-5p mimics, inhibitor and NC, and investigated *CDK1* expression using western blot analysis and qRT-PCR, overexpression of miR-378a-5p suppressed the protein expression of *CDK1*, as showed in Figures 4B–D, referring to the literature, p21 is one of the downstream targets of *CDK1* (Kreis et al., 2016). In our experiment, we found that miR-378a-5p works by reducing *CDK1* and then regulating p21. Then we performed immunofluorescence which showed that *CDK1* protein expression was decreased with transfected miR-378a-5p mimics compared with NC, as showed in Figure 4E. The conclusion of these findings is: miR-378a-5p upregulation could inhibit *CDK1* expression at the post-transcriptional level. RIP was used to analyze the protein interactions with *CDK1* mRNA. The % input detected for *CDK1* immunoprecipitation is above that detected for IgG immunoprecipitation, which means *CDK1* antibody could pull down more miR-378a-5p than the non-specific IgG antibody (Figure 4F). These results demonstrated that miR-378a-5p directly binds to the 3'-UTR of *CDK1*.

CDK1 Is Involved in VSMC Proliferation and Migration

To determine the effect of *CDK1* on the VSMC, expression of *CDK1* under PDGF-BB stimulation gradient by western blot

(Figures 5A,B). Two siRNAs were designed to knock down *CDK1*, the sequences were shown in Supplementary Table 2. The transfection efficiency and expression efficiency of si*CDK1* was detected by qRT-PCR. The result showed that *CDK1* was significantly down-regulated when transfected with si*CDK1*(#1) and si*CDK1*(#2), respectively (Figure 5C). The influence of si*CDK1* on the proliferation of cells was assessed by EdU assay (Figure 5D). Transwell assay was applied to investigate the migration ability of cells, showed in Figures 5E,F. The wound closure assay was performed to detect the migration ability of cells with or without PDGF-BB-induced (50 ng/ml) (Figures 5G,H). α -SMA and sm-MHC2 protein level were down-regulated after transfected with si*CDK1* (Figures 5I,J). The findings showed that the proliferation and migration activity of VSMCs transfected with si*CDK1* was higher than that of cells transfected with an empty plasmid with or without PDGF-BB stimulation.

MiR-378a-5p Targeted *CDK1* Expression and Enhanced Migration of VSMCs

For further confirm whether *CDK1* is a functional target gene of miR-378a-5p, wound closure (Figures 6A,B) and transwell assays (Figures 6C,D) were used to measure the migratory ability of VSMCs, compared with the NC, the migration rate of the miR-378a-5p mimic group was remarkably increased, while that of the si-*CDK1* group has no significant increase, the group with miR-378a-5p mimic

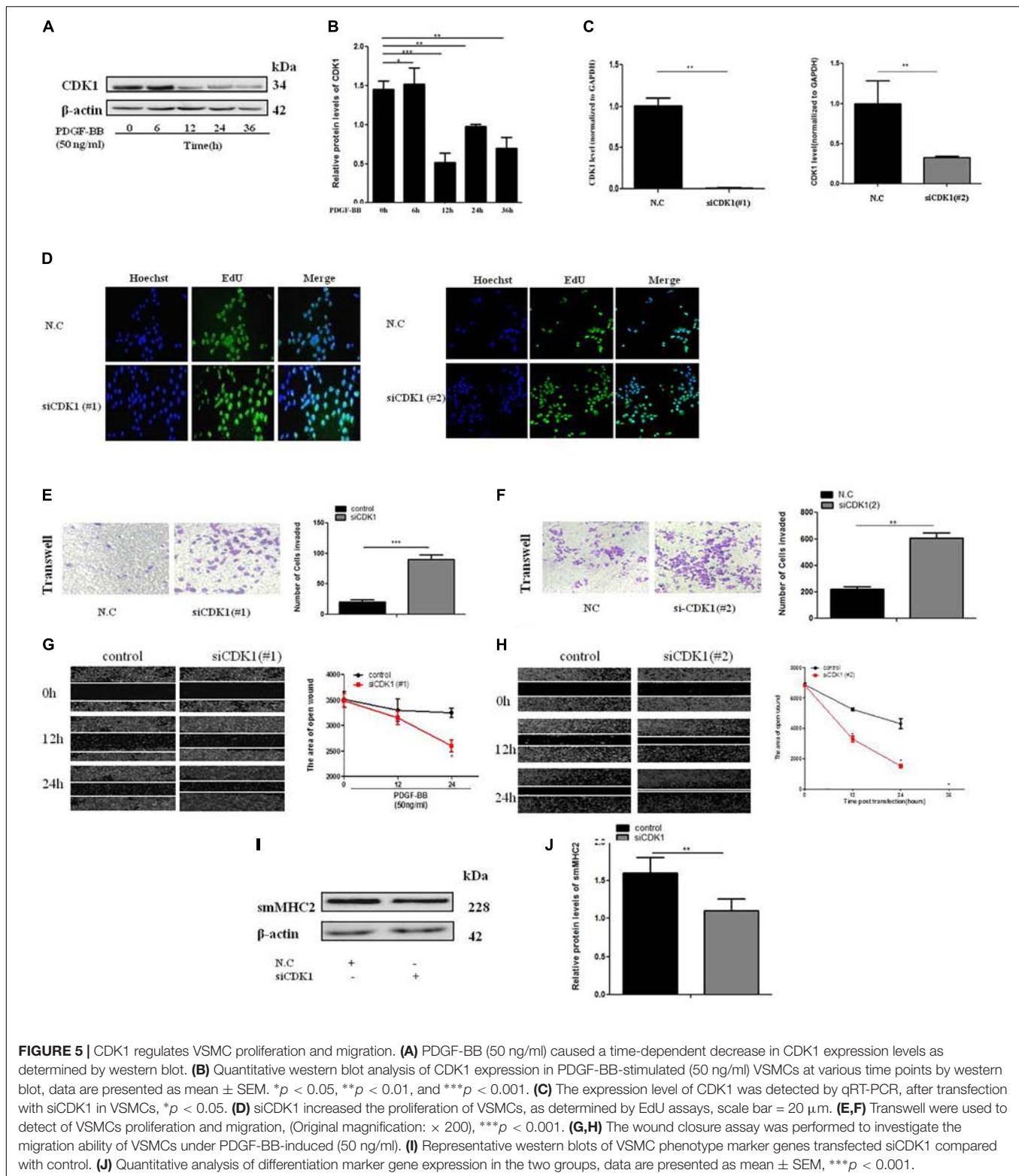


FIGURE 5 | CDK1 regulates VSMC proliferation and migration. **(A)** PDGF-BB (50 ng/ml) caused a time-dependent decrease in CDK1 expression levels as determined by western blot. **(B)** Quantitative western blot analysis of CDK1 expression in PDGF-BB-stimulated (50 ng/ml) VSMCs at various time points by western blot, data are presented as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. **(C)** The expression level of CDK1 was detected by qRT-PCR, after transfection with siCDK1 in VSMCs, $*p < 0.05$. **(D)** siCDK1 increased the proliferation of VSMCs, as determined by EdU assays, scale bar = 20 μ m. **(E,F)** Transwell were used to detect of VSMCs proliferation and migration, (Original magnification: $\times 200$), $***p < 0.001$. **(G,H)** The wound closure assay was performed to investigate the migration ability of VSMCs under PDGF-BB-induced (50 ng/ml). **(I)** Representative western blots of VSMC phenotype marker genes transfected siCDK1 compared with control. **(J)** Quantitative analysis of differentiation marker gene expression in the two groups, data are presented as mean $\pm$ SEM, $***p < 0.001$.

+ siCDK1 restored the migration ability, the number of cells migrated significantly increased. From these results, we determined that CDK1 is a functional downstream target of miR-378a-5p.

CONCLUSION

In our study, we identified that miR-378a-5p is an important modulator in the PDGF-BB stimulated proliferation and

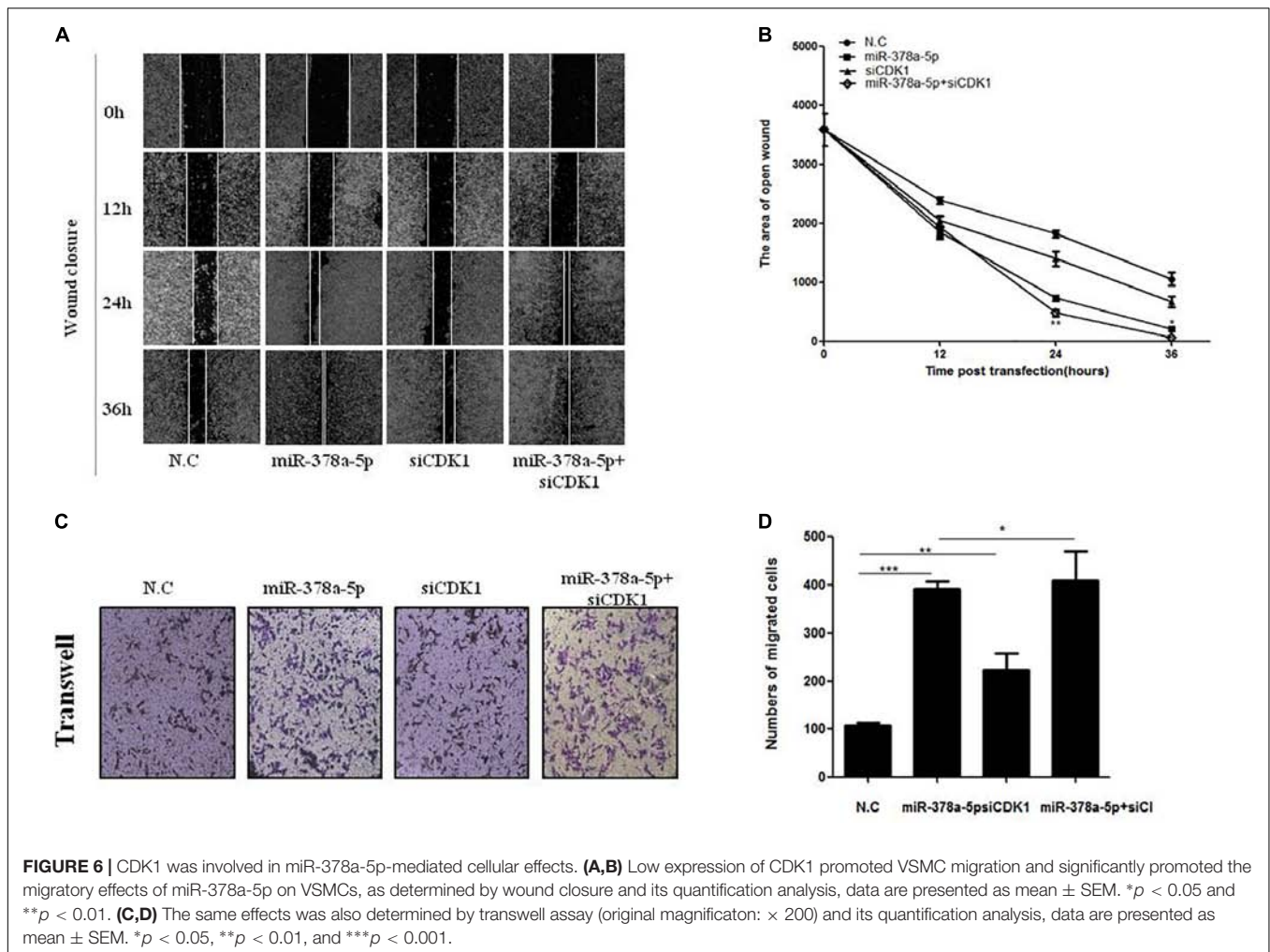


FIGURE 6 | CDK1 was involved in miR-378a-5p-mediated cellular effects. **(A,B)** Low expression of CDK1 promoted VSMC migration and significantly promoted the migratory effects of miR-378a-5p on VSMCs, as determined by wound closure and its quantification analysis, data are presented as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$. **(C,D)** The same effects was also determined by transwell assay (original magnification: $\times 200$) and its quantification analysis, data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

migration of VSMC by targeting, at least partly CDK1 pathway. Also, miR-378a-5p acts on CDK1 and then partly affects p21 to play a role in cell function. In addition, miR-378a-5p negatively regulates the expression of CDK1 after PDGF-BB serve as a stimulant to promote VSMC proliferation. SiCDK1 can partially recover the proliferation of VSMC by PDGF-BB. Furthermore, miR-378a-5p expression levels were upregulated in both human atherosclerotic vascular tissues and proliferation VSMC. The conclusion of these results is: miR-378a-5p/CDK1/p21 is a considerable pathway that can be used as a new therapeutic target in the prevention of atherosclerosis and stent restenosis.

DISCUSSION

According to the National Health and Family Planning Commission, in the year 2015 more than 500,000 patients with coronary heart disease in mainland China need PCI. PCI has become the main revascularization strategy for unstable coronary artery disease. Despite this, PCI itself still has a problem that has not been overcome, ISR. DES placement does reduce the incidence of ISR, but its incidence is still as high as 10%

(Pleva et al., 2018). ISR greatly limits the benefits of PCI. The prevention of ISR is still an important concern. A study found that the ISR of DES is mainly the result of the proliferation of VSMCs, and the high-pressure effect of post-stent expansion accelerates the proliferation of VSMCs. Proliferation, migration, and formation of the extracellular matrix of VSMCs in the middle of the blood vessels lead to intimal regeneration and stenosis of the lumen (Ko et al., 2012). Stent implantation must be performed again for patients with restenosis (Miziastec et al., 2009), so the targeted regulation of VSMC is of great significance for the treatment and prevention of post-stent restenosis.

Accumulating reports have been made to understand the effect of miRNAs in VSMCs biology (Choe et al., 2013), but the specific molecular mechanism is still unknown. In our study, we demonstrated that targeting of the miR-378a-5p/CDK1/p21 pathway may be a potential therapeutic method for stent restenosis.

MiR-378a is a small non-coding RNA molecule which has two mature chains: (1) miR-378a-3p, (2) miR-378a-5p (Krist et al., 2015). The early study of the miR-378a-5p is mainly based on its relationship with the occurrence and development of tumors. However, the role of miR-378a-5p in the regulation of VSMCs

requires deeper research. To explore the effects of miR-378a-5p in VSMCs in restenosis, we performed CCK-8 and EdU assays to detect VSMC proliferation, wound healing and transwell assays to evaluate VSMC migration. In the end, we come to this conclusion that miR-378a-5p plays a role in regulating the proliferation, migration and phenotypic transformation of VSMCs, that is miR-378a-5p participates in the abnormal VSMC biology functions that contribute to stent restenosis development.

Cyclin-dependent kinase 1 (CDK1) is a protein that regulates the cell cycle, which belongs to a serine/threonine kinase family (Malumbres and Barbacid, 2009); previous studies have proved that CDK1 acted as a key regulator for cell cycle (Yang et al., 2016), and its expression increases in several cancer growths, such as colon carcinoma (Meyer et al., 2009), non-small cell lung cancer tumor (Kim et al., 2008; Zhang et al., 2011); There are also researches that identify that inhibition of CDK1 can suppress the proliferation and migration of some tumor cells (Zhang et al., 2015). Interestingly, in oral squamous cell carcinoma (OSCC), the expression of CDK1 increases with the progression of tumor stage, but the expression of CDK1 is reduced at the stage IV and late stage of the tumor (Chen et al., 2015). There is also a report that suggests overexpression of CDK1 inhibits cell proliferation. CDK1 expression increased or decreased at different time intervals (Rui et al., 2011), this phenomenon is hard to explain from a biological view. So maybe miRNA plays different roles in regulating CDK1 at different time points; the effect of CDK1 on cell proliferation cycle needs to be further studied.

Although our research has demonstrated that miR-378a-5p can target CDK1 to regulate proliferation and migration of VSMCs, but (1) there have been studies that show one miRNA could regulate many target genes, meanwhile, one gene could be modulated by different miRNA. Therefore, targeting miR-378a-5p to treat atherosclerosis and stent restenosis may also affect other genes related to the proliferation of VSMCs, establish an interactive network of non-coding RNAs related to restenosis for early prediction and prognostic evaluation purposes. (2) Non-coding RNA also has problems in application technology and security. (3) Non-coding RNAs also present application technique and safety issues, such as how to coat miR-378a-5p

onto a scaffold, and to understand the effect of the release on human body. (4) Chemically synthesized miRNA inhibitor and mimics are used as scaffold coatings, considering factors such as *in vivo* concentration, half-life, dose, and sample specificity. (5) Another important consideration is whether external miRNAs will affect normal genes. In this research, there is still a lack of experiments on the downstream target p21 of CDK1, which needs further verification. Moreover, the selection of samples has some limitations, the number of samples is small, so the clinical sample size should be increased. The specific mechanism of miR-378a-5p for target regulation of atherosclerosis and stent restenosis remains to be further studied in the follow-up work.

AUTHOR CONTRIBUTIONS

SL and NT carried out the cell and protein analysis. YY carried out the molecular experiments. SJ, HX, RZ, and SL performed the clinical analysis. TY and SL participated in the data analysis, performed the statistical analysis, and drafted the manuscript. TY and HX conceived and designed the study, participated in the data analysis and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (31701208 and 81870331 to TY), China Postdoctoral Science Foundation (2017M612189), Natural Science Foundation of Shandong Province (ZR2017MC067), and The People's Livelihood Science and Technology Project of Qingdao (17-1-1-40-jch and 18-2-2-65-jch).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00022/full#supplementary-material>

REFERENCES

- Afzal, T. A., Luong, L. A., Chen, D., Zhang, C., Yang, F., Chen, Q., et al. (2016). NCK associated protein 1 modulated by miRNA-214 determines vascular smooth muscle cell migration, proliferation, and neointima hyperplasia. *J. Am. Heart Assoc.* 5:e004629. doi: 10.1161/JAHA.116.004629
- Alfonso, F., Perez-Vizcaino, M. J., Hernandez, R., Bethencourt, A., Marti, V., Lopez-Minguez, J. R., et al. (2006). A randomized comparison of sirolimus-eluting stent with balloon angioplasty in patients with in-stent restenosis: results of the Restenosis Intrastent: balloon angioplasty versus elective sirolimus-eluting stenting (RIBS-II) trial. *J. Am. Coll. Cardiol.* 47, 2152–2160. doi: 10.1016/j.jacc.2005.10.078
- Braun-Dullaeus, R. C., Mann, M. J., and Dzau, V. J. (1998). Cell cycle progression: new therapeutic target for vascular proliferative disease. *Circulation* 98, 82–89. doi: 10.1161/01.CIR.98.1.82
- Buccheri, D., Piraino, D., Andolina, G., and Cortese, B. (2016). Understanding and managing in-stent restenosis: a review of clinical data, from pathogenesis to treatment. *J. Thorac. Dis.* 8, E1150–E1162. doi: 10.21037/jtd.2016.10.93
- Chen, X., Zhang, F. H., Chen, Q. E., Wang, Y. Y., Wang, Y. L., He, J. C., et al. (2015). The clinical significance of CDK1 expression in oral squamous cell carcinoma. *Med. Oral Patol. Oral Cirugia Bucal.* 20, e7–e12. doi: 10.4317/medoral.19841
- Choe, N., Kwon, J. S., Kim, J. R., Eom, G. H., Kim, Y., Nam, K. I., et al. (2013). The microRNA miR-132 targets Lrrfp1 to block vascular smooth muscle cell proliferation and neointimal hyperplasia. *Atherosclerosis* 229, 348–355. doi: 10.1016/j.atherosclerosis.2013.05.009
- Feinberg, M. W. (2014). Healing the injured vessel wall using microRNA-facilitated gene delivery. *J. Clin. Invest.* 124, 3694–3697. doi: 10.1172/JCI77509
- Finn, A. V., Joner, M., Nakazawa, G., Kolodgie, F., Newell, J., John, M. C., et al. (2007). Pathological correlates of late drug-eluting stent thrombosis: strut coverage as a marker of endothelialization. *Circulation* 115, 2435–2441. doi: 10.1161/CIRCULATIONAHA.107.693739
- Gareri, C., De, R. S., and Indolfi, C. (2016). MicroRNAs for restenosis and thrombosis after vascular injury. *Circ. Res.* 118, 1170–1184. doi: 10.1161/CIRCRESAHA.115.308237
- Huang, S. C., Wang, M., Wu, W. B., Wang, R., Cui, J., Li, W., et al. (2017). Mir-22-3p inhibits arterial smooth muscle cell proliferation and migration and

- neointimal hyperplasia by targeting HMGB1 in arteriosclerosis obliterans. *Cell Physiol. Biochem.* 42, 2492–2506. doi: 10.1159/000480212
- Kim, S. J., Nakayama, S., Miyoshi, Y., Taguchi, T., Tamaki, Y., Matsushima, T., et al. (2008). Determination of the specific activity of CDK1 and CDK2 as a novel prognostic indicator for early breast cancer. *Ann. Oncol.* 19, 68–72. doi: 10.1093/annonc/mdm358
- Ko, Y. G., Kim, J. S., Kim, B. K., Choi, D., Hong, M. K., Jeon, D. W., et al. (2012). Efficacy of drug-eluting stents for treating in-stent restenosis of drug-eluting stents (from the Korean DES ISR multicenter registry study [KISS]). *Am. J. Cardiol.* 109:607. doi: 10.1016/j.amjcard.2011.10.014
- Kreis, N. N., Friemel, A., Zimmer, B., Roth, S., Rieger, M. A., Rolle, U., et al. (2016). Mitotic p21Cip1/CDKN1A is regulated by cyclin-dependent kinase 1 phosphorylation. *Oncotarget* 7, 50215–50228. doi: 10.18632/oncotarget.10330
- Krist, B., Florczyk, U., Pietraszek-Gremplewicz, K., Józkowicz, A., and Dulak, J. (2015). The role of miR-378a in metabolism, angiogenesis, and muscle biology. *Int. J. Endocrinol.* 2015, 1–13. doi: 10.1155/2015/281756
- Li, P., Zhu, N., Yi, B., Wang, N., Chen, M., You, X., et al. (2013). MicroRNA-663 regulates human vascular smooth muscle cell phenotypic switch and vascular neointimal formation. *Circ. Res.* 113:1117. doi: 10.1161/CIRCRESAHA.113.301306
- Liu, S., Yang, Y., Jiang, S., Tang, N., Tian, J., Ponnusamy, M., et al. (2018). Understanding the role of non-coding RNA (ncRNA) in stent restenosis. *Atherosclerosis* 272, 153–161. doi: 10.1016/j.atherosclerosis.2018.03.036
- Liu, X., Cheng, Y., Zhang, S., Lin, Y., Yang, J., and Zhang, C. (2009). A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ. Res.* 104, 476–487. doi: 10.1161/CIRCRESAHA.108.185363
- Luo, L., Ye, G., Nadeem, L., Fu, G., Yang, B. B., Honarparvar, E., et al. (2012). MicroRNA-378a-5p promotes trophoblast cell survival, migration and invasion by targeting Nodal. *J. Cell Sci.* 125, 3124–3132. doi: 10.1242/jcs.096412
- Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat. Rev. Cancer* 9, 153–166. doi: 10.1038/nrc2602
- McDonald, R. A., Halliday, C. A., Miller, A. M., Diver, L. A., Dakin, R. S., Montgomery, J., et al. (2015). Reducing in-stent restenosis: therapeutic manipulation of miRNA in vascular remodeling and inflammation. *J. Am. Coll. Cardiol.* 65:2314. doi: 10.1016/j.jacc.2015.03.549
- Meyer, A., Merkel, S., Brück, W., Schellerer, V., Schildberg, C., Campean, V., et al. (2009). Cdc2 as prognostic marker in stage UICC II colon carcinomas. *Eur. J. Cancer* 45, 1466–1473. doi: 10.1016/j.ejca.2009.01.010
- Miziastec, K., Gasior, Z., Haberka, M., Mizia, M., Chmiel, A., Janowska, J., et al. (2009). In-stent coronary restenosis, but not the type of stent, is associated with impaired endothelial-dependent vasodilatation. *Kardiol. Pol.* 67:9.
- Pleva, L., Kukla, P., and Hlinomaz, O. (2018). Treatment of coronary in-stent restenosis: a systematic review. *J. Geriatr. Cardiol.* 15, 173–184. doi: 10.11909/j.issn.1671-5411.2018.02.007
- Rui, L. I., Zheng, Y. L., Zhou, X. B., and Wang, W. (2011). Expression and activity of CDK1 on cell apoptosis in hepatoma HepG2. *J. Ningxia Med. Univ.* 6, 521–524.
- Rui, Y., Mengjuan, W., Fan, Y., Yuchen, S., and Lili, J. (2018). Diosbulbin B induced G2/M cell cycle arrest in hepatocytes by miRNA-186-3p and miRNA-378a-5p-mediated the decreased expression of CDK1. *Toxicol. Appl. Pharmacol.* 357, 1–9. doi: 10.1016/j.taap.2018.08.016
- Tang, N., Jiang, S., Yang, Y., Liu, S., Ponnusamy, M., Xin, H., et al. (2018). Noncoding RNAs as therapeutic targets in atherosclerosis with diabetes mellitus. *Cardiovasc. Ther.* 36:e12436. doi: 10.1111/1755-5922.12436
- Wang, H., Jiang, M., Xu, Z., Huang, H., Gong, P., Zhu, H., et al. (2015). miR-146b-5p promotes VSMC proliferation and migration. *Int. J. Clin. Exp. Pathol.* 8, 12901–12907.
- Wang, Z., Ma, B., Ji, X., Deng, Y., Zhang, T., Zhang, X., et al. (2015). MicroRNA-378-5p suppresses cell proliferation and induces apoptosis in colorectal cancer cells by targeting BRAF. *Cancer Cell Int.* 15, 40. doi: 10.1186/s12935-015-0192-2
- Wang, Z., Song, J., Liang, Z., Huang, S., Bao, L., Feng, C., et al. (2017). Increased expression of microRNA-378a-5p in acute ethanol exposure of rat cardiomyocytes. *Cell Stress Chaperones* 22, 245–252. doi: 10.1007/s12192-016-0760-y
- Xin, M., Small, E. M., Sutherland, L. B., Qi, X., Mcanally, J., Plato, C. F., et al. (2009). MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev.* 23, 2166–2178. doi: 10.1101/gad.1842409
- Yang, W., Cho, H., Shin, H. Y., Chung, J. Y., Kang, E. S., Lee, E. J., et al. (2016). Accumulation of cytoplasmic Cdk1 is associated with cancer growth and survival rate in epithelial ovarian cancer. *Oncotarget* 7, 49481–49497. doi: 10.18632/oncotarget.10373
- Zhang, C., Elkhouloun, A. G., Robertson, M., Gills, J. J., Tsurutani, J., Shih, J. H., et al. (2011). Loss of cytoplasmic CDK1 predicts poor survival in human lung cancer and confers chemotherapeutic resistance. *PLoS One* 6:e23849. doi: 10.1371/journal.pone.0023849
- Zhang, Y., Huang, W., Ran, Y., Xiong, Y., Zhong, Z., Fan, X., et al. (2015). miR-582-5p inhibits proliferation of hepatocellular carcinoma by targeting CDK1 and AKT3. *Tumour Biol.* 36, 8309–8316. doi: 10.1007/s13277-015-3582-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: MiR-378a-5p Regulates Proliferation and Migration in Vascular Smooth Muscle Cell by Targeting CDK1

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Approved by:

Frontiers in Genetics Editorial Office,
Frontiers Media SA, Switzerland

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 21 February 2019

Accepted: 22 February 2019

Published: 15 March 2019

Citation:

Liu S, Yang Y, Jiang S, Xu H, Tang N,
Lobo A, Zhang R, Liu S, Yu T and
Xin H (2019) Corrigendum:
MiR-378a-5p Regulates Proliferation
and Migration in Vascular Smooth
Muscle Cell by Targeting CDK1.
Front. Genet. 10:193.
doi: 10.3389/fgene.2019.00193

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Keywords: miR-378a-5p, vascular smooth muscle cell, stent-restenosis, proliferation, migration, atherosclerosis,
CDK1

A Corrigendum on

MiR-378a-5p Regulates Proliferation and Migration in Vascular Smooth Muscle Cell by Targeting CDK1

by Liu, S., Yang, Y., Jiang, S., Xu, H., Tang, N., Lobo, A., et al. (2019). *Front. Genet.* 10:22.
doi: 10.3389/fgene.2019.00022

In the original article, we neglected to include the funder “National Natural Science Foundation of China,” “81870331” to TY.

Additionally, there was an error in affiliation 3. Instead of “Department of Cardiology, The Affiliated Hospital of Qingdao University, Qingdao, China,” it should be “Department of Cardiology, The Affiliated Cardiovascular Hospital of Qingdao University, Qingdao, China.”

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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CircRNAs Are Here to Stay: A Perspective on the *MLL* Recombinome

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 09 October 2018

Accepted: 29 January 2019

Published: 13 February 2019

Citation:

Dal Molin A, Bresolin S, Gaffo E,
Tretti C, Boldrin E, Meyer LH,
Guglielmelli P, Vannucchi AM,
te Kronnie G and Bortoluzzi S (2019)
CircRNAs Are Here to Stay:
A Perspective on the *MLL*
Recombinome. *Front. Genet.* 10:88.
doi: 10.3389/fgene.2019.00088

Chromosomal translocations harbored by cancer genomes are important oncogenic drivers. In *MLL* rearranged acute leukemia (*MLLre*) *MLL/KMT2A* fuses with over 90 partner genes. Mechanistic studies provided clues of *MLL* fusion protein leukemogenic potential, but models failed to fully recapitulate the disease. Recently, expression of oncogenic fusion circular RNAs (f-circ) by *MLL-AF9* fusion was proven. This discovery, together with emerging data on the importance and diversity of circRNAs formed the incentive to study the circRNAs of the *MLL* recombinome. Through interactions with other RNAs, such as microRNAs, and with proteins, circRNAs regulate cellular processes also related to cancer development. CircRNAs can translate into functional peptides too. *MLL* and most of the 90 *MLL* translocation partners do express circRNAs and exploration of our RNA-seq dataset of sorted blood cell populations provided new data on alternative circular isoform generation and expression variability of circRNAs of the *MLL* recombinome. Further, we provided evidence that rearrangements of *MLL* and three of the main translocation partner genes can impact circRNA expression, supported also by preliminary observations in leukemic cells. The emerging picture underpins the view that circRNAs are worthwhile to be considered when studying *MLLre* leukemias and provides a new perspective on the impact of chromosomal translocations in cancer cells at large.

Keywords: circRNA, leukemia, *MLL* rearrangements, fusion-circRNA, translocation breakpoint region, blood cells

THE ACUTE LEUKEMIA *MLL* RECOMBINOME

Cancer genomes often harbor chromosomal translocations and specific rearrangements are recurrent in specific cancers, most often involving two specific genes or one promiscuous gene translocated with an array of different genes. In acute leukemia, the *MLL* gene (official gene symbol *KMT2A*) on chromosome 11q23 breaks and fuses with more than 90 translocation partner genes (TPGs) (Ottersbach et al., 2018), with only a few of them recurrently found in *MLL* rearrangements (*MLLre*).

Genomic studies revealed that *MLL* fusions are clonal and are considered early initiating leukemogenic events (Yip and So, 2013; Sanjuan-Pla et al., 2015), and very few additional mutations are needed to generate infant *MLLre* (Dobbins et al., 2013). Mechanistic studies

of particular MLLre showed that fusion proteins are crucial for transforming potential (Reimer et al., 2017). In addition, recent data indicated that expression of the wild type *KMT2A* is dispensable for MLLre leukemic cells, whereas deletion of *KMT2D* alone or in combination with loss of *KMT2A* reduces proliferation and induces apoptosis of *MLL-AF9* transformed cells (Chen et al., 2017).

The 2017 survey of the *MLL* recombinome consortium (Meyer et al., 2018), based on DNA sequence analysis of 2,345 MLLre acute leukemia cases and literature scrutiny, identified 135 different MLLre involving 94 TPGs, with only a few genes accounting for most cases. Association of specific partner genes and/or the breakpoint position with age classes and/or with leukemia subtypes has been described (Meyer et al., 2018). *AF4* is almost exclusively (99%) found in BCP-ALL, whereas *AF9* is slightly more frequent (60%) in myeloid leukemias. Specific *MLL* TPGs are detected exclusively in leukemias of the lymphoid (e.g., *LAF4/AFF3*) or of the myeloid lineages (e.g., *SEPT6*).

MLL breakpoints occur preferentially in three clusters (ex-in 9, ex-in 10, ex-in 11/12) (van Dongen et al., 1999; de Jonge et al., 2005). *MLL* encodes a Lysine Methyltransferase involved in tissue-specific epigenetic activation of developmental genes. Most TPGs encode proteins of complexes that affect transcriptional elongation. Even if the functional effect has not been investigated for all *MLL* fusions, MLLre leukemias display a deeply deregulated epigenetic and transcriptional state, and the contribution of *MLL* fusions to leukemia initiation and evolution, therapy resistance and relapse is still under active investigation. An array of cellular and animal models generated to study the leukemogenic mechanisms of *MLL* fusions furthered our understanding of MLLre, but failed to fully recapitulate the human disease features (Ottersbach et al., 2018). The current state-of-the-art is controversial regarding the cell-of-origin, the timing and level of *MLL* fusion protein expression. Even less is known of the transcripts expressed by *MLL* fusions. Recent reports of oncogenic fusion circular RNAs (f-circ) and data emerging on circular RNAs (circRNAs, transcripts in which the splice donor site is covalently bound to an upstream acceptor site by backsplicing) in general made the drawing more complex, but also very intriguing, and opened a series of new perspectives.

Rearranged cancer genomes (promyelocytic leukemia with *PML-RARα*, acute myeloid leukemia with *KMT2A-MLLT3* and a model of the *NPM1-ALK* fusion) (Guarnerio et al., 2016; Babin et al., 2018) express f-circ. F-circ include two sequences not present in the normal genome: the fusion junction, in which two genomic regions far apart in normal genomes are juxtaposed, and the backsplice junction, connecting in reverse order two sequences of the fusion gene. A few studies showed that f-circ can be oncogenic but only started to disclose involved mechanisms. Concurrent expression of f-circM9 and *MLL-AF9* protein contributed to leukemia progression in *in vivo* and *ex vivo* models, and f-circM9 expression increased drug resistance of leukemic cells (Guarnerio et al., 2016). F-circ generated from an *EML4-ALK* fusion promoted non-small cell lung cancer development (Tan et al., 2018). The oncogenic potential of f-circ was linked to mechanisms involving the fusion protein by the observation that proliferation and TKI resistance of *BCR-ABL1*

leukemic cells are enhanced by a f-circ that increases the fusion protein level. Nevertheless, none of these studies investigated the impact of rearrangements on circRNAs expressed by *MLL* and TPGs during normal hematopoiesis. In leukemic cells carrying translocations beyond generation of f-circ, also ablation or deregulation of circRNAs will occur with potential contribution to the disease.

Circularized transcripts of specific genes were reported since the 80's, but circRNAs were re-discovered (Salzman et al., 2012; Memczak et al., 2013, 2015; Bonizzato et al., 2016) when projects coupling RNA-seq with bioinformatics methods for reads mapping appropriate to detect backsplicing showed that thousands of circRNAs are expressed by many genes with cell type-specific expression regulation (Maass et al., 2017), and differentiation stage-specificity (Rybak-Wolf et al., 2015). As reviewed recently (Memczak et al., 2013, 2015; Bonizzato et al., 2016) circRNAs are abundantly expressed in the hematopoietic compartment. CircRNAs can play important and diverse functions, including some typical of non-coding RNAs. The first function assigned to circRNAs was sponging miRNAs (Salzman et al., 2012; Memczak et al., 2013, 2015; Bonizzato et al., 2016) and indirectly regulating miRNA-target expression. In this way, some circRNAs control key miRNA-involving axes in normal developmental processes and oncogenesis (Hansen et al., 2013; Li F. et al., 2015). Other circRNAs regulate cellular processes by interacting with RNA-binding proteins (Schneider et al., 2016), scaffolding molecular complexes, as shown for circFOXO3 that controls cell cycle progression by binding p21 and CDK1 (Du et al., 2016). As long as non-coding RNAs, also circRNAs have some coding potential, and can be translated into functional peptides according to recent reports (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017).

Taken together these discoveries of circRNA pervasiveness and functions prompted us to study the circRNAs expressed by genes of the *MLL* recombinome (*MLL-rec*) in normal blood cells. In this perspective we examined how rearrangements can result in alteration of sequences and expression level of circRNAs normally generated by *MLL* and TPGs, and provided data of MLLre leukemia in support.

CircRNAs EXPRESSED IN NORMAL HEMATOPOIESIS BY GENES OF THE *MLL* RECOMBINOME

The *MLL-rec* analyzed here included *MLL* plus 94 TPGs (**Supplementary Table 1**): 75 genes of rearrangements disclosed by Meyer et al. (2018) and 19 genes previously reported in the literature collected in the same study. Almost 93% of 2,345 leukemia cases reported in Meyer et al. (2018) resulted from *MLL* fusions with one of 12 highly recurrent TPGs (*AF4/AFF1*, *AF9/MLLT3*, *ENL/MLLT1*, *AF10/MLLT10*, *BCS1L/PTD*, *ELL*, *AFDN/AF6/MLLT4*, *EPS15*, *AF1Q/MLLT11*, *SEPT6*, *AF17/MLLT6*, and *SEPT9*), that will be cited from now on using the official gene name.

To obtain a data-driven picture of the circular transcriptome of *MLL-rec* genes in the hematopoietic compartment, we

analyzed our RNA-seq dataset regarding normal hematopoiesis (available at GEO series ID: GSE110159 and upon request): 15 samples from healthy donors, including 3 of CD34+ cells purified from cord blood, and 12 of B-, T-cells and monocytes FACS sorted from PBMCs (4 different donors per cell type). RNA-seq data were obtained from ribo-depleted RNA with Illumina® HiSeq2000 (average depth of 145 M paired end 100 nt reads per sample, 66% of reads passed quality control). CircRNAs were detected and quantified using CirComPara v0.3 (Gaffo et al., 2017), implementing 6 circRNA detection methods (CIRI, Findcirc, CIRCexplorer2+Star, CIRCexplorer2+Segemehl, CIRCexplorer2+BWA, CIRCexplorer2+TopHat). CirComPara identified 41,515 circRNAs expressed from 8,138 individual genes. We focused on the subset of 16,606 circRNAs with high expression in at least one cell type (maximum of the averages per cell type in the top 40% highest values), which derived from 5,170 genes.

For 25 of the 95 genes in the *MLL*-rec, no circRNAs were detected in our dataset and 16 had only circRNAs expressed at low level. Interestingly, 54 genes of the recombinome expressed at least one abundant circRNA, for a total of 327 circRNAs (**Supplementary Table 2**), which were further investigated.

The expression level of these circRNAs was not different from the 16,005 circRNAs expressed by the 5,116 non-*MLL*-rec genes of the human genome (**Supplementary Figure 1**). *MLL*-rec genes expressed several circRNAs at very high levels: the 33 most expressed circRNAs (**Supplementary Figure 2**) derived from 21 genes, including *AFF1* and *EPS15*, which presented circRNAs overexpressed in stem cells; and *SEPT6* and *SEPT9*, whose circRNAs were detected only in mature cells, with marked overexpression of circSEPT9 17:77402059–77402703:– in monocytes. CircAFF3 2:100006632–100008932:– and circAFF4 5:132892164–132893118:–, both from *MLL* TPGs only found in leukemias of the lymphoid lineage, were upregulated in B-cells. Four genes contributed several circRNAs to the group of the most highly expressed: 10 circPICALM were mostly upregulated in monocytes, albeit circPICALM 11:86007542–86026367:– and 11:86022367–86031611:– were more abundant in stem cells; 2 circME2 were upregulated in stem cells; 2 circARHGAP26 were upregulated in monocytes; 2 circPDS5A were upregulated in B-cells.

MLL-rec genes compared to non-*MLL*-rec genes had a significantly larger number of circular isoforms per gene (6.06 vs. 3.13 circRNAs per gene in average; *t*-test *p*-value 2.317 E–04), with a distribution of the number of circRNAs per gene significantly shifted toward higher values (Chi-squared test *p*-value 1.791 E–06; **Supplementary Figures 3A,B**). Indeed, 9 of the recombinome genes expressed only one circRNA each, and 45 (83%) had multiple circular isoforms in blood cells (**Figure 1A**). The genes with the highest numbers of isoforms were *PICALM* (31 circRNAs), *AKAP13* (19), *EPS15* (16), *PDS5A* (16), *ARHGAP26* (15), *ITPR2* (15), *EP300* (14), *ME2* (14), and *MYO1F* (10). Moreover, *MLLT10*, *SEPT6* and *AFF1* had 6 circRNAs each, and *MLL3* expressed 4 circRNAs.

Similar numbers of circRNAs were expressed in the different mature populations by *MLL*-rec (205, 244, and 210 in B-, T-cells and in monocytes, respectively), and only 94 circRNAs in the

stem population (**Supplementary Figure 4A**). Apart from 44 circRNAs expressed in all cell types, 16 from 10 genes (*CENPK*, *LPP*, *ITPR2*, *TCF12*, *CLTC*, *FNBPI*, *FRYL*, *SEPT11*, *SEPT6*, and *MLLT10*) were detected only in stem cells (circCENPK 5:65528452–65529145:– was the most abundant), and 233 had expression restricted to one or more mature populations, being mostly detected in all the mature populations (80) or in lymphocytes (40).

Most genes displayed cell type-specific alternative circularization patterns (**Supplementary Figure 4B**). Of 16 stem cell-specific circRNAs, circCLTC 17:59668941–59669222:– was expressed by a gene with circRNAs only in stem cells, and 15 derived from genes with other circular isoforms detected in mature cell types. The three most abundant of the 16 different circEPS15 had different expression profiles (**Supplementary Figure 4C**): circEPS15 1:51402435–51408332:– was expressed in all cell types with a slight upregulation in stem cells, whereas circEPS15 1:51394381–51448135:– was upregulated only in monocytes, and circEPS15 1:51405905–51408332:– was more abundant in B- and T-cells than in monocytes, and not detected in stem cells.

A scrutiny of the two main circRNA-indexing databases, CircBase and circRNADB, showed that 2,433 circRNAs were previously reported for 85 of 95 genes in the *MLL*-rec, including all the 54 with highly expressed circRNAs, and all but *ACER1* and *PFDN4* of the 70 genes with circRNAs detected in our data. Moreover, for 17 (out of 25) genes in the *MLL*-rec for which we did not observe appreciable circRNA expression in blood cells, the DBs reported circRNAs mostly in other tissues, such as brain (e.g., *ARHGEF17*) or muscle (e.g., *FLNC*). Even if a direct comparison of our data with data reported in the DBs is not feasible, findings concordantly indicate that circRNAs are expressed from the overwhelming majority of genes in the *MLL*-rec, with complex patterns of alternative circularization in many cases.

Of note, 63 new circRNAs were detected at high expression in blood populations investigated in this study, which are currently not annotated in the DBs (**Supplementary Table 2**). Newly detected circRNAs derived from 26 genes of the *MLL*-rec, including *MLLT10*, *MLLT3*, *EPS15* and *SEPT6*, and comprised 15 circPICALM and circRNAs with expression restricted to stem cells or to a specific mature population.

TRANSLOCATIONS CAN IMPACT CircRNA EXPRESSION FROM *MLL/KMT2A* AND TPGs

As described above, *KMT2A* and most of TPGs observed in acute leukemias do express circRNAs in normal blood cells (**Figure 1A**). In *MLL*re leukemias the rearrangement of *KMT2A* with a TPG leads to the formation of a “fusion gene.” When *KMT2A* is expressed, also the downstream partner gene may be expressed resulting in a transcript that potentially is translated in a fusion protein (Ayton and Cleary, 2001; So et al., 2003). Alongside also f-circ may be produced from chimeric fusion genes (Guarnerio et al., 2016), as reviewed in Bonizzato et al.

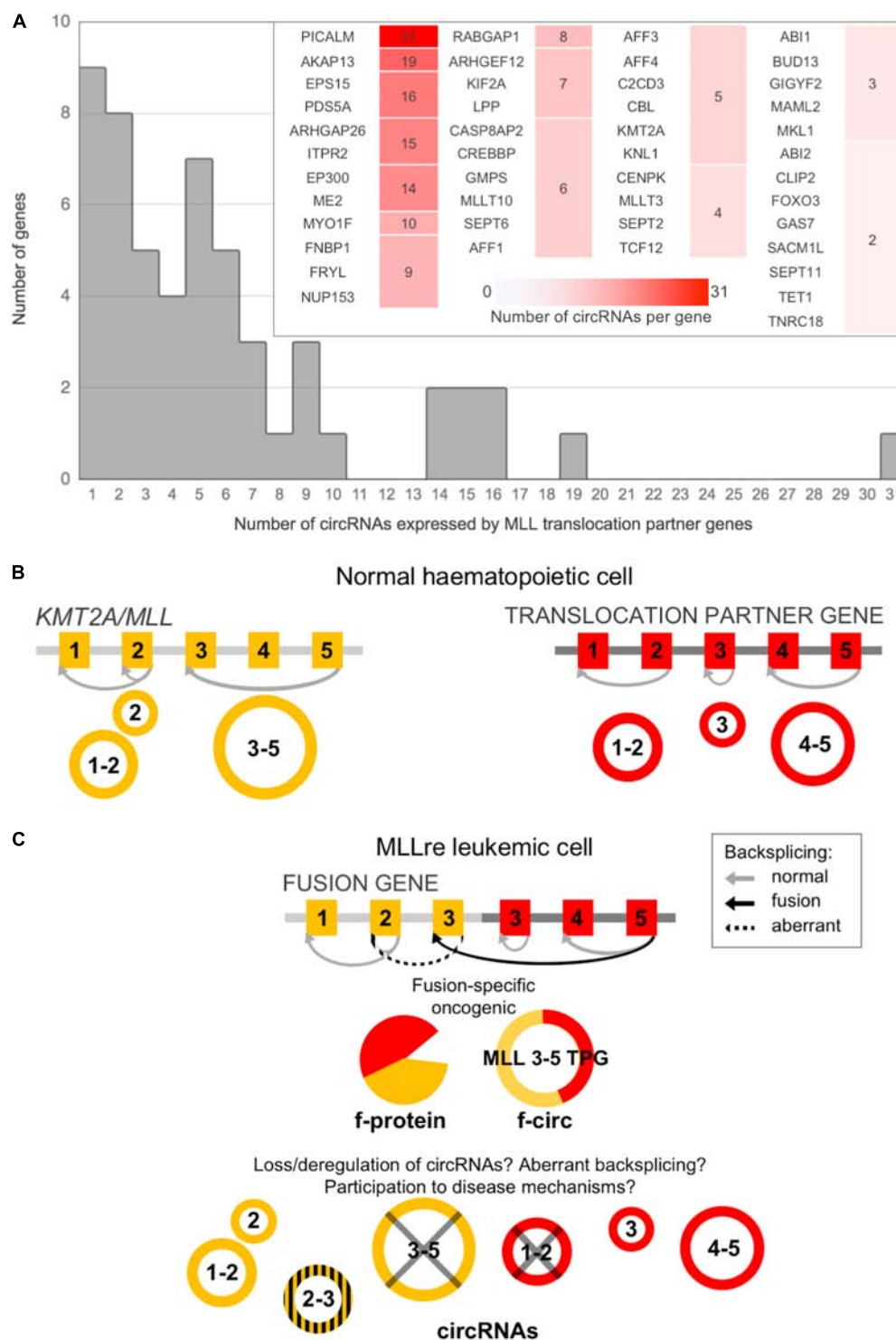


FIGURE 1 | CircRNA expression by *MLL/KMT2A* and by *MLL* translocation partner genes (TPGs) in normal hematopoiesis can be altered in leukemic cells.

(A) Distribution of the number of circRNAs per gene expressed in normal blood cells by the *MLL* recombinome (the detail shows genes with multiple isoforms); (B) *MLL/KMT2A* and a TPG can produce, in normal blood cells, several circRNAs; (C) CircRNA expression can be affected by the fusion: beyond the generation of fusion-specific products (protein and circRNAs), the absence in the fusion gene of the exons undergoing circularization and/or of the intronic sequences flanking the backsplicing with regulatory functions can deregulate or abolish circRNA expression; in addition, aberrant circRNAs can be generated by activation of “cryptic” backsplicing sites.

(2016). In addition, the breaking and fusion of *KMT2A* and TPGs may alter the formation of circRNAs from exons proximal to the breakpoint of the translocated genes. Information on the *KMT2A* and TPGs specific exons that undergo circularization in normal hematopoiesis (Figure 1B) is critical to understand how a specific rearrangement can impact circRNA expression (Figure 1C). The breakpoint position defines which genomic regions are retained in the fusion gene, determining not only if and how f-circ are generated. The breakpoint position can also affect the circRNAs “normally” generated by the partner genes influencing their expression level or possibly activating cryptic backsplicing sites.

For *KMT2A* and of three of the main TPGs we further examined in detail circRNAs expression in normal blood cells in relation to potential breakpoint regions. In addition, we provided preliminary data of MLLre leukemia using RNA-seq data of the THP1 cell line with *KMT2A-MLLT3* (*MLL-AF9*) fusion (Guarnerio et al., 2016) and unpublished data of two specimens of infant ALL with *KMT2A-MLLT1* (*MLL-ENL*) fusions (Supplementary Table 3).

In normal hematopoiesis, *KMT2A* produced 5 different circRNAs (Figure 2A). The most abundant circ*KMT2A* (11:118481715–118482495:++; exons 7–8) was expressed in all mature populations, with upregulation in both B- and T-cells, while it was absent in the stem population, in which two other circ*KMT2A* isoforms were detected. The formation of two wild type circ*KMT2A* isoforms (exons 5–8 and 7–8) are potentially perturbed in MLLre leukemias, considering that the recurrent breakpoint region includes exon 8. The other three circ*KMT2A* expressed in normal hematopoiesis (exons 12–16, 17–23, 21–23) will probably not be formed from the fusion gene, since they originate from exons not retained in most rearrangements. Thus, all circRNAs highly expressed in normal cells by *KMT2A* may be downregulated or even absent in the leukemic cells. In line, none of the circRNAs expressed in normal hematopoiesis by *KMT2A* was detected, neither in the THP1 cell line (in which two fusion circRNAs were previously detected by RT-PCR, and albeit supported by few reads, from RNA-seq data), nor in any of the two samples with *KMT2A-MLLT1* fusion. Of note, *KMT2A* exons 5 or 7 are the starting ends backspliced both in normal hematopoiesis (joined with exon 8 in two of the five observed circ*KMT2A* isoforms) and in THP1 cells (joined with *MLLT3* exon 6 in the two f-circM9 isoforms) (Guarnerio et al., 2016). If and how the reported oncogenic potential of f-circM9 (*KMT2A* exon 7 – *MLLT3* exon 6) is related to the function of the most expressed circ*KMT2A* (*KMT2A* exons 7–8), that is fully contained in the f-circM9, remains to be determined.

The three TPGs most recurrent in acute leukemias (*AFF1*, *MLLT3*, *MLLT1*) expressed one to 5 circRNAs each in normal hematopoiesis (Figures 2B–D). *AFF1* presented 5 circRNAs, with circ*AFF1* 4:87046166–87047594:++ (exons 2–3) contributing alone to 90% of circular expression of the gene. The same circ*AFF1* was one of the most expressed in the sample set, it presented a remarkable upregulation in stem cells compared to mature cells according to our data, and was found upregulated in common lymphoid precursors and in monocytes (Nicolet et al., 2018). The *AFF1* recurrent breakpoint region position in MLLre

leukemias reveals that this circ*AFF1* will likely not be generated from the chimeric gene. The expression of the other 4 circ*AFF1* will depend on the exact position of the breakpoint (Figure 2B).

MLLT3 had 4 circular isoforms, almost all specific of T-cells. The second most expressed circ*MLLT3* contains part of intron 4, as observed for other circRNAs (Li Z. et al., 2015). These circRNAs will not be generated from the fusion gene due to the position of the breakpoints, either falling within the sequence undergoing circularization or eliminating all circularized exons from the fusion transcripts (Figure 2C). None of the T-specific circ*MLLT3* isoforms was detected in *KMT2A-MLLT3* THP1 cells.

Also circ*MLLT1* 9:6230570–6274759:- (exons 2–4), expressed in mature populations, will not be generated from the *KMT2A-MLLT1* derivative, since the breakpoints of *t*(11;19) leukemias either abolish the *MLLT1* exons undergoing circularization or fall in the intron flanking the backspliced exon 2. In line with our assumption, this circRNA was not detected in leukemic cells (Figure 2D) with two different *KMT2A-MLLT1* fusions (*KMT2A* exon 9 – *MLLT1* exon 6, and *KMT2A* exon 8 – *MLLT1* exon 4 fusions, Supplementary Table 3).

Here, we analyzed one of the main *KMT2A*-TPG fusions, and the translocations indeed impacted circRNAs expressed from *KMT2A* and its TPG. Highly informative data on leukemias with *KMT2A-MLLT1* fusions were fully in agreement with observations (based on a lower depth) in THP1 cells. Little is known about the other TPG-*KMT2A* derivatives.

To generalize, based on circRNA and breakpoint respective position, four scenarios can occur: (1) The exons undergoing circularization are retained in the *KMT2A*-TPG derivative (e.g., exons in the 5' region of *KMT2A* or in the 3' region of the TPG retained in the fusion gene), circRNAs can be still generated; (2) The exons undergoing circularization are not retained, the circRNAs can be abolished; (3) The breakpoint is located between the backspliced ends, the absence in the fusion gene of one or more exons normally undergoing circularization will prevent the formation of circRNAs, possibly favoring the generation of f-circ; (4) The breakpoint is located in an intron flanking one of the backsplice ends abolishing specific *in cis* sequence elements (Alu and inverted repeats) that favor the backsplicing (Jeck et al., 2013; Bonizzato et al., 2016), and/or sequences recognized by *in trans* regulatory factors, such as QKI and ADAR1, which promotes (Conn et al., 2015) and suppresses (Rybak-Wolf et al., 2015) circularization, respectively. We are well aware that our model of how translocations can impact expression of circRNAs from fused genes is hampered by simplicity. Position-effects, as well as deep genome-wide epigenetic and transcriptional deregulation in leukemic cells can affect circRNAs expressed from the fused genes as well as circRNAs from other loci.

CONCLUSION AND FUTURE DIRECTIONS

Data emerging from recent literature and from the present study collectively show that *KMT2A* and TPGs express many circRNAs,

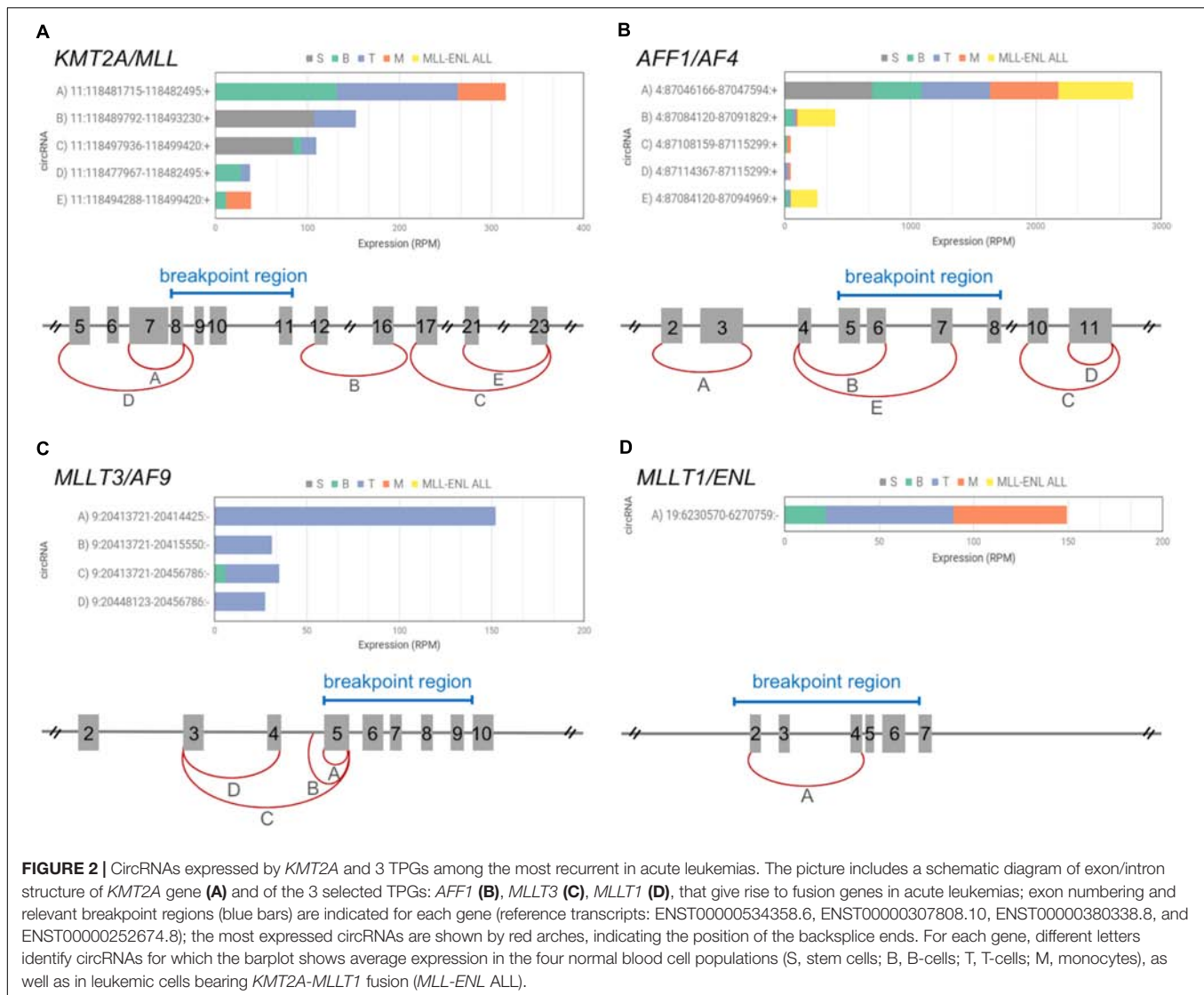


FIGURE 2 | CircRNAs expressed by *KMT2A* and 3 TPGs among the most recurrent in acute leukemias. The picture includes a schematic diagram of exon/intron structure of *KMT2A* gene (**A**) and of the 3 selected TPGs: *AFF1* (**B**), *MLLT3* (**C**), *MLLT1* (**D**), that give rise to fusion genes in acute leukemias; exon numbering and relevant breakpoint regions (blue bars) are indicated for each gene (reference transcripts: ENST00000534358.6, ENST00000307808.10, ENST00000380338.8, and ENST00000252674.8); the most expressed circRNAs are shown by red arches, indicating the position of the backsplice ends. For each gene, different letters identify circRNAs for which the barplot shows average expression in the four normal blood cell populations (S, stem cells; B, B-cells; T, T-cells; M, monocytes), as well as in leukemic cells bearing *KMT2A-MLLT1* fusion (*MLL-ENL ALL*).

possibly playing important functions and being perturbed in cells bearing rearrangements. According to our observations, depending on the position of the breakpoint relative to the backspliced sequences, translocations can impact expression of circRNAs in addition to generating f-circ. Our data on circRNAs expressed from genes of the *MLL* recombinome are instrumental to analyze alterations of circRNAs from rearranged genes of *MLL*re leukemias and f-circ generation. It is known that f-circ can be oncogenic, but the involved mechanisms remain essentially unknown. F-circ and the other circRNAs expressed from *KMT2A* and TPGs might share part of their sequences and play similar functions (e.g., through common protein or nucleic acids interactors, or due to overlapping coding potential). F-circ were previously shown to reinforce the oncogenic potential of fusion proteins, perhaps cooperating to the same mechanisms (Babin et al., 2018). A parallel line of evidence showed that loss of both *KMT2A* and *KMT2D* impair survival pathways in leukemic cells (Chen et al., 2017),

implicating them in oncogenesis. In this view, beyond f-circ, also circRNAs generated from the non-translocated alleles are critical candidates to be investigated for participation to disease mechanisms. Further, f-circ and circRNAs in general share exons with linear transcripts, and circularization is associated with exon skipping (Kelly et al., 2015). The equilibrium perturbed by translocations can involve a series of transcripts including “normal” as well as aberrant circular and linear transcripts, all linked in an intricate network of similar sequences and functions and interdependent biogenesis.

In conclusion, next to the studies of fusion proteins as oncogenic drivers of leukemias with *MLL* rearrangements, we stress the need of a molecular characterization of circRNAs expressed by fusion genes, *KMT2A* itself and its TPGs. A first direction could be to clarify the participation of circRNAs to the molecular complexes involving *KMT2A*, and to define circRNA molecular interactions with regulators, such as microRNAs, of *KMT2A* or of its interactors involved in leukemogenesis. The

perspective emerging from this pilot study on MLLre acute leukemia is presumably valid for most of the driver chromosomal fusions occurring in cancer cells.

ETHICS STATEMENT

All experiments involving human material followed the principles outlined in the Helsinki Declaration. The study has been approved by the ethics committees of Padova University Hospital, Ulm University Medical Center and of AOU Careggi (Florence) and written informed consent was obtained from all subjects.

AUTHOR CONTRIBUTIONS

GtK and StB conceived the study. ADM, EG, and StB contributed bioinformatics methods and performed data analysis. SiB, CT, and StB performed comparative public data analysis. ADM, StB,

and GtK wrote the manuscript. StB, ADM, and SiB made the figures. EB, LM, PG, and AV provided data and revised the manuscript. All authors approved the final manuscript.

ACKNOWLEDGMENTS

We acknowledge for financial support: AIRC (IG #20052 to StB and GtK, MFAG #15674 to SiB, AIRC 5 × 1000 MYNERVA project, #21267 to AV and PG), Department of Molecular Medicine, University of Padua (PRID 2017 to StB), Cariparo (Pediatric research projects to GtK and StB), Ministero della Salute GR-2011-02352109 to PG.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00088/full#supplementary-material>

REFERENCES

- Ayton, P. M., and Cleary, M. L. (2001). Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. *Oncogene* 20, 5695–5707. doi: 10.1038/sj.onc.1204639
- Babin, L., Piganeau, M., Renouf, B., Lamribet, K., Thirant, C., Deriano, L., et al. (2018). Chromosomal translocation formation is sufficient to produce fusion circular RNAs specific to patient tumor cells. *iScience* 5, 19–29. doi: 10.1016/j.isci.2018.06.007
- Bonizzato, A., Gaffo, E., Te Kronnie, G., and Bortoluzzi, S. (2016). CircRNAs in hematopoiesis and hematological malignancies. *Blood Cancer J.* 6:e483. doi: 10.1038/bcj.2016.81
- Chen, Y., Anastasiadis, K., Kranz, A., Francis Stewart, A., Arndt, K., Waskow, C., et al. (2017). MLL2, not MLL1, plays a major role in sustaining MLL rearranged acute myeloid leukemia. *Cancer Cell* 31, 755–770.e6. doi: 10.1016/j.ccell.2017.05.002
- Conn, S. J., Pillman, K. A., Toubia, J., Conn, V. M., Salmanidis, M., Phillips, C. A., et al. (2015). The RNA binding protein quaking regulates formation of circRNAs. *Cell* 160, 1125–1134. doi: 10.1016/j.cell.2015.02.014
- de Jonge, R., Hooijberg, J. H., van Zelst, B. D., Jansen, G., van Zantwijk, C. H., Kaspers, G. J. L., et al. (2005). Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia. *Blood* 106, 717–720. doi: 10.1182/blood-2004-12-4941
- Dobbins, S. E., Sherborne, A. L., Ma, Y. P., Bardini, M., Biondi, A., Cazzaniga, G., et al. (2013). The silent mutational landscape of infant MLL-AF4 pro-B acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 52, 954–960. doi: 10.1002/gcc.22090
- Du, W. W., Yang, W., Liu, E., Yang, Z., Dhaliwal, P., and Yang, B. B. (2016). Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res.* 44, 2846–2858. doi: 10.1093/nar/gkw027
- Gaffo, E., Bonizzato, A., Kronnie, G. T., and Bortoluzzi, S. (2017). CirComPara: a multi-method comparative bioinformatics pipeline to detect and study circRNAs from RNA-seq data. *Noncoding RNA* 3:8. doi: 10.3390/ncrna3010008
- Guarnerio, J., Bezzi, M., Jeong, J. C., Paffenholz, S. V., Berry, K., Naldini, M. M., et al. (2016). Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. *Cell* 165, 289–302. doi: 10.1016/j.cell.2016.03.020
- Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993
- Jeck, W. R., Sorrentino, J. A., Wang, K., Slevin, M. K., Burd, C. E., Liu, J., et al. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 19, 141–157. doi: 10.1261/rna.035667.112
- Kelly, S., Greenman, C., Cook, P. R., and Papantonis, A. (2015). Exon skipping is correlated with exon circularization. *J. Mol. Biol.* 427, 2414–2417. doi: 10.1016/j.jmb.2015.02.018
- Legnini, I., Di Timoteo, G., Rossi, F., Morlando, M., Briganti, F., Sthandier, O., et al. (2017). Circ-ZNF609 is a circular rna that can be translated and functions in myogenesis. *Mol. Cell* 66, 22–37.e9. doi: 10.1016/j.molcel.2017.02.017
- Li, F., Zhang, L., Li, W., Deng, J., Zheng, J., An, M., et al. (2015). Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/β-catenin pathway. *Oncotarget* 6, 6001–6013. doi: 10.18632/oncotarget.3469
- Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., et al. (2015). Exon-intron circular RNAs regulate transcription in the nucleus. *Nat. Struct. Mol. Biol.* 22, 256–264. doi: 10.1038/nsmb.2959
- Maass, P. G., Glazar, P., Memczak, S., Dittmar, G., Hollfinger, I., Schreyer, L., et al. (2017). A map of human circular RNAs in clinically relevant tissues. *J. Mol. Med.* 95, 1179–1189. doi: 10.1007/s00109-017-1582-9
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Memczak, S., Papavasileiou, P., Peters, O., and Rajewsky, N. (2015). Identification and characterization of circular RNAs As a new class of putative biomarkers in human blood. *PLoS One* 10:e0141214. doi: 10.1371/journal.pone.0141214
- Meyer, C., Burmeister, T., Gröger, D., Tsaar, G., Fechina, L., Renneville, A., et al. (2018). The MLL recombinome of acute leukemias in 2017. *Leukemia* 32, 273–284. doi: 10.1038/leu.2017.213
- Nicolet, B. P., Engels, S., Aglialoro, F., van den Akker, E., von Lindern, M., and Wolters, M. C. (2018). Circular RNA expression in human hematopoietic cells is widespread and cell-type specific. *Nucleic Acids Res.* 46, 8168–8180. doi: 10.1093/nar/gky721
- Ottersbach, K., Sanjuan-Pla, A., Torres-Ruiz, R., Bueno, C., Velasco-Hernández, T., and Menendez, P. (2018). The “Never-Ending” mouse models for MLL-rearranged acute leukemia are still teaching Us. *HemaSphere* 2:1. doi: 10.1097/HS9.0000000000000057
- Pamudurti, N. R., Bartok, O., Jens, M., Ashwal-Fluss, R., Stottmeister, C., Ruhe, L., et al. (2017). Translation of CircRNAs. *Mol. Cell* 66, 9–21.e7. doi: 10.1016/j.molcel.2017.02.021
- Reimer, J., Knöß, S., Labuhn, M., Charpentier, E. M., Göhring, G., Schlegelberger, B., et al. (2017). CRISPR-Cas9-induced t(11;19)/MLL-ENL translocations initiate leukemia in human hematopoietic progenitor cells

- in vivo. *Haematologica* 102, 1558–1566. doi: 10.3324/haematol.2017.164046
- Rybák-Wolf, A., Stottmeister, C., Glažar, P., Jens, M., Pino, N., Giusti, S., et al. (2015). Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol. Cell* 58, 870–885. doi: 10.1016/j.molcel.2015.03.027
- Salzman, J., Gawad, C., Wang, P. L., Lacayo, N., and Brown, P. O. (2012). Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PLoS One* 7:e30733. doi: 10.1371/journal.pone.0030733
- Sanjuan-Pla, A., Bueno, C., Prieto, C., Acha, P., Stam, R. W., Marschalek, R., et al. (2015). Revisiting the biology of infant t(4;11)/MLL-AF4 B-cell acute lymphoblastic leukemia. *Blood* 126, 2676–2685. doi: 10.1182/blood-2015-09-667378
- Schneider, T., Hung, L.-H., Schreiner, S., Starke, S., Eckhof, H., Rossbach, O., et al. (2016). CircRNA-protein complexes: IMP3 protein component defines subfamily of circRNPs. *Sci. Rep.* 6:31313. doi: 10.1038/srep31313
- So, C. W., Lin, M., Ayton, P. M., Chen, E. H., and Cleary, M. L. (2003). Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias. *Cancer Cell* 4, 99–110. doi: 10.1016/S1535-6108(03)00188-0
- Tan, S., Gou, Q., Pu, W., Guo, C., Yang, Y., Wu, K., et al. (2018). Circular RNA F-circEA produced from EML4-ALK fusion gene as a novel liquid biopsy biomarker for non-small cell lung cancer. *Cell Res.* 28, 693–695. doi: 10.1038/s41422-018-0033-7
- van Dongen, J. J., Macintyre, E. A., Gabert, J. A., Delabesse, E., Rossi, V., Saglio, G., et al. (1999). Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. *Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia* 13, 1901–1928. doi: 10.1038/sj.leu.2401592
- Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., et al. (2017). Extensive translation of circular RNAs driven by N6-methyladenosine. *Cell Res.* 27, 626–641. doi: 10.1038/cr.2017.31
- Yip, B. H., and So, C. W. E. (2013). Mixed lineage leukemia protein in normal and leukemic stem cells. *Exp. Biol. Med.* 238, 315–323. doi: 10.1177/1535370213480717

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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miR-499-5p Attenuates Mitochondrial Fission and Cell Apoptosis via p21 in Doxorubicin Cardiotoxicity

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OPEN ACCESS

Edited by:

Ge Shan,
University of Science and Technology
of China, China

Reviewed by:

Udayan Bhattacharya,
Technion – Israel Institute
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Pennsylvania State University,
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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 06 October 2018

Accepted: 22 December 2018

Published: 21 January 2019

Citation:

Wan Q, Xu T, Ding W, Zhang X,
Ji X, Yu T, Yu W, Lin Z and Wang J
(2019) miR-499-5p Attenuates
Mitochondrial Fission and Cell
Apoptosis via p21 in Doxorubicin
Cardiotoxicity. *Front. Genet.* 9:734.
doi: 10.3389/fgene.2018.00734

Doxorubicin (DOX) is a broad-spectrum anti-tumor drug, but its cardiotoxicity limits its clinical application. A better understanding of the molecular mechanisms underlying DOX cardiotoxicity will benefit clinical practice and remedy heart failure. Our present study observed that DOX caused cardiomyocyte (H9c2) apoptosis via the induction of abnormal mitochondrial fission. Notably, the expression levels of p21 increased in DOX-treated cardiomyocytes, and the silencing of p21 using siRNA greatly attenuated mitochondrial fission and apoptosis in cardiomyocytes. We also found that miR-499-5p could directly target p21 and attenuated DOX-induced mitochondrial fission and apoptosis. The role of the miR-499-5p-p21 axis in the prevention of DOX cardiotoxicity was also validated in the mice model. DOX treatment induced an upregulation of p21, which induced subsequent abnormal mitochondrial fission and myocardial apoptosis in mouse heart. Adenovirus-harboring miR-499-5p-overexpressing mice exhibited significantly reduced p21 expression, mitochondrial fission and myocardial apoptosis in hearts following DOX administration. The miR-499-5p-overexpressing mice also exhibited improved cardiomyocyte hypertrophy and cardiac function after DOX treatment. However, miR-499-5p was not involved in the DOX-induced apoptosis of cancer cells. Taken together, these findings reveal an emerging role of p21 in the regulation of mitochondrial fission program. miR-499-5p attenuated mitochondrial fission and DOX cardiotoxicity via the targeting of p21. These results provide new evidence for the miR-499-5p-p21 axis in the attenuation of DOX cardiotoxicity. The development of new therapeutic strategies based on the miR-499-5p-p21 axis is a promising path to overcome DOX cardiotoxicity as a chemotherapy for cancer treatment.

Keywords: doxorubicin, miR-499-5p, p21, cardiotoxicity, mitochondrial, apoptosis

INTRODUCTION

Doxorubicin (DOX), also known as adriamycin (ADR), exerts a killing effect on a variety of tumors, and it is a widely used anti-tumor drug (Levis et al., 2017). However, a previous study found that DOX produced heart damage in many patients that eventually led to heart failure (Gorini et al., 2018). The development of treatment strategies that avoid DOX cardiotoxicity without

affecting its anti-tumor effects is urgently needed. A previous study found that DOX-induced cardiotoxicity primarily involved the production of reactive oxygen species (ROS) (Ichikawa et al., 2014), lipid peroxidation (Yarana et al., 2018), DNA damage (Li et al., 2009), mitochondrial dysfunction (Dhingra et al., 2014), apoptosis (Kumar et al., 2001), and autophagy dysregulation (Bartlett et al., 2017). These toxicities ultimately caused cell death of cardiomyocyte. Therefore, a better understanding of the molecular mechanisms underlying DOX cardiotoxicity will improve the clinical application of DOX during cancer therapy (Kluza et al., 2004).

MicroRNAs (miRNAs) are endogenous non-coding RNAs that are highly conserved in different species (Lim et al., 2003). miRNAs functionally participate in the developmental, physiological and pathological process via negative regulation of target gene (Bartel, 2009; Chistiakov et al., 2016). Increasing evidence suggests that miRNAs are actively involved in the regulation of cardiac functions, such as electrical signal conductance, heart muscle contraction, heart growth and morphogenesis (Kozomara and Griffiths-Jones, 2011). What's more, miRNAs are also powerful regulators of cardiovascular diseases, and manipulation of miRNAs is a promising strategy for the development of novel therapeutic agents (Mette et al., 2002; Liu S. et al., 2018; Tang et al., 2018).

miR-499-5p is a recently discovered member of myosin-encoded miRNAs (Olivieri et al., 2013). Several studies reported that miR-499-5p was differentially regulated and functioned in the heart development process (Sluijter et al., 2010; Wilson et al., 2010; Fu et al., 2011; Wander et al., 2016). miR-499-5p is expressed at a high level in the heart under physiological conditions, and it attenuates the expression of the β -myosin heavy chain, which results in enhanced myocardial oxygen metabolism and tolerance. miR-499-5p is downregulated in human heart diseases and experimental models of heart failure, and it is involved in the transcriptional and posttranslational regulation of pathological hypertrophy (Chistiakov et al., 2016). Several lines of evidence suggest that miR-499-5p exerts cardioprotective effects via the protection of cardiomyocytes from stress-induced apoptosis. miR-499-5p inhibits calcineurin-mediated dephosphorylation of dynamin-related protein-1 (Drp1) via the targeting of CnA α and CnA β , which reduces Drp-1 mitochondrial aggregation and attenuates Drp-1-mediated mitochondrial fission (Wang et al., 2011). miR-499-5p also targets several regulatory factors that inhibit mitochondrial cell apoptosis and increase cell survival, such as bispecific tyrosine phosphorylation regulatory kinase 2 (Dyrk2), programmed cell death protein 4 (Pcd4) (Li Y. et al., 2016) and phosphoric acid Forint acid cluster sorting protein 2 (Pasc2) (Wang J. et al., 2014). However, the potential effects and underlying mechanisms of miR-499-5p in the protection against DOX-induced heart failure have not been revealed.

Cyclin-dependent kinase inhibitor 1a (CDKN1a), also known as p21, is a negative regulator that halts cell cycle progression at the G1/S and G2/M transition points via inhibition of CDK4,6/cyclin-D and CDK2/cyclin-E, respectively (Sharpless and Sherr, 2015). Mammalian p21 is expressed at very low levels in embryonic and neonatal hearts (Brooks et al., 1998).

Numerous studies demonstrated that p21 was involved in the pathological process of myocardial injury (Wang et al., 2015a). Inhibition of p21 prevented endothelial cell apoptosis in arsenite-induced endothelial dysfunction-related vascular diseases (Nuntharatanapong et al., 2005). Increased p21 levels in cardiac fibroblasts surrounding an infarction area is a biomarker of hyperoxia perception (Sen et al., 2006). Hyperoxia causes cardiac remodeling via the induction of p21-dependent differentiation of cardiac fibroblasts (Roy et al., 2003a,b). Abnormal p21 expression is closely related to myocardial damage and cardiac hypertrophy (Wang R. et al., 2014). Transverse aortic constriction (TAC) surgery and DOX administration concomitantly increased p21 levels in rat and mouse cardiomyocytes (Terrand et al., 2011; Koga et al., 2013). Increased p21 expression in tissues mediates myocardial fibrosis and remodeling (Kuhn et al., 2007; Megyesi et al., 2015). However, the mechanism of p21 regulation of myocardial damage and the regulation of the p21 expression in cardiomyocytes under stress have not been elucidated.

Our present study investigated the molecular mechanisms underlying DOX cardiotoxicity. We found an emerging role of p21 in promoting mitochondrial fission and cardiomyocyte apoptosis via regulation of mitochondrial fission programming induced by DOX treatment. miR-499-5p inhibited DOX-induced mitochondrial fission and apoptosis in cardiomyocytes via the targeting of p21. DOX cardiotoxicity was prevented in adenovirus-harboring miR-499-5p-overexpressing mice, and miR-499-5p overexpression improved cardiac function. Our research demonstrated that the miR-499-5p-p21 axis constitutes a new antiapoptotic pathway to attenuate DOX-induced cardiotoxicity and may provide valuable insights to prevent DOX cardiotoxicity during cancer chemotherapy.

MATERIALS AND METHODS

Cell Culture and Treatment

H9c2 cells were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). The human lung cancer cell line A-549, the human gastric cancer cell line SGC-7901, the human hepatocellular carcinoma cell line HepG-2 and the human colorectal cancer cell line SW-480 were purchased from the Chinese Academy of Sciences Cell Bank. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C. Cells were treated with 2 or 0.2 μ M DOX, unless indicated otherwise.

Animal Experiments

Mice (C57BL/6, male, 7 weeks old) were injected with DOX or saline. Briefly, mice were treated twice per week with 10 mg/kg DOX or control saline solution for 1 week (20 mg/kg cumulative dose of DOX). Heart tissues were analyzed 1 week after the last treatment. DOX doses were based on previous reports (Zhu et al., 2009). Cardiac function and ventricular remodeling were investigated 1 week after the last treatment. All procedures involving animals were reviewed and approved by

the Institutional Animal Care and Use Committee of Qingdao University Medical College.

Quantitative Real-Time PCR (qRT-PCR)

Stem-loop qRT-PCR was performed in an Applied Biosystems ABI Prism 7000 sequence detection system. Total RNA was extracted using TRIzol reagent. DNase I (Takara, Otsu, Japan) was applied, and RNA was reverse-transcribed using a reverse transcriptase kit (Takara). Mature miR-499-5p levels were measured using SYBR Green Real-time PCR Master Mix (Takara) according to the manufacturer's instructions. The same reverse primer with the sequence 5'-GTGCAGGGTCCGAGGT-3' was used for all miRNAs. The primer used is as described in Table 1.

Mitochondrial Staining

We performed mitochondrial staining as described previously (Boman et al., 2000; Wang et al., 2015b). Briefly, we plated cells onto coverslips. Cells were stained after treatment for 20 min with 0.02 μ M MitoTracker Red CMXRos (40743ES50; Yeasern, Shanghai, China). We imaged mitochondria using a laser scanning confocal microscope (Zeiss LSM510 META, Jena, Germany). We randomly measured at least 150 cells from each experiment to determine the percentage of cells undergoing mitochondrial fission.

Immunoblotting

Immunoblotting was performed to determine the expression levels of p21 and actin. Briefly, cells were lysed for 1 h at 4°C in lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM DTT, 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) containing a protease inhibitor

cocktail. Protein samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed using corresponding primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were used. Antigen-antibody complexes were visualized using enhanced chemiluminescence. Equal protein loading was controlled using Ponceau Red staining of membranes. Antigen-antibody complexes were visualized using enhanced chemiluminescence. Bands were quantitated using Image J and α -actin was used as the loading control. Fold change was normalized to the indicated control. Information on the antibodies used is described in Table 2.

Cell Death Assay

Cell death was determined using a trypan blue exclusion assay. Trypan blue-positive and trypan blue-negative cells were counted using a hemocytometer (Liu et al., 2009). We randomly measured 150 cells from each experiment to calculate the cell death rate.

Apoptosis Assays and Histology

Apoptosis was determined using terminal deoxynucleotidyl transferase-mediated TdT-mediated dUTP nick-end labeling using a kit from Yeasern (Alexa Fluor 488). Harvested hearts were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a 6- μ m thickness. Detection procedures were performed in accordance with the kit instructions (He et al., 2017). We randomly measured 150 cells from each experiment to calculate the apoptotic rate.

Echocardiographic Assessment

Echocardiography was performed as described previously. Generally, mice were mildly anesthetized, and transthoracic echocardiography was performed using a Vevo 2100 high-resolution system (VisualSonics, Toronto, ON, Canada). Two-dimensional guided M-mode tracings were recorded in parasternal long and short axis views at the level of the papillary muscles. Systolic left ventricular internal diameter (LVIDs) and diastolic left ventricular internal diameter (LVIDd) were measured. We calculated the fractional shortening (FS) of the left ventricular diameter as $(LVIDd - LVIDs)/LVIDd \times 100$. All measurements were obtained for greater than three beats and averaged. Mice were euthanized after *in vivo* evaluations of cardiac function, and hearts were harvested and weighted prior to histological examination (Coppola et al., 2016).

Electron Microscopy

Heart ultrastructural analysis was performed to quantify mitochondrial fission. Sample preparations and conventional electron microscopy were performed as described (Cadete et al., 2016). Samples were examined at a magnification of 15,000 using a JEOL JEM-1230 transmission electron microscope. Electron microscopy micrographs of thin sections were evaluated for comparisons of mitochondrial fission. The sizes of individual mitochondria were measured using Image-Pro Plus software. We defined mitochondria smaller than 0.6 μ m² as fission mitochondria (Wang et al., 2015d).

TABLE 1 | The primer used in this study.

miR-499-5p	Forward: 5'-GGTCCAGACTGGGGTCCCAGC-3' Reverse: 5'-GCATGCCGACAGTGGTTAGGGA-3'
U6	Forward: 5'-GCTTCGGCAGCACATATACTAA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
ANP	Forward: 5'-TCCGATAGATCTGCCCTCTTGAA-3' Reverse: 5'-GTACCGGAAGCTGTTGCAGCCTA-3'
β -MHC	Forward: 5'-CAGACATAGAGACCTACCTTC-3' Reverse: 5'-CAGCATGTCTAGAAGCTCAGG-3'
GAPDH	Forward: 5'-TGGAGTCTACTGGCGTCTT-3' Reverse: 5'-TGTCATATTTCTCGTGGTTCA-3'
miR-16	Forward: 5'-TAGCAGCACGTAATATTGGCG-3' Reverse: 5'-GTGCAGGGTCCGAGGT-3'
p21 3'UTR	Forward: 5'-ATGATATCCTTCTGCTGTGGGTCA-3' Reverse: 5'-GGACTAGTATTGCACGAGGGGAG-3'

TABLE 2 | The information of the antibodies used in his work.

Antibody	Source	Dilutions	Company
p21	Rabbit	1:1000	Wanleibio, China
β -action	Mouse	1:5000	Sungenebio, China
Anti-Mouse IgG	Rabbit	1:5000	Biovision, United States
Anti-Rabbit IgG	Goat	1:5000	Biovision, United States

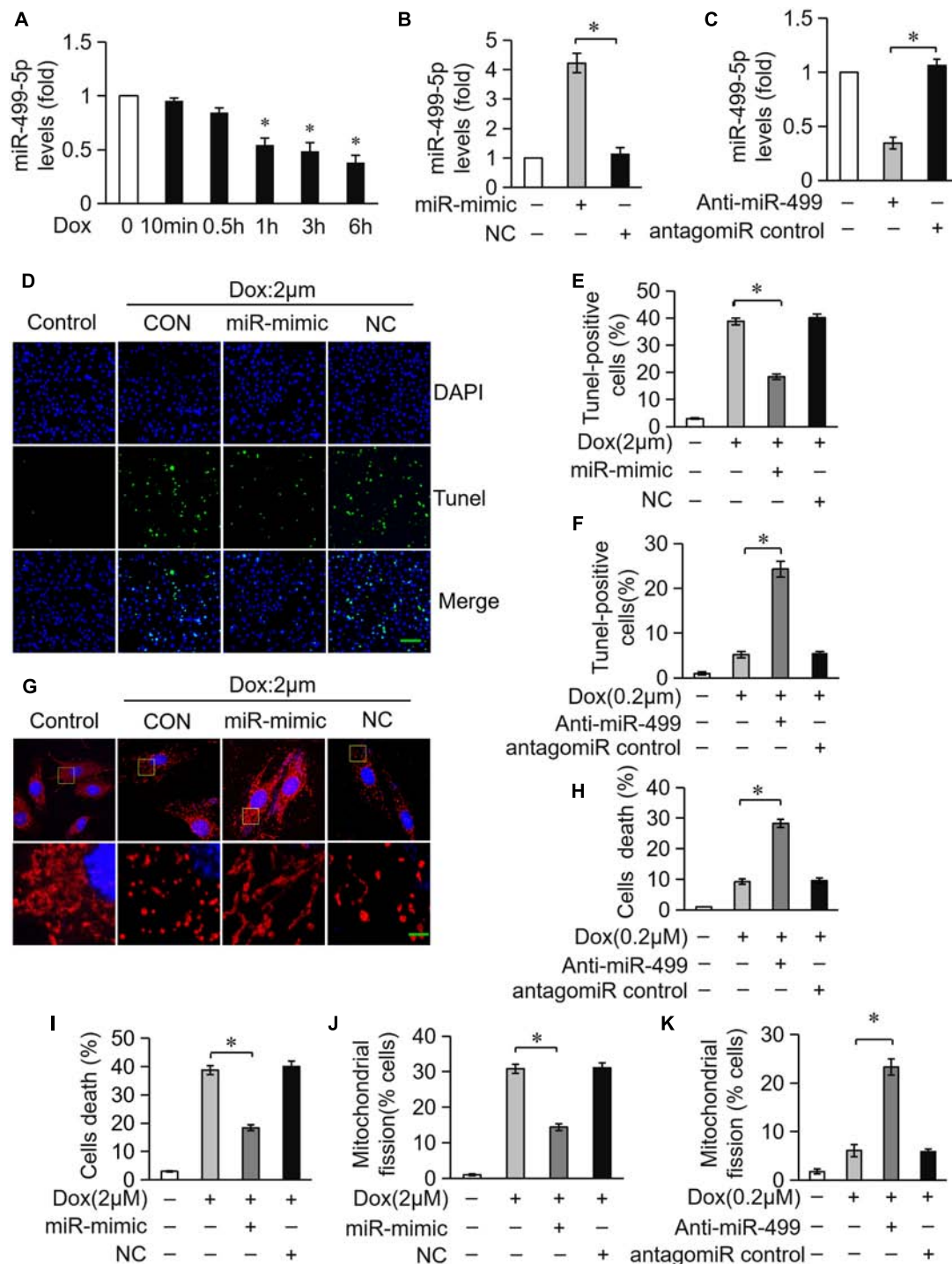


FIGURE 1 | miR-499-5p attenuates mitochondrial fission and apoptosis in cardiomyocytes treated with DOX. **(A)** Measurement of miR-499-5p mRNA levels in H9c2 cells treated with 2 μ M DOX at the indicated times. **(B)** Transfection with a miR-499-5p mimic forced the expression of miR-499-5p in cardiomyocytes. Cells were transfected with miR-499-5p mimic (miR-mimic) or negative control (NC) for 24 h. The expression levels of miR-499-5p were detected using real-time PCR. **(C)** miR-499-5p antagomiR knocked down endogenous miR-499-5p. Cells were transfected with a miR-499-5p antagomiR (Anti-miR-499) or antagomiR control for 24 h. The expression levels of miR-499-5p were detected using real-time PCR. **(D)** Overexpression of miR-499-5p inhibited 2 μ M DOX-induced apoptosis in cardiomyocytes. Cardiomyocytes were transfected with miR-499-5p mimic or negative control (NC) for 24 h and treated with 2 μ M DOX for 24 h. Cell death was detected using the TUNEL assay. Representative images show TUNEL staining results (blue, DAPI; green, TUNEL). Scale bar: 100 μ m. **(E)** Statistical analysis of TUNEL-positive cells in each group. Data are expressed as the means $\pm$ SD, $n = 3$ experiment * $P < 0.05$. **(F)** Knockdown of miR-499-5p sensitized

(Continued)

FIGURE 1 | Continued

cardiomyocytes to undergo apoptosis induced by DOX (0.2 μ M). Cell death was detected using the TUNEL assay. Data are expressed as the means $\pm$ SD; $n = 3$ experiment. * $P < 0.05$. **(G)** Overexpression of miR-499-5p inhibited DOX-induced mitochondrial fission in cardiomyocytes. Cells were transfected with a miR-499-5p mimic or negative control for 24 h. Representative images were captured using confocal microscopy. Scale bar: 2 μ M. **(H)** Knockdown of miR-499-5p sensitized cardiomyocytes to undergo 0.2 μ M DOX-induced cell death. Cell death was detected using the trypan blue assay. Data are expressed as the means $\pm$ SD, $n = 3$ experiment. * $P < 0.05$. **(I)** Overexpression of miR-499-5p inhibited 2 μ M DOX-induced cell death in cardiomyocytes. Cell death was measured using trypan blue assays. Data are expressed as the means $\pm$ SD, $n = 3$ experiment. * $P < 0.05$. **(J)** Statistical analysis of the percentage of cells undergoing mitochondrial fission. Data are expressed as the means $\pm$ SD, $n = 3$ experiment. * $P < 0.05$. Cardiomyocytes were transfected with a miR-499-5p mimic or negative control for 24 h and treated with 2 μ M DOX for 24 h. **(K)** Statistical analysis of the percentage of cells undergoing mitochondrial fission. Data are expressed as the means $\pm$ SD, $n = 3$ experiment. * $P < 0.05$. H9c2 cells were transfected with a miR-499-5p antagomiR or antagomiR control for 24 h and treated with 0.2 μ M DOX for 24 h. The percentage of cells undergoing mitochondrial fission was determined.

Reporter Construction and Luciferase Assay

The p21 3'UTR was amplified from mouse genomic DNA using PCR. The primers were as described in Table 1. PCR products were gel-purified and ligated into a pGL3 reporter vector (Promega) immediately downstream of the stop codon of the luciferase gene. Mutations of the p21 3'UTR construct were introduced using a QuikChange II XL site-directed mutagenesis kit (Stratagene). The p21 3'UTR-Mut (the wild-type p21 3'UTR site: AGUCUAAA, p21 3'UTR-Mut: AGACGGAA) was produced using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). A luciferase activity assay was performed as described previously (Wang et al., 2015c). Briefly, cells were cultured in 24-well plates, infected with miR-499-5p mimic or negative control and transfected with the plasmid construct pGL3-p21-3'UTR or pGL3-p21-3'UTR-Mut at a concentration of 200 ng/well using Lipofectamine 3000 (Invitrogen). The Renilla luciferase plasmid was cotransfected at 2.5 ng/well and served as the internal control. Cells were lysed 48 h after transfections, and luciferase activity was detected using a Dual Luciferase Reporter Assay kit (Promega). All experiments were performed in triplicate.

Construction of Adenovirus and Overexpression Vector

miR-499-5p-overexpressing adenovirus and adenovirus β -galactosidase (β -gal) were prepared as described previously (Wang et al., 2011). All adenoviruses were amplified in HEK-293 cells. Adenoviral infection of cells was performed as described previously (Wang et al., 2009). The open reading frame (ORF) of the p21 gene was generated using RT-PCR, and p21 siRNA was purchased from Genepharma (Shanghai, China). P21 was cloned into the pcDNA3.1 expression vector according to the manufacturer's guidelines (Invitrogen). The constructed sequence was further confirmed using sequencing.

Data and Statistical Analysis

All values are expressed as the means $\pm$ standard error. $n = 3$. Statistical significance was defined as $p < 0.05$. One- or two-way analysis of variance (ANOVA) was used to test each variable for differences between treatment groups. If ANOVA demonstrated a significant effect, then pairwise *post hoc* comparisons were performed using Fisher's least significant difference test.

RESULTS

miR-499-5p Attenuates Mitochondrial Fission and Apoptosis in Cardiomyocytes Treated With DOX

miR-499-5p exerts a protective role in the pathogenesis of heart diseases and miR-499-5p mRNA levels are downregulated in cardiomyocytes during apoptotic stress and in the heart under pathological conditions (Matkovich et al., 2012). We detected miR-499-5p expression levels in cardiomyocytes exposed to DOX to investigate the role of miR-499-5p in DOX-induced cardiotoxicity. miR-499-5p expression was significantly downregulated after DOX (2 μ M) treatment (Figure 1A). Cardiomyocytes were transfected with a miR-499-5p mimic. Real-time PCR demonstrated that miR-499-5p levels increased 4-fold compared to the negative control (Figure 1B). The miR-499-5p mimic efficiently inhibited mitochondrial fission (Figures 1G,J) and cell apoptosis (Figures 1D,E,I) in cardiomyocytes exposed to DOX (2 μ M). We knocked down endogenous miR-499-5p using a miR-499-5p antagomiR to mimic the DOX-induced downregulation of miR-499-5p. Real-time PCR demonstrated that the miR-499-5p antagomiR efficiently knocked down the endogenous miR-499-5p (Figure 1C). Knockdown of miR-499-5p sensitized cardiomyocytes to DOX, which induced mitochondrial fission and apoptosis in cardiomyocytes at a lower concentration (0.2 μ M) (Figures 1F,H,K). These results suggest the involvement of miR-499-5p in DOX cardiotoxicity and an attenuation of DOX-induced apoptosis in cardiomyocytes via inhibition of mitochondrial fission by miR-499-5p.

miR-499-5p Attenuates Mitochondrial Fission and Apoptosis in DOX Cardiotoxicity *in vivo*

We investigated the role of miR-499-5p in mice model to further validate its function in DOX-induced cardiotoxicity. miR-499-5p levels were significantly downregulated in DOX-treated mice heart (Figure 2A) while the serum miR-499-5p expression was significantly increased (Figure 2B). DOX administration induced abnormal mitochondrial fission and cell apoptosis in the mouse heart (Figures 2C–F). The adenovirus harboring miR-499-5p efficiently forced the expression of miR-499-5p in mouse heart (Figure 2A) and significantly attenuated cell mitochondrial fission and cell apoptosis in the heart following

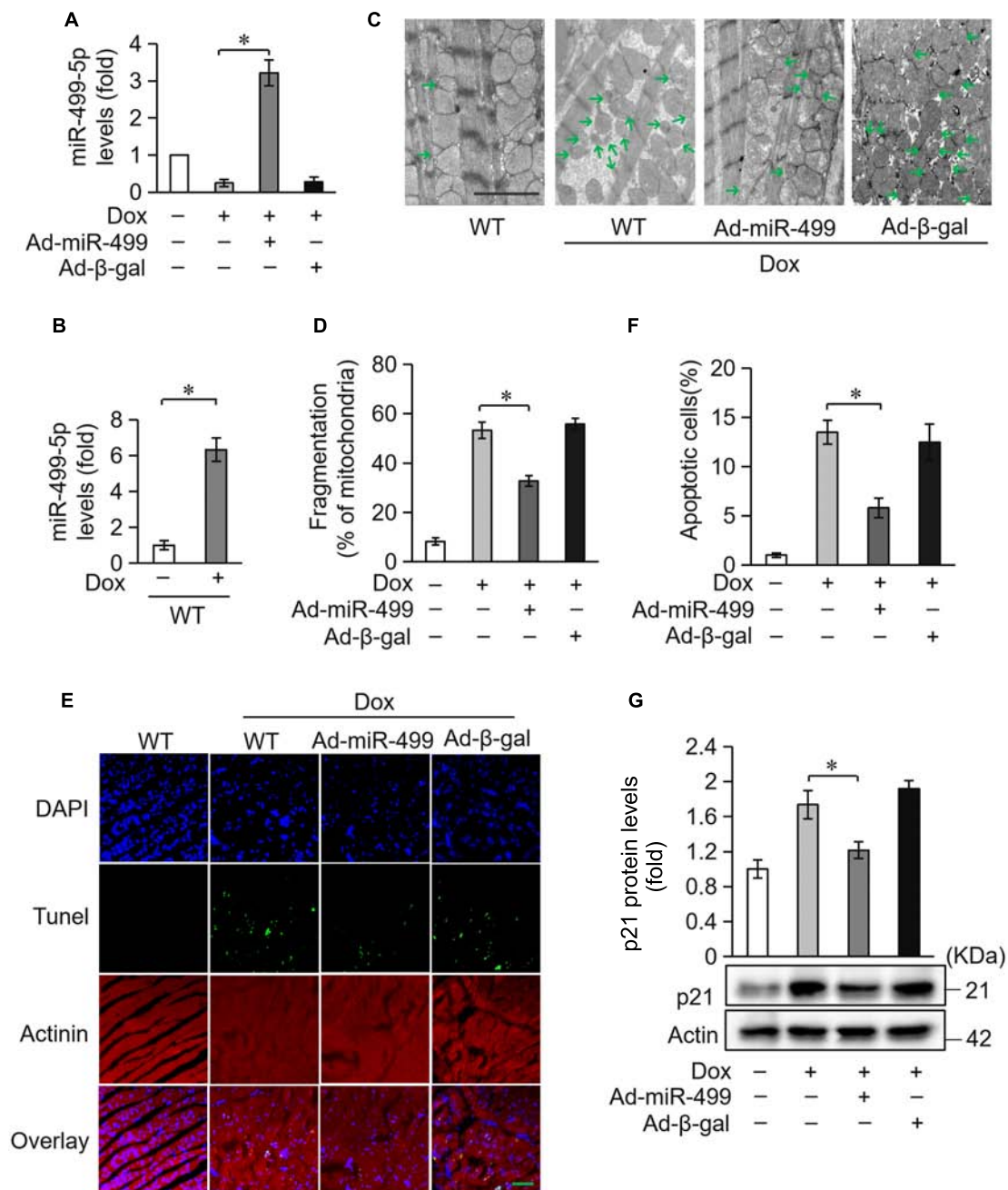


FIGURE 2 | miR-499-5p attenuates mitochondrial fission and apoptosis *in vivo*. **(A)** miR-499-5p mRNA levels in adenovirus-harboring, miR-499-5p-overexpressing mice (Ad-miR-499) and the control mice administered DOX. $n = 3$ mice per group. Data are presented as the means $\pm$ SD, $*P < 0.05$. **(B)** Serum miR-499-5p expression was detected in the mice administered DOX by real-time PCR. $n = 3$ mice per group. Data are presented as the means $\pm$ SD, $*P < 0.05$. **(C,D)** Mitochondrial fission was analyzed using scanning electron microscope (SEM) in heart tissues from miR-499-5p-overexpressing mice and the control mice administered DOX. Scale bar, 2 μ m. Arrows indicate mitochondria that underwent fission. **(E)** Statistical analyses of the percentage of mitochondrial fission. Mitochondrial fission was calculated as described in the Section "Materials and Methods." Data are presented as the means $\pm$ SD, $*P < 0.05$; $n = 5$ mice per group. **(D,E)** Representative images revealed TUNEL-positive cells in heart tissues. Green, TUNEL-positive nuclei; blue, DAPI (4,6-diamidino-2-phenylindole)-stained nuclei; red, cardiomyocytes labeled with α -actinin antibody; scale bar, 20 μ m. $n = 5$ mice per group. **(F)** Statistical analysis of TUNEL-positive cells in each group. Data are presented as the means $\pm$ SD, $*P < 0.05$. **(G)** Western blotting shows the expression of p21. Actin served as a loading control.

DOX administration (**Figures 2C–F**). Notably, we observed a significant increase in the protein levels of p21 in DOX-treated mice, which was inhibited in the miR-499-5p-overexpressing

mice (**Figure 2G**). In conclusion, miR-499-5p attenuated DOX-induced mitochondrial fission and cell apoptosis in the mouse heart *in vivo*.

miR-499-5p Attenuates DOX Cardiotoxicity in Mice

Doxorubicin treatment caused a high degree of cardiomyocytes apoptosis. Because cardiomyocytes exhibit a limited ability to regenerate, cardiomyocytes loss during treatment cannot be replenished. Cardiomyocytes undergo compensatory hypertrophy, and the heart develops dilated cardiac hypertrophy with a decrease in heart function under the treatment of DOX. ANP and β -MHC are two biomarkers for the cardiac hypertrophy (Wang et al., 2015b). We measured the expression levels of ANP and β -MHC to examine the role of miR-499-5p in DOX-induced cardiac hypertrophy. ANP and β -MHC expression levels were significantly upregulated in mice administered DOX, but these increases were significantly inhibited in the miR-499-5p-overexpressing mice (Figures 3A,B). These results indicate that miR-499-5p inhibits the cardiac hypertrophy induced by DOX cardiotoxicity. We also examined the effect of miR-499-5p on cardiac remodeling in mice. Our results demonstrated that the cardiac remodeling was ameliorated in miR-499-5p-overexpressing mice as assessed using echocardiography (Figures 3C–E). In conclusion, miR-499-5p efficiently attenuated DOX cardiotoxicity in our mouse model.

p21 Is a Target of miR-499-5p

miRNAs primarily target the 3'UTR region of target genes and negatively regulate target gene expression via the inhibition of translation or promotion of mRNA degradation (Bartel, 2009). We analyzed the potential targets of miR-499-5p using the bioinformatic program TargetScan to elucidate the molecular mechanisms by which it regulates cell apoptosis. miR-499-5p was predicted to bind to the 3'UTR region of p21, which is conserved in rats, mice and humans (Figure 4A). We also observed that p21 protein levels increased in cardiomyocytes treated with DOX in a time-dependent manner while miR-499-5p expression decreased, which suggests that p21 may be a target of miR-499-5p (Figures 1A, 4C). We forced the expression of miR-499-5p in cardiomyocytes to further analyze the regulatory role of miR-499-5p on p21. Overexpression of miR-499-5p significantly attenuated p21 expression in cardiomyocytes (Figure 4B). We used a luciferase assay system to examine whether miR-499-5p influenced p21 expression via a direct targeting of p21 3'UTR. We cloned p21 3'UTR containing the miR-499-5p binding site downstream of the luciferase reporter gene, p21 3'UTR-Wt, to examine luciferase activity driven by the 3'UTR of p21. We also generated a mutated luciferase construct, p21 3'UTR-Mut, with mutations introduced into the miR-499-5p-binding site of p21 3'UTR (Figure 4D). Both the wild-type 3'UTR of p21 (p21 3'UTR-Wt) and the mutated 3'UTR of the p21 (p21 3'UTR-Mut) showed a similar luciferase activity with the luciferase reporter gene (pGL3) (Figure 4E). However, the wild-type 3'UTR of p21 exhibited low luciferase activity in the presence of miR-499-5p while the mutated 3'UTR did not produce a significant response to miR-499-5p (Figure 4E). A luciferase activity assay in cardiomyocytes also demonstrated that DOX (2 μ M)

treatment increased the luciferase activity regulated by wild-type p21 3'UTR. The mutation of miR-499-5p-binding site slowed the increase in these luciferase activity (Figure 4F). Taken together, these results suggest that p21 is a specific target of miR-499-5p.

p21 Attenuates Mitochondrial Fission and Apoptosis in Cardiomyocytes Treated With DOX

Next, we investigated the potential role of p21 in DOX-induced cardiotoxicity. We knocked down endogenous p21 using siRNA (Figure 5A). Knockdown of endogenous p21 significantly decreased mitochondrial fission and cell death in cardiomyocytes exposed to DOX (2 μ M) (Figures 5C,D). We also overexpressed p21 using the pcDNA3.1 eukaryotic expression vector with a CMV promoter (Figure 5B). p21 overexpression induced massive mitochondrial fission and sensitized cardiomyocytes to undergo apoptosis under a lower concentration of DOX (0.2 μ M) (Figures 5E,F). Taken together, these results suggest that p21 promotes mitochondrial fission program and cell apoptosis in cardiomyocytes exposed to DOX.

miR-499-5p Attenuates Mitochondrial Fission and Apoptosis via Targeting p21

We examined whether miR-499-5p attenuated mitochondrial fission and cell apoptosis via the targeting of p21. Overexpression of miR-499-5p inhibited DOX-induced mitochondrial fission and cell death while forced expression of p21 attenuated this inhibitory effect on mitochondrial fission (Figure 6A) and cell death (Figure 6B). Knockdown of the endogenous miR-499-5p sensitized cardiomyocytes to undergo mitochondrial fission and cell death at a lower dose of DOX (0.2 μ M) while simultaneously knockdown of p21 inhibited these mitochondrial fission (Figure 6C) and cell death (Figure 6D). These data suggest that miR-499-5p attenuates DOX-induced mitochondrial fission and apoptosis in cardiomyocytes via the targeting of p21.

miR-499-5p Is Not Involved in DOX-Induced Apoptosis in Cancer Cells

Our results showed that miR-499-5p is a promising factor for preventing DOX cardiotoxicity in cancer therapy, but it is important to exclude its role in the apoptosis of tumor cells. We compared differences in miR-499-5p expression levels between cardiomyocytes and tumor cells. The results revealed that miR-499-5p was expressed at very low levels in tumor cells, including SGC-7901, A-549, SW-480 and HepG-2s, compared to cardiomyocytes (Figure 7A). We examined miR-499-5p expression levels during cell apoptosis in tumor cells treated with DOX. The results demonstrated that miR-499-5p expression was insensitive to DOX treatment in tumor cells (Figures 7B,C). Notably, miR-499-5p overexpression did not affect DOX-induced cell death in tumor cells (Figures 7D,E). These results indicate that miR-499-5p is not involved in the process of DOX-induced tumor cell apoptosis.

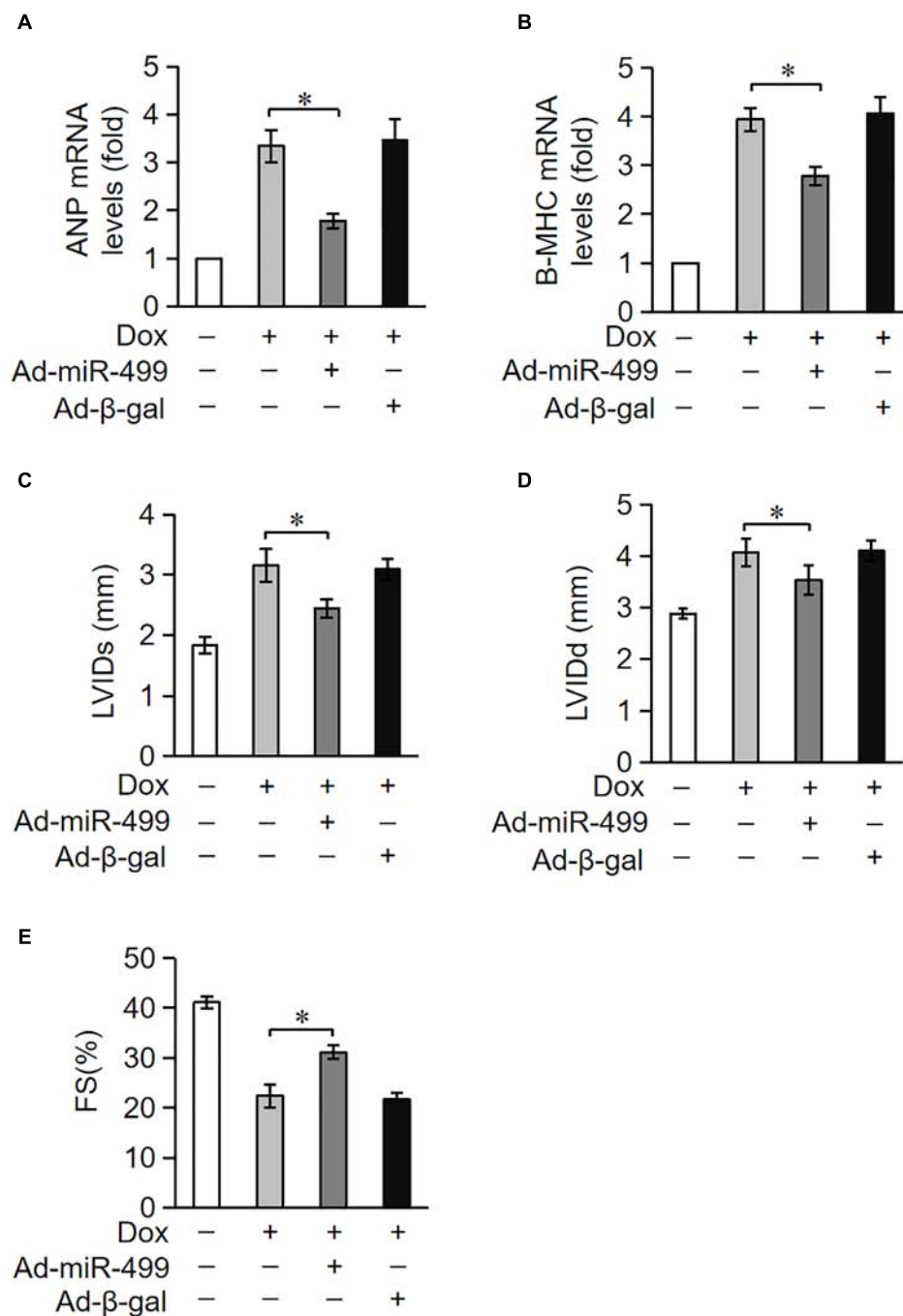


FIGURE 3 | miR-499-5p attenuates DOX cardiotoxicity in mice. **(A–E)** miR-499-5p-overexpressing mice are resistant to left ventricular remodeling after DOX treatment. **(A,B)** The expression levels of ANP and β -myosin heavy chain (B-MHC) were detected using qRT-PCR. Data are expressed as the means $\pm$ SD, $n = 3$ experiment. $*P < 0.05$. **(C–E)** miR-499-5p-overexpressing mice or control mice were exposed to DOX or saline as described in the panel, and echocardiography was used to test heart function. LVIDs, systolic left ventricular internal diameter **(C)**; LVIDd, diastolic left ventricular internal diameter **(D)**; FS, fractional shortening of the left ventricular diameter **(E)**. Data are presented as the means $\pm$ SD, $n = 8$ mice per group. $*P < 0.05$.

DISCUSSION

Doxorubicin is a powerful drug in the clinical fight against cancer. However, its cardiotoxicity is a major challenge and limits its application. A total of 20% of patients who receive DOX

treatment develop heart failure, and DOX accumulation reaches 500 mg/m² in these patients' heart (Chatterjee et al., 2010). An understanding of the molecular mechanisms will ameliorate the cardiotoxicity and increase the clinical efficacy of DOX. miRNAs play important roles in DOX-induced cardiotoxicity. miR-146a

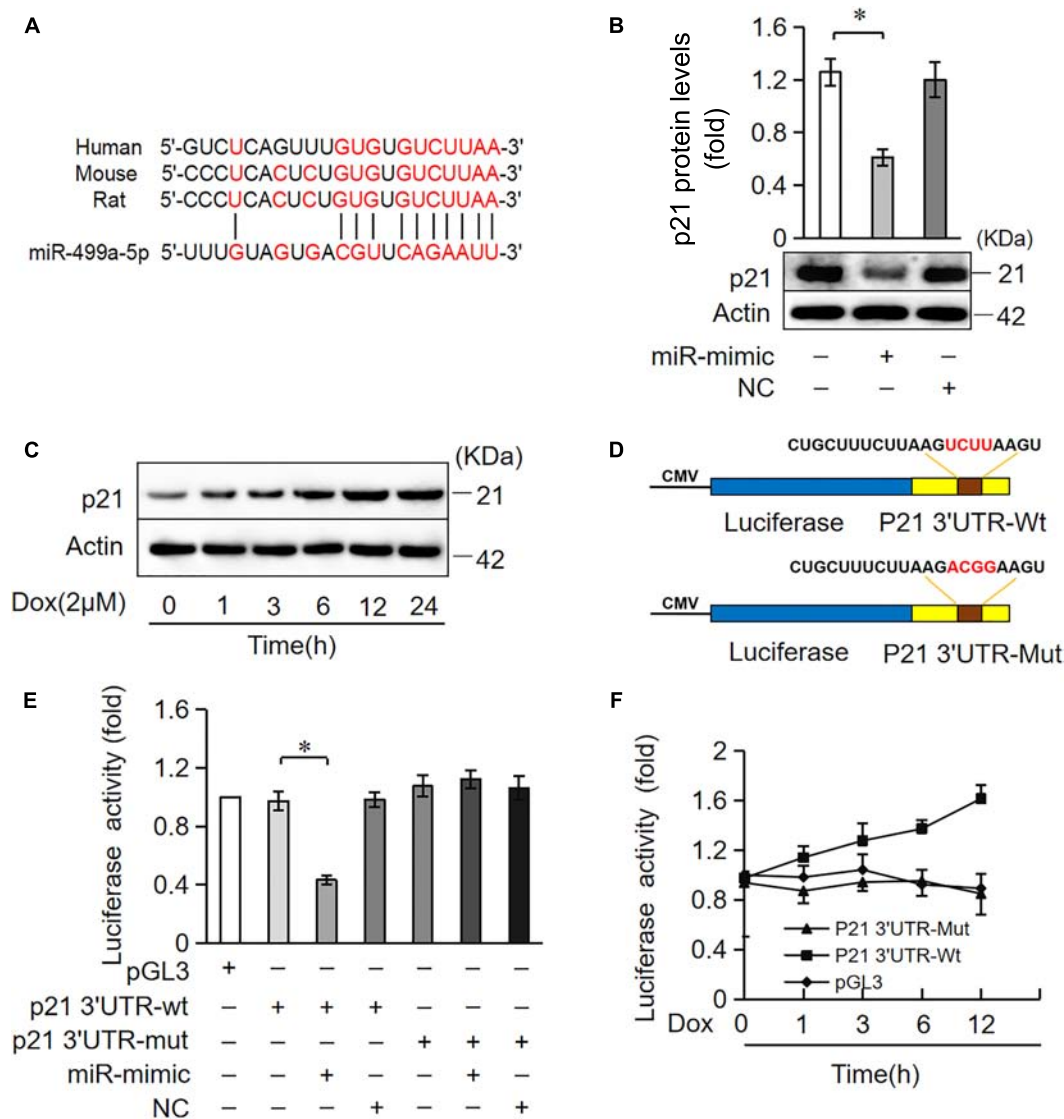


FIGURE 4 | miR-499-5p participates in the regulation of p21 expression. **(A)** Analysis of the p21 3'UTR potential binding site of miR-499-5p using RNA hybrid. Potential complementary residues are shown in red. **(B)** Cardiomyocytes were transfected with a miR-499-5p mimic or negative control for 24 h. The expression levels of p21 were determined using Western blot. **(C)** Cardiomyocytes were treated with DOX (2 μM) at the indicated time points. The expression levels of p21 were detected using Western blots. **(D–F)** miR-499-5p directly targets the p21 3'UTR. **(D)** Schematic diagram of the reporter containing the putative miR-499-5p binding in the p21 3'UTR. **(E)** Luciferase activity detected in HEK-293 cells transfected with synthesized miR-499-5p mimic or negative control along with luciferase reporter constructs, as indicated. **(F)** Luciferase activity of the luciferase construct p21 3'UTR-Wt decreased with DOX treatment in cardiomyocytes. Data are expressed as the means ± SD, $n = 3$ except in panel; * $P < 0.05$.

is involved in DOX-induced cardiomyocyte cell death via the targeting of ErbB4 (Horie et al., 2010). miR-30 targets GATA to participate in DOX-induced cardiomyocyte apoptosis (Roca-Alonso et al., 2015). MicroRNA-208 also contributes to DOX-induced cardiomyocyte cell death via the targeting of GATA4 (Tony et al., 2015). Our previous work found that miR-532-3p was involved in DOX-induced cardiotoxicity via the repression of ARC expression (Wang et al., 2015b). miR-140-5p directly targets Nrf2 and Sirt2 and increases DOX-induced oxidative damage via altering FOXO3a expression levels (Zhao et al., 2018). We reported here that miR-499-5p efficiently prevented

DOX cardiotoxicity via attenuating mitochondrial fission and cell apoptosis and targeting of p21 (Figure 6E).

Two other types of non-coding RNAs, lncRNAs and circRNAs, garnered great attention recently, and these non-coding RNAs are also involved in DOX cardiotoxicity and myocardial damage. lincRNA-p21 participates in DOX-related cardiac cell senescence via regulation of the Wnt/beta-catenin signaling pathway, and silencing of lincRNA-p21 effectively protects against DOX cardiotoxicity (Xie et al., 2018). Qki (Quaking), which is a RNA binding protein, inhibits DOX cardiotoxicity via regulation of cardiac circular RNAs (Gupta et al., 2018). Knockdown

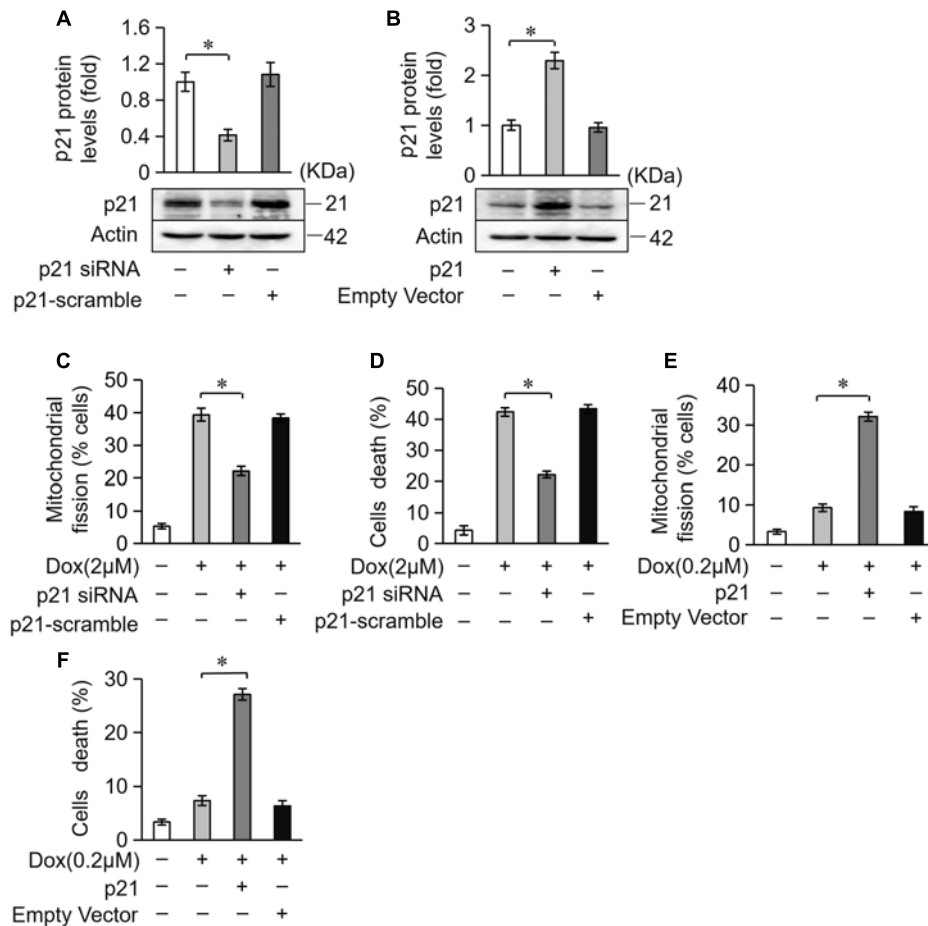


FIGURE 5 | p21 attenuates mitochondrial fission and cell death in H9c2 cells treated with DOX. **(A)** The expression levels of p21 were detected using Western blot in cardiomyocytes transfected with p21 siRNA. **(B)** The expression levels of p21 were detected using Western blot in cardiomyocytes transfected with a p21-overexpressing vector. Actin served as the loading control. **(C,D)** Knockdown of p21 inhibited 2 μ M DOX-induced mitochondrial fission and cell death. Cardiomyocytes were transfected with p21 siRNA or p21 scramble for 24 h and treated with DOX (2 μ M) for 24 h. **(E,F)** Overexpression of p21 increased mitochondrial fission and cell death induced by low-dose DOX (0.2 μ M). Cardiomyocytes were transfected with p21 or empty vector for 24 h and treated with 0.2 μ M DOX for 24 h. * P < 0.05; Data are expressed as the means $\pm$ SD, n = 3 experiment. * P < 0.05.

of MiRt1 improves cardiac function, reduces apoptosis of cardiomyocytes and attenuates inflammatory cell infiltration *in vivo* (Li et al., 2017). lncRNA-cardiac autophagy inhibitory factor (CAIF) inhibits autophagy and attenuates myocardial infarction via the targeting of p53-mediated transcription of myocardin (Liu C.Y. et al., 2018). Microarray analysis found a total of 63 differentially expressed circRNAs, including 29 upregulated and 34 downregulated circRNAs, during myocardial infarction (Wu et al., 2016). Mitochondrial fission and apoptosis-related circRNA (MFACR) regulates mitochondrial fission and apoptosis in the heart via direct targeting of miR-652-3p (Li et al., 2018). In conclusion, lncRNAs and circRNAs are potential targets for the prevention of DOX cardiotoxicity. Further study is needed to elucidate the functional role of these two non-coding RNAs.

The role of miR-499-5p in certain types of tumors is controversial. Several studies demonstrated that miR-499-5p suppressed cancer development and progression. Overexpression of miR-499-5p downregulated ETS1 expression, and it inhibited

the migration and infiltration of HepG-2 cells (Wei et al., 2012). miR-499-5p could also inhibit the growth of tumor cells and improves the effectiveness of cancer treatment (Ando et al., 2014). miR-499-5p may also act as a tumor suppressor gene via the targeting of VAV3 (Li M. et al., 2016). However, other studies revealed that miR-499-5p was abnormally expressed in non-small cell lung cancer tissues, and it may be used as a biomarker for early diagnosis and evaluation of patient prognosis (Li et al., 2014). Our study found that miR-499-5p produced no effect on tumor cells, including gastric cancer, non-small cell lung cancer, colon cancer or liver cancer, which suggests that it increases the therapeutic potential of the tumor and protects the heart without promoting tumor development. Therefore, miR-499-5p may be an effective factor in the prevention of DOX cardiotoxicity.

Single nucleotide polymorphisms (SNPs) are the most frequent type of variation in the genome. Numerous study demonstrated that functional SNPs in miRNA genes affect different signaling pathways via altering the expression levels

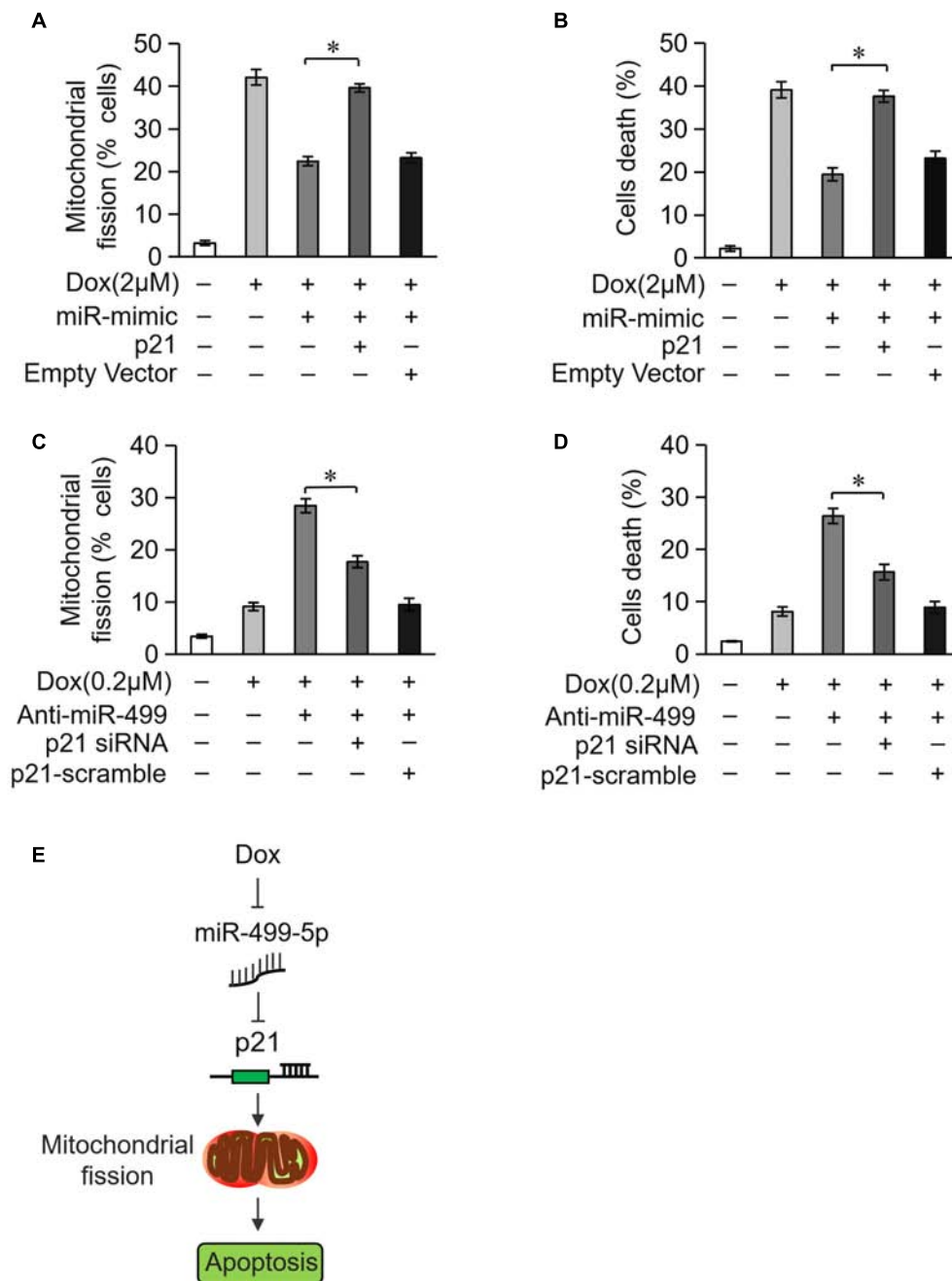


FIGURE 6 | miR-499-5p attenuates mitochondrial fission and apoptosis via targeting p21. **(A,B)** miR-499-5p attenuated the sensitivity of cardiomyocytes to DOX treatment, which was abolished by p21 overexpression. Statistical analysis of the mitochondrial fission **(A)** and cell death **(B)** in cardiomyocytes following DOX (2 μM) treatment. **(C,D)** Knockdown of miR-499-5p increased the sensitivity of cardiomyocytes to DOX (0.2 μM) treatment, which was abolished by p21 knockdown. Statistical analysis of the mitochondrial fission **(C)** and cell death **(D)** in cardiomyocytes following DOX (0.2 μM) treatment. **(E)** Schematic diagram of this study. Data are presented as the means ± SD, *n* = 3 experiment. **P* < 0.05.

of target genes (Zhi et al., 2012; Chen et al., 2014; Liu et al., 2017). Increasing evidence suggests that miRNA polymorphisms are associated with the susceptibility to heart diseases, such as myocardial infarction and coronary heart disease. Previous studies reported that miR-196a2 rs11614913 (Xu et al., 2009; Zhi et al., 2012), miR-146a rs2910164 (Chen et al., 2014), and

miR-149 rs71428439 (Ding et al., 2013) were associated with the risk of heart disease. miR-499-5p is a heart-rich miRNA under physiological conditions, and some studies demonstrated that miR-499-5p levels were downregulated under pathological conditions (van Rooij et al., 2009; Wang et al., 2011). The plasma level of miR-499-5p may help distinguish acute myocardial

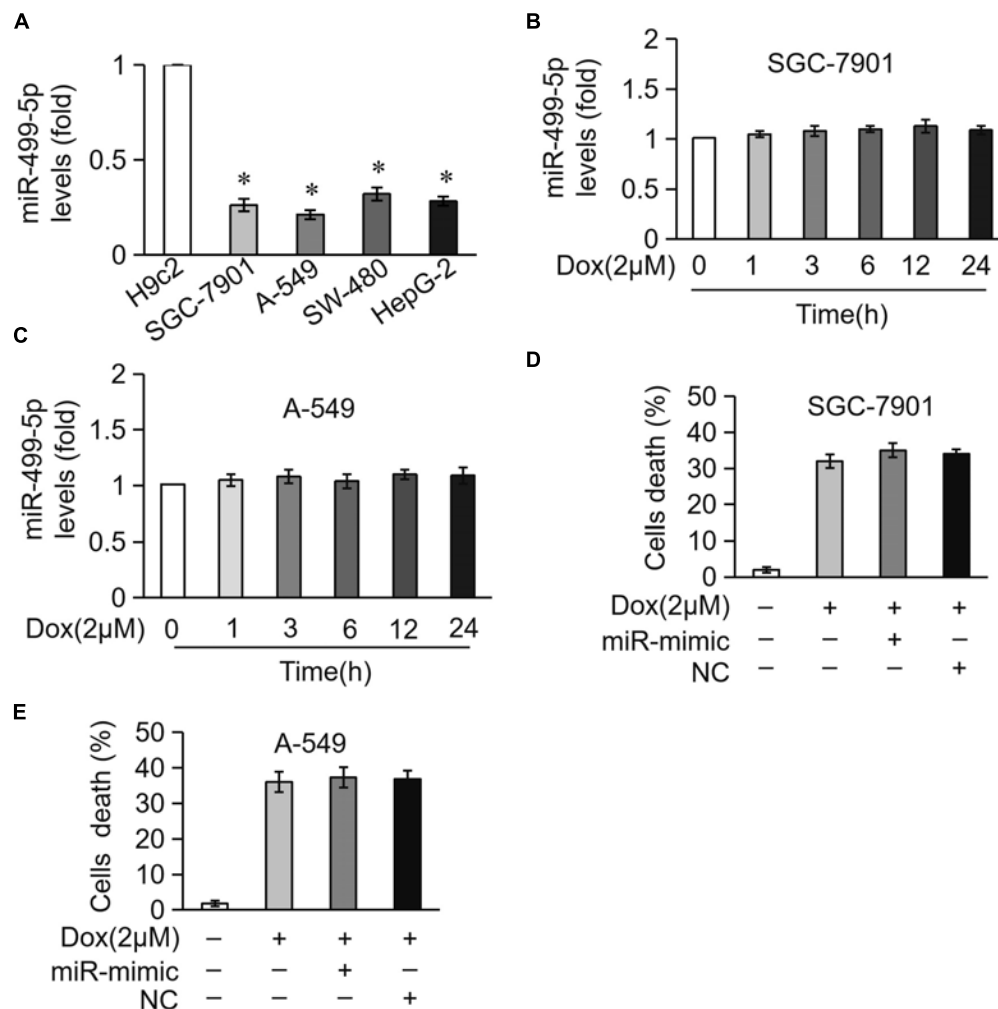


FIGURE 7 | miR-499-5p is not involved in DOX-induced apoptosis in cancer cells. **(A)** Real-time PCR results show the miR-499-5p expression levels in H9c2, SGC-7901, A-549, SW-480, and HepG-2 cells. * $P < 0.05$ versus cardiomyocytes. **(B,C)** Real-time PCR results show miR-499-5p levels in SG-7901 **(B)** and A-549 **(C)** cells treated with DOX (2 μ M) for the indicated times. **(D,E)** Statistical analysis of the cell death in SG-7901 **(D)** and A-549 **(E)** cells transfected with miR-499-5p mimic following treatment with DOX (2 μ M). Data are expressed as the means $\pm$ SD, $n = 3$ experiment.

infarction and heart disease in patients (Olivieri et al., 2013). Our previous study found that the SNP rs3746444 of the miR-499 precursor affected the expression level and anti-apoptotic function of miR-499-5p (Ding et al., 2018). Whether SNPs in miRNA-499-5p are related to the susceptibility to cardiotoxicity need to be further investigated, which is of great significance for guiding medication and overcoming drug cardiotoxicity.

Well-balanced mitochondrial dynamics play an important role in cell life (Archer, 2014). Abnormal mitochondrial fission leads to the onset of cardiomyocyte apoptosis and the development of heart failure (Lee et al., 2004; Ong and Hausenloy, 2010). DOX-induced mitochondrial dysfunction is currently the major cause of cardiotoxicity (Ichikawa et al., 2014), and it was also documented in heart failure patients (Lebrecht et al., 2005). Our present study revealed the pivotal role of p21 in the promotion of mitochondrial fission caused by DOX treatment. Drp1-mediated mitochondrial fission is an important component

of cell apoptosis (Martinou and Youle, 2006), and p21 participates in the Drp1-mediated mitochondrial fission that promotes HCC cell proliferation (Zhan et al., 2016). p21 also participated in the p53-mediated mitochondrial apoptosis program in nickel (II)-induced nasal epithelial cytotoxicity (Lee et al., 2016). However, whether p21 regulates DOX-induced mitochondrial fission via Drp1 or p53 need to be further examined.

Taken together, we report that the downregulation of miR-499-5p in cardiomyocytes exposed to DOX is involved in DOX cardiotoxicity. There is emerging evidence for the involvement of p21 in the promotion of mitochondrial fission and cell apoptosis in cardiomyocyte exposed to DOX. miR-499-5p prevented mitochondrial fission and cell apoptosis in cardiomyocytes exposed to DOX via the targeting of p21 (Figure 6E). Therefore, the development of new therapeutic strategies based on the miR-499-5p-p21 axis is promising for the overcoming of DOX cardiotoxicity in cancer treatment.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institutional Animal Care and Use Committee of Qingdao University Medical College. The protocol was approved by Institutional Animal Care and Use Committee of Qingdao University Medical College.

AUTHOR CONTRIBUTIONS

JW and TX designed the research. QW and WD performed the cellular experiments. XZ and TX performed

the animal experiments. TY and XJ constructed the reporter construct and adenoviruses. WY and ZL analyzed the cardiac function. QW and JW wrote the manuscript. All authors approved the final version of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (81622005 and 81770232 to JW) and the Natural Science Foundation of Shandong Province (JQ201815 to JW).

REFERENCES

- Ando, H., Asai, T., Koide, H., Okamoto, A., Maeda, N., Tomita, K., et al. (2014). Advanced cancer therapy by integrative antitumor actions via systemic administration of miR-499. *J. Control. Release* 181, 32–39. doi: 10.1016/j.jconrel.2014.02.019
- Archer, S. L. (2014). Mitochondrial fission and fusion in human diseases. *N. Engl. J. Med.* 370:1074. doi: 10.1056/NEJMc1316254
- Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002
- Bartlett, J. J., Trivedi, P. C., and Pulinilkunnil, T. (2017). Autophagic dysregulation in doxorubicin cardiomyopathy. *J. Mol. Cell. Cardiol.* 104, 1–8. doi: 10.1016/j.yjmcc.2017.01.007
- Boman, A. L., Zhang, C., Zhu, X., and Kahn, R. A. (2000). A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Mol. Biol. Cell* 11, 1241–1255. doi: 10.1091/mbc.11.4.1241
- Brooks, G., Poolman, R. A., and Li, J. M. (1998). Arresting developments in the cardiac myocyte cell cycle: role of cyclin-dependent kinase inhibitors. *Cardiovasc. Res.* 39, 301–311. doi: 10.1016/S0008-6363(98)00125-4
- Cadete, V. J., Deschenes, S., Cuillerier, A., Brisebois, F., Sugiura, A., Vincent, A., et al. (2016). Formation of mitochondrial-derived vesicles is an active and physiologically relevant mitochondrial quality control process in the cardiac system. *J. Physiol.* 594, 5343–5362. doi: 10.1113/JP272703
- Chatterjee, K., Zhang, J., Honbo, N., and Karliner, J. S. (2010). Doxorubicin cardiomyopathy. *Cardiology* 115, 155–162. doi: 10.1159/000265166
- Chen, C., Hong, H., Chen, L., Shi, X., Chen, Y., and Weng, Q. (2014). Association of microRNA polymorphisms with the risk of myocardial infarction in a Chinese population. *Tohoku J. Exp. Med.* 233, 89–94. doi: 10.1620/tjem.233.89
- Chistiakov, D. A., Orekhov, A. N., and Bobryshev, Y. V. (2016). Cardiac-specific miRNA in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). *J. Mol. Cell. Cardiol.* 94, 107–121. doi: 10.1016/j.yjmcc.2016.03.015
- Coppola, C., Riccio, G., Barbieri, A., Monti, M. G., Piscopo, G., Rea, D., et al. (2016). Antineoplastic-related cardiotoxicity, morphofunctional aspects in a murine model: contribution of the new tool 2D-speckle tracking. *Onco Targets Ther.* 9, 6785–6794. doi: 10.2147/OTT.S106528
- Dhingra, R., Margulets, V., Chowdhury, S. R., Thliveris, J., Jassal, D., Fernyhough, P., et al. (2014). Bnip3 mediates doxorubicin-induced cardiac myocyte necrosis and mortality through changes in mitochondrial signaling. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5537–E5544. doi: 10.1073/pnas.1414665111
- Ding, S. L., Wang, J. X., Jiao, J. Q., Tu, X., Wang, Q., Liu, F., et al. (2013). A pre-microRNA-149 (miR-149) genetic variation affects miR-149 maturation and its ability to regulate the Puma protein in apoptosis. *J. Biol. Chem.* 288, 26865–26877. doi: 10.1074/jbc.M112.440453
- Ding, W., Li, M., Sun, T., Han, D., Guo, X., Chen, X., et al. (2018). A polymorphism rs3746444 within the pre-miR-499 alters the maturation of miR-499-5p and its antiapoptotic function. *J. Cell. Mol. Med.* 22, 5418–5428. doi: 10.1111/jcmm.13813
- Fu, J. D., Rushing, S. N., Lieu, D. K., Chan, C. W., Kong, C. W., Geng, L., et al. (2011). Distinct roles of microRNA-1 and -499 in ventricular specification and functional maturation of human embryonic stem cell-derived cardiomyocytes. *PLoS One* 6:e27417. doi: 10.1371/journal.pone.0027417
- Gorini, S., De Angelis, A., Berrino, L., Malara, N., Rosano, G., and Ferraro, E. (2018). Chemotherapeutic drugs and mitochondrial dysfunction: focus on doxorubicin, trastuzumab, and sunitinib. *Oxid. Med. Cell. Longev.* 2018:7582730. doi: 10.1155/2018/7582730
- Gupta, S. K., Garg, A., Bar, C., Chatterjee, S., Foinquinos, A., Milting, H., et al. (2018). Quaking inhibits doxorubicin-mediated cardiotoxicity through regulation of cardiac circular RNA expression. *Circ. Res.* 122, 246–254. doi: 10.1161/CIRCRESAHA.117.311335
- He, X., Li, S., Liu, B., Susperreguy, S., Formoso, K., Yao, J., et al. (2017). Major contribution of the 3/6/7 class of TRPC channels to myocardial ischemia/reperfusion and cellular hypoxia/reoxygenation injuries. *Proc. Natl. Acad. Sci. U.S.A.* 114, E4582–E4591. doi: 10.1073/pnas.1621384114
- Horie, T., Ono, K., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., et al. (2010). Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. *Cardiovasc. Res.* 87, 656–664. doi: 10.1093/cvr/cvq148
- Ichikawa, Y., Ghanefar, M., Bayeva, M., Wu, R., Khechaduri, A., Naga Prasad, S. V., et al. (2014). Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *J. Clin. Invest.* 124, 617–630. doi: 10.1172/JCI72931
- Kluzza, J., Marchetti, P., Gallego, M. A., Lancel, S., Fournier, C., Loyens, A., et al. (2004). Mitochondrial proliferation during apoptosis induced by anticancer agents: effects of doxorubicin and mitoxantrone on cancer and cardiac cells. *Oncogene* 23, 7018–7030. doi: 10.1038/sj.onc.1207936
- Koga, K., Kenessey, A., and Ojamaa, K. (2013). Macrophage migration inhibitory factor antagonizes pressure overload-induced cardiac hypertrophy. *Am. J. Physiol. Heart Circ. Physiol.* 304, H282–H293. doi: 10.1152/ajpheart.00595.2012
- Kozomara, A., and Griffiths-Jones, S. (2011). miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157. doi: 10.1093/nar/gkq1027
- Kuhn, D. E., Roy, S., Radtke, J., Khanna, S., and Sen, C. K. (2007). Laser microdissection and capture of pure cardiomyocytes and fibroblasts from infarcted heart regions: perceived hyperoxia induces p21 in peri-infarct myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 292, H1245–H1253. doi: 10.1152/ajpheart.01069.2006
- Kumar, D., Kirshenbaum, L. A., Li, T., Danelisen, I., and Singal, P. K. (2001). Apoptosis in adriamycin cardiomyopathy and its modulation by probucol. *Antioxid. Redox Signal.* 3, 135–145. doi: 10.1089/152308601750100641
- Lebrecht, D., Kokkari, A., Ketelsen, U. P., Setzer, B., and Walker, U. A. (2005). Tissue-specific mtDNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. *J. Pathol.* 207, 436–444. doi: 10.1002/path.1863
- Lee, Y. J., Jeong, S. Y., Karbowski, M., Smith, C. L., and Youle, R. J. (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol. Biol. Cell* 15, 5001–5011. doi: 10.1091/mbc.e04-04-0294
- Lee, Y. J., Lim, S. S., Baek, B. J., An, J. M., Nam, H. S., Woo, K. M., et al. (2016). Nickel(II)-induced nasal epithelial toxicity and oxidative

- mitochondrial damage. *Environ. Toxicol. Pharmacol.* 42, 76–84. doi: 10.1016/j.etap.2016.01.005
- Levis, B. E., Binkley, P. F., and Shapiro, C. L. (2017). Cardiotoxic effects of anthracycline-based therapy: what is the evidence and what are the potential harms? *Lancet Oncol.* 18, e445–e456. doi: 10.1016/S1470-2045(17)30535-1
- Li, M., Ding, W., Sun, T., Tariq, M. A., Xu, T., Li, P., et al. (2018). Biogenesis of circular RNAs and their roles in cardiovascular development and pathology. *FEBS J.* 285, 220–232. doi: 10.1111/febs.14191
- Li, M., Zhang, S., Wu, N., Wu, L., Wang, C., and Lin, Y. (2016). Overexpression of miR-499-5p inhibits non-small cell lung cancer proliferation and metastasis by targeting VAV3. *Sci. Rep.* 6:23100. doi: 10.1038/srep23100
- Li, M., Zhang, Q., Wu, L., Jia, C., Shi, F., Li, S., et al. (2014). Serum miR-499 as a novel diagnostic and prognostic biomarker in non-small cell lung cancer. *Oncol. Rep.* 31, 1961–1967. doi: 10.3892/or.2014.3029
- Li, Y., Lu, J., Bao, X., Wang, X., Wu, J., Li, X., et al. (2016). MiR-499-5p protects cardiomyocytes against ischaemic injury via anti-apoptosis by targeting PDCD4. *Oncotarget* 7, 35607–35617. doi: 10.18632/oncotarget.9597
- Li, Q., Lin, S., Wang, X., Lian, G., Lu, Z., Guo, H., et al. (2009). Axin determines cell fate by controlling the p53 activation threshold after DNA damage. *Nat. Cell Biol.* 11, 1128–1134. doi: 10.1038/ncb1927
- Li, X., Zhou, J., and Huang, K. (2017). Inhibition of the lncRNA Mirt1 attenuates acute myocardial infarction by suppressing NF-kappaB activation. *Cell. Physiol. Biochem.* 42, 1153–1164. doi: 10.1159/000478870
- Lim, L. P., Lau, N. C., Weinstein, E. G., Abdelhakim, A., Yekta, S., Rhoades, M. W., et al. (2003). The microRNAs of *Caenorhabditis elegans*. *Genes Dev.* 17, 991–1008. doi: 10.1101/gad.1074403
- Liu, C. Y., Zhang, Y. H., Li, R. B., Zhou, L. Y., An, T., Zhang, R. C., et al. (2018). LncRNA CAIF inhibits autophagy and attenuates myocardial infarction by blocking p53-mediated myocardial transcription. *Nat. Commun.* 9:29. doi: 10.1038/s41467-017-02280-y
- Liu, S., Yang, Y., Jiang, S., Tang, N., Tian, J., Ponnusamy, M., et al. (2018). Understanding the role of non-coding RNA (ncRNA) in stent restenosis. *Atherosclerosis* 272, 153–161. doi: 10.1016/j.atherosclerosis.2018.03.036
- Liu, F., Fu, Y., and Meyskens, F. L. Jr. (2009). MITF regulates cellular response to reactive oxygen species through transcriptional regulation of APE-1/Ref-1. *J. Invest. Dermatol.* 129, 422–431. doi: 10.1038/jid.2008.255
- Liu, X., You, L., Zhou, R., and Zhang, J. (2017). Significant association between functional microRNA polymorphisms and coronary heart disease susceptibility: a comprehensive meta-analysis involving 16484 subjects. *Oncotarget* 8, 5692–5702. doi: 10.18632/oncotarget.14249
- Martinou, J. C., and Youle, R. J. (2006). Which came first, the cytochrome c release or the mitochondrial fission? *Cell Death Differ.* 13, 1291–1295. doi: 10.1038/sj.cdd.4401985
- Matkovich, S. J., Hu, Y., Eschenbacher, W. H., Dorn, L. E., and Dorn, G. W. II (2012). Direct and indirect involvement of microRNA-499 in clinical and experimental cardiomyopathy. *Circ. Res.* 111, 521–531. doi: 10.1161/CIRCRESAHA.112.265736
- Megyesi, J., Tarcsfalvi, A., Li, S., Hodeify, R., Seng, N. S., Portilla, D., et al. (2015). Increased expression of p21WAF1/CIP1 in kidney proximal tubules mediates fibrosis. *Am. J. Physiol. Renal Physiol.* 308, F122–F130. doi: 10.1152/ajprenal.00489.2014
- Mette, M. F., van der Winden, J., Matzke, M., and Matzke, A. J. (2002). Short RNAs can identify new candidate transposable element families in Arabidopsis. *Plant Physiol.* 130, 6–9. doi: 10.1104/pp.007047
- Nuntharatanapong, N., Chen, K., Sinhaseni, P., and Keaney, J. F. Jr. (2005). EGF receptor-dependent JNK activation is involved in arsenite-induced p21Cip1/Waf1 upregulation and endothelial apoptosis. *Am. J. Physiol. Heart Circ. Physiol.* 289, H99–H107. doi: 10.1152/ajpheart.00901.2004
- Olivieri, F., Antonicelli, R., Lorenzi, M., D'Alessandra, Y., Lazzarini, R., Santini, G., et al. (2013). Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int. J. Cardiol.* 167, 531–536. doi: 10.1016/j.ijcard.2012.01.075
- Ong, S. B., and Hausenloy, D. J. (2010). Mitochondrial morphology and cardiovascular disease. *Cardiovasc. Res.* 88, 16–29. doi: 10.1093/cvr/cvq237
- Roca-Alonso, L., Castellano, L., Mills, A., Dabrowska, A. F., Sikkel, M. B., Pellegrino, L., et al. (2015). Myocardial MiR-30 downregulation triggered by doxorubicin drives alterations in beta-adrenergic signaling and enhances apoptosis. *Cell Death Dis.* 6:e1754. doi: 10.1038/cddis.2015.89
- Roy, S., Khanna, S., Bickerstaff, A. A., Subramanian, S. V., Atalay, M., Bierl, M., et al. (2003a). Oxygen sensing by primary cardiac fibroblasts: a key role of p21(Waf1/Cip1/Sdi1). *Circ. Res.* 92, 264–271.
- Roy, S., Khanna, S., Wallace, W. A., Lappalainen, J., Rink, C., Cardounel, A. J., et al. (2003b). Characterization of perceived hyperoxia in isolated primary cardiac fibroblasts and in the reoxygenated heart. *J. Biol. Chem.* 278, 47129–47135. doi: 10.1074/jbc.M308703200
- Sen, C. K., Khanna, S., and Roy, S. (2006). Perceived hyperoxia: oxygen-induced remodeling of the reoxygenated heart. *Cardiovasc. Res.* 71, 280–288. doi: 10.1016/j.cardiores.2006.01.003
- Sharpless, N. E., and Sherr, C. J. (2015). Forging a signature of in vivo senescence. *Nat. Rev. Cancer* 15, 397–408. doi: 10.1038/nrc3960
- Sluijter, J. P., van Mil, A., van Vliet, P., Metz, C. H., Liu, J., Doevendans, P. A., et al. (2010). MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. *Arterioscler. Thromb. Vasc. Biol.* 30, 859–868. doi: 10.1161/ATVBAHA.109.197434
- Tang, N., Jiang, S., Yang, Y., Liu, S., Ponnusamy, M., Xin, H., et al. (2018). Noncoding RNAs as therapeutic targets in atherosclerosis with diabetes mellitus. *Cardiovasc. Ther.* 36:e12436. doi: 10.1111/1755-5922.12436
- Terrand, J., Xu, B., Morrissy, S., Dinh, T. N., Williams, S., and Chen, Q. M. (2011). p21(WAF1/Cip1/Sdi1) knockout mice respond to doxorubicin with reduced cardiotoxicity. *Toxicol. Appl. Pharmacol.* 257, 102–110. doi: 10.1016/j.taap.2011.08.024
- Tony, H., Yu, K., and Qiutang, Z. (2015). MicroRNA-208a silencing attenuates doxorubicin induced myocyte apoptosis and cardiac dysfunction. *Oxid. Med. Cell. Longev.* 2015:597032. doi: 10.1155/2015/597032
- van Rooij, E., Quiat, D., Johnson, B. A., Sutherland, L. B., Qi, X., Richardson, J. A., et al. (2009). A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. *Dev. Cell* 17, 662–673. doi: 10.1016/j.devcel.2009.10.013
- Wander, P. L., Enquobahrie, D. A., Pritchard, C. C., McKnight, B., Rice, K., Christiansen, M., et al. (2016). Circulating microRNAs and sudden cardiac arrest outcomes. *Resuscitation* 106, 96–101. doi: 10.1016/j.resuscitation.2016.06.038
- Wang, F., Wu, Y., Quon, M. J., Li, X., and Yang, P. (2015a). ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development. *Am. J. Physiol. Endocrinol. Metab.* 309, E487–E499. doi: 10.1152/ajpendo.00121.2015
- Wang, J. X., Zhang, X. J., Feng, C., Sun, T., Wang, K., Wang, Y., et al. (2015b). MicroRNA-532-3p regulates mitochondrial fission through targeting apoptosis repressor with caspase recruitment domain in doxorubicin cardiotoxicity. *Cell Death Dis.* 6:e1777. doi: 10.1038/cddis.2015.41
- Wang, J. X., Zhang, X. J., Li, Q., Wang, K., Wang, Y., Jiao, J. Q., et al. (2015c). MicroRNA-103/107 regulate programmed necrosis and myocardial ischemia/reperfusion injury through targeting FADD. *Circ. Res.* 117, 352–363. doi: 10.1161/CIRCRESAHA.117.305781
- Wang, K., Zhou, L. Y., Wang, J. X., Wang, Y., Sun, T., Zhao, B., et al. (2015d). E2F1-dependent miR-421 regulates mitochondrial fragmentation and myocardial infarction by targeting Pink1. *Nat. Commun.* 6:7619. doi: 10.1038/ncomms8619
- Wang, J., Jia, Z., Zhang, C., Sun, M., Wang, W., Chen, P., et al. (2014). miR-499 protects cardiomyocytes from H₂O₂-induced apoptosis via its effects on Pdc4 and Pacs2. *RNA Biol.* 11, 339–350. doi: 10.4161/rna.28300
- Wang, R., Wang, Y., Lin, W. K., Zhang, Y., Liu, W., Huang, K., et al. (2014). Inhibition of angiotensin II-induced cardiac hypertrophy and associated ventricular arrhythmias by a p21 activated kinase 1 bioactive peptide. *PLoS One* 9:e101974. doi: 10.1371/journal.pone.0101974
- Wang, J. X., Jiao, J. Q., Li, Q., Long, B., Wang, K., Liu, J. P., et al. (2011). miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. *Nat. Med.* 17, 71–78. doi: 10.1038/nm.2282
- Wang, J. X., Li, Q., and Li, P. F. (2009). Apoptosis repressor with caspase recruitment domain contributes to chemotherapy resistance by abolishing mitochondrial fission mediated by dynamin-related protein-1. *Cancer Res.* 69, 492–500. doi: 10.1158/0008-5472.CAN-08-2962
- Wei, W., Hu, Z., Fu, H., Tie, Y., Zhang, H., Wu, Y., et al. (2012). MicroRNA-1 and microRNA-499 downregulate the expression of the ets1 proto-oncogene in HepG2 cells. *Oncol. Rep.* 28, 701–706. doi: 10.3892/or.2012.1850

- Wilson, K. D., Hu, S., Venkatasubrahmanyam, S., Fu, J. D., Sun, N., Abilez, O. J., et al. (2010). Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: role for miR-499. *Circ. Cardiovasc. Genet.* 3, 426–435. doi: 10.1161/CIRCGENETICS.109.934281
- Wu, H. J., Zhang, C. Y., Zhang, S., Chang, M., and Wang, H. Y. (2016). Microarray expression profile of circular RNAs in heart tissue of mice with myocardial infarction-induced heart failure. *Cell. Physiol. Biochem.* 39, 205–216. doi: 10.1159/000445617
- Xie, Z., Xia, W., and Hou, M. (2018). Long intergenic noncoding RNA-p21 mediates cardiac senescence via the Wnt/beta-catenin signaling pathway in doxorubicin-induced cardiotoxicity. *Mol. Med. Rep.* 17, 2695–2704. doi: 10.3892/mmr.2017.8169
- Xu, J., Hu, Z., Xu, Z., Gu, H., Yi, L., Cao, H., et al. (2009). Functional variant in microRNA-196a2 contributes to the susceptibility of congenital heart disease in a Chinese population. *Hum. Mutat.* 30, 1231–1236. doi: 10.1002/humu.21044
- Yarana, C., Carroll, D., Chen, J., Chaiswing, L., Zhao, Y., Noel, T., et al. (2018). Extracellular vesicles released by cardiomyocytes in a doxorubicin-induced cardiac injury mouse model contain protein biomarkers of early cardiac injury. *Clin. Cancer Res.* 24, 1644–1653. doi: 10.1158/1078-0432.CCR-17-2046
- Zhan, L., Cao, H., Wang, G., Lyu, Y., Sun, X., An, J., et al. (2016). Drp1-mediated mitochondrial fission promotes cell proliferation through crosstalk of p53 and NF-kappaB pathways in hepatocellular carcinoma. *Oncotarget* 7, 65001–65011. doi: 10.18632/oncotarget.11339
- Zhao, L., Qi, Y., Xu, L., Tao, X., Han, X., Yin, L., et al. (2018). MicroRNA-140-5p aggravates doxorubicin-induced cardiotoxicity by promoting myocardial oxidative stress via targeting Nrf2 and Sirt2. *Redox Biol.* 15, 284–296. doi: 10.1016/j.redox.2017.12.013
- Zhi, H., Wang, L., Ma, G., Ye, X., Yu, X., Zhu, Y., et al. (2012). Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease. *Clin. Res. Cardiol.* 101, 289–296. doi: 10.1007/s00392-011-0391-3
- Zhu, W., Soonpaa, M. H., Chen, H., Shen, W., Payne, R. M., Liechty, E. A., et al. (2009). Acute doxorubicin cardiotoxicity is associated with p53-induced inhibition of the mammalian target of rapamycin pathway. *Circulation* 119, 99–106. doi: 10.1161/CIRCULATIONAHA.108.799700

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-Coding RNAs in Retinoblastoma

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Retinoblastoma (Rb) is the most common ocular pediatric malignancy that arises from the retina and is caused by a mutation of the two alleles of the tumor suppressor gene, *RB1*. Although early detection provides the opportunity of controlling the primary tumor with effective therapies, metastatic activity is fatal. Non-coding RNAs (ncRNAs) have emerged as important modifiers of a plethora of biological mechanisms including those involved in cancer. They are classified into short and long ncRNAs according to their length. Deregulation of all these molecules has also been shown to play a critical role in Rb pathogenesis and progression. It is believed that ncRNAs can provide new insights into novel regulatory mechanisms associated with clinical pathological characteristics, facilitating the development of therapeutic alternatives for the treatment of Rb. In this review, we describe a variety of ncRNAs, which capable of regulating the most likely candidate genes involved in the tumorigenesis of Rb, could prove useful in analyzing different aspects of this cancer.

OPEN ACCESS

Edited by:

Yujing Li,
Emory University, United States

Reviewed by:

Feng Wang,
Emory University School of Medicine,
United States
Alessio Naccarati,
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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 05 October 2018

Accepted: 22 October 2019

Published: 14 November 2019

Citation:

Plousiou M and Vannini I (2019) Non-
Coding RNAs in Retinoblastoma.
Front. Genet. 10:1155.
doi: 10.3389/fgene.2019.01155

Keywords: cancer, microRNAs, long ncRNAs, retinoblastoma, *RB1*

INTRODUCTION

Retinoblastoma (Rb) is the most common primary ocular paediatric malignancy and the first to be identified as having hereditary features. Inactivation of the *RB1* gene is thought to be the main reason for the development of the tumor (Ma et al., 2014). The impact of Rb is substantial, with an incidence of nearly one in 15,000–20,000 births, resulting in 9,000 new cases every year (Kivelä, 2009; Thériault et al., 2014). Although it is possible to control the primary tumor with effective therapies, metastatic disease is still fatal. The survival rate is lower in under-developed countries because the disease is often diagnosed at later stages, and the mortality rate among children is 50%–70% (Jabbour et al., 2012).

Rb can be either non-hereditary or hereditary and can affect only one eye (unilateral) or both (bilateral). In particular, the bilateral forms of heritable Rb show a germline mutation of the *RB1* gene which is followed by a second somatic inactivation of the other allele. Conversely, somatic inactivation of both *RB1* alleles in retinal cells is responsible for non-heritable cases (Thériault et al., 2014). The *RB1* gene is considered to be a central regulator of the cell cycle mechanism and is inactivated in a wide range of cancers. Its tumor suppressor function is mainly known due to the inhibition of the E2F1 transcription factor, which in the absence of *RB1* pushes cells from G1 to S phase of the cell cycle. However, *RB1* appears to function in various ways, controlling more than four types of protein interactions and taking on the role of transcriptional co-factor and adaptor protein. For example, it represses *E2F1* gene target transcription through the recruitment of chromatin remodelling complexes including HDAC, DNMT1, HP1A, and SUV39H1 to promoters. With regard to non-E2F1 transcriptional factors such as MYOD, HIF1a, and SP1, *RB1* appears to act as a transcriptional co-factor (Burkhart and Sage, 2008).

Another important gene involved in Rb progression is the enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*), which was the first overexpressed epigenetic enzyme identified in Rb samples. *EZH2* is a histone methyltransferase expressed only in Rb and is essential for tumor development because of its capacity to silence tumor suppressors such as p19/p14ARF and p16/INK4a. Targeting *EZH2* could be the basis for developing an epigenetic therapeutic approach in ocular oncology (Khan et al., 2015). However, new molecules that are involved in Rb are studied to improve the therapy.

Non-coding RNAs (ncRNAs) are transcripts that do not encode proteins. They are diffused in the human genome and abnormally dysregulated in cancer cells. Given that ncRNAs are often located in fragile sites (FRA), common breakpoint sites and in regions with loss of heterozygosity, they represent a new category of genes that participate in tumorigenesis (Calin et al., 2004; Calin et al., 2007). Some ncRNAs have an oncogenic function while others act as tumor suppressors (Sanchez Calle et al., 2018; Vannini et al., 2018a). ncRNAs are classified into two groups on the basis of the length of their sequence: short ncRNAs have a maximum length of 200 nucleotides and long ncRNAs (lncRNAs) are transcripts with sequences of over 200 nucleotides.

microRNAs (miRNAs), the most widely studied class of ncRNAs, are small molecules containing around 22 nucleotides. They regulate the expression of more than 60% of genes. miRNAs are included in the RNA-induced silencing complex (RISC), an miRNA effector machine. This complex binds the 3' untranslated region or, less frequently, the 5' untranslated region of mRNA target, determining the protein downregulation by mRNA degradation or translational repression. miRNAs also upregulate gene expression (Vasudevan et al., 2007). They are located in intergenic regions with independent promoters (Ambros et al., 2003) but can be transcribed by introns with the same promoter as that of the host gene (Lin et al., 2006).

lncRNAs are usually transcripts with 5' terminal methylguanosine cap, frequently polyadenylated, and alternatively spliced (Spizzo et al., 2012; Ulitsky and Bartel, 2013). They have a thermodynamically stable secondary structure with bulges and hairpin loops (Mercer and Mattick, 2013) that enables them to interact with proteins, mRNAs, ncRNAs, and DNA. lncRNAs regulate gene expression at varied levels, from mRNA translation to cytoplasmatic and nuclear epigenetic processes such as miRNA sponging (Vannini et al., 2018b).

The present review summarizes the most important dysregulated ncRNAs in Rb, the interaction with some of their target molecules, and the mechanisms involved in tumor progression.

miRNAs AS DIAGNOSTIC AND PROGNOSTIC BIOMARKERS IN Rb

The formation and progression of numerous cancer types is frequently correlated with an altered miRNA expression profile.

A comparison by Beta et al. between miRNA profiles in primary Rb tissues and miRNAs detected in the serum of

children with Rb revealed eight downregulated miRNAs (miR-216a, miR-217, let-7a, let-7i, let-7f, miR-9, miR-92a, miR-99b) and 25 upregulated (miR-103, miR-142-5b, miR-106b, miR-143, miR-148b, miR-17, miR-16, miR-183, miR-182, miR-19a, miR-18a, miR-29a, miR-29b, miR-29c, miR-20a, miR-30b, miR-30d, miR-34a, miR-494, miR-378, miR-513, miR-513-1, miR-513-2, miR-518c, miR-96) miRNAs. It would thus seem that these 33 RNA molecules are Rb-specific and could potentially influence tumorigenesis and tumor progression in the disease (Beta et al., 2013). Another study identified a group of 24 differentially expressed miRNAs (nine upregulated and 15 downregulated) in healthy retinal tissues and Rb tissues. Among these, 14 miRNAs including miR-20a, miR-373, miR-125b, let-7a, let-7b, let-7c, miR-25, and miR-18a proved capable of distinguishing between Rb samples and healthy tissues, thus identifying potential biomarkers of Rb (Yang and Mei, 2015). Likewise, Liu et al. demonstrated that miR-320, let-7e, and miR-21 were dysregulated in plasma of Rb patients and can thus be hypothesized as novel diagnostic biomarkers for the disease (Liu et al., 2014).

Despite the tumor heterogeneity of Rb, Castro-Magdonel et al. identified a common miRNA expression profile, highlighting miR-3613 as an interesting candidate for therapy. It was highly expressed in all of the examined samples and was also observed to have more than 36 tumor suppressor gene targets (Castro-Magdonel et al., 2017). The microenvironment was recently identified as one of the main factors influencing the background of many types of cancer. Hypoxia is considered as one of the first conditions of stress present in the tumor microenvironment. Interestingly, studies have shown that a hypoxic tumor microenvironment plays a crucial role in controlling treatment outcomes in Rb. It has, in fact, been associated with treatment failure given that it is capable of regulating various pathways including growth factor signalling, glycolysis, genetic instability, metastasis, and angiogenesis. Sudhakar et al. studied the expression of hypoxia-related proteins such as HIF-1A and survivin to understand whether hypoxia is present in Rb, observing that increased expression of these proteins induces resistance to cytotoxic therapy (Sudhakar et al., 2013). Various studies have shown that hypoxic conditions can modulate the expression of a group of miRNAs called hypoxia-regulated microRNAs (HRMs). More precisely, microarray analysis identified miR181b, miR30c-2, miR125a3p, miR497, and miR491-3p as the most important HRMs in Rb cells (Xu et al., 2011). By *in silico* and *in vitro* approaches, Venkatesan et al. identified two key miRNAs (miR486-3p, miR-532) that are downregulated in Rb. Their overexpression using mimic miRNA strategy on Rb cells led to apoptotic cell death (Venkatesan et al., 2015).

miRNA PATHWAYS IN Rb

Although the critical role played by miRNAs in cancer has been demonstrated, further research is needed to clarify the link between cancer-related miRNAs and their target genes and to identify their correlation with multiple pathways associated with tumorigenesis. Through *in silico* and *in vitro* analysis of different

cancer types it has been possible to identify and validate miRNAs that directly regulate RB1 gene (**Table 1**).

Lyu et al. observed low expression levels of miR-485 in Rb tissue and cell lines through reverse transcription-quantitative polymerase chain reaction (RT-qPCR). They also confirmed that miR-485 has a tumor suppressor function targeting Wnt family member 3A (Wnt3A) which activates the canonical Wnt signaling pathway, leading to decreased Rb proliferation, invasion, and migration (Lyu et al., 2019b).

Zhao et al. analyzed miR-361-3p expression levels by qRT-PCR in serum and tissue of Rb patients, in serum samples and normal retinal tissue from healthy controls, and in human Rb cell lines. The authors demonstrated that miR-361-3p was downregulated in Rb serum, Rb tissue and Rb cell lines compared with normal serum and normal retinal tissue. They also observed that miR-361-3p decreased Rb cell proliferation *via* targeting of GLI family zinc finger 1 and 3 (GLI 1/3) (Zhao and Cui, 2018).

Various studies have demonstrated that miR-183 is dysregulated in a great number of cancer types (Li et al., 2010; Lowery et al., 2010; Zhao et al., 2012). Interestingly, Wang et al. observed that miR-183 was downregulated in Rb cell lines and tissues with respect to healthy retinal tissues and that its forced overexpression inhibited the migration, proliferation and invasion capacity of Rb cells. Specifically, the mechanism underlying these results included low-density lipoprotein receptor-related protein (LRP6), a candidate target gene of miR-183 known to counteract the apoptotic effects of this miRNA. The authors showed that miR-183 directly targeted LRP6, downregulating its expression, and thus controlling the progression and development of Rb (Wang et al., 2014).

Another miRNA that has been shown to be involved in various cancer types, including Rb, is miR-204. Wu et al. reported that miR-204 is downregulated in Rb tissues and cell lines. The authors identified *cyclin D2* and matrix metalloproteinase *MMP-9* as its putative gene targets and focused on elucidating the mechanisms underlying this interaction (Wu et al., 2015).

MMP-9 is a protease that plays a strategic role in extracellular matrix (ECM) remodelling under normal conditions but also in

the degradation of ECM by cancer cells during the metastasis process. Cyclin D2 is involved in the phosphorylation of RB1 and numerous studies have demonstrated that its expression levels are high in various cancers. It has emerged that cyclin D2, MMP-9, and miR-204 expression are inversely correlated and that miR-204 targets cyclin D2 and MMP-9, inhibiting tumor growth in Rb (Wu et al., 2015; Golabchi et al., 2017).

miR-29a expression is inversely correlated with cyclin D1 and matrix metalloproteinase (MMP-2) being part of signal transducer and an activator of transcription 3 (STAT3) downstream genes. Liu et al. demonstrated that miR-29a expression is very low in Rb (cells and tissues) and that STAT3 is a direct target of this miRNA (Liu et al., 2018).

Wang et al. discovered that miR-504 was decreased in Rb cell lines and tissue, reporting that miR-504 overexpression suppressed Rb cell proliferation and invasion. This suggests that miR-504 explicates its tumor suppressor function by directly targeting the astrocyte elevated gene 1 (AEG-1) known to be involved in the aggressiveness of Rb (Wang et al., 2019).

Guo et al. demonstrated that miR-98 suppressed Rb invasion, migration, and cell growth through the regulation of insulin-like growth factor1 receptor (IGF1R). IGF1R is a tyrosine kinase receptor that regulates IGF1-induced signaling events and plays an important role in cellular processes including differentiation, proliferation, and migration. The authors reported that miR-98 inhibited the k-Ras/Raf/MEK/ERK signaling pathway *via* targeting of IGF1R in RB. In fact, the restoration of IGF1R reverted the effects of miR-98 on Rb migration, invasion, and cell viability (Guo L, et al., 2019).

Sun et al. demonstrated that miR-492 was upregulated in Rb cell lines and tissue. Downregulating this miRNA, they confirmed its oncogene function that leads to a decrease in Rb proliferation and invasion. Using bioinformatic analysis and luciferase reporter assay, the authors observed that miR-492 targeted large tumor-suppressor kinase 2 (LATS 2), a serine/threonine protein kinase (Sun et al., 2019).

TABLE 1 | List of validated/predicted microRNAs (miRNAs) that target the *RB1* gene.

miRNA	miRBase accession number/HGNC ID	Tumor type	Validated/Predicted miRNA (Reference/Database)
miR-661	MI0003669/ HGNC:32917	Lung cancer	Validated miRNA (Liu F, et al., 2017)
miR-215	MI0000291/HGNC:31592	Gastric cancer	Validated miRNA (Chen et al., 2017)
miR-221-3p	MI0000298/ HGNC:31601	Pancreatic cancer	Validated miRNA (Zhao et al., 2016)
miR-132	MI0000449/HGNC:31516	Pancreatic cancer	Validated miRNA (Park et al., 2011)
miR-212	MI0000288/ HGNC:31589	Pancreatic cancer	Validated miRNA (Park et al., 2011)
miR-675	MI0005416/HGNC:33351	Glioma	Validated miRNA (Zheng et al., 2017)
miR-3129-5p	MI0014146/ HGNC:38217	–	Predicted miRNA (TargetScan Human)
miR-199a-3p	MI0000242/ –	–	Predicted miRNA (TargetScan Human)
miR-199b-3p	MI0000282/ HGNC:31573	–	Predicted miRNA (TargetScan Human)

Another potential therapeutic axis was proposed by Zhang et al. for miR-125a-5p/TAZ and EGFR. The authors demonstrated that miR-125a-5p was markedly downregulated in Rb and that its overexpression inhibited cell proliferation. Transcriptional co-activator with PDZ-binding motif (*TAZ*) is a novel oncogene that promotes tumor progression and is one of the elements of the Hippo tumor suppressor pathway. *TAZ* is overexpressed in Rb and has been shown to stimulate tumor progression *via* the *EGFR* pathway. miR-125a-5p, in targeting *TAZ*, suppresses *EGFR* pathway and consequently Rb progression (Zhang et al., 2016). Whilst miR-125a-5p is downregulated in Rb, miR-125b is reported to be upregulated in both tissue and Rb cells. It has been shown to suppress cell apoptosis and promote cancer cell proliferation *via* interaction with one of its putative target genes, DNA damage regulated autophagy modulator 2 (*DRAM2*).

DRAM2 plays a crucial role in TP53-mediated apoptosis and induces cell autophagy. Studies have confirmed that *DRAM2* is downregulated in various cancer types, indicating that it may be part of the tumor signalling process. *DRAM2* gene is linked to Rb because of its involvement in the renewal and recycling mechanism of photoreceptor cells located in the retina, an essential procedure for visual function preservation.

Bai et al. showed that miR-125b exerts its oncogene biological function by directly targeting and downregulating *DRAM2* gene expression, consequently suppressing cell apoptosis (Bai et al., 2016).

The inactivation of both *TP53* and *RB1* pathways is an essential characteristic of tumorigenesis in the majority of cancer types. However, the classic *TP53* mutation does not occur in Rb. When *RB1* is inactivated, cells generally respond by activating *TP53* which induces cell cycle arrest and apoptosis. *TP53* is regulated by nuclear-localized E3 ubiquitin ligase (*MDM2*), which targets *TP53* for proteasomal degradation. In cells with a loss of *RB1*, *E2F1* is activated and induces cyclin-dependent kinase inhibitor 2A (*p14ARF*), inhibiting *MDM2* and leading to *TP53* stabilization and *TP53* target gene expression (Sherr, 2006). To et al. demonstrated that miR-24 represses *p14ARF* in Rb cells without *RB1* causing a block of activation of *TP53* tumor surveillance (To et al., 2012) (Figure 1). This might explain the lack of mutation of the *TP53* pathway in Rb (Laurie et al., 2006; Xu et al., 2009; Ksander, 2010; Turhan, 2014).

miR-21 is reported to be one of the most dysregulated miRNAs in Rb. Shen et al. focused on this cancer-related miRNA and its potential gene targets in order to shed light on the complicated regulatory network. One of the identified gene targets is programmed cell death 4 (*PDCD4*), a well-known tumor suppressor gene that promotes cell apoptosis and inhibits cell migration and proliferation. Downregulation of *PDCD4* expression has also been assessed in other cancer types including breast and lung cancer, hepatocellular carcinoma, and colorectal and squamous cell carcinoma. Shen et al. observed that miR-21 and *PDCD4* were inversely correlated and that miR-21 directly targeted *PDCD4* (Shen et al., 2014).

A regulator of Wnt signalling is Disheveled-Axin domain containing 1 (*DIXDC1*). Che et al. showed that *DIXDC1* was significantly upregulated in Rb. They also observed that the expression of *DIXDC1* and miR-186 was inversely correlated.

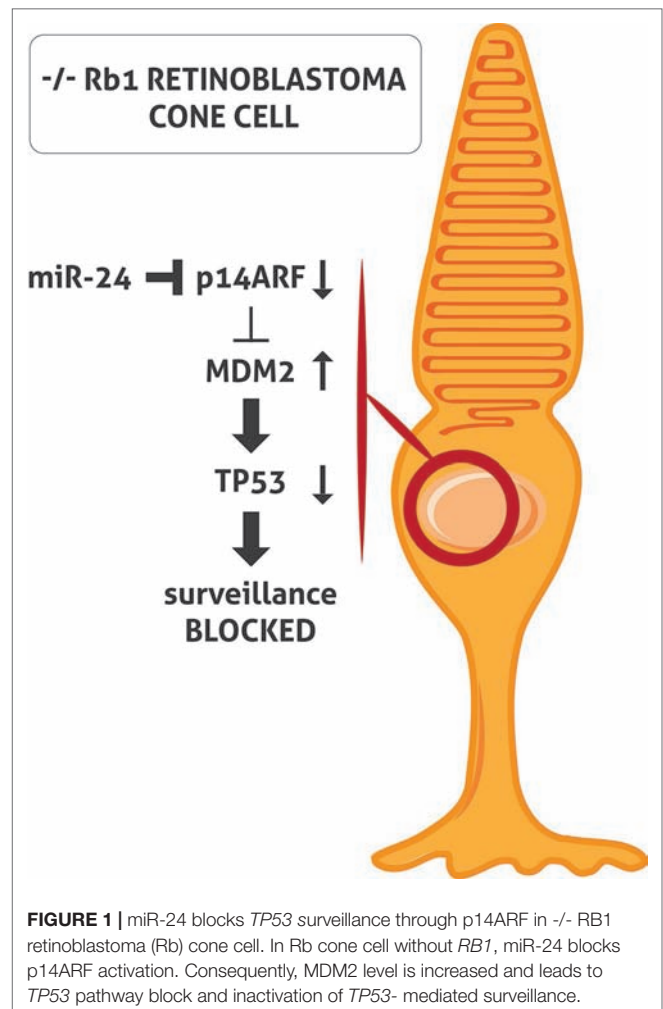


FIGURE 1 | miR-24 blocks *TP53* surveillance through *p14ARF* in *-/- RB1* retinoblastoma (Rb) cone cell. In Rb cone cell without *RB1*, miR-24 blocks *p14ARF* activation. Consequently, *MDM2* level is increased and leads to *TP53* pathway block and inactivation of *TP53*- mediated surveillance.

miR-186 overexpression caused a downregulation of *DIXDC1* and inhibited the proliferation and invasion in Rb cells (Che et al., 2018).

Wang et al. demonstrated that miR-138-5p has a tumor suppressor function, downregulating miR-138-5p and upregulating pyruvate dehydrogenase kinase 1 (*PDK1*) in Rb cells. *PDK1* caused the phosphorylation of the pyruvate dehydrogenase enzyme. miR-138-5p overexpression led to a downregulation of *PDK1* and consequently a decrease in migration, cell viability, invasion and induced apoptosis (Wang et al., 2017).

Montoya et al. elucidated the regulation of Rb proliferation by miR-31 and miR-200a, both of which were downregulated in Rb cell lines. Their induced overexpression by mimic transfection restricted the proliferative capacity of the Y-79 Rb cell line (Montoya et al., 2015).

miR-106b and its target gene Runt-related transcription factor 3 (*Runx3*) have also been identified as capable of modulating cancer progression. *Runx3* is a tumor suppressor in Rb with an important role in mammalian development. Its loss is linked to a variety of cancers such as gastric cancer, glioblastoma, and bladder tumors. Yang et al. demonstrated that miR-106b binds to the *Runx3* and downregulate its expression, which consequently

leads to the development of the malignant phenotype. Inhibition of miR-106b and thus upregulation of Runx3 could represent be a possible therapy for Rb. (Yang et al., 2017).

Moreover, given the important role of the miR-17-92 cluster in the development of Rb, it is opportune to mention the advantage of the aptamer method, elucidated by Subramanian et al. An RNA aptamer directed against the primary miR-17-92 transcript was selected by the systemic evolution of ligands by exponential enrichment (SELEX) to inhibit the biogenesis of the miRNA cluster. The authors confirmed the inhibition of cluster biogenesis and a decrease in Rb cell proliferation (Subramanian et al., 2015).

However, the described investigations focused mainly on a single element (miRNA or gene). Li et al. studied genes, miRNAs, and transcription factors as elements of the regulatory networks, analyzing their relationship in Rb. The authors focused on the interactions between genes, which regulate miRNAs, and host genes including miRNAs and miRNAs targeting genes. The authors thus constructed three regulatory networks based on the relationships called the related network, differentially expressed network and global network. Data on Rb-related genes for the related network were obtained from GeneCards database and

pertinent literature, whereas data on differentially expressed genes in Rb for the differentially expressed network were obtained from the Cancer Genetics Web. Expressed miRNAs were extracted from literatures and mir2Disease, which is a database about differentially expressed miRNAs in various human diseases. Rb-related miRNAs were collected manually from permanent literatures.

The global network was constructed from all of the interactions that have been experimentally validated, making it too complex to be used or obtaining information. In this way, the authors were able to identify the pathways of the related elements and differentially expressed elements inserted in the other two networks (Li et al., 2014).

All of the above miRNAs are summarized in **Table 2**.

lncRNAs PATHWAYS IN Rb

In the same way as miRNAs, lncRNAs are responsible for various cellular functions and play an important role in many cancer types as biomarkers, tumor regulators, and predictors of prognosis. Evidence has emerged of interesting pathways

TABLE 2 | microRNAs (miRNAs) involved in retinoblastoma (Rb) and some of their putative target genes.

miRNA	miRBase accession number/ HGNC ID	Expression in Rb	Target (reference)
miR-138-5p	MIMAT0000430	Downregulated in RB cell lines	<i>PDK1</i> (Wang et al., 2017)
miR-485	MI0002469	Downregulated in RB tissues and cell lines	<i>Wnt3A</i> (Lyu et al., 2019b)
miR-186	HGNC:32067		
	MI0000483	Downregulated in RB cell lines	<i>DIXDC1</i> (Che et al., 2018)
	HGNC:31557		
miR-504	MI0003189	Downregulated in RB tissues and cell lines	<i>AEG-1</i> (Wang et al., 2019)
	HGNC:32139		
miR-361-3p	MIMAT0004682	Downregulated in RB tissues serum samples and cell lines	<i>GLI 1/3</i> (Zhao and Cui, 2018)
miR-31	MI0000089	Downregulated in RB cell lines	(Montoya et al., 2015)
	HGNC:31630		
miR-200a	MI0000737	Downregulated in RB cell lines	(Montoya et al., 2015)
	HGNC:31578		
miR-183	MI0000273	Downregulated in RB tissues and cell lines	<i>LRP6</i> (Wang et al., 2014)
	HGNC:31554		
miR-98	MI0000100	Downregulated in RB tissues and cell lines	<i>IGF1R</i> (Guo L, et al., 2019)
	HGNC:31649		
miR-125a-5p	MIMAT0000443	Downregulated in RB tissues and cell lines	<i>TAZ</i> (Zhang et al., 2016)
miR-204	MI0000284	Downregulated in RB tissues and cell lines	<i>CyclinD2, MMP-9</i> (Wu et al., 2015; Golabchi et al., 2017)
	HGNC:31582		
miR-29a	MI0000087	Downregulated in RB tissues and cell lines	<i>STAT3</i> (Liu et al., 2018)
	HGNC:31616		
miR-125b-5p	MIMAT0000423	Upregulated in RB tissues and cell lines	<i>DRAM2</i> (Bai et al., 2016)
miR-492	MI0003131	Upregulated in RB tissues and cell lines	<i>LATS2</i> (Sun et al., 2019)
	HGNC:32081		
miR-24-3p	MIMAT0000080	Upregulated in RB tissues and cell lines	<i>p14ARF</i> (To et al., 2012)
miR-21	MI0000077	Upregulated in RB cell lines	<i>PDCD4</i> (Shen et al., 2014)
	HGNC:31586		
miR-106b	MI0000734	Upregulated in RB cell lines	<i>Runx3</i> (Yang et al., 2017)
	HGNC:31495		

involving lncRNAs and their putative gene targets in different tumors. Musahl et al. demonstrated that the depletion of the ncRNA-RB1, an lncRNA expressed by the RB1 promoter, reduced the expression of calreticulin (CARL). CARL is an endoplasmatic reticulum protein that, in pre-apoptosis, translocates to the cell surface and serves as a signal to phagocytic cells. As a result of ncRNA-RB1 depletion, tumor cell uptake by macrophages is inhibited (Musahl et al., 2015). E2F1-regulated inhibitor of cell death (ERIC) is also capable of controlling DNA damage response and subsequent apoptotic cell death, interacting with proliferation regulators such as E2F1. Specifically, Feldstein et al. showed that ERIC is regulated at transcriptional level by E2F1 and responds to DNA damage in osteosarcoma and lung cancer cell lines. The authors observed an upregulation of ERIC after DNA damage that regulated apoptosis. Consequently, the inhibition of ERIC expression led to increased apoptosis (Feldstein et al., 2013).

lncRNAs are involved in Rb progression. Li et al. observed that lncRNA 00152 (LINC00152) was upregulated in Rb cell lines and tissues. The authors also reported that Rb cells silenced for LINC00152 showed an inhibition of cell proliferation, migration, invasion, and colony formation, and increased apoptosis. Moreover, LINC00152 knockdown caused the activation of caspase-3 and caspase-8 *in vitro* and suppressed tumorigenesis in nude mouse models (Li et al., 2018).

Dong et al. reported that HOTAIR lncRNA is involved in Rb progression through the Notch signalling pathway. HOTAIR is an oncogenic lncRNA correlated with metastasis, invasion, tumorigenesis and drug resistance. It is significantly upregulated in human Rb tissues and the authors showed that lncRNA knockout impeded the proliferation of Y-79 cell line. Notch also plays an important role in tumor development processes. In particular, Dong et al. assessed the expression levels of Notch1 and Jagged 1, the most common ligand and receptor, respectively, in the Notch pathway. They found that Jagged 1 and Notch 1 expression decreased after HOTAIR knockdown, indicating that HOTAIR regulates tumor progression in Rb through the activation of the *Notch 1* pathway (Dong et al., 2016). Another mechanism of action of HOTAIR is that of a miRNA sponge. Yang et al. demonstrated that HOTAIR sponged miR-613, which subsequently did not trigger tyrosine protein kinase met (c-met), its direct target gene. *C-met* is a protooncogene and its upregulation through HOTAIR causes the progression of Rb (Yang et al., 2018).

The testis-associated highly conserved oncogenic lncRNA (THOR) has been identified as another Rb promoter. THOR is widely expressed in various cancer types including Rb, but is also restrictively expressed in healthy testis tissues. Shang et al. showed that THOR promoted the malignant phenotype of Rb by interacting with insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP1) and by controlling the mRNA stability of the c-myc oncogene. C-myc must be associated with IGF2BP1 to prevent its degradation. Thus in Rb cells, upregulated THOR promotes the association of c-myc and IGF2BP1, leading to its stabilization and consequently enhancing the malignant phenotype (Shang, 2018).

Wang et al. discovered that the lncRNA differentiation antagonizing non-protein coding RNA (DANCR) is upregulated in Rb cell lines and tissues and also overexpressed in Rb patients, leading to poor overall and disease-free survival. The authors observed that DANCR sponged miR-34c and miR-613, two miRNAs with tumor suppression function that target matrix metalloproteinase 9 (MMP9), an important protein for the breakdown of ECM. When DANCR regulation was activated, MMP9 was upregulated, leading to tumor progression (Wang et al., 2018).

A further connection between lncRNA and miRNA in Rb was demonstrated by Zhang et al. in their investigation of the association between lncRNA CCAT1 and miR-218-5p, known to occur in other cancer types. In fact, Lu et al. had previously shown that the negative regulation of miR-218-5p by CCAT1 promoted tumor progression in lung cancer (Lu et al., 2016). In the same way, Zhang et al. proved that CCAT1 was upregulated in Rb. Confirming the same mechanism of negative interaction with miR-218-5p, the authors showed that there was a reduction in apoptosis and an increase in cell proliferation and migration capacity (Zhang et al., 2017).

Actin filament-associated protein 1-antisense RNA 1 (AFAP1-AS1) is yet another lncRNA that has been hypothesized as having an oncogenic function in Rb. This lncRNA is associated with cancer progression and has been found to be overexpressed in various cell lines and tumor types such as lung cancer, ovarian cancer, oesophageal cancer, gastric cancer, hepatocellular carcinoma, nasopharyngeal carcinoma, colorectal cancer, biliary tract cancer, and pancreatic ductal adenocarcinoma (Zhang et al., 2018b). The role of this lncRNA in Rb was unknown up until recently. Hao et al. compared normal retina cell lines with Rb cells in knockdown experiments, observing that AFAP1-AS1 downregulation inhibited cell cycle progression, invasion, and migration. The authors also confirmed the oncogenic function of AFAP1-AS1 through its upregulation in Rb, reporting that it caused larger tumor size and optic nerve and choroidal invasion (Hao et al., 2018).

BDNF antisense RNA (BDNF-AS) is an lncRNAs transcribed by RNA polymerase II and has proved to be reverse regulator of BDNF. BDNF is a member of the neurotrophin family of growth factors whose role is to facilitate neuron survival and support the differentiation and growth of new synapses and neurons. Shang et al. evaluated the expression of BDNF-AS in Rb cell lines and found that it was downregulated in both Rb cell lines and tissues. Conversely, they also demonstrated that forced expression of BDNF-AS diminished cancer proliferation and metastatic potential, arresting cells in Go/G1 phase and consequently downregulating the cell cycle-associated proteins cyclin E and CDC42 (Shang et al., 2017).

Another lncRNA associated with tumorigenesis is promoter of CDKN1A antisense DNA damage activated RNA (PANDAR), and there is evidence of its upregulation in several types of cancers (Peng and Fan, 2015; Jiang et al., 2017). Sheng et al. investigated the potential clinical role of PANDAR in Rb, observing that it was overexpressed in Rb cells and tissues and that it inhibited cell apoptosis by affecting the *Bcl-2/caspase-3*

pathway. Moreover, using online databases, the authors predicted the specificity protein 1 (SP1) as a potential transcriptional factor capable of binding directly to the PANDAR promoter region and of triggering its transcription. They subsequently confirmed *in vitro* this binding ability of SP1 to the PANDAR promoter region.

The new connection discovered between PANDAR and SP1 could represent an alternative therapeutic target for Rb (Sheng et al., 2018).

In addition to the other lncRNAs, BRAF-activated non-coding RNA (BANCR) also plays an important role in the progression of various cancers, including Rb. Su et al. demonstrated that BANCR was overexpressed in Rb cell lines and tissues and that it was associated with tumor development. In fact, knocked down BANCR limited tumor invasion, metastatic capacity, and proliferation, indicating its potential usefulness as a therapeutic target for Rb (Su et al., 2015).

lncRNA H19 was among the first lncRNAs to be discovered and has different functions in different cancers. Zhang et al. hypothesized that H19 may also be involved in Rb. Initially, the authors assessed H19 expression in Rb, observing that it was substantially downregulated in Rb tissues and cell lines. They also showed that H19 has seven binding sites where it directly binds the miR-17-92 cluster in a competitive way. It has been already seen that the miR-17-92 cluster suppresses p21, an important regulator of the cell cycle, leading to STAT3 activation. Zhang et al. showed that H19 inhibited the suppressor role of the miR-17-92 cluster in p21 gene, decreasing STAT3 activation (Zhang et al., 2018a).

Cheng et al. observed that lncRNAs X inactive specific transcript (XIST) had an oncogene function in Rb. The authors discovered that XIST binds miR-101, regulating the repression of its targets, E-box binding homeobox 1 and 2 (ZEB1, ZEB2). ZEB1 and ZEB2 are transcription factors that are responsible for the malignant features of different type of cancers. The binding of miR-101 to XIST and the subsequent oncogenic activity of ZEB1

and ZEB2 led to increased proliferation, invasion and migration of Rb cells (Cheng et al., 2019).

A similar mechanism was uncovered by Liu et al. who found that the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was upregulated in Rb. The authors observed that MALAT1 silencing in cells caused the inhibition of cell viability, invasion and migration, activated apoptosis and upregulated miR-124.

They also demonstrated that MALAT1 sponged miR-124 which, consequently, did not target E-cadherin transcription repressor (Slug), its target gene. Slug activates ERK/MAPK and Wnt/ β -catenin pathways and, when downregulated by miR-124, inhibits Rb progression (Liu S, et al., 2017).

The above-mentioned lncRNAs are summarized in **Table 3**.

CIRCULAR RNAs PATHWAYS IN Rb

Circular RNAs (circ-RNAs) are an emerging group of ncRNAs present inside cells. Considering their form, they are less susceptible to RNAs degradation and are the result of a back-splicing of 5'splice position with the 3'splice position or exon skipping mechanism. Recent evidence suggests that various disorders such as nervous diseases, vascular inflammation and several types of cancer may be the result of their deregulation (Guo N, et al., 2019).

hsa_circ_0001649 is a new cancer associated circ-RNA and is reported to be the transcription product of Snf2 Histone Linker Phd Ring Helicase (SHPRH) tumor suppressor gene. Xing et al. showed *via* q-RT PCR analysis that hsa_circ_0001649 was downregulated in Rb tissue samples compared with normal tissue and that low expression of this circ-RNA was associated with aggressive phenotypes in Rb patients. To further confirm its involvement in tumorigenesis, the authors assessed hsa_circ_0001649 enhanced expression in xenografts, reporting that tumors formed from hsa_circ_0001649-transfected cells

TABLE 3 | Long ncRNAs (lncRNAs) involved in retinoblastoma (Rb) and some of their putative target genes.

lncRNA	HGNC ID	Expression in Rb	Target (reference)
H19	HGNC:4713	Downregulated in RB tissues and cell lines	<i>miR-17-92 cluster</i> (Zhang et al., 2018a)
BDNF-AS	HGNC:20608	Downregulated in RB tissues and cell lines	(Shang et al., 2017)
hsa_circ_0001649	—	Downregulated in RB tissues and cell lines	<i>AKT/mTOR pathway</i> (Xing et al., 2018)
TET1hsa_circ_0093996	—	Downregulated in RB tissues	<i>miR-183</i> (Lyu et al., 2019a)
HOTAIR	HGNC:33510	Upregulated in RB tissues and cell lines	<i>Notch</i> (Dong et al., 2016)
			<i>miR-613</i> (Yang et al., 2018)
MALAT1	HGNC:29665	Upregulated in RB cell lines	<i>miR-124</i> (Liu S, et al., 2017)
DANCR	HGNC:28964	Upregulated in RB tissues and cell lines	<i>miR-34c, miR-613</i> (Wang et al., 2018)
AFAP1-AS1	HGNC:28141	Upregulated in RB tissues and cell lines	(Hao et al, 2018)
BANCR	HGNC:43877	Upregulated in RB tissues and cell lines	(Su et al., 2015)
PANDAR	HGNC:44048	Upregulated in RB tissues and cell lines	<i>Bcl2/caspase 3 pathway</i> (Sheng et al., 2018)
LINC00152	HGNC:28717	Upregulated in RB tissues and cell lines	<i>caspase 3 and caspase 8</i> (Li et al., 2018)
THOR	HGNC:53788	Upregulated in RB tissues and cell lines	<i>c-myc</i> (Shang, 2018)
CCAT1	HGNC:45128	Upregulated in RB tissues and cell lines	<i>miR-218-5p</i> (Zhang et al., 2017)
XIST	HGNC:12810	Upregulated in RB tissues and cell lines	<i>miR-101</i> (Cheng et al., 2019)

were smaller than those of the control group. In the same study, the authors investigated the molecular mechanisms of hsa_circ_0001649 which are responsible for changes in Rb tumor cell proliferation. The Akt/mTOR apoptosis-related signalling pathway is known to be related to Rb progression, but there is still no evidence of the involvement of circ-RNA. Xing et al. suggested that the Akt/mTOR signalling pathway is regulated by hsa_circ_0001649. More precisely, after transfection of Rb cell lines with hsa_circ_0001649, the authors observed that p-AKT and p-mTOR were negatively correlated with hsa_circ_0001649 (Xing et al., 2018).

In another study, Lyu et al. analyzed the expression profile of circ-RNA in human Rb tissue and in corresponding normal retina, observing a general reduction in circ-RNA expression levels in Rb. This may have been a result of compromised back-splice machinery in the circ-RNA production or by an excessive consumption of circ-RNA, necessary for cell proliferation. In particular, TET1-hsa_circ_0093996 was significantly downregulated in Rb tissue. The authors also observed a downregulation of tumor suppressor PDCD4. Based on the *in silico* analysis, which revealed that miR-183 targeted PDCD4, the authors hypothesized that TET1-hsa_circ_0093996 sponged miR-183. In an attempt to create a regulatory axis, they assumed that TET1-hsa_circ_0093996 downregulation increased unbound miR-183, which consequently targeted PDCD4 causing enhanced cell proliferation (Lyu et al., 2019a).

CONCLUSIONS

ncRNAs are molecules physiologically present in humans where they regulate gene expression after transcription and subsequently control important mechanisms such as cell proliferation, development, and apoptosis. ncRNAs are deregulated in many types of cancer, suggesting that they are involved in carcinogenesis. In the present review, we explored short and long ncRNAs, which are deregulated in Rb, demonstrating that, in addition to *RB1*, there is a great number of other molecules involved in the development of the malignant phenotype of this type of cancer.

The evolution of genomic and genetic technologies together with the generation and development of bioinformatics have made it possible to manage the enormous quantity of accumulated data generated by large-scale high throughput analyses and basic research.

Profiling analysis is the first step to discover new mechanisms of ncRNAs involved in Rb development.

miRNA profiles in Rb have been discussed at length in this review (Beta et al., 2013; Yang and Mei, 2015). The studies in question identified miRNAs that are upregulated and downregulated in Rb with an oncogene or tumor suppressor function, respectively.

The function of miRNAs is explicated through the regulation of target genes involved in cell proliferation, migration, invasion, cell viability, and apoptosis, as demonstrated by

Wang et al. (2017). Through a comparison of miRNAs and gene profiling it is possible to identify the pathways that determine Rb progression. These pathways are carefully validated through *in vitro* and *in vivo* experiments. miRNAs directly or indirectly regulate important gene such as cyclins (Wu et al., 2015; Golabchi et al., 2017) and TP53 (To et al., 2012; Bai et al., 2016), respectively. In turn, lncRNAs regulate miRNAs through sponge mechanism (Liu S, et al., 2017; Wang et al., 2018; Yang et al., 2018; Zhang et al., 2018a). Studies show the complex ncRNA mechanisms and the prevailing pathways that determine Rb progression.

Further research into the complex Rb pathways will help to identify novel ncRNA-based therapeutic approaches to counteract the aberrations of the ncRNA that are responsible for the development of Rb. Several studies described in this review analyzed the therapeutic potential of ncRNAs, but although they highlighted the therapeutic prospects of these molecules, their clinical implementation remains a challenge. A non-toxic delivery system is needed to selectively transport ncRNA-based therapeutics to the tumor site, e.g., antisense oligonucleotide or inhibitor against an oncogene (Adams et al., 2017). Moreover, the fact that a single miRNA binds more than 100 target genes makes target specificity a problem. The recent discovery that ncRNAs contained in exosomes interact with the tumor microenvironment and affect cancer growth and metastatic potential has opened up a new chapter on the intercellular crosstalk for cancer biology (Vannini et al., 2018a). Thus, new strategies to impair the exosome-mediated ncRNA transfer affecting cancer growth and dissemination can be hypothesized. The characterization of regulatory mechanisms of lncRNAs is also a critical aspect to complement the deficiency of precision medicine.

A better understanding of the role played by ncRNAs in chemoresistance would enable patients to be spared from non-beneficial treatments but would also help to overcome the problem by modulating the expression of the ncRNAs involved in the resistance mechanisms.

Future studies will serve to clarify the role of ncRNAs as tumor suppressors or oncogenes and to design new ncRNA-based therapeutic approaches. The era of an ncRNA-based therapy for Rb is fast approaching and will provide oncologists with a powerful tool for improving patients' odds against this often deadly tumor.

AUTHOR CONTRIBUTIONS

MP found the articles that describe ncRNAs in Retinoblastoma and she wrote the manuscript. IV wrote and corrected the manuscript.

ACKNOWLEDGMENTS

We would like to thank Francesco Mazza for helping with figure design.

REFERENCES

- Adams, B. D., Parsons, C., Walker, L., Zhang, W. C., and Slack, F. J. (2017). Targeting noncoding RNAs in disease. *J. Clin. Invest.* 127, 761–771. doi: 10.1172/JCI84424
- Ambros, V., Lee, R. C., Lavanway, A., Williams, P. T., and Jewell, D. (2003). MicroRNAs and other tiny endogenous RNAs in *C. elegans*. *Curr. Biol.* 13, 807–818. doi: 10.1016/S0960-9822(03)00287-2
- Bai, S., Tian, B., Li, A., Yao, Q., Zhang, G., and Li, F. (2016). MicroRNA-125b promotes tumor growth and suppresses apoptosis by targeting DRAM2 in retinoblastoma. *Eye* 30, 1630–1638. doi: 10.1038/eye.2016.189
- Beta, M., Venkatesan, N., Vasudevan, M., Vetrivel, U., Khetan, V., and Krishnakumar, S. (2013). Identification and insilico analysis of retinoblastoma serum microRNA profile and gene targets towards prediction of novel serum biomarkers. *Bioinform. Biol. Insights* 7, 21–34. doi: 10.4137/BBI.S10501
- Burkhardt, D. L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer* 8 (9), 671–682. doi: 10.1038/nrc2399
- Calin, G. A., Liu, C., Ferracin, M., Hyslop, T., Spizzo, R., Sevignani, C., et al. (2007). Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell* 12, 215–229. doi: 10.1016/j.ccr.2007.07.027
- Calin, G. A., Sevignani, C., Dumitru, C. D., Hyslop, T., Noch, E., Yendamuri, S., et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci.* 101, 2999–3004. doi: 10.1073/pnas.0307323101
- Castro-Magdonel, B. E., Orjuela, M., Camacho, J., García-Chéquer, A. J., Cabrera-Muñoz, L., Sadowinski-Pine, S., et al. (2017). miRNome landscape analysis reveals a 30 miRNA core in retinoblastoma. *BMC Cancer* 17, 458. doi: 10.1186/s12885-017-3421-3
- Che, X., Qian, Y., and Li, D. (2018). Suppression of Dishevelled–Axin Domain Containing 1 (DIXDC1) by microRNA-186 inhibits the proliferation and invasion of retinoblastoma cells. *J. Mol. Neurosci.* 64, 252–261. doi: 10.1007/s12031-017-1017-7
- Chen, Z., Liu, K., Li, L., Chen, Y., and Du, S. (2017). miR-215 promotes cell migration and invasion of gastric cancer by targeting Retinoblastoma tumor suppressor gene 1. *Pathol. Res. Pract.* 213, 889–894. doi: 10.1016/j.prp.2017.06.006
- Cheng, Y., Chang, Q., Zheng, B., Xu, J., Li, H., and Wang, R. (2019). LncRNA XIST promotes the epithelial to mesenchymal transition of retinoblastoma via sponging miR-101. *Eur. J. Pharmacol.* 843, 210–216. doi: 10.1016/j.ejphar.2018.11.028
- Dong, C., Liu, S., Lv, Y., Zhang, C., Gao, H., Tan, L., et al. (2016). Long non-coding RNA HOTAIR regulates proliferation and invasion via activating Notch signalling pathway in retinoblastoma. *J. Biosci.* 41, 677–687. doi: 10.1007/s12038-016-9636-7
- Feldstein, O., Nizri, T., Doniger, T., Jacob, J., Rechavi, G., and Ginsberg, D. (2013). The long non-coding RNA ERIC is regulated by E2F and modulates the cellular response to DNA damage. *Mol. Cancer* 12, 131. doi: 10.1186/1476-4598-12-131
- Golabchi, K., Soleimani-Jelodar, R., Aghadoost, N., Momeni, F., Moridikia, A., Nahand, J. S., et al. (2017). MicroRNAs in Retinoblastoma: Potential diagnostic and therapeutic biomarkers. *J. Cell. Physiol.* 233, 3016–3023. doi: 10.1002/jcp.26070
- Guo, N., Xf, Liu, OP, Pant, DD, Zhou, JI, Hao, and Lu, Cw (2019). Circular RNAs: Novel Promising Biomarkers in Ocular Diseases. *Int. J. Med. Sci.* 16, 513–518. doi: 10.7150/ijms.29750
- Guo, L., Bai, Y., Ji, S., and Ma, H. (2019). MicroRNA-98 suppresses cell growth and invasion of retinoblastoma via targeting the IGF1R/k-Ras/Raf/MEK/ERK signaling pathway. *Int. J. Oncol.* 54, 807–820. doi: 10.3892/ijo.2019.4689
- Hao, F., Mou, Y., Zhang, L., Wang, S., and Yang, Y. (2018). LncRNA AFAP1-AS1 is a prognostic biomarker and serves as oncogenic role in retinoblastoma. *Biosci. Rep.* 38. doi: 10.1042/BSR20180384
- Jabbour, P., Chalouhi, N., Tjounakaris, S., Gonzalez, L. F., Dumont, A. S., Chitale, R., et al. (2012). Pearls and pitfalls of intraarterial chemotherapy for retinoblastoma. *J. Neurosurg. Pediatr.* 10, 175–81. doi: 10.3171/2012.5.PEDS1277
- Jiang, Y., Feng, E., Sun, L., Jin, W., You, Y., Yao, Y., et al. (2017). An increased expression of long non-coding RNA PANDAR promotes cell proliferation and inhibits cell apoptosis in pancreatic ductal adenocarcinoma. *Biomed. Pharmacother.* 95, 685–691. doi: 10.1016/j.biopha.2017.08.124
- Khan, M., Walters, L. L., Li, Q., Thomas, D. G., Miller, J. M. L., Zhang, Q., et al. (2015). Characterization and pharmacologic targeting of EZH2, a fetal retinal protein and epigenetic regulator, in human retinoblastoma. *Lab. Invest.* 95, 1278–1290. doi: 10.1038/labinvest.2015.104
- Kivelä, T. (2009). The epidemiological challenge of the most frequent eye cancer: Retinoblastoma, an issue of birth and death. *Br. J. Ophthalmol.* 93 (9), 1129–1131. doi: 10.1136/bjo.2008.150292
- Ksander, B. R. (2010). Development of an immunotherapy for retinoblastoma. *Retin. Today*.
- Laurie, N. A., Donovan, S. L., Shih, C. S., Zhang, J., Mills, N., Fuller, C., et al. (2006). Inactivation of the p53 pathway in retinoblastoma. *Nature* 444, 61–66. doi: 10.1038/nature05194
- Li, J., Fu, H., Xu, C., Tie, Y., Xing, R., Zhu, J., et al. (2010). miR-183 inhibits TGF-beta1-induced apoptosis by downregulation of PDCD4 expression in human hepatocellular carcinoma cells. *BMC Cancer* 10. doi: 10.1186/1471-2407-10-354
- Li, J., Xu, Z.-W., Wang, K.-H., Wang, N., Li, D.-Q., and Wang, S. (2014). Networks of MicroRNAs and Genes in Retinoblastomas. *Asian Pacific J. Cancer Prev.* 14, 6631–6636. doi: 10.7314/apjcp.2013.14.11.6631
- Li, S., Wen, D., Che, S., Cui, Z., Sun, Y., Ren, H., et al. (2018). Knockdown of long noncoding RNA 00152 (LINC 00152) inhibits human retinoblastoma progression. *Oncol. Targets Ther.* 11, 3215–3223. doi: 10.2147/OTT.S160428
- Lin, S. L., Miller, J. D., and Ying, S. Y. (2006). Intronic microRNA (miRNA). *J. Biomed. Biotechnol.* 2006 (4), 26818. doi: 10.1155/JBB/2006/26818
- Liu, S.-S., Wang, Y.-S., Sun, Y.-F., Miao, L.-X., Wang, J., Li, Y.-S., et al. (2014). Plasma microRNA-320, microRNA-let-7e and microRNA-21 as novel potential biomarkers for the detection of retinoblastoma. *Biomed. Rep.* 2, 424–428. doi: 10.3892/br.2014.246
- Liu, S., Yan, G., Zhang, J., and Yu, L. (2017). Knockdown of Long Noncoding RNA (lncRNA) Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1) Inhibits Proliferation, Migration, and Invasion and Promoted Apoptosis By Targeting miR-124 in Retinoblastoma. *Oncol. Res.* 26, 581–591. doi: 10.3727/096504017X14953948675403
- Liu, F., Cai, Y., Rong, X., Chen, J., Zheng, D., Chen, L., et al. (2017). MiR-661 promotes tumor invasion and metastasis by directly inhibiting RB1 in non small cell lung cancer. *Mol. Cancer* 16, 122. doi: 10.1186/s12943-017-0698-4
- Liu, S., Zhang, X., Hu, C., Wang, Y., and Xu, C. (2018). MiR-29a inhibits human retinoblastoma progression by targeting STAT3. *Oncol. Rep.* 39, 739–746. doi: 10.3892/or.2017.6144
- Lowery, A. J., Miller, N., Dwyer, R. M., and Kerin, M. J. (2010). Dysregulated miR-183 inhibits migration in breast cancer cells. *BMC Cancer* 10. doi: 10.1186/1471-2407-10-502
- Lyu, J., Wang, Y., Zheng, Q., Hua, P., Zhu, X., Li, J., et al. (2019a). Reduction of circular RNA expression associated with human retinoblastoma. *Exp. Eye Res.* 184, 278–285. doi: 10.1016/j.exer.2019.03.017
- Lyu, X., Wang, L., Lu, J., Zhang, H., and Wang, L. (2019b). microRNA-485 inhibits the malignant behaviors of retinoblastoma by directly targeting Wnt3a. *Oncol. Rep.* 41, 3137–3147. doi: 10.3892/or.2019.7061
- Lu, L., Xu, H., Luo, F., Liu, X., Lu, X., Yang, Q., et al. (2016). Epigenetic silencing of miR-218 by the lncRNA CCAT1, acting via BMI1, promotes an altered cell cycle transition in the malignant transformation of HBE cells induced by cigarette smoke extract. *Toxicol. Appl. Pharmacol.* 304, 30–41. doi: 10.1016/j.taap.2016.05.012
- Ma, J., Han, H., Ma, L., Liu, C., Xue, X., Ma, P., et al. (2014). The immunostimulatory effects of retinoblastoma cell supernatant on dendritic cells. *Protein Cell* 5, 307–316. doi: 10.1007/s13238-014-0029-0
- Mercer, T. R., and Mattick, J. S. (2013). Understanding the regulatory and transcriptional complexity of the genome through structure. *Genome Res.* 23 (7), 1081–1088. doi: 10.1101/gr.156612.113
- Montoya, V., Fan, H., Bryar, P. J., Weinstein, J. L., Mets, M. B., Feng, G., et al. (2015). Novel miRNA-31 and miRNA-200a-mediated regulation of retinoblastoma proliferation. *PLoS One* 10, e0138366. doi: 10.1371/journal.pone.0138366
- Musahl, A. S., Huang, X., Rusakiewicz, S., Ntini, E., Marsico, A., Kroemer, G., et al. (2015). A long non-coding RNA links calreticulin-mediated immunogenic cell removal to RB1 transcription. *Oncogene* 34, 5046–5054. doi: 10.1038/onc.2014.424
- Park, J. K., Henry, J. C., Jiang, J., Esau, C., Gusev, Y., Lerner, M. R., et al. (2011). miR-132 and miR-212 are increased in pancreatic cancer and target the

- retinoblastoma tumor suppressor. *Biochem. Biophys. Res. Commun.* 406, 518–523. doi: 10.1016/j.bbrc.2011.02.065
- Peng, W., and Fan, H. (2015). Long non-coding RNA PANDAR correlates with poor prognosis and promotes tumorigenesis in hepatocellular carcinoma. *Biomed. Pharmacother.* 72, 113–118. doi: 10.1016/j.biopha.2015.04.014
- Sanchez Calle, A., Kawamura, Y., Yamamoto, Y., Takeshita, F., and Ochiya, T. (2018). Emerging roles of long non-coding RNA in cancer. *Cancer Sci.* 109 (7), 2093–2100. doi: 10.1111/cas.13642
- Shang, W., Yang, Y., Zhang, J., and Wu, Q. (2017). Long noncoding RNA BDNF-AS is a potential biomarker and regulates cancer development in human retinoblastoma. *Biochem. Biophys. Res. Commun.* doi: 10.1016/j.bbrc.2017.01.134
- Shang, Y. (2018). LncRNA THOR acts as a retinoblastoma promoter through enhancing the combination of c-myc mRNA and IGF2BP1 protein. *Biomed. Pharmacother.* 106, 1243–1249. doi: 10.1016/j.biopha.2018.07.052
- Shen, F., Mo, M. H., Chen, L., An, S., Tan, X., Fu, Y., et al. (2014). MicroRNA-21 down-regulates RB1 expression by targeting PDCC4 in retinoblastoma. *J. Cancer* 5, 804–812. doi: 10.7150/jca.10456
- Sheng, L., Wu, J., Gong, X., Dong, D., and Sun, X. (2018). SP1-induced upregulation of lncRNA PANDAR predicts adverse phenotypes in retinoblastoma and regulates cell growth and apoptosis in vitro and in vivo. *Gene* 668, 140–145. doi: 10.1016/j.gene.2018.05.065
- Sherr, C. J. (2006). Divorcing ARF and p53: An unsettled case. *Nat. Rev. Cancer* 6 (9), 663–673. doi: 10.1038/nrc1954
- Spizzo, R., Almeida, M. I., Colombatti, A., and Calin, G. A. (2012). Long non-coding RNAs and cancer: A new frontier of translational research. *Oncogene* 31 (43), 4577–4587. doi: 10.1038/onc.2011.621
- Su, S., Gao, J., Wang, T., Wang, J., Li, H., and Wang, Z. (2015). Long non-coding RNA BANCER regulates growth and metastasis and is associated with poor prognosis in retinoblastoma. *Tumour Biol.* 36, 7205–7211. doi: 10.1007/s13277-015-3413-3
- Subramanian, N., Kanwar, J. R., Kanwar, R. K., and Krishnakumar, S. (2015). Blocking the Maturation of OncomiRNAs Using pri-miRNA-17~92 Aptamer in Retinoblastoma. *Nucleic Acid Ther.* 25, 47–52. doi: 10.1089/nat.2014.0507
- Sudhakar, J., Venkatesan, N., Lakshmanan, S., Khetan, V., Krishnakumar, S., and Biswas, J. (2013). Hypoxic tumor microenvironment in advanced retinoblastoma. *Pediatr. Blood Cancer* 60, 1598–1601. doi: 10.1002/pbc.24599
- Sun, Z., Zhang, A., and Zhang, L. (2019). Inhibition of microRNA-492 attenuates cell proliferation and invasion in retinoblastoma via directly targeting LATS2. *Mol. Med. Rep.* 19, 1965–1971. doi: 10.3892/mmr.2018.9784
- Thériault, B. L., Dimaras, H., Gallie, B. L., and Corson, T. W. (2014). The genomic landscape of retinoblastoma: A review. *Clin. Exp. Ophthalmol.* 42 (1), 33–52. doi: 10.1111/ceo.12132
- To, K. H., Pajovic, S., Gallie, B. L., and Thériault, B. L. (2012). *BMC Cancer* 12. doi: 10.1186/1471-2407-12-69
- Turhan, S. (2014). New Hopes in Retinoblastoma Treatment. *Int. J. Hematol. Oncol.* 24, 204–207. doi: 10.4999/uhod.14222
- Ulitsky, I., and Bartel, D. P. (2013). LincRNAs: Genomics, evolution, and mechanisms. *Cell* 154, 26–46. doi: 10.1016/j.cell.2013.06.020
- Vannini, I., Fanini, F., and Fabbri, M. (2018a). Emerging roles of microRNAs in cancer. *Curr. Opin. Genet. Dev.* 48, 128–133. doi: 10.1016/j.gde.2018.01.001
- Vannini, I., Wise, P. M., Challagundla, K. B., Plousiou, M., Raffini, M., Bandini, E., et al. (2018b). Publisher Correction: Transcribed ultraconserved region 339 promotes carcinogenesis by modulating tumor suppressor microRNAs. *Nat. Commun.* 9, 160. doi: 10.1038/s41467-017-02485-1
- Vasudevan, S., Tong, Y., and Steitz, J. A. (2007). Switching from repression to activation: MicroRNAs can up-regulate translation. *Sci. (80-.)* 318, 1931–4. doi: 10.1126/science.1149460
- Venkatesan, N., Deepa, P. R., Khetan, V., and Krishnakumar, S. (2015). Computational and in vitro investigation of miRNA-gene regulations in retinoblastoma pathogenesis: MiRNA mimics strategy. *Bioinform. Biol. Insights* 9, 89–101. doi: 10.4137/BBI.S21742
- Wang, J.-X., Yang, Y., and Li, K. (2018). Long noncoding RNA DANCR aggravates retinoblastoma through miR-34c and miR-613 by targeting MMP-9. *J. Cell. Physiol.* 233, 6986–6995. doi: 10.1002/jcp.26621
- Wang, J., Wang, X., Li, Z., Liu, H., and Teng, Y. (2014). MicroRNA-183 suppresses retinoblastoma cell growth, invasion and migration by targeting LRP6. *FEBS J.* 281, 1355–1365. doi: 10.1111/febs.12659
- Wang, L., Lyu, X., Ma, Y., Wu, F., and Wang, L. (2019). MicroRNA-504 targets AEG-1 and inhibits cell proliferation and invasion in retinoblastoma. *Mol. Med. Rep.* 19, 2935–2942. doi: 10.3892/mmr.2019.9923
- Wang, Z., Yao, Y.-J., Zheng, F., Guan, Z., Zhang, L., Dong, N., et al. (2017). Mir-138-5p acts as a tumor suppressor by targeting pyruvate dehydrogenase kinase 1 in human retinoblastoma. *Eur. Rev. Med. Pharmacol. Sci.* 21, 5624–5629.
- Wu, X., Zeng, Y., Wu, S., Zhong, J., Wang, Y., and Xu, J. (2015). MiR-204, down-regulated in retinoblastoma, regulates proliferation and invasion of human retinoblastoma cells by targeting CyclinD2 and MMP-9. *FEBS Lett.* 589, 645–650. doi: 10.1016/j.febslet.2015.01.030
- Xing, L., Zhang, L., Feng, Y., Cui, Z., and Ding, L. (2018). Downregulation of circular RNA hsa_circ_0001649 indicates poor prognosis for retinoblastoma and regulates cell proliferation and apoptosis via AKT/mTOR signaling pathway. *Biomed. Pharmacother.* 105, 326–333. doi: 10.1016/j.biopha.2018.05.141
- Xu, X., Jia, R., Zhou, Y., Song, X., Wang, J., Qian, G., et al. (2011). Microarray-based analysis: Identification of hypoxia-regulated microRNAs in retinoblastoma cells. *Int. J. Oncol.* 38, 1385–1393. doi: 10.3892/ijo.2011.961
- Xu, X. L., Fang, Y., Lee, T. C., Forrest, D., Gregory-Evans, C., Almeida, D., et al. (2009). Retinoblastoma Has Properties of a Cone Precursor Tumor and Depends Upon Cone-Specific MDM2 Signaling. *Cell* 137, 1018–1031. doi: 10.1016/j.cell.2009.03.051
- Yang, G., Fu, Y., Lu, X., Wang, M., Dong, H., and Li, Q. (2018). LncRNA HOTAIR/miR-613/c-met axis modulated epithelial-mesenchymal transition of retinoblastoma cells. *J. Cell. Mol. Med.* 22, 5083–5096. doi: 10.1111/jcmm.13796
- Yang, G., Fu, Y., Zhang, L., Lu, X., and Li, Q. (2017). MiR106b regulates retinoblastoma Y79 cells through Runx3. *Oncol. Rep.* 38, 3039–3043. doi: 10.3892/or.2017.5931
- Yang, Y., and Mei, Q. (2015). miRNA signature identification of retinoblastoma and the correlations between differentially expressed miRNAs during retinoblastoma progression. *Mol. Vis.* 21, 307–317.
- Zhang, A., Shang, W., Nie, Q., Li, T., and Li, S. (2018a). Long non-coding RNA H19 suppresses retinoblastoma progression via counteracting miR-17-92 cluster. *J. Cell. Biochem.* 119, 3497–3509. doi: 10.1002/jcb.26521
- Zhang, F., Li, J., Xiao, H., Zou, Y., Liu, Y., and Huang, W. (2018b). AFAP1-AS1: A novel oncogenic long non-coding RNA in human cancers. *Cell Prolif.* 51. doi: 10.1111/cpr.12397
- Zhang, H., Zhong, J., Bian, Z., Fang, X., Peng, Y., and Hu, Y. (2017). Long non-coding RNA CCAT1 promotes human retinoblastoma SO-RB50 and Y79 cells through negative regulation of miR-218-5p. *Biomed. Pharmacother.* 87, 683–691. doi: 10.1016/j.biopha.2017.01.004
- Zhang, Y., Xue, C., Zhu, X., Zhu, X., Xian, H., and Huang, Z. (2016). Suppression of microRNA-125a-5p upregulates the TAZ-EGFR signaling pathway and promotes retinoblastoma proliferation. *Cell Signal.* 28, 850–860. doi: 10.1016/j.cellsig.2016.04.002
- Zhao, D., and Cui, Z. (2018). MicroRNA-361-3p regulates retinoblastoma cell proliferation and stemness by targeting hedgehog signaling. *Exp. Ther. Med.* 17, 1154–1162. doi: 10.3892/etm.2018.7062
- Zhao, H., Guo, M., Zhao, G., Ma, Q., Ma, B., Qiu, X., et al. (2012). miR-183 inhibits the metastasis of osteosarcoma via downregulation of the expression of Ezrin in F5M2 cells. *Int. J. Mol. Med.* 30, 1013–1020. doi: 10.3892/ijmm.2012.1111
- Zhao, L., Zou, D., Wei, X., Wang, L., Zhang, Y., Liu, S., et al. (2016). MiRNA-221-3p desensitizes pancreatic cancer cells to 5-fluorouracil by targeting RB1. *Tumour Biol.* 37, 16053–16063. doi: 10.1007/s13277-016-5445-8
- Zheng, Y., Lu, X., Xu, L., Chen, Z., Li, Q., and Yuan, J. (2017). MicroRNA-675 promotes glioma cell proliferation and motility by negatively regulating retinoblastoma 1. *Hum. Pathol.* 69, 63–71. doi: 10.1016/j.humpath.2017.09.006

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptional Repression of CYP3A4 by Increased miR-200a-3p and miR-150-5p Promotes Steatosis *in vitro*

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 03 October 2018

Accepted: 06 May 2019

Published: 28 May 2019

Citation:

Huang Z, Wang M, Liu L, Peng J, Guo C, Chen X, Huang L, Tan J and Yang G (2019) Transcriptional Repression of CYP3A4 by Increased miR-200a-3p and miR-150-5p Promotes Steatosis *in vitro*. *Front. Genet.* 10:484. doi: 10.3389/fgene.2019.00484

Hepatic cytochrome P450 enzyme activities correlate with non-alcoholic fatty liver disease (NAFLD) and hepatic steatosis. The decreased activity of CYP3A4, an important drug-metabolizing enzyme, is associated with the progression of NAFLD. CYP3A4 is predicted as a target gene of miR-200a-3p and miR-150-5p by MicroInspector and TargetScan algorithms analyses. Here, we found decreased CYP3A4 and increased miR-200a-3p and miR-150-5p in LO2 cells with free fatty acid (FFA)-induced steatosis. Dual-luciferase assay confirmed that both miR-200a-3p and miR-150-5p targeted the 3'-untranslated region (3'-UTR) of CYP3A4 and that such interaction was abolished by miRNA binding site mutations in 3'-UTR of CYP3A4. Using miR-200a-3p and miR-150-5p mimics and inhibitors, we further confirmed that endogenous CYP3A4 was regulated posttranscriptionally by miR-200a-3p or miR-150-5p. Moreover, miR-200a-3p and miR-150-5p inhibitors attenuated FFA-induced steatosis in LO2 cells, and such effect was dependent on CYP3A4 expression. These results suggest that miR-200a-3p and miR-150-5p, through directly targeting 3'-UTR of CYP3A4, contribute to the development of FFA-induced steatosis.

Keywords: non-alcoholic fatty liver disease, CYP3A4, miR-200a-3p, miR-150-5p, LO2 cell line

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been the most common chronic liver disease in the world for the past 30 years (Younossi et al., 2018) with an estimated prevalence of 10–40% (Younossi et al., 2016). NAFLD includes a spectrum of disease from simple liver steatosis to non-alcoholic steatohepatitis (NASH). NASH is considered as a leading indication for liver transplants in the near future, as it increases the risk of hepatocarcinoma (HCC) (Sattar et al., 2014; Diehl and Day, 2017).

Several genetic defects and environmental factors have been implicated in the pathogenesis of NAFLD. Specifically, a few studies have reported that NAFLD is associated with decreased expression and function of CYP3A4, a major member of the hepatic cytochrome P450 superfamily contributing to the metabolism of 45–60% of all drugs used in the clinical setting

(Wojnowski and Kamdem, 2006; Anglicheau et al., 2007). Donato et al. (2006) observed a significantly reduced CYP3A4 activity in both human liver tissue with steatosis and fat-overloaded hepatocytes cultured *in vitro*. Similar results were shown in nutritionally obese mice (Yoshinari et al., 2006; Maximos et al., 2017). Compared with healthy controls, NAFLD patients showed higher plasma concentration of CYP3A4 substrate, indicating impaired CYP3A4 function in NAFLD patients (Woolsey et al., 2015). Decreased protein expression and activity of CYP3A4 were observed with NAFLD development (Fisher et al., 2009). A recent study further confirmed reduced protein level and activity of CYP3A4 in liver tissues of NAFLD patients as compared to those of controls (Jamwal et al., 2018). However, despite all these observations, the underlying mechanism regulating the expression and function of CYP3A4 in NAFLD remains unclear.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs that negatively regulate gene expression through binding to the 3'-untranslated region (3'-UTR) of mRNAs, thus altering the expression and function of various genes, including CYP3A4 (Pan et al., 2009; Swathy et al., 2017; Yan et al., 2017). miRNA-34a, miRNA-122, and miRNA-192 are considered as potential biomarkers of NAFLD staging (Liu et al., 2018). Importantly, high-throughput sequencing revealed that expressions of miR-150-5p and miR-200a-3p were significantly higher in NAFLD with fibrosis than in NAFLD without fibrosis (Leti et al., 2015). Therefore, we hypothesize that significant change of hepatic miRNAs in NAFLD could regulate CYP3A4 expression posttranscriptionally. Here, we report that miR-150-5p and miR-200a-3p directly regulate CYP3A4 and are involved in free fatty acid (FFA)-induced steatosis.

MATERIALS AND METHODS

Reagents

Sodium salts of palmitic acid (PA) (P9767) and oleic acid (OA) (O7501), fatty acid (FA) free bovine serum albumin (BSA) (A8806), and BODIPY 493/503 (790389) were purchased from Sigma-Aldrich (MO, United States), and RPMI 1640 (11875-093) medium and fetal bovine serum (FBS) (26140079) were from GIBCO (Invitrogen, CA, United States). Lipofectamine™ 2000 (11668019) was from Invitrogen (CA, United States). Oligonucleotide primers for CYP3A4 were synthesized by Sangon Biotech (Shanghai, China). All miRNA mimics, miRNA mimic negative controls, miRNA inhibitors, miRNA inhibitor negative controls, and primers for miRNA RT-qPCR were purchased from RiboBio (Guangzhou, China). All other chemicals and solvents were of the highest commercial grades.

Cell Culture Model of Hepatic Steatosis *in Vitro*

LO2 cells were provided by Stem Cell Bank, Chinese Academy of Sciences and cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C/5% CO₂. Steatosis was induced as previously described (Feldstein et al., 2004;

Ricchi et al., 2009). PA and OA were codissolved in 10% FA-free BSA prepared in H₂O. In accordance with previous studies (Wang et al., 2014), LO2 cells were exposed to a mixture of 1 mM OA and PA (final ratio 2:1) for 24 h. After 24 h of incubation, lipid droplets were stained by BODIPY according to a previously reported protocol (Qiu and Simon, 2016).

In silico Identification of Putative miRNA Binding Sites

The 3'-UTR sequences of human CYP3A4 (GenBank sequence NM_017460) were searched for the antisense matches to individual miRNAs using MicroInspector (Rusinov et al., 2005) and Target Scan (Lewis et al., 2005).

Transfection

All transfections were performed by Lipofectamine™ 2000 according to the manufacturer's instructions.

Real-Time PCR

Small RNA was extracted with an E.Z.N.A miRNA kit (Omega BIO-TEK, GA, United States) and reverse transcribed using PrimeScript RT Reagent Kit (Roche, Basel, Switzerland) with primers for specified miRNAs. The oligonucleotide sequences of STEM-LOOP RT primers were designed (Kramer, 2011) as follows: miR-200a-3p, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACacatcgtt-3'; miR-150-5p, 5'-GTCGTATCCAGTGCAGGTCCGAGGTATTCGCACTGGATACGACctgtcccc-3'; U6, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaaaatat-3'. Real-time PCR was performed with SYBR Green PCR Master Mix (Roche, Basel, Switzerland) using the following conditions: 95°C for 10 min followed by 40 cycles of amplification at 95°C for 10 s and 59°C for 30 s. Mature miR-200a-3p and miR-150-5p levels were normalized with U6. The oligonucleotide sequences of qPCR primers were as follows: miR-200a-3p, 5'-CACGCataacactgtctggttaa-3'; miR-150-5p, 5'-CACGCActgttacaggcctgg-3'; U6, 5'-CACGCAGcaaggatgacacgcaa-3'; general reverse primer, 5'-CACGCATGGAAGGACGGG-3'.

To inhibit or induce miR-150-5p or miR-200a-3p, transient transfection of miRNA inhibitors or mimics (100 nM) was performed in LO2 cells using Lipofectamine™ 2000. Specific miRNA-150 or miRNA-200a inhibitors or mimics were commercially purchased from RiboBio (Guangzhou, China), including anti-miRNA-150 (target sequence 5'-CUGGUA CAGGCCUGGGGACAG-3'), anti-miRNA-200a (target sequence 5'-UAACACUGUCUGGUAACGAUGU-3'), syn-miRNA-150 (target sequence 5'-CUGGUACAGGCCUGGGGACAG-3'), and syn-miRNA-200a (target sequence 5'-UAACACUGUCUGGUAACGAUGU-3'). miScript inhibitor negative control (100 nM) (RiboBio, Guangzhou, China) was used as internal reference for normalization.

Twenty-four hours after transfection, cells were treated with a mixture of 1 mM OA and PA (final ratio 2:1) for

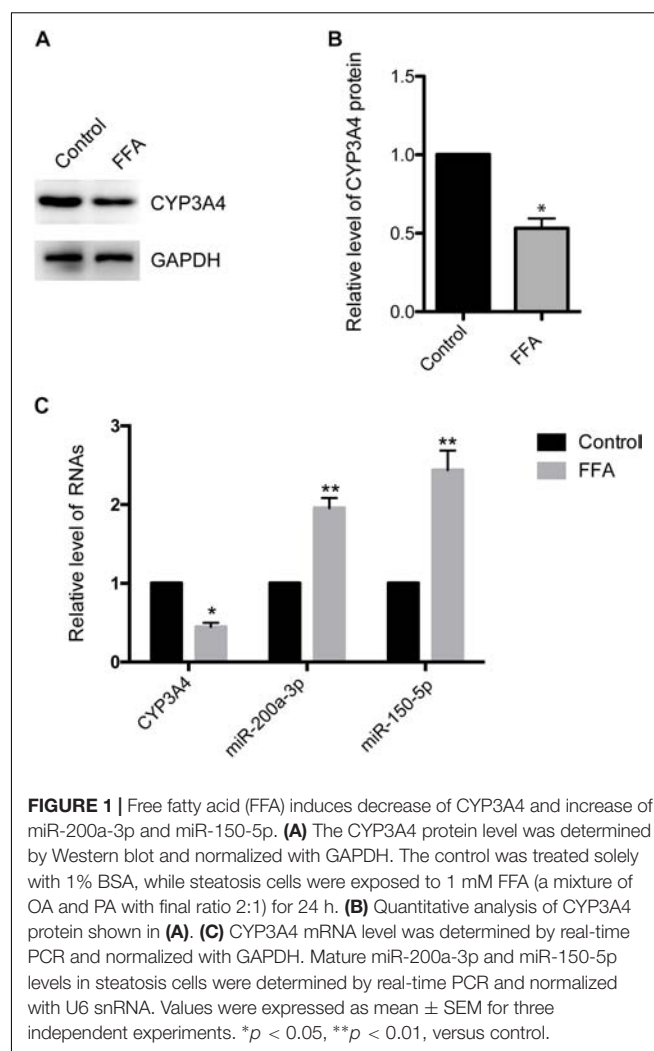
24 h. To confirm the effect of miR-150-5p or miR-200a-3p inhibition or induction on CYP3A4 mRNA, total RNAs were extracted from LO2 cells with TRIzol (Life Technologies, CA, United States). Total mRNAs were reverse transcribed into cDNAs by the PrimeScript RT Reagent kit. Real-time PCR was performed by the SYBR Green PCR Master Mix using the following conditions: 95°C for 10 min followed by 40 cycles of amplification at 95°C for 10 s and 59°C for 30 s. GAPDH (forward primer: AGAAGGCTGGGGCTCATTTG; reverse primer: AGGGGCCATCCACAGTCTTC) was used as an internal control to normalize CYP3A4 expression (forward primer: CCCGTTGTTCTAAAGGTTGA; reverse primer: TCTGGTGTTCAGGCACAG). qPCR was quantified using the formula $2^{-\Delta\Delta CT}$ and plotted as x-fold to the control.

Western Blot

Cells in six-well plates were harvested posttransfection and treatment, and whole-cell lysates were prepared with RIPA lysis buffer (Beyotime, Beijing, China) supplemented with complete protease inhibitor and phenylmethanesulfonyl fluoride (Beyotime, Beijing, China). Protein concentrations were determined with the BCA Protein Assay Kit (Beyotime, Beijing, China). Whole-cell protein (20 µg) was separated on SDS-PAGE and electrophoretically transferred onto PVDF membrane (Millipore, CA, United States). The membrane was incubated with a selective rabbit anti-human CYP3A4 polyclonal antibody (Millipore, CA, United States) or mouse anti-human GAPDH antibodies (Zhongshan Inc., Guangzhou, China), and subsequently with the secondary antibody of HRP goat anti-rabbit IgG (Zhongshan Inc., Guangzhou, China) or rabbit anti-mouse IgG (Zhongshan Inc., Guangzhou, China). Images were acquired with GE Healthcare ImageQuant 350, and band densities were quantified with GeneTools (SynGene, Cambridge, United Kingdom).

Construction of Reporter Plasmids

The 3'-UTR of CYP3A4 gene corresponding to 1,620–2,792 nt (1,173 bp; accession no. NM_001202855) was cloned into pmiR-RB-REPORTTM vector via *XhoI* and *NotI* restriction sites. The primers used for construction of wild-type CYP3A4 3'-UTR reporter plasmid were as follows: h-CYP3A4-F, 5'-CTTGACTCGAGATTTTCCTAAGGACTTCTGC-3'; h-CYP3A4-R, 5'-ATTGCGGCCGCGAGGCTTATGCTCAATC-3'. The sequence of the recombinant clones was confirmed by DNA sequencing and named as pmiR-RB-REPORTTM CYP3A4 3'-UTR WT. miRNA-200a binding site mutant (AGTGTTA changed to TGTGCCA) and miRNA-150 binding site double mutant (TTCCCAG changed to ATCCGAT and TGGGAGA changed to AGGCATA) were constructed using Q5[®] Site-Directed Mutagenesis Kit (NEB, MA, United States) and confirmed by DNA sequencing. The firefly luciferase gene used 3'-UTR of CYP3A4 as the report luciferase, with Renilla luciferase gene as an internal control.



Dual-Luciferase Assay

The LO2 cells were seeded into 24-well plates. Firefly luciferase (0.1 µg) containing 3'-UTR of CYP3A4 in pmiR-RB-REPORTTM vector, along with miR-200a-3p or miR-150-5p mimic, was transfected into LO2 cells with Lipofectamine 2000. After 24 h of incubation, luciferase activities were measured with a luminometer (Tecan Infinite 200 Pro, Switzerland) using the Dual-Luciferase Reporter Assay System (Promega, Valencia, CA, United States). Firefly luciferase activity was normalized by Renilla luciferase activity and compared between different treatments.

Statistical Analysis

All values were expressed as the mean $\pm$ SEM. Comparisons of variables between groups were performed with an unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

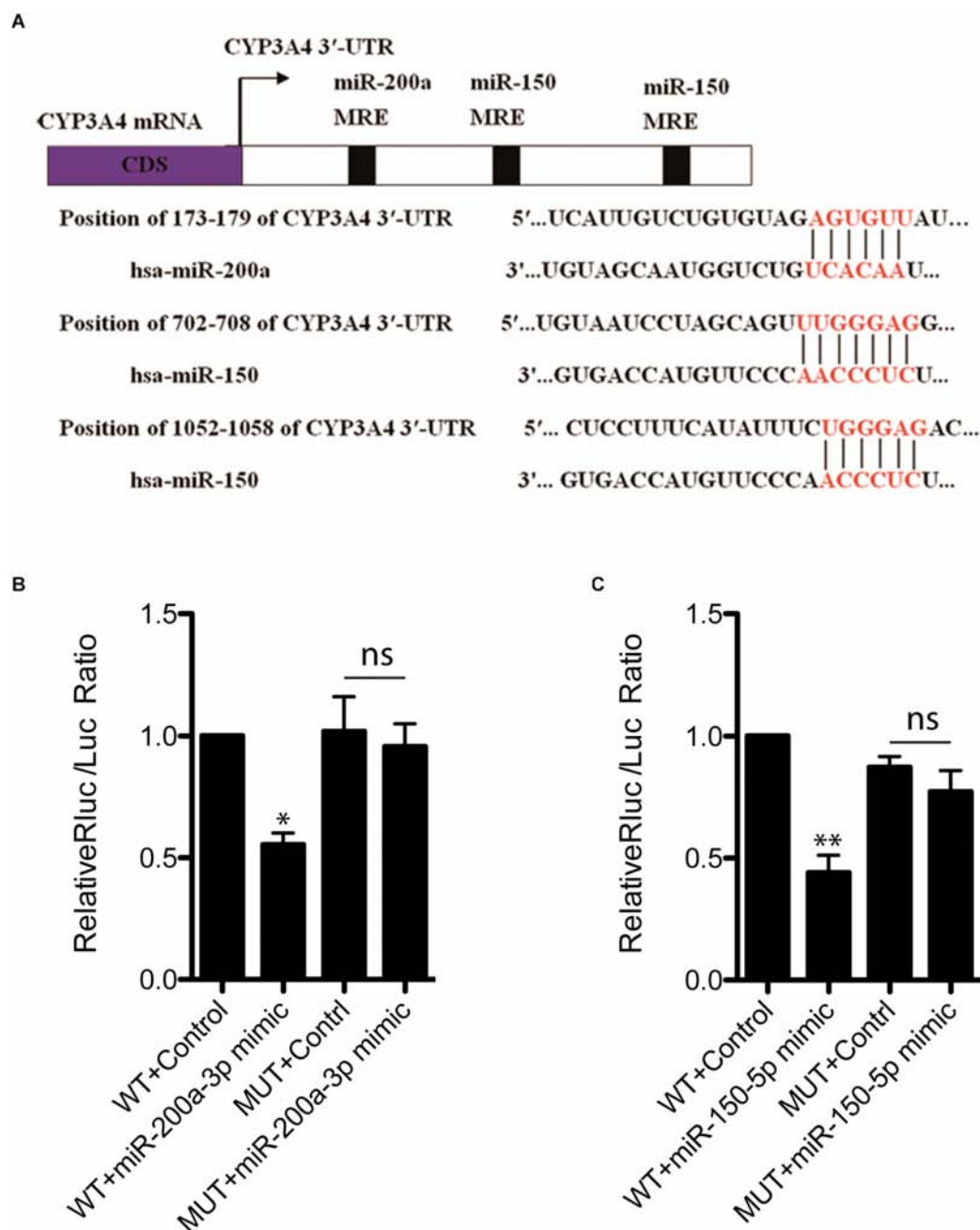


FIGURE 2 | miR-200a-3p and miR-150-5p mimics down-regulate CYP3A4 expression by binding to its 3'-UTR. **(A)** The miR-200a-3p and miR-150-5p MRE sites (miRNA recognition element) within human CYP3A4 3'-UTR predicted by MicroInspector and/or TargetScan are both conserved. **(B)** Evaluation of binding ability of miR-200a-3p **(B)** or miR-150-5p **(C)** mimics to CYP3A4 3'-UTR-Luc WT and miRNA binding site mutants (MUT). LO2 cells were cotransfected with a firefly luciferase reporter vector containing the indicated CYP3A4 3'-UTR constructs, a Renilla luciferase reporter as internal control, and treated with miR-200a-3p **(B)** or miR-150-5p **(C)** mimics. Values were mean $\pm$ SEM for three independent experiments. * $p < 0.05$, ** $p < 0.01$, versus control; ns, not significant.

RESULTS

CYP3A4 Expression Is Decreased in FFA-Induced Steatosis Cells

CYP3A4 activity is reduced in human NAFLD as well as in mouse and *in vitro* cell models of the disease (Kolwankar et al., 2007;

Woolsey et al., 2015). Here, we investigated CYP3A4 expression in FFA-induced steatosis cells *in vitro*. As shown in **Figures 1A,B**, CYP3A4 protein level was decreased in FFA-induced steatosis cells compared to control. Similarly, a significant decrease of CYP3A4 mRNA was observed in FFA-induced steatosis cells compared to control (**Figure 1C**).

Increased Mature miR-200a-3p and miR-150-5p in FFA-Induced Steatosis Cells

To investigate whether decreased CYP3A4 in FFA-induced steatosis cells was due to miRNAs regulation, we used MicroInspector and TargetScan algorithms to screen antisense matches of CYP3A4 3'-UTR against human miRNAs. CYP3A4 was predicted to be a target gene of miR-200a-3p and miR-150-5p (Figure 2A). Next, we determined the expression levels of mature miR-200a-3p and miR-150-5p in FFA-induced steatosis cells and found that both mature miR-200a-3p and miR-150-5p were higher in FFA-induced steatosis cells than in control cells (Figure 1C).

miR-200a-3p and miR-150-5p Regulate CYP3A4 Expression by Targeting Its 3'-UTR

To investigate whether CYP3A4 can be directly regulated by mature miR-200a-3p and miR-150-5p, CYP3A4 3'-UTR WT and the two miRNA binding site mutants were cloned separately into pmiR-RB-REPORTTM vector for dual-luciferase assay. We showed that both miR-200a-3p and miR-150-5p interacted with CYP3A4 3'-UTR WT, but not the two miRNA binding site mutants (Figures 2B,C).

miR-200a-3p and miR-150-5p Down-Regulate CYP3A4 Expression in FFA-Induced Steatosis Cells

To investigate the effect of miR-200a-3p and miR-150-5p on CYP3A4 expression, we examined changes of CYP3A4 mRNA and protein levels in response to inhibition or induction of miR-200a-3p or miR-150-5p. miR-200a-3p or miR-150-5p inhibitor increased CYP3A4 protein (Figures 3A–D) and mRNA (Figure 3E) levels. In addition, CYP3A4 protein and mRNA (Figure 3F) levels were decreased after miR-200a-3p or miR-150-5p mimic treatment.

miR-200a-3p or miR-150-5p Inhibitor Regulates FFA-Induced Steatosis via CYP3A4

We have demonstrated that miR-200a-3p or miR-150-5p negatively regulated CYP3A4 expression. Because of the importance of CYP3A4 activity in steatosis development (Donato et al., 2006; Hu et al., 2014), we then determined whether CYP3A4 mediated the regulatory effect of miR-200a-3p or miR-150-5p inhibitor on FFA-induced steatosis. miR-200a-3p or miR-150-5p inhibitor increased CYP3A4 expression, which was abolished by CYP3A4 knockdown (Figures 4A–D). More importantly, miR-200a-3p or miR-150-5p inhibitor reduced FFA-induced steatosis assessed by the BODIPY 493/503 staining, and this effect was abrogated by CYP3A4 gene silencing (Figures 4E,F). This suggested that miR-200a-3p or miR-150-5p inhibitor, through up-regulating CYP3A4, protected against FFA-induced steatosis.

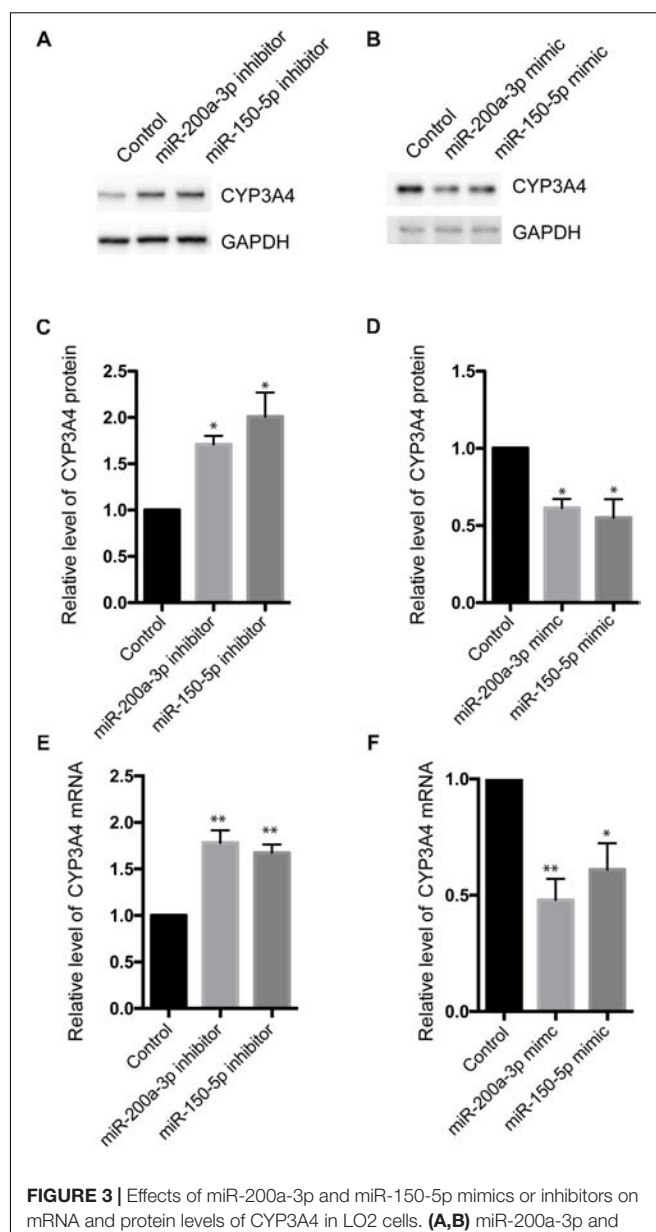


FIGURE 3 | Effects of miR-200a-3p and miR-150-5p mimics or inhibitors on mRNA and protein levels of CYP3A4 in LO2 cells. (A,B) miR-200a-3p and miR-150-5p inhibitors (A) or mimics (B) were transfected into LO2 cells. After 24 h, the cells were harvested. CYP3A4 protein level was determined by Western blot and normalized with GAPDH. (C,D) Quantitative analysis of CYP3A4 shown in (A,B), respectively. (E,F) The mRNA level of CYP3A4 was determined by real-time PCR and normalized with GAPDH in cells transfected with miR-200a-3p or miR-150-5p inhibitors (E) or mimics (F). Values were mean $\pm$ SEM for three independent experiments. * $p < 0.05$, ** $p < 0.01$, versus control.

DISCUSSION

In recent years, NAFLD has emerged as a major public health concern characterized by elevated serum FFAs and hepatocyte lipoapoptosis (Feldstein et al., 2003). Several reports have indicated the importance of both quantitative and qualitative (e.g., saturated versus unsaturated FAs) changes in dietary FAs as underlying causative mechanisms for NAFLD in both rodent

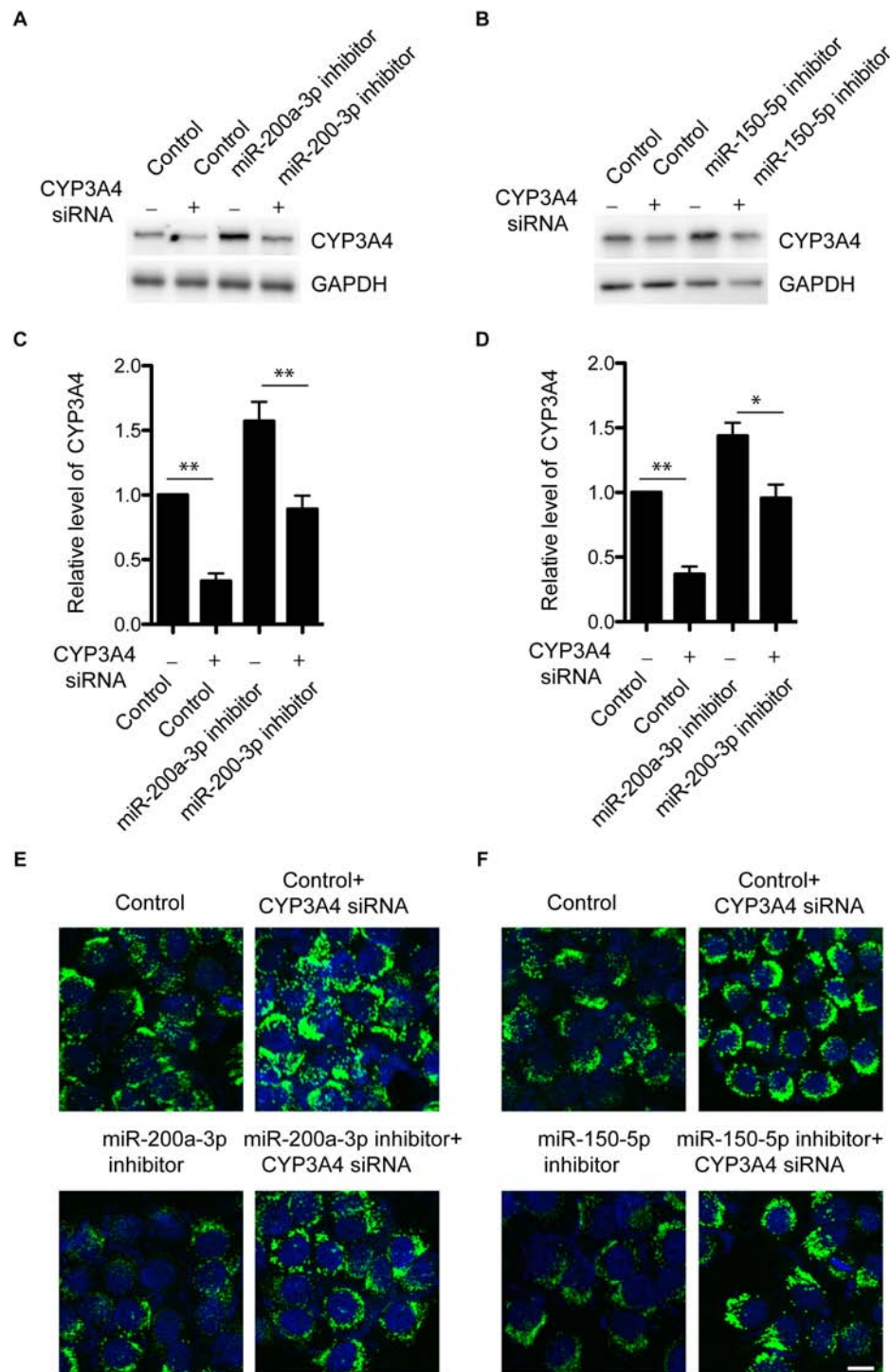


FIGURE 4 | Knockdown of CYP3A4 abolishes reduced FFA-induced steatosis mediated by miR-200a-3p and miR-150-5p inhibitors. **(A,B)** miR-200a-3p **(A)** and miR-150-5p **(B)** inhibitors were transfected into LO2 cells with or without knockdown of CYP3A4. After 24 h, cells were harvested. CYP3A4 protein level was determined by Western blot and normalized with GAPDH. **(C,D)** Quantitative analysis of CYP3A4 protein shown in **(A,B)**, respectively. **(E,F)** miR-200a-3p and miR-150-5p inhibitors were transfected into LO2 cells with or without knockdown of CYP3A4. After 24 h, cells were exposed to 1 mM FFA or vehicle. The BODIPY 493/503 staining was performed to assess cellular steatosis. Green fluorescence indicated lipid droplets. The cell nucleus was stained by DAPI (Blue). * $p < 0.05$, ** $p < 0.01$, bar = 10 μm .

models and humans (Nehra et al., 2001; Musso et al., 2003; Wang et al., 2006). Increased supply of FAs or decreased lipid clearance in hepatocytes can set off esterification of FAs and formation of lipid droplets (Anderson and Borlak, 2008). In terms of mechanism, enzymes, important regulators of plasma and tissue FA composition, signaling pathways, and transcriptional factors controlling FA synthesis and gluconeogenesis have all been implicated in NAFLD (Malhi and Gores, 2008; Mota et al., 2016). Here, we demonstrated that miR-200a-3p and miR-150-5p, through directly targeting 3'-UTR of CYP3A4, contributed to the development of FFA-induced steatosis *in vitro*.

Previous studies have evaluated the relationship between NAFLD and hepatic CYP3A activity. Fisher et al. (2009) explored the relationship between hepatic CYP3A4 and non-alcoholic hepatic steatosis in human liver samples. It was found that CYP3A4 protein expression and activity decreased with the progression of NAFLD. Other studies (Kolwankar et al., 2007; Jamwal et al., 2018) showed that liver samples with steatosis had significantly lower hepatic CYP3A4 activity than those without steatosis, which was consistent with *in vitro* findings in long-chain FFA-induced steatosis. CYP3A4 mRNA and protein also decreased significantly in fatty mice induced by a high-fat diet (Yoshinari et al., 2006).

Although most of the CYPs are found within endoplasmic reticulum, CYP3A4 localizes in mitochondria and synthesizes arachidonic acid (AA)-derived epoxyeicosatrienoic acids (EETs), which promotes electron transport chain/respiration and mitochondrial function (Guo et al., 2017). It is well known that CYP enzyme activities are affected by steatosis, which poses major impact on drug metabolism and drug-induced hepatotoxicity (Gomez-Lechon et al., 2009). However, our study suggests that increased CYP3A4 expression prevents LO2 cells from FFA-induced steatosis. Our results demonstrate that CYP3A4 activity impairment is not only induced by hepatic steatosis but also a factor further promoting hepatic steatosis. This could explain the phenotype of cyp3a-null male mice,

including increases in weight, liver triglycerides, and total lipids (Kumar et al., 2018).

It was previously shown that CYP3A4 could be regulated by miRNAs *via* direct and indirect targeting (Takagi et al., 2008; Pan et al., 2009; Swathy et al., 2017; Yan et al., 2017). Hepatic miRNA changes in NAFLD have been confirmed in several studies of dietary NASH in mice and rats (Pogribny et al., 2010; Alisi et al., 2011), as well as serum and liver tissue of NAFLD patients (Leti et al., 2015; Liu et al., 2018). In this study, we reported two new CYP3A4 regulators, including miR-200a-3p and miR-150-5p, in FFA-induced steatosis. Our results showed that expressions of miR-200a-3p and miR-150-5p increased significantly in hepatocytes with steatosis as compared to normal hepatocytes, which is consistent with previous studies reporting an increase of miR-200a-3p in NAFLD (Cheung et al., 2008; Alisi et al., 2011). More importantly, miR-200a-3p or miR-150-5p inhibitor can alleviate FFA-induced steatosis. Thus, our findings provide a potential therapeutic target for the use of miR-200a-3p and miR-150-5p inhibitors to protect against steatosis development.

AUTHOR CONTRIBUTIONS

ZH and MW performed the experiments and wrote the manuscript. LL, JP, CG, XC, LH, and JT performed the experiments. ZH and GY designed the project and provided supervision and support for the project.

FUNDING

This work was supported by National Scientific Foundation of China (Nos. 81673520 and 81673519) and the New Xiangya Talent Project of the Third Xiangya Hospital of Central South University (No. 20180302).

REFERENCES

- Alisi, A., Da Sacco, L., Bruscalupi, G., Piemonte, F., Panera, N., De Vito, R., et al. (2011). Mirnome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. *Lab Invest.* 91, 283–293. doi: 10.1038/labinvest.2010.166
- Anderson, N., and Borlak, J. (2008). Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol. Rev.* 60, 311–357. doi: 10.1124/pr.108.00001
- Anglicheau, D., Legendre, C., Beaune, P., and Thervet, E. (2007). Cytochrome P450 3A polymorphisms and immunosuppressive drugs: an update. *Pharmacogenomics* 8, 835–849. doi: 10.2217/14622416.8.7.835
- Cheung, O., Puri, P., Eicken, C., Contos, M. J., Mirshahi, F., Maher, J. W., et al. (2008). Nonalcoholic steatohepatitis is associated with altered hepatic microRNA expression. *Hepatology* 48, 1810–1820. doi: 10.1002/hep.22569
- Diehl, A. M., and Day, C. (2017). Cause, pathogenesis, and treatment of nonalcoholic steatohepatitis. *N. Engl. J. Med.* 377, 2063–2072. doi: 10.1056/nejmra1503519
- Donato, M. T., Lahoz, A., Jimenez, N., Perez, G., Serralta, A., Mir, J., et al. (2006). Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab. Dispos.* 34, 1556–1562. doi: 10.1124/dmd.106.009670
- Feldstein, A. E., Canbay, A., Guicciardi, M. E., Higuchi, H., Bronk, S. F., and Gores, G. J. (2003). Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. *J. Hepatol.* 39, 978–983. doi: 10.1016/s0168-8278(03)00460-4
- Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. E., Bronk, S. F., Rydzewski, R., et al. (2004). Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology* 40, 185–194. doi: 10.1002/hep.20283
- Fisher, C. D., Lickteig, A. J., Augustine, L. M., Ranger-Moore, J., Jackson, J. P., Ferguson, S. S., et al. (2009). Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab. Dispos.* 37, 2087–2094. doi: 10.1124/dmd.109.027466
- Gomez-Lechon, M. J., Jover, R., and Donato, M. T. (2009). Cytochrome p450 and steatosis. *Curr. Drug Metab.* 10, 692–699. doi: 10.2174/138920009789895543
- Guo, Z., Sevrioukova, I. F., Denisov, I. G., Zhang, X., Chiu, T. L., Thomas, D. G., et al. (2017). Heme binding biguanides target cytochrome P450-dependent cancer cell mitochondria. *Cell Chem. Biol.* 24:1314.
- Hu, N., Hu, M., Duan, R., Liu, C., Guo, H., Zhang, M., et al. (2014). Increased levels of fatty acids contributed to induction of hepatic CYP3A4 activity induced by diabetes—in vitro evidence from HepG2 cell and Fa2N-4 cell lines. *J. Pharmacol. Sci.* 124, 433–444. doi: 10.1254/jphs.13212fp
- Jamwal, R., De La Monte, S. M., Ogasawara, K., Adusumalli, S., Barlock, B. B., and Akhlaghi, F. (2018). Nonalcoholic fatty liver disease and diabetes are associated

- with decreased CYP3A4 protein expression and activity in human liver. *Mol. Pharm.* 15, 2621–2632. doi: 10.1021/acs.molpharmaceut.8b00159
- Kolwankar, D., Vuppalanchi, R., Ethell, B., Jones, D. R., Wrighton, S. A., Hall, S. D., et al. (2007). Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin. Gastroenterol. Hepatol.* 5, 388–393. doi: 10.1016/j.cgh.2006.12.021
- Kramer, M. F. (2011). Stem-loop RT-qPCR for miRNAs. *Curr. Protoc. Mol. Biol.* 15:10. doi: 10.1002/0471142727.mb1510s95
- Kumar, R., Litoff, E. J., Boswell, W. T., and Baldwin, W. S. (2018). High fat diet induced obesity is mitigated in Cyp3a-null female mice. *Chem. Biol. Interact.* 289, 129–140. doi: 10.1016/j.cbi.2018.05.001
- Leti, F., Malenica, I., Doshi, M., Courtright, A., Van Keuren-Jensen, K., Legendre, C., et al. (2015). High-throughput sequencing reveals altered expression of hepatic microRNAs in nonalcoholic fatty liver disease-related fibrosis. *Transl. Res.* 166, 304–314. doi: 10.1016/j.trsl.2015.04.014
- Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035
- Liu, C. H., Ampuero, J., Gil-Gomez, A., Montero-Vallejo, R., Rojas, A., Munoz-Hernandez, R., et al. (2018). miRNAs in patients with non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J. Hepatol.* 69, 1335–1348. doi: 10.1016/j.jhep.2018.08.008
- Malhi, H., and Gores, G. J. (2008). Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin. Liver Dis.* 28, 360–369. doi: 10.1055/s-0028-1091980
- Maximos, S., Chamoun, M., Gravel, S., Turgeon, J., and Michaud, V. (2017). Tissue specific modulation of cyp2c and cyp3a mRNA levels and activities by diet-induced obesity in mice: the impact of type 2 diabetes on drug metabolizing enzymes in liver and extra-hepatic tissues. *Pharmaceutics* 9:40. doi: 10.3390/pharmaceutics9040040
- Mota, M., Banini, B. A., Cazanave, S. C., and Sanyal, A. J. (2016). Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. *Metabolism* 65, 1049–1061. doi: 10.1016/j.metabol.2016.02.014
- Musso, G., Gambino, R., De Micheli, F., Cassader, M., Rizzetto, M., Durazzo, M., et al. (2003). Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* 37, 909–916. doi: 10.1053/jhep.2003.50132
- Nehra, V., Angulo, P., Buchman, A. L., and Lindor, K. D. (2001). Nutritional and metabolic considerations in the etiology of nonalcoholic steatohepatitis. *Dig. Dis. Sci.* 46, 2347–2352.
- Pan, Y. Z., Gao, W., and Yu, A. M. (2009). MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab. Dispos.* 37, 2112–2117. doi: 10.1124/dmd.109.027680
- Pogribny, I. P., Starlard-Davenport, A., Tryndyak, V. P., Han, T., Ross, S. A., Rusyn, I., et al. (2010). Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab. Invest.* 90, 1437–1446. doi: 10.1038/labinvest.2010.113
- Qiu, B., and Simon, M. C. (2016). BODIPY 493/503 Staining of neutral lipid droplets for microscopy and quantification by flow cytometry. *Bio. Protoc.* 6:e1912. doi: 10.21769/BioProtoc.1912
- Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., et al. (2009). Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J. Gastroenterol. Hepatol.* 24, 830–840. doi: 10.1111/j.1440-1746.2008.05733.x
- Rusinov, V., Baev, V., Minkov, I. N., and Tabler, M. (2005). Microinspector: a web tool for detection of miRNA binding sites in an RNA sequence. *Nucleic Acids Res.* 33, W696–W700.
- Sattar, N., Forrest, E., and Preiss, D. (2014). Non-alcoholic fatty liver disease. *BMJ* 349:g4596.
- Swathy, B., Saradalekshmi, K. R., Nair, I. V., Nair, C., and Banerjee, M. (2017). Pharmacoeigenomic responses of antipsychotic drugs on pharmacogenes are likely to be modulated by miRNAs. *Epigenomics* 9, 811–821. doi: 10.2217/epi-2016-0181
- Takagi, S., Nakajima, M., Mohri, T., and Yokoi, T. (2008). Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J. Biol. Chem.* 283, 9674–9680. doi: 10.1074/jbc.M709382200
- Wang, D., Wei, Y., and Pagliassotti, M. J. (2006). Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 147, 943–951. doi: 10.1210/en.2005-0570
- Wang, J. W., Wan, X. Y., Zhu, H. T., Lu, C., Yu, W. L., Yu, C. H., et al. (2014). Lipotoxic effect of p21 on free fatty acid-induced steatosis in L02 cells. *PLoS One* 9:e96124. doi: 10.1371/journal.pone.0096124
- Wojnowski, L., and Kamdem, L. K. (2006). Clinical implications of CYP3A polymorphisms. *Expert Opin. Drug Metab. Toxicol.* 2, 171–182.
- Woolsey, S. J., Mansell, S. E., Kim, R. B., Tirona, R. G., and Beaton, M. D. (2015). CYP3A activity and expression in nonalcoholic fatty liver disease. *Drug Metab. Dispos.* 43, 1484–1490. doi: 10.1124/dmd.115.065979
- Yan, L., Liu, J., Zhao, Y., Nie, Y., Ma, X., Kan, Q., et al. (2017). Suppression of miR-628-3p and miR-641 is involved in rifampin-mediated CYP3A4 induction in HepaRG cells. *Pharmacogenomics* 18, 57–64. doi: 10.2217/pgs-2016-0088
- Yoshinari, K., Takagi, S., Yoshimasa, T., Sugatani, J., and Miwa, M. (2006). Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. *Pharm. Res.* 23, 1188–1200. doi: 10.1007/s11095-006-0071-6
- Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., et al. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat. Rev. Gastroenterol. Hepatol.* 15, 11–20. doi: 10.1038/nrgastro.2017.109
- Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., and Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64, 73–84. doi: 10.1002/hep.28431

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Autophagy-Modulating Long Non-coding RNAs (LncRNAs) and Their Molecular Events in Cancer

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OPEN ACCESS

Edited by:

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Emory University, United States

Reviewed by:

Kaushendra Tripathi,
University of Alabama at Birmingham,
United States
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Specialty section:

This article was submitted to
RNA,

a section of the journal
Frontiers in Genetics

Received: 02 October 2018

Accepted: 31 December 2018

Published: 14 January 2019

Citation:

Islam Khan MZ, Tam SY and
Law HKW (2019)
Autophagy-Modulating Long
Non-coding RNAs (LncRNAs)
and Their Molecular Events in Cancer.
Front. Genet. 9:750.
doi: 10.3389/fgene.2018.00750

Cancer is a global threat of health. Cancer incidence and death is also increasing continuously because of poor understanding of diseases. Although, traditional treatments (surgery, radiotherapy, and chemotherapy) are effective against primary tumors, death rate is increasing because of metastasis development where traditional treatments have failed. Autophagy is a conserved regulatory process of eliminating proteins and damaged organelles. Numerous research revealed that autophagy has dual sword mechanisms including cancer progressions and suppressions. In most of the cases, it maintains homeostasis of cancer microenvironment by providing nutritional supplement under starvation and hypoxic conditions. Over the past few decades, stunning research evidence disclosed significant roles of long non-coding RNAs (lncRNAs) in the regulation of autophagy. LncRNAs are RNA containing more than 200 nucleotides, which have no protein-coding ability but they are found to be expressed in most of the cancers. It is also proved that, autophagy-modulating lncRNAs have significant impacts on pro-survival or pro-death roles in cancers. In this review, we highlighted the recently identified autophagy-modulating lncRNAs, their signaling transduction in cancer and mechanism in cancer. This review will explore newly emerging knowledge of cancer genetics and it may provide novel targets for cancer therapy.

Keywords: autophagy, long non-coding RNAs, cancer, therapy, biomarkers

Abbreviations: ATGs, autophagy-related genes; BANCER, BRAF-activated long non-coding RNA; BDC, bladder cancer; BRC, breast cancer; BT, brain tumor; CASC2, cancer susceptibility candidate gene 2; CRC, colorectal cancer; CTA, lncRNA CTA; DFS, disease-free survival; EMT, epithelial-mesenchymal transition; ENDC, endometrial cancer; GAS5, growth arrest-specific 5; GC, gastric cancer; GMEC, glioma microvascular endothelial cancer; HCC, hepatocellular carcinoma; HMGB1, high-mobility group box 1 protein; HNC, head and neck cancer; HNF 1A-AS1, a natural antisense transcript of HNF 1A; HOTAIR, Hox transcript antisense RNA; HULC, highly up-regulated in liver cancer; LC, lung cancer; LCPAT1, lung cancer progression associated transcript 1; LEUK, leukemia; lncRNAs, long non-coding RNAs; LNM, lymphatic node metastasis; LVC, liver cancer; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MEG3, maternally expressed gene 3; MM, multiple myeloma; MPM, malignant pleural mesothelioma; ncRNAs, non-coding RNAs; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; OS, overall survival; OSTs, osteosarcoma; OVC, ovarian cancer; PCA3, prostate cancer antigen 3; PDA, pancreatic ductal adenocarcinoma; PKM2, pyruvate kinase isozymes 2; PNC, pancreatic cancer; PPTC, papillary thyroid carcinoma; PRKD3, protein kinase D3; PTC, prostate cancer; PTENP1, pseudogene of tumor suppressor gene PTEN; PVT1, plasmacytoma variant translocation 1; ROR, regulator of reprogramming; RTB, retinoblastoma; SB, sleeping beauty; sirt1, silent information regulator 1; STAT3, signal transducer and activator of transcription 3; TNM, tumor node metastasis.

INTRODUCTION

Cancer is a global threat of public health because of its high mortality rate. More than 14 million of new cancer cases and ~60% of death were accounted in 2012 by GLOBOCAN data (**Figure 1A**). It is also assumed that more than 20 million new cancer cases will be counted in the year 2025 (Ferlay et al., 2015). Cancer death is increasing day by day, 8.8 million death were counted in 2015 where abundant death was reduced in low to moderate income countries (**Figure 1B**) (GBD 2015 Risk Factors Collaborators, 2016; WHO, 2018). The cancer cases in 10 Asian countries are also estimated to increase from 6.1 million to 10.7 million in the year 2008 to 2030 respectively. The consecutive death was estimated to raise from 4.1 million to 7.5 million (Sankaranarayanan et al., 2014).

Metastatic tumors are the leading cause of death and pose a great challenge for cancer treatments. Although the molecular basis of carcinogenesis is different in different cancers, the metastasis developmental process is almost similar in all solid tumor cells (Bogenrieder and Herlyn, 2003). The aim of traditional treatments of cancer is either removing or destroying cancerous cells by surgery, radiation therapy, chemotherapy and sometimes personalized treatment. Theoretically, these traditional treatments are very promising but practically none of these are able to cure effectively and efficiently because of poor understating of mechanisms and development of metastasis (Wang Z. et al., 2016).

Autophagy is a highly conserved and critical regulatory process for cells to maintain homeostasis by lysosomal degradation of various proteins and damaged organelles. Dynamic roles of autophagy have been identified in cancers where it participates in cancer progression, prevention, as well as, drug resistance mechanisms (Santana-Codina et al., 2017). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is sometimes also referred to as autophagy which is the major autophagic pathways and most extensively studied compared to microautophagy and chaperone-mediated autophagy. In macroautophagy, phagophore is initially formed and matured to autophagosome. Subsequently, autophagosome fused with a lysosome to degrade the internal materials in autolysosome (**Figure 2**) (Mizushima and Komatsu, 2011). Although autophagy may suppress tumors (Kung et al., 2011) in most cases, the induction of autophagy promotes tumorigenesis by providing survival capability of tumor under microenvironmental stress (Kung et al., 2011; Avalos et al., 2014; White, 2015). Autophagy promotes cancer by inhibiting tumor suppressor protein p53 and controlling the metabolism of cells (Amaravadi et al., 2016). Cellular metabolism and homeostasis are encoded by more than 30 ATGs, their translational products and transduction of signals (**Figure 2**) (Kim and Lee, 2014; Cicchini et al., 2015; Kistakis and Tooze, 2016). Tumorigenesis of both benign and malignant tumors are controlled by either single or group of ATG genes (Blessing et al., 2017; Mowers et al., 2017). Thus, in most of the cancers, autophagy is key therapeutic choices in clinical trials (Mowers et al., 2016).

There is increasing evidence that a large number of ncRNAs are actively transcribed from the human genome, controlling diverse cellular metabolic process in growth and development of cancers through regulating various gene expressions (Lin and He, 2017). Most diverse ncRNAs, specifically lncRNAs, are largely involved in cancer initiation, maturation, metastasis and resistance against chemotherapy (Lin and He, 2017; Hu et al., 2018). lncRNAs are >200 nucleotides in length and are mostly transcribed by RNA polymerase II. According to GENCODE (version 18), 13,562 lncRNAs have been identified (Huang X. et al., 2017). lncRNAs have independent expressions in different tissues and different cancers and are responsible for various cancers including BRC, CRC, GC, glioma, HCC, leukemia, LVC, LC, OVC, PTC, and retinoblastoma (He et al., 2016; Huang J. et al., 2017; Huang X. et al., 2017; Li et al., 2017b,d,e; Luo et al., 2017; Ma et al., 2017d; Jiang et al., 2018; Li H. et al., 2018).

Over the past decades, advance research evidence has shown that lncRNAs regulate most of the cancers by means of controlling the autophagy process and modulating the transcriptional and post-transcriptional ATGs (Mowers et al., 2017; Zhang J. et al., 2017). In this review, we aim to provide an overview of recently identified autophagy-modulating lncRNAs autophagy in cancers, their mechanisms and future directions on therapeutic intervention.

The field of targeted therapy is rapidly developing with advanced genetic study and their successful applications. Previously considered junk molecules such as lncRNAs are now extensively studied to be established as novel diagnostic biomarkers and therapeutic targets. The biological and physiological roles of autophagy-modulating lncRNAs in carcinogenesis are being unveiled recently. The expression of lncRNAs greatly impacts on the extent of autophagy at different carcinogenic stages, mostly in advanced metastatic stages. A number of research articles suggested that lncRNAs induce or suppress autophagy through ATGs and their signaling pathways (**Table 1**). The complex process of autophagy modulation by expressions of lncRNAs may suppress or promote carcinogenesis under diverse physiological conditions (**Figure 3**). Here we described below the recently characterized lncRNAs and their mechanisms through inducing or inhibiting autophagy in different cancers. We also summarized some autophagy-modulating lncRNA which worth further investigation in the field of cancer.

AUTOPHAGY-INDUCING lncRNAs IN CANCER

Majority of the autophagy-modulating lncRNAs have a positive relationship with the induction of autophagy. Hence, increase expression of these lncRNA in tumor induced autophagy and decrease expression of these lncRNA suppressed autophagy. Some examples are listed as follow.

HOX Transcript Antisense RNA

HOX transcript antisense RNA contains 2158 nucleotides and was discovered by Rinn et al. (2007). It is located on intergenic

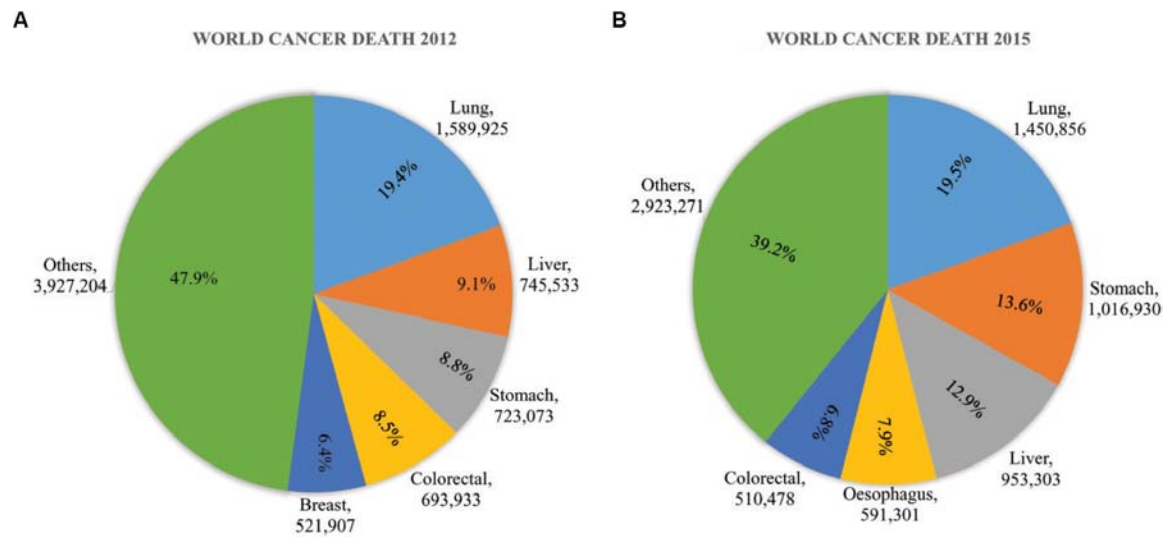


FIGURE 1 | Global cancer impact in (A) 2012 and (B) 2015. In 2012, the death toll 8.2 million from 14 million new cases. It raised gradually to 8.8 million in 2015. The growth of cancer death was more than 7% from 2012 to 2015. The most common cancers were Lung cancer, liver cancer, stomach cancer, colorectal cancer, esophageal cancer, and breast cancer was a major threat of women cancer death worldwide. These figures were based on data published by WHO and GLOBOCAN (Ferlay et al., 2015; GBD 2015 Risk Factors Collaborators, 2016; WHO, 2018).

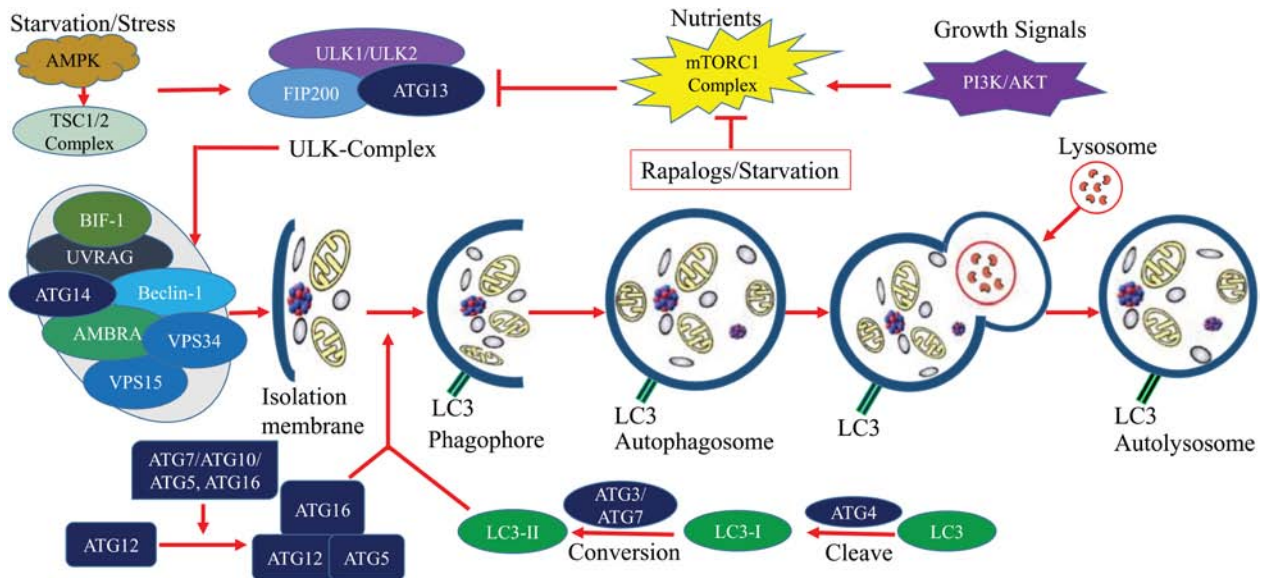


FIGURE 2 | The molecular signaling of the autophagy process. Autophagy is a critical process of cells, it can be simplified into several steps including, Formation of isolation membrane; Nucleation of phagophore; Maturation into autophagosome and Autolysosome. Under starvation/stress, ATP/AMP activates AMPK which further activates TSC1/2 complex. This complex induces autophagy through the blocking of mTOR. Elongation is controlled by ATG7 and ATG10 mediated covalent conjugation with ATG12 and ATG5. ATG5/ATG12 non-covalently bind with ATG16 to form an ATG12/ATG5/ATG16 complex which subsequently adds LC3 to phagophoric barrier. Circular autophagosome formation is carried out by recycling of LC3 (conversion of LC3I to LC3II by ATG4). Mature autophagosome gradually fuses with lysosome for selective degradation and recycling of nutrients. This figure was based on published data (Kim and Lee, 2014; Cicchini et al., 2015; Ktistakis and Tooze, 2016).

space of HOXC11 and HOXC12 in chromosome 12q13.13 (Rinn et al., 2007). Abnormal expression of HOTAIR has been noticed for most of the cancers including, BT, BRC, CRC, GC, LVC, NSCLC, OVC, and PNC (Loewen et al., 2014; Zhou et al., 2014; Miao et al., 2016; Gerardo et al., 2017). Several

research groups have reported HOTAIR association in different cancers evolutionary processes including, EMT, TNM, prognosis, drug resistance, metastasis, DFS, OS, and tumor development (Loewen et al., 2014; Zhou et al., 2014). Recently, a growing number of studies have revealed HOTAIR contexts in the

TABLE 1 | List of autophagy-modulating lncRNAs and their roles in various cancer.

LncRNAs	Expression in cancer	Autophagy modulation	Functions	Sources	Reference
AC023115.3	Up-regulated	Inhibit autophagy	Increase cisplatin -mediated apoptosis in glioma	Glioma cell lines	Ma et al., 2017b
BANCR	Up-regulated	Induce autophagy	Promote PPTC proliferation, growth and reduce cell population at G1 phase.	Cell line and tumor samples	Wang et al., 2014
CASC2	Down-regulated	Induce autophagy	Glioma progression and temozolomide resistance through controlling mTOR	Glioma cells, tissues and peritumoral brain edema tissues	Jiang et al., 2018
CTA	Down-regulated	Induce autophagy	Promote TNM, larger tumor depth and reduced chemosensitivity in OSTS	OSTS cells and tissues (without pre-operative therapy)	Wang Z. et al., 2017
GAS5	Down-regulated	Induce autophagy/inhibit autophagy	Progression of NSCLC and cisplatin resistance; progression of glioma and cisplatin resistance by inducing autophagy	NSCLC cell lines and stage III tumor tissues; Glioma cell lines	Zhang et al., 2016; Huo and Chen, 2018
H19	Up-regulated	Induce autophagy	Promote PPTC pathogenesis by promoting proliferation	PPTC cells and tissues	Li M. et al., 2018
HNF1A-AS1	Up-regulated	Induce autophagy	Promote TNM, increase growth and apoptosis in HCC	HCC cells and surgically resected tissues	Liu et al., 2016
HOTAIR	Up-regulated	Induce autophagy	Cancer progression and drug resistance in HCC and ENDC	HCC cells and tissues; ENDC cell lines	Yang et al., 2016; Bao et al., 2017; Sun et al., 2017
HULC	Up-regulated	Induce autophagy/Inhibit autophagy	Promote GC proliferation, migration, and invasion, and reduced apoptosis by inducing autophagy; chemosensitivity of HCC is promoted by inhibiting autophagy	GC cells and tissues; HCC cell lines and tissues	Zhao et al., 2014; Xiong et al., 2017
LCPAT1	Up-regulated	Induce autophagy	Promote carcinogenesis of LC and knockdown gradually reduce <i>in vivo</i> tumor size	LC cell lines and patients sample	Yu et al., 2018
LncRNA-p21	Up-regulated	Induce autophagy	Promote proliferation, motility, reduce apoptosis and radio-sensitivity in hepatoma and glioma	Glioma cells and urine samples	Işın et al., 2015; Shen et al., 2017
MALAT1	Up-regulated	Induce autophagy	Promote cell proliferation, metastasis, and autophagy induced drug resistance in MM, PTC, and GC	MM cell lines; PNC cells and pancreatic ductal adenocarcinoma tissues; GC cell lines	Li L. et al., 2016; Yuan et al., 2016; Fu et al., 2017; Gao D. et al., 2017; Huang J. et al., 2017; YiRen et al., 2017
MEG3	Down-regulated	Induce autophagy/inhibit autophagy	Reduced expression promote BDC proliferation, colony formation, and progression through inducing autophagy; while upregulation lead to OVC proliferation and progression is raised via inhibiting autophagy	BDC cells and tissues; Epithelial OVC cell and tissues with different stages	Ying et al., 2013; Xiu et al., 2017
PCA3	Up-regulated	Inhibit autophagy	Increase proliferation, migration, and invasion in PTC	PTC cells and tissues	He et al., 2016
POU3F3	Up-regulated	Inhibit autophagy	Promote CRC proliferation and migration	CRC cells and tissues	Shan et al., 2016
PTENP1	Down-regulated	Inhibit autophagy	Promote proliferation and migration in HCC	HCC cell lines	Chen et al., 2015
PVT1	Up-regulated	Induce autophagy	Promote GMEC proliferation migration and angiogenesis	Cerebral microvascular endothelial cell line	Ma et al., 2017c
ROR	Up-regulated	Inhibit autophagy/induce autophagy	Promote proliferation, invasion, migration, tamoxifen resistance and reduced gemcitabine- induced cell death in BRC; up-regulated in PNC to reduce chemosensitivity	BRC cells and tissues (without any therapy); PNC cells and tissues	Chen Y.M. et al., 2016; Li C. et al., 2016; Li et al., 2017f

BDC, bladder cancer; BRC, breast cancer; CRC, colorectal cancer; ENDC, endometrial cancer; GC, gastric cancer; GMEC, glioma microvascular endothelial cancer; HCC, hepatocellular carcinoma; LC, lung cancer; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OSTS, osteosarcoma; OVC, ovarian cancer; PNC, pancreatic cancer; PPTC, papillary thyroid cancer; PTC, prostate cancer; TNM, tumor node metastasis.

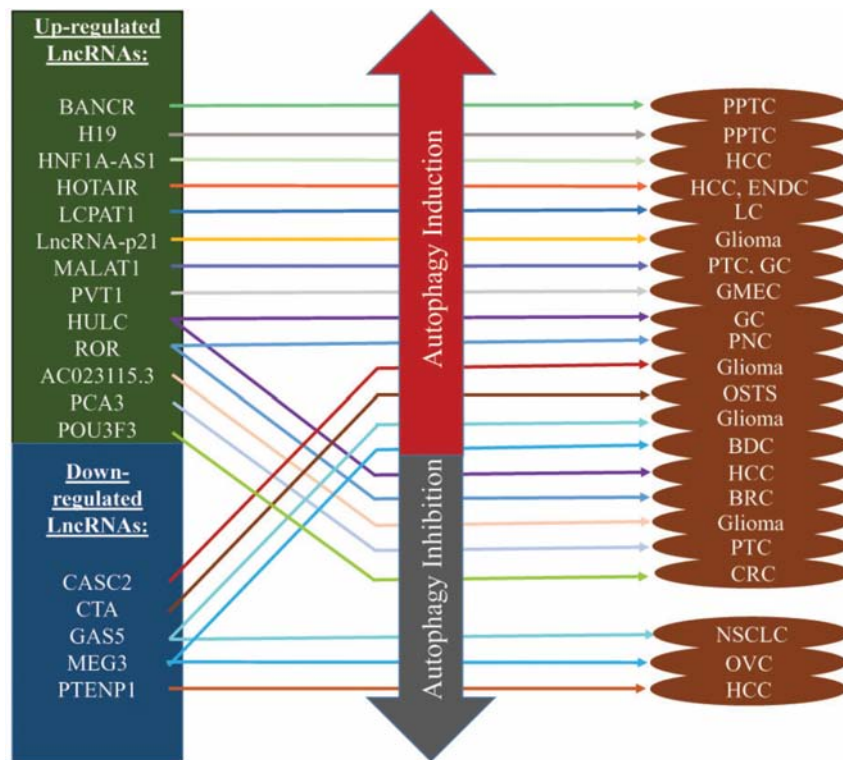


FIGURE 3 | Autophagy-modulating lncRNAs in cancer. Most of the lncRNAs are overexpressed and they induce autophagy to promote/inhibit carcinogenesis. Some of them play key roles in drug resistance while some inhibit autophagy to maintain homeostasis in cancer micro-environments. In the contrary, down-regulated lncRNAs induce/suppress autophagy to promote/inhibit cancer progression.

regulation of autophagy, cancer progression, and drug resistance (Yang et al., 2016; Bao et al., 2017; Sun et al., 2017). Liu's research group proved that, upregulated HOTAIR in HCC cells and tissues induce autophagy by promoting two major ATG3 and ATG7 (Yang et al., 2016). It is also proved that, HOTAIR expression increase along with STAT3 and ATG12 (key of autophagosome formation) through suppressing cancer suppressing micro RNA miR-454-3p in chondrosarcoma (Bao et al., 2017). Sun et al. (2017) revealed that HOTAIR abundance in ENDC cells significantly induce autophagy which controls the development of chemo-resistance toward cisplatin through the expression of Beclin-1 and P-glycoprotein.

Metastasis-Associated Lung Adenocarcinoma Transcript 1

Metastasis-associated lung adenocarcinoma transcript 1 is located on chromosome 11q13, containing over 8.7 kb nucleotides and was first identified in NSCLC since 2003 (Ji et al., 2003). It has been proven that MALAT1 plays significant roles in the development, proliferation, invasion, and metastasis of BDC, BRC, CRC, HCC, LC, NSCLC, and osteosarcoma (OSTS) (Ji et al., 2003; Gutschner et al., 2013; Hou et al., 2017; Li et al., 2017c; Zuo et al., 2017; Xiong et al., 2018). Up-regulated MALAT1 promote proliferation and metastasis of PTC cells and tissues (Li L. et al., 2016) via inducing autophagy. To facilitate

the process, MALAT1 interacts with RNA binding protein HuR to activate autophagy via controlling post-transcriptional effects of cytotoxic granule-associated RNA binding protein TIA1 (Li L. et al., 2016). It has also been postulated that, aberrant expression of MALAT1 modulate autophagy in various cancers including glioma, GC, HCC, and RTB by controlling micro RNAs miR-216b, miR-101, miR-124, and miR-23b-3p (Yuan et al., 2016; Fu et al., 2017; Huang X. et al., 2017; YiRen et al., 2017). To maintain homeostasis of the cancer micro-environments, up-regulated MALAT1 induce conserved autophagy process directly or indirectly to take part in the progression of chemo-resistance and multi-drug resistance (Yuan et al., 2016; YiRen et al., 2017). More recently, YiRen et al. (2017) revealed that MALAT1 regulate GC progression and autophagy-mediated chemo-resistance via controlling micro RNA miR-23b-3p (YiRen et al., 2017). Gao D. et al. (2017) demonstrated that MALAT1 is highly expressed in MM along with HMGB1 to promote carcinogenesis by significantly expressing two key autophagy regulatory proteins LC3B and Beclin 1 (The mammalian ortholog of yeast ATG6). Their *in vivo* investigation suggests that knockdown of MALAT1 would be an effective target of MM growth inhibition by autophagy suppression (Gao D. et al., 2017).

Plasmacytoma Variant Translocation 1

Plasmacytoma variant translocation 1 was first identified in murine leukemia virus-mediated T lymphomas. It contains 1716

nucleotides and is located on chromosome 8q24.21 (Zeidler et al., 1994). After its discovery, the roles of PVT1 have been identified in various cancers, including BDC, BRC, LC, MPM, and NSCLC (Cui et al., 2016; Li et al., 2017d; Lu et al., 2017; Zhang X.W. et al., 2017; Zhou et al., 2017; Guo et al., 2018; Li H. et al., 2018). Ma and coworkers proved that PVT1 is significantly up-regulate in GMEC and promote Atg7 and Beclin-1 expression. They reported that excessive endothelial cell proliferation and migration is mediated by PTV1/Atg7/Beclin-1 (Ma et al., 2017c). Huang and co-workers revealed that expression of PVT1 directly activates ULK1, an autophagy activating protein, in PDA cells, patients sample, and *in vivo* xenograft model. PVT1 promotes pathogenesis by regulating miR-20a-5p (Huang et al., 2018). Thus, PVT1/ULK1/autophagy/miR-20a-5p may lead to being a novel therapeutic target of PDA.

H19

Maternally expressed non-protein coding transcript H19 is lying on the imprinted region of chromosome 11p15.5 and it is 2.3 kb in length (Cui et al., 2002). H19 is transcribed by RNA polymerase II and dysregulation of H19 is associated with BRC, CRC, GC, Glioblastoma, HCC, HNC, LC and NSCLC (Hu et al., 2016; Matouk et al., 2016; Chen et al., 2017b; Luo et al., 2017). Brannan et al. (1990) first discovered H19 as a riboregulator. To date, numerous evidence has been established for H19's association in various human cancers through distinctive molecular pathways (Chen T. et al., 2016; Chen et al., 2017b). Expression of H19 was found to be increased in both PPTC cells and tissues along with estrogen receptor β which may trigger autophagy through regulating ROS and ERK1/2 pathways. Higher expression of H19 promotes PPTC pathogenesis where further investigation may lead to better understanding of PPTC carcinogenesis through H19/autophagy regulation (Li M. et al., 2018).

Others

Increasing number of autophagy-modulating lncRNAs are being identified but some of them attracted less attention. Wang et al. (2014) described, BANCR activate autophagy and contribute to proliferation and apoptosis of both PPTC cells and tissues. The overexpression of BANCR promote conversion of LC3-II/LC3-I, activated autophagy promote cells growth and reduce apoptosis in G1 phase (Wang et al., 2014). lncRNA-p21 is a hypoxia-responsive intergenic non-coding RNA which is highly expressed in hepatoma and glioma (İşin et al., 2015; Shen et al., 2017). Overexpression of lncRNA-p21 is associated with autophagy induction in hepatoma and glioma cells through HIF-1/Akt/mTOR/P70S6K pathways, resulting in excessive proliferation, motility, reduced apoptosis, and reduced radiosensitivity. Therefore, knockdown of lncRNA-p21 is a new target of radiotherapy as its knockdown potentially alter the molecular events and increase radiosensitivity of hypoxic tumor cells (Shen et al., 2017). HNF 1A-AS1 is located on chromosome 12. It is associated with larger tumor size, advanced TNM stage, excessive growth and apoptosis process of HCC cells and tissues through sponging tumor suppressor miR-30b-5p and inducing autophagy (Liu et al., 2016). Yu et al. (2018), recently noticed LCPAT1 lncRNA in LC which is directly regulated with

autophagic flux. The overexpression of LCPAT1 and LC3 was found to be in both LC cells and tumor samples which accelerate the autophagic flux formation to promote carcinogenesis. Whereas, knockdown of LCPAT1 can significantly reduce *in vivo* tumor size by reducing LC3, ATG3, ATG5, ATG7, ATG12, ATG14, and Beclin1 expression (Yu et al., 2018). Chen and co-workers shown that PTENP1 is a lncRNA which is downregulated in HCC and SB based hybrid baculovirus vectors mediated insertion of PTENP1 potentially work as targeted anti-tumor agent in HCC cells by reducing proliferation and migration by activating autophagy in PI3K/AKT pathways (Chen et al., 2015).

AUTOPHAGY-INHIBITING lncRNAs IN CANCER

Some lncRNA has an inverse relationship with autophagy and one example that has been studied extensively is MEG3. MEG3 is an imprinted gene which was first identified in 2000 (Miyoshi et al., 2000). It contains ~1600 nucleotides and is found in 14q32.3 chromosomal position (Ma et al., 2018). Extensive research demonstrated that MEG3 expression is significantly reduced in cancer and it affects the proliferation, migration, and metastasis of most cancers including BRC, CRC, GC, glioma, HCC, LC, NSCLC, and PNC (Gong and Huang, 2017; He et al., 2017; Wang P. et al., 2017; Wei and Wang, 2017; Zhang C.Y. et al., 2017; Ma et al., 2018). Ying et al. (2013) speculated that MEG3 inversely regulate cellular autophagy process via the p53 pathways, and reduced MEG3 induce autophagy to promote BDC proliferation and progression. Down-regulation of MEG3 promote tumorigenesis and progression of epithelial OVC cells proliferation and colony formation through inhibiting autophagy process (Xiu et al., 2017). On the other hand, up-regulation of MEG3 inhibits the expression of autophagy-related proteins LC3, ATG3, and LAMP1 (Xiu et al., 2017). These findings led to the development of MEG3 as a potential biomarker of early diagnosis and treatment of OVC. More recently, Ma et al. (2017a) also proved that MEG3 is association with cisplatin-induced glioma cells death by regulating autophagy.

Others

Jiang et al. (2018) explored a novel relationship of CASC2 in temozolomide (chemotherapy drug) resistance of glioma. CASC2 is negatively downregulated with miR-193a-5p in temozolomide resistant glioma tissues and induce autophagy by controlling mTOR expression to promote drug resistance (Jiang et al., 2018). Ma et al. (2017b) have described lncRNA AC023115.3 up-regulated in glioma cells after cisplatin treatment and induce cisplatin-mediated apoptosis by inhibiting autophagy process via miR-26a/GSK3 β axis. Wang and colleagues showed that lncRNA CTA (CTA) is significantly downregulated in OSTs cells and cancer tissues in contrast with the adjacent normal tissues. CTA downregulated expression is also associated with the advanced TNM stage, larger tumor size and reduced chemosensitivity of doxorubicin through autophagy process (Wang Z. et al., 2017). Micro-RNA miR-210 is negatively regulated with CTA in OSTs and promote apoptosis of OSTs cells, whilst overexpression of

CTA inhibit autophagy and sensitize to doxorubicin subsequently in both *in vitro* and *in vivo* (Wang Z. et al., 2017). PCA3 is a newly identified lncRNA, located on chromosome 9q21-22 and highly specific for PTC (Popa et al., 2007). He et al. (2016) reported that PCA3 is overexpressed in PTC to promote proliferation, migration, and invasion by sponging miR-1261 through inhibiting PRKD3 and blocking protective autophagy. On the other hand, silencing of PCA3 is able to induce protective autophagy and lessen the PTC progression which could be a novel target of personalized treatment (He et al., 2016). Shan's team established that, silencing of lncRNA POU3F3 significantly reduce CRC cells proliferation, migration, and activate autophagy process by enhancing the expression of autophagy-related genes and proteins Beclin-1, ATG5, ATG7, and LC3 II which is novel therapeutic target of CRC (Shan et al., 2016).

AUTOPHAGY-MODULATING lncRNAs EITHER INDUCE OR INHIBIT AUTOPHAGY IN CANCER

In the literature, we found some reports describing the same lncRNA but with opposite relationship with autophagy. Here, we include three examples which have been studied extensively.

Highly Up-Regulated in Liver Cancer

Highly up-regulated in liver cancer was an lncRNA originally characterized in HCC as a significantly overexpressed lncRNA (Panzitt et al., 2007). HULC contains two exons, 1.6k nucleotides in length, located on chromosome 6p24.3. It is significantly dysregulated in most of the cancers including CRC, GC, HCC, OSTs and PNC (Panzitt et al., 2007; Chen et al., 2017c; Li et al., 2017g; Ma et al., 2017d; Shaker et al., 2017; Yu et al., 2017). A number of groups addressed HULC dysregulation and its molecular mechanisms in various cancer cells proliferation, migration, apoptosis, and metastasis but limited reports have focused on autophagy. Zhao et al. (2014) established that overexpression of HULC is clinically correlated with the developmental process of GC by promoted proliferation, migration, invasion, and reduced cellular apoptosis by inducing autophagy. Xiong et al. (2017) found that HULC overexpression induces autophagy and resulting in reduced chemosensitivity of potent chemo drugs 5-fluorouracil, oxaliplatin, and pirarubicin in HCC cells. Moreover, inhibition of protective autophagy by silencing of HULC sensitize these three drugs activity through controlling sirt1 protein in HCC (Xiong et al., 2017). In the contrary, Chen et al. (2017a) reported that HULC suppresses *in vitro* apoptosis and *in vivo* tumor development through autophagy blockage in epithelial ovarian carcinoma. Up-regulated HULC inhibits expression of ATG7, LC3-II, and LAMP1 while activates SQSTM1/p62 to promote carcinogenic events (Chen et al., 2017a).

Growth Arrest-Specific 5

lncRNA GAS5 was first identified in 1988. It is a tumor suppressor lncRNA which contains 630 nucleotides and encoded

at chromosome 1q25 (Schneider et al., 1988). So far, it is well-established that GAS5 plays key roles in diverse molecular functions in cancers (Pickard and Williams, 2015; Ma et al., 2016; Gao Q. et al., 2017). Meta-analysis of GAS5 shown that, it is associated with DFS, OS, LNM and tumor stages (I, II, III, IV) (Gao Q. et al., 2017). GAS5 is well-known for the negative regulation of most cancer cells survival (Song et al., 2014). Zhang and co-workers have demonstrated that down-regulating GAS5 would inhibit autophagy in NSCLC and facilitate drug resistance. Overexpression of GAS5 through vector mediated transfection induced autophagy and promote chemotherapy (cisplatin) response in NSCLC cells (Zhang et al., 2016). Gu et al. (2018) reported that GAS5 expression and autophagy were both downregulated in BRC cells and patients sample GAS5 expression is negatively correlated with depth tumor size, advanced TNM and poor prognosis of diseases. Interestingly, vector-mediated overexpression of GAS5 triggers autophagy and increases LC3, ATG3, and p62 expressions through sponging miR-23a. These findings may be developed into a targeted therapy for BRC through GAS5/miR-23a/ATG3 axis (Gu et al., 2018). However, another experiment conducted by Huo's group also demonstrated reduced GAS5 expression in cisplatin-resistant glioma cell lines. Further investigations on the mechanisms have shown that GAS5 down-regulated glioma cells become resistant to cisplatin by increasing autophagosomes formation (Huo and Chen, 2018).

Regulator of Reprogramming

The lncRNA ROR was first identified in pluripotent stem cells. It contains four exons, totally 2.6 kb in length and is located on chromosome 18q21.31 (Loewer et al., 2010; Zhan et al., 2016). ROR has been reported to be involved in isolated cellular processes, including growth, proliferation, migration, apoptosis, autophagy and metastasis of BRC, CRC, GC, HCC, NPC, and PNC cancers (Takahashi et al., 2014; Pan et al., 2016; Wang S. et al., 2016; Zhan et al., 2016; Li et al., 2017a; Peng et al., 2017; Wang Y. et al., 2017). Chen Y.M. et al. (2016) proved that ROR suppress autophagy and gemcitabine-induced cell death (apoptosis) in BRC cells by regulating miR-34a. In another study, however, Li's team showed that ROR is up-regulated in PNC to promote basal autophagy which suppresses PKM2 and reduce chemo-sensitivity (gemcitabine) of cells (Li C. et al., 2016). Li et al. (2017f) also identified ROR overexpression reduce autophagy to increase proliferation, invasion, migration, and tamoxifen resistance in BRC cells and tissues. On the other hand, silencing of ROR effectively increases the sensitivity of tamoxifen, proliferation, and migration by inducing autophagy (Li et al., 2017f).

DISCUSSION

Advanced technologies have improved our understanding of the role of lncRNAs in cancer. At this stage, it is hard to consider lncRNAs as cancer biomarker because the sensitivity and specificity are still not at the desired level. However, researchers may consider using the autophagy-modulating lncRNAs for the

development of biomarkers and targeted cancer therapy. From the growing research based knowledge on lncRNAs, autophagy and cancers, we may declare that most lncRNAs are involved in tumorigenesis through inducing or inhibiting the autophagy pathway.

Autophagy is a conserved regulatory process which is essential for both normal and cancer microenvironments under stress and hypoxic conditions (Xu et al., 2017). Advances in NGS technologies identified more than 3500 putative lncRNAs and the epigenetic roles of lncRNAs have been identified in many diseases including cancers. The discovery of lncRNAs and their molecular signals in cancers through autophagy process has drawn keen attention by scientists as biomarkers and targets for cancer therapy of lncRNAs/autophagy modulated diseases (Xu et al., 2017). However, autophagy and lncRNAs research is still in its fancy compared to its quantity, limited research methods of relationship establishment studies and inadequate therapeutic and clinical trials. Therefore, a major part is still under investigations. The effects of autophagy-modulating lncRNAs are very controversial from its discovery. Both autophagy and lncRNAs may accelerate the carcinogenesis or suppress the cancers (Wang et al., 2015; Liu et al., 2016; Lu et al., 2017; Ma et al., 2017b; Xu et al., 2017). In addition, the dual roles playing autophagy and lncRNAs made of difficult to comprehend the regulatory mechanisms in cancers (Zhang J. et al., 2017).

The regulatory process of autophagy composed of initiation, phagophore nucleation, autophagosome structure formation, and autolysosomal fusion to degrade the unwanted proteins or cytoplasmic components from the body (Mizushima, 2007). It is now well-established that lncRNAs participate in cellular regulatory autophagy pathways (Xu et al., 2017; Yang et al., 2017; Sun, 2018), for example, H19, BRCA1, MEG3, PTENP1, and MALAT1 are involved in initiation; ROR, loc146880, and AC023115.3 are involved in nucleation; TGF β 2-OT1, GAS5, HNF1A-AS1, PCGEM1, and HULC are involved in elongation; Chast and MALAT1 are involved in lysosomal fusion process (Yang et al., 2017). With the perspective of ATGs genes, lncRNAs provided a new paradigm of gene expression in the autophagy pathway. An increasing number of lncRNAs have been identified in cancer which triggers autophagy to either promotion or suppression of carcinogenesis (Xu et al., 2017; Sun, 2018). It is now a precious research question that, does autophagy impact on the expression of lncRNAs to promote or suppress cancer? So far, PVT1 is the only reported lncRNA which is regulated by autophagy in diabetes (Li Z. et al., 2016). To address this question in cancer we are currently working on autophagy regulated lncRNAs and their impact on CRC pathogenesis.

REFERENCES

- Amaravadi, R., Kimmelman, A. C., and White, E. (2016). Recent insights into the function of autophagy in cancer. *Genes Dev.* 30, 1913–1930. doi: 10.1101/gad.287524.116
- Avalos, Y., Canales, J., Bravo-Sagua, R., Criollo, A., Lavandero, S., and Quest, A. F. (2014). Tumor suppression and promotion by autophagy. *Biomed. Res. Int.* 2014:603980. doi: 10.1155/2014/603980

CONCLUSION AND INSIGHTS

Cancer is the second leading cause of death worldwide. Incidences and deaths are increasing because of poor understanding of the disease, diagnostic techniques, and proper treatments. Progression of treatments remains dissatisfactory because most of the cancer patients were diagnosed after development of metastasis. Recently circulating lncRNAs has been considered for diagnostic and therapeutic purpose even though their mechanisms remain unclear (Qi et al., 2016; Sun, 2017). In this review, we have described the roles autophagy-regulated lncRNAs on cancer (Table 1), and their impact on autophagy regulation through the distinct network (Figure 3). At present, the research on the lncRNAs involvement on autophagy pathways is still in its primary stage. Although every day new lncRNAs are reporting on various cancer type, most of the researchs mainly focused on their impact on cancer pathogenesis. By considering the lncRNAs impact on autophagy mechanisms (Yang et al., 2017), more critical functions underlying autophagy are waiting to be demonstrated. Some autophagy-modulated lncRNAs described in this review are very specific with identical tissue types, for example HULC, PCA3, PVT1 may serve as a potential biomarkers and target therapy in identical cancer types (He et al., 2016; Ma et al., 2017c; Xiong et al., 2017). Therefore, extensive studies are needed to address the interaction between lncRNAs and the complex regulatory autophagy process with selective target genes and signaling transductions for the discovery of new targets, prognostic and diagnostic biomarkers and target therapy.

AUTHOR CONTRIBUTIONS

All authors have participated sufficiently in the work to take public responsibility for the content. We have worked together in the conception, drafting and revising the articles to provide an intellectual content of critical importance to the work described and final approval of the version to be published.

FUNDING

This work was supported by Departmental Start-up and Seed Funding for HL, and Departmental Postgraduate Funds for MK and ST, The Hong Kong Polytechnic University.

- Bao, X., Ren, T., Huang, Y., Sun, K., Wang, S., Liu, K., et al. (2017). Knockdown of long non-coding RNA HOTAIR increases miR-454-3p by targeting Stat3 and Atg12 to inhibit chondrosarcoma growth. *Cell Death Dis.* 8:e2605. doi: 10.1038/cddis.2017.31
- Blessing, A. M., Rajapakse, K., Reddy Bollu, L., Shi, Y., White, M. A., Pham, A. H., et al. (2017). Transcriptional regulation of core autophagy and lysosomal genes by the androgen receptor promotes prostate cancer progression. *Autophagy* 13, 506–521. doi: 10.1080/15548627.2016.1268300

- Bogenrieder, T., and Herlyn, M. (2003). Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene* 22, 6524–6536. doi: 10.1038/sj.onc.1206757
- Brannan, C. I., Dees, E. C., Ingram, R. S., and Tilghman, S. M. (1990). The product of the H19 gene may function as an RNA. *Mol. Cell Biol.* 10, 28–36. doi: 10.1128/MCB.10.1.28
- Chen, C. L., Tseng, Y. W., Wu, J. C., Chen, G. Y., Lin, K. C., Hwang, S. M., et al. (2015). Suppression of hepatocellular carcinoma by baculovirus-mediated expression of long non-coding RNA PTENP1 and MicroRNA regulation. *Biomaterials* 44, 71–81. doi: 10.1016/j.biomaterials.2014.12.023
- Chen, S., Wu, D.-D., Sang, X.-B., Wang, L.-L., Zong, Z.-H., Sun, K.-X., et al. (2017a). The lncRNA HULC functions as an oncogene by targeting ATG7 and ITGB1 in epithelial ovarian carcinoma. *Cell Death Dis.* 8:e3118. doi: 10.1038/cddis.2017.486
- Chen, S., Zhu, J., Wang, F., Guan, Z., Ge, Y., Yang, X., et al. (2017b). lncRNAs and their role in cancer stem cells. *Oncotarget* 8, 110685–110692. doi: 10.18632/oncotarget.22161
- Chen, T., Yang, P., and He, Z. Y. (2016). Long non-coding RNA H19 can predict a poor prognosis and lymph node metastasis: a meta-analysis in human cancer. *Minerva Med.* 107, 251–258.
- Chen, X., Lun, L., Hou, H., Tian, R., Zhang, H., and Zhang, Y. (2017c). The value of lncRNA HULC as a prognostic factor for survival of cancer outcome: a meta-analysis. *Cell. Physiol. Biochem.* 41, 1424–1434. doi: 10.1159/000468005
- Chen, Y.-M., Liu, Y., Wei, H.-Y., Lv, K.-Z., and Fu, P.-F. (2016). Large intergenic non-coding RNA-ROR reverses gemcitabine-induced autophagy and apoptosis in breast cancer cells. *Oncotarget* 7, 59604–59617. doi: 10.18632/oncotarget.10730
- Cicchini, M., Karantza, V., and Xia, B. (2015). Molecular pathways: autophagy in cancer—a matter of timing and context. *Clin. Cancer Res.* 21, 498–504. doi: 10.1158/1078-0432.CCR-13-2438
- Cui, H., Onyango, P., Brandenburg, S., Wu, Y., Hsieh, C. L., and Feinberg, A. P. (2002). Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. *Cancer Res.* 62, 6442–6446.
- Cui, M., You, L., Ren, X., Zhao, W., Liao, Q., and Zhao, Y. (2016). Long non-coding RNA PVT1 and cancer. *Biochem. Biophys. Res. Commun.* 471, 10–14. doi: 10.1016/j.bbrc.2015.12.101
- Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., et al. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* 136, E359–E386. doi: 10.1002/ijc.29210
- Fu, Z., Luo, W., Wang, J., Peng, T., Sun, G., Shi, J., et al. (2017). Malat1 activates autophagy and promotes cell proliferation by sponging miR-101 and upregulating STMN1, RAB5A and ATG4D expression in glioma. *Biochem. Biophys. Res. Commun.* 492, 480–486. doi: 10.1016/j.bbrc.2017.08.070
- Gao, D., Lv, A. E., Li, H. P., Han, D. H., and Zhang, Y. P. (2017). lncRNA MALAT-1 Elevates HMGB1 to promote autophagy resulting in inhibition of tumor cell apoptosis in multiple myeloma. *J. Cell Biochem.* 118, 3341–3348. doi: 10.1002/jcb.25987
- Gao, Q., Xie, H., Zhan, H., Li, J., Liu, Y., and Huang, W. (2017). Prognostic values of long noncoding RNA GAS5 in various carcinomas: an updated systematic review and meta-analysis. *Front. Physiol.* 8:814. doi: 10.3389/fphys.2017.00814
- GBD 2015 Risk Factors Collaborators (2016). Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 388, 1659–1724. doi: 10.1016/S0140-6736(16)31679-8
- Gerardo, B., Laura, M., Maria Gabriella, M., Annamaria, A., Chiara, B., Renato, F., et al. (2017). lncRNA HOTAIR as prognostic circulating marker and potential therapeutic target in patients with tumor diseases. *Curr. Drug Targets* 18, 27–34.
- Gong, X., and Huang, M. (2017). Long non-coding RNA MEG3 promotes the proliferation of glioma cells through targeting Wnt/beta-catenin signal pathway. *Cancer Gene Ther.* 24, 381–385. doi: 10.1038/cgt.2017.32
- Gu, J., Wang, Y., Wang, X., Zhou, D., Wang, X., Zhou, M., et al. (2018). Effect of the lncRNA GAS5-MiR-23a-ATG3 axis in regulating autophagy in patients with breast cancer. *Cell. Physiol. Biochem.* 48, 194–207. doi: 10.1159/000491718
- Guo, D., Wang, Y., Ren, K., and Han, X. (2018). Knockdown of lncRNA PVT1 inhibits tumorigenesis in non-small-cell lung cancer by regulating miR-497 expression. *Exp. Cell Res.* 362, 172–179. doi: 10.1016/j.yexcr.2017.11.014
- Gutschner, T., Hämmerle, M., and Diederichs, S. (2013). MALAT1 — a paradigm for long noncoding RNA function in cancer. *J. Mol. Med.* 91, 791–801. doi: 10.1007/s00109-013-1028-y
- He, J. H., Li, B. X., Han, Z. P., Zou, M. X., Wang, L., Lv, Y. B., et al. (2016). Snail-activated long non-coding RNA PCA3 up-regulates PRKD3 expression by miR-1261 sponging, thereby promotes invasion and migration of prostate cancer cells. *Tumour Biol.* doi: 10.1007/s13277-016-5450-y [Epub ahead of print].
- He, Y., Luo, Y., Liang, B., Ye, L., Lu, G., and He, W. (2017). Potential applications of MEG3 in cancer diagnosis and prognosis. *Oncotarget* 8, 73282–73295. doi: 10.18632/oncotarget.19931
- Hou, Z., Xu, X., Zhou, L., Fu, X., Tao, S., Zhou, J., et al. (2017). The long non-coding RNA MALAT1 promotes the migration and invasion of hepatocellular carcinoma by sponging miR-204 and releasing SIRT1. *Tumour Biol.* 39:1010428317718135. doi: 10.1177/1010428317718135
- Hu, M.-C., He, J.-Y., Hu, Y.-H., Hong, J.-W., and Zhang, J. (2016). Long non-coding RNA H19 as a prognostic marker in human cancer: a meta-analysis. *Int. J. Clin. Exp. Pathol.* 9, 5015–5024.
- Hu, X., Sood, A. K., Dang, C. V., and Zhang, L. (2018). The role of long noncoding RNAs in cancer: the dark matter matters. *Curr. Opin. Genet. Dev.* 48, 8–15. doi: 10.1016/j.gde.2017.10.004
- Huang, F., Chen, W., Peng, J., Li, Y., Zhuang, Y., Zhu, Z., et al. (2018). lncRNA PVT1 triggers Cyto-protective autophagy and promotes pancreatic ductal adenocarcinoma development via the miR-20a-5p/ULK1 Axis. *Mol. Cancer* 17:98. doi: 10.1186/s12943-018-0845-6
- Huang, J., Yang, Y., Fang, F., and Liu, K. (2017). MALAT1 modulates the autophagy of retinoblastoma cell through miR-124-mediated stx17 regulation. *J. Cell Biochem.* 119, 3853–3863. doi: 10.1002/jcb.26464
- Huang, X., Xiao, R., Pan, S., Yang, X., Yuan, W., Tu, Z., et al. (2017). Uncovering the roles of long non-coding RNAs in cancer stem cells. *J. Hematol. Oncol.* 10:62. doi: 10.1186/s13045-017-0428-9
- Huo, J. F., and Chen, X. B. (2018). Long noncoding RNA growth arrest-specific 5 facilitates glioma cell sensitivity to cisplatin by suppressing excessive autophagy in an mTOR-dependent manner. *J. Cell Biochem.* doi: 10.1002/jcb.27900 [Epub ahead of print].
- Işın, M., Uysaler, E., Özgür, E., Köseoğlu, H., Şanlı, Ö., Yücel, Ö. B., et al. (2015). Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. *Front. Genet.* 6:168. doi: 10.3389/fgene.2015.00168
- Ji, P., Diederichs, S., Wang, W., Böing, S., Metzger, R., Schneider, P. M., et al. (2003). MALAT-1, a novel noncoding RNA, and thymosin β_4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* 22, 8031–8041. doi: 10.1038/sj.onc.1206928
- Jiang, C., Shen, F., Du, J., Fang, X., Li, X., Su, J., et al. (2018). Upregulation of CASC2 sensitized glioma to temozolomide cytotoxicity through autophagy inhibition by sponging miR-193a-5p and regulating mTOR expression. *Biomed. Pharmacother.* 97, 844–850. doi: 10.1016/j.biopha.2017.10.146
- Kim, K. H., and Lee, M.-S. (2014). Autophagy—a key player in cellular and body metabolism. *Nat. Rev. Endocrinol.* 10, 322–337. doi: 10.1038/nrendo.2014.35
- Ktistakis, N. T., and Tooze, S. A. (2016). Digesting the expanding mechanisms of autophagy. *Trends Cell Biol.* 26, 624–635. doi: 10.1016/j.tcb.2016.03.006
- Kung, C. P., Budina, A., Balaburski, G., Bergenstock, M. K., and Murphy, M. (2011). Autophagy in tumor suppression and cancer therapy. *Crit. Rev. Eukaryot. Gene Expr.* 21, 71–100. doi: 10.1615/CritRevEukaryotGeneExpr.v21.i1.50
- Li, C., Zhao, Z., Zhou, Z., and Liu, R. (2016). linc-ROR confers gemcitabine resistance to pancreatic cancer cells via inducing autophagy and modulating the miR-124/PTBP1/PKM2 axis. *Cancer Chemother. Pharmacol.* 78, 1199–1207. doi: 10.1007/s00280-016-3178-4
- Li, H., Chen, S., Liu, J., Guo, X., Xiang, X., Dong, T., et al. (2018). Long non-coding RNA PVT1-5 promotes cell proliferation by regulating miR-126/SLC7A5 axis in lung cancer. *Biochem. Biophys. Res. Commun.* 495, 2350–2355. doi: 10.1016/j.bbrc.2017.12.114
- Li, H., Jiang, X., and Niu, X. (2017a). Long non-coding RNA Reprogramming (ROR) promotes cell proliferation in colorectal cancer via affecting P53. *Med. Sci. Monit.* 23, 919–928.

- Li, J., Yang, C., Li, Y., Chen, A., Li, L., and You, Z. (2017b). LncRNA GAS5 suppresses ovarian cancer by inducing inflammasome formation. *Biosci. Rep.* 38: BSR20171150. doi: 10.1042/BSR20171150
- Li, L., Chen, H., Gao, Y., Wang, Y.-W., Zhang, G.-Q., Pan, S.-H., et al. (2016). Long noncoding RNA MALAT1 promotes aggressive pancreatic cancer proliferation and metastasis via the stimulation of autophagy. *Mol. Cancer Ther.* 15, 2232–2243. doi: 10.1158/1535-7163.MCT-16-0008
- Li, M., Chai, H.-F., Peng, F., Meng, Y.-T., Zhang, L.-Z., Zhang, L., et al. (2018). Estrogen receptor β upregulated by lncRNA-H19 to promote cancer stem-like properties in papillary thyroid carcinoma. *Cell Death Dis.* 9, 1120–1120. doi: 10.1038/s41419-018-1077-9
- Li, S., Mei, Z., and Hu, H. B. (2017c). The lncRNA MALAT1 contributes to non-small cell lung cancer development via modulating miR-124/STAT3 axis. *J. Cell. Physiol.* 233, 6679–6688. doi: 10.1002/jcp.26325
- Li, X., Chen, W., Wang, H., Wei, Q., Ding, X., and Li, W. (2017d). Amplification and the clinical significance of circulating cell-free DNA of PVT1 in breast cancer. *Oncol. Rep.* 38, 465–471. doi: 10.3892/or.2017.5650
- Li, Y., Gu, J., and Lu, H. (2017e). The GAS5/miR-222 axis regulates proliferation of gastric cancer cells through the PTEN/Akt/mTOR pathway. *Dig. Dis. Sci.* 62, 3426–3437. doi: 10.1007/s10620-017-4831-4
- Li, Y., Jiang, B., Zhu, H., Qu, X., Zhao, L., Tan, Y., et al. (2017f). Inhibition of long non-coding RNA ROR reverses resistance to Tamoxifen by inducing autophagy in breast cancer. *Tumour Biol.* 39:1010428317705790. doi: 10.1177/1010428317705790
- Li, Z., Dou, P., Liu, T., and He, S. (2017g). Application of long noncoding RNAs in osteosarcoma: biomarkers and therapeutic targets. *Cell. Physiol. Biochem.* 42, 1407–1419. doi: 10.1159/000479205
- Li, Z., Hao, S., Yin, H., Gao, J., and Yang, Z. (2016). Autophagy ameliorates cognitive impairment through activation of PVT1 and apoptosis in diabetes mice. *Behav. Brain Res.* 305, 265–277. doi: 10.1016/j.bbr.2016.03.023
- Lin, C.-P., and He, L. (2017). Noncoding RNAs in cancer development. *Annu. Rev. Cancer Biol.* 1, 163–184. doi: 10.1146/annurev-cancerbio-050216-034443
- Liu, Z., Wei, X., Zhang, A., Li, C., Bai, J., and Dong, J. (2016). Long non-coding RNA HNF1A-AS1 functioned as an oncogene and autophagy promoter in hepatocellular carcinoma through sponging hsa-miR-30b-5p. *Biochem. Biophys. Res. Commun.* 473, 1268–1275. doi: 10.1016/j.bbrc.2016.04.054
- Loewen, G., Jayawickramarajah, J., Zhuo, Y., and Shan, B. (2014). Functions of lncRNA HOTAIR in lung cancer. *J. Hematol. Oncol.* 7:90. doi: 10.1186/s13045-014-0090-4
- Loewer, S., Cabili, M. N., Guttman, M., Loh, Y. H., Thomas, K., Park, I. H., et al. (2010). Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. *Nat. Genet.* 42, 1113–1117. doi: 10.1038/ng.710
- Lu, D., Luo, P., Wang, Q., Ye, Y., and Wang, B. (2017). lncRNA PVT1 in cancer: a review and meta-analysis. *Clin. Chim. Acta* 474, 1–7. doi: 10.1016/j.cca.2017.08.038
- Luo, J., Qu, J., Wu, D.-K., Lu, Z.-L., Sun, Y.-S., and Qu, Q. (2017). Long non-coding RNAs: a rising biotarget in colorectal cancer. *Oncotarget* 8, 22187–22202. doi: 10.18632/oncotarget.14728
- Ma, B., Gao, Z., Lou, J., Zhang, H., Yuan, Z., Wu, Q., et al. (2017a). Long noncoding RNA MEG3 contributes to cisplatin-induced apoptosis via inhibition of autophagy in human glioma cells. *Mol. Med. Rep.* 16, 2946–2952. doi: 10.3892/mmr.2017.6897
- Ma, B., Yuan, Z., Zhang, L., Lv, P., Yang, T., Gao, J., et al. (2017b). Long non-coding RNA AC023115.3 suppresses chemoresistance of glioblastoma by reducing autophagy. *Biochim. Biophys. Acta* 1864, 1393–1404. doi: 10.1016/j.bbamcr.2017.05.008
- Ma, Y., Wang, P., Xue, Y., Qu, C., Zheng, J., Liu, X., et al. (2017c). PVT1 affects growth of glioma microvascular endothelial cells by negatively regulating miR-186. *Tumour Biol.* 39:1010428317694326. doi: 10.1177/1010428317694326
- Ma, Z., Huang, H., Xu, Y., He, X., Wang, J., Hui, B., et al. (2017d). Current advances of long non-coding RNA highly upregulated in liver cancer in human tumors. *Onco Targets Ther.* 10, 4711–4717. doi: 10.2147/OTT.S136915
- Ma, C., Shi, X., Zhu, Q., Li, Q., Liu, Y., Yao, Y., et al. (2016). The growth arrest-specific transcript 5 (GAS5): a pivotal tumor suppressor long noncoding RNA in human cancers. *Tumor Biol.* 37, 1437–1444. doi: 10.1007/s13277-015-4521-9
- Ma, L., Wang, F., Du, C., Zhang, Z., Guo, H., Xie, X., et al. (2018). Long non-coding RNA MEG3 functions as a tumour suppressor and has prognostic predictive value in human pancreatic cancer. *Oncol. Rep.* 39, 1132–1140. doi: 10.3892/or.2018.6178
- Matouk, I. J., Halle, D., Raveh, E., Gilon, M., Sorin, V., and Hochberg, A. (2016). The role of the oncofetal H19 lncRNA in tumor metastasis: orchestrating the EMT-MET decision. *Oncotarget* 7, 3748–3765. doi: 10.18632/oncotarget.6387
- Miao, Z., Ding, J., Chen, B., Yang, Y., and Chen, Y. (2016). HOTAIR overexpression correlated with worse survival in patients with solid tumors. *Minerva Med.* 107, 392–400.
- Miyoshi, N., Wagatsuma, H., Wakana, S., Shiroishi, T., Nomura, M., Aisaka, K., et al. (2000). Identification of an imprinted gene, Meg3/Gtl2 and its human homologue MEG3, first mapped on mouse distal chromosome 12 and human chromosome 14q. *Genes Cells* 5, 211–220. doi: 10.1046/j.1365-2443.2000.00320.x
- Mizushima, N. (2007). Autophagy: process and function. *Genes Dev.* 21, 2861–2873. doi: 10.1101/gad.1599207
- Mizushima, N., and Komatsu, M. (2011). Autophagy: renovation of cells and tissues. *Cell* 147, 728–741. doi: 10.1016/j.cell.2011.10.026
- Mowers, E. E., Sharifi, M. N., and Macleod, K. F. (2016). Autophagy in cancer metastasis. *Oncogene* 36, 1619–1630. doi: 10.1038/onc.2016.333
- Mowers, E. E., Sharifi, M. N., and Macleod, K. F. (2017). Autophagy in cancer metastasis. *Oncogene* 36, 1619–1630. doi: 10.1038/onc.2016.333
- Pan, Y., Li, C., Chen, J., Zhang, K., Chu, X., Wang, R., et al. (2016). The Emerging roles of long noncoding RNA ROR (lincRNA-ROR) and its possible mechanisms in human cancers. *Cell. Physiol. Biochem.* 40, 219–229. doi: 10.1159/000452539
- Panzitt, K., Tschernatsch, M. M., Guelly, C., Moustafa, T., Stradner, M., Strohmaier, H. M., et al. (2007). Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA. *Gastroenterology* 132, 330–342. doi: 10.1053/j.gastro.2006.08.026
- Peng, W. X., Huang, J. G., Yang, L., Gong, A. H., and Mo, Y. Y. (2017). Linc-RoR promotes MAPK/ERK signaling and confers estrogen-independent growth of breast cancer. *Mol. Cancer* 16:161. doi: 10.1186/s12943-017-0727-3
- Pickard, M. R., and Williams, G. T. (2015). Molecular and cellular mechanisms of action of tumour suppressor GAS5 lncRNA. *Genes* 6, 484–499. doi: 10.3390/genes6030484
- Popa, I., Fradet, Y., Beaudry, G., Hovington, H., Beaudry, G., and Têtu, B. (2007). Identification of PCA3 (DD3) in prostatic carcinoma by in situ hybridization. *Mod. Pathol.* 20, 1121–1127. doi: 10.1038/modpathol.3800963
- Qi, P., Zhou, X. Y., and Du, X. (2016). Circulating long non-coding RNAs in cancer: current status and future perspectives. *Mol. Cancer* 15:39. doi: 10.1186/s12943-016-0524-4
- Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Bruggmann, S. A., et al. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129, 1311–1323. doi: 10.1016/j.cell.2007.05.022
- Sankaranarayanan, R., Ramadas, K., and Qiao, Y.-L. (2014). Managing the changing burden of cancer in Asia. *BMC Med.* 12:3. doi: 10.1186/1741-7015-12-3
- Santana-Codina, N., Mancias, J. D., and Kimmelman, A. C. (2017). The role of autophagy in cancer. *Annu. Rev. Cancer Biol.* 1, 19–39. doi: 10.1146/annurev-cancerbio-041816-122338
- Schneider, C., King, R. M., and Philipson, L. (1988). Genes specifically expressed at growth arrest of mammalian cells. *Cell* 54, 787–793. doi: 10.1016/S0092-8674(88)91065-3
- Shaker, O. G., Senousy, M. A., and Elbaz, E. M. (2017). Association of rs6983267 at 8q24, HULC rs7763881 polymorphisms and serum lncRNAs CCAT2 and HULC with colorectal cancer in Egyptian patients. *Sci. Rep.* 7:16246. doi: 10.1038/s41598-017-16500-4
- Shan, T. D., Xu, J. H., Yu, T., Li, J. Y., Zhao, L. N., Ouyang, H., et al. (2016). Knockdown of linc-POU3F3 suppresses the proliferation, apoptosis, and migration resistance of colorectal cancer. *Oncotarget* 7, 961–975. doi: 10.18632/oncotarget.5830
- Shen, Y., Liu, Y., Sun, T., and Yang, W. (2017). LincRNA-p21 knockdown enhances radiosensitivity of hypoxic tumor cells by reducing autophagy through HIF-1/Akt/mTOR/P70S6K pathway. *Exp. Cell Res.* 358, 188–198. doi: 10.1016/j.yexcr.2017.06.016

- Song, J., Ahn, C., Chun, C.-H., and Jin, E.-J. (2014). A long non-coding RNA, GAS5, plays a critical role in the regulation of miR-21 during osteoarthritis. *J. Orthop. Res.* 32, 1628–1635. doi: 10.1002/jor.22718
- Sun, M. Y., Zhu, J. Y., Zhang, C. Y., Zhang, M., Song, Y. N., Rahman, K., et al. (2017). Autophagy regulated by lncRNA HOTAIR contributes to the cisplatin-induced resistance in endometrial cancer cells. *Biotechnol. Lett.* 39, 1477–1484. doi: 10.1007/s10529-017-2392-4
- Sun, T. (2017). Long noncoding RNAs act as regulators of autophagy in cancer. *Pharmacol. Res.* 129, 151–155. doi: 10.1016/j.phrs.2017.11.009
- Sun, T. (2018). Long noncoding RNAs act as regulators of autophagy in cancer. *Pharmacol. Res.* 129, 151–155. doi: 10.1016/j.phrs.2017.11.009
- Takahashi, K., Yan, I. K., Kogure, T., Haga, H., and Patel, T. (2014). Extracellular vesicle-mediated transfer of long non-coding RNA ROR modulates chemosensitivity in human hepatocellular cancer. *FEBS Open Biol.* 4, 458–467. doi: 10.1016/j.fob.2014.04.007
- Wang, K., Liu, C. Y., Zhou, L. Y., Wang, J. X., Wang, M., Zhao, B., et al. (2015). APF lncRNA regulates autophagy and myocardial infarction by targeting miR-188-3p. *Nat. Commun.* 6:6779. doi: 10.1038/ncomms7779
- Wang, P., Chen, D., Ma, H., and Li, Y. (2017). LncRNA MEG3 enhances cisplatin sensitivity in non-small cell lung cancer by regulating miR-21-5p/SOX7 axis. *Onco Targets Ther.* 10, 5137–5149. doi: 10.2147/OTT.S146423
- Wang, S., Liu, F., Deng, J., Cai, X., Han, J., and Liu, Q. (2016). Long noncoding RNA ROR regulates proliferation, invasion, and stemness of gastric cancer stem cell. *Cell Reprogram.* 18, 319–326. doi: 10.1089/cell.2016.0001
- Wang, Y., Guo, Z., Zhao, Y., Jin, Y., An, L., Wu, B., et al. (2017). Genetic polymorphisms of lncRNA-p53 regulatory network genes are associated with concurrent chemoradiotherapy toxicities and efficacy in nasopharyngeal carcinoma patients. *Sci. Rep.* 7:8320. doi: 10.1038/s41598-017-08890-2
- Wang, Z., Liu, Z., and Wu, S. (2017). Long non-coding RNA CTA sensitizes osteosarcoma cells to doxorubicin through inhibition of autophagy. *Oncotarget* 8, 31465–31477. doi: 10.18632/oncotarget.16356
- Wang, Z., Yang, H., Wang, X., Wang, L., Cheng, Y., Zhang, Y., et al. (2016). The molecular mechanism and regulatory pathways of cancer stem cells. *Cancer Transl. Med.* 2, 147–153. doi: 10.4103/2395-3977.192932
- Wang, Y., Guo, Q., Zhao, Y., Chen, J., Wang, S., Hu, J., et al. (2014). BRAF-activated long non-coding RNA contributes to cell proliferation and activates autophagy in papillary thyroid carcinoma. *Oncol. Lett.* 8, 1947–1952. doi: 10.3892/ol.2014.2487
- Wei, G. H., and Wang, X. (2017). lncRNA MEG3 inhibit proliferation and metastasis of gastric cancer via p53 signaling pathway. *Eur. Rev. Med. Pharmacol. Sci.* 21, 3850–3856.
- White, E. (2015). The role for autophagy in cancer. *J. Clin. Invest.* 125, 42–46. doi: 10.1172/JCI73941
- WHO (2018). *Cancer (Fact Sheet) [Online]. World Health Organization.* Available: <http://www.who.int/mediacentre/factsheets/fs297/en/> [accessed Feb 26, 2018]
- Xiong, H., Ni, Z., He, J., Jiang, S., Li, X., He, J., et al. (2017). LncRNA HULC triggers autophagy via stabilizing Sirt1 and attenuates the chemosensitivity of HCC cells. *Oncogene* 36, 3528–3540. doi: 10.1038/onc.2016.521
- Xiong, Y., Wang, J., Zhu, H., Liu, L., and Jiang, Y. (2018). Chronic oxymatrine treatment induces resistance and epithelialmesenchymal transition through targeting the long non-coding RNA MALAT1 in colorectal cancer cells. *Oncol. Rep.* 39, 967–976. doi: 10.3892/or.2018.6204
- Xiu, Y. L., Sun, K. X., Chen, X., Chen, S., Zhao, Y., Guo, Q. G., et al. (2017). Upregulation of the lncRNA Meg3 induces autophagy to inhibit tumorigenesis and progression of epithelial ovarian carcinoma by regulating activity of ATG3. *Oncotarget* 8, 31714–31725. doi: 10.18632/oncotarget.15955
- Xu, Z., Yan, Y., Qian, L., and Gong, Z. (2017). Long non-coding RNAs act as regulators of cell autophagy in diseases (Review). *Oncol. Rep.* 37, 1359–1366. doi: 10.3892/or.2017.5416
- Yang, L., Wang, H., Shen, Q., Feng, L., and Jin, H. (2017). Long non-coding RNAs involved in autophagy regulation. *Cell Death Dis.* 8:e3073. doi: 10.1038/cddis.2017.464
- Yang, L., Zhang, X., Li, H., and Liu, J. (2016). The long noncoding RNA HOTAIR activates autophagy by upregulating ATG3 and ATG7 in hepatocellular carcinoma. *Mol. Biosyst.* 12, 2605–2612. doi: 10.1039/c6mb00114a
- Ying, L., Huang, Y., Chen, H., Wang, Y., Xia, L., Chen, Y., et al. (2013). Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer. *Mol. Biosyst.* 9, 407–411. doi: 10.1039/c2mb25386k
- YiRen, H., Yingcong, Y., Sunwu, Y., Keqin, L., Xiaochun, T., Senrui, C., et al. (2017). Long noncoding RNA MALAT1 regulates autophagy associated chemoresistance via miR-23b-3p sequestration in gastric cancer. *Mol. Cancer* 16:174. doi: 10.1186/s12943-017-0743-3
- Yu, X., Ye, X., Lin, H., Feng, N., Gao, S., Zhang, X., et al. (2018). Knockdown of long non-coding RNA LCPAT1 inhibits autophagy in lung cancer. *Cancer Biol. Med.* 15, 228–237. doi: 10.20892/j.issn.2095-3941.2017.0150
- Yu, X., Zheng, H., Chan, M. T. V., and Wu, W. K. K. (2017). HULC: an oncogenic long non-coding RNA in human cancer. *J. Cell. Mol. Med.* 21, 410–417. doi: 10.1111/jcmm.12956
- Yuan, P., Cao, W., Zang, Q., Li, G., Guo, X., and Fan, J. (2016). The HIF-2 α -MALAT1-miR-216b axis regulates multi-drug resistance of hepatocellular carcinoma cells via modulating autophagy. *Biochem. Biophys. Res. Commun.* 478, 1067–1073. doi: 10.1016/j.bbrc.2016.08.065
- Zeidler, R., Lipp, M., Joos, S., Delecluse, H.-J., Bornkamm, G. W., Klobbeck, G., et al. (1994). Breakpoints of burkitt's lymphoma t(8;22) translocations map within a distance of 300 kb downstream of MYC. *Genes Chromosomes Cancer* 9, 282–287. doi: 10.1002/gcc.2870090408
- Zhan, H. X., Wang, Y., Li, C., Xu, J. W., Zhou, B., Zhu, J. K., et al. (2016). LincRNA-ROR promotes invasion, metastasis and tumor growth in pancreatic cancer through activating ZEB1 pathway. *Cancer Lett.* 374, 261–271. doi: 10.1016/j.canlet.2016.02.018
- Zhang, C. Y., Yu, M. S., Li, X., Zhang, Z., Han, C. R., and Yan, B. (2017). Overexpression of long non-coding RNA MEG3 suppresses breast cancer cell proliferation, invasion, and angiogenesis through AKT pathway. *Tumour Biol.* 39: 1010428317701311. doi: 10.1177/1010428317701311
- Zhang, J., Wang, P., Wan, L., Xu, S., and Pang, D. (2017). The emergence of noncoding RNAs as Heracles in autophagy. *Autophagy* 13, 1004–1024. doi: 10.1080/15548627.2017.1312041
- Zhang, N., Yang, G. Q., Shao, X. M., and Wei, L. (2016). GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells. *Eur. Rev. Med. Pharmacol. Sci.* 20, 2271–2277.
- Zhang, X. W., Liu, L., Zhang, X. Z., and Bo, P. (2017). Kanglaite inhibits the expression of drug resistance genes through suppressing PVT1 in cisplatin-resistant gastric cancer cells. *Exp. Ther. Med.* 14, 1789–1794. doi: 10.3892/etm.2017.4650
- Zhao, Y., Guo, Q., Chen, J., Hu, J., Wang, S., and Sun, Y. (2014). Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation. *Oncol. Rep.* 31, 358–364. doi: 10.3892/or.2013.2850
- Zhou, D.-D., Liu, X.-F., Lu, C.-W., Pant, O. P., and Liu, X.-D. (2017). Long non-coding RNA PVT1: Emerging biomarker in digestive system cancer. *Cell Proliferation* 50:e12398. doi: 10.1111/cpr.12398
- Zhou, X., Chen, J., and Tang, W. (2014). The molecular mechanism of HOTAIR in tumorigenesis, metastasis, and drug resistance. *Acta Biochim. Biophys. Sin.* 46, 1011–1015. doi: 10.1093/abbs/gmu104
- Zuo, Y., Li, Y., Zhou, Z., Ma, M., and Fu, K. (2017). Long non-coding RNA MALAT1 promotes proliferation and invasion via targeting miR-129-5p in triple-negative breast cancer. *Biomed. Pharmacother.* 95, 922–928. doi: 10.1016/j.biopha.2017.09.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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miRNA–mRNA Associated With Survival in Endometrial Cancer

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
RNA, a section of the journal
Frontiers in Genetics

Received: 29 September 2018

Accepted: 16 July 2019

Published: 20 August 2019

Citation:

Xu X, Liu T, Wang Y, Fu J, Yang Q,
Wu J and Zhou H (2019) miRNA–
mRNA Associated With Survival
in Endometrial Cancer.
Front. Genet. 10:743.
doi: 10.3389/fgene.2019.00743

Although various factors may contribute to its initiation and progression, the etiology and prognostic factors of endometrial carcinoma (EC) remains not fully understood. We sought to understand the role of changes in transcriptome during the progress of EC by exploring public datasets. The aberrant expression characteristics of EC based on RNA-Seq and miRNA-seq data from The Cancer Genome Atlas (TCGA) were analyzed. Kaplan–Meier analysis was performed to assess the relationship between differently expressed genes (DEGs) and patient survival. As a result, 320 out of 4,613 differently expressed mRNAs (DE mRNAs) and 68 out of 531 differently expressed miRNAs (DE miRNAs) with a significantly poorer survival were determined. We predicted eight paired DE miRNAs and DE mRNAs through TargetScan. Patients with three out of the eight paired low rate of miRNA/mRNA (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) expression had a significantly poorer survival. Furthermore, the simultaneous presence of these selected low miRNA/mRNA pairs occurred in most patients and resulted in a significantly poorer survival rate. Luciferase reporter assay identified that EMX1 was a direct target of miR-497. Both low expression of miR-497 and overexpression of EMX1 were significantly associated with more advanced clinicopathologic characteristics (stage, tumor status, grade, and histology) besides survival (all P values < 0.05). Multivariate analysis also demonstrated that miR-497 remained an independent prognostic variable for overall survival. In summary, we identified that a series of DE mRNAs and miRNAs, including eight paired DE miRNAs and mRNAs, were associated with survival in EC. Clinical evaluation of downregulated miR-497 and paired upregulated EMX1 confirmed the value of our prediction analysis. The simultaneous presence of low rate of these selected low miRNA/mRNA pairs (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) might have a better prediction value. Therefore, further studies are required to validate these findings.

Keywords: endometrial carcinoma, miR-497, EMX1, survival, TCGA

INTRODUCTION

Endometrial cancer (EC) is one of the most prevalent gynecological malignancies in the world (Siegel et al., 2018). The incidence has increased by 1.2% per year from 2005 to 2014, along with the mortality rates, which similarly increased during this period (Smith et al., 2018). Importantly, EC is one of only two common cancers that defy the general trend of improvement in morbidity and mortality, with a worse survival rate today than in the 1970s (Siegel et al., 2018). The American

Cancer Society (ACS) estimates that 63,230 women will be diagnosed with EC and that more than 11,000 women will die from this disease in 2018 (Siegel et al., 2018). Clearly, the burden of EC is increasing in the USA and worldwide, hence increasing the need to investigate its causes and improve prevention, early diagnosis, and treatment (Binder and Mutch, 2014). Although various endocrine, genetic, and external factors may contribute to its initiation and progression, the etiology and prognostic factors of endometrial carcinoma remain not fully understood.

Previous analysis of the human genome revealed that while ~85% is transcribed, only ~1% is protein-coding mRNA (Dunham et al., 2012; Djebali et al., 2012; Dykes and Emanueli, 2017). With time, more and more attention was being paid to the research of non-coding RNAs, including microRNAs (miRNAs), which have important regulatory roles in cancer cellular biology. Studies demonstrated that mature miRNAs can regulate the expression of their target genes by impeding complementation to the 3'-untranslated regions (UTRs), 5'-UTRs, and even coding sequences of the mRNAs to repress their translation (Ambros, 2004; Duursma et al., 2008; Sacco and Masotti, 2012). Srivastava et al. reviewed the amount of miRNAs differentially expressed in EC versus normal endometrial tissue, including the increased expression of miR-9, miR-92a, miR-141, miR-182, miR-183, miR-186, miR-200a, miR-205a, miR-222, miR-223, miR-410, miR-429, miR-449, and miR-1228 and downregulation of miR-99b, miR-143, miR-145, miR-193b, and miR-204 (Srivastava et al., 2017). Numerous miRNAs could regulate EC cells by silencing their target genes (Zhou et al., 2012; Chen et al., 2016). Though expression patterns of several miRNAs were found to be associated with the International Federation of Gynaecology and Obstetrics (FIGO) stage, grade, relapse, and nodal metastases in EC (Torres et al., 2013; Canlorbe et al., 2016; Srivastava et al., 2017), few studies focused on the relationship between both miRNAs and their target genes with patient survival.

The Cancer Genome Atlas (TCGA) studies have defined the molecular genetic landscape of EC and highlighted the molecular genetic diversity of both endometrioid and non-endometrioid cancers (Burk et al., 2017). Recently, accompanied by the advent era of sharing information, more and more cancer researches including EC were carried out based on TCGA database (Dellinger et al., 2016; Reyes et al., 2017; Wang et al., 2018; Wu and Zhang, 2018). However, the previous studies mainly focused on the single factor, such as differentially expressed mRNAs (DE mRNAs), differentially expressed miRNAs (DE miRNAs), or differentially expressed long non-coding RNAs (DE lncRNAs) to find the potential etiology and prognostic factor of tumor.

In this study, considering the silence effect to their target genes of miRNAs, we performed survival analysis on both DE mRNAs and DE miRNAs comparing EC and normal samples from TCGA database. As a result, we revealed a group of DE mRNAs and DE miRNAs associated with survival. Furthermore, we identified that low expression of miR-497 and overexpression of its potential target gene Empty Spiracles Homeobox 1 (EMX1) were both related to more advanced clinicopathologic characteristics.

METHODS

Data Collection

Expression profiles of RNA-Seq and miRNA-seq for TCGA-UCEC project were downloaded from TCGA official website (<https://cancergenome.nih.gov>) in July 2018. RNA expression data of 543 EC cases and 35 normal cases were downloaded from the database, while miRNA expression data of 539 EC and 33 normal samples were included. The corresponding clinical information was downloaded from <http://www.cbioportal.org>. Data were collated and extracted for analysis.

Identification of Differently Expressed Genes (DEGs)

We applied the expression profiles to the edgeR package in R language and calculated for differential expression between tumor and normal group samples after normalization and filtration. The adjusted *P* values (adj. *P*) were applied to correct the false-positive results. Then the significant DEGs (adj. *P* < 0.05 and fold-change value larger than 2) were selected out for the next step analysis.

Survival Analysis

In this study, survival analysis refers to the overall survival (OS) Kaplan–Meier estimate. We performed Kaplan–Meier analysis (R package “survival”) to assess survival and relapse difference across cases with DEGs. For each gene, patients were divided into high-expression group and low-expression group according to the median expression level. Based on the survival curves of each group, these upregulated and downregulated DEGs with a poorer survival rate were within our consideration. *P* value < 0.05 was set as the cutoff point.

Functional and Pathway Enrichment Analysis of DEGs

The Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>) is a biological database regularly used to facilitate functional annotation and pathway analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis aims to identify and visualize significantly enriched pathways of molecular interactions, reactions, and relations. Gene Ontology (GO) analysis uses hypergeometric tests to perform enrichment analysis on gene sets (Falcon and Gentleman, 2007). We uploaded selected DEGs associated with survival to DAVID to perform KEGG pathway and GO enrichment analysis. The human genome was selected as the background list parameter, and *P* value < 0.05 was set as the cutoff point.

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether a predefined set of genes shows significant differences between two biological phenotypes under study (Subramanian et al., 2005). Gene set permutations were performed 1,000 times for each analysis. The nominal *P* value (NOM *P* value) and normalized enrichment score (NES) were used to sort the pathways enriched in each phenotype.

Prediction of DE miRNA-Targeted DE mRNAs Associated With OS

In order to improve the validity of our search results, we further excavated the relationship between the selected DE mRNAs and DE miRNAs through TargetScan (http://www.targetscan.org/vert_72/). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved sites that match the seed region of each miRNA (Fromm et al., 2015). In brief, we not only searched for the presence of conserved sites among these DE mRNA that matched the seed region of DE miRNA but also searched for DE miRNA containing matched seed region for DE mRNA. These poorly conserved sites were excluded. Only these enrolled miRNA-mRNA pairs with opposite expression levels in EC samples as compared with normal tissues both presented in our dataset were adapted for further study.

Cell Culture and Luciferase Reporter Assay

Cell culture and Luciferase reporter assay were very similar with those in our previous study (Zhou et al., 2012). Briefly, 293T cell and human endometrial cancer Ishikawa cell were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin (100 U/ml). The 300-nt-long 3'-UTR (wild type or mutant) of EMX1 containing the predicted conserved binding sites for miR-497 was cloned into H306 pMIR-LUC vectors purchased from Obio Technology (Shanghai, China). Cells were plated at a density of 1×10^5 in 12-well plate. After 24 h, the pMIR-LUC reporters were co-transfected with either miR-497 mimics or control using Lipofectamine 3000. Luciferase activity normalized to the Renilla Luciferase was measured by the Dual-Luciferase assay (Promega, USA) according to the manufacturer's instructions after 48 h on the Luminometer (Promega, USA). The assay was repeated three times.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from cells with isolator reagent (Vazyme, China). After measurement of the RNA concentration, cDNAs were generated from reverse transcription with the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China). The expression level of miR-497 was measured according to the instructions of the ChamQ™ Universal SYBR qPCR Master Mix kit (Vazyme, China) using the ABI-7300 Real-Time PCR Detection System (Applied Biosystems, USA). The bulge-loop™ miRNA Primer Sets (one RT primer and a pair of qPCR primers) specific for miR-497 were purchased from RiboBio (Guangzhou, China). The level of miR-497 was normalized to U6. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Each plate was run in triplicate.

Statistical Analysis

Logistic regression and *t* test were used to evaluate the relationship between gene expression and clinical-pathologic features. For gene expression level, the cutoff value was determined by its absolute median expression level (high- and low-expression groups). For the analysis of survival for patients with paired

miRNA/mRNA, the median rate of miRNA/mRNA expression was used to divide groups for low and high rates. In logistic regression, the absolute gene expression level (high- and low-expression group) was used as categorical dependent variable, while clinical-pathologic feature was used as independent variable. Among all the clinical-pathologic features, age and BMI were calculated as continuous variables and the rest as categorical variables. Kaplan-Meier method and Cox regression were used to analyze clinicopathologic characteristics associated with OS. The influence of gene expression on survival along with other clinical characteristics was determined by multivariate Cox analysis. *P* value < 0.05 was set as the cutoff point. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

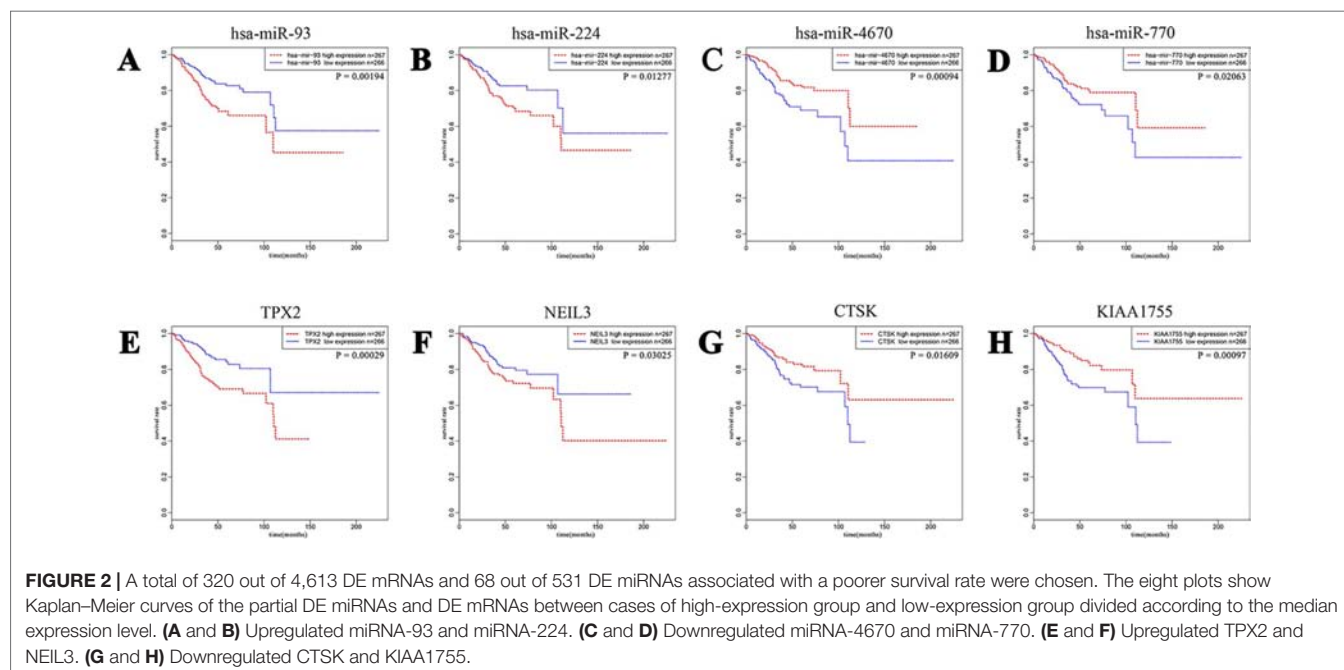
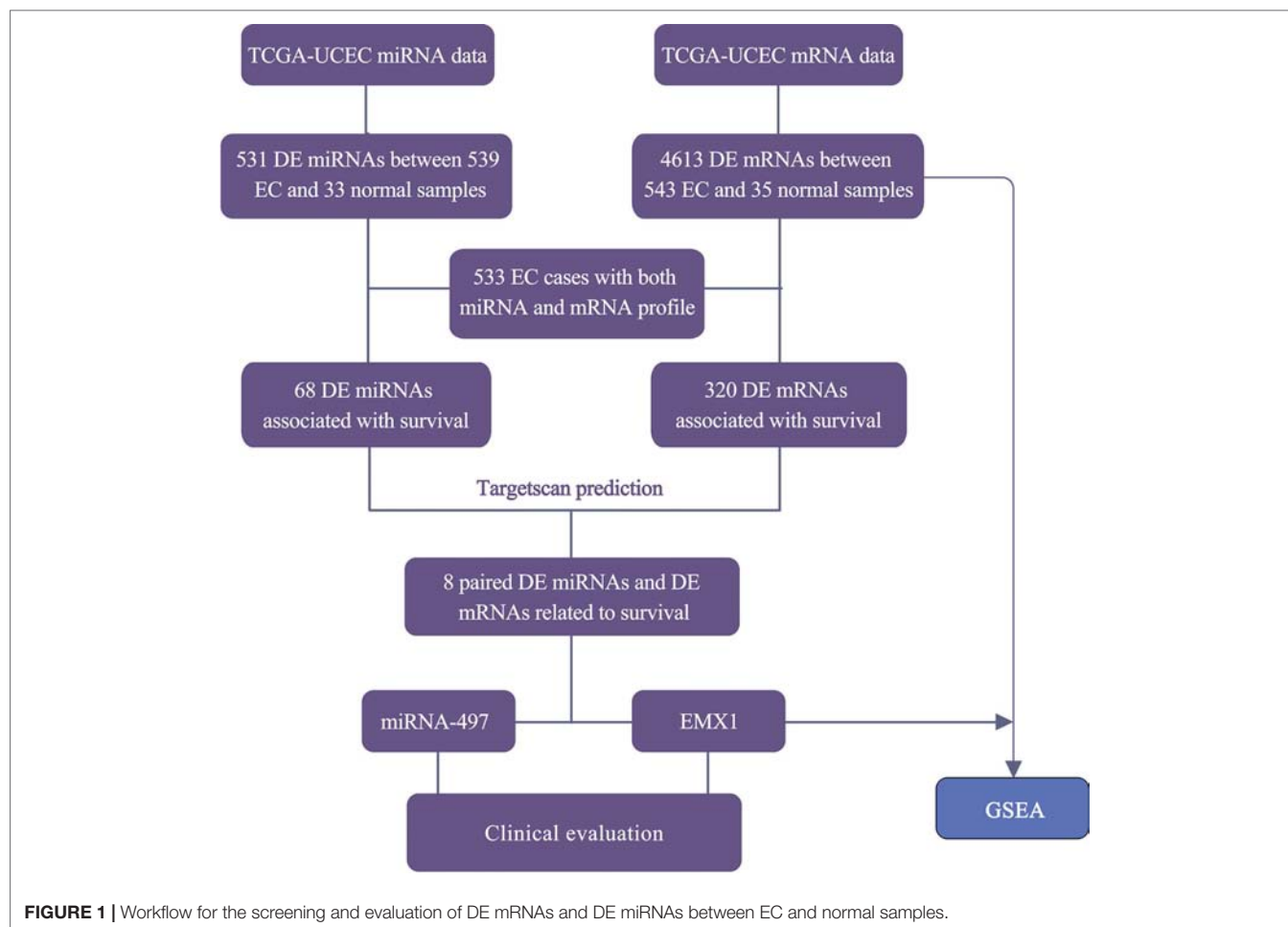
Identification of DE mRNAs and DE miRNAs With a Poorer Survival in EC

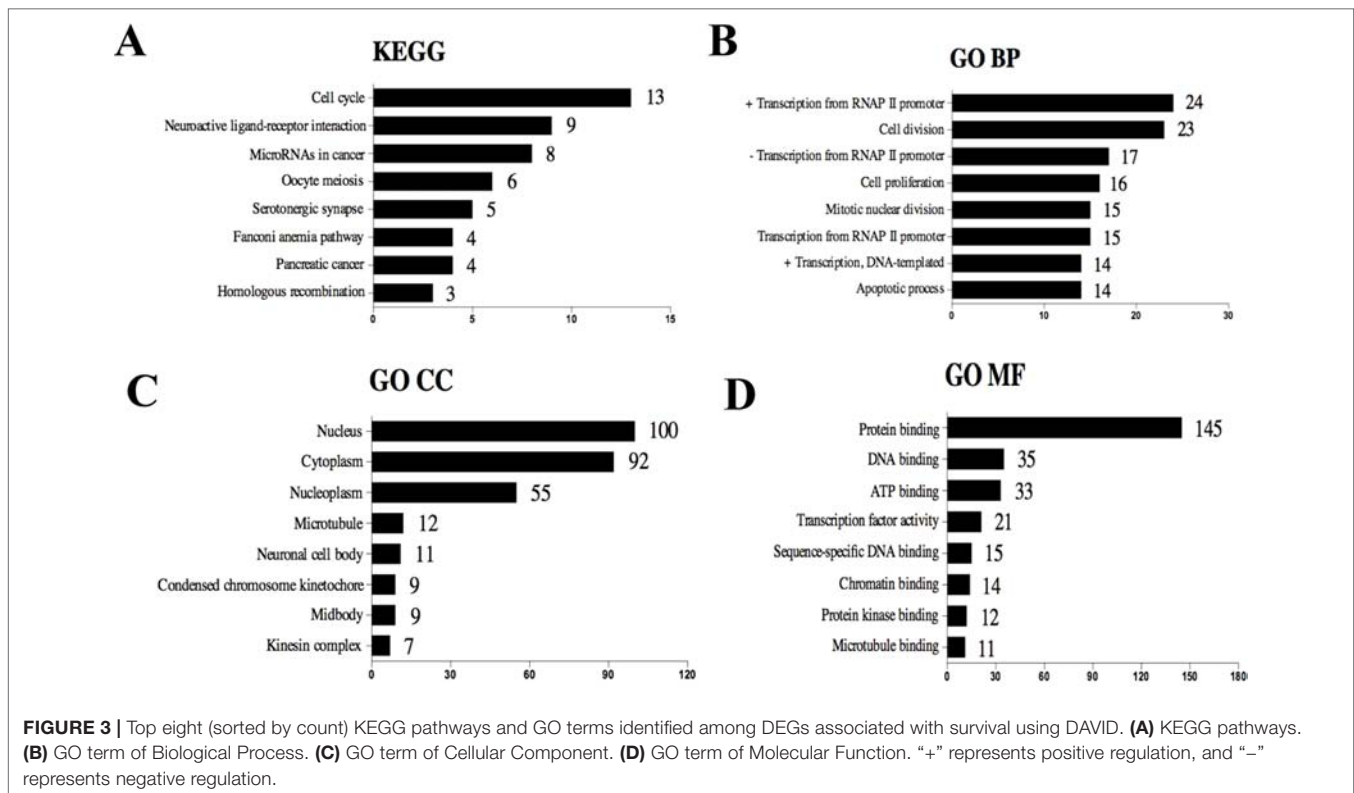
The RNA-Seq profile data of 543 EC and 35 normal cases were downloaded from TCGA database along with the miRNA-seq data of 539 EC and 33 normal samples. Among them, 533 patients shared two expression profiles (Figure 1). Under the threshold of adj. *P* < 0.05 and fold-change value larger than 2, a total of 4,613 DE mRNAs (3,221 upregulated and 1,392 downregulated) and 531 DE miRNAs (374 upregulated and 157 downregulated) were identified in EC compared with normal samples. The heatmaps and volcano plots are shown in Supplementary Figure 1.

To have a better understanding of the relationship between DEGs and patient survival, we conducted the “survival” package in R language to draw Kaplan-Meier curves according to the median expression level of DEGs among 533 patients. These upregulated and downregulated genes with a significantly poorer survival (*P* < 0.05), respectively, were determined. A total of 320 (280 upregulated and 40 downregulated) out of 4,613 DE mRNAs and 68 (43 upregulated and 25 downregulated) out of 531 DE miRNAs were chosen for further study (Supplementary Table 1). Kaplan-Meier curves of the partial genes between the high-expression group and low-expression group are shown in Figure 2.

Functional and Pathway Enrichment Analysis

The selected 320 DE mRNAs associated with survival were uploaded to DAVID to perform KEGG pathways and GO enrichment analysis. KEGG analysis revealed that these DEGs were mostly enriched in cell cycle, neuroactive ligand-receptor interaction, microRNAs in cancer, oocyte meiosis, and serotonergic synapse signaling pathways (Figure 3A). The top five enriched GO biological process (BP) terms included positive regulation of transcription from RNA polymerase II promoter, cell division, negative regulation of transcription from RNA polymerase II promoter, cell proliferation, and mitotic nuclear division. Nucleus, cytoplasm, nucleoplasm, microtubule, and neuronal cell body were the five most enriched GO terms for cellular component (CC). The top five enriched GO molecular function (MF) terms were protein binding, DNA binding, ATP binding, transcription factor activity,





and sequence-specific DNA binding (Figures 3B–D). The most significantly enriched pathways and enrichment terms are also shown in **Supplementary Table 2**.

Prediction Roles of Identified DE miRNAs and Their Potential Targeted mRNAs Associated With Survival

In order to better understand the role of DE miRNAs and their potential targeted DE mRNAs related to survival, we made target predictions through TargetScan. These genes containing conserved target sites, which matched the seed regions of miRNAs, were in our consideration. Besides, we only accepted these DE miRNAs and DE mRNAs with opposite expression levels in view of the silence effect of miRNA targeting. As a result, we predicted that 5 out of 68 DE miRNAs could interact with 8 out of 320 DE mRNAs (Figure 4), with one upregulated miRNA paired with two downregulated mRNAs and four downregulated miRNAs paired with six upregulated mRNAs (Table 1). The survival curves are also shown in Figure 5.

We further tried to assess the differences between high and low rates of expression levels for the eight miRNA/mRNA pairs under the cutoff of median rate. As shown in Figure 6A, patients with low rate of three paired miRNA/mRNA (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) expression had a significantly poorer survival, while the rest seemed no different (data were not shown). Furthermore, under each cutoff of median rate, there were 156 patients carrying low rates of both miR-497/EMX1 and miR-23c/DMBX1 expression, 135

patients carrying low rates of both miR-497/EMX1 and miR-670/KCNS1 expression, 160 patients carrying low rates of both miR-670/KCNS1 and miR-23c/DMBX1 expression, and 92 patients carrying low rates of all the three miRNA/mRNA pairs (Table 2). Surprisingly, these patients with the simultaneous presence of these two or three low miRNA/mRNA pairs had a significantly poorer survival rate (Figure 6B). These results strongly verified the validity and prediction capacity of our analysis.

Clinical Evaluation of miR-497 and EMX1 Expression in EC Patient Characteristics

We downloaded the clinical data of 533 primary EC from <http://www.cbioportal.org>. Patient characteristics are shown in Table 3. Median age at diagnosis of our study cohort was 64 year old (range 31–90 years old); 72.6% of the study group were white, with the rest scattered among other races. Most of the patients were endometrioid endometrial adenocarcinoma (EA; $n = 402$, 75.4%), 20.5% ($n = 109$) were serous, and 22 (4.1%) were mixed serous and endometrioid. The histopathologic distribution of EC was well differentiated (18.2%), moderately differentiated (22.5%), and poorly differentiated (59.3%). The cancer status included 419 cases without tumor (84.5%) and 77 cases with tumor (15.5%). In all patients, 32.7% had deep myometrial invasion, and 14.2% had positive peritoneal washings. About 16.8% of all patients had positive pelvic lymph nodes, while 10.3% had positive para-aortic lymph nodes. Stages I, II, III, and IV comprised 62.9%, 9.0%, 22.8%, and 5.3%, respectively. Median

Paired DE miRNAs and DE mRNAs	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score	Weighted context++ score	Conserved branch length	P _{cr}
Position 614-621 of GDF7 3' UTR	5' ...CCUGCUUUAUUUUAAGGAA... hsa-miR-211 3' ...UCCGCUUCCUACUGUUCUU	8mer	-0.10	79	-0.10	1.897	<0.1
Position 250-256 of SGCZ 3' UTR	5' ...CAUGACUUUGUCGAAAGGAG... hsa-miR-211 3' ...UCCGCUUCCUACUGUUCUU	7mer-m8	-0.11	81	-0.11	3.736	0.19
Position 353-359 of DMBX1 3' UTR	5' ...UAAGUCUACGUGGAAUGUGAG... hsa-miR-23c 3' ...CCAUUAGUACCGUUAACUA	7mer-m8	-0.02	37	-0.02	6.353	0.28
Position 614-621 of DNMT3B 3' UTR	5' ...UGCAACGAUCUCAAUUGAA... hsa-miR-23c 3' ...CCAUUAGUACCGUUAACUA	8mer	-0.47	98	-0.47	2.265	N/A
Position 356-363 of KCNS1 3' UTR	5' ...UCCCCACUUCACAGUAGGAAA... hsa-miR-670 3' ...AGGACUACUUAUACUUU	8mer	-0.03	43	-0.03	1.893	N/A
Position 203-209 of TEX19 3' UTR	5' ...GGCAGCAGUGAAUUGUCUG... hsa-miR-497 3' ...UGUUGUGUCACACGAC	7mer-m8	-0.22	91	-0.22	3.601	0.57
Position 32-38 of EMX1 3' UTR	5' ...CCCACAGGGCAGUGUCUU... hsa-miR-497 3' ...UGUUGUGUCACACGAC	7mer-m8	-0.15	81	-0.15	4.024	0.63
Position 259-266 of FADS6 3' UTR	5' ...UGGACCUACGAUUAUUA... hsa-miR-4770 3' ...UCGAUGACAGUAGAGU	8mer	-0.20	93	-0.20	2.562	<0.1

FIGURE 4 | Eight predicted consequential pairings of DE miRNAs and target regions of DE mRNAs associated with survival. These enrolled miRNA-mRNA pairs not only contained conserved binding sites but also had opposite expression levels in EC samples as compared with normal tissues.

TABLE 1 | DE miRNAs and their potential target DE mRNAs associated with survival.

DE miRNA	P value*	DE mRNA	P value*
Upregulated		Downregulated	
hsa-miR-211	0.03387	GDF7	0.02457
		SGCZ	0.04658
Downregulated		Upregulated	
hsa-miR-670	0.00427	KCNS1	0.00563
hsa-miR-4770	0.01774	FADS6	0.04633
hsa-miR-23c	0.02062	DMBX1	0.01274
		DNMT3B	0.03376
hsa-miR-497	0.00505	EMX1	0.03575
		TEX19	0.04040

*P value < 0.05 represented a significantly different OS rate between patients grouped according to the median level of gene expression. The bolded miR-497/EMX1 was selected as a representative of eight miRNA/mRNA pairs for subsequent study.

follow-up for subjects alive at last contact was 23.5 months (range 0–190 months).

miR-497, EMX1 Expression, and Association With Clinicopathologic Variables

Univariate analysis using logistic regression revealed that miR-497 expression as a categorical dependent variable (based on median expression value) was associated with adverse prognostic clinical pathological features (Table 4). Decreased miR-497 expression in EC was significantly associated with high grade (OR = 0.228 for G1, G2 vs. G3), stage (OR = 0.470 for I vs. II, III, and IV), histology (OR = 0.175 for EA vs. non-EA), tumor status (OR = 0.391 for tumor free vs. with tumor), pelvic lymph node metastasis (OR = 0.294 for positive vs. negative), para-aortic lymph node metastasis (OR = 0.232 for positive vs. negative) and peritoneal washings (OR = 0.510 for positive vs. negative) (all P values < 0.05, Figures 7A–D).

On the contrary, high EMX1 expression was associated with poor prognostic clinicopathologic characteristics (Table 5). Overexpressed EMX1 in EC was associated with clinically advanced stage (OR = 1.668 for I vs. II, III, and IV), high grade (OR = 2.325 for G1, G2 vs. G3), histology

(OR = 3.060 for EA vs. non-EA), tumor status (OR = 2.342 for tumor free vs. with tumor), and para-aortic lymph node metastasis (OR = 2.547 for positive vs. negative) (all P values < 0.05, Figures 7E–H).

These results suggested that EC patients with low expression of miR-497 and high expression of EMX1 were prone to progress to a more advanced stage than those with high expression of miR-497 and low expression of EMX1.

Survival Outcomes and Multivariate Analysis

Kaplan–Meier survival analysis showed that EC with low expression of miR-497 had a worse prognosis than that with high expression (Figure 5G). The univariate analysis revealed that reduced miR-497 correlated significantly with a poor OS (HR: 0.536; 95% CI: 0.345–0.831; P = 0.005).

Meanwhile, Kaplan–Meier survival analysis suggested that patients with high EMX1 expression also had a poorer prognosis than those with low EMX1 expression (Figure 5I). And, univariate analysis revealed that high EMX1 expression correlated significantly with a poorer OS rate (HR: 1.563, 95% CI: 1.019–2.396; P = 0.041).

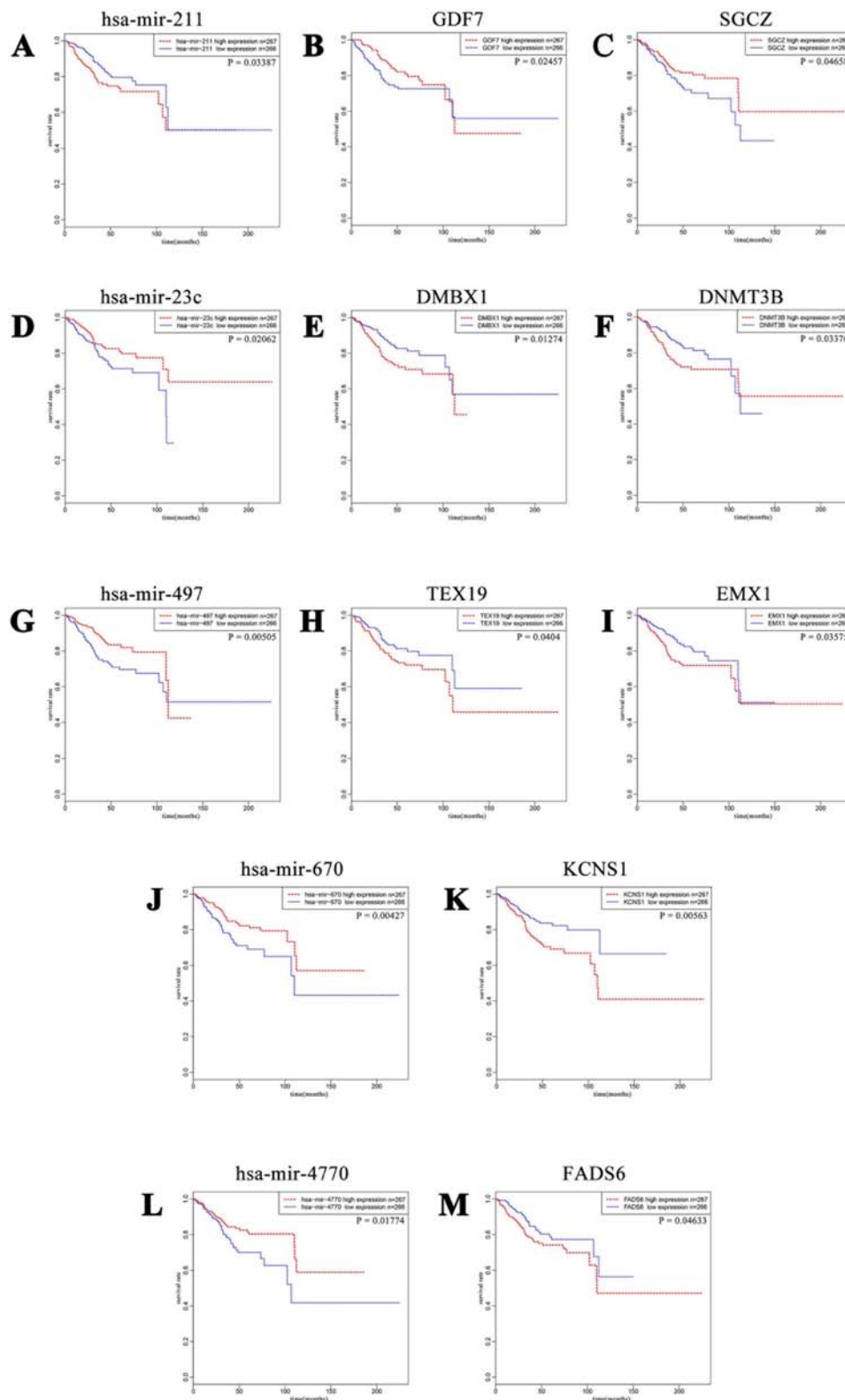


FIGURE 5 | Kaplan–Meier curves of five DE miRNAs and eight potential target DE mRNAs, with one upregulated miRNA **(A)** miR-211 paired with two downregulated mRNAs **(B)** GDF7 and **(C)** SGCZ and four downregulated miRNAs **(D)** miR-23c, **(G)** miR-497, **(J)** miR-670, and **(L)** miR-4770 paired with six upregulated mRNAs **(E)** DMXB1, **(F)** DNMT3B, **(H)** TEX19, **(I)** EMX1, **(K)** KCNS1, and **(M)** FADS6. For each gene, patients were divided into high-expression group and low-expression group according to the median expression level.

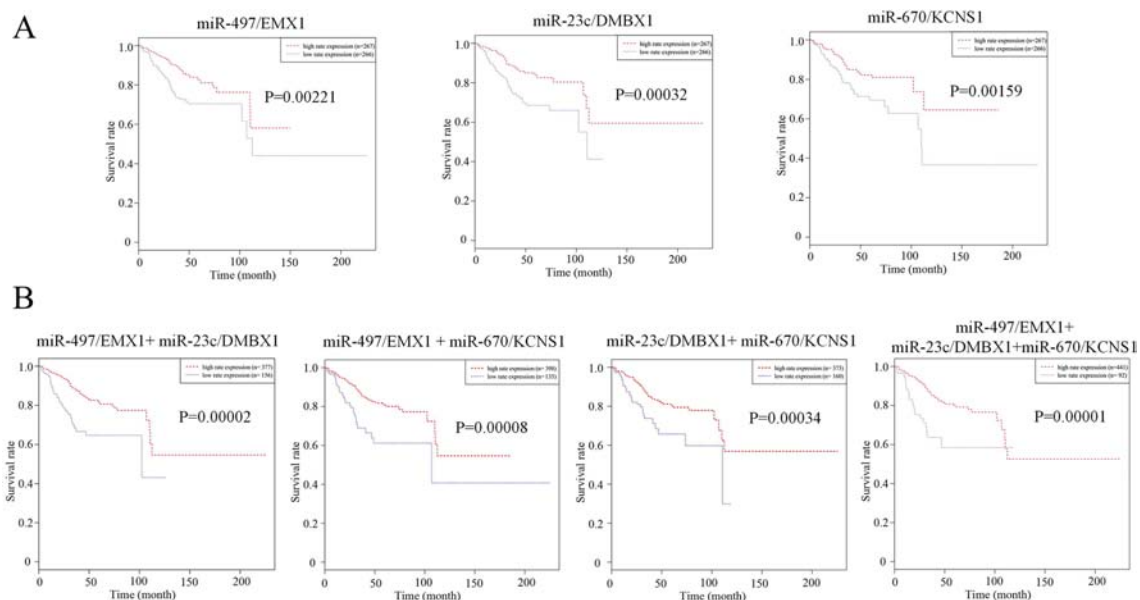


FIGURE 6 | Prediction roles of identified DE miRNAs and their potential targeted mRNAs associated with survival. **(A)** Kaplan-Meier curves for patients with rate of three single paired miRNA/mRNA (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) expression. **(B)** Kaplan-Meier curves for patients with simultaneous presence of rates for more than one miRNA/mRNA pair among these three selected pairs. The median rate of miRNA/mRNA expression was used to divide groups into low and high rates.

TABLE 2 | Patients with the simultaneous presence of low rates of miRNA/mRNA pairs.

Low rate of miRNA/mRNA pairs	N	%	P value*
miR-497/EMX1 + miR-23c/DMBX1	156	29.27	0.00002
miR-497/EMX1 + miR-670/KCNS1	135	25.33	0.00008
miR-23c/DMBX1 + miR-670/KCNS1	160	30.02	0.00034
miR-497/EMX1 + miR-23c/DMBX1 + miR-670/KCNS1	92	17.26	0.00001

*P value represented a significantly different OS rate between patients with low and high rates of miRNA/mRNA expression.

Other clinicopathologic factors associated with poor survival include high grade, histology, advanced stage, myometrial invasion, peritoneal washings, and lymph node metastasis (Table 6A).

In multivariate analysis (Table 6b), miR-497 remained an independent prognostic variable for OS, with an HR of 0.587 (CI: 0.363–0.951, $P = 0.030$), along with myometrial invasion.

EMX1 Was a Direct Target of miR-497

To explore whether EMX1 was a direct target of miR-497, we first detected the miR-497 levels in 293T and Ishikawa cells. As shown in Figure 8A, the miR-497 level in Ishikawa cell was very low, while it was much higher in 293T cell. We next performed a Dual-Luciferase reporter assay. The binding sites for miR-497 with EMX1 wild-type 3'-UTR (EMX1 3'-UTR) and EMX1 mutant 3'-UTR (EMX1 mu3'-UTR) are shown in Figure 8B. There was no loss of luciferase activity in Ishikawa cells with co-transfection of miR-497 mimics and mutated 3'-UTR of EMX1 plasmid, while a significant luciferase activity decrease was observed in cells co-transfected with miR-497

mimics and EMX1 3'-UTR plasmid (Figure 8C, *** $P = 0.0001$). We also performed the luciferase assay in 293T cells, which showed an increased relative luciferase activity of the construct having the mutated binding site compared with that of the wild-type site (Figure 8D, ** $P < 0.01$). These data indicated that EMX1 was a direct target of miR-497.

GSEA Identified EMX1-Related Oncogenic Signaling Pathways

The GSEA method is used to deal with continuous data and identify gene sets that are enriched at the top (overexpressed vs. control) or bottom (underexpressed) of a ranked gene list (Subramanian et al., 2005). We performed GSEA with the ordered list of preliminary screening DEGs according to their correlation with EMX1 expression. MSigDB Collection (c6.all.v6.2.symbols.gmt), which represents signatures of cellular pathways often dysregulated in cancer, was applied to our GSEA analysis at the phenotype of EMX1 expression level. Twenty-one signaling pathways were significantly enriched based on their NES (NOM P value < 0.05 , FDR q -value < 0.25 , Table 7). Supplementary Figure 2 shows that part of those oncogenic gene-involved cellular pathways, including PIGF, SRC, JNK KRAS.AMP.LUNG, PTEN, and E2F3, were differentially enriched in EMX1 high-expression phenotype.

DISCUSSION

In the recent years, more and more evidence has revealed that miRNAs play important roles in the development and progression of tumors. They may serve as molecular indicators of prognosis

TABLE 3 | Clinical characteristics of TCGA EC patients.

Clinical characteristics		Total	%
Age at diagnosis (years)		64 (31–90)	
Race	White	363	72.6
	Black or African American	105	20.9
	Asian	20	3.9
	Other	13	2.6
		504	
BMI	Median BMI (range)	32.05 (17.36–81.64)	
Menopause status	Premenopausal	34	6.6
	Postmenopausal	438	86.6
	Peri-menopausal	17	3.4
	Indeterminate	17	3.4
Histologic grade	G1	97	18.2
	G2	120	22.5
	G3	316	59.3
Status	Tumor free	419	84.5
	With tumor	77	15.5
Histology	Serous endometrial adenocarcinoma	109	20.5
	Mixed serous and endometrioid	22	4.1
	Endometrioid endometrial adenocarcinoma	402	75.4
Stage	I	335	62.9
	II	48	9.0
	III	122	22.8
	IV	28	5.3
Myometrial invasion	Superficial ($\leq 50\%$)	311	67.3
	Deep ($> 50\%$)	151	32.7
Peritoneal cytology	Negative	343	85.8
	Positive	57	14.2
Pelvic lymph nodes	Negative	361	83.2
	Positive	73	16.8
Para-aortic lymph nodes	Negative	322	89.7
	Positive	37	10.3

TABLE 4 | Univariate miR-497 expression* association with clinicopathologic characteristics (logistic regression).

Clinicopathologic variable	Total (N)	Odds ratio	P value
Age (continuous)	531	0.971 (0.955–0.986)	0.080
Race (white vs. other)	501	0.471 (0.315–0.704)	0.000
BMI (continuous)	503	1.039 (1.018–1.061)	0.000
Grade (G1, G2 vs. G3)	533	0.228 (0.157–0.331)	0.000
Status (tumor free vs. with tumor)	496	0.391 (0.234–0.654)	0.000
Histology (EA vs. non-EA)	533	0.175 (0.110–0.280)	0.000
Stage (I vs. II, III, IV)	533	0.470 (0.328–0.672)	0.000
Myometrial invasion (deep vs. superficial)	462	0.736 (0.498–1.087)	0.123
Peritoneal cytology (positive vs. negative)	400	0.510 (0.286–0.910)	0.023
Pelvic lymph nodes (positive vs. negative)	434	0.294 (0.169–0.512)	0.000
Para-aortic lymph nodes (positive vs. negative)	359	0.232 (0.103–0.522)	0.000

*Categorical dependent variable, greater or less than the median expression level.

and targets for oncotherapy (Jonas and Izaurralde, 2015). In endometrial cancer, a number of studies have looked at the miRNA profiles using tumor tissues or blood samples with a goal to identify disease-specific biomarkers (Srivastava et al., 2017). However, previous studies usually focused on one or two specific miRNAs and their target genes based on their potential to regulate diverse biological processes. Few researchers carried out studies on the relationship between whole miRNA profile and their potential target mRNAs based on their impact on patient survival.

A miRNA-based research data source of which was also a TCGA-UCEC project was carried out not long ago (Wang et al., 2018). It identified miRNAs that were correlated with the occurrence and progression of EC and established a six-miRNA (miR-15a. MIMAT0000068, miR-142. MIMAT0000433, miR-142. MIMAT0000434, miR-3170. MIMAT0015045, miR-1976. MIMAT0009451, and miR-146a. MIMAT0000449) expression signature as a predictor for the OS of patients with EC. In this study, the six-miRNA model was based on these 144 DE miRNAs resulting only from 15 patients with corresponding miRNA expression of the paired adjacent tissues. Another miRNA-based study on TCGA-UCEC project focused on the difference of miRNA profile between metastatic and nonmetastatic ECs (Zhu et al., 2018). It revealed that four miRNAs (miR-1247 miR-3200, miR-150, and miR-301b) were differently expressed between two groups, miR-1247 was associated with metastasis of EC to the lung, and miR-3200 is associated with the clinical stage of EC. The researchers also performed functional enrichment analysis with these predicted potential target genes of the four miRNAs, which showed that they might be involved in multiple pathways of cancer, including the Wnt, NOTCH, and TGF- β signaling pathways and signaling pathways regulating pluripotency of stem cells. However, miRNAs can target multiple genes, and a single gene can be targeted by multiple miRNAs (Ambros, 2004). Unilateral research is not enough to illustrate the problem.

In this study, for the first time, we performed Kaplan–Meier analysis for all the DE mRNAs and DE miRNAs in a larger number of EC cases from TCGA project with the help of bioinformation technology. As a result, 320 out of 4,613 DE mRNAs and 68 out of 531 DE miRNAs with a significantly poorer survival were determined. For the selected DE mRNAs associated with survival, KEGG pathway and GO enrichment analysis revealed that these selected DE mRNAs were significantly enriched in several functional pathways, including microRNAs in cancer. We further made target predictions through TargetScan and found that 5 out of 68 DE miRNAs could interact with eight DE mRNAs from 320 DE mRNAs. However, since the cutoff value for dividing groups (high expression and low expression) was based on the absolute median, most *P* values for different OS rates of the selected paired genes were of borderline statistical significance. Xie et al. (2018) used median mRNA expression level of ALKBH1 as a separation for high- and low-expression groups based on another TCGA project of glioblastoma ($n = 488$), which also resulted in a minor difference ($P = 0.0386$) and provided evidence for ALKBH1 to be a potential therapeutically targetable node. Another way to perform analysis was by using the computing cutoff expression

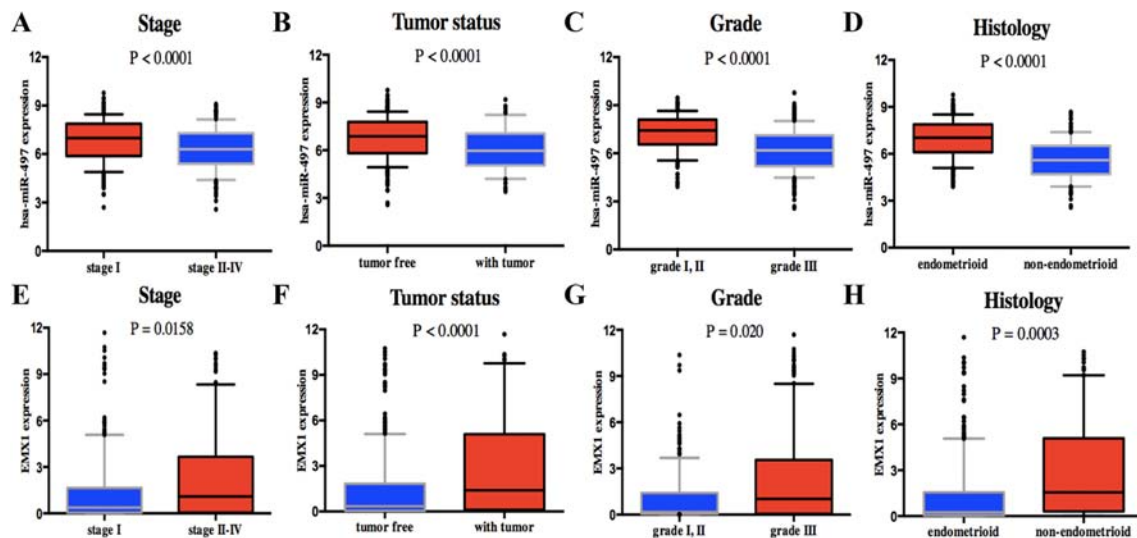


FIGURE 7 | Association with DEG expression and clinicopathologic characteristics, including has-miR-497 expression associated with (A) stage, (B) tumor status, (C) grade, and (D) histology and EMX1 expression associated with (E) stage, (F) tumor status, (G) grade, and (H) histology. *P* values were calculated using *t* test.

TABLE 5 | Univariate EMX1 expression* association with clinicopathologic characteristics (logistic regression).

Clinicopathologic variable	Total (N)	Odds ratio	<i>P</i> value
Age (continuous)	531	1.021 (1.006–1.037)	0.143
Race (white vs. other)	501	1.476 (0.995–2.190)	0.053
BMI (continuous)	503	0.966 (0.947–0.986)	0.001
Grade (G1, G2 vs. G3)	533	2.325 (1.632–3.313)	0.000
Status (tumor free vs. with tumor)	496	2.342 (1.406–3.899)	0.001
Histology (EA vs. non-EA)	533	3.060 (2.004–4.672)	0.000
Stage (I vs. II, III, IV)	533	1.668 (1.170–2.379)	0.005
Myometrial invasion (deep vs. superficial)	462	1.377 (0.932–2.034)	0.108
Peritoneal cytology (positive vs. negative)	400	1.365 (0.776–2.400)	0.280
Pelvic lymph nodes (positive vs. negative)	434	1.456 (0.876–2.420)	0.147
Para-aortic lymph nodes (positive vs. negative)	359	2.547 (1.217–5.327)	0.013

*Categorical dependent variable, greater or less than the median expression level.

level for the best separation with the smallest *P* value on survival and different numbers in two groups for each gene, which was also our initial design and resulted in a much larger number of DE mRNAs and miRNAs related to survival. To narrow the results of genes associated with survival, we finally chose the median truncation value, though some genes with potential predictability may be lost in our study.

Since each group of patients with a single miRNA or mRNA was made independently and their composition might be different, it would be interesting to determine differential expression of more than one miRNA or mRNA simultaneously in the patients. We tried to assess the differences between high and low rates of expression levels for the eight miRNA/mRNA pairs. As a result, patients with a low rate of three paired miRNA/mRNA (miR-497/EMX1, miR-23c/DMBX1, and miR-670/KCNS1) had a significantly poorer survival, while the rest seemed no different. As the presence of a single miRNA/mRNA

TABLE 6 | a. Overall survival and associations with clinicopathologic characteristics using Cox regression. **b.** Multivariate survival model after variable selection.

Clinicopathologic variable	Hazard ratio (95% CI)	<i>P</i> value
a.		
Age (continuous)	1.035 (1.015–1.056)	0.001
has-miR-497 expression (low vs. high)	0.536 (0.345–0.831)	0.005
EMX1 expression (high vs. low)	1.563 (1.019–2.396)	0.041
Race (white vs. other)	0.944 (0.585–1.521)	0.812
BMI (continuous)	0.989 (0.965–1.013)	0.360
Grade (G1, G2 vs. G3)	3.636 (2.084–6.342)	0.000
Status (tumor free vs. with tumor)	8.553 (5.524–13.242)	0.000
Histology (EA vs. non-EA)	2.944 (1.930–4.492)	0.000
Stage (I vs. II, III, IV)	3.775 (2.429–5.866)	0.000
Myometrial invasion (deep vs. superficial)	3.976 (2.490–6.347)	0.000
Peritoneal cytology (positive vs. negative)	4.842 (2.937–7.982)	0.000
Pelvic lymph nodes (positive vs. negative)	4.672 (2.841–7.682)	0.000
Para-aortic lymph nodes (positive vs. negative)	3.906 (2.083–7.325)	0.000
b.		
has-miR-497 expression (low vs. high)	0.587 (0.363–0.951)	0.030
EMX1 expression (high vs. low)	1.479 (0.929–2.353)	0.099
Myometrial invasion (deep vs. superficial)	3.618 (2.260–5.792)	0.000

pair is clearly not sufficient to predict the outcome of a patient having EC, we further verified the simultaneous presence of low rate of more than one miRNA/mRNA pair among these three selected pairs in the same group of patients. Surprisingly, the

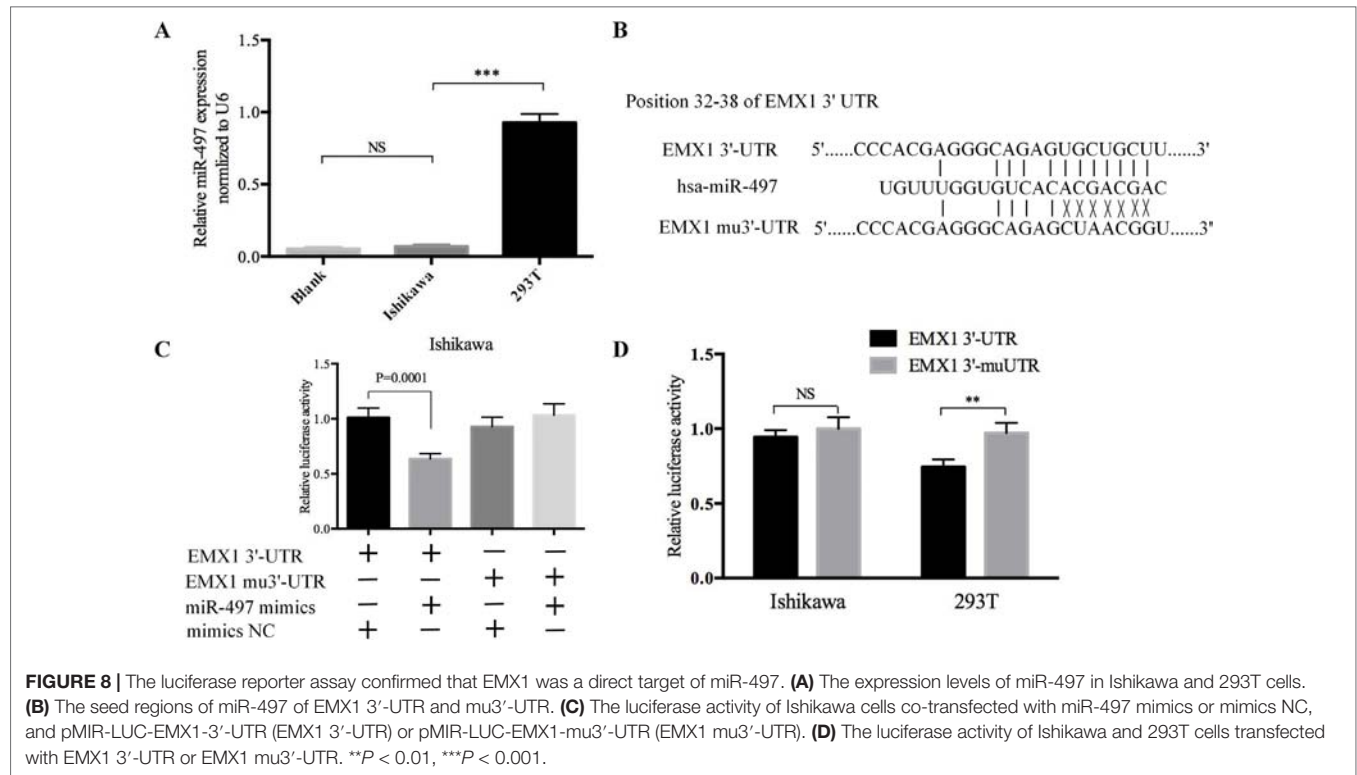


TABLE 7 | Gene Set Enrichment Analysis in EMX1 phenotype.

MSigDB collection	Gene set name	NES	NOM P val	FDR q -val
c6.all.v6.2.symbols.gmt	PIGF_UP.V1_DN	1.808	0.0027	0.2133
	SRC_UP.V1_UP	1.756	0.0071	0.1653
	JNK_DN.V1_DN	1.661	0.0107	0.2138
	KRAS.AMP.LUNG_UP.V1_DN	1.739	0.0116	0.1629
	PTEN_DN.V2_DN	1.648	0.0175	0.1952
	E2F3_UP.V1_UP	1.671	0.0217	0.2194
	KRAS.LUNG_UP.V1_DN	1.806	0.0218	0.1624
	KRAS.50_UP.V1_DN	1.799	0.0235	0.1374
	ATM_DN.V1_UP	1.628	0.0236	0.1827
	PRC2_EZH2_UP.V1_DN	1.620	0.0245	0.1810
	NOTCH_DN.V1_UP	1.560	0.0265	0.2069
	PRC2_SUZ12_UP.V1_DN	1.539	0.0288	0.1912
	RB_P107_DN.V1_UP	1.632	0.0339	0.2043
	P53_DN.V2_UP	1.628	0.0356	0.1952
	NOTCH_DN.V1_DN	1.547	0.0372	0.1962
	ATF2_UP.V1_UP	1.579	0.0396	0.2249
	KRAS.KIDNEY_UP.V1_DN	1.557	0.0396	0.2005
	PKCA_DN.V1_UP	1.484	0.0417	0.2356
	JAK2_DN.V1_UP	1.568	0.0435	0.2168
	CAHOY_NEURONAL	1.674	0.0464	0.2415
	RB_P130_DN.V1_UP	1.574	0.0490	0.2190

NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate. Gene sets with NOM P val < 0.05 and FDR q -val < 0.25 are considered significant.

simultaneous presence of these selected low miRNA/mRNA pairs occurred in most EC patients and resulted in a significantly poorer survival rate, which strongly verified the validity and prediction capacity of our analysis. The experimental luciferase reporter assay confirmed that EMX1 was a direct target of miR-497. Clinical evaluation was assessed on miR-497 and EMX1 expression levels. Interestingly, they had an opposite significant

relationship with several clinicopathologic characteristics besides survival. Multivariate analysis also demonstrated that miR-497 remained independent prognostic variables for OS. These data also provided evidence for the value of our prediction analysis in EC.

Among 68 DE miRNAs related to a poorer survival, 43 miRNAs were upregulated and 25 were downregulated. In 2013, Torres

et al. conducted a study that aimed to reveal the relationship between DE miRNAs and EC (Torres et al., 2013). They found that upregulated miR-1228 was significantly associated with a poorer survival, which was consistent with our research result. Another research revealed that lower expression of miR-101, miR-10b, miR-139-5p, miR-152, miR-29b, and miR-455-5p was significantly correlated with poor OS and that decreased expression of miR-152 was a statistically independent risk factor for OS in EC (Hiroki et al., 2010). Mitamura et al. found that miR-31 was significantly upregulated in the EC patients with a high risk of recurrence compared with that observed in the low-risk patients, and this higher expression correlated with a poorer progression-free survival (Mitamura et al., 2014).

miRNAs mainly function and result in silence effect at posttranscriptional level by base pairing with the 3'-UTR of their target mRNAs completely or incompletely (Moran et al., 2017). In the present study, we predicted eight potential target DE mRNAs for the five DE miRNAs, including miR-211, miR-23c, miR-670, miR-497, and miR-4770. To our knowledge, there is no basic experimental evidence for the eight pairs of miRNAs and mRNAs predicted. One of the mostly studied miRNAs, miR-497, was recognized as a tumor suppressor in many cancers (Zhao et al., 2013; Du et al., 2015; Zhao et al., 2015; Yang et al., 2016), which was also consistent with our result in EC. During our manuscript preparation, a meta-analysis (Feng et al., 2018) on the prognostic role of miR-497 in different cancer patients revealed that high-expression levels of miR-497 are less possible to have lymph node metastasis and have better overall survival, which indicated that miR-497 might be a potential biomarker and could be used to predict the better prognosis of different cancer types. A recent study showed that miR-497 negatively regulated glioma cells by targeting oncogene *Wnt3a* and that reduced expression of miR-497 was associated with poor disease-free and overall survival rates (Lu et al., 2018). It could also suppress clear cell renal cell carcinoma by targeting PD-L1, which was an immune-related oncogene (Qu et al., 2018). miR-497 could target SERPINE-1 and induce reversion of epithelial-to-mesenchymal transition in cutaneous squamous cell carcinoma (Mizrahi et al., 2018). However, these targets of miR-497 did not appear as a DE mRNA associated with survival in the present study. *EMX1*, a potential target of miR-497 predicted through TargetScan, also had a very close relationship with EC clinicopathologies and patient survival, which was in contrast to the miR-497 in our study. Asada et al. revealed that a high quartile of *EMX1* methylation level had a significant univariate HR and a multivariate-adjusted HR of developing authentic metachronous gastric cancers (Asada et al., 2015). Its methylation level was also differently expressed in hepatocellular carcinoma (HCC), which showed a potential role in the development of HCC (Sun et al., 2018). However, most of the research on *EMX1* was focused on brain development (Lim et al., 2015; Kobeissy et al., 2016). The relationship between miR-497/*EMX1* and cancer needs further study.

Testis-expressed 19 (*TEX19*) was another potential target gene of miR-497 in our study. Zhong et al. revealed that *TEX19* exhibited increased expression in high-grade tumors and might

represent a novel cancer-testis gene related to the progression of bladder cancer (Zhong et al., 2016). *TEX19* was also required to drive cell proliferation in a range of cancer cell types, and its expression was linked to a poor prognosis for breast cancer, kidney cancer, prostate cancer, and glioma cancer (Planells-Palop et al., 2017). However, further study on its role in EC is needed.

Bu Y. et al. identified that miR-211 directly targets to *Bmal1* and *Clock* in Burkitt's lymphoma, thereby suppressing both circadian oscillation and ongoing protein synthesis to facilitate tumor progression (Bu et al., 2018). miR-23c, as a target of lncRNA *MALAT1*, could directly repress its target *ELAVL1* and inhibit hyperglycemia-induced cell pyroptosis (Li et al., 2017). Shi C. et al. showed evidence that miR-670 could induce cell proliferation in hepatocellular carcinoma by targeting *PROX1* (Shi and Xu, 2016), while our study predicted that it might play an important role in EC progression via targeting Potassium Voltage-Gated Channel Modifier Subfamily S Member 1 (*KCNS1*), which was also a DE mRNA associated with survival in our study. Previous studies on *KCNS1* mainly focused on pain (Tsantoulas et al., 2018; Wonkam et al., 2018). Besides the downregulated expression level of *KCNS1* in metastatic breast carcinoma (Savci-Heijink et al., 2016), little research has been carried out on the relationship between *KCNS1* and cancer.

DNA methyltransferase 3B (*DNMT3B*), one of the eight paired genes in our study, was a methyltransferase responsible for *de novo* DNA methylation. A previous study also showed that it was significantly upregulated in both grade I and grade III ECs as compared with normal controls (Jin et al., 2005). *DNMT3B*-involved divergent DNA methylation pathways and protein synthesis required for posttranscriptional regulation may be implicated in the development of type I and type II ECs (Xiong et al., 2005a; Xiong et al., 2005b). However, little is known about the relationships between EC and the rest of the potential miRNA-targeted DE mRNAs, including growth differentiation factor 7 (*GDF7*), sarcoglycan zeta (*SGCZ*), diencephalon/mesencephalon homeobox 1 (*DMBX1*), and fatty acid desaturase 6 (*FADS6*). Further research is also needed.

There were emerging researches on EC using TCGA data. Studies revealed that *L1CAM* (Dellinger et al., 2016) and *TFAP2B* (Wu and Zhang, 2018) were the two DEGs associated with advanced clinicopathologic characteristics and independent predictors for survival in EC based on TCGA-UCEC data. However, the massive narrowing down process for the DEG screening was not shown. Another study focused on the mutation-expression profile and looked for guidance for EC drug discovery (Wong et al., 2016). Thus, while these studies and ours were all based on TCGA data for EC, the different focus and data analysis approaches led to the discovery of different aspects of the disease.

In this study, we identified a series of DE mRNAs and DE miRNAs associated with survival in EC. Furthermore, we predicted eight pairs of DE miRNAs and their potential target DE mRNAs related to survival. Clinical evaluation of downregulated miR-497 and paired upregulated *EMX1* confirmed the value of our prediction analysis in EC. The simultaneous presence of low rate of these selected low miRNA/mRNA pairs (miR-497/

EMX1, miR-23c/DMBX1, and miR-670/KCNS1) might have a better prediction value. Therefore, further studies are required to validate these findings.

AUTHOR CONTRIBUTIONS

XX, JW, and HZ designed the study, checked the data, and prepared the manuscript. TL, YW, JF, and QY performed data collection and statistical analysis. TL searched the literature and took part in the manuscript preparation. JW and HZ conceived and supervised the project.

FUNDING

Funding was provided by the Natural Science Foundation of Jiangsu Province (BK20151096) and the Key Projects of National

Health and Family Planning Commission of Nanjing City (ZKX17015) to HZ and the Fundamental Research Funds for the Central Universities (3332018179) to JW.

ACKNOWLEDGMENTS

We thank Juan Chen from CCN Department of ZTE for her computer technology support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00743/full#supplementary-material>

REFERENCES

- Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431, 350–355. doi: 10.1038/nature02871
- Asada, K., Nakajima, T., Shimazu, T., Yamamichi, N., Maekita, T., Yokoi, C., et al. (2015). Demonstration of the usefulness of epigenetic cancer risk prediction by a multicentre prospective cohort study. *Gut* 64, 388–396. doi: 10.1136/gutjnl-2014-307094
- Binder, P. S., and Mutch, D. G. (2014). Update on prognostic markers for endometrial cancer. *Women's Heal.* 10, 277–288. doi: 10.2217/WHE.14.13
- Bu, Y., Yoshida, A., Chitnis, N., Altman, B. J., Tameire, F., Oran, A., et al. (2018). A PERK-miR-211 axis suppresses circadian regulators and protein synthesis to promote cancer cell survival. *Nat. Cell Biol.* 20, 104–115. doi: 10.1038/s41556-017-0006-y
- Burk, R. D., Chen, Z., Saller, C., Tarvin, K., Carvalho, A. L., Scapulatempo-Neto, C., et al. (2017). Integrated genomic and molecular characterization of cervical cancer. *Nature* 543, 378–384. doi: 10.1038/nature21386
- Canlorbe, G., Wang, Z., Laas, E., Bendifallah, S., Castela, M., Lefevre, M., et al. (2016). Identification of microRNA expression profile related to lymph node status in women with early-stage grade 1–2 endometrial cancer. *Mod. Pathol.* 29, 391–401. doi: 10.1038/modpathol.2016.30
- Chen, S., Sun, K., Liu, B., Zong, Z., and Zhao, Y. (2016). MicroRNA-505 functions as a tumor suppressor in endometrial cancer by targeting TGF- α . *Mol. Cancer* 15, 11. doi: 10.1186/s12943-016-0496-4
- Dellinger, T. H., Smith, D. D., Ouyang, C., Warden, C. D., Williams, J. C., and Han, E. S. (2016). L1CAM is an independent predictor of poor survival in endometrial cancer—an analysis of The Cancer Genome Atlas (TCGA). *Gynecol. Oncol.* 141, 336–340. doi: 10.1016/j.ygyno.2016.02.003
- Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., et al. (2012). Landscape of transcription in human cells. *Nature* 489, 101–108. doi: 10.1038/nature11233
- Du, M., Shi, D., Yuan, L., Li, P., Chu, H., Qin, C., et al. (2015). Circulating miR-497 and miR-663b in plasma are potential novel biomarkers for bladder cancer. *Sci. Rep.* 5, 10437. doi: 10.1038/srep10437
- Dunham, I., Kundaje, A., Aldred, S. F., Collins, P. J., Davis, C. A., Doyle, F., et al. (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74. doi: 10.1038/nature11247
- Duursma, A. M., Kedde, M., Schrier, M., le Sage, C., and Agami, R. (2008). miR-148 targets human DNMT3b protein coding region. *RNA* 14, 872–877. doi: 10.1261/rna.972008
- Dykes, I. M., and Emanuelli, C. (2017). Transcriptional and post-transcriptional gene regulation by long non-coding RNA. *Genomics Proteomics Bioinformatics* 15, 177–186. doi: 10.1016/j.gpb.2016.12.005
- Falcon, S., and Gentleman, R. (2007). Using GOSTats to test gene lists for GO term association. *Bioinformatics* 23, 257–258. doi: 10.1093/bioinformatics/btl567
- Feng, J., Gu, X., Liu, L., Lu, M., Ma, X., Cao, Y., et al. (2018). Prognostic role of microRNA-497 in cancer patients: a meta-analysis. *J. Cancer* 9, 3334–3342. doi: 10.7150/jca.25514
- Fromm, B., Billipp, T., Peck, L. E., Johansen, M., Tarver, J. E., King, B. L., et al. (2015). A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome. *Annu. Rev. Genet.* 49, 213–242. doi: 10.1146/annurev-genet-120213-092023
- Hiroki, E., Akahira, J., Suzuki, F., Nagase, S., Ito, K., Suzuki, T., et al. (2010). Changes in microRNA expression levels correlate with clinicopathological features and prognoses in endometrial serous adenocarcinomas. *Cancer Sci.* 101, 241–249. doi: 10.1111/j.1349-7006.2009.01385.x
- Jin, F., Dowdy, S. C., Xiong, Y., Eberhardt, N. L., Podratz, K. C., and Jiang, S. (2005). Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. *Gynecol. Oncol.* 96, 531–538. doi: 10.1016/j.ygyno.2004.10.039
- Jonas, S., and Izaurralde, E. (2015). Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* 16, 421–433. doi: 10.1038/nrg3965
- Kobeissy, F. H., Hansen, K., Neumann, M., Fu, S., Jin, K., and Liu, J. (2016). Deciphering the role of Emx1 in neurogenesis: a neuroproteomics approach. *Front. Mol. Neurosci.* 9, 1–16. doi: 10.3389/fnmol.2016.00098
- Li, X., Zeng, L., Cao, C., Lu, C., Lian, W., Han, J., et al. (2017). Long noncoding RNA MALAT1 regulates renal tubular epithelial pyroptosis by modulated miR-23c targeting of ELAVL1 in diabetic nephropathy. *Exp. Cell Res.* 350, 327–335. doi: 10.1016/j.yexcr.2016.12.006
- Lim, J. W. C., Donahoo, A.-L. S., Bunt, J., Edwards, T. J., Fenlon, L. R., Liu, Y., et al. (2015). EMX1 regulates NRP1-mediated wiring of the mouse anterior cingulate cortex. *Development* 142, 3746–3757. doi: 10.1242/dev.119909
- Lu, F., Ye, Y., Zhang, H., He, X., Sun, X., Yao, C., et al. (2018). miR-497/Wnt3a/c-jun feedback loop regulates growth and epithelial-to-mesenchymal transition phenotype in glioma cells. *Int. J. Biol. Macromol.* 120, 985–991. doi: 10.1016/j.ijbiomac.2018.08.176
- Mitamura, T., Watari, H., Wang, L., Kanno, H., Kitagawa, M., Hassan, M., et al. (2014). microRNA 31 functions as an endometrial cancer oncogene by suppressing Hippo tumor suppressor pathway. *Mol. Cancer* 13, 97. doi: 10.1186/1476-4598-13-97
- Mizrahi, A., Barzilai, A., Gur-Wahnon, D., Ben-Dov, I. Z., Glassberg, S., Meningher, T., et al. (2018). Alterations of microRNAs throughout the malignant evolution of cutaneous squamous cell carcinoma: the role of miR-497 in epithelial to mesenchymal transition of keratinocytes. *Oncogene* 37, 218–230. doi: 10.1038/onc.2017.315
- Moran, Y., Agron, M., Praher, D., and Technau, U. (2017). The evolutionary origin of plant and animal microRNAs. *Nat. Ecol. Evol.* 1, 0027. doi: 10.1038/s41559-016-0027
- Planells-Palop, V., Hazazi, A., Feichtinger, J., Jezkova, J., Thallinger, G., Alsiwiehri, N. O., et al. (2017). Human germ/stem cell-specific gene TEX19 influences cancer

- cell proliferation and cancer prognosis. *Mol. Cancer* 16, 84. doi: 10.1186/s12943-017-0653-4
- Qu, F., Ye, J., Pan, X., Wang, J., Gan, S., Chu, C., et al. (2018). MicroRNA-497-5p down-regulation increases PD-L1 expression in clear cell renal cell carcinoma. *J. Drug Target.* 0, 1–8. doi: 10.1080/1061186X.2018.1479755
- Reyes, H. D., Miecznikowski, J., Gonzalez-Bosquet, J., Devor, E. J., Zhang, Y., Thiel, K. W., et al. (2017). High stathmin expression is a marker for poor clinical outcome in endometrial cancer: An NRG oncology group/gynecologic oncology group study. *Gynecol. Oncol.* 146, 247–253. doi: 10.1016/j.ygyno.2017.05.017
- Sacco, L., and Masotti, A. (2012). Recent insights and novel bioinformatics tools to understand the role of micrornas binding to 5' untranslated region. *Int. J. Mol. Sci.* 14, 480–495. doi: 10.3390/ijms14010480
- Savci-Heijink, C. D., Halfwerk, H., Koster, J., and van de Vijver, M. J. (2016). A novel gene expression signature for bone metastasis in breast carcinomas. *Breast Cancer Res. Treat.* 156, 249–259. doi: 10.1007/s10549-016-3741-z
- Shi, C., and Xu, X. (2016). MiR-670-5p induces cell proliferation in hepatocellular carcinoma by targeting PROX1. *Biomed. Pharmacother.* 77, 20–26. doi: 10.1016/j.biopha.2015.07.030
- Siegel, R. L., Miller, K. D., and Jemal, A. (2018). Cancer statistics, 2018. *CA. Cancer J. Clin.* 68, 7–30. doi: 10.3322/caac.21442
- Smith, R. A., Andrews, K. S., Brooks, D., Fedewa, S. A., Manassaram-Baptiste, D., Saslow, D., et al. (2018). Cancer screening in the United States, 2018: a review of current American Cancer Society guidelines and current issues in cancer screening. *CA. Cancer J. Clin.* 68, 297–316. doi: 10.3322/caac.21446
- Srivastava, S. K., Ahmad, A., Zubair, H., Miree, O., Singh, S., Rocconi, R. P., et al. (2017). MicroRNAs in gynecological cancers: small molecules with big implications. *Cancer Lett.* 407, 123–138. doi: 10.1016/j.canlet.2017.05.011
- Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* 102, 15545–15550. doi: 10.1073/pnas.0506580102
- Sun, X.-J., Wang, M.-C., Zhang, F.-H., and Kong, X. (2018). An integrated analysis of genome-wide DNA methylation and gene expression data in hepatocellular carcinoma. *FEBS Open Bio.* 8, 1093–1103. doi: 10.1002/2211-5463.12433
- Torres, A., Torres, K., Pesci, A., Ceccaroni, M., Paszkowski, T., Cassandrini, P., et al. (2013). Diagnostic and prognostic significance of miRNA signatures in tissues and plasma of endometrioid endometrial carcinoma patients. *Int. J. Cancer* 132, 1633–1645. doi: 10.1002/ijc.27840
- Tsantoulas, C., Denk, F., Signore, M., Nassar, M. A., Futai, K., and McMahon, S. B. (2018). Mice lacking Kcns1 in peripheral neurons show increased basal and neuropathic pain sensitivity. *Pain* 159, 1641–1651. doi: 10.1097/j.pain.0000000000001255
- Wang, Y., Xu, M., and Yang, Q. (2018). A six-microRNA signature predicts survival of patients with uterine corpus endometrial carcinoma. *Curr. Probl. Cancer.* 43, 1–10. doi: 10.1016/j.cuprob.2018.02.002
- Wong, H. S.-C., Juan, Y.-S., Wu, M.-S., Zhang, Y.-F., Hsu, Y.-W., Chen, H.-H., et al. (2016). Integrative bioinformatic analyses of an oncogenomic profile reveal the biology of endometrial cancer and guide drug discovery. *Oncotarget* 7, 5909–5923. doi: 10.18632/oncotarget.6716
- Wonkam, A., Mnika, K., Ngo Bitoungui, V. J., Chetcha Chemegni, B., Chimusa, E. R., Dandara, C., et al. (2018). Clinical and genetic factors are associated with pain and hospitalisation rates in sickle cell anaemia in Cameroon. *Br. J. Haematol.* 180, 134–146. doi: 10.1111/bjh.15011
- Wu, H., and Zhang, J. (2018). Decreased expression of TFAP2B in endometrial cancer predicts poor prognosis: a study based on TCGA data. *Gynecol. Oncol.* 149, 592–597. doi: 10.1016/j.ygyno.2018.03.057
- Xie, Q., Wu, T., Gimple, R., Li, Z., Prager, B., Wu, Q., et al. (2018). N⁶-methyladenine DNA modification in glioblastoma. *Cell.* 175, 1–16. doi: 10.1016/j.cell.2018.10.006
- Xiong, Y., Dowdy, S. C., Podratz, K. C., Jin, F., Attewell, J. R., Eberhardt, N. L., et al. (2005a). Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate *de novo* DNA methyltransferase activity in human endometrial cells. *Cancer Res.* 65, 2684–2689. doi: 10.1158/0008-5472.CAN-04-2843
- Xiong, Y., Dowdy, S. C., Xue, A., Shujuan, J., Eberhardt, N. L., Podratz, K. C., et al. (2005b). Opposite alterations of DNA methyltransferase gene expression in endometrioid and serous endometrial cancers. *Gynecol. Oncol.* 96, 601–609. doi: 10.1016/j.ygyno.2004.11.047
- Yang, G., Xiong, G., Cao, Z., Zheng, S., You, L., Zhang, T., et al. (2016). miR-497 expression, function and clinical application in cancer. *Oncotarget* 7, 21–23. doi: 10.18632/oncotarget.10152
- Zhao, W., Wang, Y., An, Z., Shi, C., Zhu, G., Wang, B., et al. (2013). Downregulation of miR-497 promotes tumor growth and angiogenesis by targeting HDGF in non-small cell lung cancer. *Biochem. Biophys. Res. Commun.* 435, 466–471. doi: 10.1016/j.bbrc.2013.05.010
- Zhao, X., Zhao, Z., Xu, W., Hou, J., and Du, X. (2015). Down-regulation of miR-497 is associated with poor prognosis in renal cancer. *Int. J. Clin. Exp. Pathol.* 8, 758–764.
- Zhong, J., Chen, Y., Liao, X., Li, J., Wang, H., Wu, C., et al. (2016). Testis expressed 19 is a novel cancer-testis antigen expressed in bladder cancer. *Tumor Biol.* 37, 7757–7765. doi: 10.1007/s13277-015-4567-8
- Zhou, H., Xu, X., Xun, Q., Yu, D., Ling, J., Guo, F., et al. (2012). microRNA-30c negatively regulates endometrial cancer cells by targeting metastasis-associated gene-1. *Oncol. Rep.* 27, 807–812. doi: 10.3892/or.2011.1574
- Zhu, L., Shu, Z., and Shun, X. (2018). Bioinformatic analysis of four miRNAs relevant to metastasis-regulated processes in endometrial carcinoma. *Cancer Manag. Res. Volume* 10, 2337–2346. doi: 10.2147/CMAR.S168594

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Long Non-coding RNA DLEU1 Promotes Proliferation and Invasion by Interacting With miR-381 and Enhancing HOXA13 Expression in Cervical Cancer

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OPEN ACCESS

Edited by:

Yujing Li,
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Reviewed by:

Young-Ho Ahn,
Ewha Womans University,
South Korea
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Emory University, United States

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 29 September 2018

Accepted: 23 November 2018

Published: 07 December 2018

Citation:

Liu C, Tian X, Zhang J and Jiang L
(2018) Long Non-coding RNA DLEU1
Promotes Proliferation and Invasion
by Interacting With miR-381
and Enhancing HOXA13 Expression
in Cervical Cancer.
Front. Genet. 9:629.
doi: 10.3389/fgene.2018.00629

Although growing evidence has demonstrated that the long non-coding RNA DLEU1 is involved in the progression of various cancers, its functional role and underlying mechanisms have not been explored in cervical cancer (CC). In this study, we found that DLEU1 was up-regulated in both CC tissues and CC cell lines, and overexpression of DLEU1 was significantly correlated with shorter patient survival. Knockdown of DLEU1 suppressed CC cell proliferation and invasion, whereas overexpression of DLEU1 promoted the proliferation and invasion of CC cells. Bioinformatics analysis was used to elucidate the potential correlation between DLEU1 and miR-381. Moreover, qRT-PCR analysis, luciferase reporter assay and RNA immunoprecipitation assay confirmed that DLEU1 inhibited the expression of miR-381, and revealed a direct interaction between DLEU1 and miR-381. In addition, we demonstrated that miR-381 directly targeted HOXA13 in CC cells. The restoration of HOXA13 expression reversed DLEU1 knockdown or miR-381 overexpression-mediated suppression of cell proliferation and invasion. These results suggested that DLEU1 can promote CC cell proliferation and invasion via the miR-381/HOXA13 axis.

Keywords: DLEU1, long non-coding RNA, cervical cancer, miR-381, HOXA13

INTRODUCTION

Cervical cancer (CC) is the second most common cause of death among women with various cancers, with over 500,000 new patients diagnosed and approximately 280,000 deaths each year (Torre et al., 2015). Although CC is curable if diagnosed at an early stage, many still present with an advanced or metastatic disease and have a worse prognosis. Metastasis is responsible for as much as 90% of cancer-induced mortality (Chaffer and Weinberg, 2011). Consequently, a better understanding of the molecular mechanism in metastatic CC is essential for the development of effective therapeutic strategies against CC.

MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been shown to have crucial roles in carcinogenesis, metastasis and drug resistance (Han Li and Chen, 2015). MiRNAs

post-transcriptionally regulate the expression of oncogenes and tumor suppressor genes, thus modulating the biological behaviors of tumor cells (Fabian et al., 2010). LncRNAs are known to function as scaffolds or guides to regulate interactions between protein and genes, and as decoys to bind proteins or miRNAs to modulate the expression of their target genes (Sanchez Calle et al., 2018). Importantly, lncRNAs act as competing endogenous RNAs (ceRNAs) and participate in a miRNA-dependent crosstalk by competitively binding miRNAs, representing a critical mechanism of tumor development and metastasis (de Giorgio et al., 2013; Gao et al., 2017).

LncRNA DLEU1, located on chromosome 13q14.3, has been reported to be dysregulated in chronic lymphocytic leukemia, multiple myeloma, breast cancer, gastric cancer and ovarian cancer (Garding et al., 2013; Dowd et al., 2015; Wu et al., 2015; Wang et al., 2017; Li et al., 2018). High expression of DLEU1 was associated with poor prognosis of gastric cancer and contributes to cell proliferation (Li et al., 2018). DLEU1 was also shown to promote ovarian cancer cell proliferation and invasion by interacting with miR-490-3p (Wang et al., 2017). However, little is known about the role and the upstream regulatory mechanism of DLEU1 in CC.

In our current study, we found that DLEU1 was highly expressed in CC tissues and CC cell lines. Moreover, silencing DLEU1 expression obviously inhibited the proliferative and invasive ability of CC cells. Mechanistic analysis demonstrated that DLEU1 served as a ceRNA by sponging miR-381 and upregulating HOXA13 expression, thus promoting CC cell proliferation and invasion. The DLEU1/miR-381/HOXA13 axis might be a promising therapeutic target for CC.

MATERIALS AND METHODS

Cell Cultures and Transfection

Human CC cell lines (SiHa cells and HeLa cells) and human normal cervical cells (Academia Sinica Cell Bank, Shanghai, China) were conserved in DMEM/F12 (GIBCO-BRL) mediums with ten percent of fetal bovine serum (FBS) under a wet condition at 37°C with 5% of CO₂. Two different DLEU1 siRNAs (Genepharma, Shanghai, China) were used for knockdown of DLEU1 expression. Scrambled siRNA was used as a negative control (Genepharma, Shanghai, China). miR-381 mimic, control mimic, miR-381 inhibitor and control inhibitor were purchased from Genepharma (Shanghai, China). The DLEU1 expression plasmid pcDNA3.1-DLEU1 and the HOXA13 expression plasmid pcDNA3.1-HOXA13 were constructed by Genepharma (Shanghai, China). The empty vector pcDNA3.1 was used as negative control. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) was used for cell transfection following the guidance of the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

The expression level of DLEU1 was detected by real-time RT-PCR. In brief, total RNA from CC cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States)

according to the manufacturer's instructions. We reversely transcribed 1 µg of RNA into cDNA in virtue of a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed using SYBR-Green-quantitative real-time PCR Master Mix kit (Toyobo Co., Osaka, Japan). The mirVana™ qRT-PCR microRNA Detection Kit (Ambion Inc., Austin, TX, United States) was used for miR-381 detection according to the manufacturer's instructions. A specific stem-loop RT primer was used for miR-381 detection. The primer sequences used have been reported (Lee et al., 2017). MiR-381 was normalized to U6. DLEU1 expression data were normalized to GAPDH.

Western Blot Analysis

The entire protein lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane (Millipore, Bedford, MA, United States). Then the above PVDF membranes were incubated with the corresponding primary antibody HOXA13 (1:2000, Abcam, Cambridge, United Kingdom) and GAPDH (1:1000, Santa Cruz Biotech, Santa Cruz, CA, United States) overnight, followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz, CA, United States). Protein bands were detected using an ECL western blotting kit (Amersham Biosciences, Buckinghamshire, United Kingdom). GAPDH was used as the loading control.

CCK-8 Assay

Cervical cancer cells (5×10^3 cells per well in 96-well plates) were transfected as described above. Cell proliferation was measured 72 h after transfection using a CCK-8 (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm by a microplate reader (Bio-Rad, Hercules, CA, United States).

In vitro Invasion Assay

The invasive ability of the cells was measured using transwell chambers (Corning, New York, United States), as described previously (Dong P. et al., 2017). In brief, cells (5×10^4) suspended in serum-free medium were transferred to the upper chamber. The medium containing 10% FBS was added as chemokine in the lower chamber. After 24 h, the invaded cells on the membrane lower surface were fixed with 75% methanol, and stained with crystal violet. Evaluation of invasive capacity was performed by counting invading cells under a microscope, and five random fields of view were analyzed for each chamber. All experiments were performed in triplicate.

Luciferase Reporter Assay

The wild-type DLEU1 (DLEU1-WT), mutant DLEU1 (DLEU1-MUT), wild-type HOXA13 3'-UTR (HOXA13-WT), and mutant HOXA13 3'-UTR (HOXA13-MUT) were synthesized and cloned into pMIR-GLO™ Luciferase vectors (Promega, Madison, WI, United States). For the luciferase reporter assay, CC cells were co-transfected with the above luciferase reporter

vectors containing DLEU1 (WT or MUT) or HOXA13 3'-UTR (WT or MUT) and miR-381 mimic, miR-381 inhibitor or their respective controls using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured after 48 h. The relative luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, China). Firefly luciferase activity was normalized to that of Renilla luciferase.

RNA Immunoprecipitation Assay (RIP)

To verify the interaction between DLEU1 and miR-381, RNA immunoprecipitation assay was conducted using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, CC cells at 80% confluency were harvested and lysed in complete RIP lysis buffer. Then, the whole cell extract was co-immunoprecipitated with RIP buffer containing magnetic beads conjugated with anti-Agonate2 (Ago2) antibody (Millipore, Bedford, MA, United States) or normal mouse IgG (Millipore) as a negative control. Samples were digested with proteinase K, and RNAs were isolated from the immunoprecipitation products were subjected to qRT-PCR analysis of DLEU1 and miR-381 expression.

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 statistical software (IBM, Armonk, NY, United States). Data are presented as the mean $\pm$ standard deviation (SD) from at least three experiments. The significant differences were analyzed using Student's *t*-test or one-way ANOVA. *P*-values were based on

the two-sided statistical analysis, and a *P*-value < 0.05 from a two-tailed test was considered significant.

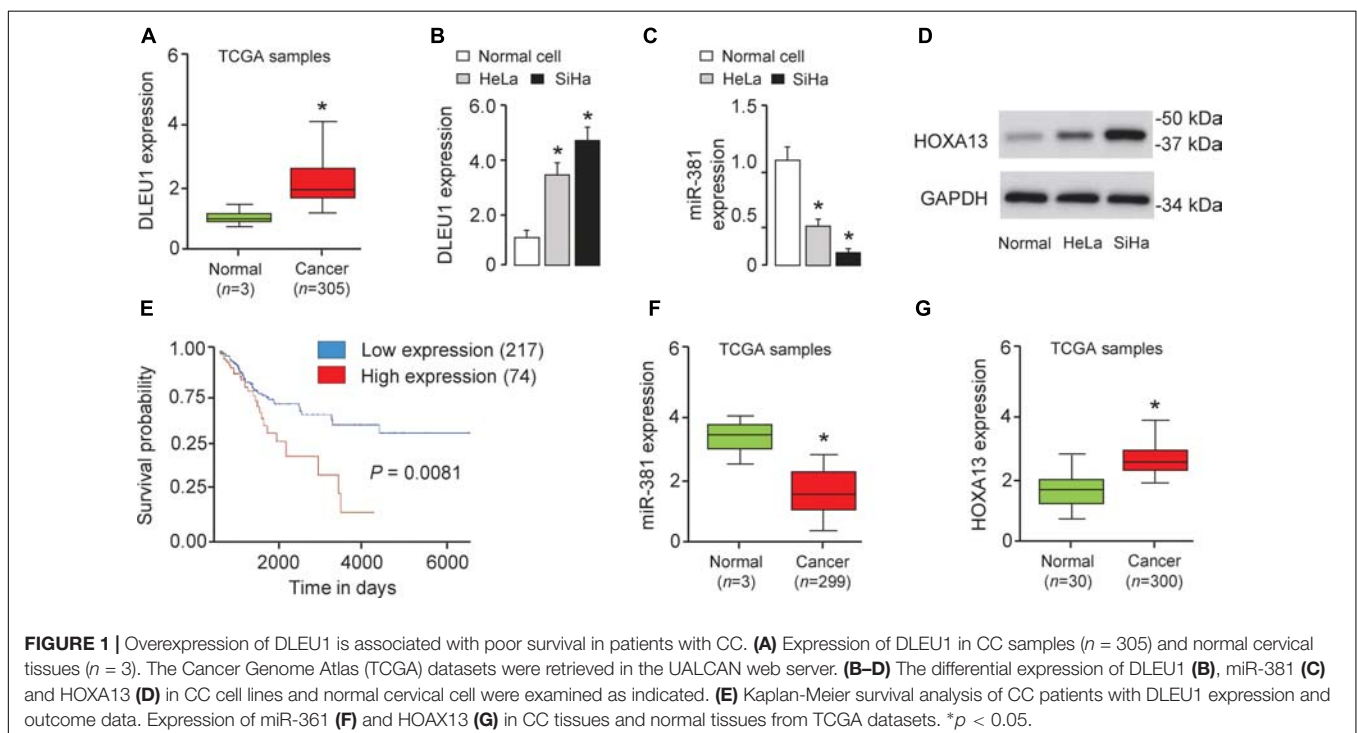
Data Availability

All data generated during this study are available from the corresponding author on reasonable request.

RESULTS

DLEU1 Is Upregulated in CC Tissues and CC Cell Lines and Significantly Correlated With Shorter Patient Survival

To investigate the role of lncRNA in CRC metastasis, we first analyzed the expression of DLEU1 in CC tissues and normal cervical tissues using the TCGA database (Chandrashekar et al., 2017). We found that the level of DLEU1 was markedly increased in CC tissues in comparison with that in the normal samples (Figure 1A). Subsequently, we investigated the expression of DLEU1 in CC cell lines (SiHa cells and HeLa cells) and human normal cervical cells. Our qRT-PCR analysis suggested that the level of DLEU1 was significantly higher in CC cells compared with the normal cervical cells (Figure 1B), indicating that DLEU1 might have an oncogenic role in CC progression. To determine the association between DLEU1 expression and patient survival, we examined the expression of DLEU1 in TCGA dataset via the UALCAN web server. For the cohort of 291 patients, overexpression of DLEU1 was associated with poor survival (Figure 1E). The difference in survival is significant ($P = 0.008$). These data indicate that DLEU1



expression may be an important prognostic factor for patients with CC.

Overexpression of DLEU1 Promotes CC Cell Proliferation and Invasion

To test the biological function of DLEU1 in CC cells, two different DLEU1-specific siRNAs were used to silence DLEU1 expression in SiHa cells, which exhibits the high level of DLEU1. The qRT-PCR analysis confirmed down-regulation of DLEU1 levels in SiHa cells. Both siRNA-1 and siRNA-2 resulted in a significant down-regulation of DLEU1 expression (Figure 2A). The transfection with siRNA-1 was more effective than the transfection with siRNA-2 in terms of downregulating the DLEU1 level. We

therefore chose to use DLEU1-siRNA-1 for all subsequent experiments.

Furthermore, HeLa cells with low expression of DLEU1 were selected in the following experiments. We stably overexpressed DLEU1 and found that DLEU1 level was significantly elevated after transfecting with pcDNA3.1-DLEU1 in HeLa cells (Figure 2A). To assess the influence of DLEU1 on cell proliferation and invasion, either loss-of-function or gain-of-function assays were performed. Results of CCK-8 assay and invasion assay showed that knockdown of DLEU1 significantly weakened the proliferative and invasive ability of SiHa cells (Figures 2B,C). However, overexpression of DLEU1 increased the proliferation and invasion of HeLa cells, as measured using the CCK-8 assay and invasion assay (Figures 2B,D). These data indicated

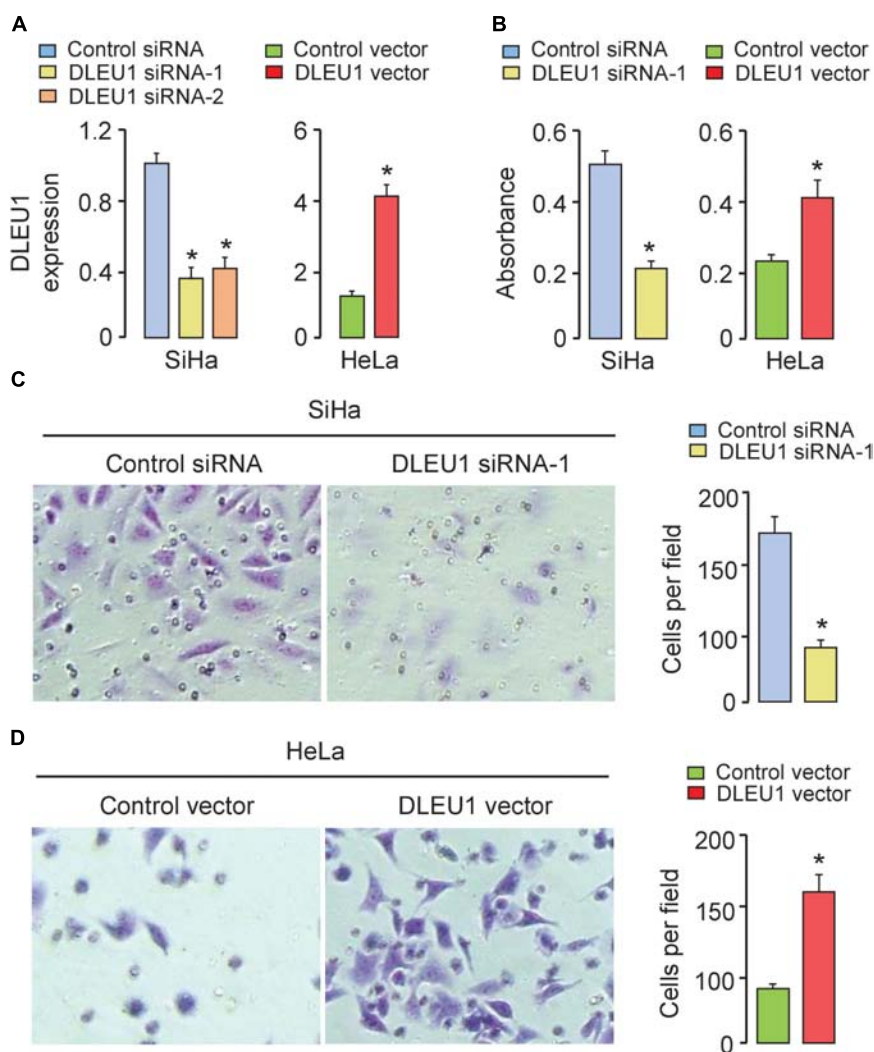


FIGURE 2 | Silencing of DLEU1 inhibited, whereas overexpression of DLEU1 promoted proliferation and invasion of CC cells. **(A)** qRT-PCR analysis of DLEU1 expression in SiHa cells transfected with DLEU1 siRNA-1, DLEU1-siRNA-2 or control siRNA, and in HeLa cells transfected with pcDNA3.1-DLEU1 or empty vector pcDNA3.1. **(B)** CCK-8 assay was used to measure cell proliferation in SiHa cells transfected with DLEU1 siRNA-1 or control siRNA, and in HeLa cells transfected with pcDNA3.1-DLEU1 or empty vector pcDNA3.1. **(C,D)** Transwell invasion assay was performed to assess invasiveness in SiHa cells transfected with DLEU1 siRNA-1 or control siRNA **(C)**, and in HeLa cells transfected with pcDNA3.1-DLEU1 or empty vector pcDNA3.1 **(D)**. * $p < 0.05$.

that overexpression of DLEU1 promoted CC cell growth and invasion.

DLEU1 Acts as a ceRNA by Sponging miR-381 in CC Cells

To investigate whether DLEU1 functions as a molecular sponge of miRNA to liberate mRNA transcript targeted by miRNA, thereby contributing to CC progression, we used the public prediction algorithm StarBase V2.0 (Li et al., 2014) and identified miR-381 with complementary sequences to the DLEU1 transcript (Figure 3A). Some ceRNAs will degrade their miRNA binding partners (Liu et al., 2017).

To study the clinical relevance of miR-381 to human CC, we examined miR-381 expression in normal cells and CC cell lines. The level of miR-381 was significantly downregulated in SiHa and HeLa cells than the normal cells (Figure 1C). Using the BioExpress database¹, we analyzed the TCGA

¹ <https://hive.biochemistry.gwu.edu/bioexpress/about>

data to evaluate miR-381 expression in human CC tissues and normal tissues. We found that miR-381 expression was markedly downregulated in CC tissues compared with normal tissues (Figure 1F). These results suggested a negative correlation between DLEU1 and miR-381 expression in CC (Figure 1A).

To examine whether DLEU1 has an impact on miR-381 expression, qRT-PCR was used to investigate the effect of DLEU1 knockdown or overexpression on miR-381 expression in CC cells. MiR-381 expression was up-regulated in SiHa cells with DLEU1 knockdown (Figure 3B). In contrast, overexpression of DLEU1 reduced the expression of miR-381 in HeLa cells (Figure 3B).

To further whether DLEU1 directly interacts with miR-381, we constructed luciferase reporter vectors containing wild-type or mutated DLEU1 and performed luciferase reporter assay. As shown in Figure 3C, ectopic expression of miR-381 resulted in a significant reduction in luciferase activity of wild-type DLEU1 in SiHa cells (Figure 3C), but had no

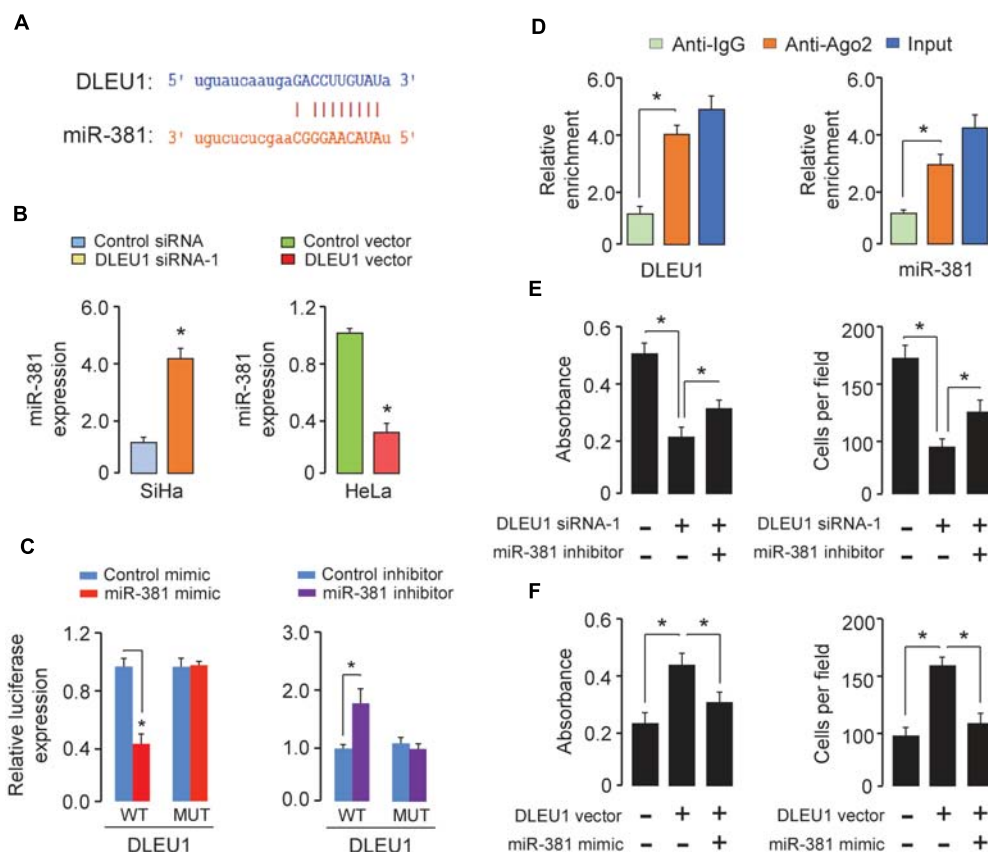


FIGURE 3 | DLEU1 acts as a ceRNA by sponging miR-381. **(A)** The predicted binding site of miR-381 to the DLEU1 sequence was shown. **(B)** qRT-PCR analysis of miR-381 in SiHa cells transfected with DLEU1 siRNA-1 or control siRNA, and in HeLa cells transfected with pcDNA3.1-DLEU1 or empty vector pcDNA3.1. **(C)** The relative luciferase activity in SiHa cells cotransfected with luciferase reporter vectors containing wild-type (WT) DLEU1 or mutant (MUT) DLEU1 and control mimic or miR-381 mimic, and in HeLa cells cotransfected with luciferase reporter vectors containing wild-type DLEU1 or mutated DLEU1 and miR-381 inhibitor or control inhibitor. **(D)** RIP assay was performed in SiHa cells. DLEU1 and miR-381 expression was detected using qRT-PCR. **(E)** Cell proliferation (left panel) and invasion (right panel) assessed in SiHa cells transfected with control siRNA + control inhibitor, DLEU1 siRNA-1 + control inhibitor, or DLEU1 siRNA-1 + miR-381 inhibitor. **(F)** Cell proliferation (left panel) and invasion (right panel) assessed in HeLa cells transfected with control vector + control mimic, DLEU1 vector + control mimic, or DLEU1 vector + miR-381 mimic. * $p < 0.05$.

evident inhibitory effect on mutant DLEU1. In addition, the transfection with miR-381 inhibitor led to a notable increase in the luciferase activity of wild-type DLEU1 in HeLa cells, while miR-381 did not affect the luciferase activity of mutated DLEU1 (Figure 3C).

In order to further verify the direct binding between miR-381 and DLEU1 at endogenous levels, RIP assay was performed to pull down endogenous miRNAs associated with DLEU1 in SiHa cells using the antibody against Ago2. We found that DLEU1 and miR-381 were specifically enriched in Ago2 pellets of SiHa cell extracts relative to the IgG control group (Figure 3D).

To determine whether DLEU1 exerted its function through miR-381 in CC cells, we performed the rescue experiments by transfecting DLEU1 siRNA in combination with miR-381 inhibitor into SiHa cells, or by transfecting DLEU1 vector in combination with miR-381 mimic into HeLa cells. The knockdown of DLEU1 significantly impeded cell proliferation and invasion of SiHa cells, while the inhibition of miR-381 significantly abrogated these effects (Figure 3E). Conversely, the induction in proliferative capacity and invasion ability caused by overexpressing DLEU1 could be largely reversed by the restoration of miR-381 in HeLa cells (Figure 3F). Taken together, our results indicated that DLEU1 could serve as a ceRNA by binding miR-381 in CC cells.

miR-381 Targeted HOXA13 in CC Cells

To further explore the potential targets of miR-381 in CC cells, we performed the bioinformatic-based target prediction analysis using TargetScan². Among the potential target genes, HOXA13 was predicted to contain the binding sequence of miR-381 (Figure 4A), and its upregulation was shown to enhance cancer cell proliferation and invasion (He et al., 2017; Yu et al., 2018). Next, we explored the protein expression of HOXA13 in CC cells and normal cells. As demonstrated in Figure 1D, in comparison with that in normal cells, the expression of HOXA13 in CC cells was much higher, as measured using Western blot analysis, indicating that miR-381 directly targets HOXA13. We examined HOXA13 expression in TCGA CC datasets using the publicly available tool MethHC³, and found higher HOXA13 mRNA levels in CC tissues compared with normal tissues (Figure 1G), suggesting a positive correlation between DLEU1 in CC (Figure 1A).

Dual-luciferase reporter assay further showed that HOXA13 was the direct target of miR-381 (Figure 4B). As shown in Figure 4C, HOXA13 expression was decreased after transfecting with miR-381 mimic, but was increased after transfecting with miR-381 inhibitor.

Restoration of HOXA13 Reversed the Effects of DLEU1 Knockdown or miR-381 Overexpression in CC Cells

Then, we conducted the rescue assays to further affirm the regulatory role of DLEU1 silencing and miR-381 inhibition on

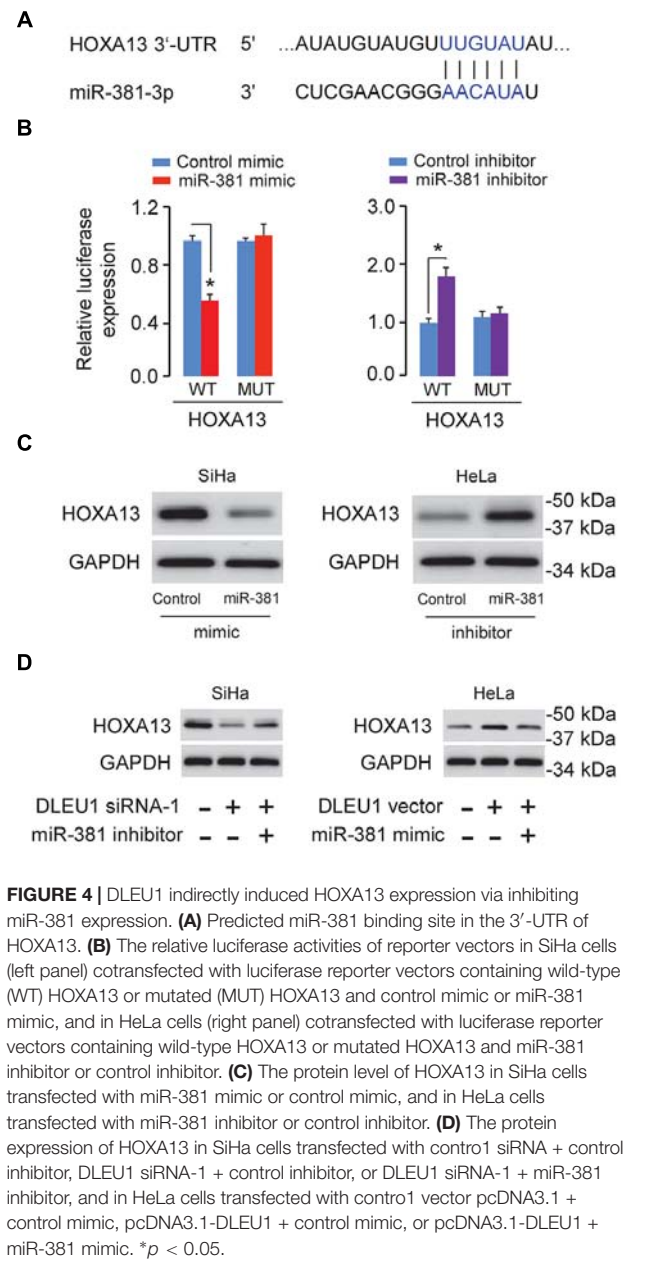


FIGURE 4 | DLEU1 indirectly induced HOXA13 expression via inhibiting miR-381 expression. (A) Predicted miR-381 binding site in the 3'-UTR of HOXA13. (B) The relative luciferase activities of reporter vectors in SiHa cells (left panel) cotransfected with luciferase reporter vectors containing wild-type (WT) HOXA13 or mutated (MUT) HOXA13 and control mimic or miR-381 mimic, and in HeLa cells (right panel) cotransfected with luciferase reporter vectors containing wild-type HOXA13 or mutated HOXA13 and miR-381 inhibitor or control inhibitor. (C) The protein level of HOXA13 in SiHa cells transfected with miR-381 mimic or control mimic, and in HeLa cells transfected with miR-381 inhibitor or control inhibitor. (D) The protein expression of HOXA13 in SiHa cells transfected with control siRNA + control inhibitor, DLEU1 siRNA-1 + control inhibitor, or DLEU1 siRNA-1 + miR-381 inhibitor, and in HeLa cells transfected with control vector pcDNA3.1 + control mimic, pcDNA3.1-DLEU1 + control mimic, or pcDNA3.1-DLEU1 + miR-381 mimic. * $p < 0.05$.

the protein expression of HOXA13 using Western blot analysis. The knockdown of DLEU1 reduced the protein level of HOXA13 in SiHa cells, while the transfection with miR-381 inhibitor apparently abolished this effect (Figure 4D). The induction of HOXA13 expression caused by DLEU1 overexpression could be largely suppressed by the transfection of miR-381 mimic in HeLa cells (Figure 4D), suggesting that DLEU1 liberated HOXA13 expression by competitively binding to miR-381.

Furthermore, CCK-8 and invasion assay showed that knockdown of DLEU1 or miR-381 overexpression significantly suppressed SiHa cell proliferation and invasion, while forced HOXA13 expression could partially rescue the anti-proliferation and anti-invasion effects mediated by the knockdown of DLEU1

²<http://www.targetscan.org>

³<http://methhc.mbc.nctu.edu.tw/php/index.php>

or miR-381 overexpression (Figure 5). These data revealed that DLEU1 might promote CC cell growth and invasion by upregulating HOXA13 via competitively interacting with miR-381.

DISCUSSION

Emerging studies have revealed that lncRNA plays an important role in cancer progression, and they are aberrantly expressed in a variety of tumors including CC (Dong J. et al., 2017). For instance, lncRNA NORAD (Huo et al., 2018), TPT1-AS1 (Jiang et al., 2018) and NEAT1 (Guo et al., 2018) could promote cell growth and metastasis in CC. HOXD-AS1 motivates doxorubicin resistance in CC (Hou et al., 2018). HOTAIR and CCHE1 were overexpressed in CC tissues, and elevated expression of HOTAIR and CCHE1 were negative prognostic factors in CC (Kim et al., 2015; Chen et al., 2017). Up to now, studies have shown that DLEU1 functioned as an oncogenic lncRNA in several tumors (Garding et al., 2013; Dowd et al., 2015; Wu et al., 2015; Wang et al., 2017; Li et al., 2018), but the contribution of DLEU1 to CC cell proliferation and invasion and the underlying mechanisms still remained to be elucidated. In this study, we found that DLEU1 level was significantly increased in the CC tissues and CC cell lines. By performing loss-of-function and gain-of-function assays, our

results revealed the oncogenic role of DLEU1 in promoting cellular proliferation and invasiveness. Thus, our study provided a new insight into the molecular mechanism of DLEU1 in CC progression.

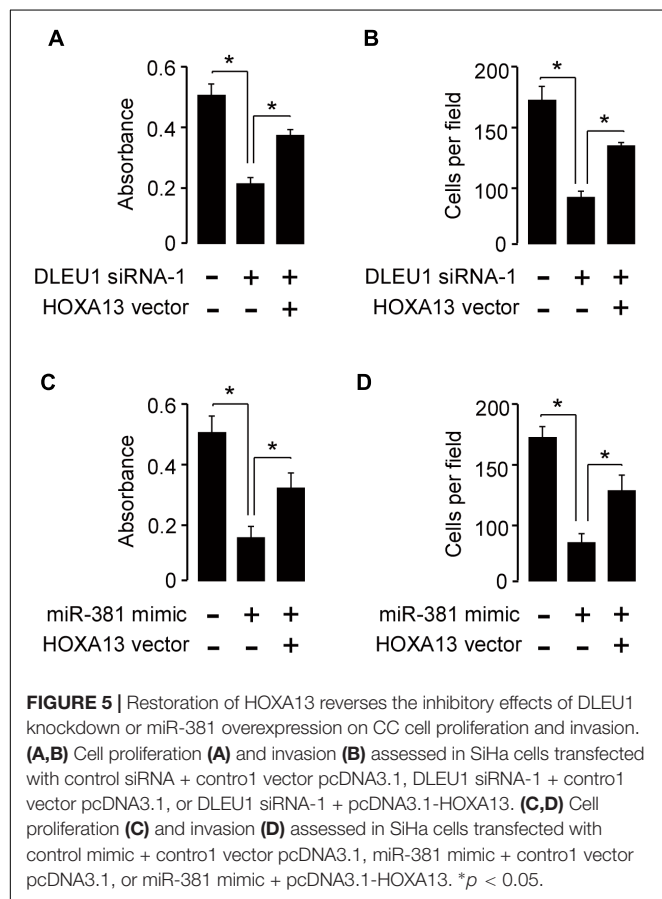
Although lncRNAs are involved in many pathological processes, the underlying molecular mechanisms remain largely unknown. Recently, a novel mechanism was proposed in which lncRNAs could serve as ceRNAs for miRNAs in cancer (Salmena et al., 2011). For example, DLEU1 functions as a ceRNA for miR-490-3p, thereby up-regulating the expression of CDK1, CCND1 and SMARCD1 and subsequently promoting the development and progression of CC (Wang et al., 2017). Here, we show that DLEU1 acts as an oncogene *in vitro* through binding miR-381. Our luciferase activity assays and RIP assays further confirmed that DLEU1 served as a molecular sponge for miR-381 to upregulate the expression of its target HOXA13, thus promoting CC pathogenesis.

Previously, miR-381 was reported to be dysregulated in many cancers, including gastric cancer (Zhang et al., 2017), oral squamous cell carcinoma (Yang et al., 2017), breast cancer (Xue et al., 2017), colorectal cancer (He et al., 2016) and ovarian cancer (Xia et al., 2016). Moreover, miR-381 acts as a tumor suppressor and was known to widely participate in tumor cell proliferation, invasion, metastasis and chemoresistance (He et al., 2016; Xia et al., 2016; Cao et al., 2017; Xue et al., 2017; Yang et al., 2017; Zhang et al., 2017; Huang et al., 2018). However, little was known about its function in CC. In the present study, we reported that miR-381 could significantly suppress CC cell growth and invasion. Furthermore, we showed the endogenous interaction between DLEU1 and miR-381 by RIP assays in CC cells, thus our results also indicated that the effects of miR-381 on proliferation and invasion are possibly due to its direct interaction with DLEU1.

HOXA13 has been reported to be upregulated and has been demonstrated to play an oncogenic role in tumorigenesis and progression in various tumors (Dong Y. et al., 2017; He et al., 2017). Although HOXA13 was highly expressed in CC tissues compared to normal cervical tissues (Saha et al., 2017), its role in the proliferation and invasion of CC cells has not been reported. Through the bioinformatic analysis, we predicted the potential miRNA binding site in HOXA13. Importantly, we found that miR-381 directly interacted with HOXA13 and downregulated its expression in CC cells. The ectopic HOXA13 expression could partially rescued the suppressive effects of miR-381 mimic or DLEU1 knockdown on cell proliferation and invasion. These experiments supported that HOXA13 acts as an oncogene in CC, and DLEU1 exerted its function via miR-381/HOXA13 axis in CC cells.

CONCLUSION

Taken together, these results revealed that DLEU1 might facilitate the progression of CC partially through the miR-381/HOXA13



axis. The DLEU1/miR-381/HOXA13 axis should be considered as a potential therapeutic target against CC.

AUTHOR CONTRIBUTIONS

CL provided the direction. CL, XT, and JZ performed the experiments. CL wrote the manuscript. LJ made significant

revisions to the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by a grant from Cancer Hospital Affiliated to Zhengzhou University.

REFERENCES

- Cao, Q., Liu, F., Ji, K., Liu, N., He, Y., Zhang, W., et al. (2017). MicroRNA-381 inhibits the metastasis of gastric cancer by targeting TMEM16A expression. *J. Exp. Clin. Cancer Res.* 36:29. doi: 10.1186/s13046-017-0499-z
- Chaffer, C. L., and Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science* 331, 1559–1564. doi: 10.1126/science.1203543
- Chandrashekar, D. S., Bachel, B., Balasubramanya, S. A. H., Creighton, C. J., Ponce-Rodriguez, I., Chakravarthi, B. V. S. K., et al. (2017). UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* 19, 649–658. doi: 10.1016/j.neo.2017.05.002
- Chen, Y., Wang, C. X., Sun, X. X., Wang, C., Liu, T. F., and Wang, D. J. (2017). Long non-coding RNA CCHE1 overexpression predicts a poor prognosis for cervical cancer. *Eur. Rev. Med. Pharmacol. Sci.* 21, 479–483.
- de Giorgio, A., Krell, J., Harding, V., Stebbing, J., and Castellano, L. (2013). Emerging roles of competing endogenous RNAs in cancer: insights from the regulation of PTEN. *Mol. Cell. Biol.* 33, 3976–3982. doi: 10.1128/MCB.0068313
- Dong, J., Su, M., Chang, W., Zhang, K., Wu, S., and Xu, T. (2017). Long non-coding RNAs on the stage of cervical cancer (Review). *Oncol. Rep.* 38, 1923–1931. doi: 10.3892/or.2017.5905
- Dong, P., Xiong, Y., Hanley, S. J. B., Yue, J., and Watari, H. (2017). Musashi-2, a novel oncoprotein promoting cervical cancer cell growth and invasion, is negatively regulated by p53-induced miR-143 and miR-107 activation. *J. Exp. Clin. Cancer Res.* 36:150. doi: 10.1186/s13046-017-0617-y4
- Dong, Y., Cai, Y., Liu, B., Jiao, X., Li, Z. T., Guo, D. Y., et al. (2017). HOXA13 is associated with unfavorable survival and acts as a novel oncogene in prostate carcinoma. *Future Oncol.* 13, 1505–1516. doi: 10.2217/fon-2016-0522
- Dowd, A. A., Homeida, S., and Elkarem, H. A. (2015). Detection of chromosome 13 (13q14) deletion among sudanese patients with multiple myeloma using a molecular genetics fluorescent in situ hybridization technique (FISH). *Malays J. Pathol.* 37, 95–100.
- Fabian, M. R., Sundermeier, T. R., and Sonenberg, N. (2010). Understanding how miRNAs post-transcriptionally regulate gene expression. *Prog. Mol. Subcell. Biol.* 50, 1–20. doi: 10.1007/978-3-642-03103-8_1
- Gao, Y. L., Zhao, Z. S., Zhang, M. Y., Han, L. J., Dong, Y. J., and Xu, B. (2017). Long noncoding RNA PVT1 facilitates cervical cancer progression via negative regulating of miR-424. *Oncol. Res.* 25, 1391–1398. doi: 10.3727/096504017X14881559833562
- Garding, A., Bhattacharya, N., Claus, R., Ruppel, M., Tschuch, C., Filarsky, K., et al. (2013). Epigenetic upregulation of lncRNAs at 13q14.3 in leukemia is linked to the in Cis downregulation of a gene cluster that targets NF-κB. *PLoS Genet.* 9:e1003373. doi: 10.1371/journal.pgen.1003373
- Guo, H. M., Yang, S. H., Zhao, S. Z., Li, L., Yan, M. T., and Fan, M. C. (2018). LncRNA NEAT1 regulates cervical carcinoma proliferation and invasion by targeting AKT/PI3K. *Eur. Rev. Med. Pharmacol. Sci.* 22, 4090–4097. doi: 10.26355/eurrev_201807_15400
- Han Li, C., and Chen, Y. (2015). Small and long non-coding RNAs: novel targets in perspective cancer therapy. *Curr. Genomics.* 16, 319–326. doi: 10.2174/1389202916666150707155851
- He, X., Wei, Y., Wang, Y., Liu, L., Wang, W., and Li, N. (2016). MiR-381 functions as a tumor suppressor in colorectal cancer by targeting Twist1. *Oncol. Targets Ther.* 7, 1231–1239. doi: 10.2147/OTT.S99228
- He, Y. X., Song, X. H., Zhao, Z. Y., and Zhao, H. (2017). HOXA13 upregulation in gastric cancer is associated with enhanced cancer cell invasion and epithelial-to-mesenchymal transition. *Eur. Rev. Med. Pharmacol. Sci.* 21, 258–265.
- Hou, W., Mao, M., Shen, Z., Chen, Y., and Chi, C. (2018). HOXD-AS1 exerts oncogenic functions and promotes chemoresistance in cisplatin-resistant cervical cancer cells. *Hum. Gene Ther.* doi: 10.1089/hum.2017.256 [Epub ahead of print].
- Huang, R. S., Zheng, Y. L., Zhao, J., and Chun, X. (2018). microRNA-381 suppresses the growth and increases cisplatin sensitivity in non-small cell lung cancer cells through inhibition of nuclear factor-κB signaling. *Biomed. Pharmacother.* 98, 538–544. doi: 10.1016/j.biopha.2017.12.092
- Huo, H., Tian, J., Wang, R., Li, Y., Qu, C., and Wang, N. (2018). Long non-coding RNA NORAD upregulate SIP1 expression to promote cell proliferation and invasion in cervical cancer. *Biomed. Pharmacother.* 24, 1454–1460. doi: 10.1016/j.biopha.2018.07.101
- Jiang, H., Huang, G., Zhao, N., Zhang, T., Jiang, M., He, Y., et al. (2018). Long non-coding RNA TPT1-AS1 promotes cell growth and metastasis in cervical cancer via acting AS a sponge for miR-324-5p. *J. Exp. Clin. Cancer Res.* 37:169. doi: 10.1186/s13046-018-0846-8
- Kim, H. J., Lee, D. W., Yim, G. W., Nam, E. J., Kim, S., Kim, S. W., et al. (2015). Long non-coding RNA HOTAIR is associated with human cervical cancer progression. *Int. J. Oncol.* 46, 521–530. doi: 10.3892/ijo.2014.2758
- Lee, S., Luo, W., Shah, T., Yin, C., O'Connell, T., Chung, T. H., et al. (2017). The effects of DLEU1 gene expression in burkitt lymphoma (BL): potential mechanism of chemioimmunotherapy resistance in BL. *Oncotarget* 8, 27839–27853. doi: 10.18632/oncotarget.15711
- Li, J. H., Liu, S., Zhou, H., Qu, L. H., and Yang, J. H. (2014). Starbase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res.* 42, D92–D97. doi: 10.1093/nar/gkt1248
- Li, X., Li, Z., Liu, Z., Xiao, J., Yu, S., and Song, Y. (2018). Long non-coding RNA DLEU1 predicts poor prognosis of gastric cancer and contributes to cell proliferation by epigenetically suppressing KLF2. *Cancer Gene Ther.* 25, 58–67. doi: 10.1038/s41417-017-0007-9
- Liu, F., Chen, N., Gong, Y., Xiao, R., Wang, W., and Pan, Z. (2017). The long non-coding RNA NEAT1 enhances epithelial-to-mesenchymal transition and chemoresistance via the miR-34a/c-Met axis in renal cell carcinoma. *Oncotarget* 8, 62927–62938. doi: 10.18632/oncotarget.17757
- Saha, S. S., Chowdhury, R. R., Mondal, N. R., Roy, S., and Sengupta, S. (2017). Expression signatures of HOX cluster genes in cervical cancer pathogenesis: impact of human papillomavirus type 16 oncoprotein E7. *Oncotarget* 8, 36591–36602. doi: 10.18632/oncotarget.16619
- Salmena, L., Poliseno, L., Tay, Y., Kats, L., and Pandolfi, P. P. (2011). A ceRNA hypothesis: the rosetta stone of a hidden RNA language? *Cell* 146, 353–358. doi: 10.1016/j.cell.2011.07.014
- Sanchez Calle, A., Kawamura, Y., Yamamoto, Y., Takeshita, F., and Ochiya, T. (2018). Emerging roles of long non-coding RNA in cancer. *Cancer Sci.* 109, 2093–2100. doi: 10.1111/cas.13642
- Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. *CA Cancer J. Clin.* 65, 87–108. doi: 10.3322/caac.21262
- Wang, L. L., Sun, K. X., Wu, D. D., Xiu, Y. L., Chen, X., Chen, S., et al. (2017). DLEU1 contributes to ovarian carcinoma tumorigenesis and development by interacting with miR-490-3p and altering CDK1 expression. *J. Cell. Mol. Med.* 21, 3055–3065. doi: 10.1111/jcmm.13217
- Wu, Q., Guo, L., Jiang, F., Li, L., Li, Z., and Chen, F. (2015). Analysis of the miRNA-mRNA-lncRNA networks in ER+ and ER- breast cancer cell lines. *J. Cell Mol. Med.* 19, 2874–2887. doi: 10.1111/jcmm.12681

- Xia, B., Li, H., Yang, S., Liu, T., and Lou, G. (2016). MiR-381 inhibits epithelial ovarian cancer malignancy via YY1 suppression. *Tumour Biol.* 37, 9157–9167. doi: 10.1007/s13277-016-4805-8
- Xue, Y., Xu, W., Zhao, W., Wang, W., Zhang, D., and Wu, P. (2017). miR-381 inhibited breast cancer cells proliferation, epithelial-to-mesenchymal transition and metastasis by targeting CXCR4. *Biomed. Pharmacother.* 86, 426–433. doi: 10.1016/j.biopha.2016.12.051
- Yang, X., Ruan, H., Hu, X., Cao, A., and Song, L. (2017). miR-381-3p suppresses the proliferation of oral squamous cell carcinoma cells by directly targeting FGFR2. *Am. J. Cancer Res.* 1, 913–922.
- Yu, H., Xu, Y., Zhang, D., and Liu, G. (2018). Long noncoding RNA LUCAT1 promotes malignancy of ovarian cancer through regulation of miR-612/HOXA13 pathway. *Biochem. Biophys. Res. Commun.* 503, 2095–2100. doi: 10.1016/j.bbrc.2018.07.165
- Zhang, M., Huang, S., and Long, D. (2017). MiR-381 inhibits migration and invasion in human gastric carcinoma through downregulatedting SOX4. *Oncol. Lett.* 14, 3760–3766. doi: 10.3892/ol.2017.6637
- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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The Functions of Non-coding RNAs in rRNA Regulation

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Ribosomes are ribonucleoprotein machines that decode the genetic information embedded in mRNAs into polypeptides. Ribosome biogenesis is tightly coordinated and controlled from the transcription of pre-rRNAs to the assembly of ribosomes. Defects or disorders in rRNA production result in a number of human ribosomopathy diseases. During the processes of rRNA synthesis, non-coding RNAs, especially snoRNAs, play important roles in pre-rRNA transcription, processing, and maturation. Recent research has started to reveal that other long and short non-coding RNAs, including risiRNA, LoNA, and SLERT (among others), are also involved in pre-rRNA transcription and rRNA production. Here, we summarize the current understanding of the mechanisms of non-coding RNA-mediated rRNA generation and regulation and their biological roles.

Keywords: rRNA, non-coding RNA, PAPAS, SLERT, 5S-OT, risiRNA, LoNA

OPEN ACCESS

Edited by:

Thomas S. Wingo,
Emory University, United States

Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 29 September 2018

Accepted: 18 March 2019

Published: 05 April 2019

Citation:

Yan Q, Zhu C, Guang S and
Feng X (2019) The Functions
of Non-coding RNAs in rRNA
Regulation. *Front. Genet.* 10:290.
doi: 10.3389/fgene.2019.00290

INTRODUCTION

In humans, there are approximately 300–400 copies of rDNA genes per haploid genome that are distributed over five chromosomes (Henderson et al., 1973; Boisvert et al., 2007). Each rDNA unit is ~43 kb long. rDNAs are transcribed by RNA polymerase I (Pol I) to generate pre-rRNAs that subsequently undergo multiple modifications and processing steps to remove the external transcribed spacers (ETSs) and internal transcribed spacers (ITSs) to produce mature 18S, 5.8S, and 28S rRNAs (McStay, 2016). Pol I activity is a key determinant for ribosome abundance and is essential for cell growth and proliferation (McStay, 2006; Srivastava et al., 2016). Interestingly, only some rDNA units are transcriptionally active. Uncontrolled rRNA synthesis by dysregulated Pol I is associated with aberrant cell proliferation and oncogenesis (Peltonen et al., 2014; Nguyen le et al., 2015).

Non-coding RNAs play essential roles in rRNA regulation. The small nucleolar RNA (snoRNA) is widely known to guide the nucleotide modifications and processing (Cech and Steitz, 2014; Sloan et al., 2017). Recently, increasingly more studies have started to reveal the roles of other classes of non-coding RNAs in regulating rRNA transcription and nucleolar function (Srivastava et al., 2016; Bazin et al., 2017). In this review, we will focus on recent work investigating how several long non-coding RNAs (lncRNAs) and antisense ribosomal siRNA (risiRNA) regulate rRNA expression and their potential biological roles in anti-stress reactions (Table 1).

LncRNAs REGULATE rRNA TRANSCRIPTION IN THE NUCLEUS

Long non-coding RNAs comprise a fast-growing classes of RNA molecules with sizes greater than 200 nt. Most lncRNAs are first transcribed by polymerase II (Pol II), then capped, polyadenylated, and spliced after transcription (Cech and Steitz, 2014). lncRNAs localize in distinct subcellular compartments, including the nucleus, nucleolus and cytoplasm. Nuclear-localized lncRNAs, such

as MANTIS and Xist, may function as transcriptional or posttranscriptional regulators or structural scaffolds for nuclear domains (Sun et al., 2017). The nucleolar localization of lncRNAs suggests that they may modulate rRNA transcription and maturation (Cech and Steitz, 2014; Chen, 2016). Several studies revealed that lncRNAs regulate rDNA transcription by altering rDNA epigenetic status or by acting as “decoys” to inhibit transcription factor activity. Interestingly, some lncRNAs might contain short open reading frames that can be translated (Ji et al., 2015).

Binding of pRNAs to TIP5 Induces Heterochromatin Formation of rDNA Genes

There are several clusters of tandemly arrayed rDNA genes exist in each mammalian genome, yet not all of these repeats are transcribed. rDNA exists in two types of chromatin – a euchromatic conformation that is actively transcribed and a heterochromatic conformation that is transcriptionally inactive. Silent rDNA repeats are marked by heterochromatic histone modifications and CpG methylation at the rDNA promoter (Schmitz et al., 2010). Silencing of rDNA depends on NoRC, a chromatin-remodeling complex that directs heterochromatin formation. NoRC function requires RNA that is complementary to the rDNA promoter, which is termed as promoter-associated RNAs (pRNA). pRNAs are 150~300 nt long and are produced from rDNA promoters. TIP5 (TIF interacting protein 5), the large subunit of NoRC, binds to pRNAs. pRNA interacts with regulatory elements in the rDNA promoter, forms a DNA:RNA triplex, and is recognized by the DNA methyltransferase DNMT3b (Figure 1; Mayer et al., 2008; Bierhoff et al., 2010). Thus, the binding of NoRC to the rDNA promoter represses rDNA transcription through recruitment of histone modifying and DNA methylating enzymes (Santoro et al., 2002; Mayer et al., 2006; Guetg et al., 2010).

Interestingly, by using mature pRNAs to tether heterochromatin at nucleoli in embryonic stem cells, Savić et al. (2014) found that localized heterochromatin condensation of rDNA genes initiates establishment of highly condensed chromatin structures outside of the nucleolus. Meanwhile, the formation of such highly condensed, transcriptionally inactive heterochromatin promotes transcriptional activation of differentiation genes and loss of pluripotency of embryonic stem cells. NoRC safeguards genome stability by triggering heterochromatin formation at telomeres and centromeres (Postepska-Igielska et al., 2013). Whether and how pRNA and NoRC function together to maintain rDNA stability requires further investigation.

LoNA Modulates rRNA to Promote Learning and Memory

When mice are trained with a Morris water maze, both rRNA and pre-rRNA levels are significantly elevated (Li et al., 2018). The trained mice exhibit decreased expression of the lncRNA LoNA in the hippocampus. LoNA is synthesized by Pol II and specifically enriched in nucleolus, and it can suppress the

transcription of pre-rRNAs (Figure 2). The 5′ portion of LoNA interacts with nucleolin (NCL), while its 3′ portion contains a snoRNA that binds to fibrillarin (FBL). NCL can remodel rDNA loci, and can, therefore, modulate pre-rRNA transcription (Abdelmohsen and Gorospe, 2012; Durut and Saez-Vasquez, 2015). LoNA binds to NCL and inactivates the chromatin status of rDNA region. FBL is a component of C/D box small nucleolar ribonucleoproteins (snoRNPs), which direct 2′-O-methylation of rRNAs and participate in rRNA processing (Newton et al., 2003). LoNA competes with snoRNAs to bind to FBL, thereby altering the methylation status of rRNAs. By binding to both NCL and FBL proteins, LoNA suppresses rRNA production and alters ribosome heterogeneity (Li et al., 2018).

Nucleolar stress is accompanied by decreased rRNA synthesis and failures in ribosome biogenesis and functions, which are considered to be cellular stress events associated with aging and neurodegenerative diseases (Boulon et al., 2010; Wu et al., 2018). In patients with Alzheimer disease (AD), rRNA production decreases (Dönmez-Altuntaş et al., 2005). In an AD animal model, LoNA expression is significantly increased in the mouse brain, which is accompanied by decreased rRNA levels (Li et al., 2018). Mice lacking LoNA are more efficient in locating the hidden platform in Morris water maze tests than are the control animals. LoNA-deficient AD mice show rescue of the learning and memory defects compared to the control animals in Morris water mazes and in object context discrimination behavioral tests. These results suggest that hippocampal LoNA is involved in learning and memory and may represent a potential therapeutic target for AD treatment.

SLERT Regulates DDX21 Rings Associated With Pol I Transcription

SLERT is a box H/ACA snoRNA-ended lncRNA (Xing et al., 2017). SLERT contains 694 nt and is highly expressed in many human cell lines, especially in human embryonic stem cells and ovarian carcinoma cells. SLERT mainly accumulates in the nucleolus, and its localization depends on its box H/ACA snoRNA ends. SLERT depletion results in decreased levels of the 18S and 28S rRNAs, indicating that SLERT promotes rRNA production.

Mass spectrometry (MS) data of SLERT-associated proteins identified DDX21, a DEAD-box RNA helicase that is involved in multiple steps of ribosome biogenesis (Holmström et al., 2008; Calo et al., 2015; Sloan et al., 2015). SLERT depletion enhances the interaction between DDX21 and Pol I by tightening the DDX21 rings surrounding Pol I complexes, thereby suppressing rDNA transcription (Xing et al., 2017). Dysregulated rRNA synthesis by Pol I is associated with uncontrolled cancer cell proliferation (Nguyen le et al., 2015). The interaction between SLERT and DDX21, therefore, represents a potential therapeutic target for future anti-cancer drug discovery (Peltonen et al., 2014).

PAPAS Responds to Environment Stresses to Maintain rRNA Suppression

Upon stress, cells utilize various strategies to suppress rDNA transcription to promote survival, for example, by inactivating

TABLE 1 | Non-coding RNAs regulate rRNA production.

RNA	Classification	Synthesis	Organisms	Length	Function	References
snoRNA	snoRNA	Pol II	Eukaryotes	70 nt	Essential for pre-rRNA processing and modification by serving as a guide RNA	Cech and Steitz, 2014
risiRNA	siRNA	RdRP	<i>C. elegans</i>	22 nt	Suppresses pre-rRNA via the nuclear RNAi pathway to inhibit the accumulation of erroneous rRNAs	Zhou et al., 2017b; Zhu et al., 2018
pRNA	lncRNA	Pol I	Mouse, human	150~300 nt	Complementary to rDNA promoters, required for the NoRC complex to suppress rRNA transcription	Bierhoff et al., 2010, 2014
LoNA	lncRNA	Pol II	Mouse	~26 kb	Regulates rRNA transcription and methylation, involved in learning and memory	Li et al., 2018
SLERT	lncRNA	Pol II	Human	694 nt	Regulates DDX21 rings associated with Pol I transcription	Xing et al., 2017
PAPAS	lncRNA	Pol II	Mouse, human	17 kb	Interacts with chromatin remodelers at rDNA loci, responds to various environmental stresses	Bierhoff et al., 2014; Zhao et al., 2016a,b
5S-OT	lncRNA	Pol II	Fission yeast and mammals	847 nt	Regulates 5S rRNA production in cis and alternative splicing in trans	Hu et al., 2016

certain transcription factors and inducing chromatin remodeling (Bierhoff et al., 2014; Holland et al., 2016). Furthermore, a class of lncRNAs, PAPAS, is expressed to inhibit pre-rRNA transcription (**Figure 3**). PAPAS is transcribed by RNA polymerase II from a fraction of the rDNA units in an antisense orientation, and, therefore, it is called “promoter and pre-rRNA antisense” (PAPAS) (Bierhoff et al., 2010, 2014). PAPAS comprises a heterogeneous population of 12–16 kb lncRNAs that are complementary to both the pre-rRNA coding region and the rDNA promoter. PAPAS responds to distinct stresses and modulates pre-rRNA synthesis accordingly.

In density-arrested or serum-deprived cells, pre-rRNA synthesis is suppressed and rDNA is enriched with H4K20me3 marks, while PAPAS is upregulated. After starved cells are refed with serum, the levels of H4K20me3 modifications and PAPAS decrease. RNA immunoprecipitation (RIP) experiments revealed that quiescence-induced PAPAS recruits Suv4-20h2

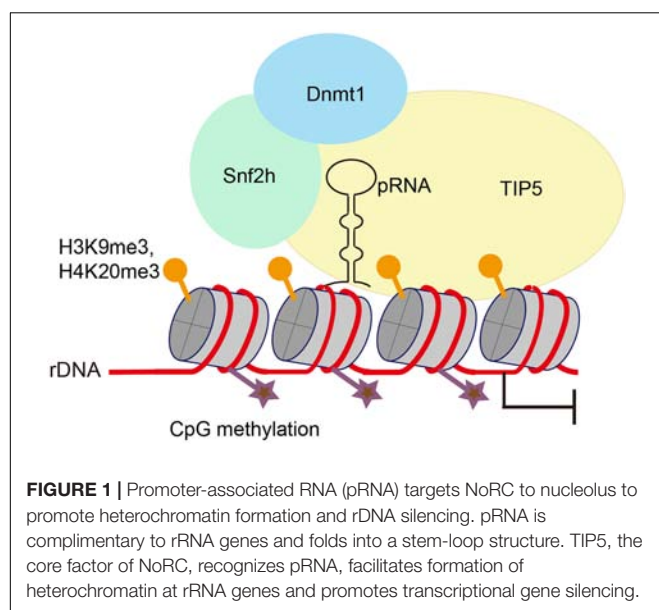
to transcription-competent rRNA genes to trigger H4K20me3 modification and chromatin compaction. Furthermore, siRNA-mediated knockdown of endogenous PAPAS decreased the level of H4K20me3 modifications, but not the level of H3K9me3 modifications (Bierhoff et al., 2014).

Hypo-osmotic stress also upregulates PAPAS and inhibits rDNA transcription. However, unlike serum deprivation, hypo-osmotic shock does not increase the Suv4-20h2 occupancy and H4K20me3 abundance at rDNA loci, but rather induces the degradation of Suv4-20h2 (Zhao et al., 2016a). The Mi-2/nucleosome remodeling and deacetylase factor (NuRD) is a multisubunit protein complex containing the HDAC1 histone deacetylase and the ATP-dependent remodeling enzyme CHD4 (Xue et al., 1998; Torchy et al., 2015). Upon hypo-osmotic stress, the elevated PAPAS associates with CHD4/NuRD and recruits them to rDNA regions where they deacetylate histone H4, remodel the promoter-bound nucleosomes, and reinforce transcriptional repression (Zhao et al., 2016a).

Similar to hypotonicity, heat shock also increases PAPAS expression, induces the degradation of Suv4-20h2, recruits NuRD to rDNA, and turns off transcription of pre-rRNA (Zhao et al., 2016b). Recent studies revealed the molecular mechanism of how PAPAS recruits CHD4/NuRD to rDNA. CHD4 is an RNA-binding protein that associates with both DNA and RNA via its N-terminal PHD and chromo-domains. Heat-shock elicits CHD4 dephosphorylation to facilitate its association with PAPAS (Zhao et al., 2018). PAPAS binds to the adjacent rDNA sequence via the formation of a DNA-RNA triplex, thereby directing CHD4/NuRD to rDNA, where it remodels the chromatin into a transcription refractory state (Zhao et al., 2016b, 2018).

5S-OT Plays a Cis Role in Regulating the Transcription of 5S rRNA and a Trans Role in Alternative Splicing of mRNAs

Unlike other Pol I-transcribed rRNAs, 5S rRNAs are transcribed by Pol III. 5S rRNA genes are clustered as tandem repeats with intergenic sequences, and they are located on distinct chromosomes (Ciganda and Williams, 2011). A number of



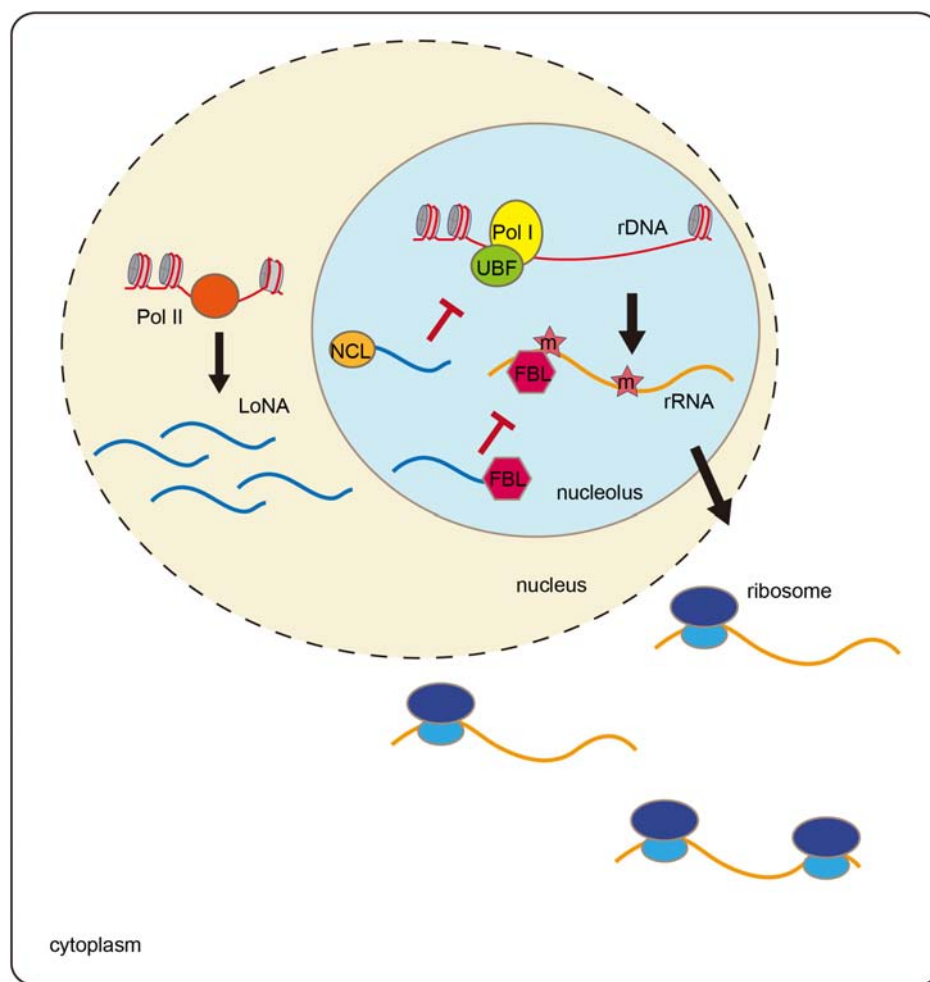


FIGURE 2 | LoNA modulates rRNA and promotes learning and memory. LoNA is synthesized by Pol II. LoNA interacts with nucleolin (NCL) and inactivates the chromatin status of rDNA region via reducing the loading of UBF and Pol I to rDNA loci. Meanwhile, LoNA competes with snoRNAs to bind to FBL, thereby altering the methylation status of rRNAs. Therefore, by binding to both NCL and FBL proteins, LoNA suppresses rRNA production and alters ribosome heterogeneity.

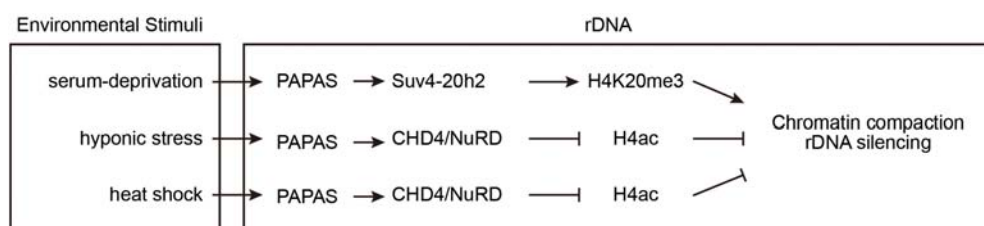


FIGURE 3 | PAPAS responds to environmental stimuli to suppress rDNA transcription. At rDNA clusters, the lncRNA PAPAS directs Suv4-20h2 for repressive mark H4K20me3 deposition after growth factor deprivation. Upon hypotonic and heat-shock stresses, PAPAS recruits NuRD to rDNA and induces a nucleosomal “off” position to repress rDNA transcription.

studies have revealed Pol II binding sites adjacent to Pol III-transcribed genes, including the 5S rRNA genes (Oler et al., 2010; Hu et al., 2012). These cryptic Pol II transcripts may therefore modulate the transcription of neighboring 5S rRNAs.

Hu et al. (2016) identified a lncRNA, 5S rRNA overlapped transcripts (5S-OT), that is transcribed by RNA polymerase

II and is complementary to the 5S rRNA. 5′ and 3′ rapid amplification of cDNA ends (RACE) experiments demonstrated that this transcript contains 847 and 354 nt in mice and humans, respectively. Chromatin immunoprecipitation (ChIP) experiments indicated that Pol II binds to the promoter at the 5S-OT transcription start site in both mouse and human cells.

Inhibition of Pol II with α -amanitin results in a decreased level of 5S-OT transcripts, which further leads to a reduction of nascent 5S rRNAs. Consistently, knocking down 5S-OT by siRNAs also inhibits the production of nascent 5S rRNAs. It was suggested that in mammalian cells, the lncRNA 5S-OT associates with 5S rDNA clusters where it promotes the transcription of 5S rRNAs, thus providing a mechanism to couple Pol II and Pol III transcription.

Furthermore, human 5S-OT contains an antisense Alu element at its 3' end (Hu et al., 2016). Alu is a primate-specific transposable element. The Alu element in the human 5S-OT gene belongs to the AluY subfamily (Batzer and Deininger, 2002). In human cells, 5S-OT regulates alternative splicing of multiple genes in trans via Alu/anti-Alu pairing with targeted genes and by interacting with the splicing factor U2AF65.

Therefore, the lncRNA 5S-OT modifies 5S rRNA and mRNAs via cis and trans mechanisms, respectively. Since 5S-OT is relatively conserved in eukaryotes from fission yeast to humans, it will be interesting to examine whether similar mechanisms are applicable in other organisms.

SMALL REGULATORY RNAs INHIBIT PRE-rRNA VIA THE NUCLEAR RNAi PATHWAY

The gene silencing capacity of small interfering RNAs (siRNAs) was first described in *Caenorhabditis elegans* two decades ago (Fire et al., 1991). siRNAs silence complementary nucleic acids in

both the cytoplasm and nucleus. Previous research has focused on the mechanism of siRNA-dependent regulation of mRNAs. In the cytoplasm, siRNAs can direct the degradation of targeted RNAs and inhibit protein translation (Ipsaro and Joshua-Tor, 2015). In the nucleus, siRNAs can guide heterochromatin formation and inhibit transcription elongation (Feng and Guang, 2013; Rechavi and Lev, 2017). Here, we will summarize our recent work that begins to illustrate the function of siRNAs in the regulation of ribosomal RNAs (Figure 4; Zhou et al., 2017a; Zhu et al., 2018).

Antisense ribosomal siRNAs (risiRNAs) are widely present in various organisms. In *Schizosaccharomyces pombe* lacking Cid14, rRNAs become substrates for the RNAi pathway, giving rise to siRNAs targeting rRNA (Buhler et al., 2008). In *Neurospora crassa*, DNA damage induces the expression of the Argonaute protein QDE-2 and a class of RNAs that interact with it (qiRNAs) from the ribosomal DNA locus (Lee et al., 2009).

In *C. elegans*, upon exposure to low temperature treatment or ultraviolet (UV) light, risiRNAs accumulate (Zhou et al., 2017b). risiRNAs are complementary to the 18S and 26S rRNAs, contain 22 nt, and start with a 5' guanosine. risiRNAs belong to a class of 22G-RNAs that are synthesized by the RNA-dependent RNA polymerases (RdRPs) in *C. elegans*. risiRNAs associate with the Argonaute protein NRDE-3 and translocate to nucleolus, where they suppress pre-rRNA expression (Zhou et al., 2017b; Zhu et al., 2018).

Ribosomal siRNAs act to surveil erroneous rRNAs and maintain rRNA homeostasis. Misprocessed rRNAs are usually detected and degraded by multiple surveillance machineries, including the exosome and Trf4/Air2/Mtr4p

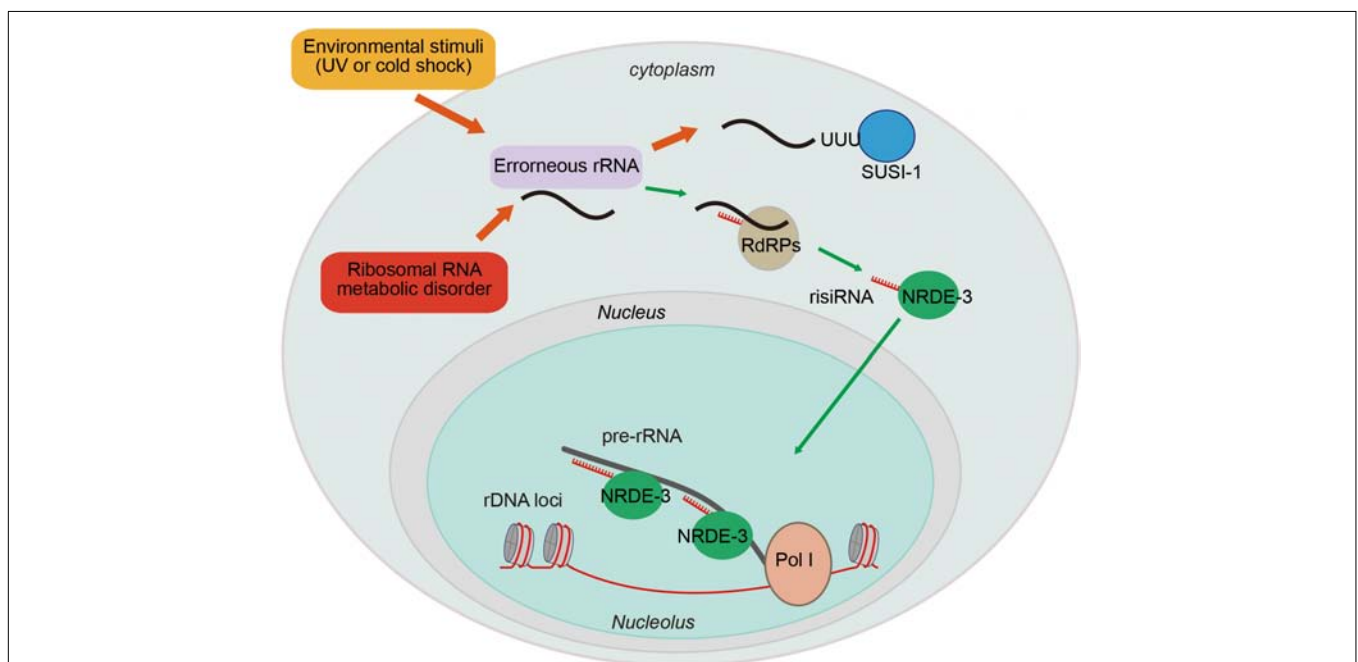


FIGURE 4 | Ribosomal siRNAs silence pre-rRNAs via the nuclear RNAi pathway. rRNA biogenesis is a multistep process, including a series of pre-rRNA processing, folding, and modification steps. Endogenous metabolic stresses and environmental stimuli can cause RNA processing disorders, leading to the accumulation of erroneous RNAs. This accumulation leads to the recruitment of RdRPs to synthesize risiRNA, subsequently turning on the nuclear RNAi-mediated gene silencing pathway to inhibit pre-rRNA expression.

polyadenylation (TRAMP) complexes (Schmidt and Butler, 2013). The exonuclease SUSI-1 (ceDIS3L2) is involved in the 3′–5′ degradation of oligouridylated rRNA fragments (Astuti et al., 2012; Zhou et al., 2017b). When the rRNA modification or processing steps are disrupted, or upon cold shock treatment or UV exposure, erroneous rRNAs are oligouridylated and recognized by RdRPs to generate risiRNAs via a poorly understood mechanism (Zhou et al., 2017b; Zhu et al., 2018). risiRNAs in turn silence rRNAs via the RNAi machinery to prohibit the accumulation of erroneous rRNAs.

Downregulation of rRNA transcription is one of the major strategies to preserve cellular homeostasis upon encountering stress conditions and to limit energy consumption under unfavorable conditions (Parlato and Liss, 2014). The risiRNA/RNAi-directed feedback loop, therefore, may compensate for dysfunctions in the exoribonuclease-engaged degradation of erroneous rRNAs. Consistently, when rRNA modification and processing steps are defective, the animals grow more slowly because of the presence of risiRNAs (Zhu et al., 2018).

PERSPECTIVES

Ribosome biogenesis is tightly coordinated and controlled from the transcription of pre-rRNAs to the assembly of ribosomes, a process that is influenced by many developmental programs and environmental stress challenges (McStay, 2016; Sloan et al., 2017). Cells respond to these signals by modulating the transcription, processing, maturation of rRNAs and the assembly and usage of ribosomes. Defects or disorders in any of these steps lead to a number of human diseases. In addition to protein factors, small and long regulatory RNAs also play important roles in the regulation of pre-rRNA transcription and rRNA maturation. The regulatory RNAs may act to sense developmental signals and environmental stresses. For example, PAPAS can sense nutrition deprivation, heat-shock, and hypo-osmotic stresses. risiRNAs respond to cold shock and UV and further down regulate pre-rRNA transcription. risiRNAs also surveil the fidelity and precision of rRNA modifications and processing to avoid the accumulation of erroneous rRNAs expressed during the development of organisms. More interestingly, the lncRNA LoNA is involved in learning and memory by modulating rRNA transcription.

REFERENCES

- Abdelmohsen, K., and Gorospe, M. (2012). RNA-binding protein nucleolin in disease. *RNA Biol.* 9, 799–808. doi: 10.4161/rna.19718
- Astuti, D., Morris, M. R., Cooper, W. N., Staals, R. H., Wake, N. C., Few, G. A., et al. (2012). Germline mutations in DIS3L2 cause the Perlman syndrome of overgrowth and Wilms tumor susceptibility. *Nat. Genet.* 44, 277–284. doi: 10.1038/ng.1071
- Batzler, M. A., and Deininger, P. L. (2002). Alu repeats and human genomic diversity. *Nat. Rev. Genet.* 3, 370–379. doi: 10.1038/nrg798
- Bazin, J., Baerenfaller, K., Gosai, S. J., Gregory, B. D., Crespi, M., and Bailey-Serres, J. (2017). Global analysis of ribosome-associated noncoding RNAs unveils new modes of translational regulation. *Proc.*

Many questions still remain to be addressed to fully understand the mechanisms and roles of non-coding RNAs in anti-stress pathways and rRNA regulation. For example, how do these lncRNAs surveil distinct environmental stresses? Cold shock could induce untemplated addition of oligouridylation at the 3′ ends of 26S rRNAs to elicit the production of risiRNAs (Zhou et al., 2017b). Heat-shock elicits CHD4 dephosphorylation to facilitate its association with PAPAS (Zhao et al., 2018). Were these surveillance mechanisms conserved among different organisms during evolution? Do poikilotherms and homeotherms use similar mechanisms to sense temperature alterations in the environment? Beside nuclear non-coding RNAs, cytoplasmic lncRNAs are frequently bound to and degraded at ribosomes (Carlevaro-Fita et al., 2016). Whether these cytoplasmic lncRNAs can in turn regulate rRNAs need further investigation. In particular, what are the biological roles of non-coding RNAs in regulating rRNAs during developmental processes? With new emerging technologies, many novel discoveries will help to answer these important questions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by grants from the National Key R&D Program of China (2018YFC1004500 and 2017YFA0102903), the National Natural Science Foundation of China (Nos. 31671346, 91640110, 31870812, and 31871300), the Major/Innovative Program of Development Foundation of Hefei Center for Physical Science and Technology (2017FXZY005), and CAS Interdisciplinary Innovation Team.

ACKNOWLEDGMENTS

We are grateful to the members of the SG's lab for their comments.

- Natl. Acad. Sci. U.S.A.* 114, E10018–E10027. doi: 10.1073/pnas.1708433114
- Bierhoff, H., Dammert, M. A., Brocks, D., Dambacher, S., Schotta, G., and Grummt, I. (2014). Quiescence-induced lncRNAs trigger H4K20 trimethylation and transcriptional silencing. *Mol. Cell* 54, 675–682. doi: 10.1016/j.molcel.2014.03.032
- Bierhoff, H., Schmitz, K., Maass, F., Ye, J., and Grummt, I. (2010). Noncoding transcripts in sense and antisense orientation regulate the epigenetic state of ribosomal RNA genes. *Cold Spring Harb. Symp. Quant. Biol.* 75, 357–364. doi: 10.1101/sqb.2010.75.060
- Boisvert, F. M., van Koningsbruggen, S., Navascues, J., and Lamond, A. I. (2007). The multifunctional nucleolus. *Nat. Rev. Mol. Cell Biol.* 8, 574–585. doi: 10.1038/nrm2184

- Boulon, S., Westman, B. J., Hutten, S., Boisvert, F. M., and Lamond, A. I. (2010). The nucleolus under stress. *Mol. Cell* 40, 216–227. doi: 10.1016/j.molcel.2010.09.024
- Buhler, M., Spies, N., Bartel, D. P., and Moazed, D. (2008). TRAMP-mediated RNA surveillance prevents spurious entry of RNAs into the *Schizosaccharomyces pombe* siRNA pathway. *Nat. Struct. Mol. Biol.* 15, 1015–1023. doi: 10.1038/nsmb.1481
- Calo, E., Flynn, R. A., Martin, L., Spitale, R. C., Chang, H. Y., and Wysocka, J. (2015). RNA helicase DDX21 coordinates transcription and ribosomal RNA processing. *Nature* 518, 249–253. doi: 10.1038/nature13923
- Carlevaro-Fita, J., Rahim, A., Guigo, R., Vardy, L. A., and Johnson, R. (2016). Cytoplasmic long noncoding RNAs are frequently bound to and degraded at ribosomes in human cells. *RNA* 22, 867–882. doi: 10.1261/rna.053561.115
- Cech, T. R., and Steitz, J. A. (2014). The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 157, 77–94. doi: 10.1016/j.cell.2014.03.008
- Chen, L. L. (2016). Linking Long Noncoding RNA Localization and Function. *Trends Biochem. Sci.* 41, 761–772. doi: 10.1016/j.tibs.2016.07.003
- Ciganda, M., and Williams, N. (2011). Eukaryotic 5S rRNA biogenesis. *Wiley Interdiscip. Rev. RNA* 2, 523–533. doi: 10.1002/wrna.74
- Dönmez-Altuntaş, H., Akalin, H., Karaman, Y., Demirtaş, H., Imamoğlu, N., and Ozkul, Y. (2005). Evaluation of the nucleolar organizer regions in Alzheimer's disease. *Gerontology* 51, 297–301. doi: 10.1159/000086365
- Durut, N., and Saez-Vasquez, J. (2015). Nucleolin: dual roles in rDNA chromatin transcription. *Gene* 556, 7–12. doi: 10.1016/j.gene.2014.09.023
- Feng, X., and Guang, S. (2013). Small RNAs, RNAi and the inheritance of gene silencing in *Caenorhabditis elegans*. *J. Genet. Genomics* 40, 153–160. doi: 10.1016/j.jgg.2012.12.007
- Fire, A., Albertson, D., Harrison, S. W., and Moerman, D. G. (1991). Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. *Development* 113, 503–514.
- Guett, C., Lienemann, P., Sirri, V., Grummt, I., Hernandez-Verdun, D., Hottiger, M. O., et al. (2010). The NoRC complex mediates the heterochromatin formation and stability of silent rRNA genes and centromeric repeats. *EMBO J.* 29, 2135–2146. doi: 10.1038/emboj.2010.17
- Henderson, A. S., Warburton, D., and Atwood, K. C. (1973). Letter: ribosomal DNA connectives between human acrocentric chromosomes. *Nature* 245, 95–97. doi: 10.1038/245095b0
- Holland, M. L., Lowe, R., Caton, P. W., Gemma, C., Carbajosa, G., Danson, A. F., et al. (2016). Early-life nutrition modulates the epigenetic state of specific rDNA genetic variants in mice. *Science* 353, 495–498. doi: 10.1126/science.aaf7040
- Holmström, T. H., Mialon, A., Kallio, M., Nymalm, Y., Mannervaa, L., Holm, T., et al. (2008). c-Jun supports ribosomal RNA processing and nucleolar localization of RNA helicase DDX21. *J. Biol. Chem.* 283, 7046–7053. doi: 10.1074/jbc.M709613200
- Hu, S., Wang, X., and Shan, G. (2016). Insertion of an Alu element in a lncRNA leads to primate-specific modulation of alternative splicing. *Nat. Struct. Mol. Biol.* 23, 1011–1019. doi: 10.1038/nsmb.3302
- Hu, S., Wu, J., Chen, L., and Shan, G. (2012). Signals from noncoding RNAs: unconventional roles for conventional pol III transcripts. *Int. J. Biochem. Cell Biol.* 44, 1847–1851. doi: 10.1016/j.biocel.2012.07.013
- Ipsaro, J. J., and Joshua-Tor, T. (2015). From guide to target: molecular insights into eukaryotic RNA-interference machinery. *Nat. Struct. Mol. Biol.* 22, 20–28. doi: 10.1038/nsmb.2931
- Ji, Z., Song, R., Regev, A., and Struhl, K. (2015). Many lncRNAs, 5'UTRs, and pseudogenes are translated and some are likely to express functional proteins. *eLife* 4:e08890. doi: 10.7554/eLife.08890
- Lee, H. C., Chang, S. S., Choudhary, S., Aalto, A. P., Maiti, M., Bamford, D. H., et al. (2009). qiRNA is a new type of small interfering RNA induced by DNA damage. *Nature* 459, 274–277. doi: 10.1038/nature08041
- Li, D., Zhang, J., Wang, M., Li, X., Gong, H., Tang, H., et al. (2018). Activity dependent LoNA regulates translation by coordinating rRNA transcription and methylation. *Nat. Commun.* 9:1726. doi: 10.1038/s41467-018-04072-4
- Mayer, C., Neubert, M., and Grummt, I. (2008). The structure of NoRC-associated RNA is crucial for targeting the chromatin remodelling complex NoRC to the nucleolus. *EMBO Rep.* 9, 774–780. doi: 10.1038/embo.2008.109
- Mayer, C., Schmitz, K. M., Li, J., Grummt, I., and Santoro, R. (2006). Intergenic transcripts regulate the epigenetic state of rRNA genes. *Mol. Cell* 22, 351–361. doi: 10.1016/j.molcel.2006.03.028
- McStay, B. (2006). Nucleolar dominance: a model for rRNA gene silencing. *Genes Dev.* 20, 1207–1214. doi: 10.1101/gad.1436906
- McStay, B. (2016). Nucleolar organizer regions: genomic 'dark matter' requiring illumination. *Genes Dev.* 30, 1598–1610. doi: 10.1101/gad.283838.116
- Newton, K., Petfalski, E., Tollervey, D., and Caceres, J. F. (2003). Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse. *Mol. Cell. Biol.* 23, 8519–8527. doi: 10.1128/MCB.23.23.8519-8527.2003
- Nguyen le, X. T., Raval, A., Garcia, J. S., and Mitchell, B. S. (2015). Regulation of ribosomal gene expression in cancer. *J. Cell Physiol.* 230, 1181–1188. doi: 10.1002/jcp.24854
- Oler, A. J., Alla, R. K., Roberts, D. N., Wong, A., Hollenhorst, P. C., Chandler, K. J., et al. (2010). Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. *Nat. Struct. Mol. Biol.* 17, 620–628. doi: 10.1038/nsmb.1801
- Parlato, R., and Liss, B. (2014). How Parkinson's disease meets nucleolar stress. *Biochim. Biophys. Acta* 1842, 791–797. doi: 10.1016/j.bbdis.2013.12.014
- Peltonen, K., Colis, L., Liu, H., Trivedi, R., Moubarek, M. S., Moore, H. M., et al. (2014). A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. *Cancer Cell* 25, 77–90. doi: 10.1016/j.ccr.2013.12.009
- Postepska-Igielska, A., Kronic, D., Schmitt, N., Greulich-Bode, K. M., Boukamp, P., and Grummt, I. (2013). The chromatin remodelling complex NoRC safeguards genome stability by heterochromatin formation at telomeres and centromeres. *EMBO Rep.* 14, 704–710. doi: 10.1038/embo.2013.87
- Rechavi, O., and Lev, I. (2017). Principles of transgenerational small RNA inheritance in *Caenorhabditis elegans*. *Curr. Biol.* 27, R720–R730. doi: 10.1016/j.cub.2017.05.043
- Santoro, R., Li, J., and Grummt, I. (2002). The nucleolar remodeling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. *Nat. Genet.* 32, 393–396. doi: 10.1038/ng1010
- Savić, N., Bär, D., Leone, S., Frommel, S. C., Weber, F. A., Vollenweider, E., et al. (2014). lncRNA maturation to initiate heterochromatin formation in the nucleolus is required for exit from pluripotency in ESCs. *Cell Stem Cell* 15, 720–734. doi: 10.1016/j.stem.2014.10.005
- Schmidt, K., and Butler, J. S. (2013). Nuclear RNA surveillance: role of TRAMP in controlling exosome specificity. *Wiley Interdiscip. Rev. RNA* 4, 217–231. doi: 10.1002/wrna.1155
- Schmitz, K. M., Mayer, C., Postepska, A., and Grummt, I. (2010). Interaction of noncoding RNA with the rDNA promoter mediates recruitment of DNMT3b and silencing of rRNA genes. *Genes Dev.* 24, 2264–2269. doi: 10.1101/gad.590910
- Sloan, K. E., Leisegang, M. S., Doebele, C., Ramirez, A. S., Simm, S., Saffertal, C., et al. (2015). The association of late-acting snoRNPs with human pre-ribosomal complexes requires the RNA helicase DDX21. *Nucleic Acids Res.* 43, 553–564. doi: 10.1093/nar/gku1291
- Sloan, K. E., Warda, A. S., Sharma, S., Entian, K. D., Lafontaine, D. L. J., and Bohnsack, M. T. (2017). Tuning the ribosome: the influence of rRNA modification on eukaryotic ribosome biogenesis and function. *RNA Biol.* 14, 1138–1152. doi: 10.1080/15476286.2016.1259781
- Srivastava, R., Srivastava, R., and Ahn, S. H. (2016). The epigenetic pathways to ribosomal DNA silencing. *Microbiol. Mol. Biol. Rev.* 80, 545–563. doi: 10.1128/MMBR.00005-16
- Sun, Q., Hao, Q., and Prasanth, K. V. (2017). Nuclear long noncoding RNAs: key regulators of gene expression. *Trends Genet.* 34, 142–157. doi: 10.1016/j.tig.2017.11.005
- Torchy, M. P., Hamiche, A., and Klaholz, B. P. (2015). Structure and function insights into the NuRD chromatin remodeling complex. *Cell. Mol. Life Sci.* 72, 2491–2507. doi: 10.1007/s00018-015-1880-8
- Wu, J., Jiang, X., Li, Y., Zhu, T., Zhang, J., Zhang, Z., et al. (2018). PHA-4/FoxA senses nucleolar stress to regulate lipid accumulation in *Caenorhabditis elegans*. *Nat. Commun.* 9:1195. doi: 10.1038/s41467-018-03531-2
- Xing, Y. H., Yao, R. W., Zhang, Y., Guo, C. J., Jiang, S., Xu, G., et al. (2017). SLERT regulates DDX21 rings associated with Pol I transcription. *Cell* 169, 664–678.e16. doi: 10.1016/j.cell.2017.04.011
- Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Côté, J., and Wang, W. (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling

- and histone deacetylase activities. *Mol. Cell* 2, 851–861. doi: 10.1016/S1097-2765(00)80299-3
- Zhao, Z., Dammert, M. A., Grummt, I., and Bierhoff, H. (2016a). lncRNA-Induced nucleosome repositioning reinforces transcriptional repression of rRNA genes upon hypotonic stress. *Cell Rep.* 14, 1876–1882. doi: 10.1016/j.celrep.2016.01.073
- Zhao, Z., Dammert, M. A., Hoppe, S., Bierhoff, H., and Grummt, I. (2016b). Heat shock represses rRNA synthesis by inactivation of TIF-IA and lncRNA-dependent changes in nucleosome positioning. *Nucleic Acids Res.* 44, 8144–8152. doi: 10.1093/nar/gkw496
- Zhao, Z., Senturk, N., Song, C., and Grummt, I. (2018). lncRNA PAPAS tethered to the rDNA enhancer recruits hypophosphorylated CHD4/NuRD to repress rRNA synthesis at elevated temperatures. *Genes Dev.* 32, 836–848. doi: 10.1101/gad.311688.118
- Zhou, X., Chen, X., Wang, Y., Feng, X., and Guang, S. (2017a). A new layer of rRNA regulation by small interference RNAs and the nuclear RNAi pathway. *RNA Biol.* 14, 1492–1498. doi: 10.1080/15476286.2017.1341034
- Zhou, X., Feng, X., Mao, H., Li, M., Xu, F., Hu, K., et al. (2017b). RdRP-synthesized antisense ribosomal siRNAs silence pre-rRNA via the nuclear RNAi pathway. *Nat. Struct. Mol. Biol.* 24, 258–269. doi: 10.1038/nsmb.3376
- Zhu, C., Yan, Q., Weng, C., Hou, X., Mao, H., Liu, D., et al. (2018). Erroneous ribosomal RNAs promote the generation of antisense ribosomal siRNA. *Proc. Natl. Acad. Sci. U.S.A.* 115, 10082–10087. doi: 10.1073/pnas.1800974115
- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Systematic Analysis of Intestinal MicroRNAs Expression in HCC: Identification of Suitable Reference Genes in Fecal Samples

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to RNA,
a section of the journal
Frontiers in Genetics

Received: 03 September 2018

Accepted: 01 July 2019

Published: 13 August 2019

Citation:

Wang H, Lv Y, Wang C, Leng D,
Yan Y, Blessing Fasae M,
Madiha Zahra S, Jiang Y,
Wang Z, Yang B and Bai Y (2019)
Systematic Analysis of Intestinal
MicroRNAs Expression in HCC:
Identification of Suitable Reference
Genes in Fecal Samples.
Front. Genet. 10:687.
doi: 10.3389/fgene.2019.00687

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Hepatocellular carcinoma (HCC) is an extremely fatal malignancy. Intestinal microRNAs, which can be detected in fecal samples in humans may be involved in the pathological process of HCC. Therefore, screening for functional intestinal microRNAs in fecal samples and investigating their potential roles in the molecular progression of HCC are necessary. Quantitative real-time PCR (qRT-PCR) has been widely used in microRNA expression studies. However, few genes have been reported as reference genes for intestinal microRNAs in fecal samples. In order to obtain a more accurately analyzed intestinal microRNAs expression, we first searched for reliable reference genes for intestinal microRNAs expression normalization during qRT-PCR, using three software packages (GeNorm, NormFinder, and Bestkeeper). Next we screened and predicted the target genes of the differentially intestinal microRNAs of control and HCC mice through quantitative RT-PCR or miRtarBase. Finally, we also analyzed the mRNA targets for enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the DAVID Bioinformatic Resources database. This study has successfully screened relatively suitable reference genes and we have discovered that the differential intestinal microRNAs play significant roles in the development of HCC. The top reference genes identified in this study could provide a theoretical foundation for the reasonable selection of a suitable reference gene. Furthermore, the detection of intestinal microRNAs expression may serve as a promising therapeutic target for the diagnosis and treatment of HCC.

Keywords: intestinal microRNAs, quantitative real-time PCR assays, reference genes, hepatocellular carcinoma, feces

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, and the overall 5-year survival rate for HCC is less than 12% (Torre et al., 2015). It is usually diagnosed at the late stage and is characterized by a low resection rate, high postoperative recurrence, and poor drug treatment results (Galun et al., 2015). At present, the only drug approved for the treatment of liver cancer is sorafenib. Oxaliplatin has been shown to be effective in the treatment of advanced liver cancer, but the drug resistance potentially limits its efficacy (Petrelli et al., 2014). Finding sensitive and specific early diagnostic biomarkers, and understanding the mechanisms involved in the development and progression of HCC are a major issue in need of urgent resolution.

MicroRNAs are small non-coding RNAs, with transcripts that are just 18–25 nucleotides in length, and they bind mainly to the 3′ untranslated regions (3′ UTRs) of target RNAs through the microRNAs' seed sequences (microRNA nucleotides 2–7) (Parodi et al., 2016). They are highly conservative and tissue-specific and exists widely in nematodes, drosophila, and plants, as well as in humans (Gillan et al., 2017; You et al., 2018). MicroRNAs regulate the expression of protein-coding gene expression in a sequence-specific manner through cleavage or translational repression in the genomes (Kagiya, 2016; Xiao et al., 2018). The abnormal expression of microRNAs is related to many diseases, including cancer, and miRNAs can also be used as tumor-suppressor genes or oncogenes. During the development and progression of human cancers, microRNAs have been observed to regulate cell proliferation, survival, differentiation, invasion and metastasis (Davis-Dusenbery and Hata, 2010). In patients with HCC caused by a hepatitis B virus infection, mir-29c could control cell proliferation and leads to apoptosis by targeting TNFAIP3 (Wang, Wang et al., 2011); mir-101 produces a pro-apoptotic effect by targeting Mcl-1 (Su et al., 2009). Since the discovery of their essential roles in the occurrence and development of diseases, microRNAs have been intensely studied as prognostic and diagnostic biomarkers and predictors of drug responses (Li et al., 2014).

Past research has focused mainly on post-transcriptional and translational controls regulated by non-coding RNAs (Ranjha and Paul, 2013). MicroRNAs could efficiently interrupt the synthesis of proteins excluding further transcriptional activation and any subsequent mRNA processing steps. Therefore, it provides cells with a more accurate, direct and energy-efficient way to manipulate protein expression (Xi et al., 2007; Ge et al., 2015; Connerty et al., 2016). Liu et al. (2016) have illustrated that microRNAs in feces, which originate from intestinal cells, can enter into bacteria and regulate their gene expression and growth. These special microRNAs are called intestinal microRNAs. They specifically show that mir-515-5p and mir-1226-5p can promote the growth of *Fn* and *E. coli*, respectively. Thus, knowing the differential expressions of intestinal microRNAs in fecal samples could be critical to understand physiological and pathological conditions. They could even be used as a potential marker for a new therapeutic strategy. However, the expression profiles and functions of the intestinal microRNAs present in the fecal samples of patients with HCC remains unclear.

Due to the instability of microRNAs, their low expression level and minor differences in sequence, highly sophisticated analytical methods are required. Quantitative real-time PCR (qRT-PCR) plays an important role in studying the biological functions of microRNA with strong specificity and high sensitivity (Daud and Scott, 2008). With the development of qRT-PCR, the selection of reference genes is crucial for the accuracy of the relative quantification of gene expressions (Liu et al., 2018; Zhou et al., 2018). However, only a few studies have been published that systematically evaluated the normalization targets in intestinal microRNAs qRT-PCR assays. Successfully screening for suitable reference genes first will provide reliable evidence for the quantitative analysis of intestinal microRNAs in fecal samples. Once the expression profile of intestinal microRNAs in HCC is confirmed by qRT-PCR, the functions of differentiated intestinal microRNAs could be investigated during the biological process.

This study aimed to find relatively stable reference genes and to clarify whether intestinal microRNAs from fecal samples play an important role in indicating HCC development. RNA was extracted from mouse fecal samples and microRNA expressions were detected by qRT-PCR. Four conventional housekeeping genes and 18 microRNAs were selected as candidate genes. Their expression stability was evaluated using GeNorm, NormFinder, and Bestkeeper software. These statistical analysis software programs were primarily used to identify appropriate reference genes in qRT-PCR experiments. We identified three genes (*mir-23a*, *GAPDH* and *let-7i*) which may be used as suitable reference genes for intestinal microRNAs. Next, we detected the expression levels of some intestinal microRNAs, which had already been observed in humans and mice, between the control and HCC group. Finally, we analyzed the mRNA targets of the differentiated intestinal microRNAs. Then GO terms and KEGG pathways were built using these databases. In this study, relatively suitable reference genes of intestinal microRNAs in fecal samples are first screened for, with a potential diagnosis and treatment for patients with HCC is then proposed.

MATERIALS AND METHODS

Samples Preparation and the Ethics Approval

Professor Feng Hai from Harbin Medical University supplied fecal samples from five control and five HCC-suffering mice. The C57BL/6 mice (14th postnatal days) were injected DEN at a single intraperitoneal dose of 25 mg/kg and the animals were observed until 10 months. At 10 months of age, mice injected with DEN developed striking liver HCCs with tumors that could be clearly observed. The mice were allowed to defecate normally, and the first three fecal pellets of each animal were collected into an empty 1.5 ml tube with a sterile toothpick. A new toothpick was used for each mouse. The tubes were closed immediately and placed into liquid nitrogen, finally transferred into a −80°C refrigerator for storage. The methods were performed in accordance with the National Guidelines for Experimental Animal Welfare (the Ministry of Science and Technology, People's Republic of China,

2006). All experimental protocols were pre-approved by the Experimental Animal Ethics Committee of Harbin Medical University, China.

Total RNA Extraction

One ml of Trizol isolation reagent (Invitrogen, Carlsbad, CA) was added per 40–60 mg of frozen fecal samples and total RNAs was extracted according to the manufacturer's protocol. The only difference from the usual RNA extraction protocol used in fecal samples was the need for repeated extraction using chloroform. The concentrations and purity of the total RNA extracted were measured by ultraviolet spectrophotometer NanoDro 2000 (Thermo Scientific, Waltham, USA). Purity requirements were considered to be met if the extracted RNA had an A260/A280 value between 1.80 and 2.00.

Quantitative Real-Time PCR Assays

For reverse transcription, 1 µg of total RNA was reverse transcribed using a ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). The reverse transcription used a 20 µl reaction with random primers or gene-specific stem-loop primers, designed as described previously (Chen et al., 2005). All microRNA RT primer sequences are shown in **Table S1**. At the beginning of the experiment, the amplification efficiencies of the primers were first tested. According to the relative standard curve, the amplification efficiency of primers was about 93–116%. The cDNA was amplified by real-time PCR using SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) on the ABI 7500 fast Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The primer sequences of the primers (Invitrogen, Shanghai, China) are listed in **Table S1**. The quantitative RT-PCR reaction mixture was run in a 20 µl volume reaction. The mixture consisted of 10 µl SYBR Green PCR Master Mix, 1.0 µl of each specific primer, 2.0 µl cDNA template, and 6.0 µl RNase-free water. The reactions performed were as follows: 1 min at 95°C for pre-denaturation, 40 cycles of 15 s at 95°C for denaturation, 15 s at 60°C for annealing, 45 s at 72°C for extension.

Data Preprocessing

Bestkeeper uses the cycle threshold (Ct) value directly from the qRT-PCR to analyze the stability of the gene, whereas the Ct value needs to be converted to a Q value before analysis by GeNormer and NormFinder. The formula for calculating the Q value is: $Q = 2^{-(Ct/sample - Ct/min)}$. The calculated results were then imported into Excel according to the rules of GeNormer and NormFinder as they only identify a specific format (Vandesompele et al., 2002, Andersen et al., 2004).

MicroRNA Target Prediction and Functional Classification

The miRTarBase database (<http://miRTarBase.mbc.nctu.edu.tw/>) is a commonly used database for microRNA-target interactions. Using miRTarBase, all the validated mRNA targets

of the differentially expressed intestinal microRNAs of control and HCC mice were listed. To create more accurate and reliable predicted results, only target genes validated by at least one experimental method were chosen. The DAVID Bioinformatic Resources database (<https://david.ncifcrf.gov/>) was then used to analyze the mRNA targets for enrichment of GO terms and KEGG pathways. A P value of <0.05 was used as the significance cut-off.

Statistical Analysis

All measurement data were presented as mean ± SEM and analyzed by GraphPad Prism 5. Differences between groups were analyzed by Student's t-test, where P < 0.05 was considered as indicative of a statistically significant difference.

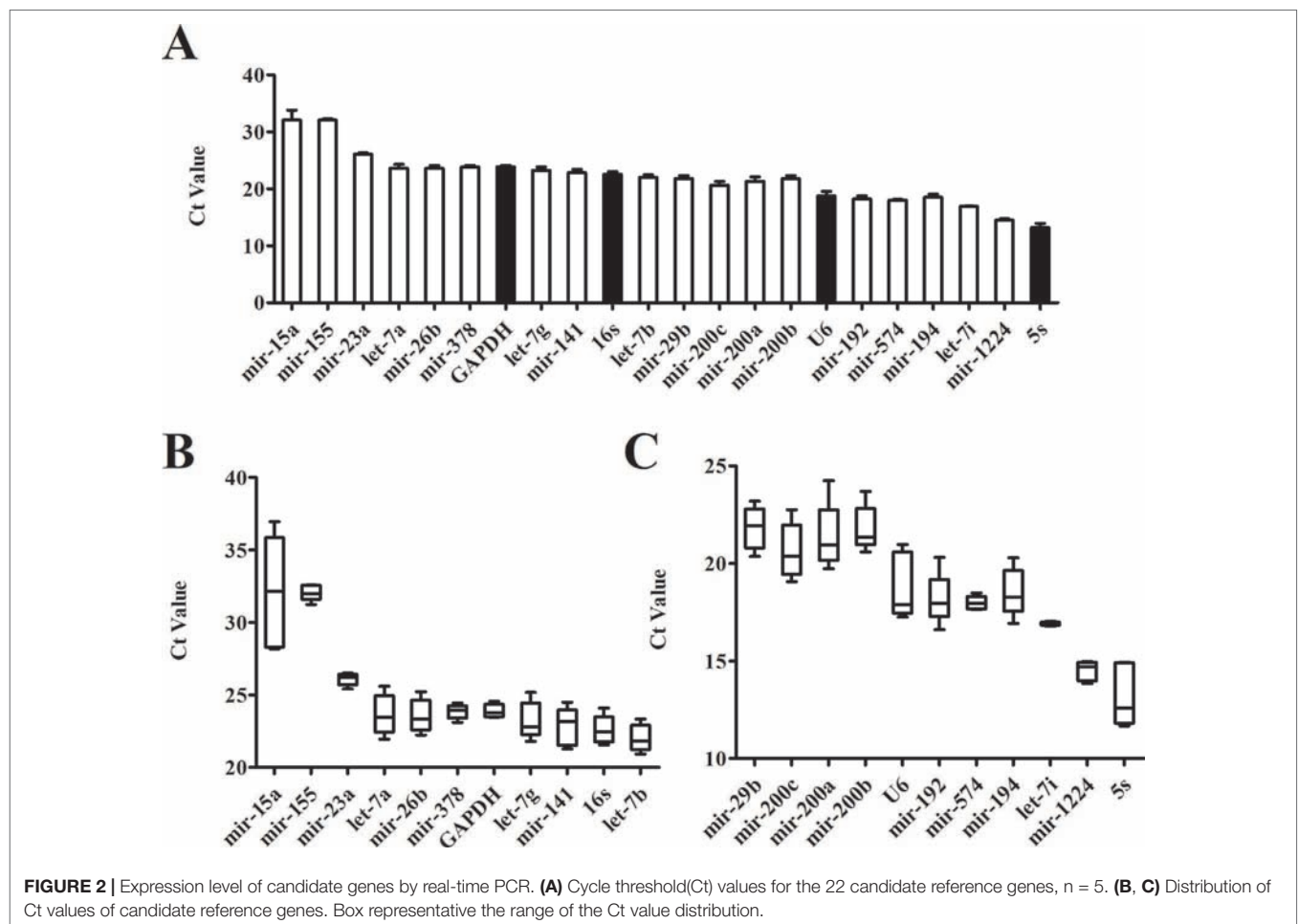
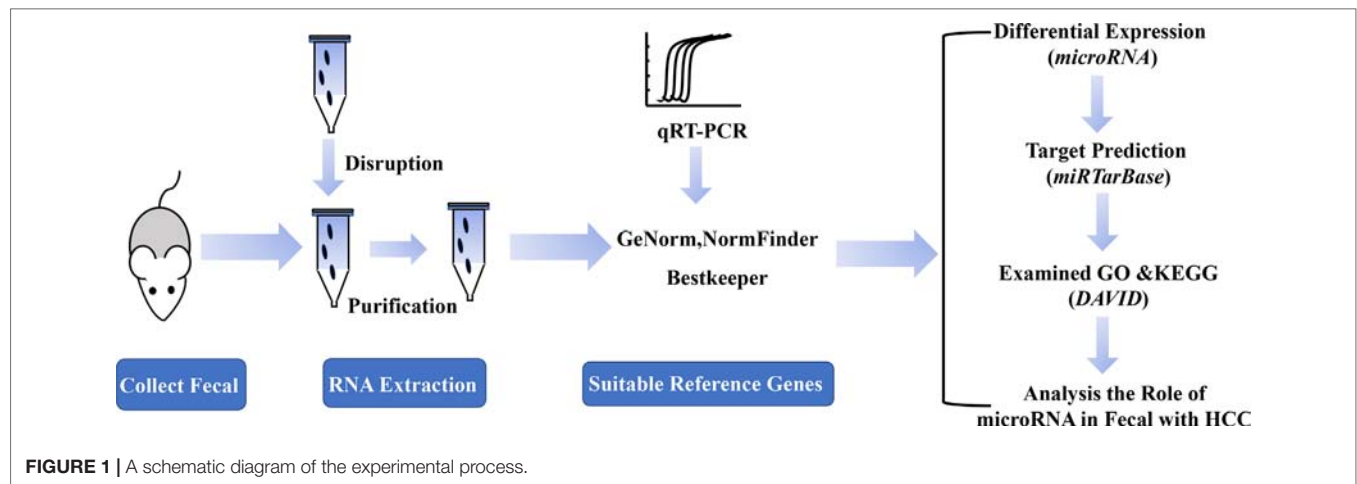
RESULTS

A Schematic Diagram for the Experimental Process

In order to identify the suitable reference genes for intestinal microRNAs in fecal samples and to systematically analyze intestinal microRNAs expression in HCC, a schematic flow diagram of sample processing and microRNA analysis was first designed (**Figure 1**). Due to the novelty and scarcity of data in this area of study, 17 intestinal microRNAs, commonly expressed both in human and mouse fecal samples, were selected as candidate genes (Liu et al., 2016). Generally, *GAPDH*, *U6 snRNA*, *16S rRNA* and *5S rRNA* were also selected as they are usually used as reference genes.

Comparison of Ct Values Between Candidate Reference Genes

Even in different tissues or different growth stages of the same organ, there may be differences in the expression of the same gene. The Ct value of the gene is inversely proportional to the amount of target nucleic acid present. The lower the Ct value, the higher the amount of the gene present. According to its Ct value from real-time PCR, we analyzed the expression of each candidate reference gene (**Figure 2A**). The Ct values of 22 candidate reference genes ranged between 11 and 37. Large differences and a wide distribution range were found between the expressions of the candidate genes. The traditional reference genes *5S rRNA* had the highest expression level, where the Ct value was between 11.67–14.94; the expressions of *U6 snRNA*, *mir-192*, *mir-574*, *mir-194*, *let-7i*, and *mir-1224* were relatively high; *Mir-15a* and *mir-155* showed a larger Ct value with low expressions. Additionally, the box plots illustrated that the expression range of each candidate reference gene varied significantly (**Figures 2B, C**). The Ct value of *mir-155*, *mir-23a*, *mir-378*, *GAPDH*, *mir-574*, *let-7i* and *mir-1224* had fluctuating ranges that were comparatively small with a more concentrated distribution. Preliminary Ct values showed that the expression of *mir-23a*, *GAPDH*, *mir-574*, *let-7i*, and *mir-1224* was higher and had few differences between the samples.



Expression Stability of Candidate Reference Genes

Three software packages (GeNorm, NormFinder, and Bestkeeper) were used for analyzing the average expression stabilities of the candidate reference genes. GeNorm and NormFinder software was specifically used for selecting reference genes. Before the analysis,

it was necessary to convert the qRT-PCR result (Ct value) to a Q value. GeNorm software was written by Vandesompele in 2002 for screening reference genes where two or more reference genes can be chosen (Vandesompele et al., 2002). For the screening and selection of reference genes, an M value is usually displayed according to the output file of the GeNorm program. The default

critical M value of the software is 1.5, where an M value of less than 1.5 indicates that the gene expression is relatively stable. The smaller the M value, the more stable the gene is. Through GeNorm software, we analyzed the M values of 22 candidate reference genes (**Figure 3A**). The data showed that the M value of *mir-200b* was the largest, whereas *GAPDH* and *mir-23a* were the smallest, indicating that *GAPDH* and *mir-23a* were the most stable genes. However, the traditional reference genes *U6 snRNA* and *5S rRNA* remained rather unstable. Another program, NormFinder, was written in 2004 by Claus, and was used to screen the stability of qualitative reference genes (Andersen et al., 2004). The calculation principle of this program is similar to that of GeNorm and the stability value used is also the M value. The results showed that *mir-23a*, *mir-378*, and *GAPDH* were relatively more constant (**Figure 3B**). *Mir-200b* and *mir-15a* were discarded in the results since they had M values that were too large. These results were similar to those obtained from the GeNorm software. The Bestkeeper software was also used to evaluate the stability of the candidate genes (Pfaffl et al., 2004). The Ct values of 22 candidate reference genes were calculated, along with the standard deviation (SD) and coefficient of variation (CV). Our results showed that higher gene stability is related to a smaller SD and CV. The data showed that *let-7i* had the lowest SD and CV values of 0.06 and 0.36, respectively, followed by

mir-23a, *mir-574*, and *GAPDH* (**Figure 3C**, **Table S2**). The results from Bestkeeper were somewhat similar to those from GeNorm and NormFinder.

Comprehensive Evaluation of Candidate Reference Genes in the Three Software Programs

When comparing the results of the three software programs, we found that despite the similar results obtained, there were still differences among them. A geometric mean method was thus used to evaluate the stability of each candidate reference gene. The candidate genes' stabilities were ranked in each of the software programs and then the rankings were averaged across the three software programs (**Table 1**). The top three genes were *mir-23a*, *GAPDH* and *let-7i*. These three genes were found to be the most suitable reference genes for intestinal microRNAs. Considering that the expression and distribution of *let-7i* was the most concentrated and the highest, it was selected as a reference gene for the subsequent analysis. To further identify the stability of *let-7i* as a reference gene, a validated experiment was carried out in different animal models. We collected the fecal samples from some disease models whose incidence is higher, such as HCC, Type 2 diabetes (T2DM) and Myocardial Infarction (MI). The Ct value dispersion of the *let-7i*

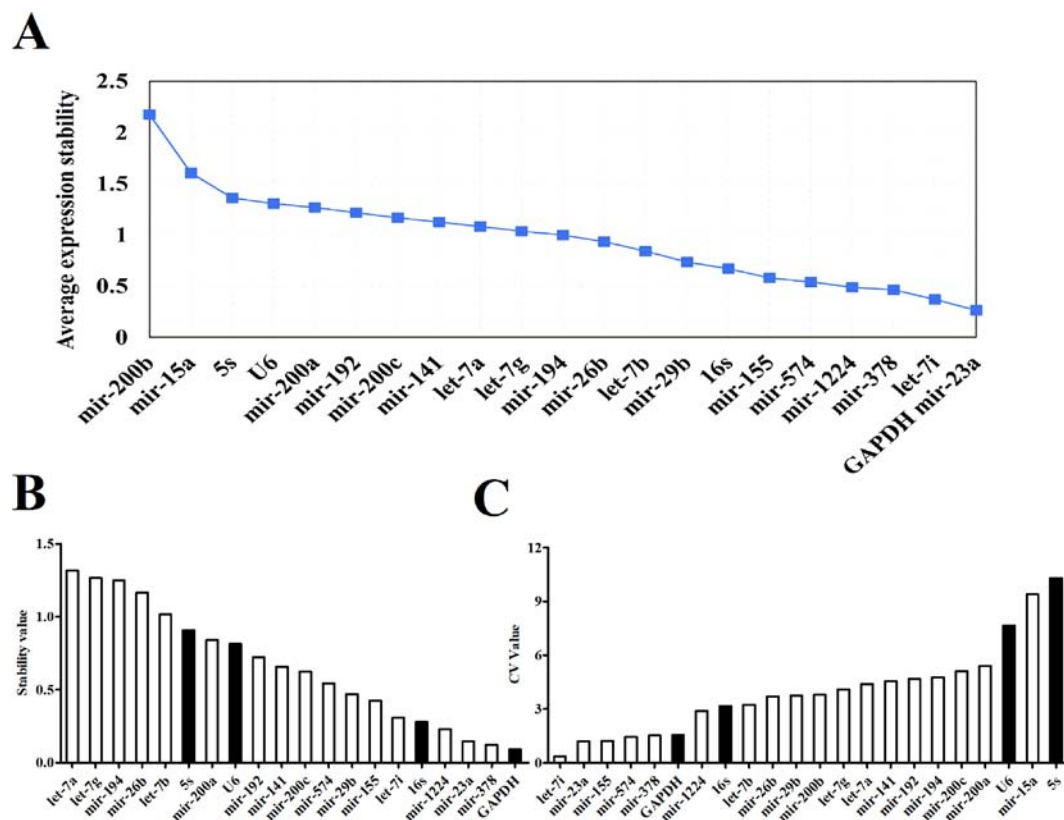


FIGURE 3 | The expression stabilities of candidate reference genes analyzed by software. **(A)** Average expression M values analysis of candidate reference genes by geNorm software. **(B)** Average expression stability values analysis of candidate reference genes by NormFinder software. **(C)** Average expression CV values analysis of candidate reference genes by Bestkeeper software.

TABLE 1 | The overall ranking of expression stability for candidate reference genes.

Gene name	GeNorm	NormFinder	BestKeeper	Average	Comprehensive rankings
mir-23a	1	3	2	2	1
GAPDH	1	1	6	2.667	2
let-7i	3	6	1	3.333	3
mir-378	4	2	5	3.667	4
mir-1224	5	4	7	5.333	5
mir-155	7	7	3	5.667	6
mir-574	6	9	4	6.333	7
16S rRNA	8	5	8	7	8
mir-29b	9	8	11	9.333	9
let-7b	10	16	9	11.667	10
mir-26b	11	17	10	12.667	11
mir-141	15	11	15	13.667	12
mir-200c	16	10	18	14.667	13
mir-192	17	12	16	15	14
let-7g	13	19	13	15	15
mir-194	12	18	17	15.667	16
let-7a	14	20	14	16	17
mir-200a	18	14	19	17	18
U6 snRNA	19	13	20	17.333	19
mir-200b	22	22	12	18.667	20
5s rRNA	20	15	22	19	21
mir-15a	21	21	21	21	22

was detected by qRT-PCR. The result showed that there was no significant difference between different disease animal models (**Figure S1A**). Additionally, the boxplot also indicated that the distribution of *let-7i* was concentrated within groups (**Figure S1B**). These assays demonstrated that *let-7i* displayed higher stability which could be used as a reference gene for microRNA qRT-PCR in fecal samples.

Altered Intestinal MicroRNAs Expression Profiles in HCC Mice

In order to explore the functional intestinal microRNAs in HCC, we detected the expression levels of microRNAs which had been observed both in humans and mice. According to the comprehensive evaluation of candidate reference genes in the three software programs and their expression levels (Ct value), we chose *let-7i* as an internal reference gene. We demonstrated the presence of 11 miRNAs that displayed a decrease (*miR-155*, *miR-378*, *mir-23a*, *mir-26b*, *mir-29b*, *mir-194*, *mir-192*, *mir-15a*, *let-7a*, *mir-200a*, and *mir-200b*), in the HCC group compared with the control group, while *mir-200c*, *mir-1224*, *mir-574*, *mir-141*, *let-7b*, and *let-7g* showed no significant differences between the groups (**Figure 4**).

Enrichment of Predicted mRNA Targets for Differentiated Intestinal MicroRNAs

To clarify downstream target gene networks for the intestinal microRNAs with HCC, we first listed all the potential mRNA targets of the differentiated intestinal microRNAs. These were predicted using miRTarBase. In order to improve the accuracy of the predicted mRNA targets, we only chose targets with strong evidence which had been supported by the reporter assay, western blotting or qPCR. The GO terms and KEGG pathways were then

examined using the DAVID database. The results obtained from GO terms showed that the major processes in the pathology of HCC includes apoptosis, cell proliferation and the Wnt signaling pathway (**Figure 5A**). The KEGG pathway analysis explained that mRNA targets were mainly concentrated in the Jak-STAT pathway, apoptosis, and the p53 signaling pathway (**Figure 5B**). These results suggested that the differential intestinal microRNAs are likely to indicate the occurrence of HCC through predicted targets and pathways.

DISCUSSION

MicroRNAs are expected to become potential biomarkers for cancer diagnosis and new targets for disease treatments as aberrant expressions of microRNAs may be related to specific diseases. In recent years, quantitative RT-PCR assays have become the main method of studying the relative expression of microRNA. However, the quality of the results may be affected by some non-specific variables, such as the quality of RNA, efficiency of PCR amplification and the selection of reference genes (Yang et al., 2017). Appropriate normalization is a key part of quantitative gene expression analysis which is often ignored. The reference gene is also known as a housekeeping gene and is usually used to correct experimental errors when testing the gene expression levels. The ideal internal reference gene needs to satisfy the following conditions: the gene's expression should be stable in different tissues, experimental conditions and growth stages (Lv et al., 2017, Molina et al., 2018). By using stable reference genes, the data in quantitative RT-PCR assays can be standardized. However, no reference genes have been found to be stable under all experimental conditions. Therefore, when studying specific gene expressions, it is necessary to select the most stable gene as the reference gene to correct the data and to ensure the accuracy of the results.

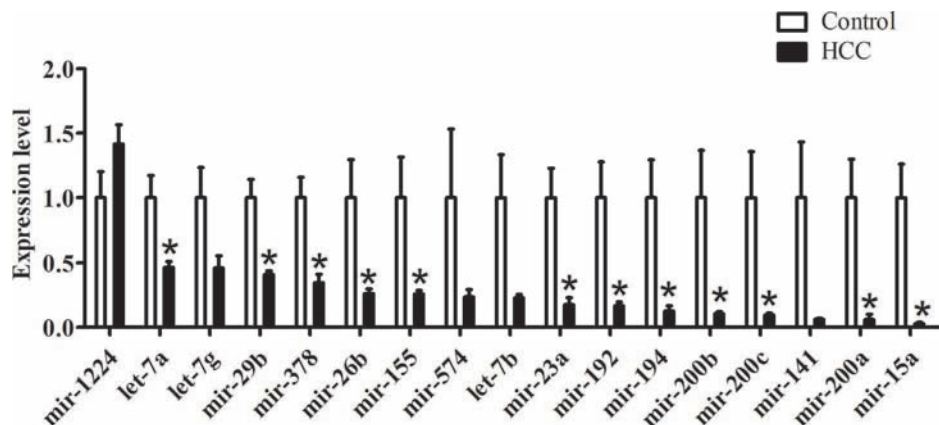


FIGURE 4 | qRT-PCR validations of intestinal microRNA expression within HCC mice, $n = 5$, $^*P < 0.05$.

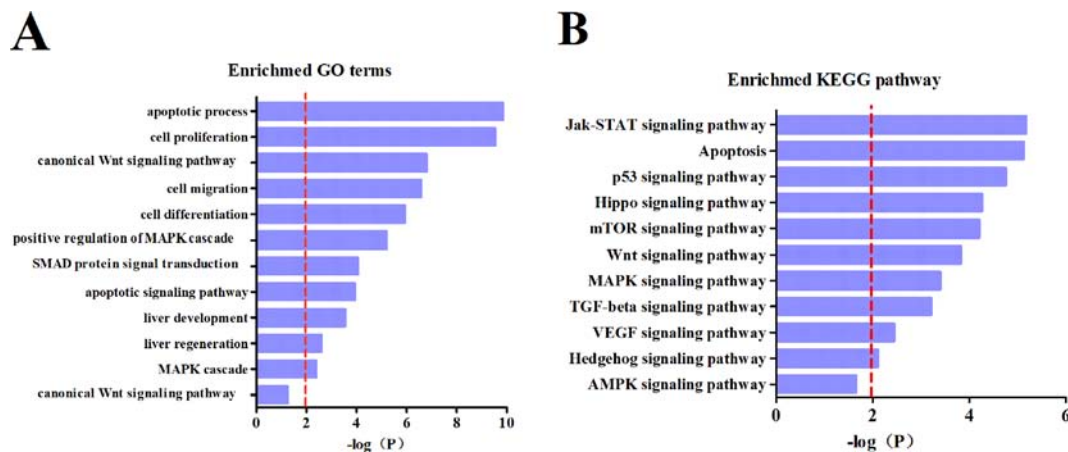


FIGURE 5 | Subpathway enrichment analysis of microRNA target. **(A)** Enrichment of GO terms. **(B)** Enrichment of KEGG pathways.

Interestingly, a new study suggests that fecal microRNAs could be identified as potential markers for intestinal malignancy (Ahmed et al., 2009). These fecal microRNAs exist in the gut lumen and feces and are called intestinal microRNAs. Some specific microRNAs that have been found in HCC cells and tissues may participate in tumor development, or could be a notable biological feature (Nelson and Weiss, 2008). The expression of microRNA in feces is therefore likely to become a new diagnostic marker in HCC. To achieve this potential, relatively accurate expression profiles of intestinal microRNA in HCC is a critical requirement. Because the reference gene of intestinal microRNA has not been identified yet, our first process step was to seek out a stable normalizer for the subsequent microRNA qRT-PCR experiments. Beginning with screening potentially stable reference genes, four traditional reference genes (*5S rRNA*, *U6 snRNA*, *GAPDH* and *16S rRNA*) and 22 intestinal microRNAs co-expressed in humans and mice were used as candidate genes. Considering that normal samples are necessary in all experiments and conditions, we initiated our studies using control mice. First of all, the dispersion of the candidate genes' Ct values was detected by qRT-PCR. There was a particularly large range of Ct values from 11 to 37 (Figure 2A). The boxplot indicated

that Ct value distribution of *let-7i* was the most concentrated whereas *mir-23a*, *mir-378* and *GAPDH* had slightly dispersed Ct values (Figures 2B, C). Preliminary assays demonstrated that these four genes with little dispersion also displayed higher stability.

However, the Ct value distribution map could only roughly assess the stability of the candidate reference genes. In order to determine the stability of a reference gene more accurately, it is necessary to further evaluate it using reference gene analysis software. There are a number of software programs available for analyzing reference genes, including GeNorm, NormFinder and Bestkeeper. They are three different statistical models that are widely used to select the most appropriate reference gene (Shen et al., 2011). Both GeNorm and NormFinder evaluates the stability of the gene by measuring the M value. When the maximum expression of the gene's stability is achieved, it will be accompanied by a low M value. Bestkeeper is a comprehensive evaluation software program which depends on the SD and CV. The principle of determination is that the smaller the SD and CV is, the better the stability of the reference gene is. When SD is greater than one, it directly indicates that the expression of the reference gene is unstable. In our study, the stability of reference genes was analyzed

by all three of these software programs. According to the qRT-PCR results, NormFinder showed that *GAPDH* was the most stable reference gene with the smallest M value (**Figure 3B**). This result was consistent with GeNorm software, with only one difference, GeNorm had the two best candidate reference genes and the other one was *mir-23a* (**Figure 3A**). This is in accordance with previous studies where *mir-23a* was considered to be a novel microRNA normalizer in profiling studies of cervical tissues (Shen et al., 2011). Nevertheless, the results of Bestkeeper were slightly different from those of GeNorm and NormFinder. The results showed that *let-7i* was one of the best reference genes with the smallest SD and CV (**Figure 3C**, **Table S1**), and was thus considered the most stable gene by the Bestkeeper software. Since different software programs have different algorithms, some differences are expected between the results obtained from each program. Therefore, to obtain relatively stable reference genes, we comprehensively analyzed the results from the three software programs. As presented in **Table 1**, it can be seen that the stabilities of the expression levels of the 22 candidate reference genes from the highest to the lowest were *mir-23a* > *GAPDH* > *let-7i* > *mir-378* > *mir-1224* > *mir-155* > *mir-574* > *16S Rrna* > *mir-29b* > *let-7b* > *mir-26b* > *mir-141* > *mir-200c* > *mir-192* > *let-7g* > *mir-194* > *let-7a* > *mir-200a* > *U6 snRNA* > *mir-200b* > *5s Rrna* > *mir-15a*. Commonly used conventional reference genes in our microRNA qRT-PCR assays, like *5S rRNA* and *U6 snRNA* were particularly unstable. This phenomenon indicates that traditional reference genes are not applicable to all samples. Therefore, gene stability should always be evaluated and verified before use. Conclusively, *mir-23a*, *GAPDH* and *let-7i* are the optimal reference genes for intestinal microRNA in normal mouse feces. This information could provide recommendations for intestinal microRNA qRT-PCR studies with diseases statuses or other conditions.

MicroRNAs could modulate multiple target mRNA degradations or translations in the same pathway. Therefore, it has been proposed to identify miRNA regulatory profiles through predicted target genes and established miRNA-mRNA expression modules (Fu et al., 2012). In order to explore the possible mechanism of intestinal microRNAs regulating HCC, we constructed the intestinal miRNA-mRNA expression modules. Intestinal microRNA expression levels with HCC were first detected by quantitative RT-PCR. *Let-7i* was chosen as the reference gene since it showed a higher expression ($Ct < 15$) than *mir-23a* and *GAPDH* (**Figure 2A**). Results showed that there were differential intestinal microRNAs in the HCC group compared to the control group (**Figure 4**). Unexpectedly, *mir-23a* displayed a significant change in the HCC group, even though it has never been reported that *mir-23a* is a novel microRNA normalizer for relative quantification in human uterine cervical tissues. Indeed, this also clarified that the expression of reference genes is not always stable, and an appropriate reference gene under one experimental condition may not be suitable for all experimental conditions.

To better understand the biological functions of dysregulation intestinal microRNAs in HCC, a functional analysis of miRNA-mRNA was performed. Thus, an analysis of the differential microRNAs for GO terms and KEGG pathways were conducted. The targets of the intestinal microRNAs were significantly enriched in cell proliferation, migration and differentiation, which is closely related to the TGF- β , Wnt and VEGF signaling pathway that

is involved in tumorigenesis, metastasis and therapy (**Figure 5A**). *miR-29b* plays a protective role in cardiac remodeling by targeting the TGF- β /Smad3 pathway (Zhang et al., 2014). mTOR is the key point of various important signaling pathways in cells, as well as being activated in a subset of HCCs (Fikret et al., 2004). The enriched KEGG pathway indicated that differential intestinal microRNA targets were likely to be involved in hepatocellular carcinogenesis through the mTOR pathway (**Figure 5B**). Previous research has clarified that *mir-155* enhances mTOR activity in the progression of breast carcinomas (Martin et al., 2014). It is evident that mRNA targets of differential intestinal microRNAs were significantly enriched in the apoptotic process and apoptosis pathway (**Figure 5**), where apoptosis was indicated to be involved in liver cancer development. Low *mir-15a* expression has shown negative regulation by targeting *Bcl-2* in human leukemia that is directly or indirectly involved in the biological process of apoptosis (Calin et al., 2008). The above evidence supports that intestinal miRNA could be a potential oncogene and antioncogene that is involved in HCC development.

In conclusion, the top three genes that were comprehensively ranked (*mir-23a*, *GAPDH* and *let-7i*), provide recommendations for intestinal microRNA qRT-PCR studies with disease statuses or other conditions. Additionally, differential intestinal microRNAs in fecal samples were predicted to be a potential oncogene or antioncogene involved in HCC development and may provide a novel strategy for diagnosing HCC and other related diseases.

AUTHOR CONTRIBUTIONS

HW and YL conceptualized and designed the experiments in this study. CW, DL, and YY performed experimental procedures related to the study. MB, SM, and YJ proofread and discussed articles relating to this research. ZW provides great help for revise article. YB and BY provided financial support and reviewed experimental directions. All authors contributed to the generation of experimental data for this study.

ACKNOWLEDGMENTS

We would like to extend our gratitude to Howard L. Weiner and Shirong Liu from Brigham and Women's Hospital, Harvard Medical School for providing help in intestinal microRNA isolation. Also, to Professor Feng Hai from Harbin Medical University for supplying fecal samples from HCC mice. We also thank Chiam Kai Wen Rachel for modifying the language used in this article. This project was supported by the National Natural Science Foundation of China (Grant No. 81674326 and Grant No. 81730012).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00687/full#supplementary-material>

FIGURE S1 | The expression level of *let-7i* by real-time PCR. (A) Cycle threshold (Ct) values for *let-7i*, $n=5$. (B) Distribution of Ct values of *let-7i*. Box representative the range of the Ct value distribution.

REFERENCES

- Ahmed, F. E., Jeffries, C. D., Vos, P. W., Flake, G., Nuovo, G. J., Sinar, D. R., et al. (2009). Diagnostic MicroRNA markers for screening sporadic human colon cancer and active ulcerative colitis in stool and tissue. *Cancer Genomics Proteomics* 6 (5), 281.
- Andersen, C. L., Jensen, J. L., and Orntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64 (15), 5245–5250. doi: 10.1158/0008-5472.CAN-04-0496
- Calin, G. A., Amelia, C., Muller, F., Manuela, F., Wojcik, S. E., Masayoshi, S., et al. (2008). MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc. Natl. Acad. Sci. U. S. A.* 105 (13), 5166–5171. doi: 10.1073/pnas.0800121105
- Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 33 (20), e179. doi: 10.1093/nar/gni178
- Connerty, P., Bajan, S., Remenyi, J., Fuller-Pace, F. V., and Hutvagner, G. (2016). The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells. *Biochim. Biophys. Acta* 1859 (10), 1299–1305. doi: 10.1016/j.bbaggm.2016.07.013
- Daud, I. I., and Scott, M. E. (2008). Validation of reference genes in cervical cell samples from human papillomavirus-infected and -uninfected women for quantitative reverse transcription-PCR assays. *Clin. Vaccine Immunol.* 15 (9), 1369–1373. doi: 10.1128/CVI.00074-08
- Davis-Dusenbery, B. N., and Hata, A. (2010). MicroRNA in Cancer: the involvement of aberrant MicroRNA biogenesis regulatory pathways. *Genes Cancer* 1 (11), 1100–1114. doi: 10.1177/1947601910396213
- Fikret, S., Rajesh, K., Onikepe, A., Jianzhou, W., Gloria, S., and Michael, T. (2004). mTOR and P70 S6 kinase expression in primary liver neoplasms. *Clin. Cancer Res.* 10 (24), 8421–8425. doi: 10.1158/1078-0432.CCR-04-0941
- Fu, J., Tang, W., Du, P., Wang, G., Chen, W., Li, J., et al. (2012). Identifying microRNA-mRNA regulatory network in colorectal cancer by a combination of expression profile and bioinformatics analysis. *BMC Syst. Biol.* 6, 68. doi: 10.1186/1752-0509-6-68
- Galun, D., Basaric, D., Zuvela, M., Bulajic, P., Bogdanovic, A., Bidzic, N., et al. (2015). Hepatocellular carcinoma: from clinical practice to evidence-based treatment protocols. *World J. Hepatol.* 7 (20), 2274–2291. doi: 10.4254/wjh.v7.i20.2274
- Ge, Y., Yan, X., Jin, Y., Yang, X., Yu, X., Zhou, L., et al. (2015). MiRNA-192 [corrected] and miRNA-204 Directly Suppress lncRNA HOTTIP and Interrupt GLS1-Mediated Glutaminolysis in Hepatocellular Carcinoma. *PLoS Genet.* 11 (12), e1005726. doi: 10.1371/journal.pgen.1005726
- Gillan, V., Maitland, K., Laing, R., Gu, H., Marks, N. D., Winter, A. D., et al. (2017). Increased expression of a MicroRNA correlates with anthelmintic resistance in parasitic nematodes. *Front. Cell. Infect. Microbiol.* 7, 452. doi: 10.3389/fcimb.2017.00452
- Kagiya, T. (2016). MicroRNAs: potential biomarkers and therapeutic targets for alveolar bone loss in periodontal disease. *Int. J. Mol. Sci.* 17 (8), 1317. doi: 10.3390/ijms17081317
- Li, X., Yang, W., Ye, W., Jin, L., He, J., and Lou, L. (2014). microRNAs: novel players in hepatitis C virus infection. *Clin. Res. Hepatol. Gastroenterol.* 38 (6), 664–675. doi: 10.1016/j.clinre.2014.04.008
- Liu, S., da Cunha, A. P., Rezende, R. M., Cialic, R., Wei, Z., Bry, L., et al. (2016). The host shapes the gut microbiota via fecal MicroRNA. *Cell Host Microbe* 19 (1), 32–43. doi: 10.1016/j.chom.2015.12.005
- Liu, S., Meng, C., Xu, G., Jian, H., and Wang, F. (2018). Validation of reference genes for reverse transcription real-time quantitative PCR analysis in the deep-sea bacterium *Shewanella psychrophila* WP2. *FEMS Microbiol. Lett.* 365 (7), fny048. doi: 10.1093/femsle/fny048
- Lv, Y., Zhao, S. G., Lu, G., Leung, C. K., Xiong, Z. Q., Su, X. W., et al. (2017). Identification of reference genes for qRT-PCR in granulosa cells of healthy women and polycystic ovarian syndrome patients. *Sci. Rep.* 7 (1), 6961. doi: 10.1038/s41598-017-07346-x
- Martin, E. C., Rhodes, L. V., Elliott, S., Krebs, A. E., Nephew, K. P., Flemington, E. K., et al. (2014). microRNA regulation of mammalian target of rapamycin expression and activity controls estrogen receptor function and RAD001 sensitivity. *Mol. Cancer* 13 (1), 229. doi: 10.1186/1476-4598-13-229
- Molina, C. E., Jacquet, E., Ponien, P., Munoz-Guijosa, C., Baczko, I., Maier, L. S., et al. (2018). Identification of optimal reference genes for transcriptomic analyses in normal and diseased human heart. *Cardiovasc. Res.* 114 (2), 247–258. doi: 10.1093/cvr/cvx182
- Nelson, K. M., and Weiss, G. J. (2008). MicroRNAs and cancer: past, present, and potential future. *Mol. Cancer Ther.* 7 (12), 3655–3660. doi: 10.1158/1535-7163.MCT-08-0586
- Parodi, F., Carosio, R., Ragusa, M., Di Pietro, C., Maugeri, M., Barbagallo, D., et al. (2016). Epigenetic dysregulation in neuroblastoma: a tale of miRNAs and DNA methylation. *Biochim. Biophys. Acta* 1859 (12), 1502–1514. doi: 10.1016/j.bbaggm.2016.10.006
- Petrelli, F., Coinu, A., Borgonovo, K., Cabiddu, M., Ghilardi, M., Lonati, V., et al. (2014). Oxaliplatin-based chemotherapy: a new option in advanced hepatocellular carcinoma. A systematic review and pooled analysis. *Clin. Oncol.* 26 (8), 488–496. doi: 10.1016/j.clon.2014.04.031
- Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: bestKeeper – excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26 (6), 509–515. doi: 10.1023/B:BILE.0000019559.84305.47
- Ranjha, R., and Paul, J. (2013). Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. *Inflamm. Res.* 62 (4), 343–355. doi: 10.1007/s00011-013-0600-9
- Shen, Y., Li, Y., Ye, F., Wang, F., Wan, X., Lu, W., et al. (2011). Identification of miR-23a as a novel microRNA normalizer for relative quantification in human uterine cervical tissues. *Exp. Mol. Med.* 43 (6), 358–366. doi: 10.3858/emmm.2011.43.6.039
- Su, H., Yang, J. R., Xu, T., Huang, J., Xu, L., Yuan, Y., et al. (2009). MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *Cancer Res.* 69 (3), 1135–1142. doi: 10.1158/0008-5472.CAN-08-2886
- Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistic, 2012. *CA Cancer J. Clin.* 65 (2), 87–108. doi: 10.3322/caac.21262
- Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3 (7), research0034.1–research0034.11. doi: 10.1186/gb-2002-3-7-research0034
- Wang, C. M., Wang, Y., Fan, C. G., Xu, F. F., Sun, W. S., Liu, Y. G., et al. (2011). miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.* 411 (3), 586–592. doi: 10.1016/j.bbrc.2011.06.191
- Xi, Y., Nakajima, G., Gavin, E., Morris, C. G., Kudo, K., Hayashi, K., et al. (2007). Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA* 13 (10), 1668–1674. doi: 10.1261/rna.642907
- Xiao, S., Wang, R., Wu, X., Liu, W., and Ma, S. (2018). The Long Noncoding RNA TP73-AS1 Interacted with miR-124 to modulate glioma growth by targeting inhibitor of apoptosis-stimulating protein of p53. *DNA Cell Biol.* 37 (2), 117–125. doi: 10.1089/dna.2017.3941
- Yang, Y., Li, Z., Cao, J., Li, Y., Li, H., Yang, Q., et al. (2017). Identification and evaluation of suitable reference genes for normalization of MicroRNA expression in *helicoverpa armigera* (Lepidoptera: Noctuidae) using quantitative real-time PCR. *J. Insect Sci.* 17 (2), 1–10. doi: 10.1093/jisesa/iex007
- You, S., Fulga, T. A., Van Vactor, D., and Jackson, F. R. (2018). Regulation of circadian behavior by astroglial MicroRNAs in drosophila. *Genetics* 208 (3), 1195–1207. doi: 10.1534/genetics.117.300342
- Zhang, Y., Huang, X. R., Wei, L. H., Chung, A. C., Yu, C. M., and Lan, H. Y. (2014). miR-29b as a therapeutic agent for angiotensin II-induced cardiac fibrosis by targeting TGF-beta/Smad3 signaling. *Mol. Ther.* 22 (5), 974–985. doi: 10.1038/mt.2014.25
- Zhou, L., Chen, F., Ye, J., and Pan, H. (2018). Selection of reliable reference genes for RT-qPCR analysis of *Bursaphelenchus mucronatus* gene expression from different habitats and developmental stages. *Front. Genet.* 9, 269. doi: 10.3389/fgene.2018.00269

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RNA Expression Profile and Potential Biomarkers in Patients With Spinocerebellar Ataxia Type 3 From Mainland China

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OPEN ACCESS

Edited by:

Yujing Li,
Emory University, United States

Reviewed by:

Matteo Chiara,
University of Milan, Italy
Venugopal Thayanithy,
University of Minnesota, United States

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 01 September 2018

Accepted: 29 May 2019

Published: 13 June 2019

Citation:

Li T, Hou X, Chen Z, Peng Y, Wang P, Xie Y, He L, Yuan H, Peng H, Qiu R, Xia K, Tang B and Jiang H (2019) RNA Expression Profile and Potential Biomarkers in Patients With Spinocerebellar Ataxia Type 3 From Mainland China. *Front. Genet.* 10:566. doi: 10.3389/fgene.2019.00566

Long non-coding RNAs (lncRNAs) play an important role in growth, development, and reproduction and undoubtedly contribute to the pathogenesis and progression of diseases. Emerging evidence suggests the involvement of lncRNAs as regulatory factors in pathological conditions, including some neurodegenerative diseases. Spinocerebellar Ataxia Type 3/Machado–Joseph Disease (SCA3/MJD) has a prominent prevalence in China. Because the role of lncRNAs in SCA3/MJD pathogenesis has not yet been investigated, we conducted a pilot study to investigate the expression profile of lncRNAs by high-throughput sequencing in 12 patients and 12 healthy individuals. The sequencing analysis detected 5,540 known and 2,759 novel lncRNAs. Six lncRNAs were confirmed to be differentially expressed in peripheral blood mononuclear cells between SCA3/MJD patients and healthy individuals and were further validated in cerebellar tissue. Based on these results, NONHSAT022144.2 and NONHSAT165686.1 may be involved in the pathogenesis of SCA3/MJD and may be potential biomarkers for SCA3/MJD. Together with NONHSAT022144.2 and NONHSAT165686.1, the other four novel lncRNAs increase our understanding of lncRNA expression profile.

Keywords: Spinocerebellar Ataxia Type 3, Machado–Joseph disease, long non-coding RNAs, expression profile, biomarker

INTRODUCTION

Long non-coding RNAs (lncRNAs) play an essential role in regulating the expression of genes involved in almost all metabolic pathways and other biological macromolecules at both the transcriptional and post-transcriptional levels. lncRNAs also play a role in the regulation of growth, development, and reproduction (Wapinski and Chang, 2011; Khorkova et al., 2015; Lardenoije et al., 2015; Engreitz et al., 2016). A growing body of evidence suggests that lncRNAs are involved in the pathogenesis and progression of some neurodegenerative diseases, such as Alzheimer's Disease (AD) and Huntington's disease (HD) (Lin et al., 2014; Tan et al., 2014; Salta and De Strooper, 2017; Sunwoo et al., 2017; Zhou et al., 2018).

Alteration in the expression levels of some lncRNAs contributes to the pathogenesis of neurodegenerative diseases (Lin et al., 2014; Roberts et al., 2014; Tan et al., 2014; Zhang et al., 2016; Salta and De Strooper, 2017; Sunwoo et al., 2017; Gagliardi et al., 2018). For example, lnc-sca7 (*ATXN7L3B*) is highly conserved in the central nervous system of human and adult mice and transcriptionally regulates the expression of *ATXN7*, which is the pathogenic gene of Spinocerebellar Ataxia Type 7 (SCA7). Indeed, knockout of lnc-sca7 in N2a cells leads to a significant decrease in *ATXN7* expression at the translational level. Accordingly, overexpression of lnc-sca7 significantly enhances transcription of *ATXN7* (Tan et al., 2014; Salta and De Strooper, 2017). A more detailed study showed that the lncRNA TUNA has elevated expression in the thalamus and striatum. Gene expression analysis in brain tissue of 44 HD patients and 36 healthy individuals confirmed that TUNA expression in the caudate nucleus might be involved in the pathophysiology of HD (Lin et al., 2014; Salta and De Strooper, 2017). Additionally, the lncRNA NEAT1 is associated with the damage mechanism of HD (Sunwoo et al., 2017).

Polyglutamine disease (PolyQ disease) is a type of disease caused by dynamic mutation of a trinucleotide sequence; PolyQ diseases include spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, and 17; dentatorubral–pallidoluysian atrophy (DRPLA); spinal and bulbar muscular atrophy (SBMA), and HD (Matos et al., 2011). Spinocerebellar Ataxia Type 3/Machado–Joseph Disease, one of the PolyQ diseases similar to SCA7 and HD (Wang et al., 2015), is the most common SCA subtype in mainland China (Chen et al., 2018). It is mainly caused by the abnormal expansion of the trinucleotide sequence CAG in the *ATXN3* gene and has a broad age of onset ranging from 4- to over 70-years-old (Peng et al., 2014; Wang et al., 2015; Chen Z. et al., 2016; Chen et al., 2017). Although various pathogenic hypotheses have been proposed and supported experimentally for SCA3/MJD, no effective strategies are available for surveillance, delay progression, or treatment of the disease (Costa Mdo and Paulson, 2012). Given that some lncRNAs are involved in SCA7 and HD pathogenesis (Lin et al., 2014; Roberts et al., 2014; Tan et al., 2014; Zhang et al., 2016; Salta and De Strooper, 2017; Sunwoo et al., 2017; Gagliardi et al., 2018) and that we previously detected abnormal expression of three lncRNAs in the SCA3/MJD mouse model (Vidal et al., 2000), we speculated that some lncRNAs also contribute to SCA3/MJD pathogenesis. Largely because of their accessibility and ease of operation, peripheral blood mononuclear cells (PBMCs) are widely used to establish suitable biomarkers for neurodegenerative and other diseases (Coppola et al., 2011; Zhang et al., 2016; Gagliardi et al., 2018). To investigate the possible mechanisms of lncRNAs in SCA3/MJD pathogenesis and to explore potential SCA3/MJD biomarkers, we isolated PBMCs from patients and healthy individuals for high-throughput sequencing. Through bioinformatics analysis, we identified 5,540 known and 2,759 novel lncRNAs in patients and normal individuals. Validation including expression analysis in human brains enabled us to target six lncRNAs. This provides useful information for understanding the pathogenesis of SCA3/MJD and enabling new treatment strategy options.

MATERIALS AND METHODS

Sample Collection and PBMC Isolation

All participants, including the 12 patients and 12 healthy individuals from Xiangya Hospital of Central South University, were required to sign consent forms prior to collection of their blood samples. The subjects included six males and six females ranging in age from 18- to 62-years-old. All of the patients met the clinical manifestations of SCA3/MJD, had been diagnosed with SCA3/MJD by neurologists and geneticists, and were confirmed to not have any other known brain or neurological disorder. By matching age and gender, we found suitable healthy individuals from the physical examination center at Xiangya Hospital. Ten milliliters of peripheral blood was collected for isolation of PBMCs. The blood samples collected from participants were processed within 2 h to isolate PBMCs using lymphocyte solution (Ficoll–Hypaque) based on the standard protocol instructions.

RNA Extraction and High-Throughput Sequencing

TRIzolTM reagent (Invitrogen, Carlsbad, CA, United States) was added to isolated PBMCs for total RNA extraction, and genomic DNA was removed with the RNeasy kit (Qiagen, Hilden, Germany). Ribosomal RNA removal was conducted using a biotin-labeled specific probe from the Ribo-ZeroTM rRNA Removal Kit (Epicentre)[®] prior to RNA library preparation. The first strand cDNA was then synthesized using random primers and reverse transcriptase in the TruSeq[®] Stranded kit (Illumina) followed by synthesis of the double-stranded cDNA using DNA polymerase I and RNaseH. The adaptor-ligated cDNA fragments were amplified to generate the final cDNA library followed by library purification. High-throughput sequencing was carried out by BGI Genomics with the Illumina Hi-seq X-Ten platform.

High-throughput sequencing obtained raw reads as data. Subsequently, we used the short reads alignment tool SOAP¹ to compare reads to the ribosomal database and filtered data by removing the ribosomal reads on the alignment, which allows for up to five mismatches. Then, reads containing only adapter dimers or reads with 10% or more N or low-quality bases were filtered out. After that, we obtained the clean reads. Clean reads were aligned to the reference genome with the HISAT software² and assembled with StringTie³. The Hg19 genome assembly version was used for alignment and assembly. By aligning all reads to the genome and constructing all possible transcripts in each alignment region, only the transcripts with fragments per kilobase of transcript per million reads mapped (FPKM) values ≥ 0.5 , coverage values > 1 , and length values > 200 bp were retained for further quantitative analysis. To compare the expression levels between samples, it was necessary to standardize the expression level of the gene. Therefore, we aligned the clean reads to the reference sequence with Bowtie2⁴, and expression

¹<https://github.com/BGI-flexlab/SOAPnuke>

²<http://www.ccb.jhu.edu/software/hisat>

³<http://ccb.jhu.edu/software/stringtie>

⁴<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>

TABLE 1 | The classification of overlap.

Overlap class	LncRNA number	mRNA number	Pair number
Lnc-Overlap-mRNA	624	664	812
Lnc-AntiOverlap-mRNA	188	189	204
Lnc-Completein-mRNAExon	154	156	159
Lnc-AntiCompletein-mRNAExon	8	8	8
mRNA-Completein-LncExon	42	46	47
mRNA-AntiCompletein-LncExon	2	2	2
Lnc-Completein-mRNAIntron	212	213	235
Lnc-AntiCompletein-mRNAIntron	63	56	64
mRNA-Completein-LncIntron	14	16	19
mRNA-AntiCompletein-LncIntron	15	10	18

Lnc-Overlap-mRNA means lncRNA on the same strand as mRNA, and lncRNA has an overlap with mRNA.

Lnc-AntiOverlap-mRNA means that lncRNA and mRNA are on different strands, and there is an overlap between lncRNA and mRNA.

Lnc-Completein-mRNAExon means that lncRNA and mRNA are on the same strand, and the lncRNA completely falls in the Exon region of mRNA.

Lnc-AntiCompletein-mRNAExon means lncRNA and mRNA are on different strands, and the lncRNA completely falls in the Exon region of mRNA.

mRNA-Completein-LncExon means that lncRNA and mRNA are on the same strand, and the mRNA completely falls in the Exon region of lncRNA.

mRNA-AntiCompletein-LncExon means that lncRNA and mRNA are on different strands, and mRNA completely falls in the Exon region of lncRNA.

Lnc-Completein-mRNAIntron means that lncRNA and mRNA are on the same strand, and the lncRNA completely falls in the Intron region of mRNA.

Lnc-AntiCompletein-mRNAIntron means lncRNA and mRNA are on different strands, and the lncRNA completely falls in the Intron region of mRNA.

mRNA-Completein-LncIntron means that lncRNA and mRNA are on the same strand, and mRNA completely falls in the Intron region of lncRNA.

mRNA-AntiCompletein-LncIntron means that lncRNA and mRNA are on different strands, and mRNA completely falls in the Intron region of lncRNA.

levels of genes and transcripts were calculated by using RSEM⁵. The standardized method of RSEM is FPKM. Its calculation method is $FPKM(A) = 10^6 C / (NL/10^3)$, where FPKM(A) is the expression level of gene A, C is the number of fragments that are uniquely aligned to gene A, N is the total fragment number that is aligned to the reference gene, and L is the number of bases of the gene A coding region. We needed to predict whether transcripts had coding ability to determine if they were lncRNAs. Three prediction programs – CPC⁶, txCdsPredict⁷, and

⁵<http://deweylab.biostat.wisc.edu/rsem>

⁶<http://CPC.cbi.pku.edu.cn>

⁷<http://hgdownload.soe.ucsc.edu/admin/jksrc.zip>

CNCI⁸ – were used to score the transcripts' coding ability to distinguish mRNA and lncRNA by different score ranges. The thresholds in the CPC and CNCI software were set to 0, and mRNAs had scores greater than 0, while lncRNAs had scores less than 0. The txCdspredict threshold was set to 500, and mRNA transcripts had scores over 500, lncRNAs had scores while less than 500. Additionally, transcripts were compared to the protein database Pfam⁹ to classify coding RNAs (mRNAs) and lncRNAs. We considered transcripts lncRNAs only if they met the criteria of at least three of the four software and databases, and then we analyzed their predicted target gene functional annotation. Moreover, we considered a fold change ≥ 2.00 and false discovery rate ≤ 0.001 significant in DEGseq (Wang et al., 2010) to compare lncRNA expression differences the SCA3/MJD and control group. All data alignments, assembly and analysis were conducted by BGI Genomics.

Target Gene Prediction

We determined the Spearman and Pearson correlation coefficients to determine lncRNA targets; only mRNAs with a Spearman correlation ≥ 0.6 and Pearson correlation ≥ 0.6 were considered target genes of a given lncRNA. When the lncRNA overlapped with the target gene, we classified the lncRNAs and their target mRNAs based on their positional relationship.

Gene Function Annotation

We analyzed differentially expressed genes (DEGs) and their target genes with the GO database¹⁰ to explore possible functional enrichment analyses of DEGs and identify functional modules enriched in DEGs. To determine the pathways that DEGs and their target genes are concentrated in, we performed enrichment analysis using the KEGG database¹¹.

Quantitative Real-Time PCR Verification in PBMCs and Human Brain Tissue

Reverse transcription was carried out with the GoldenstarTM RT6 cDNA Synthesis Kit (Beijing TsingKe Biotech Co. Ltd., China). Based on the analysis of the high-throughput sequencing, 20 lncRNAs were selected for quantitative real-time PCR (qRT-PCR) to confirm their differential expression between the case

⁸<https://github.com/www-bioinfo-org/CNCI>

⁹<http://pfam.xfam.org/>

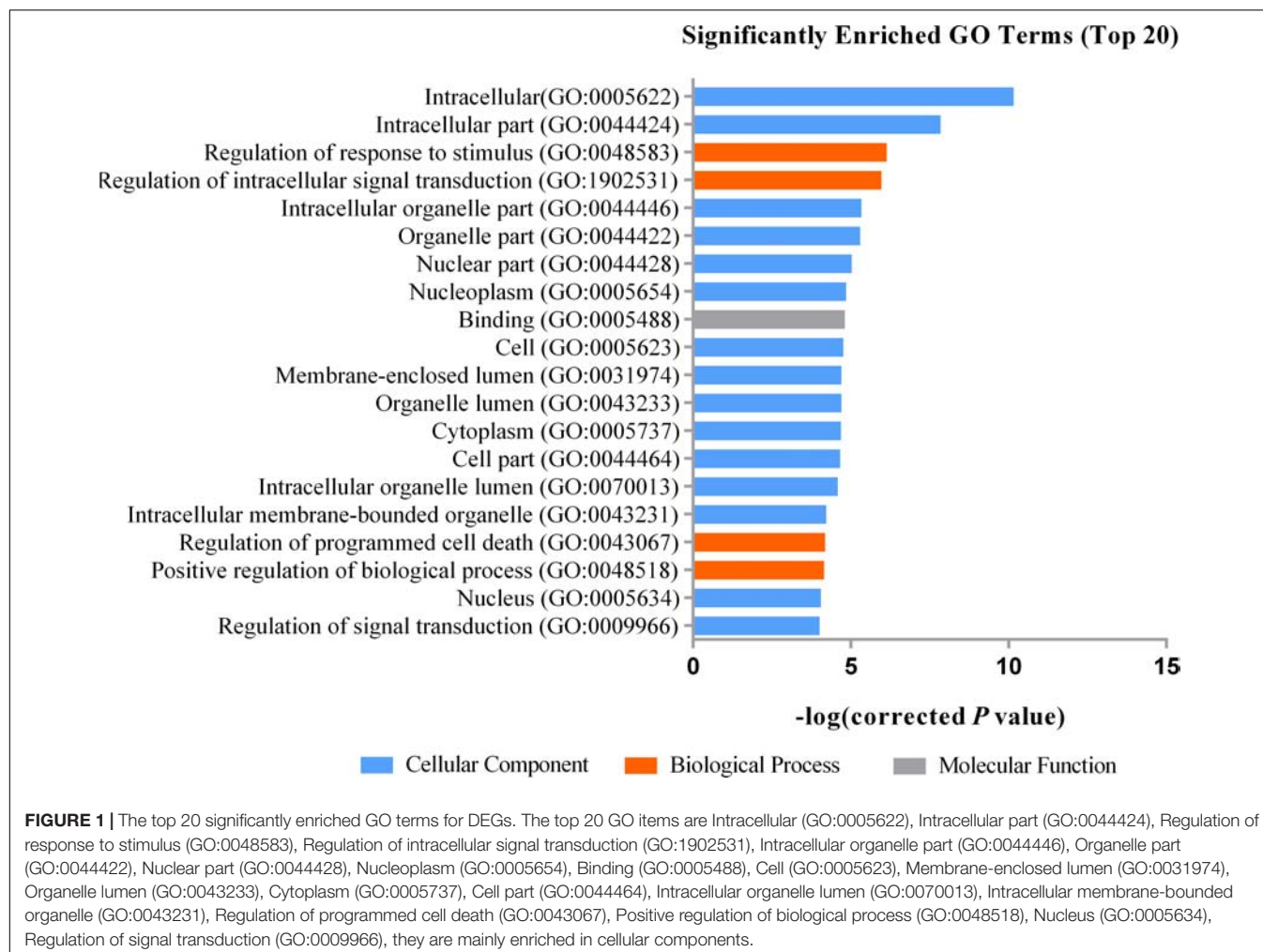
¹⁰<http://www.geneontology.org/>

¹¹<https://www.kegg.jp/>

TABLE 2 | The classification of overlap of DEGs.

LncRNA ID	Target mRNA	Overlap class	The value of Pearson correlation	The value of Spearman correlation
LTCONS_00174904	MTCONS_00174903	Lnc-Completein-mRNAIntron	0.9031	0.7902
LTCONS_00175021	MTCONS_00174902	NA	0.8225	0.8433
LTCONS_00051791	MTCONS_00051780	Lnc-Overlap-mRNA	0.9743	0.7005
NONHSAT007220.2	NM_001184763	Lnc-Completein-mRNAExon	0.6652	0.7045
NONHSAT177312.1	NM_001291470	NA	0.8275	0.6707

The details of overlap class included in **Table 1**, and NA means the lncRNA and their target gene are on different chromosomes.



and control groups. Further validation was performed in 28 SCA3/MJD patients and 28 healthy individuals as mentioned in the section “Materials and Methods.” Primers for RT-PCR listed in **Supplementary Table S1** were designed by Primer 5, and the qRT-PCR was performed per the instructions of the 2 × T5 Fast qPCR Mix (SYBR Green I) Kit. We used GAPDH as an internal control and the $2^{-\Delta\Delta C_t}$ method to calculate the quantitative expression of lncRNAs. The Wilcoxon rank-sum test was used to compare statistical significance in the expression levels of DEGs between groups, and p -values < 0.05 were considered statistically significant. For further validation, statistically significant lncRNAs were validated by qRT-PCR in cerebellar tissue from a SCA3/MJD patient and a healthy individual.

RESULTS

Expression Profiles of lncRNAs and mRNAs

A total of 124,394 transcripts was detected by high-throughput sequencing, including 15,926 novel lncRNAs, 13,651 novel

mRNAs, 61,335 known lncRNAs, and 33,482 known mRNAs. After data filtering and DEG analysis with DEGseq, we identified 5,540 known lncRNAs and 2,759 novel lncRNAs with differential expression. Additionally, we identified 4,701 known mRNAs and 2,517 novel mRNAs.

Summary of the Cis- and Trans-Regulation and Classification of the Overlap

Through target gene prediction analyses, we predicted the possible regulatory patterns of lncRNAs. All of the positional information and the complementarity between mRNAs and lncRNAs are shown in **Supplementary Material II**, and we indicate how lncRNAs may act on their target genes. The overlap is divided into 10 classes (**Table 1**), including lnc-Overlap-mRNA with 812 pairs, lnc-AntiOverlap-mRNA with 204 pairs, lnc-Complete In-mRNAExon with 153 pairs, lnc-AntiComplete In-mRNAExon with eight pairs, mRNA-CompleteIn-lncExon with 47 pairs, and mRNA-AntiCompleteIn-lncExon with 2 pairs. lnc-CompleteIn-mRNA Intron with 235 pairs, lnc-AntiComplete In-mRNA Intron with 64 pairs, mRNA-Complete

In-LncIntron with 19 pairs, and mRNA-AntiComplete In-LncIntron with 18 pairs. As listed in **Table 2**, we predicted the target genes for 5 of 20 tested lncRNAs in this study.

Gene Function Annotation

Gene Ontology (GO) analysis showed that DEGs were significantly enriched in certain functional terms, and the top 20 GO terms are listed in **Figure 1**. Similarly, we performed GO analysis of target genes of DEGs and listed significant enriched GO terms in **Figure 2**. KEGG analysis indicated that DEGs and their target genes were enriched in different pathways. We listed the top 20 terms in **Figures 3, 4**.

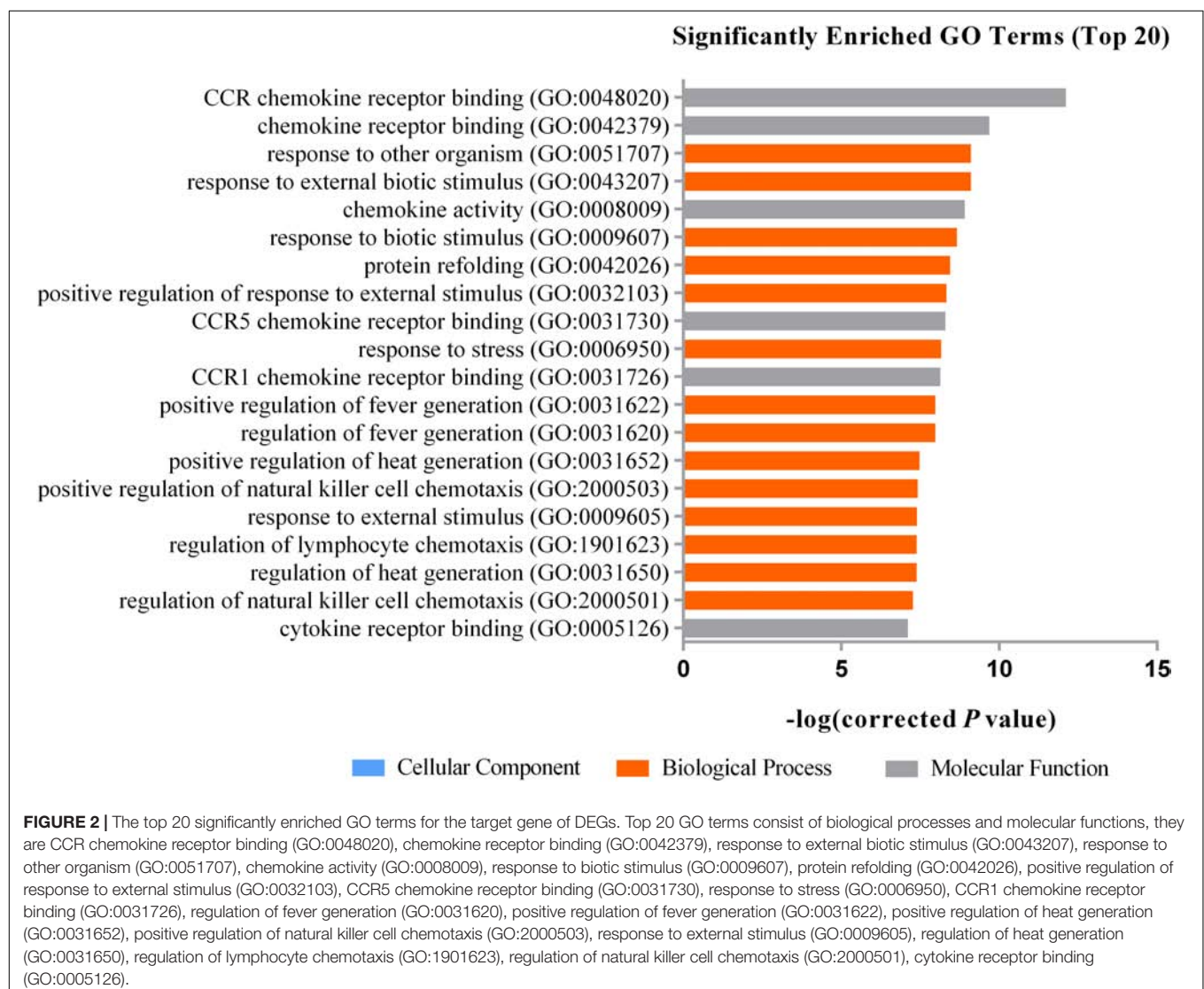
Validation of lncRNAs

Among the 20 lncRNAs we selected, we verified that 6 lncRNAs are statistically different in PBMCs. The NONHSAT165686.1, LTCONS_00051791, LTCONS_00175021, and LTCONS_00175040 lncRNAs were up-regulated by 5.6,

4.8, 2.0, and 2.4-fold, respectively. There were also two down-regulated lncRNAs. The expression of NONHSAT022144.2 in the SCA3/MJD group was approximately one-quarter of the control group, while the expression of LTCONS_00176188 in the SCA3/MJD group was approximately two-thirds of the control group (**Figure 5**). All six lncRNAs were verified in cerebellum tissue (**Figure 6**). The dysregulation of these four lncRNAs, NONHSAT165686.1, LTCONS_00051791, NONHSAT022144.2, and LTCONS_00176188, in cerebellum tissue was consistent with the results in PBMCs. In contrast, the expression trends for LTCONS_00175021 and LTCONS_00175040 in cerebellum tissue differed from those in PBMCs.

DISCUSSION

To explore possible biomarkers for SCA3/MJD, we performed high-throughput sequencing of 12 SCA3/MJD patients and





Significantly Enriched Pathway Terms (Top 20)

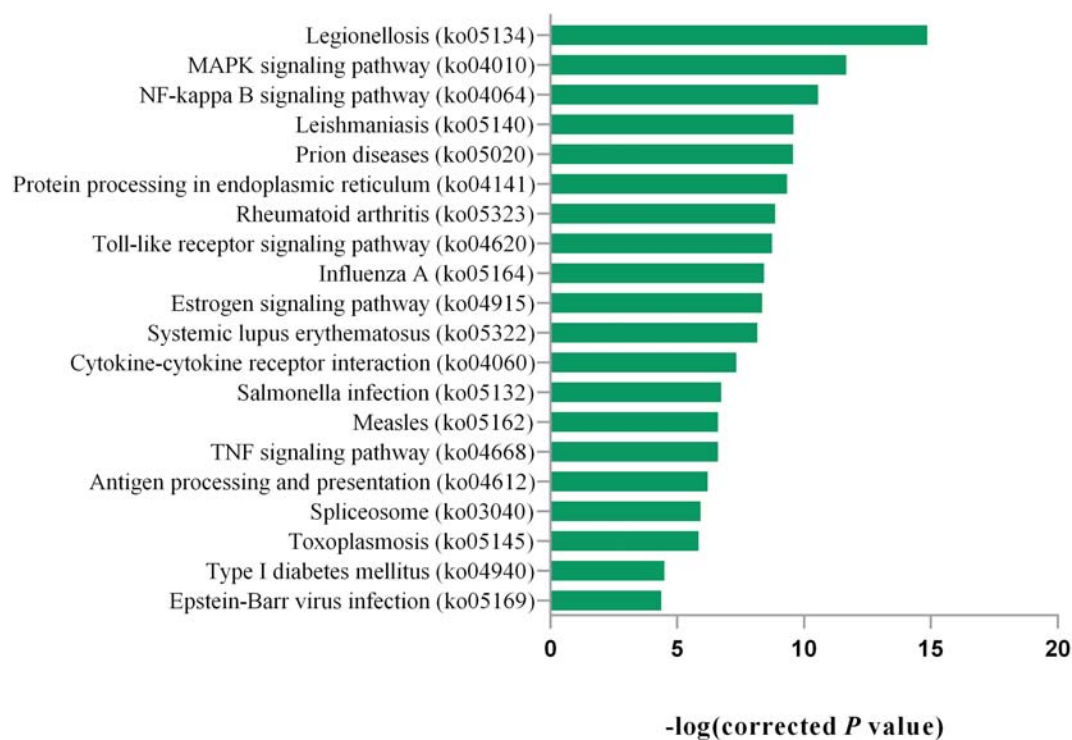


FIGURE 4 | The top 20 significantly enriched pathway terms for the target gene of DEGs. The top 20 KEGG pathway terms are Legionellosis (ko05134), MAPK signaling pathway (ko04010), NF-kappa B signaling pathway (ko04064), Leishmaniasis (ko05140), Prion diseases (ko05020), Protein processing in endoplasmic reticulum (ko04141), Rheumatoid arthritis (ko05323), Toll-like receptor signaling pathway (ko04620), Influenza A (ko05164), Estrogen signaling pathway (ko04915), Systemic lupus erythematosus (ko05322), Cytokine-cytokine receptor interaction (ko04060), Salmonella infection (ko05132), Measles (ko05162), TNF signaling pathway (ko04668), Antigen processing and presentation (ko04612), Spliceosome (ko03040), Toxoplasmosis (ko05145), Type I diabetes mellitus (ko04940), Epstein-Barr virus infection (ko05169).

potential therapeutic molecule for SCA3/MJD. Additionally, we found that the expression level of this lncRNA was significantly decreased in SCA3/MJD patients relative to healthy individuals, suggesting its potential as a biomarker and therapeutic molecule.

NONHSAT165686.1 is located on Chr13:48233221–48261860, as described in the hg38 and NONCODE database¹⁴, and it aligns to the *ITM2B*, NM_021999.4 transcript. To our knowledge, mutation of this gene leads to two autosomal dominant neurodegenerative diseases: familial British dementia (FBD) and familial Danish dementia (FDD). FBD is characterized by progressive dementia, cerebellar ataxia, and spasticity and is partially similar to SCA3/MJD (Vidal et al., 1999). Coincidentally, FDD shares similar symptoms such as progressive ataxia (Vidal et al., 2000). FBD also shares certain similarities with SCA3/MJD pathogenesis. Mitochondrial dysfunction has been implicated in the pathogenesis of cerebral amyloidosis such as FBD (Todd et al., 2014). Similarly, mitochondrial dysfunction is involved in the pathogenesis of SCA3/MJD, and ataxin-3 proteolysis produces

toxic fragments, leading to mitochondrial defects (Harmuth et al., 2018). Expression of *ITM2B* induces apoptosis and is associated with loss of mitochondrial membrane potential, release of cytochrome c, and induction of apoptosis by a caspase-dependent mitochondrial pathway (Fleischer et al., 2002). Our results show that the expression of NONHSAT165686.1 is 5.6-fold higher in SCA3/MJD than the control group. This suggests that NONHSAT165686.1 might be associated with the pathogenesis and clinical features of SCA3/MJD.

Although only two human cerebellar tissues were used for validation, it seems that these lncRNAs are differentially expressed in cerebellar tissue between SCA3/MJD patients and healthy individuals. More importantly, we detected a significant decrease in LTCONS_00175040 expression in cerebellar tissue of SCA3/MJD patients relative to the healthy individual. We only used two human samples for validation due to the limitation of human brain sample collection. Even though we cannot exclude the possibility that the difference between these two human brain samples (the SCA3/MJD patients and the healthy individuals) was caused by individual variation, this result at least confirms the expression of these lncRNAs in the cerebellum. Thus,

¹⁴http://www.noncode.org/show_rna.php?id=NONHSAT165686&version=1&utd=1#

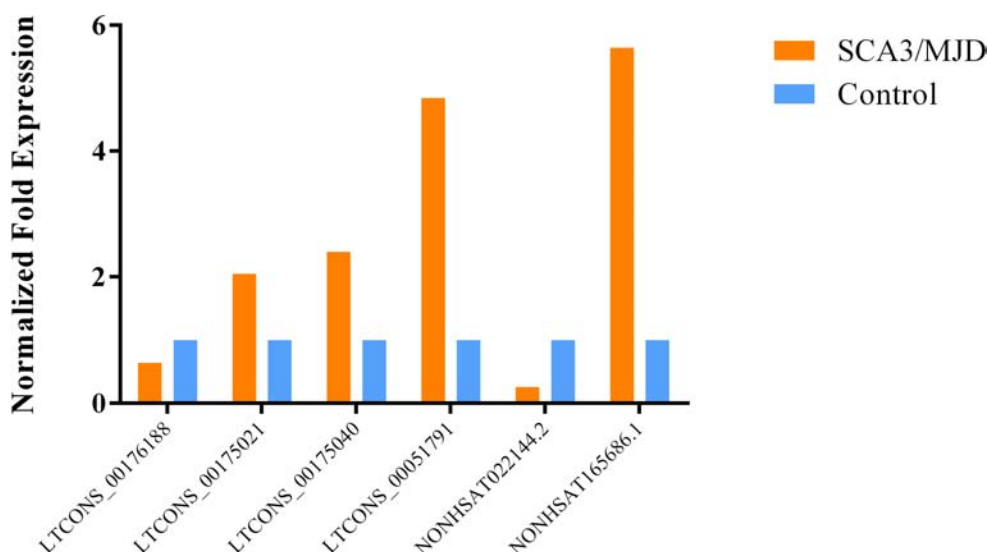


FIGURE 5 | The expression level of statistically significant DEGs in PBMCs. The expression levels of NONHSAT165686.1, LTCONS_00175040, LTCONS_00175021, and LTCONS_00051791 are up-regulated. Compared with the control group, the expression level of NONHSAT165686.1 is about 5.6 times ($p = 0.036$), the expression of LTCONS_00051791 is about 4.8 times ($p = 0.018$), and LTCONS_00175021 and LTCONS_00175040 are both up more than twice ($p = 0.027$ of LTCONS_00175021 and $p = 0.032$ of LTCONS_00175040). Contrary to this, both known lncRNA NONHSAT022144.2 and novel lncRNA LTCONS_00176188 are down-regulated, their expression levels are 0.259 times ($p = 0.000$) and 0.640 times ($p = 0.043$) that of the control group, respectively.

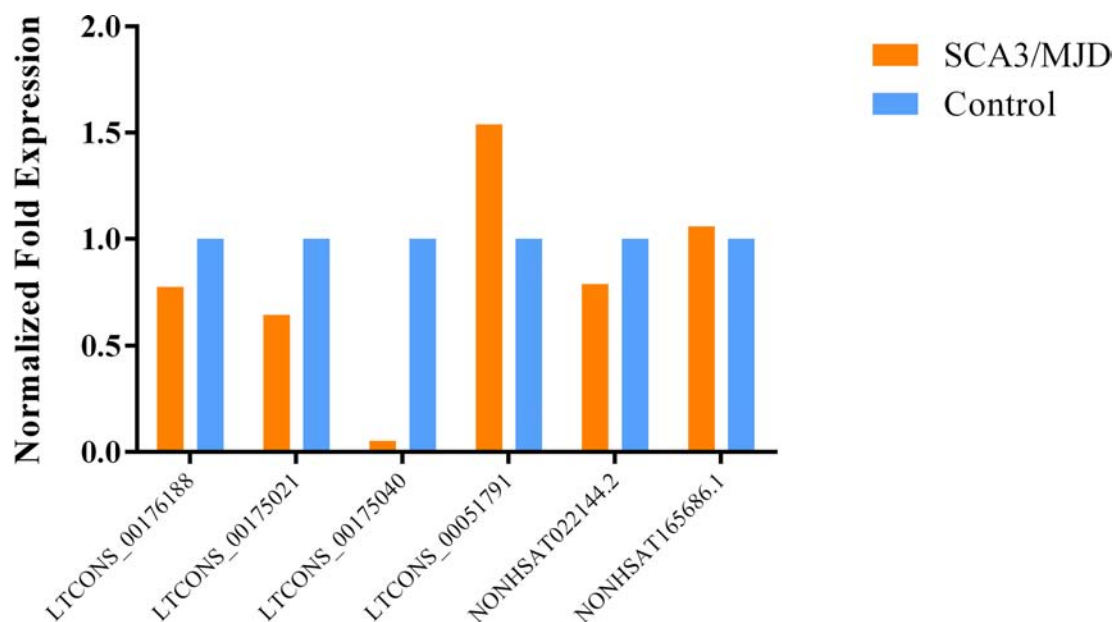


FIGURE 6 | The expression level of statistically significant DEGs in cerebellum tissue. The expression levels of LTCONS_00176188 and NONHSAT022144.2 decreased by about a quarter in the cerebellar tissues of SCA3/MJD patients, while the expression levels of LTCONS_00175021 decreased by about a third in the cerebellar tissues of SCA3/MJD patients. Surprisingly, the expression of LTCONS_00175040 is only 5% of the control. Among the up-regulated genes, the expression level of NONHSAT165686.1 was only 5% higher than that of the control group, while the expression level of LTCONS_00051791 was 1.5 times that of the control group.

further validation using cerebellar tissues from more patients and healthy individuals is indispensable for concluding that LTCONS_00175040 is an important molecule. Additionally, more than 10 other lncRNAs have been identified near the

LTCONS_00175040 lncRNA locus, indicating that this is a lncRNA-enriched region. Furthermore, four novel lncRNAs contribute to the expansion and improvement of the lncRNA expression profile.

In summary, our study may open a new angle for dissecting SCA3/MJD pathogenesis based on lncRNA analysis. By further exploring potential functions and pathways in which the lncRNAs are involved, we anticipate that lncRNAs such as NONHSAT022144.2 and NONHSAT165686.1 will be biomarkers or even potential therapeutic targets for SCA3/MJD treatment.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Xiangya Hospital of Central South University in China (equivalent to an Institutional Review Board), and written informed consent was obtained from all of the patients.

AUTHOR CONTRIBUTIONS

All the authors contributed to the experimental design and sample collection. TL completed the statistical analysis and wrote the manuscript.

FUNDING

This work was supported by the National Key Research and Development Program of China (Nos. 2016YFC0905100

and 2016YFC0901504 to HJ), the National Natural Science Foundation of China (No. 81771231 to HJ and No. 81600995 to Yuting Shi), Scientific Research Foundation of Health Commission of Hunan Province (No. B2019183 to HJ), the Key Research and Development Program of Hunan Province (No. 2018SK2092 to HJ), the Clinical and Rehabilitation Fund of Peking University Weiming Biotech Group (No. xywm2015I10 to HJ), Youth Foundation of Xiangya Hospital (No. 2017Q03 to ZC), and Independent Exploration and Innovation Project of Graduate Students of Central South University (No. 1053320170177 to TL).

ACKNOWLEDGMENTS

The authors sincerely thank all of the patients and healthy individuals who volunteered for and supported this study. The authors also thank those who helped conduct this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00566/full#supplementary-material>

REFERENCES

- Assali, A., Akhavan, O., Mottaghi, F., Adeli, M., Dinarvand, R., Razzazan, S., et al. (2018). Cationic graphene oxide nanoplateform mediates miR-101 delivery to promote apoptosis by regulating autophagy and stress. *Int. J. Nanomed.* 13, 5865–5886. doi: 10.2147/ijn.s162647
- Chen, L., Feng, P., Zhu, X., He, S., Duan, J., and Zhou, D. (2016). Long non-coding RNA Malat1 promotes neurite outgrowth through activation of ERK/MAPK signalling pathway in N2a cells. *J. Cell. Mol. Med.* 20, 2102–2110. doi: 10.1111/jcmm.12904
- Chen, Z., Zheng, C., Long, Z., Cao, L., Li, X., Shang, H., et al. (2016). (CAG)n loci as genetic modifiers of age-at-onset in patients with Machado-Joseph disease from mainland China. *Brain* 139(Pt 8):e41. doi: 10.1093/brain/aww087
- Chen, Z., Wang, C., Zheng, C., Long, Z., Cao, L., Li, X., et al. (2017). Ubiquitin-related network underlain by (CAG)n loci modulate age at onset in Machado-Joseph disease. *Brain* 140:e25. doi: 10.1093/brain/aww028
- Chen, Z., Wang, P., Wang, C., Peng, Y., Hou, X., Zhou, X., et al. (2018). Updated frequency analysis of spinocerebellar ataxia in China. *Brain* 141:e22. doi: 10.1093/brain/awy016
- Cheng, X. R., Cui, X. L., Zheng, Y., Zhang, G. R., Li, P., Huang, H., et al. (2013). Nodes and biological processes identified on the basis of network analysis in the brain of the senescence accelerated mice as an Alzheimer's disease animal model. *Front. Aging Neurosci.* 5:65. doi: 10.3389/fnagi.2013.00065
- Coppola, G., Burnett, R., Perlman, S., Versano, R., Gao, F., Plasterer, H., et al. (2011). A gene expression phenotype in lymphocytes from Friedreich ataxia patients. *Ann. Neurol.* 70, 790–804. doi: 10.1002/ana.22526
- Costa Mdo, C., and Paulson, H. L. (2012). Toward understanding Machado-Joseph disease. *Prog. Neurobiol.* 97, 239–257. doi: 10.1016/j.pneurobio.2011.11.006
- Engreitz, J. M., Haines, J. E., Perez, E. M., Munson, G., Chen, J., Kane, M., et al. (2016). Local regulation of gene expression by lncRNA promoters, transcription and splicing. *Nature* 539, 452–455. doi: 10.1038/nature20149
- Fleischer, A., Ayllon, V., and Rebollo, A. (2002). ITM2BS regulates apoptosis by inducing loss of mitochondrial membrane potential. *Eur. J. Immunol.* 32, 3498–3505. doi: 10.1002/1521-4141(200212)32:12<3498::aid-immu3498>3.0.co;2-c
- Gagliardi, S., Zucca, S., Pandini, C., Diamanti, L., Bordoni, M., Sproviero, D., et al. (2018). Long non-coding and coding RNAs characterization in peripheral blood mononuclear cells and spinal cord from amyotrophic lateral sclerosis patients. *Sci. Rep.* 8:2378. doi: 10.1038/s41598-018-20679-5
- Harmuth, T., Prell-Schicker, C., Weber, J. J., Gellerich, F., Funke, C., Driessen, S., et al. (2018). Mitochondrial morphology, function and homeostasis are impaired by expression of an n-terminal calpain cleavage fragment of ataxin-3. *Front. Mol. Neurosci.* 11:368. doi: 10.3389/fnmol.2018.00368
- Khorkova, O., Hsiao, J., and Wahlestedt, C. (2015). Basic biology and therapeutic implications of lncRNA. *Adv. Drug Deliv. Rev.* 87, 15–24. doi: 10.1016/j.addr.2015.05.012
- Lardenoije, R., Iatrou, A., Kenis, G., Kompotis, K., Steinbusch, H. W., Mastroeni, D., et al. (2015). The epigenetics of aging and neurodegeneration. *Prog. Neurobiol.* 131, 21–64. doi: 10.1016/j.pneurobio.2015.05.002
- Li, Z., Xu, C., Ding, B., Gao, M., Wei, X., and Ji, N. (2017). Long non-coding RNA MALAT1 promotes proliferation and suppresses apoptosis of glioma cells through derepressing Rap1B by sponging miR-101. *J. Neurooncol.* 134, 19–28. doi: 10.1007/s11060-017-2498-5
- Lin, N., Chang, K. Y., Li, Z., Gates, K., Rana, Z. A., Dang, J., et al. (2014). An evolutionarily conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. *Mol. Cell* 53, 1005–1019. doi: 10.1016/j.molcel.2014.01.021
- Matos, C. A., de Macedo-Ribeiro, S., and Carvalho, A. L. (2011). Polyglutamine diseases: the special case of ataxin-3 and Machado-Joseph disease. *Prog. Neurobiol.* 95, 26–48. doi: 10.1016/j.pneurobio.2011.06.007
- Peng, H., Wang, C., Chen, Z., Sun, Z., Jiao, B., Li, K., et al. (2014). APOE epsilon2 allele may decrease the age at onset in patients with spinocerebellar ataxia type 3 or Machado-Joseph disease from the Chinese Han population. *Neurobiol. Aging* 35, e2115–e2178. doi: 10.1016/j.neurobiolaging.2014.03.020
- Roberts, T. C., Morris, K. V., and Wood, M. J. (2014). The role of long non-coding RNAs in neurodevelopment, brain function and neurological disease.

- Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130507. doi: 10.1098/rstb.2013.0507
- Salta, E., and De Strooper, B. (2017). Noncoding RNAs in neurodegeneration. *Nat. Rev. Neurosci.* 18, 627–640. doi: 10.1038/nrn.2017.90
- Sunwoo, J. S., Lee, S. T., Im, W., Lee, M., Byun, J. I., Jung, K. H., et al. (2017). Altered expression of the long noncoding RNA NEAT1 in huntington's disease. *Mol. Neurobiol.* 54, 1577–1586. doi: 10.1007/s12035-016-9928-9
- Tan, J. Y., Vance, K. W., Varela, M. A., Sirey, T., Watson, L. M., Curtis, H. J., et al. (2014). Cross-talking noncoding RNAs contribute to cell-specific neurodegeneration in SCA7. *Nat. Struct. Mol. Biol.* 21, 955–961. doi: 10.1038/nsmb.2902
- Todd, K., Fossati, S., Ghiso, J., and Rostagno, A. (2014). Mitochondrial dysfunction induced by a post-translationally modified amyloid linked to a familial mutation in an alternative model of neurodegeneration. *Biochim. Biophys. Acta* 1842(12 Pt A), 2457–2467. doi: 10.1016/j.bbadis.2014.09.010
- Vidal, R., Frangione, B., Rostagno, A., Mead, S., Révész, T., Plant, G., et al. (1999). A stop-codon mutation in the BRI gene associated with familial British dementia. *Nature* 399, 776–781. doi: 10.1038/21637
- Vidal, R., Revesz, T., Rostagno, A., Kim, E., Holton, J. L., Bek, T., et al. (2000). A decamer duplication in the 3' region of the BRI gene originates an amyloid peptide that is associated with dementia in a Danish kindred. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4920–4925. doi: 10.1073/pnas.080076097
- Wang, C., Chen, Z., Yang, F., Jiao, B., Peng, H., Shi, Y., et al. (2015). Analysis of the GGGGCC Repeat Expansions of the C9orf72 Gene in SCA3/MJD Patients from China. *PLoS One* 10:e0130336. doi: 10.1371/journal.pone.0130336
- Wang, L., Feng, Z., Wang, X., Wang, X., and Zhang, X. (2010). DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* 26, 136–138. doi: 10.1093/bioinformatics/btp612
- Wapinski, O., and Chang, H. Y. (2011). Long noncoding RNAs and human disease. *Trends Cell Biol.* 21, 354–361. doi: 10.1016/j.tcb.2011.04.001
- Zhang, F., Gao, C., Ma, X. F., Peng, X. L., Zhang, R. X., Kong, D. X., et al. (2016). Expression profile of long noncoding RNAs in peripheral blood mononuclear cells from multiple sclerosis patients. *CNS Neurosci. Ther.* 22, 298–305. doi: 10.1111/cns.12498
- Zhou, M., Zhao, H., Wang, X., Sun, J., and Su, J. (2018). Analysis of long noncoding RNAs highlights region-specific altered expression patterns and diagnostic roles in Alzheimer's disease. *Brief. Bioinform.* [Epub ahead of print]

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past collaboration with several of the authors HJ and BT.

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Long Non-coding RNA NEAT1: A Novel Target for Diagnosis and Therapy in Human Tumors

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OPEN ACCESS

Edited by:

Ge Shan,
University of Science and Technology
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Reviewed by:

Jernej Ule,
University College London,
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Karolinska Institutet (KI), Sweden

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 09 August 2018

Accepted: 24 September 2018

Published: 15 October 2018

Citation:

Dong P, Xiong Y, Yue J, Hanley SJB,
Kobayashi N, Todo Y and Watari H
(2018) Long Non-coding RNA NEAT1:
A Novel Target for Diagnosis and
Therapy in Human Tumors.
Front. Genet. 9:471.
doi: 10.3389/fgene.2018.00471

The nuclear paraspeckle assembly transcript 1 (NEAT1, a long non-coding RNA) is frequently overexpressed in human tumors, and higher NEAT1 expression is correlated with worse survival in cancer patients. NEAT1 drives tumor initiation and progression by modulating the expression of genes involved in the regulation of tumor cell growth, migration, invasion, metastasis, epithelial-to-mesenchymal transition, stem cell-like phenotype, chemoresistance and radioresistance, indicating the potential for NEAT1 to be a novel diagnostic biomarker and therapeutic target. Mechanistically, NEAT1 functions as a scaffold RNA molecule by interacting with EZH2 (a subunit of the polycomb repressive complex) to influence the expression of downstream effectors of EZH2, it also acts as a microRNA (miRNA) sponge to suppress the interactions between miRNAs and target mRNAs, and affects the expression of miR-129 by promoting the DNA methylation of the miR-129 promoter region. Knockdown of NEAT1 via small interfering RNA or short hairpin RNA inhibits the malignant behavior of tumor cells. In this review, we highlight the latest insights into the expression pattern, biological roles and mechanisms underlying the function and regulation of NEAT1 in tumors, and especially focus on its clinical implication as a new diagnostic biomarker and an attractive therapeutic target for cancers.

Keywords: NEAT1, nuclear paraspeckle assembly transcript 1, long non-coding RNA, cancer diagnosis, cancer treatment, EMT, microRNA

INTRODUCTION

Although most of the genome (around 80%) is actively transcribed into RNA, <2% is actually translated into functional proteins (ENCODE Project Consortium, 2012), suggesting that the dominant fraction of the transcriptome consists of non-coding RNAs (ncRNAs). ncRNAs can be classified into small ncRNAs (<200 nt) or long ncRNAs (>200 nt). One class of small ncRNAs, microRNAs (miRNAs), bind to the 3'-untranslated region of the target gene mRNA, thereby repressing target mRNA translation or inducing mRNA degradation. In contrast to well-studied miRNAs, the function and mechanism of long ncRNAs (lncRNAs) in human cancers are poorly characterized.

CATEGORIES OF LNCRNAS

According to genomic location, lncRNAs can be further subdivided into exon or intron sense-overlapping lncRNAs, intergenic lncRNAs, antisense lncRNAs, bidirectional lncRNAs and enhancer lncRNAs (Esteller, 2011; Thum and Condorelli, 2015; **Figure 1**).

FUNCTIONS AND MECHANISMS OF LNCRNAS

The function of lncRNA depends on their subcellular location (Chen L. L., 2016). Up to 30% of lncRNAs are found exclusively in the nucleus, ~15% are found exclusively in the cytoplasm, while the remaining lncRNAs are present in both nucleus and cytoplasm (Kapranov et al., 2007). In the nucleus, lncRNAs are important transcriptional and epigenetic modulators of nuclear functions, whereas cytoplasmic lncRNAs target mRNA transcripts and modulate mRNA stability and translation (Mercer and Mattick, 2013).

lncRNAs could form complex secondary and tertiary structures, which are proposed to provide multiple binding sites for other molecules (Liu et al., 2017b). lncRNAs regulates the expression of target genes by interacting with other molecules such as protein, DNA and RNA (**Figure 2**). Some lncRNAs can guide site-specific recruitment of transcriptional activators or suppressors to genomic sites and regulate gene expression (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016). A number of lncRNAs may act as scaffolding proteins by recruiting chromatin remodeling complexes, including the polycomb repressive complex 1 (PRC1) and PRC2, to silence target-specific genes (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016). Furthermore, lncRNAs can scaffold HBXIP and LSD1 to form a complex that activates transcription of c-Myc target genes (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016). lncRNAs can bind to transcription factors as decoys to sequester them away from their targets, thereby affecting gene transcription (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016). lncRNAs may serve as molecular sponges by harboring binding sites for miRNAs and sequester them away from their mRNA targets (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016). Certain lncRNAs can regulate RNA splicing either by interacting with splicing factors or by binding the splicing junctions of pre-mRNA (Mercer et al., 2009; Tripathi et al., 2010; Kotake et al., 2011; Wang and Chang, 2011; Li Y. et al., 2016; Xie et al., 2016).

THE ROLES OF LNCRNAS IN CANCER

Increasing number of studies have shown that lncRNAs are either up- or down-regulated in cancers. Some lncRNAs are expressed

in a cell type-specific manner during differentiation and in certain cancers, whereas several lncRNAs such as MALAT1 are widely overexpressed in various cancers (Prensner and Chinnaiyan, 2011; Wang Y. et al., 2016). Their dysregulation is associated with tumorigenesis, cancer progression, metastasis and prognosis of various tumors (Schmitt and Chang, 2016), suggesting that lncRNAs may have roles as potential biomarkers for cancer. Actually, lncRNAs have been identified as prognostic and diagnostic biomarkers for lymph node metastasis and distant metastasis in early-stage cancer (Chen J. et al., 2016). Furthermore, extracellular lncRNAs can be stable and detectable in bodily fluids (such as blood and urine), thus the levels of circulating lncRNAs can be considered as promising non-invasive biomarkers over conventional biomarkers (Shi et al., 2016).

Through the mechanisms mentioned in **Figure 2**, lncRNAs exert their oncogenic or tumor suppressive functions in human tumors and they are key regulators of pathways involving all hallmarks of cancer (Prensner and Chinnaiyan, 2011; Schmitt and Chang, 2016; Wang Y. et al., 2016). Due to the massive involvement of lncRNAs in the mechanistic, functional and translational aspects of cancer biology, they have been explored as therapeutic targets. For example, aberrant expression of the lncRNA H19 occurs in ovarian cancer and other types of cancers (Yoshimura et al., 2018). The toxin vector DTA-H19 is a plasmid that expresses the diphtheria toxin A chain under the control of the *H19* gene regulatory sequences (Mizrahi et al., 2009). The intra-tumoral injection of DTA-H19 caused significant inhibition of tumor growth in ovarian cancer xenograft models (Mizrahi et al., 2009). The safety, tolerability, and efficacy of DTA-H19 have been verified in a phase 1/2a clinical trial for the treatment of H19-overexpressing bladder cancer (Sidi et al., 2008).

Recent studies have demonstrated a complicated interplay and cross-regulation among different species of non-coding RNAs (Deng et al., 2016). lncRNAs and miRNAs can directly interact with and regulate each other through at least four mechanisms (Deng et al., 2016): (i) miRNA can decrease the abundance of lncRNA by reducing its stability (Yoon et al., 2012); (ii) lncRNAs can serve as sponges or decoys for miRNAs to decrease the available levels of miRNAs (Cesana et al., 2011); (iii) lncRNAs may compete with miRNAs for binding to mRNAs (Faghihi et al., 2010); and (iv) lncRNAs generate miRNAs from their exons and introns (Cesana et al., 2011).

NEAT1, A NOVEL PLAYER IN TUMOR

Nuclear paraspeckle assembly transcript 1 (NEAT1, a lncRNA) is transcribed from the familial tumor syndrome multiple endocrine neoplasia (MEN) type 1 loci on chromosome 11q13.1 and encodes two transcriptional variants, NEAT1-1 (3756 bp) and NEAT1-2 (22,743 bp) (Bond and Fox, 2009; **Figure 3A**). NEAT1 is enriched in the nucleus but also found in the cytoplasm (van Heesch et al., 2014). NEAT1 appears to be dispensable for normal embryonic development and adult life, as mice lacking

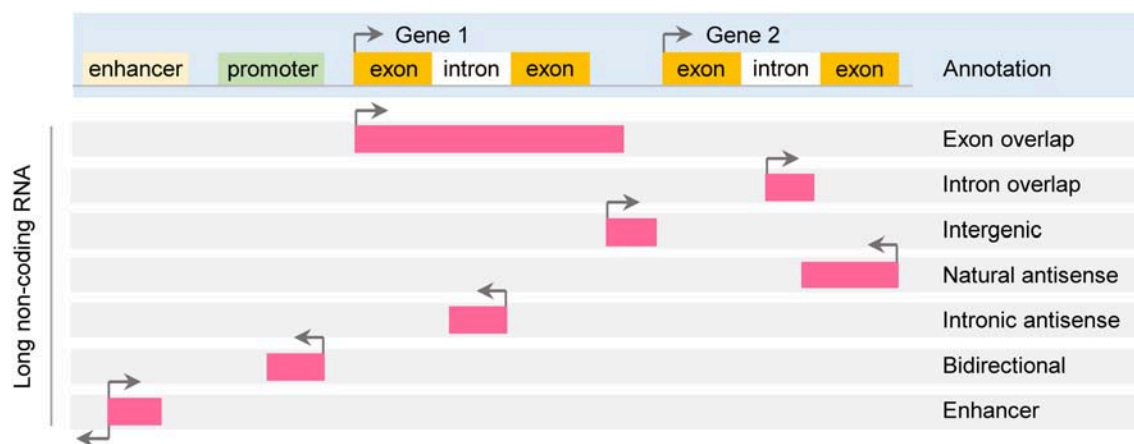


FIGURE 1 | Classification of long noncoding RNA. Long non-coding RNAs (lncRNAs) are roughly classified based on their position relative to protein-coding genes: exon or intron sense-overlapping lncRNAs, intergenic lncRNAs, antisense lncRNAs, bidirectional lncRNAs and enhancer lncRNAs.

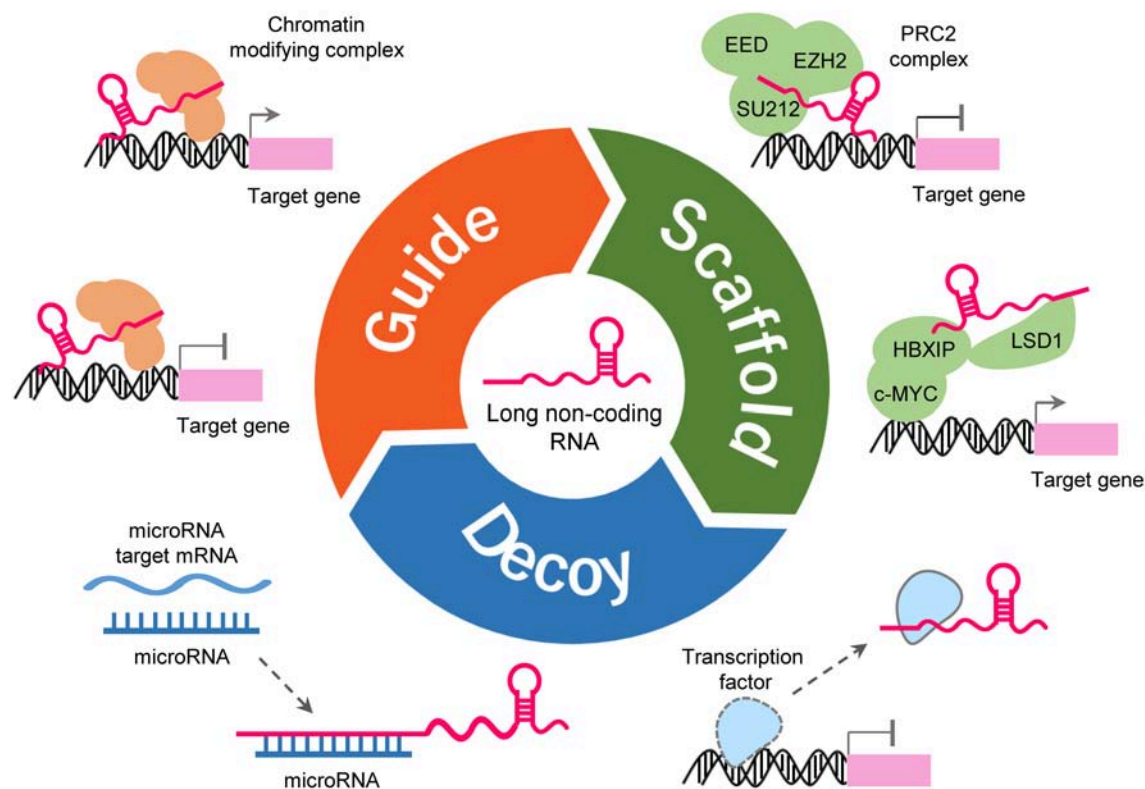
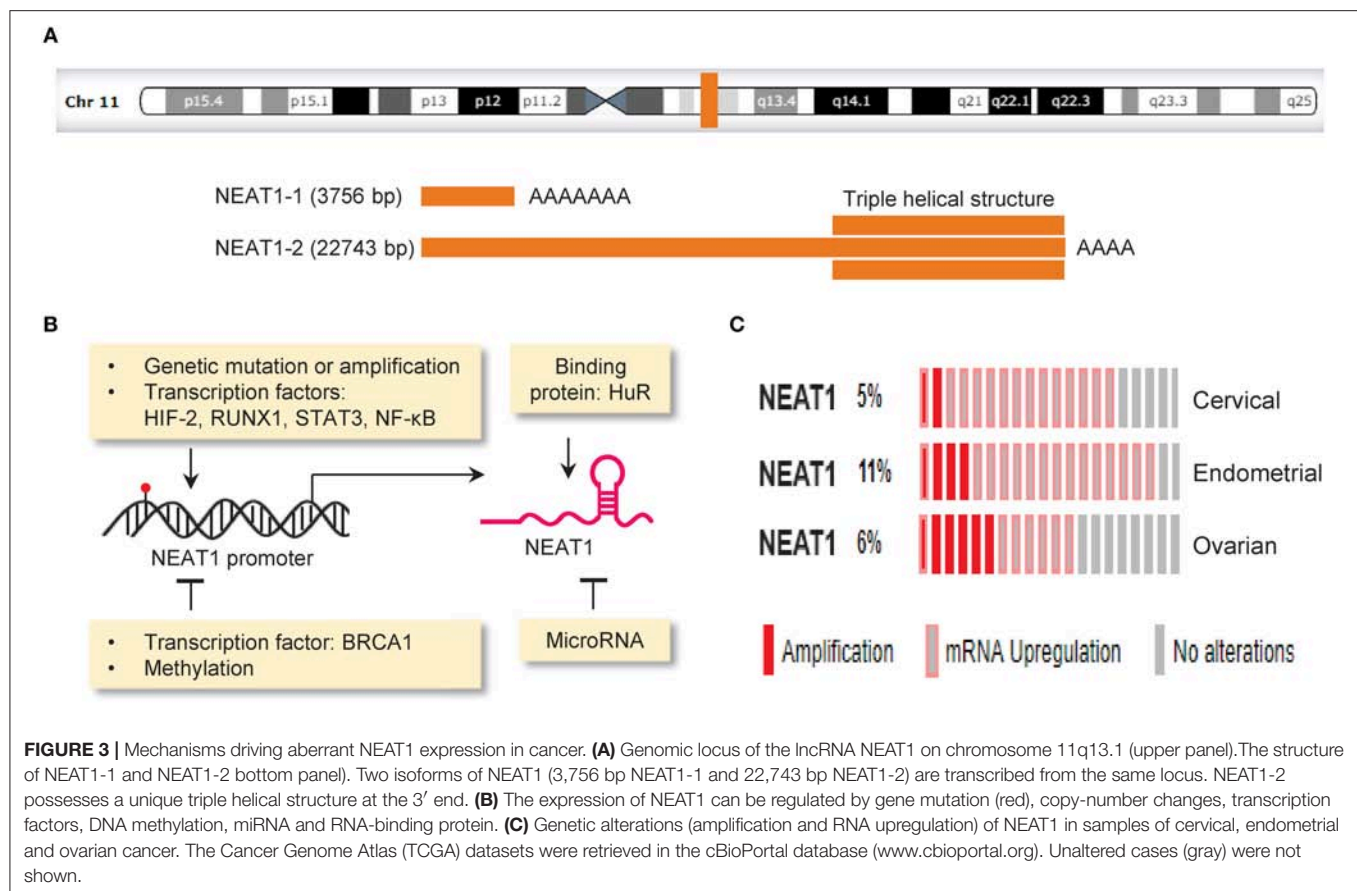


FIGURE 2 | Functions and mechanisms of lncRNA. lncRNA can guide transcription factors to specific genomic locations for the regulation of gene expression (**upper-left**). lncRNA works as a scaffold to facilitate the assembling of chromatin remodeling complexes (**upper-right**). lncRNA can also serve as a sponge to titrate miRNAs out from their mRNA targets (**lower-left**). lncRNA can bind to transcription factors or other proteins as a decoy and sequester them away from chromatin (**lower-right**).

NEAT1 develop normally (Nakagawa et al., 2011). However, another study reported that genetic ablation of NEAT1 resulted in aberrant mammary gland morphogenesis and lactation defects (Standaert et al., 2014). Whether the loss of NEAT1 is compatible

with normal cell viability and normal development should be further evaluated.

NEAT1 displays typical characteristics of cancer drivers, because it is responsible for tumor initiation and progression,



and its frequent dysregulation in cancers correlates with clinical features such as metastasis, recurrence rate and patient survival (Lanzós et al., 2017).

Increasing evidence suggested that NEAT1 is overexpressed in many solid tumors, including non-small cell lung cancer (Pan et al., 2015; Sun et al., 2016), ovarian cancer (Kim et al., 2010; Chen Z. J. et al., 2016), cervical cancer (Han et al., 2017), hepatocellular carcinoma (Guo et al., 2015; Liu Z. et al., 2017), colorectal cancer (Li et al., 2015; Peng et al., 2017), gastric cancer (Ma et al., 2016), esophageal squamous cell carcinoma (Chen et al., 2015), endometrial cancer (Li Z. et al., 2016; Wang J. et al., 2017), cholangiocarcinoma (Zhang C. et al., 2018), laryngeal squamous cell cancer (Wang P. et al., 2016), pancreatic cancer (Huang et al., 2017), thyroid carcinoma (Li J. H. et al., 2017), oral squamous cell carcinoma (Huang et al., 2018), nasopharyngeal carcinoma (Cheng and Guo, 2017; Liu F. et al., 2018), osteosarcoma (Wang H. et al., 2017; Hu et al., 2018), breast cancer (Zhang et al., 2017; Zhao et al., 2017), glioma (Zhen et al., 2016) and renal cell carcinoma (Liu et al., 2017a; Ning et al., 2017). However, its expression was reduced in acute promyelocytic leukemia (Zeng et al., 2014), indicating that the role of NEAT1 may vary with cancer types.

The overexpression of NEAT1 was significantly associated with poor overall survival in non-small cell lung cancer (Pan et al., 2015), ovarian cancer (Chen Z. J. et al., 2016), cervical cancer (Han et al., 2017), colorectal cancer (Li et al., 2015;

Peng et al., 2017), hepatocellular carcinoma (Liu Z. et al., 2017), esophageal squamous cell carcinoma (Chen et al., 2015), pancreatic cancer (Huang et al., 2017), oral squamous cell carcinoma (Huang et al., 2018), breast cancer (Zhao et al., 2017), gastric cancer (Fu et al., 2016), breast cancer (Zhang et al., 2017), and renal cell carcinoma (Ning et al., 2017). Moreover, higher expression levels of NEAT1 were positively correlated with cancer stage and metastasis in endometrial cancer (Li Z. et al., 2016), ovarian cancer (Chen Z. J. et al., 2016), non-small cell lung cancer (Pan et al., 2015; Sun et al., 2017), hepatocellular carcinoma (Sun et al., 2016), esophageal squamous cell carcinoma (Chen et al., 2015), laryngeal squamous cell cancer (Wang P. et al., 2016), breast cancer (Zhao et al., 2017), gastric cancer (Fu et al., 2016), and osteosarcoma (Hu et al., 2018).

MECHANISMS OF NEAT1 DYSREGULATION IN CANCER

The expression of NEAT1 in cancer cells is controlled by the following mechanisms: genetic alterations (such as copy number gain and gene mutation), transcription factors, DNA methylation, miRNAs and RNA-binding protein (**Figure 3B**).

The Cancer Genome Atlas (TCGA, a large-scale cancer genomics project) has revealed molecular alterations such as gene mutations, copy-number changes, upregulation or

downregulation of mRNA and lncRNA across diverse tumor types. A comprehensive study analyzing lncRNA alterations in the TCGA datasets covering 5,860 tumor samples from 13 cancer types revealed that on average 13.16% of lncRNAs underwent copy number gains and 13.53% of lncRNAs underwent copy number loss (Yan et al., 2015). Here, we used the TCGA cervical, endometrial and ovarian cancer datasets to annotate genetic alterations of NEAT1 in these gynecological cancers via the cBioPortal online application (<http://www.cbioportal.org>). Consistent with previous observations (Kim et al., 2010; Chen Z. J. et al., 2016; Li Z. et al., 2016; Han et al., 2017; Wang J. et al., 2017), we found that genetic alterations occur in 5–11% of cervical, endometrial and ovarian cancers, and the predominant alterations were amplification and RNA upregulation (**Figure 3C**). The presence of copy number gain in *NEAT1* gene might provide a possible explanation for high NEAT1 expression in these cancers. In addition, deep sequencing analysis of breast cancers has indicated mutations in the *NEAT1* promoter region (Rheinbay et al., 2017). Another group also found an elevation of mutations in the *NEAT1* promoter in renal cell carcinoma, and these mutations were associated with increased NEAT1 expression and unfavorable patient survival (Li S. et al., 2017). Although the functional impact of these mutations is unknown, mutations that occur in the *NEAT1* promoter might affect the binding of a protein to the *NEAT1* promoter and alter its expression (Rheinbay et al., 2017).

Several studies reported that transcription factors such as hypoxia-inducible factor (HIF)-2 and RUNX1 bind to the locus of *NEAT1* and induce its expression in breast cancer (Choudhry et al., 2015; Barutcu et al., 2016). STAT3 and NF- κ B, two downstream effectors of EGFR signaling, could bind to and activate the *NEAT1* promoter in glioblastoma (Chen et al., 2018). On the other hand, BRCA1 inhibits NEAT1 expression through binding to its genomic binding site upstream of the *NEAT1* gene in breast cancer (Lo et al., 2016). At the post-transcriptional level, NEAT1 is physically associated with and stabilized by an RNA-binding protein HuR (Chai et al., 2016).

Epigenetic mechanisms such as aberrant DNA methylation and miRNA dysregulation account for the aberrant expression of lncRNAs in tumors (Choudhry et al., 2015; Yan et al., 2015). The expression of NEAT1 was increased by the treatment with 5-AZA in hepatocellular carcinoma cells, indicating that DNA methylation is an important determinant of NEAT1 expression (Fang et al., 2017). Numerous miRNAs that directly target lncRNAs have identified in tumor cells (Braconi et al., 2011), and miRNAs that directly interact with NEAT1 will be discussed below. How epigenetic mechanisms (such as histone modifications) contribute to the transcriptional control of NEAT1 expression warrants further investigation.

NEAT1 CONTROLS CANCER INITIATION AND PROGRESSION

NEAT1 inhibits cell cycle arrest and apoptosis, but promotes migration, invasion, metastasis, epithelial-to-mesenchymal transition (EMT), stem cell-like phenotype, chemoresistance

and radioresistance, through at least three different molecular mechanisms (**Figure 4** and **Table 1**): (i) NEAT1 functions as a scaffold RNA molecule by interacting with EZH2 (a subunit of the polycomb repressive complex) to influence the expression of downstream effectors of EZH2, (ii) NEAT1 acts as miRNA sponges to antagonize the interactions between multiple tumor suppressor miRNAs and target mRNAs, and (iii) NEAT1 suppresses the expression of miR-129 by promoting the DNA methylation of the miR-129 promoter region.

In gastric cancer, laryngeal squamous cell cancer, pancreatic cancer, oral squamous cell carcinoma, nasopharyngeal carcinoma and breast cancer, knockdown of NEAT1 via small interfering RNA (siRNA) inhibited cell proliferation (Ma et al., 2016; Wang P. et al., 2016; Cheng and Guo, 2017; Huang et al., 2017, 2018; Qian et al., 2017). Similarly, the inhibition of NEAT1 via siRNA reduced cancer cell proliferation, migration and invasion in esophageal squamous cell carcinoma, endometrial cancer, glioma, hepatocellular carcinoma and renal cell carcinoma (Chen et al., 2015; Li Z. et al., 2016; Zhen et al., 2016; Ning et al., 2017; Wang Z. et al., 2017). Furthermore, short hairpin RNA (shRNA)-mediated silencing of NEAT1 significantly impaired cell proliferation, migration and invasion in cholangiocarcinoma (Zhang C. et al., 2018). Conversely, forced expression of NEAT1 enhanced cell growth, migration and invasion in endometrial cancer, papillary thyroid cancer, nasopharyngeal carcinoma, ovarian cancer, non-small cell lung cancer and breast cancer (Li Z. et al., 2016; Sun et al., 2016; Cheng and Guo, 2017; Ding et al., 2017; Wang J. et al., 2017; Zhao et al., 2017; Zhang H. et al., 2018).

EMT is a complex process in which epithelial cells acquire the characteristics of invasive mesenchymal cells and has been shown to contribute to tumorigenesis, invasion, metastasis, resistance to conventional chemotherapy, radiotherapy and small-molecule-targeted therapy (Dong et al., 2016; Nieto et al., 2016; Huo et al., 2017). Cancer stem cells (CSCs) are a class of pluripotent cells that possess a capacity for self-renewal and are resistant to chemotherapy and radiotherapy (Ayob and Ramasamy, 2018). Previous studies have established a link between EMT and CSC formation (Mani et al., 2008; Dong et al., 2014). NEAT1 overexpression promotes EMT and invasion in breast cancer, renal cell carcinoma, renal cell carcinoma, hepatoblastoma and nasopharyngeal carcinoma (Lu et al., 2016; Fu et al., 2017; Liu et al., 2017a; Li W. et al., 2017; Ning et al., 2017; Zhang et al., 2017; Zheng et al., 2018). Importantly, NEAT1 was found to be overexpressed in CD133⁺ glioma stem cells and knockdown of NEAT1 by siRNA reduced the ability of these cells to form colonies in soft agar (Yang et al., 2017). Glioma stem cells transfected with NEAT1 shRNA exhibited weaker proliferation, migration and invasion than that of those cells transfected with control shRNA (Gong et al., 2016). In sphere-forming cells generated from certain non-small cell lung cancer cell lines, downregulation of NEAT1 resulted in a significant decrease in the expression of CSC markers (CD133, CD44, ABCG2, Sox2, Nanog, and Oct-4) (Jiang et al., 2018). Not surprisingly, NEAT1 silencing by siRNA sensitized tumor cells to anti-cancer drugs such as sorafenib (Liu et al., 2017a), cisplatin (Hu et al., 2018; Liu F. et al., 2018), dexamethasone (Wu and Wang, 2018), and paclitaxel (An et al., 2017). shRNA-mediated reduction of NEAT1

enhanced the *in vitro* radiosensitivity of cancer cell lines to radiation therapy (Lu et al., 2016; Han et al., 2017).

NEAT1-1 and NEAT1-2 appear to have distinct roles in regulating the phenotypes of cancer cells. For example, in colorectal cancer cell lines, knockdown of NEAT1-1 could inhibit cell invasion and proliferation, whereas knockdown of NEAT1-2 promoted cell growth (Wu et al., 2015). The same study showed that expression of NEAT1-1 was significantly higher in liver metastatic lesions compared with adjacent normal colorectal tissues and primary colorectal cancer tissues (Wu et al., 2015). These findings suggested that NEAT1-1 may act as a carcinogenic factor, while NEAT1-2 may be a tumor suppressor in colorectal cancer. It appears that the expression of NEAT1 isoforms is regulated in a cell type-specific manner. For example, in adult mouse tissues, NEAT1-1 is expressed in a broad range of cell types (Nakagawa et al., 2011). However, NEAT1-2 expression is largely restricted to the epithelial layers of digestive tissues (Nakagawa et al., 2011). The compositions of NEAT1 isoforms may vary in different cancer types. These dynamic changes in NEAT1 isoform expression may affect the main function of NEAT1 as an oncogene or a tumor suppressor. Therefore, elucidating the exact contribution of NEAT1 isoforms to the development of human tumors would be an exciting direction for future studies.

Mechanistically, NEAT1 recruits EZH2 to form the PRC2 complex and mediate the expression of EZH2 target genes, thereby promoting tumor cell growth and invasion in

glioblastoma and cholangiocarcinoma (Chen et al., 2018; Zhang C. et al., 2018; **Figure 4** and **Table 1**).

Additionally, by sponging a set of miRNAs, such as let-7a (Liu F. et al., 2018), let-7e (Gong et al., 2016), miR-34a (Ding et al., 2017; Liu et al., 2017a), miR-34c (Hu et al., 2018), miR-101 (Qian et al., 2017; Wang Y. et al., 2017), miR-106b (Sun et al., 2018), miR-107 (Wang P. et al., 2016; Yang et al., 2017), miR-124 (Chai et al., 2016; Cheng and Guo, 2017; Liu X. et al., 2018), miR-193a (Wu and Wang, 2018), miR-193b (Han et al., 2017), miR-194 (An et al., 2017; Wang H. et al., 2017), miR-204 (Lu et al., 2016), miR-211 (Li X. et al., 2017), miR-214 (Li J. H. et al., 2017; Wang J. et al., 2017), miR-218 (Zhao et al., 2017), miR-365 (Huang et al., 2018), miR-377 (Sun et al., 2016), miR-449 (Zhen et al., 2016), miR-506 (Huang et al., 2017), and miR-613 (Wang Z. et al., 2017), NEAT1 could abolish miRNAs-mediated suppression of their target genes, therefore promoting tumor cell growth, migration, invasion, metastasis, EMT, stem cell-like phenotype, chemoresistance and radioresistance (**Figure 4** and **Table 1**).

Changes in miRNA expression through altered DNA methylation affect tumor progression. NEAT1 was shown to silence the expression of miR-129 by increasing the DNA methylation level in its promoter region in breast cancer cells (Lo et al., 2016; **Table 1**).

Of note, NEAT1 overexpression induced the activity of the Wnt/ β -catenin signaling, either by inhibiting the expression of miR-214 (Wang J. et al., 2017) or by interacting with EZH2 to

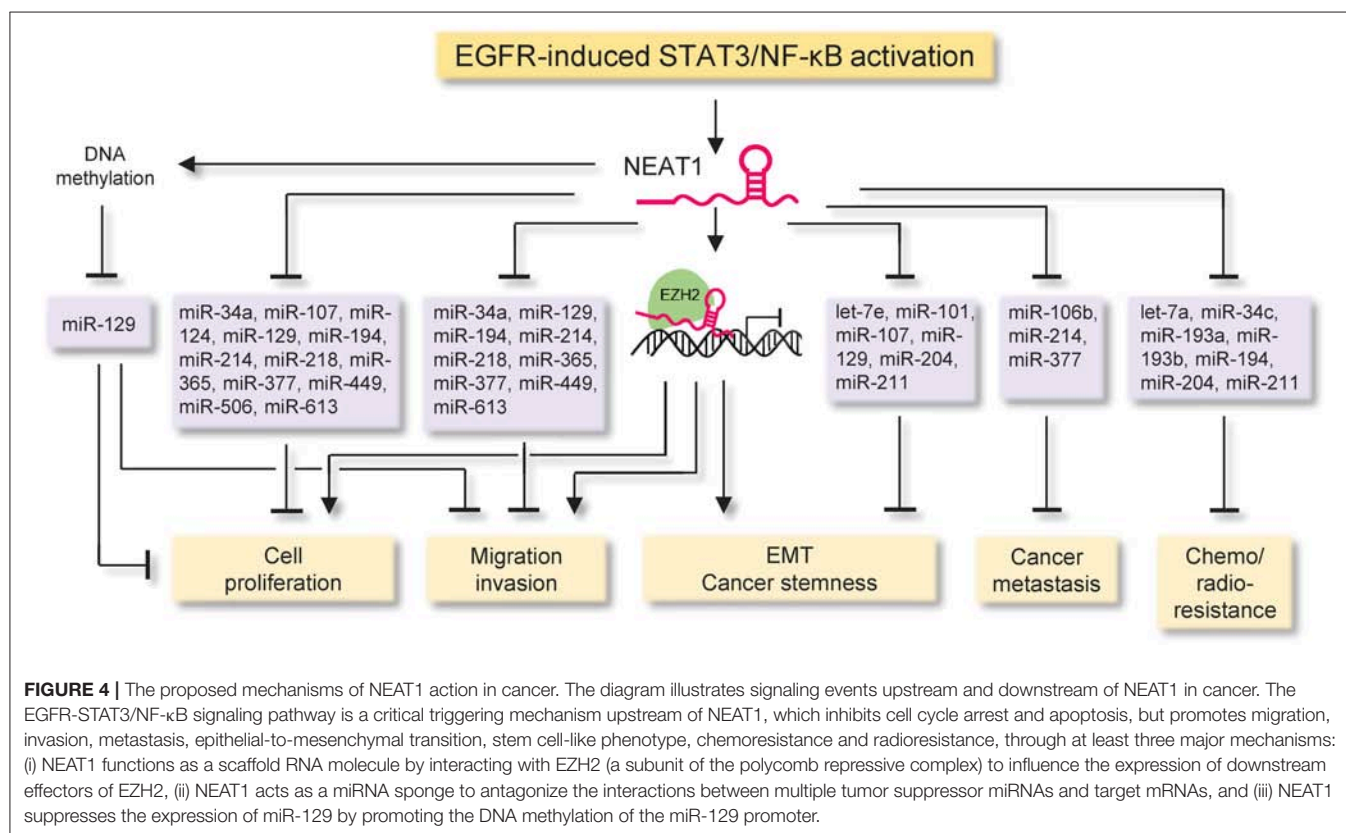


TABLE 1 | NEAT1 is a key regulator of cancer initiation and progression.

Cancer type	Function	Cell lines	Assay methods	Isoforms	Molecular mechanisms	References
Non-small cell lung cancer	Oncogene	A549, SPC-A1, H1299, 95D, SK-MES-1, NCI-H520	Colony formation, CCK8, migration, invasion, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-377	(Sun et al., 2016)
Non-small cell lung cancer	Oncogene	A549, H1299, SPCA1, H358	CCK8, migration, invasion assay	NA	Activating the Wnt/ β -catenin signaling pathway	(Sun et al., 2017)
Non-small cell lung cancer	Oncogene	A549, H460, H1299	Flow cytometry analysis	NA	Activating the Wnt/ β -catenin signaling pathway and EMT-related pathway	(Jiang et al., 2018)
Cervical cancer	Oncogene	HeLa, SiHa	MTT, Flow cytometry analysis, Radiation sensitivity assay	NA	Functioning as a competing endogenous RNA for miR-193	(Han et al., 2017)
Colorectal Cancer	Oncogene	HCT116, LoVo, HT29, SW480	MTT, Flow cytometry analysis,	NA	Activating the AKT pathway	(Peng et al., 2017)
Colorectal Cancer	Oncogene or tumor suppressor	HCT116, LoVo	CCK8, invasion assay	NEAT1-1 or NEAT-2	NA	(Wu et al., 2015)
Esophageal squamous cell carcinoma	Oncogene	KYSE140, KYSE150	CCK8, Colony formation, migration, invasion assay	NA	NA	Chen et al., 2015
Endometrial cancer	Oncogene	HEC-59, KLE, AN3CA, HEC-1-A, HEC-1-B	CCK8, Colony formation, Cell cycle and cell apoptosis analysis, migration, invasion assay	NA	Up-regulating the expression of c-Myc, IFG1, MMP-2 and MMP-7	Li Z. et al., 2016
Endometrial cancer	Oncogene	HEC-1A	WST-1, migration, invasion assay	NA	Functioning as a competing endogenous RNA for miR-214	Wang J. et al., 2017
Cholangiocarcinoma	Oncogene	QBC939, RBE, HuCCT1, TFK1	CCK8, Colony formation, in vivo tumor formation assay	NA	Repressing E-cadherin transcription through binding with EZH2	(Zhang C. et al., 2018)
Laryngeal squamous cell cancer	oncogene	Hep-2	CCK8, Wound-healing, invasion assay, cell apoptosis analysis, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-107	Wang P. et al., 2016
Pancreatic cancer	Oncogene	AsPC-1, BxPC-3, SW1990, PANC-1	CCK8 assay, Cell cycle and cell apoptosis analysis	NA	Functioning as a competing endogenous RNA for miR-506	(Huang et al., 2017)
Thyroid carcinoma	Oncogene	TPC-1	CCK8, migration, invasion assay, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-214	Li J. H et al., 2017
Oral squamous cell carcinoma	Oncogene	Unknown	CCK8 assay, Cell cycle and cell apoptosis analysis	NA	Functioning as a competing endogenous RNA for miR-365	(Huang et al., 2018)
Nasopharyngeal carcinoma	Oncogene	CNE1, CNE2, SUNE1, 6-10B, SUNE2	CCK8, Colony formation assay, cell apoptosis analysis	NA	Functioning as a competing endogenous RNA for miR-124	(Cheng and Guo, 2017)
Nasopharyngeal carcinoma	Oncogene	CNE-2, HONE-1, 5-8F, SUNE-1	Colony formation, apoptosis assay	NA	Functioning as a competing endogenous RNA for miR-204	(Lu et al., 2016)
Nasopharyngeal carcinoma	Oncogene	5-8F, CNE1, CNE2, S26, HNE1, SUNE1, HONE1	MTT, Drug resistance, cell apoptosis, wound healing assay	NA	Functioning as a competing endogenous RNA for miR-101	(Wang H. et al., 2017)
Osteosarcoma	Oncogene	MG63, U2OS	MTT, invasion assay, cell apoptosis analysis	NA	Functioning as a competing endogenous RNA for miR-194	Wang Y. et al., 2017
Osteosarcoma	Oncogene	MG63, 143B, HOS, Saos2	CCK8 assay, cell apoptosis analysis, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-34c	(Hu et al., 2018)
Breast cancer	Oncogene	Unknown	CCK8, colony formation, invasion assay	NA	Functioning as a competing endogenous RNA for miR-218	(Zhao et al., 2017)
Breast cancer	Oncogene	MCF-7, MDA-MB-231, MDAMB-468, T47D, BT-547	MTT, wound Healing assay	NA	Activating the β -catenin signaling, and triggering EMT	(Zhang et al., 2017)
Breast cancer	Oncogene	MCF-7, SKBR3, MDA-MB-453, T47D, DU4475	MTT, BrdU incorporation assay	NA	Functioning as a competing endogenous RNA for miR-101	(Qian et al., 2017)

(Continued)

TABLE 1 | Continued

Cancer type	Function	Cell lines	Assay methods	Isoforms	Molecular mechanisms	References
Breast cancer	Oncogene	MCF-7, MDA-MB-231, T47D, ZR-75-1	Migration, invasion, in vivo tumor formation and metastasis assay	NA	Functioning as a competing endogenous RNA for miR-211	Li X. et al., 2017
Breast cancer	Oncogene	MCF-7, HCC1937	Migration, invasion, Soft agar colony formation assay, in vivo tumor formation assay	NA	Silencing miR-129-5p expression by promoting the DNA methylation of the CpG island in the miR-129 gene	(Lo et al., 2016)
Glioma	Oncogene	U87, U373, U251	MTT, migration, invasion assay, cell apoptosis analysis, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-449b	(Zhen et al., 2016)
Glioma	Oncogene	U87, Glioma primary cultured cells	Sphere formation, Soft agar colony formation assay, cell cycle and cell apoptosis analysis	NA	Functioning as a competing endogenous RNA for miR-107	(Yang et al., 2017)
Clear cell renal cell carcinoma	Oncogene	Unknown	CCK8, migration, invasion assay, cell apoptosis analysis	NA	Inducing the EMT process	(Ning et al., 2017)
Renal cell carcinoma	Oncogene	ACHN, 786-O, A498, Caki-1	EdU, migration, invasion assay, cell cycle analysis	NA	Functioning as a competing endogenous RNA for miR-34c	(Liu et al., 2017a)
Acute promyelocytic leukemia	Tumor suppressor	NB4, NB4-R2, U937-PR9	Flow cytometry analysis	NEAT1-1 and NEAT1-2	NA	(Zeng et al., 2014)
Gastric cancer	Oncogene	NCI-N87, SGC-7901, MKN-45, AGS	Colony formation, migration, invasion assay	NA	Inducing the EMT process	(Fu et al., 2016)
Gastric cancer	Oncogene	MKN-45, BGC823, MGC803, SGC7901, AGS, MKN28	WST-1, migration assay	NA	NA	(Ma et al., 2016)
Glioblastoma	Oncogene	N5, N9, and N33 patient-derived cells	CCK8, colony formation, invasion assay, cell apoptosis assay, in vivo tumor formation assay	NA	Recruiting EZH2 to form the PRC2 complex and mediate the expression of EZH2 target genes	Chen et al., 2018
Hepatocellular carcinoma	Oncogene	MHCC97H, MHCC97L, SMCC7721, Huh7	MTT, invasion assay	NA	Functioning as a competing endogenous RNA for miR-613	Wang Z. et al., 2017
Thyroid cancer	Oncogene	NPA8, TPC-1, KAT-5	Colony formation, wound healing, invasion assay, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-129	(Zhang H. et al., 2018)
Thyroid cancer	Oncogene	K1, TPC-1	CCK8, wound healing, migration, invasion assay, cell apoptosis assay	NEAT1-2	Functioning as a competing endogenous RNA for miR-106b	(Sun et al., 2018)
Ovarian cancer	Oncogene	OVCAR3, SKOV3, HO8910, OV90	MTT, caspase-3 activity assay	NA	Functioning as a competing endogenous RNA for miR-34a	(Ding et al., 2017)
Ovarian cancer	Oncogene	SKOV3, HeyA-8	Drug resistance assay, cell apoptosis assay, in vivo tumor formation assay	NA	Functioning as a competing endogenous RNA for miR-194	(An et al., 2017)
Multiple myeloma	Oncogene	RPMI8226, JJN-3, U266, ANBL6, OPM-2, MM1S, MM1R	MTT, cell apoptosis assay	NA	Functioning as a competing endogenous RNA for miR-193a	(Wu and Wang, 2018)

NA, Undetermined.

reduce the expression of negative regulators of WNT/ β -catenin signaling such as ICAT, GSK3B and Axin2 (Chen et al., 2018 Table 1).

NEAT1 AS A POTENTIAL DIAGNOSTIC BIOMARKER OF CANCER

Cancer type- or subtype-specific expression patterns and the sensitive and inexpensive quantitative detection methods for ncRNAs make lncRNAs suitable as hopeful biomarkers for cancer diagnosis and prognosis (Brunner et al., 2012; Bijnsdorp et al., 2017). LncRNAs can exhibit different expression patterns

between subtypes of the same cancer (Su et al., 2014). Several lncRNAs (such as PCA3, PCGEM1 and PCAT-1) are highly specific to prostate cancer (Crea et al., 2014) and have been used to identify primary tumors.

Although lncRNAs are commonly expressed at lower levels than protein-coding mRNAs, some lncRNAs (including NEAT1) exhibit moderate to high levels of expression in clinical samples. Moreover, lncRNAs can be detected in body fluids such as serum, plasma, urine and saliva of cancer patients (Chandra Gupta and Nandan Tripathi, 2017). The diagnostic value of NEAT1 was validated in a cohort of colorectal cancer patients, because the whole-blood NEAT1 expression was significantly increased in cancer patients than persons without cancer (Wang Y. et al.,

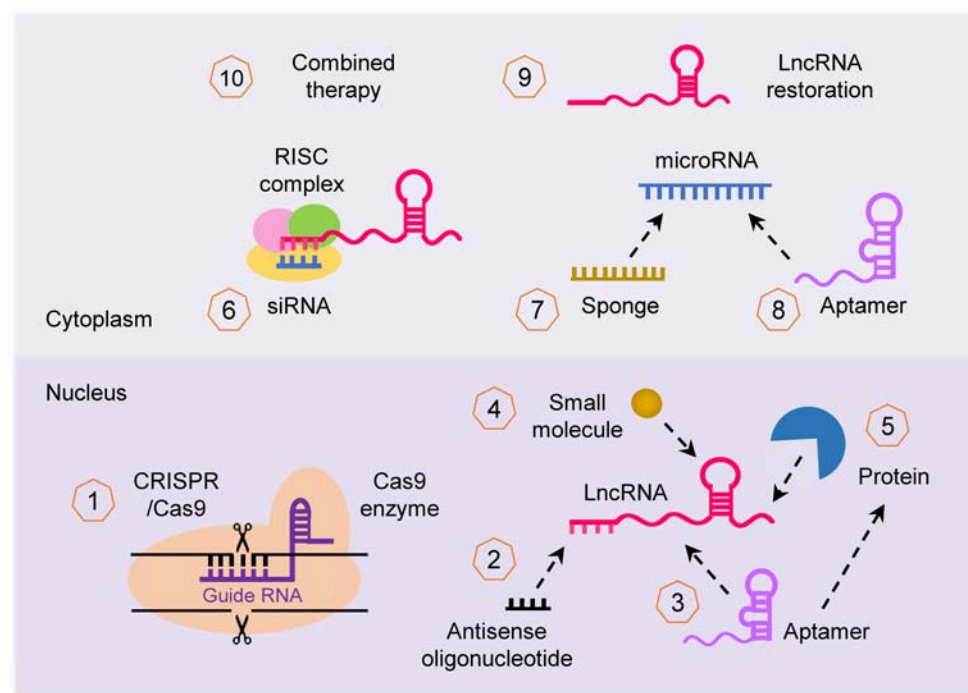


FIGURE 5 | Therapeutic strategies to target lncRNA. Several approaches can be applied to target lncRNA in cancer for therapeutic purposes: (1) inhibition of lncRNA loci using the CRISPR/Cas-9 system, (2) knockdown of lncRNA transcript using antisense oligonucleotide, (3, 4) interfering with the binding of lncRNA with their binding partner (protein, DNA and RNA) with small synthetic molecule, peptide and aptamer, (5) repression of lncRNA that function through chromatin remodeling using selective PRC1/2 inhibitors, (6) silencing of lncRNA using specific siRNA, (7, 8) indirect targeting of lncRNA through modulating the function of miRNAs via miRNA mimics or anti-miRNA inhibitors such as aptamer, (9) restoring the expression of tumor suppressor lncRNA with synthetic lncRNA mimics, and (10) lncRNA can be applied for enhancing the effectiveness of other forms of therapy (such as chemotherapy and radiotherapy).

2017). Therefore, NEAT1 may be possibly used as a biomarker for the existence of primary cancer. The same study also reported that the whole-blood expression of NEAT1-1 was significantly higher in colorectal cancer patients with distant metastasis than in those without metastasis (Wang Y. et al., 2017). Thus, the whole-blood NEAT1 expression might serve as a biomarker for prediction of distant metastasis.

NEAT1 AS A POTENTIAL THERAPEUTIC TARGET IN CANCER

Given the importance of the lncRNA-mediated networks that broadly affect cancer initiation and progression, lncRNAs are of interest for developing novel therapeutics. lncRNAs can be targeted by multiple approaches (Parasramka et al., 2016; Worku et al., 2017; Arun et al., 2018; **Figure 5**): (i) CRISPR/Cas-9 system can be used to knockout lncRNA; (ii) lncRNA transcript can be knocked down using lncRNA-specific siRNA and antisense oligonucleotide (ASO); (iii) small synthetic molecules/peptides/aptamers can be designed to block and antagonize the binding of lncRNAs with their binding partners (such as protein, DNA, RNA, or other interacting complexes); (iv) selective PRC1/2 inhibitors such as GSK343 (Ihira et al., 2017) can be used to inhibit the activity of lncRNA that

function through chromatin remodeling; (v) miRNA regulates the expression of lncRNA, thus restoration or blocking of a specific miRNA using miRNA mimics or anti-miRNA inhibitor (such as locked nucleic acid and miRNA sponge) can indirectly alter the expression of lncRNA; (vi) synthetic lncRNA mimics could be used to restore the expression of tumor suppressor lncRNA; and (vii) approaches to target lncRNAs can be used in combination with other therapies such as chemotherapy and radiotherapy to enhance their effectiveness. For example, targeted depletion of NEAT1 by siRNA (Li J. H et al., 2017) or shRNA (Zhang C. et al., 2018; Zhang H. et al., 2018) displayed a significant therapeutic effect *in vivo*. *In vivo* studies also confirmed that knockdown of NEAT1 sensitized tumor cells to cisplatin/paclitaxel- or radio-induced tumor regression (An et al., 2017; Han et al., 2017; Hu et al., 2018).

Apart from these strategies, targeting the upstream signaling pathways of NEAT1 or its binding partners would be another method to modulate NEAT1 expression or function in malignant tumors. Despite their potential side effects, blocking of NF- κ B and STAT3 activity may represent a good approach to combat NEAT1-overexpressing tumors (Grivennikov and Karin, 2010; Liby et al., 2010). In addition, hypoxia is frequently observed in solid tumors and HIF-1/-2 has been potential targets for developing novel cancer therapeutics. A number of small molecule inhibitors of HIF-2 have been developed

and might be used to downregulate the expression of NEAT1 (Yu et al., 2017). HuR is overexpressed in a wide variety of cancer types and stabilizes a large subset of mRNAs, which encode proteins implicated in tumor cell proliferation, survival, angiogenesis, invasion and metastasis (Kotta-Loizou et al., 2016). High-throughput screening methods have been established to identify small molecular molecules against HuR (Meisner et al., 2007). Thus, HuR inhibition may offer new conceptual routes to treat cancers expressing high levels of NEAT1.

PERSPECTIVES AND CHALLENGES

The current research on NEAT1 is still at a very early stage, but growing evidence has clearly identified NEAT1 as an attractive biomarker of cancer and an ideal candidate for lncRNA therapeutics. Although the broad implications of NEAT1 in cancers have been described, several key challenges associated with NEAT1 still exist: (i) The exact mechanisms for NEAT1-mediated carcinogenesis and metastasis remain elusive, although the interactions between PRC2 complex and miRNAs appears to be at least involved in these processes. High-throughput technologies such as ChIRP, PAR-CLIP and iCLIP have been used to reveal NEAT1-DNA or NEAT1-protein interactions (Hafner et al., 2010; Zhao et al., 2010; Chu et al., 2011; Tollervey et al., 2011; Yoon et al., 2014). (ii) The precise mechanisms by which the genetic and epigenetic factors contribute to NEAT1

dysregulation, as well as the downstream signaling pathways of NEAT1, are still far from clear. (iii) The *NEAT1* loci might be targeted for therapeutic purposes using CRISPR/Cas-9 genome editing technology. However, a challenge is the safety evaluation of CRISPR/Cas-9-based therapeutics, such as the risk of disease occurrence due to unwanted mutations, the immune response to the delivery system and other toxic side effects. Taken together, NEAT1 has the potential to provide a new biomarker for the diagnosis and monitoring of cancers and will need to be taken into consideration in the development of NEAT1-targeting therapeutics (possibly in combination with chemotherapy and radiotherapy).

AUTHOR CONTRIBUTIONS

PD and HW provided direction. PD, YX, and HW wrote the manuscript. JY, SH, NK, and YT made significant revisions to the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by a grant from JSPS Grant-in-Aid for Scientific Research (C) (16K11123 and 18K09278), the Science and Technology Planning Project of Guangdong Province, China (2014A020212124) and an NIH/NCI grant 1R21CA216585-01A1 to JY.

REFERENCES

- An, J., Lv, W., and Zhang, Y. (2017). LncRNA NEAT1 contributes to paclitaxel resistance of ovarian cancer cells by regulating ZEB1 expression via miR-194. *Onco. Targets. Ther.* 10, 5377–5390. doi: 10.2147/OTT.S147586
- Arun, G., Diermeier, S. D., and Spector, D. L. (2018). Therapeutic targeting of long non-coding RNAs in cancer. *Trends Mol. Med.* 24, 257–277. doi: 10.1016/j.molmed.2018.01.001
- Ayob, A. Z., and Ramasamy, T. S. (2018). Cancer stem cells as key drivers of tumour progression. *J. Biomed. Sci.* 25:20. doi: 10.1186/s12929-018-0426-4
- Barutcu, A. R., Hong, D., Lajoie, B. R., McCord, R. P., van Wijnen, A. J., Lian, J. B., et al. (2016). RUNX1 contributes to higher-order chromatin organization and gene regulation in breast cancer cells. *Biochim. Biophys. Acta* 1859, 1389–1397. doi: 10.1016/j.bbaggm.2016.08.003
- Bijnsdorp, I. V., van Royen, M. E., Verhaegh, G. W., and Martens-Uzunova, E. S. (2017). The non-coding transcriptome of prostate cancer: implications for clinical practice. *Mol. Diagn. Ther.* 21, 385–400. doi: 10.1007/s40291-017-0271-2
- Bond, C. S., and Fox, A. H. (2009). Paraspeckles: nuclear bodies built on long noncoding RNA. *J. Cell Biol.* 186, 637–644. doi: 10.1083/jcb.200906113
- Braconi, C., Kogure, T., Valeri, N., Huang, N., Nuovo, G., Costinean, S., et al. (2011). microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. *Oncogene* 30, 4750–4756. doi: 10.1038/ncr.2011.193
- Brunner, A. L., Beck, A. H., Edris, B., Sweeney, R. T., Zhu, S. X., Li, R., et al. (2012). Transcriptional profiling of long non-coding RNAs and novel transcribed regions across a diverse panel of archived human cancers. *Genome Biol.* 13:R75. doi: 10.1186/gb-2012-13-8-r75
- Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., et al. (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 147, 358–369. doi: 10.1016/j.cell.2011.09.028
- Chai, Y., Liu, J., Zhang, Z., and Liu, L. (2016). HuR-regulated lncRNA NEAT1 stability in tumorigenesis and progression of ovarian cancer. *Cancer Med.* 5, 1588–1598. doi: 10.1002/cam4.710
- Chandra Gupta, S., and Nandan Tripathi, Y. (2017). Potential of long non-coding RNAs in cancer patients: from biomarkers to therapeutic targets. *Int. J. Cancer* 140, 1955–1967. doi: 10.1002/ijc.30546
- Chen, J., Chen, Y., Gu, L., Li, X., Gao, Y., Lyu, X., et al. (2016). LncRNAs act as prognostic and diagnostic biomarkers in renal cell carcinoma: a systematic review and meta-analysis. *Oncotarget* 7, 74325–74336. doi: 10.18632/oncotarget.11101
- Chen, L. L. (2016). Linking long noncoding RNA localization and function. *Trends Biochem. Sci.* 41, 761–772. doi: 10.1016/j.tibs.2016.07.003
- Chen, Q., Cai, J., Wang, Q., Wang, Y., Liu, M., Yang, J., et al. (2018). Long noncoding RNA NEAT1, regulated by the EGFR pathway, contributes to glioblastoma progression through the WNT/ β -Catenin Pathway by scaffolding EZH2. *Clin. Cancer Res.* 24, 684–695. doi: 10.1158/1078-0432.CCR-17-0605
- Chen, X., Kong, J., Ma, Z., Gao, S., and Feng, X. (2015). Up regulation of the long non-coding RNA NEAT1 promotes esophageal squamous cell carcinoma cell progression and correlates with poor prognosis. *Am. J. Cancer Res.* 5, 2808–2815.
- Chen, Z. J., Zhang, Z., Xie, B. B., and Zhang, H. Y. (2016). Clinical significance of up-regulated lncRNA NEAT1 in prognosis of ovarian cancer. *Eur. Rev. Med. Pharmacol. Sci.* 20, 3373–3377.
- Cheng, N., and Guo, Y. (2017). Long noncoding RNA NEAT1 promotes nasopharyngeal carcinoma progression through regulation of miR-124/NF- κ B pathway. *Onco. Targets. Ther.* 10, 5843–5853. doi: 10.2147/OTT.S151800
- Choudhry, H., Albukhari, A., Morotti, M., Haider, S., Moralli, D., Smythies, J., et al. (2015). Tumor hypoxia induces nuclear paraspeckle formation through HIF-2 α dependent transcriptional activation of NEAT1 leading to cancer cell survival. *Oncogene* 34, 4482–4490. doi: 10.1038/ncr.2014.378

- Chu, C., Qu, K., Zhong, F. L., Artandi, S. E., and Chang, H. Y. (2011). Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol. Cell* 44, 667–678. doi: 10.1016/j.molcel.2011.08.027
- Crea, F., Watahiki, A., Quagliata, L., Xue, H., Pikor, L., Parolia, A., et al. (2014). Identification of a long non-coding RNA as a novel biomarker and potential therapeutic target for metastatic prostate cancer. *Oncotarget* 5, 764–774. doi: 10.18632/oncotarget.1769
- Deng, K., Wang, H., Guo, X., and Xia, J. (2016). The cross talk between long, non-coding RNAs and microRNAs in gastric cancer. *Acta Biochim. Biophys. Sin. (Shanghai)* 48, 111–116. doi: 10.1093/abbs/gmv120
- Ding, N., Wu, H., Tao, T., and Peng, E. (2017). NEAT1 regulates cell proliferation and apoptosis of ovarian cancer by miR-34a-5p/BCL2. *Onco. Targets. Ther.* 10, 4905–4915. doi: 10.2147/OTT.S142446
- Dong, P., Konno, Y., Watari, H., Hosaka, M., Noguchi, M., and Sakuragi, N. (2014). The impact of microRNA-mediated PI3K/AKT signaling on epithelial-mesenchymal transition and cancer stemness in endometrial cancer. *J. Transl. Med.* 12:231. doi: 10.1186/s12967-014-0231-0
- Dong, P., Xiong, Y., Watari, H., Hanley, S. J., Konno, Y., Ihira, K., et al. (2016). Suppression of iASP-dependent aggressiveness in cervical cancer through reversal of methylation silencing of microRNA-124. *Sci. Rep.* 6:35480. doi: 10.1038/srep35480
- ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74. doi: 10.1038/nature11247
- Esteller, M. (2011). Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12, 861–874. doi: 10.1038/nrg3074
- Faghihi, M. A., Zhang, M., Huang, J., Modarresi, F., Van der Brug, M. P., Nalls, M. A., et al. (2010). Evidence for natural antisense transcript-mediated inhibition of microRNA function. *Genome Biol.* 11:R56. doi: 10.1186/gb-2010-11-5-r56
- Fang, L., Sun, J., Pan, Z., Song, Y., Zhong, L., Zhang, Y., et al. (2017). Long non-coding RNA NEAT1 promotes hepatocellular carcinoma cell proliferation through the regulation of miR-129-5p-VCP-IκB. *Am. J. Physiol. Gastrointest. Liver Physiol.* 313, G150–G156. doi: 10.1152/ajpgi.00426.2016
- Fu, J. W., Kong, Y., and Sun, X. (2016). Long noncoding RNA NEAT1 is an unfavorable prognostic factor and regulates migration and invasion in gastric cancer. *J. Cancer Res. Clin. Oncol.* 142, 1571–1579. doi: 10.1007/s00432-016-2152-1
- Fu, M. C., Yuan, L. Q., Zhang, T., Yan, X. M., Zhou, Y., Xia, H. L., et al. (2017). Nuclear paraspeckle assembly transcript 1 promotes the metastasis and epithelial-mesenchymal transition of hepatoblastoma cells by inhibiting miR-129-5p. *Oncol. Lett.* 14, 5773–5778. doi: 10.3892/ol.2017.6995
- Gong, W., Zheng, J., Liu, X., Ma, J., Liu, Y., and Xue, Y. (2016). Knockdown of NEAT1 restrained the malignant progression of glioma stem cells by activating microRNA let-7e. *Oncotarget* 7, 62208–62223. doi: 10.18632/oncotarget.11403
- Grivennikov, S. I., and Karin, M. (2010). Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev.* 21, 11–19. doi: 10.1016/j.cytogfr.2009.11.005
- Guo, S., Chen, W., Luo, Y., Ren, F., Zhong, T., Rong, M., et al. (2015). Clinical implication of long non-coding RNA NEAT1 expression in hepatocellular carcinoma patients. *Int. J. Clin. Exp. Pathol.* 8, 5395–5402.
- Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., et al. (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141. doi: 10.1016/j.cell.2010.03.009
- Han, D., Wang, J., and Cheng, G. (2017). LncRNA NEAT1 enhances the radio-resistance of cervical cancer via miR-193b-3p/CCND1 axis. *Oncotarget* 9, 2395–2409. doi: 10.18632/oncotarget.23416
- Hu, Y., Yang, Q., Wang, L., Wang, S., Sun, F., Xu, D., et al. (2018). Knockdown of the oncogene lncRNA NEAT1 restores the availability of miR-34c and improves the sensitivity to cisplatin in osteosarcoma. *Biosci. Rep.* 38:BSR20180375. doi: 10.1042/BSR20180375
- Huang, B., Liu, C., Wu, Q., Zhang, J., Min, Q., Sheng, T., et al. (2017). Long non-coding RNA NEAT1 facilitates pancreatic cancer progression through negative modulation of miR-506-3p. *Biochem. Biophys. Res. Commun.* 482, 828–834. doi: 10.1016/j.bbrc.2016.11.120
- Huang, G., He, X., and Wei, X. L. (2018). LncRNA NEAT1 promotes cell proliferation and invasion by regulating miR-365/RGS20 in oral squamous cell carcinoma. *Oncol. Rep.* 39, 1948–1956. doi: 10.3892/or.2018.6283
- Huo, W., Zhao, G., Yin, J., Ouyang, X., Wang, Y., Yang, C., et al. (2017). Lentiviral CRISPR/Cas9 vector mediated miR-21 gene editing inhibits the epithelial to mesenchymal transition in ovarian cancer cells. *J. Cancer* 8, 57–64. doi: 10.7150/jca.16723
- Ihira, K., Dong, P., Xiong, Y., Watari, H., Konno, Y., Hanley, S. J., et al. (2017). EZH2 inhibition suppresses endometrial cancer progression via miR-361/Twist axis. *Oncotarget* 8, 13509–13520. doi: 10.18632/oncotarget.14586
- Jiang, P., Chen, A., Wu, X., Zhou, M., Ul Haq, I., Mariyam, Z., et al. (2018). NEAT1 acts as an inducer of cancer stem cell-like phenotypes in NSCLC by inhibiting EGCG-upregulated CTR1. *J. Cell. Physiol.* 233, 4852–4863. doi: 10.1002/jcp.26288
- Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Dutttagupta, R., Willingham, A. T., et al. (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* 316, 1484–1488. doi: 10.1126/science.1138341
- Kim, Y. S., Hwan, J. D., Bae, S., Bae, D. H., and Shick, W. A. (2010). Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma. *BMC Cancer* 10:576. doi: 10.1186/1471-2407-10-576
- Kotake, Y., Nakagawa, T., Kitagawa, K., Suzuki, S., Liu, N., Kitagawa, M., et al. (2011). Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. *Oncogene* 30, 1956–1962. doi: 10.1038/onc.2010.568
- Kotta-Loizou, I., Vasilopoulos, S. N., Coutts, R. H., and Theocharis, S. (2016). Current evidence and future perspectives on HuR and breast cancer development, prognosis, and treatment. *Neoplasia* 18, 674–688. doi: 10.1016/j.neo.2016.09.002
- Lanzós, A., Carlevaro-Fita, J., Mularoni, L., Reverter, F., Palumbo, E., Guigó, R., et al. (2017). Discovery of cancer driver long noncoding RNAs across 1112 tumour genomes: new candidates and distinguishing features. *Sci. Rep.* 7:41544. doi: 10.1038/srep41544
- Li, J. H., Zhang, S. Q., Qiu, X. G., Zhang, S. J., Zheng, S. H., and Zhang, D. H. (2017). Long non-coding RNA NEAT1 promotes malignant progression of thyroid carcinoma by regulating miRNA-214. *Int. J. Oncol.* 50, 708–716. doi: 10.3892/ijo.2016.3803
- Li, S., Shuch, B. M., and Gerstein, M. B. (2017). Whole-genome analysis of papillary kidney cancer finds significant noncoding alterations. *PLoS Genet.* 13:e1006685. doi: 10.1371/journal.pgen.1006685
- Li, W., Zhang, Z., Liu, X., Cheng, X., Zhang, Y., Han, X., et al. (2017). The FOXN3-NEAT1-SIN3A repressor complex promotes progression of hormonally responsive breast cancer. *J. Clin. Invest.* 127, 3421–3440. doi: 10.1172/JCI94233
- Li, X., Wang, S., Li, Z., Long, X., Guo, Z., Zhang, G., et al. (2017). The lncRNA NEAT1 facilitates cell growth and invasion via the miR-211/HMGA2 axis in breast cancer. *Int. J. Biol. Macromol.* 105(Pt 1), 346–353. doi: 10.1016/j.ijbiomac.2017.07.053
- Li, Y., Li, Y., Chen, W., He, F., Tan, Z., Zheng, J., et al. (2015). NEAT expression is associated with tumor recurrence and unfavorable prognosis in colorectal cancer. *Oncotarget* 6, 27641–27650. doi: 10.18632/oncotarget.4737
- Li, Y., Wang, Z., Shi, H., Li, H., Li, L., Fang, R., et al. (2016). HBXIP and LSD1 scaffolded by lncRNA hotair mediate transcriptional activation by c-Myc. *Cancer Res.* 76, 293–304. doi: 10.1158/0008-5472.CAN-14-3607
- Li, Z., Wei, D., Yang, C., Sun, H., Lu, T., and Kuang, D. (2016). Overexpression of long noncoding RNA, NEAT1 promotes cell proliferation, invasion and migration in endometrial endometrioid adenocarcinoma. *Biomed. Pharmacother.* 84, 244–251. doi: 10.1016/j.biopha.2016.09.008
- Liby, K. T., Royce, D. B., Risingsong, R., Williams, C. R., Maitra, A., Hruban, R. H., et al. (2010). Synthetic triterpenoids prolong survival in a transgenic mouse model of pancreatic cancer. *Cancer Prev. Res(Phila)* 3, 1427–1434. doi: 10.1158/1940-6207.CAPR-10-0197
- Liu, F., Chen, N., Gong, Y., Xiao, R., Wang, W., and Pan, Z. (2017a). The long non-coding RNA NEAT1 enhances epithelial-to-mesenchymal transition and chemoresistance via the miR-34a/c-Met axis in renal cell carcinoma. *Oncotarget* 8, 62927–62938. doi: 10.18632/oncotarget.17757
- Liu, F., Somarowthu, S., and Pyle, A. M. (2017b). Visualizing the secondary and tertiary architectural domains of lncRNA RepA. *Nat. Chem. Biol.* 13, 282–289. doi: 10.1038/nchembio.2272
- Liu, F., Tai, Y., and Ma, J. (2018). LncRNA NEAT1/let-7a-5p axis regulates the cisplatin resistance in nasopharyngeal carcinoma by targeting Rsf-1

- and modulating the Ras-MAPK pathway. *Cancer Biol. Ther.* 19, 534–542. doi: 10.1080/15384047.2018.1450119
- Liu, X., Liang, Y., Song, R., Yang, G., Han, J., Lan, Y., et al. (2018). Long non-coding RNA NEAT1-modulated abnormal lipolysis via ATGL drives hepatocellular carcinoma proliferation. *Mol. Cancer* 17:90. doi: 10.1186/s12943-018-0838-5
- Liu, Z., Chang, Q., Yang, F., Liu, B., Yao, H. W., Bai, Z. G., et al. (2017). Long non-coding RNA NEAT1 overexpression is associated with unfavorable prognosis in patients with hepatocellular carcinoma after hepatectomy: a Chinese population-based study. *Eur. J. Surg. Oncol.* 43, 1697–1703. doi: 10.1016/j.ejso.2017.06.013
- Lo, P. K., Zhang, Y., Wolfson, B., Gernapudi, R., Yao, Y., Duru, N., et al. (2016). Dysregulation of the BRCA1/long non-coding RNA NEAT1 signaling axis contributes to breast tumorigenesis. *Oncotarget* 7, 65067–65089. doi: 10.18632/oncotarget.11364
- Lu, Y., Li, T., Wei, G., Liu, L., Chen, Q., Xu, L., et al. (2016). The long non-coding RNA NEAT1 regulates epithelial to mesenchymal transition and radioresistance in through miR-204/ZEB1 axis in nasopharyngeal carcinoma. *Tumour Biol.* 37, 11733–11741. doi: 10.1007/s13277-015-4773-4
- Ma, Y., Liu, L., Yan, F., Wei, W., Deng, J., and Sun, J. (2016). Enhanced expression of long non-coding RNA NEAT1 is associated with the progression of gastric adenocarcinomas. *World J. Surg. Oncol.* 14:41. doi: 10.1186/s12957-016-0799-3
- Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133, 704–715. doi: 10.1016/j.cell.2008.03.027
- Meisner, N. C., Hintersteiner, M., Mueller, K., Bauer, R., Seifert, J. M., Naegeli, H. U., et al. (2007). Identification and mechanistic characterization of low-molecular-weight inhibitors for HuR. *Nat. Chem. Biol.* 3, 508–515. doi: 10.1038/nchembio.2007.14
- Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155–159. doi: 10.1038/nrg2521
- Mercer, T. R., and Mattick, J. S. (2013). Structure and function of long noncoding RNAs in epigenetic regulation. *Nat. Struct. Mol. Biol.* 20, 300–307. doi: 10.1038/nsmb.2480
- Mizrahi, A., Czerniak, A., Levy, T., Amiur, S., Gallula, J., Matouk, I., et al. (2009). Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. *J. Transl. Med.* 7:69. doi: 10.1186/1479-5876-7-69
- Nakagawa, S., Naganuma, T., Shioi, G., and Hirose, T. (2011). Paraspeckles are subpopulation-specific nuclear bodies that are not essential in mice. *J. Cell Biol.* 193, 31–39. doi: 10.1083/jcb.201011110
- Nieto, M. A., Huang, R. Y., Jackson, R. A., and Thiery, J. P. (2016). EMT: 2016. *Cell* 166, 21–45. doi: 10.1016/j.cell.2016.06.028
- Ning, L., Li, Z., Wei, D., Chen, H., and Yang, C. (2017). LncRNA, NEAT1 is a prognosis biomarker and regulates cancer progression via epithelial-mesenchymal transition in clear cell renal cell carcinoma. *Cancer Biomark.* 19, 75–83. doi: 10.3233/CBM-160376
- Pan, L. J., Zhong, T. F., Tang, R. X., Li, P., Dang, Y. W., Huang, S. N., et al. (2015). Upregulation and clinicopathological significance of long non-coding NEAT1 RNA in NSCLC tissues. *Asian Pac. J. Cancer Prev.* 16, 2851–2855. doi: 10.7314/APJCP.2015.16.7.2851
- Parasramka, M. A., Maji, S., Matsuda, A., Yan, I. K., and Patel, T. (2016). Long non-coding RNAs as novel targets for therapy in hepatocellular carcinoma. *Pharmacol. Ther.* 161, 67–78. doi: 10.1016/j.pharmthera.2016.03.004
- Peng, W., Wang, Z., and Fan, H. (2017). LncRNA NEAT1 impacts cell proliferation and apoptosis of colorectal cancer via regulation of Akt signaling. *Pathol. Oncol. Res.* 23, 651–656. doi: 10.1007/s12253-016-0172-4
- Prensner, J. R., and Chinnaiyan, A. M. (2011). The emergence of lncRNAs in cancer biology. *Cancer Discov.* 1, 391–407. doi: 10.1158/2159-8290.CD-11-0209
- Qian, K., Liu, G., Tang, Z., Hu, Y., Fang, Y., Chen, Z., et al. (2017). The long non-coding RNA NEAT1 interacted with miR-101 modulates breast cancer growth by targeting EZH2. *Arch. Biochem. Biophys.* 615, 1–9. doi: 10.1016/j.abb.2016.12.011
- Rheinbay, E., Parasuraman, P., Grimsby, J., Tiao, G., Engreitz, J. M., Kim, J., et al. (2017). Recurrent and functional regulatory mutations in breast cancer. *Nature* 547, 55–60. doi: 10.1038/nature22992
- Schmitt, A. M., and Chang, H. Y. (2016). Long noncoding RNAs in cancer pathways. *Cancer Cell* 29, 452–463. doi: 10.1016/j.ccell.2016.03.010
- Shi, T., Gao, G., and Cao, Y. (2016). Long noncoding RNAs as novel biomarkers have a promising future in cancer diagnostics. *Dis. Markers* 2016:9085195. doi: 10.1155/2016/9085195
- Sidi, A. A., Ohana, P., Benjamin, S., Shalev, M., Ransom, J. H., Lamm, D., et al. (2008). Phase I/II marker lesion study of intravesical BC-819 DNA plasmid in H19 over expressing superficial bladder cancer refractory to bacillus Calmette-Guerin. *J. Urol.* 180, 2379–2383. doi: 10.1016/j.juro.2008.08.006
- Standaert, L., Adriaens, C., Radaelli, E., Van Keymeulen, A., Blanpain, C., Hirose, T., et al. (2014). The long noncoding RNA Neat1 is required for mammary gland development and lactation. *RNA* 20, 1844–1849. doi: 10.1261/rna.047332.114
- Su, X., Malouf, G. G., Chen, Y., Zhang, J., Yao, H., Valero, V., et al. (2014). Comprehensive analysis of long non-coding RNAs in human breast cancer clinical subtypes. *Oncotarget* 5, 9864–9876. doi: 10.18632/oncotarget.2454
- Sun, C., Li, S., Zhang, F., Xi, Y., Wang, L., Bi, Y., et al. (2016). Long non-coding RNA NEAT1 promotes non-small cell lung cancer progression through regulation of miR-377-3p-E2F3 pathway. *Oncotarget* 7, 51784–51814. doi: 10.18632/oncotarget.10108
- Sun, S. J., Lin, Q., Ma, J. X., Shi, W. W., Yang, B., and Li, F. (2017). Long non-coding RNA NEAT1 acts as oncogene in NSCLC by regulating the Wnt signaling pathway. *Eur. Rev. Med. Pharmacol. Sci.* 21, 504–510.
- Sun, W., Lan, X., Zhang, H., Wang, Z., Dong, W., He, L., et al. (2018). NEAT1_2 functions as a competing endogenous RNA to regulate ATAD2 expression by sponging microRNA-106b-5p in papillary thyroid cancer. *Cell Death Dis.* 9:380. doi: 10.1038/s41419-018-0418-z
- Thum, T., and Condorelli, G. (2015). Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ. Res.* 116, 751–762. doi: 10.1161/CIRCRESAHA.116.303549
- Tollervey, J. R., Curk, T., Rogelj, B., Briese, M., Cereda, M., Kayikci, M., et al. (2011). Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. *Nat. Neurosci.* 14, 452–458. doi: 10.1038/nn.2778
- Tripathi, V., Ellis, J. D., Shen, Z., Song, D. Y., Pan, Q., Watt, A. T., et al. (2010). The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol. Cell* 39, 925–938. doi: 10.1016/j.molcel.2010.08.011
- van Heesch, S., van Iterson, M., Jacobi, J., Boymans, S., Essers, P. B., de Bruijn, E., et al. (2014). Extensive localization of long noncoding RNAs to the cytosol and mono- and polyribosomal complexes. *Genome Biol.* 15:R6. doi: 10.1186/gb-2014-15-1-r6
- Wang, H., Yu, Y., Fan, S., and Luo, L. (2017). Knockdown of long non-coding RNA NEAT1 inhibits proliferation and invasion and induces apoptosis of osteosarcoma by inhibiting miR-194 expression. *Yonsei Med. J.* 58, 1092–1100. doi: 10.3349/ymj.2017.58.6.1092
- Wang, J., Zhao, X., Guo, Z., Ma, X., Song, Y., and Guo, Y. (2017). Regulation of NEAT1/miR-214-3p on the growth, migration and invasion of endometrial carcinoma cells. *Arch. Gynecol. Obstet.* 295, 1469–1475. doi: 10.1007/s00404-017-4365-1
- Wang, K. C., and Chang, H. Y. (2011). Molecular mechanisms of long noncoding RNAs. *Mol. Cell* 43, 904–914. doi: 10.1016/j.molcel.2011.08.018
- Wang, P., Wu, T., Zhou, H., Jin, Q., He, G., Yu, H., et al. (2016). Long noncoding RNA NEAT1 promotes laryngeal squamous cell cancer through regulating miR-107/CDK6 pathway. *J. Exp. Clin. Cancer Res.* 35:22. doi: 10.1186/s13046-016-0297-z
- Wang, Y., Wang, C., Chen, C., Wu, F., Shen, P., Zhang, P., et al. (2017). Long non-coding RNA NEAT1 regulates epithelial membrane protein 2 expression to repress nasopharyngeal carcinoma migration and irradiation-resistance through miR-101-3p as a competing endogenous RNA mechanism. *Oncotarget* 8, 70156–70171. doi: 10.18632/oncotarget.19596
- Wang, Y., Xue, D., Li, Y., Pan, X., Zhang, X., Kuang, B., et al. (2016). The long noncoding RNA MALAT-1 is a novel biomarker in various cancers: a meta-analysis based on the GEO database and literature. *J. Cancer* 7, 991–1001. doi: 10.7150/jca.14663
- Wang, Z., Zou, Q., Song, M., and Chen, J. (2017). NEAT1 promotes cell proliferation and invasion in hepatocellular carcinoma by negative regulating miR-613 expression. *Biomed. Pharmacother.* 94, 612–618. doi: 10.1016/j.biopha.2017.07.111
- Worku, T., Bhattarai, D., Ayers, D., Wang, K., Wang, C., Rehman, Z. U., et al. (2017). Long non-coding RNAs: the new horizon of gene regulation

- in ovarian cancer. *Cell. Physiol. Biochem.* 44, 948–966. doi: 10.1159/000485395
- Wu, Y., and Wang, H. (2018). LncRNA NEAT1 promotes dexamethasone resistance in multiple myeloma by targeting miR-193a/MCL1 pathway. *J. Biochem. Mol. Toxicol.* 32:e22008. doi: 10.1002/jbt.22008
- Wu, Y., Yang, L., Zhao, J., Li, C., Nie, J., Liu, F., et al. (2015). Nuclear-enriched abundant transcript 1 as a diagnostic and prognostic biomarker in colorectal cancer. *Mol. Cancer* 14, 191. doi: 10.1186/s12943-015-0455-5
- Xie, S. S., Jin, J., Xu, X., Zhuo, W., and Zhou, T. H. (2016). Emerging roles of non-coding RNAs in gastric cancer, Pathogenesis and clinical implications. *World J. Gastroenterol.* 22, 1213–1223. doi: 10.3748/wjg.v22.i3.1213
- Yan, X., Hu, Z., Feng, Y., Hu, X., Yuan, J., Zhao, S. D., et al. (2015). Comprehensive genomic characterization of long non-coding RNAs across human cancers. *Cancer Cell* 28, 529–540. doi: 10.1016/j.ccell.2015.09.006
- Yang, X., Xiao, Z., Du, X., Huang, L., and Du, G. (2017). Silencing of the long non-coding RNA NEAT1 suppresses glioma stem-like properties through modulation of the miR-107/CDK6 pathway. *Oncol. Rep.* 37, 555–562.
- Yoon, J. H., Abdelmohsen, K., Srikantan, S., Yang, X., Martindale, J. L., De, S., et al. (2012). LincRNA-p21 suppresses target mRNA translation. *Mol. Cell* 47, 648–655. doi: 10.1016/j.molcel.2012.06.027
- Yoon, J. H., De, S., Srikantan, S., Abdelmohsen, K., Grammatikakis, I., Kim, J., et al. (2014). PAR-CLIP analysis uncovers AUF1 impact on target RNA fate and genome integrity. *Nat. Commun.* 5:5248. doi: 10.1038/ncomms6248
- Yoshimura, H., Matsuda, Y., Yamamoto, M., Kamiya, S., and Ishiwata, T. (2018). Expression and role of long non-coding RNA H19 in carcinogenesis. *Front. Biosci. (Landmark Ed.)* 23, 614–625. doi: 10.2741/4608
- Yu, T., Tang, B., and Sun, X. (2017). Development of inhibitors targeting hypoxia-inducible factor 1 and 2 for cancer therapy. *Yonsei Med. J.* 58, 489–496. doi: 10.3349/ymj.2017.58.3.489
- Zeng, C., Xu, Y., Xu, L., Yu, X., Cheng, J., Yang, L., et al. (2014). Inhibition of long non-coding RNA NEAT1 impairs myeloid differentiation in acute promyelocytic leukemia cells. *BMC Cancer* 14:693. doi: 10.1186/1471-2407-14-693
- Zhang, C., Li, J. Y., Tian, F. Z., Zhao, G., Hu, H., Ma, Y. F., et al. (2018). Long noncoding RNA NEAT1 promotes growth and metastasis of cholangiocarcinoma cells. *Oncol. Res.* 26, 879–888. doi: 10.3727/096504017X15024935181289
- Zhang, H., Cai, Y., Zheng, L., Zhang, Z., Lin, X., and Jiang, N. (2018). Long noncoding RNA NEAT1 regulate papillary thyroid cancer progression by modulating miR-129-5p/CLK7 expression. *J. Cell. Physiol.* 233, 6638–6648. doi: 10.1002/jcp.26425
- Zhang, M., Wu, W. B., Wang, Z. W., and Wang, X. H. (2017). LncRNA NEAT1 is closely related with progression of breast cancer via promoting proliferation and EMT. *Eur. Rev. Med. Pharmacol. Sci.* 21, 1020–1026.
- Zhao, D., Zhang, Y., Wang, N., and Yu, N. (2017). NEAT1 negatively regulates miR-218 expression and promotes breast cancer progression. *Cancer Biomark.* 20, 247–254. doi: 10.3233/CBM-170027
- Zhao, J., Ohsumi, T. K., Kung, J. T., Ogawa, Y., Grau, D. J., Sarma, K., et al. (2010). Genome-wide identification of polycomb-associated RNAs by RIP-seq. *Mol. Cell* 40, 939–953. doi: 10.1016/j.molcel.2010.12.011
- Zhen, L., Yun-Hui, L., Hong-Yu, D., Jun, M., and Yi-Long, Y. (2016). Long noncoding RNA NEAT1 promotes glioma pathogenesis by regulating miR-449b-5p/c-Met axis. *Tumour Biol.* 37, 673–683. doi: 10.1007/s13277-015-3843-y
- Zheng, X., Zhang, Y., Liu, Y., Fang, L., Li, L., Sun, J., et al. (2018). HIF-2 α activated lncRNA NEAT1 promotes hepatocellular carcinoma cell invasion and metastasis by affecting the epithelial-mesenchymal transition. *J. Cell. Biochem.* 119, 3247–3256. doi: 10.1002/jcb.26481

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Interpreting Non-coding Genetic Variation in Multiple Sclerosis Genome-Wide Associated Regions

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Edited by:

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Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 31 July 2018

Accepted: 30 November 2018

Published: 17 December 2018

Citation:

Paraboschi EM, Cardamone G,
Soldà G, Duga S and Asselta R
(2018) Interpreting Non-coding
Genetic Variation in Multiple Sclerosis
Genome-Wide Associated Regions.
Front. Genet. 9:647.
doi: 10.3389/fgene.2018.00647

Multiple sclerosis (MS) is the most common neurological disorder in young adults. Despite extensive studies, only a fraction of MS heritability has been explained, with association studies focusing primarily on protein-coding genes, essentially for the difficulty of interpreting non-coding features. However, non-coding RNAs (ncRNAs) and functional elements, such as super-enhancers (SE), are crucial regulators of many pathways and cellular mechanisms, and they have been implicated in a growing number of diseases. In this work, we searched for possible enrichments in non-coding elements at MS genome-wide associated loci, with the aim to highlight their possible involvement in the susceptibility to the disease. We first reconstructed the linkage disequilibrium (LD) structure of the Italian population using data of 727,478 single-nucleotide polymorphisms (SNPs) from 1,668 healthy individuals. The genomic coordinates of the obtained LD blocks were intersected with those of the top hits identified in previously published MS genome-wide association studies (GWAS). By a bootstrapping approach, we hence demonstrated a striking enrichment of non-coding elements, especially of circular RNAs (circRNAs) mapping in the 73 LD blocks harboring MS-associated SNPs. In particular, we found a total of 482 circRNAs (annotated in publicly available databases) vs. a mean of 194 ± 65 in the random sets of LD blocks, using 1,000 iterations. As a proof of concept of a possible functional relevance of this observation, we experimentally verified that the expression levels of a circRNA derived from an MS-associated locus, i.e., hsa_circ_0043813 from the *STAT3* gene, can be modulated by the three genotypes at the disease-associated SNP. Finally, by evaluating RNA-seq data of two cell lines, SH-SY5Y and Jurkat cells, representing tissues relevant for MS, we identified 18 (two novel) circRNAs derived from MS-associated genes. In conclusion, this work showed for the first time that MS-GWAS top hits map in LD blocks enriched in circRNAs, suggesting circRNAs as possible novel contributors to the disease pathogenesis.

Keywords: multiple sclerosis, single-nucleotide polymorphism, association, long non-coding RNA, circular RNA, micro RNA, super-enhancer

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system, characterized by demyelination and progressive neurological impairment (Brownlee et al., 2017). Epidemiological studies showed both an important role of the environment in determining MS risk (Ramagopalan et al., 2010), and a strong contribution of genetic components (Belbasis et al., 2015). To date, besides the human leukocyte antigen (HLA) gene cluster (Patsopoulos et al., 2013), genome-wide association studies (GWAS) identified several common variants contributing to disease pathogenesis with mild effects on risk, many of which located within or close to genes displaying primarily immunologic functions (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2007, 2011, 2013; Aulchenko et al., 2008; Comabella et al., 2008; Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene), 2009; Baranzini et al., 2009; de Jager et al., 2009; Jakkula et al., 2010; Nischwitz et al., 2010; Sanna et al., 2010; Patsopoulos et al., 2011; Martinelli-Boneschi et al., 2012; Matesanz et al., 2012). Despite these extensive efforts, the identified GWAS variants explain only 28% of the sibling recurrence risk (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2013), thus implicating that the complete spectrum of MS genetic determinants is still far from being complete. These studies focused primarily on protein-coding genes, due to the difficulty of interpreting non-coding features. However, advances in the systematic annotation of non-coding genes and non-coding functional elements are revolutionizing genetic approaches and are paving the way to build a map that can help reveal “hidden” processes underlying disease associations (Ward and Kellis, 2012).

In this frame, non-coding RNAs (ncRNAs) have recently emerged as crucial regulators of many pathways and cellular mechanisms (Vidigal and Ventura, 2015; Barrett and Salzman, 2016; Quinn and Chang, 2016), and they have been implicated in a growing number of diseases (Mendell and Olson, 2012; Vučićević et al., 2014). Many long ncRNAs (lncRNAs), for instance, were shown to contribute to the pathogenesis of neurological and psychiatric conditions in different ways, from regulation of transcription to modulation of RNA processing and translation (Vučićević et al., 2014). In addition, microRNAs (miRNAs) dysregulation was associated with several disorders, such as different kinds of cancers and immune-related diseases (Mendell and Olson, 2012). Another group of ncRNAs with regulatory functions is represented by circular RNAs (circRNAs), a novel class of RNAs generated from the back-splicing of exons or introns (Jeck et al., 2013). By acting as miRNA sponges, or by binding to RNA-associated proteins, circRNAs regulate gene expression at the transcriptional or post-transcriptional level, although their exact mechanism of action still needs to be clarified (Greene et al., 2017). Moreover, they have been associated with human diseases such as ischemic heart disease, Alzheimer's disease, diabetes, cancer, as well as MS (Cardamone et al., 2017; Greene et al., 2017; Iparraguirre et al., 2017).

Among non-coding functional elements, also super-enhancers (SEs) have been described as key gene expression regulators

(Pott and Lieb, 2015). SEs are genomic regions characterized by a strong enrichment in binding sites both for transcriptional coactivators, specifically the Mediator protein, and for factors generally associated with enhancer activity, such as RNA polymerase II and chromatin factors (Pott and Lieb, 2015). Very interestingly, many SE regions are significantly enriched in disease-associated single-nucleotide polymorphisms (SNPs), including those related to autoimmunity, and more specifically to MS (Hnisz et al., 2013; Farh et al., 2015). The enrichment in GWAS variants within enhancers suggests that they influence the disease risk by altering gene regulation. However, only a few disease-associated SNPs directly alter a transcription factor motif; many trait-associated SNPs instead modulate the enhancer activity by changing nearby nucleotides, resulting in slight but critical alterations of gene expression (Farh et al., 2015).

In this work, we aim at identifying ncRNAs and SEs mapping in proximity of MS GWAS-significant signals that could point to so-far unexplored mechanisms involved in the susceptibility to the disease.

MATERIALS AND METHODS

Defining the Linkage Disequilibrium (LD) Structure of the Italian Population

The global LD structure of the Italian population was explored by using genome-wide genotyping data (727,478 quality-checked markers, genotyped with the Affymetrix 6.0 GeneChip platform; Affymetrix, Santa Clara, CA, United States) obtained from 1,668 healthy controls (for genotyping details see Myocardial Infarction Genetics Consortium et al., 2009). Haplotype blocks were estimated with the Plink program (Purcell et al., 2007) following the default procedure described for the Haploview software (Barrett et al., 2005). Pairwise LD was calculated for SNPs within 200 kb for autosomal chromosomes. Chromosome X was excluded from this analysis, leading the total number of SNPs used for LD studies to 699,676.

To verify whether the Italian LD structure was comparable to the European one, we analyzed the 1000 Genomes data on European subjects (phase 1 project) (1000 Genomes Project Consortium et al., 2015). This test was performed on chromosome 22 data, by selecting only those SNPs whose genetic information was available both in the Italian and 1000 Genome populations. These were used to calculate the European LD structure.

Retrieving the Reference Files for ncRNAs and Regulatory Elements

Reference files for the analysis were retrieved for lncRNAs, miRNAs, circRNAs, and SEs. In particular: (1) The reference gene transfer format (GTF) file for lncRNAs was obtained from GENCODE (Harrow et al., 2012), selecting the comprehensive gene annotation of lncRNA genes on the reference chromosomes, version 25¹. (2) The miRNA reference file was downloaded from miRBase² (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008;

¹https://www.encodegenes.org/human/release_25lift37.html

²<http://www.mirbase.org/>

Kozomara and Griffiths-Jones, 2011, 2014) version 20. (3) The circRNA reference file was obtained from the circBase database³ (Glažar et al., 2014), by downloading data from all the available studies on humans (Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013; Zhang et al., 2013; Rybak-Wolf et al., 2015). (4) The SE reference file was downloaded from the SEA: Super-Enhancer Archive⁴ (Wei et al., 2016) based on studies on humans.

In all cases, genome version hg19 was considered; databases were accessed on April 2016.

Defining Overlapping Regions Between LD Blocks, MS Genome-Wide Significant SNPs, and ncRNAs/SEs

Multiple sclerosis-associated SNPs, excluding those mapping in the highly complex HLA region, were retrieved from the literature (**Supplementary Table 1**) (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2013). Their genomic coordinates were crossed with those of the LD blocks, to identify the blocks in which each single SNP resides.

The next step was searching for partial/total overlapping between LD blocks containing the genome-wide associated SNPs and the different classes of ncRNAs (lncRNAs, miRNAs and circRNAs) or SE elements. The overlaps were identified on the basis of the genomic coordinates of each LD block (using as borders the physical positions of the most 5' and 3' SNPs belonging to the block) and of each ncRNAs/SE elements (for these genomic features, coordinates were extracted from the reference files described in the previous section). The final list includes both the elements completely contained within the LD blocks and those showing only a partial overlap. Filtering for redundancy was used to eliminate multiple annotations referring to the same element. All procedures were performed using awk command line (described in the section **Supplementary Material**).

Enrichment Analysis

To determine if the MS-related LD blocks are significantly enriched in ncRNA genes and SE elements, a bootstrapping strategy was adopted.

First, a set of random SNPs was extracted from the “Genome-Wide Human SNP array 6.0” manifest (copy number variants were excluded), which is one of the most used genotyping arrays in MS GWAS. The number of SNPs to be extracted was chosen in order to obtain either a number of LD blocks similar to the one of the MS-related analysis (Random set I) or an overall genomic region of equal length (i.e., 3.8 Mb; Random set II). Again, the HLA region and X chromosome were avoided. Moreover, since about half of the MS-associated SNPs are located in introns (**Supplementary Table 1**) (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2013), the random SNP sets were constructed to mirror the proportion of intronic SNPs of the MS list. More in particular, to perform this step,

two complete lists of SNPs from the “Genome-Wide Human SNP array 6.0” manifest were generated: one containing only SNPs annotated as intronic in the manifest file, the second containing only extragenic SNPs. SNPs were chosen from both lists with a randomized procedure (using the gshuf Unix command), respecting the constraints above mentioned.

Then, the LD blocks in which the random SNPs reside were identified, and a search for overlapping regions between LD blocks and ncRNAs/SE regions was performed, as described above.

Finally, the results were filtered to avoid redundancy, and the total number of lncRNAs, miRNAs, circRNAs, and SEs was annotated. The entire procedure was repeated 1,000 times for random set I and II, and the outputs of each set averaged, in order to compare the resulting means with the result obtained with the MS SNP set. The comparison was based on the % of times in which the same (or a larger) number of lncRNAs, circRNAs, miRNAs, or SEs was obtained in the 1,000 iterations respect to the MS dataset. Enrichment *p*-values were calculated according to Davison and Hinkley method (Davison and Hinkley, 1997).

All analyses were performed using in-house developed Perl scripts (listed in the section **Supplementary Material**).

The entire procedure is schematized in **Supplementary Figure 1**.

Replication on an Unrelated Disease

To test the specificity of the analysis on MS, we repeated the entire workflow considering a disease with a completely different etiology, i.e., coronary artery disease (CAD).

The list of CAD-associated SNPs was derived from the literature (**Supplementary Table 2**) (Nikpay et al., 2015).

Genotype-Dependent Analysis of circRNA Expression

DNA samples were extracted from whole blood of 35 healthy donors using an automated DNA extractor (Maxwell 16 System; Promega, Madison, WI, United States). All subjects gave written informed consent in accordance with the Declaration of Helsinki. To genotype the MS-associated SNP rs2293152, PCR amplifications (GoTaq; Promega) and Sanger sequencing, using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 and an ABI-3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, United States), were performed following standard protocols.

Peripheral blood mononuclear cells (PBMCs) of the same healthy donors were isolated by means of centrifugation on a Lympholyte Cell separation medium (Cederlane Laboratories Limited, Hornby, ON, Canada) gradient. RNA extraction was performed using the EuroGold Trifast kit (Euroclone, Wetherby, United Kingdom). RNA was reverse-transcribed using the Superscript-III Reverse Transcriptase (Thermo Fisher Scientific) and random hexamers (Promega), according to the manufacturers' instructions.

Semi-quantitative real-time RT-PCRs to detect the expression levels of circRNA hsa_circ_0043813 were performed by using divergent primers (5'-ACATTCTGGGCACAAACACA-3' and

³<http://www.circbase.org/>

⁴<https://sea.nebulagene.com/SEA/index.html>

5'-CCTCTGAGAGCTGCAACG-3'), the FastStart SYBR Green Master mix (Roche, Basel, Switzerland), and a LightCycler 480 (Roche). *HMBS* (hydroxymethylbilane synthase) was used as housekeeping gene; reactions were performed in triplicate, and expression data were analyzed using the GeNorm software (Vandesompele et al., 2002).

CircRNA Analysis by RNA Sequencing

RNA was extracted using the Maxwell 16 LEV simplyRNA Cells Kit (Promega) from SH-SY5Y (human neuroblastoma) and Jurkat E6-1 (human T lymphocyte) cell lines. RNA quality was assessed by the LabChip GX Touch instrument (PerkinElmer, Waltham, MA, United States). RNA sequencing was performed using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, United States), following the manufacturer's instructions and a paired-end sequencing strategy. SH-SY5Y and Jurkat samples underwent a high-coverage paired-end 75- and 150-bp strand-specific sequencing, respectively, using a NextSeq 500 platform (Illumina).

The circRNA analysis was then performed using the DCC software (Cheng et al., 2016). In detail, raw reads were first aligned to the hg19 version of the genome using STAR (Dobin et al., 2013), switching on the detection of chimeric alignments to detect reads containing backspliced products, as suggested by the DCC manual. In a first step, reads were mapped using both mates; subsequently, an additional separate mate mapping was performed. After mapping, DCC was used to analyze the chimeric reads to detect circRNAs. Only those circRNAs supported by at least five reads were considered for further analyses. CircRNAs mapping on mitochondrial DNA or in repetitive regions of the genome were filtered out.

Data Repository

Raw sequence files of SH-SY5Y have been deposited in NCBI Sequence Read Archive (SRA) under the following Bioproject ID: PRJNA483101, and with the accession number SRP155458; raw sequence files of Jurkat cells have been deposited in the GEO database (Accession No.: GSE110525).

RESULTS

The Global LD Structure of the Italian Population Is Not Different From the European One

The LD structure of the Italian population was built by using data on 699,676 genotyped SNPs on 1,668 healthy subjects. A total of 96,666 LD blocks (with an average length of 17.48 kb; range 0.01–200 kb) were identified in autosomes, ranging from 1,421 blocks of chromosome 22 to 8,148 of chromosome 2 (on average: 4,394 blocks per chromosome).

The comparison of the LD structure of chromosome 22 of the Italian and European populations confirmed a substantial similarity in the blocks distribution (Pearson's χ^2 $p = 0.79$), thus suggesting that the Italian population is a good representation of the European LD structure, and confirming the data previously obtained by Mueller et al. (2005). The substantial overlap between the structures of the Italian and of the European populations was also confirmed by a principal component analysis performed using genotype data of our cohort and those from the 1000 Genome project (503 available individuals; **Supplementary Figure 2**).

MS-Associated Regions Are Significantly Enriched in circRNAs and SEs

With the aim of identifying ncRNAs and SE regulatory elements mapping in LD blocks harboring MS GWAS-significant signals, we selected through literature data mining all those SNPs that reached a genome-wide significant threshold in MS GWAS studies and meta-analyses (**Supplementary Table 1**). We excluded from this list all SNPs mapping in the HLA region as well as those located on the X chromosome. The list of 97 SNPs was intersected with that of LD blocks inferred for the Italian population. Our automated pipeline allowed the identification of 73 LD blocks, each harboring a single genome-wide significant SNP. For 24 out of the 97 SNPs used for the analysis, it was not possible to establish a precise LD block.

TABLE 1 | Results of the analysis performed on the MS-associated loci (upper part) and on 1,000 random sets (middle and lower part).

List	<i>n</i> Blocks	Blocks\$(kb)	DNA content (Mb)	<i>n</i> LncRNAs	<i>n</i> CircRNAs	<i>n</i> MiRNAs	<i>n</i> SEs
MS set	73	52.2	3.8	30	482	7	23
SD	–	54.8	–	–	–	–	–
Random sets (I)*	73.8	40.6	2.9	22.6	193.8	2.1	2.4
SD	4.0	5.7	0.4	5.6	65.0	2.1	1.8
%**	–	–	–	11.0	0	3.6	0
<i>p</i> -value	–	–	–	0.11	9.9×10^{-4}	0.36	9.9×10^{-4}
Random sets (II)*	96.0	40.2	3.8	29.3	249.3	2.7	3.1
SD	4.6	4.8	0.5	6.3	76.4	2.5	2.0
%**	–	–	–	46.4	0.6	6.3	0
<i>p</i> -value	–	–	–	0.46	0.006	0.06	9.9×10^{-4}

\$Average length of the LD blocks. *For the random sets analysis, the average values calculated on 1,000 iterations are indicated. **% of times in which the same or a larger number of lncRNAs, circRNAs, miRNAs, or SEs was obtained in the 1,000 iterations as compared to the MS dataset. *p*-Values were calculated as described (Davison and Hinkley, 1997); in detail: $p\text{-value} = [1 + \sum(s > s_0)] / (N + 1)$, where s is the observed value in the random set, s_0 is the value of the observed MS-specific result, and N is the number of bootstraps. *n*, number; SD, standard deviation.

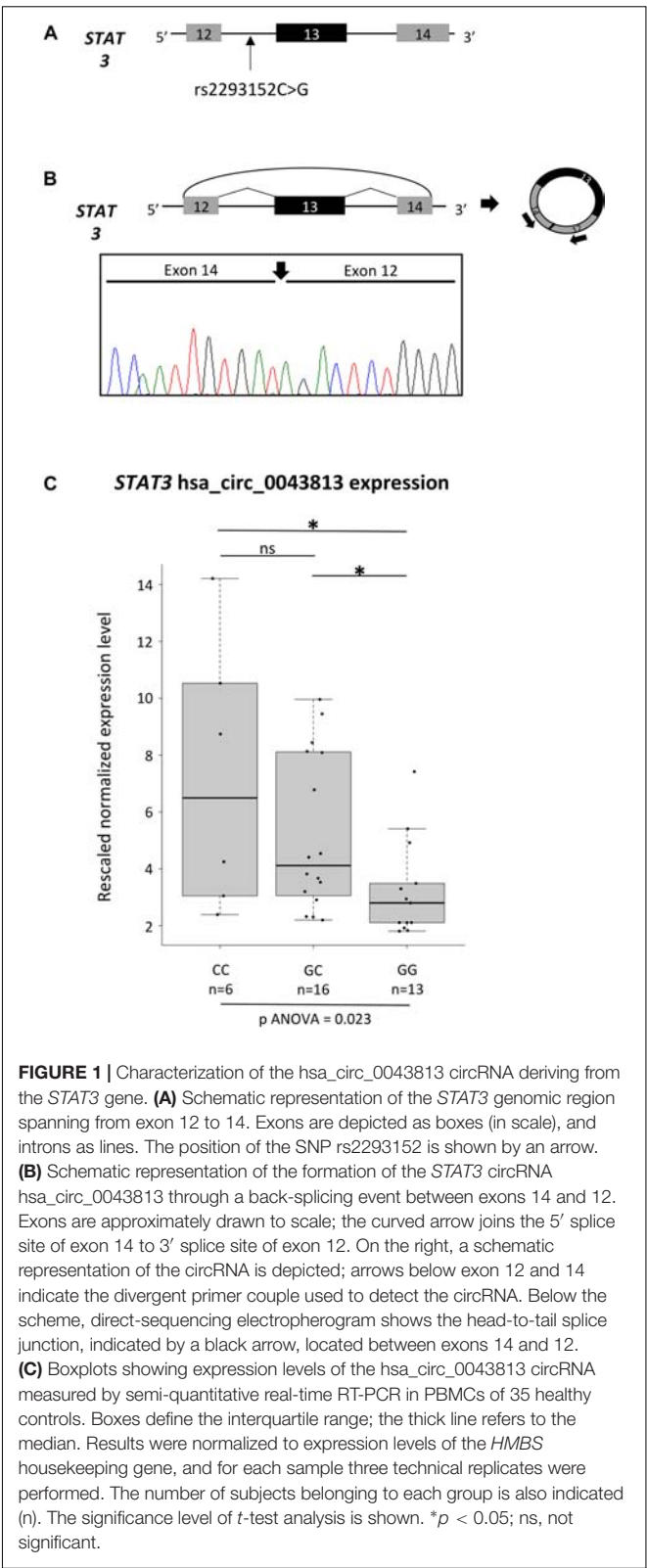
The genomic coordinates of the 73 LD blocks were hence intersected with those of ncRNAs and SEs annotated in public genomic databases. This analysis evidenced the presence of 30 lncRNAs, 482 circRNAs, 7 miRNAs, and 23 SEs partially or totally overlapping the 73 identified blocks (Table 1). To test for possible significant enrichments in non-coding elements, the same workflow was applied to randomly selected SNPs (Random set I). To this aim, subsets of 94 SNPs were randomly selected from the genome, and the process was repeated 1,000 times. The pipeline identified, on average, 73.8 LD blocks (median value: 74) in which 22.6 lncRNAs (median: 22), 193.8 circRNAs (median: 185), 2.1 miRNAs (median: 2), and only 2.4 SEs (median: 2) were located (Table 1). Comparing the random set I results with the MS dataset, we obtained the same or a larger number of lncRNAs, circRNAs, and miRNAs in the 11, 0, and 3.6%, of the iterations, respectively, thus indicating a very strong enrichment in circRNAs in MS dataset. None of the randomly selected SNP subsets evidenced the same or a larger number of SE hits when compared to the MS list, thus confirming the enrichment in these regulatory elements previously reported by Farh et al. (2015) (observations that, however, were focused specifically on immune-related genes). Since the average length of the LD blocks in the Random set I was 22% lower than the one of MS LD blocks, we repeated the enrichment analysis on a genomic region spanning the same length as the MS dataset (3.8 Mb; Random set II). Also in this case, 1,000 iterations confirmed a strong enrichment in circRNAs and SEs in MS LD blocks (Table 1).

To test the specificity of the results, we applied the same pipeline on another dataset, composed of SNPs associated with CAD, a disease with a different etiology from MS. In this case, 55 SNPs were retrieved from the literature and used for the analysis, and 36 LD blocks were identified (Table 2). The workflow evidenced the presence of 19 lncRNAs, 122 circRNAs, 1 miRNA, and 2 SEs mapping within the corresponding blocks. When compared to the CAD dataset, the random bootstrapping strategy applied 1,000 times evidenced the same (or a larger) number of lncRNAs, circRNAs, miRNAs, and SEs in the 4.6, 12.4, 45.9, and 26.7% of the iterations, respectively (Table 2), thus suggesting only a slight enrichment in lncRNAs in the CAD dataset. No significant enrichment was evidenced in circRNAs and SEs.

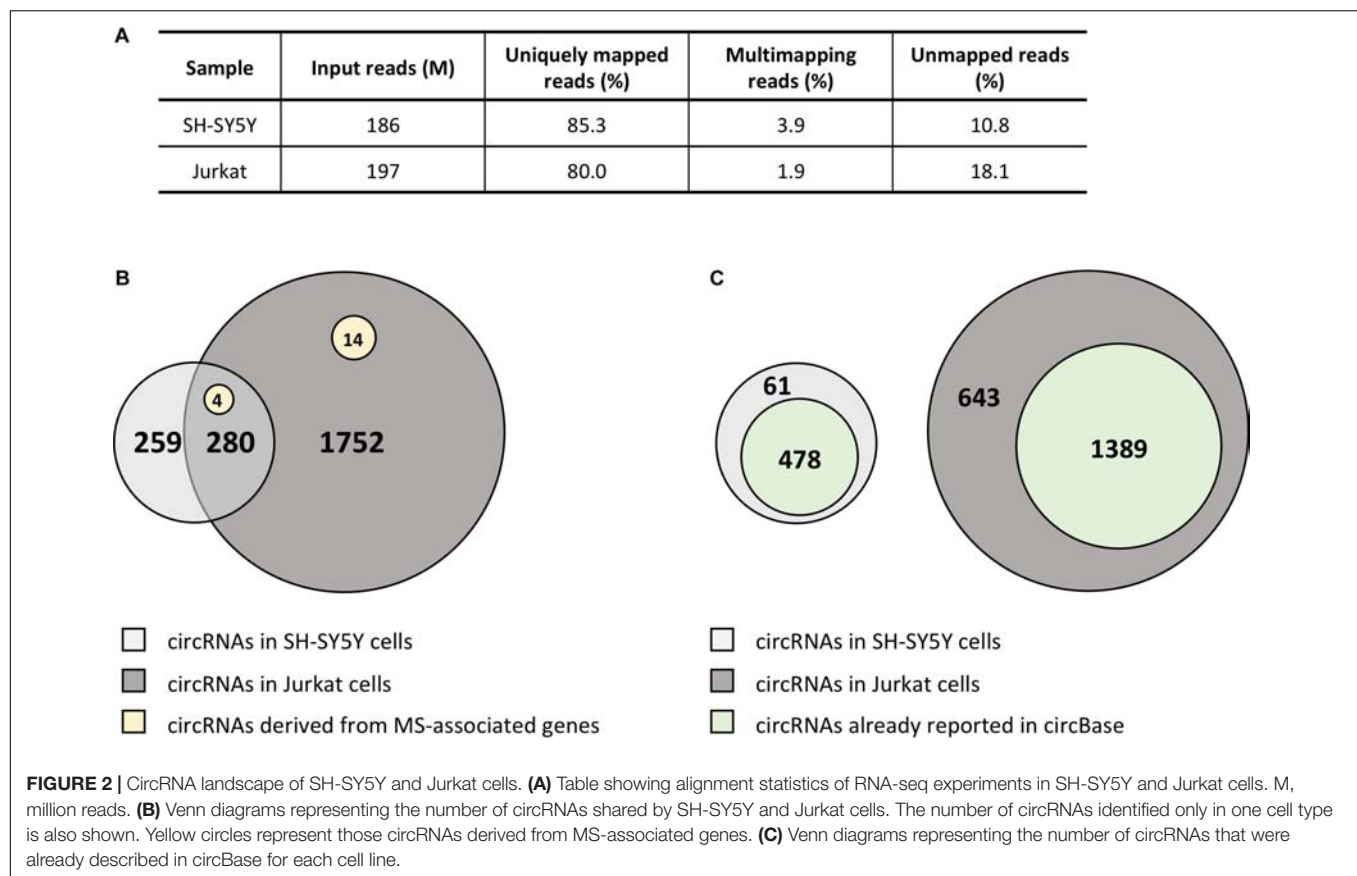
TABLE 2 | Results of the analysis performed on the CAD-associated loci (upper part) and on 1,000 random sets (lower part).

List	n Blocks	n LncRNAs	n CircRNAs	n MiRNAs	n SEs
CAD set	36	19	122	1	2
Random sets*	35.3	11.3	73.4	1.0	1.0
SD	2.7	4.1	42.5	1.4	1.1
%**	–	4.6	12.4	45.9	26.7
p-value	–	0.05	0.12	0.46	0.27

*For the random sets analysis, the average values calculated on 1,000 iterations are indicated. n, number; SD, standard deviation. **% of times in which the same or a larger number of lncRNAs, circRNAs, miRNAs, or SEs was obtained in the 1,000 iterations as compared to the CAD dataset. p-Values were calculated as described in the footnote of Table 1.



The list of circRNAs/SE elements mapping within MS- or CAD-specific blocks is given in the Supplementary Table 3.



SNP rs2293152 Genotype Influences *STAT3* hsa_circ_0043813 Expression Levels

The newly observed enrichment in circRNAs in MS led us to test, as a proof of concept, whether the different genotypes of an MS-associated SNP could influence the expression levels of a circRNA mapping in the corresponding LD block. To this aim, we decided to better characterize a circRNA deriving from *STAT3* (Signal Transducer and Activator of Transcription 3), a gene necessary for pro-inflammatory cytokines signaling (Adamson et al., 2009) and that it is required for differentiation and expansion of Th17 cells, key players of MS disease activity (Brucklacher-Waldert et al., 2009). In particular, four different SNPs mapping in *STAT3* have been described as associated with MS in GWA studies: rs744166 (Jakkula et al., 2010), rs2293152 (Patsopoulos et al., 2011), rs9891119 (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2011), and rs4796791 (International Multiple Sclerosis Genetics Consortium [IMSGC] et al., 2013). Among them, rs2293152 is in close proximity to a protein-coding exon, being located 50 nt upstream of *STAT3* exon 14 (Figure 1A), and could in theory affect the expression levels of the circRNAs hsa_circ_0043813 (CircBase, chr17:40481427-40481794, hg19), which is composed of exons 12, 13, and 14 (Refseq NM_003150).

To confirm the existence/expression of hsa_circ_0043813, we first performed a RT-PCR assay with a divergent primer couple

tagging exons 12 and 13 on RNA extracted from PBMCs of two healthy controls. Direct sequencing of the circRNA product confirmed the presence of the backspliced exons 12 and 14, joined by a head-to-tail splice junction (Figure 1B). The analysis of the expression levels of the hsa_circ_0043813 circRNA was hence performed on a total of 35 healthy subjects: 6 homozygous for the CC genotype, 16 heterozygous, and 13 homozygous GG (Figure 1C). Our data showed significant different expression levels upon genotype stratification, with the CC subjects showing the highest levels of expression (one-way ANOVA $p = 0.023$).

CircRNA Landscape in SH-SY5Y and Jurkat T Cell Lines

Due to the striking enrichment in circRNAs mapping in the regions associated with MS, we looked at the circRNA landscape of two cell lines, SH-SY5Y and Jurkat cells, representing tissues relevant for MS, by analyzing high-coverage RNA-seq data already available in our lab. We obtained ~186 and 197 million reads for SH-SY5Y and Jurkat cells, respectively (Figure 2A). The circRNA analysis detected the presence of 539 circRNAs supported by at least five reads in SH-SY5Y cells, and of 2,032 circRNAs in Jurkat cells. About half (52%) of circRNAs identified in SH-SY5Y cells were also present in the Jurkat sample (Figure 2B). Most of the detected circRNAs were already annotated in circBase (89% for SH-SY5Y, 68% for Jurkat cells; Figure 2C). In **Supplementary Table 3** we listed the 61 and 643

TABLE 3 | CircRNAs identified in Jurkat and SH-SY5Y cell lines within MS-associated genes.

Chr	Start*	End*	CircRNA ID**	Gene	Jurkat	SH-SY5Y
3	119219542	119222868	hsa_circ_0001329	<i>TIMMDC1</i>	×	×
3	119219542	119232566	hsa_circ_0006884	<i>TIMMDC1</i>	×	
3	119219542	119236162	hsa_circ_0066874	<i>TIMMDC1</i>	×	
4	103610731	103651893	hsa_circ_0001431	<i>MANBA</i>	×	
4	103635595	103651893	hsa_circ_0142051	<i>MANBA</i>	×	
4	106155054	106158508	hsa_circ_0070562	<i>TET2</i>	×	×
7	50358644	50367353	hsa_circ_0001708	<i>IKZF1</i>	×	
7	50358644	50459561	novel	<i>IKZF1</i>	×	
7	50444231	50459561	novel	<i>IKZF1</i>	×	
16	11063018	11076848	hsa_circ_0004179	<i>CLEC16A</i>	×	
16	11114050	11145498	hsa_circ_0002086	<i>CLEC16A</i>	×	
16	11114050	11154879	hsa_circ_0000672	<i>CLEC16A</i>	×	
16	11114050	11220003	hsa_circ_0007846	<i>CLEC16A</i>	×	×
17	57808782	57816308	hsa_circ_0006508	<i>VMP1</i>	×	×
17	57808782	57851246	hsa_circ_0005077	<i>VMP1</i>	×	
22	22153301	22162135	hsa_circ_0004872	<i>MAPK1</i>	×	
22	22160139	22162135	hsa_circ_0008870	<i>MAPK1</i>	×	

*circRNA position is according to DCC output and referred to version hg19 of the genome. **circRNA identifier is referred to circBase nomenclature.

circRNAs that were newly identified in SH-SY5Y and Jurkat cells, respectively. Interestingly, we identified 18 (two novel) and 4 circRNAs derived from the MS-associated genes in Jurkat and SH-SY5Y cells, respectively (**Figure 2B** and **Table 3**).

Finally, *UBAP2* and *WHSC1*, and *MPP6* and *ZNF124* were the genes giving origin to the highest number of different circRNAs species in Jurkat and SH-SY5Y, respectively (**Supplementary Figure 2**). These genes show completely different genomic structures, going from 29 exons distributed on a region of 127 kb in the case of the *UBAP2* gene, to 4 exons spread over 16 kb for the *ZNF124* gene. Instead, for all these genes we observed highest level of expression in cell lines of lymphoid origin and in SH-SY5Y when compared to other cell lines (source⁵).

DISCUSSION

New classes of ncRNAs have been described over the last years; they all display regulatory functions, being part of a large RNA communication network that ultimately regulates the fundamental cellular functions (Adams et al., 2017). Many of them have in fact emerged as regulators of crucial mechanisms (Vidigal and Ventura, 2015; Barrett and Salzman, 2016; Quinn and Chang, 2016), and evidence suggests their implication in various diseases (Mendell and Olson, 2012; Vučićević et al., 2014). Given this background, in this work we aimed at identifying possible enrichments in non-coding elements at MS genome-wide associated loci, that could point to their involvement in the susceptibility to the disease.

By taking advantage of the top hits identified in MS GWASs and of the LD structure of the Italian population, we demonstrated a striking enrichment of circRNAs in the LD blocks

harboring MS-associated SNPs. This result suggests that this class of ncRNAs could play an important role in the disease predisposition and supports emerging evidence in the literature indicating that a dysregulation of the back-splicing process could be a signature of the disease. More specifically, our group identified in MS patients, for the first time, one dysregulated circRNA (Cardamone et al., 2017) derived from *GSDMB*, a gene associated with susceptibility to asthma and autoimmune diseases. Subsequently, Iparraguirre et al. (2017) performed a microarray analysis identifying 406 differentially expressed circRNAs and validating two of them (both deriving from the *ANXA2* gene). As the biogenesis of circRNAs competes with pre-mRNA splicing (Ashwal-Fluss et al., 2014), alterations in the back-splicing process may also interfere with alternative splicing (AS), a mechanism already demonstrated to be dysregulated in MS (Evsyukova et al., 2010; Paraboschi et al., 2015).

Considering that AS dysregulation has been described as a possible pathogenic mechanism underlying autoimmune diseases (Evsyukova et al., 2010; Paraboschi et al., 2015; Juan-Mateu et al., 2016), and given the tight interconnection between AS and back-splicing, we hypothesized that an enrichment in circRNAs could be a signature also for other autoimmune disorders. Recent findings showed that immune-mediated diseases have a complex network of shared genetic architecture, with ~70% of the associated loci for each disease being shared with other autoimmune disorders (Farh et al., 2015). We hence investigated whether we could identify a ncRNA signature also in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), taking advantage of the GWAS top hits for these diseases (Okada et al., 2014; Bentham et al., 2015). By applying the same pipeline used for MS, we observed in SLE a circRNA enrichment in the LD blocks corresponding to GWAS signals (**Supplementary Tables 3, 4**). This result is in line with a growing body of evidence in the literature of an involvement of circRNAs in the

⁵<https://www.proteinatlas.org/>

disease: circHLA-C was in fact shown to be increased in patients affected by lupus nephritis (LN), a kidney disease caused by SLE. In addition, circHLA-C was correlated with clinical disease activities and was suggested to act as sponge for miR-150 (which, in turn, positively correlates with renal chronicity index in LN patients) (Luan et al., 2018). Regarding RA, we could not find any circRNA enrichment in the LD blocks corresponding to genome-wide associated loci (Supplementary Table 5). This finding, however, may not be so surprising: systematic reviews on familial clustering of autoimmune disorders found evidence of an inverse clustering of RA and MS, suggesting that these two pathologies might be less closely related than other autoimmune diseases (Richard-Miceli and Criswell, 2012).

In our work, by studying *STAT3* hsa_circ_0043813, we also showed that the expression level of specific circRNAs may be influenced by the genotype of disease-associated SNPs (which might be defined as circ-eQTL). This observation could be very useful in understanding the functional impact of disease-associated SNPs, a task that still remains a key challenge of the post-GWAS era. Our hypothesis is that some variants associated with MS may impact on the biogenesis or on the sequence of circRNAs. This is in line with what has been reported for circANRIL, the only example of circRNA for which a link between disease associated SNPs and circRNA biogenesis has been demonstrated (Holdt et al., 2016). CircANRIL derives from the lncRNA ANRIL (Burd et al., 2010), transcribed from the CAD risk locus on chromosome 9p21. Holdt et al. (2016) demonstrated that carriers of the CAD-protective haplotype at this locus have significantly increased expression of circANRIL, and this is inversely correlated with the expression of linear ANRIL (linANRIL). Moreover, highest circANRIL:linANRIL ratios are found in CAD-free patients, thus implying an atheroprotective role of circANRIL. It is therefore likely that SNPs contained in the 9p21 haplotype are responsible for differential circANRIL formation, and that subtle genotype-directed gene expression differences may modulate the risk to develop the disease (Holdt et al., 2016). On the basis of this example, we can speculate that there might be other cases in which a disease-associated SNP exerts its functional effect by modulating the levels of specific circRNAs and, hence, modifying the ratio of the circular:linear isoforms. Of note, the RNA-seq analysis of the circRNA landscape in Jurkat cells highlighted

the existence of 18 circRNAs deriving from seven MS-associated genes (~8% of the total number of genes here considered; Supplementary Table 1). This group of circRNAs, together with their linear counterparts, could be a good starting point for an in-depth analysis of circular:linear isoform ratio in PBMCs of MS patients vs. controls, also in the perspective to find novel, simple, and reliable biomarkers for MS susceptibility and progression.

We are aware that our work has the potential limitation of comparing MS-associated loci, which are by definition non-random, with randomly sampled genomic regions. However, we think we have accounted for the main sources of bias by considering regions of equal length and exon density, and by performing a large number of iterations.

In conclusion, this work showed for the first time that MS-GWAS top hits map in LD blocks enriched in circRNAs, suggesting that this feature could be shared by other autoimmune diseases, and pointing to circRNAs as possible novel contributors to the disease pathogenesis.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Humanitas Research Hospital and conducted according to the Declaration of Helsinki. All subject signed an appropriate informed consent.

AUTHOR CONTRIBUTIONS

EP and RA conceived and designed the experiments. GC and EP performed the experiments. RA and EP analyzed the data. EP drafted the paper. GC, GS, SD, and RA critically revised the manuscript. RA supervised the entire study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00647/full#supplementary-material>

REFERENCES

- 1000 Genomes Project Consortium, Auton, A., Brooks, L. D., Durbin, R. M., Garrison, E. P., Kang, H. M., et al. (2015). A global reference for human genetic variation. *Nature* 526, 68–74. doi: 10.1038/nature15393
- Adams, B. D., Parsons, C., Walker, L., Zhang, W. C., and Slack, F. J. (2017). Targeting noncoding RNAs in disease. *J. Clin. Invest.* 127, 761–771. doi: 10.1172/JCI84424
- Adamson, A. S., Collins, K., Laurence, A., and O'Shea, J. J. (2009). The Current STATUS of lymphocyte signaling: new roles for old players. *Curr. Opin. Immunol.* 21, 161–166. doi: 10.1016/j.coi.2009.03.013
- Ashwal-Fluss, R., Meyer, M., Pamudurti, N. R., Ivanov, A., Bartok, O., Hanan, M., et al. (2014). circRNA biogenesis competes with pre-mRNA splicing. *Mol. Cell.* 56, 55–66. doi: 10.1016/j.molcel.2014.08.019
- Aulchenko, Y. S., Hoppenbrouwers, I. A., Ramagopalan, S. V., Broer, L., Jafari, N., Hillert, J., et al. (2008). Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. *Nat. Genet.* 40, 1402–1403. doi: 10.1038/ng.251
- Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene) (2009). Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12, and 20. *Nat. Genet.* 41, 824–828. doi: 10.1038/ng.396
- Baranzini, S. E., Wang, J., Gibson, R. A., Galwey, N., Naegelin, Y., Barkhof, F., et al. (2009). Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. *Hum. Mol. Genet.* 18, 767–778. doi: 10.1093/hmg/ddn388
- Barrett, J. C., Fry, B., Maller, J., and Daly, M. J. (2005). Haploview: analysis and visualization of LD and haplotype

- maps. *Bioinformatics* 21, 263–265. doi: 10.1093/bioinformatics/bth457
- Barrett, S. P., and Salzmann, J. (2016). Circular RNAs: analysis, expression and potential functions. *Development* 143, 1838–1847. doi: 10.1242/dev.128074
- Belbasis, L., Bellou, V., Evangelou, E., Ioannidis, J. P., and Tzoulaki, I. (2015). Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. *Lancet Neurol.* 14, 263–273. doi: 10.1016/S1474-4422(14)70267-4
- Bentham, J., Morris, D. L., Graham, D. S. C., Pinder, C. L., Tombleson, P., Behrens, T. W., et al. (2015). Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus. *Nat. Genet.* 47, 1457–1464. doi: 10.1038/ng.3434
- Brownlee, W. J., Hardy, T. A., Fazekas, F., and Miller, D. H. (2017). Diagnosis of multiple sclerosis: progress and challenges. *Lancet* 389, 1336–1346. doi: 10.1016/S0140-6736(16)30959-X
- Brucklacher-Waldert, V., Stuermer, K., Kolster, M., Wolthausen, J., and Tolosa, E. (2009). Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* 132, 3329–3341. doi: 10.1093/brain/awp289
- Burd, C. E., Jeck, W. R., Liu, Y., Sanoff, H. K., Wang, Z., and Sharpless, N. E. (2010). Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. *PLoS Genet.* 6:e1001233. doi: 10.1371/journal.pgen.1001233
- Cardamone, G., Paraboschi, E. M., Rimoldi, V., Duga, S., Soldà, G., and Asselta, R. (2017). The characterization of GSDMB splicing and backsplicing profiles identifies novel isoforms and a circular RNA that are dysregulated in multiple sclerosis. *Int J Mol Sci.* 18:E576. doi: 10.3390/ijms18030576
- Cheng, J., Metge, F., and Dieterich, C. (2016). Specific identification and quantification of circular RNAs from sequencing data. *Bioinformatics* 32, 1094–1096. doi: 10.1093/bioinformatics/btv656
- Comabella, M., Craig, D. W., Carminha-Tato, M., Morcillo, C., Lopez, C., Navarro, A., et al. (2008). Identification of a novel risk locus for multiple sclerosis at 13q31.3 by a pooled genome-wide scan of 500,000 single nucleotide polymorphisms. *PLoS One* 3:e3490. doi: 10.1371/journal.pone.0003490
- Davison, A. C., and Hinkley, D. V. (1997). *Bootstrap Methods and Their Application*. New York, NY: Cambridge University Press. doi: 10.1017/CBO9780511802843
- de Jager, P. L., Jia, X., Wang, J., de Bakker, P. I., Ottoboni, L., Aggarwal, N. T., et al. (2009). Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nat. Genet.* 41, 776–782. doi: 10.1038/ng.401
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635
- Evsyukova, I., Somarelli, J. A., Gregory, S. G., and Garcia-Blanco, M. A. (2010). Alternative splicing in multiple sclerosis and other autoimmune diseases. *RNA Biol.* 7, 462–467. doi: 10.4161/rna.7.4.12301
- Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., et al. (2015). Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 518, 337–343. doi: 10.1038/nature13835
- Glažar, P., Papavasileiou, P., and Rajewsky, N. (2014). circBase: a database for circular RNAs. *RNA* 20, 1666–1670. doi: 10.1261/rna.043687.113
- Greene, J., Baird, A. M., Brady, L., Lim, M., Gray, S. G., McDermott, R., et al. (2017). Circular RNAs: biogenesis, function and role in human diseases. *Front. Mol. Biosci.* 4:38. doi: 10.3389/fmolb.2017.00038
- Griffiths-Jones, S. (2004). The microRNA Registry. *Nucleic Acids Res.* 32, D109–D111. doi: 10.1093/nar/gkh023
- Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A., and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34, D140–D144. doi: 10.1093/nar/gkj112
- Griffiths-Jones, S., Saini, H. K., van Dongen, S., and Enright, A. J. (2008). miRBase: tools for microRNA genomics. *Nucleic Acids Res.* 36, D154–D158. doi: 10.1093/nar/gkm952
- Harrow, J., Frankish, A., Gonzalez, J. M., Tapanari, E., Diekhans, M., Kokocinski, F., et al. (2012). GENCODE: the reference human genome annotation for The ENCODE Project. *Genome Res.* 22, 1760–1774. doi: 10.1101/gr.135350.111
- Hnisz, D., Abraham, B. J., Lee, T. I., Lau, A., Saint-André, V., Sigova, A. A., et al. (2013). Super-enhancers in the control of cell identity and disease. *Cell* 155, 934–947. doi: 10.1016/j.cell.2013.09.053
- Holdt, L. M., Stahringer, A., Sass, K., Pichler, G., Kulak, N. A., Wilfert, W., et al. (2016). Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. *Nat. Commun.* 7:12429. doi: 10.1038/ncomms12429
- International Multiple Sclerosis Genetics Consortium [IMSGC], Beecham, A. H., Patsopoulos, N. A., Xifara, D. K., Davis, M. F., Kempainen, A., et al. (2013). Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat. Genet.* 45, 1353–1360. doi: 10.1038/ng.2770
- International Multiple Sclerosis Genetics Consortium [IMSGC], Hafler, D. A., Compston, A., Sawcer, S., Lander, E. S., Daly, M. J., et al. (2007). Risk alleles for multiple sclerosis identified by a genomewide study. *N. Engl. J. Med.* 357, 2373–2383. doi: 10.1056/NEJMoa073493
- International Multiple Sclerosis Genetics Consortium [IMSGC], Wellcome Trust Case Control Consortium, Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., et al. (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 476, 214–219. doi: 10.1038/nature10251
- Iparraguirre, L., Muñoz-Culla, M., Prada-Luengo, I., Castillo-Triviño, T., Olascoaga, J., and Otaegui, D. (2017). Circular RNA profiling reveals that circular RNAs from ANXA2 can be used as new biomarkers for multiple sclerosis. *Hum. Mol. Genet.* 26, 3564–3572. doi: 10.1093/hmg/ddx243
- Jakkula, E., Leppä, V., Sulonen, A. M., Varilo, T., Kallio, S., Kempainen, A., et al. (2010). Genome-wide association study in a high-risk isolate for multiple sclerosis reveals associated variants in STAT3 Gene. *Am. J. Hum. Genet.* 86, 285–291. doi: 10.1016/j.ajhg.2010.01.017
- Jeck, W. R., Sorrentino, J. A., Wang, K., Slevin, M. K., Burd, C. E., Liu, J., et al. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 19, 141–157. doi: 10.1261/rna.035667.112
- Juan-Mateu, J., Villate, O., and Eizirik, D. L. (2016). MECHANISMS IN ENDOCRINOLOGY: alternative splicing: the new frontier in diabetes research. *Eur. J. Endocrinol.* 174, R225–R238. doi: 10.1530/EJE-15-0916
- Kozomara, A., and Griffiths-Jones, S. (2011). miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157. doi: 10.1093/nar/gkq1027
- Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42, D68–D73. doi: 10.1093/nar/gkt1181
- Luan, J., Jiao, C., Kong, W., Fu, J., Qu, W., Chen, Y., et al. (2018). circHLA-C plays an important role in lupus nephritis by sponging miR-150. *Mol. Ther. Nucleic Acids* 10, 245–253. doi: 10.1016/j.omtn.2017.12.006
- Martinelli-Boneschi, F., Esposito, F., Brambilla, P., Lindström, E., Lavorgna, G., Stankovich, J., et al. (2012). A genome-wide association study in progressive multiple sclerosis. *Mult. Scler.* 18, 1384–1394. doi: 10.1177/1352458512439118
- Matesanz, F., González-Pérez, A., Lucas, M., Sanna, S., Gayán, J., Urcelay, E., et al. (2012). Genome-wide association study of multiple sclerosis confirms a novel locus at 5p13.1. *PLoS One* 7:e36140. doi: 10.1371/journal.pone.0036140
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Mendell, J. T., and Olson, E. N. (2012). MicroRNAs in stress signaling and human disease. *Cell* 148, 1172–1187. doi: 10.1016/j.cell.2012.02.005
- Mueller, J. C., Lohmussaar, E., Mägi, R., Remm, M., Bettecken, T., Lichtner, P., et al. (2005). Linkage disequilibrium patterns and tagSNP transferability among European populations. *Am. J. Hum. Genet.* 76, 387–398. doi: 10.1086/427925
- Myocardial Infarction, Genetics Consortium, Kathiresan, S., Voight, B. F., Purcell, S., Musunuru, K., et al. (2009). Genome-wide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants. *Nat. Genet.* 41, 334–341. doi: 10.1038/ng.327
- Nikpay, M., Goel, A., Won, H. H., Hall, L. M., Willenborg, C., Kanoni, S., et al. (2015). A comprehensive 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. *Nat. Genet.* 47, 1121–1130. doi: 10.1038/ng.3396
- Nischwitz, S., Cepok, S., Kroner, A., Wolf, C., Knop, M., Müller-Sarnowski, F., et al. (2010). Evidence for VAV2 and ZNF433 as susceptibility genes for multiple

- sclerosis. *J. Neuroimmunol.* 227, 162–166. doi: 10.1016/j.jneuroim.2010.06.003
- Okada, Y., Wu, D., Trynka, G., Raj, T., Terao, C., Ikari, K., et al. (2014). Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature* 506, 376–381. doi: 10.1038/nature12873
- Paraboschi, E. M., Cardamone, G., Rimoldi, V., Gemmati, D., Spreafico, M., Duga, S., et al. (2015). Meta-analysis of multiple sclerosis microarray data reveals dysregulation in RNA Splicing Regulatory Genes. *Int. J. Mol. Sci.* 16, 23463–23481. doi: 10.3390/ijms161023463
- Patsopoulos, N. A., Barcellos, L. F., Hintzen, R. Q., Schaefer, C., van Duijn, C. M., Noble, J. A., et al. (2013). Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects. *PLoS Genet.* 9:e1003926. doi: 10.1371/journal.pgen.1003926
- Patsopoulos, N. A., Bayer Pharma, M. S., Genetics Working Group, Steering Committees of Studies Evaluating IFN(beta)-1b, and a CCR1-Antagonist, ANZgene Consortium, GeneMSA, International Multiple Sclerosis Genetics Consortium, et al. (2011). Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci. *Ann. Neurol.* 70, 897–912. doi: 10.1002/ana.22609
- Pott, S., and Lieb, J. D. (2015). What are super-enhancers? *Nat Genet.* 47, 8–12. doi: 10.1038/ng.3167
- Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., et al. (2007). PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am. J. Hum. Genet.* 81, 559–575. doi: 10.1086/519795
- Quinn, J. J., and Chang, H. Y. (2016). Unique features of long non-coding RNA biogenesis and function. *Nat. Rev. Genet.* 17, 47–62. doi: 10.1038/nrg.2015.10
- Ramagopalan, S. V., Dobson, R., Meier, U. C., and Giovannoni, G. (2010). Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet Neurol.* 9, 727–739. doi: 10.1016/S1474-4422(10)70094-6
- Richard-Miceli, C., and Criswell, L. A. (2012). Emerging patterns of genetic overlap across autoimmune disorders. *Genome Med.* 4:6. doi: 10.1186/gm305
- Rybak-Wolf, A., Stottmeister, C., Glažar, P., Jens, M., Pino, N., Giusti, S., et al. (2015). Circular RNAs in the mammalian brain are highly abundant. Conserved, and Dynamically Expressed. *Mol Cell.* 58, 870–885. doi: 10.1016/j.molcel.2015.03.027
- Salzman, J., Chen, R. E., Olsen, M. N., Wang, P. L., and Brown, P. O. (2013). Cell-type specific features of circular RNA expression. *PLoS Genet.* 9:e1003777. doi: 10.1371/journal.pgen.1003777
- Sanna, S., Pitzalis, M., Zoledziewska, M., Zara, I., Sidore, C., Murru, R., et al. (2010). Variants within the immunoregulatory CBLB gene are associated with multiple sclerosis. *Nat. Genet.* 42, 495–497. doi: 10.1038/ng.584
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034
- Vidigal, J. A., and Ventura, A. (2015). The biological functions of miRNAs: lessons from in vivo studies. *Trends Cell Biol.* 25, 137–147. doi: 10.1016/j.tcb.2014.11.004
- Vučičević, D., Schrewe, H., and Orom, U. A. (2014). Molecular mechanisms of long ncRNAs in neurological disorders. *Front. Genet.* 5:48. doi: 10.3389/fgene.2014.00048
- Ward, L. D., and Kellis, M. (2012). Interpreting noncoding genetic variation in complex traits and human disease. *Nat. Biotechnol.* 30, 1095–1096. doi: 10.1038/nbt.2422
- Wei, Y., Zhang, S., Shang, S., Zhang, B., Li, S., Wang, X., et al. (2016). SEA: a super-enhancer archive. *Nucleic Acids Res.* 44, D172–D179. doi: 10.1093/nar/gkv1243
- Zhang, Y., Zhang, X. O., Chen, T., Xiang, J. F., Yin, Q. F., Xing, Y. H., et al. (2013). Circular intronic long noncoding RNAs. *Mol. Cell.* 51, 792–806. doi: 10.1016/j.molcel.2013.08.017

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association of mir-196a-2 rs11614913 and mir-149 rs2292832 Polymorphisms With Risk of Cancer: An Updated Meta-Analysis

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OPEN ACCESS

Edited by:

Yujing Li,
Emory University, United States

Reviewed by:

Peter Igaz,
Simmelweis University, Hungary
Graziella Curtale,
The Scripps Research Institute,
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equally to this work

Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 29 July 2018

Accepted: 19 February 2019

Published: 15 March 2019

Citation:

Choupani J, Nariman-Saleh-Fam Z,
Saadatian Z, Ouladsahebmadarek E,
Masotti A and Bastami M (2019)
Association of mir-196a-2
rs11614913 and mir-149 rs2292832
Polymorphisms With Risk of Cancer:
An Updated Meta-Analysis.
Front. Genet. 10:186.
doi: 10.3389/fgene.2019.00186

Background: Accumulating evidence suggests that functional dysregulations of miRNAs, especially miR-196a-2 and miR-149, in cancers could be attributed to polymorphisms in miRNA sequences. This study was aimed at clarifying the association of mir-196a-2 rs11614913 and mir-149 rs2292832 with cancer risk by performing an updated meta-analysis of genetic association studies.

Methods: PubMed, Embase, Scopus, and ScienceDirect databases were searched until 9 April 2018 to identify eligible studies. Studies should meet the following criteria to be included in the meta-analysis: evaluation of genetic association between rs11614913 and/or rs2292832 and susceptibility to cancer; A case-control design; Written in English; Availability of sufficient data for estimating odds ratio (OR) and its 95% confidence interval (95%CI). Studies that met the following criteria were excluded: review articles, meta-analysis, abstracts or conference papers; duplicate publications; studies on animals or cell-lines; studies without a case-control design; studies that did not report genotype frequencies. Pooled ORs and 95% CIs were estimated using a total of 111 studies (41,673 cases and 49,570 controls) for mir-196a rs11614913 and 44 studies (15,954 cases and 19,594 controls) for mir-149 rs2292832. Stratified analysis according to quality scores, genotyping method, ethnicity, broad cancer category and cancer type was also performed.

Results: Mir-196a-2 rs11614913T allele was associated with decreased cancer risk in overall population. The association was only significant in Asians but not Caucasians. In subgroup analysis, significant associations were found in high quality studies, gynecological cancers, ovarian, breast, and hepatocellular cancer. Mir-149 rs2292832 was not associated with cancer risk in overall population and there were no differences between Asians and Caucasians. However, the T allele was associated with a decrease risk of gastrointestinal tract cancers under the heterozygote model and an increased risk of colorectal cancer under the recessive model.

Conclusions: The present meta-analysis suggests that mir-196a-2 rs11614913 may contribute to the risk of cancer especially in Asians. Mir-149 rs2292832 may modulate the risk of gastrointestinal tract cancers especially colorectal cancer. This study had some limitations such as significant heterogeneity in most contrasts, limited number of studies enrolling Africans or Caucasians ancestry and lack of adjustment for covariates and environmental interactions.

Keywords: microRNA, polymorphism, meta-analysis, cancer, mir-196a-2, mir-149

INTRODUCTION

Despite remarkable recent progress in clinical management, diagnosis and treatment, cancer has remained one of the major causes of death worldwide. According to the recent World Health Organization (WHO) report, about one in six deaths were caused by cancer in 2015. It is predicted that cancer-related death will increase up to 13.2 million by 2030 worldwide (Ferlay et al., 2010; Bray et al., 2012). Complex genetic and environmental risk factors and also interactions between these components contribute to the etiopathology of different cancers. Until recent years, much effort has been devoted to link the alteration of protein coding genes to tumorigenesis. However, latest evidence has demonstrated the emerging role of noncoding RNAs in cancer development and, especially, introduced microRNAs (miRNAs) as new players in pathobiology of cancers (Peng and Croce, 2016). MiRNAs are short noncoding functional RNAs that are involved in the regulation of transcriptome (Ha and Kim, 2014). They modulate important cellular processes both in normal physiology and disease state and are involved in almost all cellular processes altered during tumorigenesis (Osada and Takahashi, 2007; Li et al., 2009). Human mir-196a (*MIR196A2*, HGNC:31568) and mir-149 (*MIR149*, HGNC: 31536) are well-studied miRNAs that may function either as oncomiRs, by targeting tumor suppressor genes, or as tumor suppressors, by targeting oncogenes, in different conditions (Lu et al., 2016; He J. et al., 2018; Ow et al., 2018). It has been shown that single nucleotide polymorphism (SNP) in miRNA genes, such as hsa-mir-196a-2 rs11614913 and hsa-mir-149 rs2292832, may influence their functions through altering miRNA expression, maturation and/or efficiency of targeting and, thereby, contribute to the risk of cancer (Hu et al., 2008; Hoffman et al., 2009; Tu et al., 2012; Nariman-Saleh-Fam et al., 2016, 2017). Several association studies in a range of populations evaluated the contribution of mir-196a-2 rs11614913 and mir-149 rs2292832 to cancer risk; but results are inconclusive. Therefore, this study was aimed at clarifying the association of mir-196a-2 rs11614913 and mir-149 rs2292832 with cancer risk by performing an updated meta-analysis of genetic association studies.

Abbreviations: BC, breast cancer; BLC, bladder cancer; CRC, colorectal cancer; ESCC, esophageal squamous cell carcinoma; GC, gastric cancer; GI, cancers of digestive system; GYC, Gynecologic cancers; HCC, Hepatocellular cancer; HM, Hematological malignancies; HNC, Head and neck carcinoma; HWE, Hardy-Weinberg equilibrium; HWD, Hardy-Weinberg deviation; LC, lung cancer; UG, Urogenital cancers; OC, Oral cancer; OR, Odds ratio; OvC, ovarian cancer; PC, prostate cancer.

MATERIALS AND METHODS

Publication Search

To identify all potentially eligible publications, PubMed, Embase, Scopus and ScienceDirect databases were searched, with respect to specific search tips of each database, using following keywords. ("microRNA 196a2" OR "miRNA-196a2" OR "mir-196a2" OR "mir196a" OR "mir-196a-2" OR "pre-mir-196a" OR "pre-mir196a" OR "196a" OR "rs11614913") OR ("microRNA 149" OR "miRNA-149" OR "mir-149" OR "mir149" OR "pre-mir-149" OR "pre-mir149" OR "rs2292832") AND ("single nucleotide polymorphism" OR "SNP" OR "variant" OR "variation" OR "polymorphism" OR "mutation" OR "locus") AND ("neoplasm" OR "cancer" OR "tumor" OR "carcinoma" OR "sarcoma" OR "lymphoma" OR "adenoma" OR "leukemia" OR "leucemia" OR "malignancy" OR "malignance" OR "malignant" OR "glioma"). Last search was performed on 9 April 2018. References of the relevant literature and review articles were also evaluated to identify all potentially eligible articles. This meta-analysis carried out in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Liberati et al., 2009).

Inclusion and Exclusion Criteria

Studies should meet the following criteria to be included in the meta-analysis: (1) evaluation of genetic association between rs11614913 and/or rs2292832 and susceptibility to cancer; (2) a case-control design; (3) Written in English; (4) Availability of sufficient data for estimating odds ratio (OR) and its 95% confidence interval (95%CI). Studies that met the following criteria were excluded: (1) review articles, meta-analysis, abstracts or conference papers; (2) duplicate publications; (3) studies on animals or cell-lines; (4) studies without a case-control design (5) studies that did not report genotype frequencies.

Data Extraction

Data was extracted from each eligible study and manually checked. Then, items were recorded for each eligible study: the first author, publication year, category of cancer, type of cancer, country, ethnicity, source of controls, genotyping method, number of subjects in the case and the control groups, genotype counts for each SNP in the case and the control groups. A broad cancer category was assigned for each study according to the following scheme: gastrointestinal tract cancers (GI, including gastric, esophageal, colorectal, bladder, pancreatic, or hepatocellular cancers), head and neck squamous cell

carcinoma (HNC, including oral, non-oral, and nasopharyngeal cancers), gynecologic cancer (GyC, including endometrial, ovarian, and cervical cancers), hematological malignancies (HM, including leukemia and lymphomas), urogenital cancers (UG, including prostate, renal cell, and bladder cancers), or other cancers.

Quality Assessment

The quality of each study was assessed using quality assessment criteria for genetic association studies used elsewhere (Thakkestian et al., 2011; Xue et al., 2015). This score is based on seven items including representativeness of cases, representativeness of controls, ascertainment of cancer case, control selection, genotyping examination, Hardy-Weinberg Equilibrium (HWE) status in controls, and total sample size. Quality scores ranged from 0 (lowest) to 15 (highest). Studies which were scored equal to or less than eight were regarded as low quality, while those with scores of greater than eight were regarded as high quality.

Statistical Analysis

The Meta package for R was used to perform meta-analysis (Schwarzer, 2007). Association of rs11614913 and rs2292832 with cancer was estimated by calculating pooled ORs and their 95% CIs assuming homozygote, heterozygote, dominant, recessive, and allelic models. Heterogeneity was assessed using the Chi-squared based Q test (Lau et al., 1997). In the presence of a significant heterogeneity (i.e., P -value of Q-test < 0.05 or $I^2 > 50\%$), the random effect (RE) model (DerSimonian and Laird, 1986) was used to calculate pooled ORs and 95% CIs. Otherwise, the fixed effect (FE) model was used (Mantel and Haenszel, 1959). Significance of the pooled OR was determined by the Z-test ($P < 0.05$ was considered significant). In cases of remarkable heterogeneity (i.e., $I^2 > 50\%$), the potential sources of heterogeneity across studies was explored using univariate meta-regression and stratified analysis. Moreover, subgroup analyses based on genotyping method, study quality, ethnicities, broad cancer categories, and cancer types were carried out. To assess consistency of results and influence of each study on the pooled OR, sensitivity analysis was done by omitting one study at a time and recalculating summary OR and 95% CI. Publication bias was evaluated by the Begg's rank correlation test of funnel plot asymmetry (Begg and Mazumdar, 1994) the "Trim and Fill" approach was used to correct for asymmetry in cases of significant rank correlation test (Duval and Tweedie, 2000a,b). All P -values were two-sided and P -value < 0.05 was considered statistically significant. All statistical analyses were performed in R (version 3.3.1).

Dealing With HWD (Departure From Hardy-Weinberg Equilibrium)

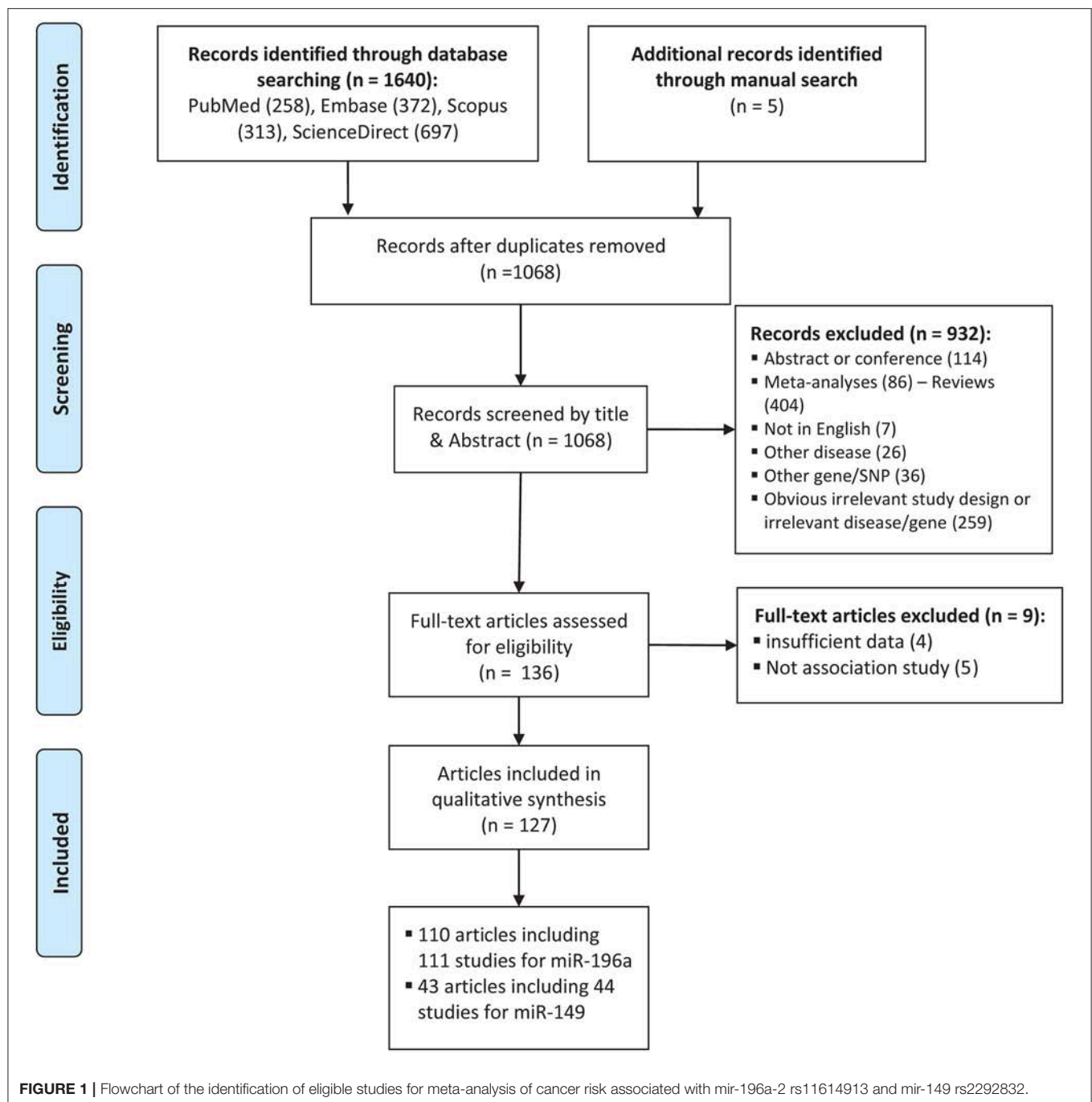
Departure from Hardy-Weinberg equilibrium (HWE) may be caused by a range of factors, among which genotyping error is more importantly relevant to the association study context. Currently there is no consensus on the way of handling association studies with the controls not in HWE,

but it has been recommended that such studies should not be excluded from meta-analysis (Minelli et al., 2008). However, sensitivity analysis should be performed to evaluate the possible effects of such studies on the pooled estimates (Attia et al., 2003; Thakkestian et al., 2005; Zintzaras and Lau, 2008; Wang X. B. et al., 2014). In the present meta-analysis, the following approach with regards to HWE-deviated studies was followed. Departure of genotype distributions from HWE (i.e., HWD) in the control group of each study was evaluated using the Chi-squared or the exact goodness of fit test. Meta-analyses, including the overall and subgroup analyses, were performed considering all eligible studies including HWD studies. However, to evaluate possible impacts of HWE-deviated studies, HWD sensitivity analysis was performed by evaluating the influence of excluding these studies on point estimates and identifying the influenced genotype contrasts. In cases that excluding HWD studies altered the result of meta-analysis, ORs of such studies were adjusted for HWE deviation by means of incorporating the HWE-expected genotype counts in the control group as recommended (Trikalinos et al., 2006; Zintzaras et al., 2006; Zintzaras, 2008; Zintzaras and Lau, 2008; Srivastava and Srivastava, 2012) and the HWD-adjusted pooled ORs were calculated in genotype contrasts.

RESULTS

Study Characteristics

The process of selecting eligible studies is depicted in **Figure 1**. A total of 1,645 articles were found from different sources outlined in materials and methods and screened by reading titles and abstracts. A total of 1,509 articles were excluded in which 577 articles were duplicates, 114 articles were abstracts or conference meetings, 86 articles were meta-analysis, 404 were review articles, 7 articles were not written in English, 26 articles were related to other diseases, 36 articles were related to other genes, or polymorphisms and 259 more articles had either obvious irrelevant study design or irrelevant disease/gene. The full text of the remaining 136 articles were evaluated and 9 more articles were also excluded as they did not have either sufficient data to calculate ORs and 95% CIs ($n: 4$) or an association study design ($n: 5$). Finally, a total of 127 eligible articles remained (Horikawa et al., 2008; Yang et al., 2008, 2016; Hoffman et al., 2009; Hu et al., 2009, 2013; Tian et al., 2009; Catucci et al., 2010; Christensen et al., 2010; Dou et al., 2010; Kim et al., 2010, 2012; Kontorovich et al., 2010; Li et al., 2010, 2014; Liu et al., 2010, 2013, 2014; Okubo et al., 2010; Peng et al., 2010; Qi et al., 2010, 2014, 2015; Srivastava et al., 2010, 2017; Wang et al., 2010, 2013, 2016; Akkiz et al., 2011; George et al., 2011; Hong et al., 2011; Jedlinski et al., 2011; Mittal et al., 2011; Vinci et al., 2011, 2013; Zhan et al., 2011; Zhou et al., 2011, 2014; Alshatwi et al., 2012; Chen et al., 2012; Chu et al., 2012, 2014; Hezova et al., 2012; Linhares et al., 2012; Min et al., 2012; Tu et al., 2012; Zhang M. et al., 2012; Zhang M. W. et al., 2012; Zhu et al., 2012; Ahn et al., 2013; Han et al., 2013; Huang et al., 2013, 2017; Lv et al., 2013; Ma et al., 2013; Pavlakis et al., 2013; Umar et al., 2013; Wei et al., 2013, 2014; Zhang et al., 2013; Bansal et al., 2014;



Dikeakos et al., 2014; Du et al., 2014; Hao et al., 2014; Kou et al., 2014; Kupcinskis et al., 2014a,b; Omrani et al., 2014; Parlayan et al., 2014; Pu et al., 2014; Qu et al., 2014; Roy et al., 2014; Tong et al., 2014; Wang N. et al., 2014; Wang R. et al., 2014; Wang X. H. et al., 2014; Deng et al., 2015; Dikaiakos et al., 2015; Dong et al., 2015; He et al., 2015; Li T. et al., 2015; Liu, 2015; Li X. et al., 2015; Martin-Guerrero et al., 2015; Nikolić et al., 2015; Pratedrat et al., 2015; Sodhi et al., 2015; Sushma et al., 2015; Yan et al., 2015; Yin et al., 2015, 2016, 2017; Dai et al., 2016; Gu and Tu, 2016; Hashemi et al., 2016; Jiang et al., 2016;

Li H. et al., 2016; Li J. et al., 2016; Li M. et al., 2016; Miao et al., 2016; Morales et al., 2016; Ni and Huang, 2016; Peckham-Gregory et al., 2016; Shen et al., 2016; Song et al., 2016; Su et al., 2016; Sun et al., 2016; Toraih et al., 2016a,b; Zhang L. H. et al., 2016; Zhao et al., 2016; Afsharzadeh et al., 2017; Bodal et al., 2017; Cimpeanu et al., 2017; Poltronieri-Oliveira et al., 2017; Rakmanee et al., 2017; Rogoveanu et al., 2017; Tandon et al., 2017; Zhang E. et al., 2017; Abdel-Hamid et al., 2018; Damodaran et al., 2018; Doulah et al., 2018; He J. et al., 2018; He Y. et al., 2018; Mashayekhi et al., 2018; Minh et al., 2018; Ranjbar

TABLE 1 | Main characteristics of studies evaluating cancer risk associated with miR-196a-2 rs11614913 C/T which included in the current meta-analysis.

N	References	Country	Ethnicity	Genotyping Method	Design	Cases/ Controls	Genotype distribution ^a		Cancer Type	Quality Score	P _{HWE}
							Cases	Controls			
1	Abdel-Hamid et al., 2018	Egypt	African	PCR-RFLP	PB	50/50	21/26/3	24/20/6	HCC	10	0.53
2	Damodaran et al., 2018	India	Asian	PCR-RFLP	HB	100/100	32/51/17	47/36/17	PC	7	0.054
3	Doulah et al., 2018	Iran	Asian	ARMS-PCR	HB	98/100	33/51/14	25/62/13	BC	3	0.015
4	He J. et al., 2018	China	Asian	TaqMan	HB	393/812	94/192/107	183/399/230	NB	11	0.73
5	Mashayekhi et al., 2018	Iran	Asian	PCR-RFLP	HB	353/353	142/169/42	149/158/46	BC	10	0.75
6	Minh et al., 2018	Vietnam	Vietnamese	HRMA	HB	113/127	48/35/30	31/64/32	BC	5	0.95
7	Afsharzadeh et al., 2017	Iran	Asian	ARMS-PCR	Not mentioned	100/150	34/52/14	38/93/19	BC	3	0.002
8	Bodal et al., 2017	India	Asian	PCR-RFLP	HB	95/99	48/47/0	64/35/0	BC	8	0.051
9	Huang et al., 2017	China	Asian	PCR-RFLP	PB	165/284	22/81/62	39/134/111	HCC	9	0.95
10	Poltronieri-Oliveira et al., 2017	Brazil	Mixed	PCR-RFLP	PB	149/246	64/57/28	105/120/21	GC	9	0.12
11	Rakmanee et al., 2017	Thailand	Asian	PCR-RFLP	HB	104/180	48/43/13	49/78/53	ALL	9	0.09
12	Rogoveanu et al., 2017	Romania	Caucasian	TaqMan	HB	142/288	61/63/18	121/128/39	GC	9	0.64
13	Srivastava et al., 2017	India	Asian	PCR-RFLP	HB	184/164	20/93/71	21/81/62	CSCC	1-	0.57
14	Yin et al., 2017	China	Asian	TaqMan	HB	1,003/1,003	252/555/196	221/496/286	LC	1-	0.86
15	Zhang E. et al., 2017	China	Asian	TaqMan	HB	340/340	71/169/100	88/155/97	OC	11	0.12
16	Dai et al., 2016	China	Asian	Mass-ARRAY	HB	560/583	197/265/98	155/284/144	BC	10	0.58
17	Gu and Tu, 2016	China	Asian	PCR-RFLP	HB	186/186	39/96/51	57/98/31	GC	7	0.36
18	Hashemi et al., 2016	Iran	Asian	t-ARMS-PCR	HB	169/182	64/88/17	77/93/12	PC	7	0.029
19	Li M. et al., 2016	China	Asian	MassARRAY	HB	182/182	24/83/75	11/79/92	GC	8	0.33
20	Li J. et al., 2016	China	Asian	Sequencing	Not mentioned	109/105	25/64/20	18/52/35	HCC	8	0.95
21	Miao et al., 2016	China	Asian	Array (Illumina Infinium1)	HB	576/1,550	130/284/162	292/755/503	HNSCC	12	0.80
22	Morales et al., 2016	Chile	Mixed	TaqMan	PB	440/807	192/191/57	342/351/114	BC	12	0.13
23	Ni and Huang, 2016	China	Asian	PCR-RFLP	HB	155/342	32/82/41	66/176/100	OvC	9	0.51
24	Peckham-Gregory et al., 2016	USA	Caucasian	Allele-specific PCR Array	PB	179/529	72/88/19	196/257/76	NHL	13	0.62
25	Shen et al., 2016	China	Asian	SNaPshot	PB	1,400/2,185	295/698/407	392/1121/672	ESCC	10	0.046
26	Song et al., 2016	China	Asian	PCR-RFLP	PB	479/431	121/247/111	86/203/142	OvC	13	0.42
27	Su et al., 2016	China	Asian	PCR-RFLP	HB	245/315	83/128/34	119/158/38	GC	10	0.22
28	Sun et al., 2016	China	Asian	PCR-RFLP	HB	134/227	29/66/39	34/116/77	OvC	7	0.42
29	Toraih et al., 2016b	Egypt	African	TaqMan	HB	209/100	84/93/32	55/35/10	Cancer	8	0.30
30	Toraih et al., 2016a	Egypt	African	TaqMan	PB	125/150	48/63/14	80/53/17	Cancer	11	0.11
31	Wang et al., 2017	China	Asian	Mass-ARRAY	PB	283/283	73/158/52	65/124/94	BIC	10	0.066
32	Yin et al., 2016	China	Asian	TaqMan	HB	575/608	128/298/149	133/297/178	LC	14	0.70

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TABLE 1 | Continued

N	References	Country	Ethnicity	Genotyping Method	Design	Cases/ Controls	Genotype distribution ^a		Cancer Type	Quality Score	P _{HWE}
							Cases	Controls			
33	Zhang L. H. et al., 2016	China	Asian	PCR-RFLP	PB	175/302	25/85/65	42/138/122	HCC	9	0.83
34	Zhao et al., 2016	China	Asian	Sequencing	HB	114/114	31/50/33	28/61/25	BC	9	0.53
35	Zhou et al., 2011	China	Asian	PCR-RFLP	HB	226/309	46/123/57	58/169/82	CSCC	12	0.09
36	Deng et al., 2015	China	Asian	PCR-RFLP	PB	159/298	41/66/52	56/166/76	BIC	9	0.048
37	Dikaiakos et al., 2015	Greece	Caucasian	PCR-RFLP	PB	157/299	19/69/69	33/149/117	CRC	10	0.18
38	He et al., 2015	China	Asian	Mass-ARRAY	PB	450/450	81/233/136	93/223/134	BC	11	0.94
39	Li T. et al., 2015	China	Asian	PCR-RFLP	PB	318/320	61/146/111	42/134/144	NHL	13	0.26
40	Liu, 2015	China	Asian	PCR-RFLP	PB	266/266	84/131/51	113/123/30	HCC	12	0.76
41	Liu, 2015	China	Asian	PCR-RFLP	HB	216/100	25/133/58	23/49/28	GyC	5	0.95
42	Martin-Guerrero et al., 2015	Spain	Caucasian	TaqMan	PB	104/345	35/40/29	137/159/49	CLL	9	0.86
43	Nikolić et al., 2015	Serbia	Caucasian	HRMA	PB	351/309	150/161/40	121/147/41	PC	10	0.79
44	Qi et al., 2015	China	Asian	TaqMan	PB	321/290	34/119/168	17/88/185	BC	11	0.18
45	Sodhi et al., 2015	India	Asian	PCR-RFLP	PB	250/255	70/161/19	101/146/8	LC	12	5.46e-7
46	Sushma et al., 2015	India	Asian	PCR-RFLP	PB	100/102	22/10/68	6/15/81	OC	8	0.001
47	Yan et al., 2015	China	Asian	PCR-RFLP	HB	274/328	46/147/81	27/165/136	HCC	9	0.02
48	Yin et al., 2015	China	Asian	TaqMan	HB	258/310	50/141/67	63/150/97	LC	11	0.78
49	Bansal et al., 2014	India	Asian	PCR-RFLP	PB	121/165	68/41/12	85/59/21	BC	9	0.058
50	Chu et al., 2014	Taiwan	Asian	PCR-RFLP	PB	188/337	41/81/66	70/167/100	HCC	12	0.94
51	Dikeakos et al., 2014	Greece	Caucasian	PCR-RFLP	HB	163/480	102/46/15	79/229/172	GC	9	0.9
52	Hao et al., 2014	China	Asian	PCR-RFLP	PB	235/282	77/126/32	67/160/55	HCC	11	0.027
53	Kou et al., 2014	China	Asian	PCR-RFLP	PB	271/532	84/150/37	125/304/103	HCC	9	0.001
54	Kupcinskask et al., 2014b	Latvia	Caucasian	TaqMan	HB	363/350	144/184/35	159/145/46	GC	8	0.18
55	Kupcinskask et al., 2014a	Latvia	Caucasian	TaqMan	HB	193/427	79/87/27	199/174/54	CRC	8	0.12
56	Li et al., 2014	China	Asian	TaqMan	PB	1,020/1,006	209/489/322	218/518/270	NC	13	0.32
57	Du et al., 2014	China	Asian	TaqMan	PB	1,000/1,022	149/514/337	211/497/314	RCC	13	0.61
58	Omran et al., 2014	Iran	Asian	T-ARMS-PCR	PB	236/203	218/18/0	178/25/0	BC	11	0.70
59	Parlayan et al., 2014	Japan	Asian	TaqMan	HB	827/524	174/409/244	108/270/146	Cancer	11	0.44
60	Pu et al., 2014	China	Asian	PCR-RFLP	HB	159/511	39/95/25	101/324/86	GC	9	1.7e-9
61	Qi et al., 2014	China	Asian	HRMA	PB	314/406	45/209/60	71/214/121	HCC	12	0.17
62	Qu et al., 2014	China	Asian	PCR-RFLP	PB	381/426	126/207/48	133/211/82	ESCC	13	0.96
63	Roy et al., 2014	India	Asian	TaqMan	HB	451/448	218/187/46	242/168/38	OC	10	0.29
64	Tong et al., 2014	China	Asian	TaqMan	PB	570/673	103/308/159	129/307/237	ALL	14	0.11
65	Wang N. et al., 2014	China	Asian	PCR-LDR	PB	597/597	128/307/162	145/298/154	ESCC	13	0.97
66	Zhou et al., 2014	China	Asian	PCR-RFLP	HB	266/281	93/139/34	66/160/55	HCC	10	0.02
67	Ahn et al., 2013	South Korea	Asian	PCR-RFLP	PB	461/447	100/242/119	87/232/128	GC	12	0.35
68	Han et al., 2013	China	Asian	TaqMan	PB	1,017/1,009	207/505/305	220/485/304	HCC	10	0.33
69	Hu et al., 2013	China	Asian	Sequencing	HB	680/690	185/314/181	138/342/210	Glioma	14	0.98

(Continued)

TABLE 1 | Continued

N	References	Country	Ethnicity	Genotyping Method	Design	Cases/ Controls	Genotype distribution ^a		Cancer Type	Quality Score	P _{HWE}
							Cases	Controls			
70	Liu et al., 2013	Taiwan	Asian	PCR-RFLP	NA	315/92	64/147/104	26/36/30	OC	6	0.055
71	Lv et al., 2013	China	Asian	PCR-RFLP	PB	347/531	10/223/114	109/331/91	CRC	10	1.5e-8
72	Ma et al., 2013	China	Asian	Mass-ARRAY	PB	190/187	44/92/54	49/79/59	BC	11	0.047
73	Min et al., 2012	South Korea	Asian	PCR-RFLP	PB	446/502	120/201/125	100/254/148	CRC	12	0.68
74	Pavlikis et al., 2013	Greece	Caucasian	PCR-RFLP	PB	93/122	12/33/48	14/58/50	PNC	10	0.75
75	Umar et al., 2013	India	Asian	PCR-RFLP	PB	289/309	146/121/22	171/122/16	ESCC	12	0.39
76	Vinci et al., 2013	Italy	Caucasian	HRMA	HB	160/178	62/86/12	83/84/11	CRC	9	0.11
77	Wang et al., 2013	China	Asian	TaqMan	HB	1,689/1,946	319/851/519	482/940/524	GC	10	0.14
78	Wei et al., 2013	China	Asian	Mass-ARRAY	HB	367/370	65/196/106	87/170/113	ESCC	12	0.16
79	Zhang et al., 2013	China	Asian	Mass-ARRAY	PB	996/995	214/488/294	165/502/328	HCC	10	0.26
80	Alshatwi et al., 2012	Saudi Arabia	Asian	TaqMan	HB	100/100	35/63/2	46/50/4	BC	4	0.051
81	Chen et al., 2012	China	Asian	PCR-LDR	HB	126/407	27/64/35	94/206/107	CRC	12	0.84
82	Chu et al., 2012	Taiwan	Asian	PCR-RFLP	PB	470/425	57/277/136	87/206/132	OC	11	0.68
83	Hezova et al., 2012	Czech Republic	Caucasian	TaqMan	PB	197/212	82/89/26	87/103/22	CRC	9	0.34
84	Kim et al., 2012	South Korea	Asian	PCR-RFLP	PB	159/201	34/84/41	45/107/49	HCC	11	0.41
85	Linhares et al., 2012	Brazil	Mixed	TaqMan	HB	388/388	94/177/117	127/165/96	BC	7	0.005
86	Zhang M. et al., 2012	China	Asian	PCR-RFLP	PB	248/243	11/89/148	17/93/133	BC	13	0.96
87	Zhu et al., 2012	China	Asian	TaqMan	HB	573/588	140/303/130	121/295/172	CRC	12	0.83
88	Akkiz et al., 2011	Turkey	Asian	PCR-RFLP	HB	185/185	77/86/22	58/87/40	HCC	12	0.56
89	George et al., 2011	India	Asian	PCR-RFLP	PB	159/230	55/101/3	106/114/10	PC	10	0.003
90	Hong et al., 2011	South Korea	Asian	TaqMan	HB	406/428	86/224/96	96/198/134	LC	12	0.18
91	Jedlinski et al., 2011	Australia	Caucasian	PCR-RFLP	PB	187/171	68/86/33	58/82/31	BC	9	0.91
92	Mittal et al., 2011	India	Asian	PCR-RFLP	HB	212/250	76/131/5	109/127/14	BIC	10	0.004
93	Vinci et al., 2011	Italy	Caucasian	HRMA	HB	101/129	35/54/12	58/61/10	LC	9	0.34
94	Zhan et al., 2011	China	Asian	PCR-RFLP	HB	252/543	68/128/56	113/267/163	CRC	11	0.89
95	Catucci et al., 2010	Italy	Caucasian	TaqMan	PB	751/1243	334/330/87	532/550/161	BC	11	0.33
96	Christensen et al., 2010	Germany	Caucasian	TaqMan	PB	1,101/1,496	432/512/157	584/696/216	BC	11	0.74
		USA	Caucasian	TaqMan	PB	484/555	182/224/78	188/279/88	HNSCC	14	0.39
97	Dou et al., 2010	China	Asian	PCR-LDR	HB	643/656	111/343/189	143/305/208	Glioma	11	0.13
98	Li et al., 2010	China	Asian	PCR-RFLP	HB	310/222	78/150/82	42/102/78	HCC	9	0.46
99	Liu et al., 2010	USA	Caucasian	PCR-RFLP	HB	1,109/1,130	350/565/194	383/545/202	HNSCC	13	0.77
100	Okubo et al., 2010	Japan	Asian	PCR-RFLP	HB	552/697	105/281/166	124/350/223	GC	11	0.54
101	Peng et al., 2010	China	Asian	PCR-RFLP	HB	213/213	76/94/43	56/107/50	GC	10	0.96
102	Qi et al., 2010	China	Asian	PCR-LDR	PB	361/391	82/179/100	92/197/102	HCC	10	0.92

(Continued)

TABLE 1 | Continued

N	References	Country	Ethnicity	Genotyping Method	Design	Cases/Controls	Genotype distribution ^a		Cancer Type	Quality Score	P _{HWE}
							Cases	Controls			
103	Srivastava et al., 2010	India	Asian	PCR-RFLP	PB	230/230	119/95/16	136/75/19	GBC	12	0.09
104	Wang et al., 2010	China	Asian	SNaPshot	HB	458/489	148/262/48	128/250/111	ESCC	11	0.64
105	Kim et al., 2010	Korea	Asian	PCR-RFLP	PB	654/640	187/305/162	155/300/185	LC	14	0.14
106	Hoffman et al., 2009	USA	Caucasian	Mass-ARRAY	HB	426/466	181/209/36	166/229/71	BC	9	0.63
107	Hu et al., 2009	China	Asian	PCR-RFLP	PB	1,009/1,093	239/483/287	218/517/358	BC	14	0.22
108	Tian et al., 2009	China	Asian	PCR-RFLP	PB	1,058/1,035	253/512/293	209/519/307	LC	14	0.73
109	Horikawa et al., 2008	USA	Caucasian	Capillary electrophoresis	PB	276/277	105/126/45	101/117/59	RCC	11	0.03
110	Yang et al., 2008	USA	Caucasian	Capillary electrophoresis	PB	736/731	255/348/133	257/342/132	BIC	13	0.35

Genotype distributions are sorted as CC/CT/TT.

^aGenotype distributions are sorted as CC/CT/TT. PB, population-based design; HB, hospital-based design; NC, Not clear; ALL, Acute myeloblastic leukemia; BC, Breast cancer; BIC, Bladder cancer; CLL, Chronic lymphoblastic leukemia; CRC, Colorectal cancer; CESC, Cervical Squamous Cell Carcinoma; ESCC, Esophageal squamous cell carcinoma; GBC, Gallbladder carcinoma; GC, Gastric cancer; HCC, Hepatocellular carcinoma; HNSCC, Head and neck squamous cell carcinoma; LC, Lung cancer; NB, Neuroblastoma; NC, Nasopharyngeal carcinoma; NHL, non-Hodgkin lymphoma; OC, Oral cancer; OvC, Ovarian cancer; PC, Prostate cancer; PNC, Pancreatic cancer; RCC, Renal cell carcinoma.

et al., 2018). Characteristics of the included studies are tabulated in **Tables 1, 2**.

A total of 110 articles which included 111 studies (41,673 cases and 49,570 controls) evaluated the association of miR-196a-2 rs11614913 and cancer risk (**Table 1**). The article by Catucci et al. included two studies on separate populations (Catucci et al., 2010). In a study on head and neck carcinoma the genotype frequencies were not reported in the paper and data were retrieved by contacting the authors (Christensen et al., 2010). The final meta-analysis of mir-196a-2 rs11614913 and cancer risk included 111 studies (including 41,673 patients and 49,570 controls), among which 93 were scored greater than eight in the quality assessment and regarded as high quality studies. In 93 out of 111 studies, the genotype distribution of rs11614913 in the control group was concordant with HWE. Mir-196a-2 rs11614913 were genotype using a range of techniques with the most common being PCR-RFLP ($n = 53$). With regard to the ethnicity, 85 studies were performed in Asians, 20 were performed in Caucasians and the remaining six studies were included either Africans or individuals from different ancestries (mixed ancestry). In four studies the patients were subgrouped into multiple cancer types. Namely, Liu evaluated both ovarian and endometrial cancers (Liu, 2015), Parlayan evaluated gastric, lung, colorectal, prostate, and acute leukemia (Parlayan et al., 2014), Toraih studied both GI and non-GI cancers in a study (Toraih et al., 2016b) and hepatic and renal cancers in another study (Toraih et al., 2016a). For these studies genotype distribution of all patients were used to calculate point estimates in the overall analysis. However, in subgroup analysis, as these studies were assigned to more than one subgroup, the genotype distribution of patients with the relevant cancer category/type was used for pooling data. When studies were subgrouped according to the broad cancer category, there were

49 gastrointestinal tract cancers (GI), nine head and neck squamous cell carcinoma (HNSCC), six gynecologic cancers (GyC), six hematological malignancies (HM), 12 urogenital cancers (UG), and 34 other cancers. When studies subgrouped according to cancer type, there were 21 breast cancer (BC), 18 hepatocellular carcinoma (HCC), 13 gastric cancer (GC), ten colorectal cancer (CRC), nine lung cancer (LC), four bladder cancer (BIC), five prostate cancer (PC), six oral carcinoma (OC), four ovarian cancer (OvC), six esophageal cancer (ESCC), and 22 other cancer types.

Moreover, 43 articles comprising 44 studies (15,954 cases and 19,594 controls) evaluated the association of mir-149 rs2292832 and cancer risk (**Table 2**), among which 39 studies were evaluated as being high quality (quality score > 8). The genotype distribution of rs2292832 in the control groups of 34 studies were in agreement with HWE. The main genotyping technique was PCR-RFLP (29 studies). Most studies ($n: 39$) were performed in Asian populations and only few studies ($n: 5$) had focused on Caucasians. According to the broad cancer category, there were 22 GI studies, seven HNC studies and 15 studies on other cancer types. When studies were subgrouped by cancer type, there were four studies on BC, nine studies on HCC, eight studies on GC, five studies on CRC, three studies on LC and 15 studies on other types of cancer.

Quantitative Synthesis

Association of mir-196a2 rs11614913 and Cancer Risk

Statistically significant associations between mir-196a2 rs11614913 and cancer risk were observed assuming the homozygote (TT vs. CC, OR_{RE} [95% CI]: 0.88 [0.79–0.98], $P: 0.027$) and the recessive (TT vs. CC+CT, OR_{RE} [95%

TABLE 2 | Main characteristics of studies evaluating cancer risk associated with miR-149 rs2292832 C/T which included in the current meta-analysis.

N	References	Country	Ethnicity	Genotyping method	Design	Cases/Controls	Genotype distribution ^a		Cancer type	Quality score	P _{HWE}
							Cases	Controls			
1	He J. et al., 2018	China	Asian	TaqMan	HB	380/791	32/62/286	59/172/560	NB	10	2.98e-14
2	Ranjbar et al., 2018	Iran	Asian	PCR-RFLP	PB	73/70	8/14/51	4/24/42	CRC	8	0.92
3	Cimpeanu et al., 2017	Romania	Caucasian	TaqMan	HB	142/288	70/49/23	126/120/42	GC	10	0.15
4	Tandon et al., 2017	India	Asian	PCR-RFLP	HB	200/200	140/37/23	174/16/10	OC	9	7.96e-12
5	Gu and Tu, 2016	China	Asian	PCR-RFLP	HB	186/186	55/89/42	60/87/39	GC	7	0.54
6	Hashemi et al., 2016	Iran	Asian	t-ARMS-PCR	HB	169/182	77/68/24	101/57/24	PC	6	0.002
7	Jiang et al., 2016	China	Asian	MassARRAY	PB	869/955	65/164/640	66/204/685	GC	12	8.88e-16
8	Li H. et al., 2016	China	Asian	TaqMan	PB	555/395	49/252/254	42/176/177	LC	13	0.92
9	Miao et al., 2016	China	Asian	Array	HB	575/1,548	61/231/283	175/647/726	HNSCC	12	0.10
10	Ni and Huang, 2016	China	Asian	PCR-RFLP	HB	155/342	47/82/26	108/179/55	OvC	9	0.20
11	Su et al., 2016	China	Asian	PCR-RFLP	HB	245/315	108/112/25	149/140/26	GC	10	0.44
12	Yang et al., 2016	China	Asian	PCR-RFLP	PB	30/98	3/16/11	9/49/40	KS	9	0.35
13	Dong et al., 2015	China	Asian	MassARRAY	PB	369/751	63/175/131	91/339/321	PTC	12	0.96
14	He et al., 2015	China	Asian	Mass-ARRAY	PB	450/450	36/183/231	60/188/202	BC	11	0.14
15	Liu, 2015	China	Asian	PCR-RFLP	PB	266/266	91/130/45	108/124/34	HCC	12	0.76/
16	Pratedrat et al., 2015	Thailand	Asian	TaqMan	HB	104/95	11/27/66	9/24/62	HCC	8	0.018
17	Sushma et al., 2015	India	Asian	PCR-RFLP	PB	100/102	72/22/6	90/12/0	OC	9	0.79
18	Yan et al., 2015	China	Asian	PCR-RFLP	HB	274/328	66/133/75	72/156/100	HCC	10	0.50
19	Chu et al., 2014	Taiwan	Asian	PCR-RFLP	PB	188/337	13/36/139	27/64/246	HCC	11	8.9e-10
20	Dikeakos et al., 2014	Greece	Caucasian	PCR-RFLP	HB	163/480	21/73/69	33/198/249	GC	9	0.50
21	Du et al., 2014	China	Asian	TaqMan	PB	355/362	47/163/145	37/148/177	RCC	12	0.52
22	Kou et al., 2014	China	Asian	PCR-RFLP	PB	270/532	113/122/35	202/253/77	HCC	10	0.93
23	Liu et al., 2014	China	Asian	PCR-RFLP	PB	327/327	84/143/100	56/138/133	HCC	11	0.066
24	Pu et al., 2014	China	Asian	PCR-RFLP	HB	187/459	22/31/134	48/103/308	GC	9	8.5e-13
25	Wang R. et al., 2014	China	Asian	MassARRAY	HB	772/717	83/318/371	56/309/352	HCC	9	0.33
26	Wang X. H. et al., 2014	China	Asian	PCR-RFLP	PB	152/304	13/72/67	43/148/113	HCC	11	0.68
27	Wei et al., 2014	China	Asian	MassARRAY	PB	838/1,006	105/354/379	86/424/496	PTC	14	0.77
28	Ahn et al., 2013	South Korea	Asian	PCR-RFLP	PB	461/447	44/176/241	40/187/220	GC	12	0.94
29	Hu et al., 2013	China	Asian	Sequencing	HB	680/690	70/297/313	78/302/310	Glioma	14	0.78
30	Huang et al., 2013	China	Asian	PCR-RFLP	PB	158/242	22/67/69	39/113/90	NPC	11	0.8
31	Lv et al., 2013	China	Asian	PCR-RFLP	PB	347/459	30/64/253	48/103/308	CRC	10	8.5e-13
32	Min et al., 2012	South Korea	Asian	PCR-RFLP	PB	446/502	48/177/221	51/219/232	CRC	12	0.97
33	Vinci et al., 2013	Italy	Caucasian	HRMA	HB	160/178	79/58/23	86/75/17	CRC	9	0.95
34	Chu et al., 2012	Taiwan	Asian	PCR-RFLP	PB	470/425	37/88/345	26/84/315	OC	10	1.1e-7
35	Kim et al., 2012	South Korea	Asian	PCR-RFLP	PB	159/201	14/64/81	21/97/83	HCC	11	0.40
36	Tu et al., 2012	Taiwan	Asian	PCR-RFLP	PB	273/122	20/129/124	21/52/49	HNSCC	7	0.33
37	Zhang M. et al., 2012	China	Asian	PCR-RFLP	PB	245/229	23/102/120	24/113/92	BC	13	0.25

(Continued)

TABLE 2 | Continued

N	References	Country	Ethnicity	Genotyping method	Design	Cases/Controls	Genotype distribution ^a		Cancer type	Quality score	<i>P</i> _{HWE}
							Cases	Controls			
38	Zhang M. W. et al., 2012	China	Asian	PCR-RFLP	PB	443/435	50/190/203	46/202/187	CRC	14	0.48
					PB	274/269	41/101/132	35/120/114	GC	14	0.77
39	Vinci et al., 2011	Italy	Caucasian	HRMA	HB	101/129	44/41/16	65/53/11	LC	9	0.89
40	Kontorovich et al., 2010	Israel	Asian	MassARRAY	PB	167/122	87/40/40	53/30/39	Cancer	9	6.5e-8
41	Liu et al., 2010	USA	Caucasian	PCR-RFLP	HB	1,109/1,130	580/441/88	586/445/99	HNSCC	13	0.29
42	Hu et al., 2009	China	Asian	PCR-RFLP	PB	1,009/1,093	450/460/99	482/503/108	BC	14	0.17
43	Tian et al., 2009	China	Asian	PCR-RFLP	PB	1,058/1,035	123/472/463	112/453/470	LC	14	0.89

Genotype distributions are sorted as CC/CT/TT.

^aGenotype distributions are sorted as CC/CT/TT. PB, population-based design; HB, hospital-based design; BC, Breast cancer; CRC, Colorectal cancer; GC, Gastric cancer; HCC, Hepatocellular carcinoma; HNSCC, Head and neck squamous cell carcinoma; LC, Lung cancer; NB, Neuroblastoma; NC, Nasopharyngeal carcinoma; NSCLC, non-Small cell lung carcinoma; OC, Oral cancer; OvC, Ovarian cancer; PC, Prostate cancer; PTC, Papillary thyroid carcinoma; RCC, Renal cell carcinoma; KS, Kaposi sarcoma.

TABLE 3 | Summary of the results of meta-analysis of cancer risk associated with miR-196a-2 rs11614913 and miR-149 rs2292832.

Genetic models	n ^a	Cases/controls	OR ^b (95% CI)	<i>P</i> ^c	<i>P</i> _{Het} ^d	<i>I</i> ²	<i>P</i> _{bias} ^e
miR-196a2 rs11614913							
Homozygote (TT vs. CC)	109	41,342/49,268	0.88 (0.79–0.98)	0.027	<0.0001	77.1%	0.370
Heterozygote (CT vs. CC)	111	41,673/49,570	1.00 (0.92–1.07)	0.960	<0.0001	70.4%	0.832
Dominant (TT+CT vs. CC)	111	41,673/49,570	0.96 (0.89–1.04)	0.429	<0.0001	76.8%	0.666
Recessive (TT vs. CT+CC)	109	41,342/49,268	0.89 (0.83–0.95)	0.001	<0.0001	68.6%	0.496
Allelic (T vs. C)	111	41,673/49,570	0.95 (0.90–1.00)	0.051	<0.0001	78.9%	0.148
miR-149 rs2292832							
Homozygote ^f (TT vs. CC)	44	15,954/19,594	0.89 (0.77–1.02)	0.119	<0.0001	64.2	0.004
Heterozygote (CT vs. CC)	44	15,954/19,594	0.96 (0.87–1.05)	0.427	0.008	37.1	0.478
Dominant (CT+TT vs. CC)	44	15,954/19,594	1.00 (0.90–1.11)	0.955	<0.0001	53.5	0.175
Recessive ^f (TT vs. CC+CT)	44	15,954/19,594	1.00 (0.92–1.08)	0.907	<0.0001	48.6	0.045
Allelic (T vs. C) ^f	44	15,954/19,594	0.96 (0.88–1.04)	0.358	<0.0001	73.6	0.026

^aNumber of studies in each contrast.

^bPooled OR and 95% CI (Random-effect model).

^c*P*-value of the Z-test.

^d*P*-value of the Q-test.

^e*P*-value of the Begg's test.

^fFor these models (homozygote, recessive, and allelic comparisons of rs2292832) "trim and fill" adjusted results are shown (consult with **Supplementary Figure S4** for forest plots and **Supplementary Figure S11** for funnel plots of these models).

Significant associations are shown in boldface.

CI]: 0.89 [0.83–0.95], *P*: 0.001) models (Table 3). Mir-196a-2 rs11614913 was not associated with cancer risk in the heterozygote and dominant models and there was a non-significant borderline association in the allele contrast (Table 3). **Supplementary Figure S1** shows the forest plots of association of mir-196a-2 rs11614913 and cancer risk in five models. The results of subgroup analysis for mir-196a-2 rs11614913 are shown in Table 4. Decreased risk of cancer was found in high quality studies under the homozygote (TT vs. CC, OR_{RE} [95% CI]: 0.87 [0.77–0.97], *P*: 0.017), the recessive (TT vs. CC+CT, OR_{RE} [95% CI]: 0.88 [0.81–0.95], *P*: 0.001) and the allele contrasts (T vs. C, OR_{RE} [95% CI]: 0.93 [0.88–0.99], *P*: 0.020). In subgroup analysis by genotyping method, the only significant

association was observed under the recessive model for studies which used a method other than PCR-RFLP (TT vs. CC+CT, OR_{RE} [95% CI]: 0.89 [0.82–0.96], *P*: 0.007). When sub-grouped by ethnicity, decreased risks of cancer under the homozygote (TT vs. CC, OR_{RE} [95% CI]: 0.86 [0.77–0.97], *P*: 0.016), the recessive (TT vs. CC+CT, OR_{RE} [95% CI]: 0.87 [0.81–0.93], *P*: 0.0004) and the allelic (T vs. C, OR_{RE} [95% CI]: 0.93 [0.89–0.98], *P*: 0.015) models were found only in Asians but not in Caucasians or the African/mixed ancestry subgroups (Table 4, Figure 2). Subgrouping by broad cancer categories indicated that mir-196a-2 rs11614913 was associated by a decreased risk of gynecologic cancer (GyC) assuming the recessive model (TT vs. CC+CT, OR_{FE} [95% CI]: 0.80 [0.68–0.95], *P*: 0.010) and the allelic contrast

TABLE 4 | Meta-analysis of miR-196a-2 rs11614913 and cancer risk.

Groups	Cases/Controls ^a	Homozygote (TT vs. CC)			Heterozygote (CT vs. CC)			Dominant (TT+CT vs. CC)			Recessive (TT vs. CT+CC)			Allelic (T vs. C)		
		OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²
All studies	41,673/43,570	0.88 (0.79-0.98)	<1e-4	77.1	1.00 (0.93-1.08)	<1e-4	70.4	0.97 (0.89-1.05)	<1e-4	76.8	0.89 (0.83-0.96)	<1e-4	68.6	0.95 (0.90-1.00)	<1e-4	78.9
Controls in HWE ^d	36,620/42,501	0.88 (0.80-0.98)	<1e-4	74.6	1.00 (0.93-1.08)	<1e-4	66.3	0.97 (0.89-1.04)	<1e-4	74.3	0.88 (0.82-0.95)	<1e-4	67.7	0.94 (0.89-0.99)	<1e-4	77.3
QUALITY																
High (>8)	38,503/46,453	0.87 (0.77-0.97)	<1e-4	78.1	0.98 (0.91-1.06)	<1e-4	69.6	0.94 (0.87-1.03)	<1e-4	76.7	0.88 (0.81-0.95)	<1e-4	70.9	0.93 (0.88-0.99)	<1e-4	79.7
Low (≤8)	3170/3117	0.98 (0.70-1.38)	<1e-4	68.3	1.09 (0.80-1.48)	<1e-4	71.9	1.07 (0.80-1.44)	<1e-4	74	0.97 (0.79-1.18)	0.025	44.5	1.03 (0.87-1.22)	<1e-4	71
GENOTYPING																
PCR-RFLP	15,282/18,081	0.85 (0.69-1.04)	<1e-4	81.4	0.96 (0.83-1.11)	<1e-4	77.8	0.93 (0.80-1.09)	<1e-4	82.9	0.89 (0.78-1.00)	<1e-4	71.1	0.93 (0.85-1.02)	<1e-4	84
Others	26,391/31,489	0.91 (0.82-1.01)	<1e-4	70.3	1.03 (0.96-1.11)	<1e-4	57.2	1.00 (0.92-1.07)	<1e-4	64.8	0.89 (0.82-0.96)	<1e-4	66.5	0.96 (0.91-1.01)	<1e-4	70.7
ETHNICITIES																
Asian	33,039/38,092	0.86 (0.77-0.97)	<1e-4	76.4	1.0 (0.92-1.09)	<1e-4	68.3	0.96 (0.88-1.05)	<1e-4	74.1	0.87 (0.81-0.93)	<1e-4	68.2	0.93 (0.89-0.98)	<1e-4	76.4
Caucasian	7273/9737	0.86 (0.63-1.18)	<1e-4	81.1	0.92 (0.75-1.13)	<1e-4	78.3	0.91 (0.72-1.15)	<1e-4	85.1	0.92 (0.73-1.15)	<1e-4	71.2	0.94 (0.78-1.13)	0.00	86.5
Africans/mixed	1361/1741	1.40 (0.89-2.21)	0.058	53.1	1.27 (0.87-1.86)	0.01	63.1	1.31 (0.95-1.80)	0.02	60.5	1.24 (0.77-2.0)	0.05	54.3	1.21 (0.99-1.49)	0.047	55.3
CANCER CATEGORIES																
GI	16,537/21,226	0.87 (0.71-1.06)	<1e-4	83.7	0.96 (0.84-1.10)	<1e-4	76.8	0.93 (0.80-1.08)	<1e-4	82.9	0.90 (0.79-1.02)	<1e-4	76.1	0.94 (0.85-1.03)	<1e-4	85
HNC	4,865/5,648	1.05 (0.76-1.46)	0.001	68.9	1.10 (0.79-1.54)	<1e-4	75	1.08 (0.78-1.49)	<1e-4	76.2	0.99 (0.90-1.09)	0.085	42.3	1.01 (0.85-1.21)	5e-4	71.2
GyC	1,394/1,573	0.85 (0.53-1.36)	0.042	56.6	1.02 (0.66-1.58)	0.061	52.5	0.96 (0.61-1.50)	0.030	59.5	0.80 (0.68-0.95)	0.346	10.8	0.88 (0.79-0.98)	0.089	47.6
HIM	1,347/2,571	0.71 (0.34-1.49)	1e-4	80.4	0.91 (0.77-1.09)	0.071	50.7	0.79 (0.52-1.19)	0.008	67.5	0.80 (0.42-1.51)	0.00	80.2	0.83 (0.57-1.21)	0.00	80.5
UG	3,599/4,356	0.98 (0.74-1.30)	0.002	61.5	1.19 (0.96-1.48)	0.003	60.5	1.15 (0.94-1.42)	0.002	61.3	0.91 (0.70-1.19)	0.001	63.8	1.047 (0.91-1.19)	0.002	61.5
Others	13,710/16,018	0.86 (0.76-0.98)	<1e-4	57.2	0.98 (0.88-1.10)	<1e-4	56.7	0.95 (0.85-1.06)	<1e-4	62.4	0.87 (0.79-0.95)	0.003	44.6	0.94 (0.87-1.00)	<1e-4	62.3

(Continued)

TABLE 4 | Continued

Groups	Cases/Controls ^a	Homozygote (TT vs. CC)			Heterozygote (CT vs. CC)			Dominant (TT+CT vs. CC)			Recessive (TT vs. CT+CC)			Allelic (T vs. C)		
		OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²
CANCER TYPES																
BC ^e	7,401/8,828	0.85 (0.71–1.01)	0.001	56.4	0.93 (0.81–1.08)	0.002	53.3	0.91 (0.78–1.06)	2e–4	60.8	0.89 (0.79–1.01)	0.024	43	0.93 (0.84–1.02)	<1e–4	62.7
HCC ^e	5,401/6,326	0.73 (0.57–0.94)	<1e–4	69.5	0.92 (0.78–1.09)	0.002	55.2	0.87 (0.73–1.04)	<1e–4	64.7	0.79 (0.66–0.95)	2e–4	63.2	0.88 (0.78–0.98)	<1e–4	68.8
GC	4,664/6,385	0.80 (0.47–1.36)	<1e–4	90.3	0.81 (0.56; 1.15)	<1e–4	88.6	0.80 (0.53–1.20)	<1e–4	92.1	0.91 (0.65–1.29)	<1e–4	81.5	0.88 (0.66–1.17)	<1e–4	92.4
CRC ^e	2,567/4,211	1.21 (0.65–2.27)	<1e–4	87.6	1.12 (0.71–1.75)	<1e–4	81.1	1.13 (0.70–1.85)	<1e–4	84.7	1.08 (0.83–1.42)	<1e–4	78.6	1.06 (0.86–1.29)	<1e–4	86.4
LC ^e	4,453/4,932	0.86 (0.63–1.17)	0.009	60.2	1.01 (0.91–1.12)	0.057	46.9	1.00 (0.82–1.21)	0.015	57.7	0.82 (0.65–1.03)	0.012	59	0.93 (0.81–1.08)	0.004	64.5
BIC	1,390/1,562	0.75 (0.41–1.39)	0.060	59.3	1.01 (0.53–1.89)	0.017	70.4	0.97 (0.62–1.52)	0.088	54	0.77 (0.30–1.97)	3e–4	84.3	0.94 (0.68–1.29)	0.036	64.7
PC	868/1,345	1.01 (0.74–1.39)	0.396	1.8	1.27 (0.83–1.95)	0.057	56.4	1.23 (0.83–1.83)	0.069	54	0.94 (0.71–1.24)	0.485	0	1.06 (0.93–1.22)	0.241	27.1
OC	2,138/2,957	0.98 (0.49–1.95)	<1e–4	81.6	1.14 (0.57–2.29)	<1e–4	82.3	1.07 (0.53–2.12)	<1e–4	85	0.87 (0.76–1.00)	0.189	32.8	0.96 (0.66–1.40)	<1e–4	82
OvC	843/1,100	0.81 (0.28–2.37)	0.023	68.4	1.03 (0.38–2.78)	0.029	66.6	0.95 (0.34–2.63)	0.017	70.4	0.73 (0.60–0.90)	0.332	12.1	0.88 (0.58–1.32)	0.050	61.6
ESOC	3,492/4,376	0.85 (0.49–1.47)	<1e–4	82.4	1.04 (0.83–1.31)	0.039	57.2	0.99 (0.76; 1.29)	0.008	67.8	0.81 (0.51–1.28)	<1e–4	81.3	0.94 (0.76–1.16)	6e–4	76.8
Others	8,214/11,444	0.98 (0.80–1.20)	<1e–4	68.5	1.05 (0.91–1.22)	<1e–4	61.5	1.03 (0.88–1.21)	<1e–4	68.3	0.96 (0.83–1.11)	<1e–4	61.7	1.00 (0.89–1.11)	<1e–4	72

The overall, HWD-sensitivity and subgroup analyses.

^an represents number of cases and controls in each group.

^bPooled ORs and 95% confidence intervals.

^cP-value of the heterogeneity test.

^dMeta-analysis of all studies excluding those with the control group not in HWE.

^eThese subgroups were found to be influenced by departure from HWE. Please consult with the **Supplementary Table S2** for details on HWD sensitivity and adjustments.

GI, cancers of digestive system; HNC, Head and neck carcinoma; GYC, Gynecologic cancers; HM, Hematological malignancies; UG, Urogenital cancers; BC, breast cancer; HCC, Hepatocellular cancer; GC, gastric cancer; CRC, colorectal cancer; LC, lung cancer; BIC, bladder cancer; PC, prostate cancer; OC, Oral cancer; OVC, ovarian cancer; ESCC, esophageal squamous cell carcinoma.

Significant associations are shown in boldface.

(T vs. C, OR_{FE} [95% CI]: 0.88 [0.79–0.98], P : 0.021) (**Figure 3**). No significant findings were observed for gastrointestinal, head and neck, hematological or urogenital cancers (**Table 4**). **Supplementary Figure S2** presents the forest plots for subgroups according to the broad cancer categories. Further subgrouping by cancer type revealed significant association of mir-196a-2 rs11614913 with hepatocellular carcinoma (**Figure 4**) under the homozygote model (TT vs. CC, OR_{RE} [95% CI]: 0.73 [0.57–0.94], P : 0.017), the recessive model (TT vs. CC+CT, OR_{RE} [95% CI]: 0.79 [0.66–0.95], P : 0.017) and the allele contrast (T vs. C, OR_{FE} [95% CI]: 0.88 [0.78–0.98], P : 0.030), and with ovarian cancer (**Figure 5**) under the recessive model (TT vs. CC+CT, OR_{FE} [95% CI]: 0.73 [0.60–0.90], P : 0.003). **Supplementary Figure S3** presents the forest plots for subgroups according to cancer type.

HWD sensitivity analysis (i.e., excluding studies with controls deviated from HWE) revealed stable results in the overall analysis under the homozygote, heterozygote, dominant, and recessive models (**Table 4**). However, excluding HWD studies made the borderline allele contrast statistically significant (**Table 4**). Moreover, excluding HWE violating studies had no dramatic effects on subgroup meta-analyses using quality of studies, genotyping methods, the ethnicity and the broad cancer category (**Supplementary Table S1**). In meta-analysis subgrouped by cancer type, the results were also stable for gastric, bladder, oral, ovarian, prostate, and esophageal cancer subgroups after excluding HWD studies (**Supplementary Table S1**). However, excluding such studies altered the results for the breast, hepatocellular, colorectal, and lung cancer subgroups. Therefore, for these subgroups, pooled ORs were estimated to account for departures from HWE (denoted as HWD-adjusted ORs) (**Supplementary Table S2**). When corrected for HWD, mir-196a-2 rs11614913 was found to be significantly associated with breast cancer under the homozygote (TT vs. CC, HWD-adjusted OR_{RE} [95% CI]: 0.75 [0.61–0.93], P : 0.011) and recessive (TT vs. CC+CT, HWD-adjusted OR_{RE} [95% CI]: 0.84 [0.71–0.98], P : 0.030) models (**Figure 6**). The association with hepatocellular cancer under the homozygote and the recessive models was remained significant after adjustment for HWD (TT vs. CC, HWD-adjusted OR_{RE} [95% CI]: 0.69 [0.53–0.91], P : 0.011 and TT vs. CC+CT, HWD-adjusted OR_{RE} [95% CI]: 0.72 [0.57–0.90], P : 0.008). Furthermore, adjustment for HWD confirmed that mir-196a-2 rs11614913 is not associated with colorectal or lung cancer assuming any genetic model (**Supplementary Table S2**).

Association of mir-149 rs2292832 and Cancer Risk

The overall analysis showed no significant association with cancer risk under any genetic model (**Table 3**). **Supplementary Figure S4** shows the forest plots for the association of mir-149 rs2292832 and cancer risk under different genetic models. However, in subgroup analyses (**Table 5**) significant association of rs2292832 with cancer risk was observed in studies which used a genotyping method other than PCR-RFLP (CT vs. CC, OR_{FE} [95% CI]: 0.88 [0.79–0.98], P : 0.025). When subgrouped by broad cancer category, a decreased risk of gastrointestinal tract cancers was found in the heterozygote model (**Figure 7A**, CT vs. CC, OR_{FE}

[95% CI]: 0.87 [0.79–0.97], P : 0.011). Subgrouping by cancer type, however, revealed an increased risk of colorectal cancer for individuals carrying TT genotype compared to those who carry at least one C allele (**Figure 7B**, TT vs. CT+CC, OR_{FE} [95% CI]: 1.21 [1.04–1.40], P : 0.011). No significant association was observed for other comparisons (**Table 5**). **Supplementary Figures S5, S6** show the forest plots for subgroup analysis according to the broad cancer category and cancer type, respectively. Sensitivity analysis revealed that HWD studies had no significant effect on point estimates in the overall meta-analysis of mir-149 rs2292832 and cancer risk, and still no significant association was observed in overall analysis (**Table 5**). Moreover, most subgroup analyses were also stable after removing HWD studies (**Supplementary Table S3**). However, removing studies with HWD controls influenced comparisons in three subgroups: (i) non-PCR-RFLP subgroup (heterozygote model); (ii) the breast cancer subgroup (recessive model); (iii) the colorectal cancer subgroup (recessive model). Therefore, for these subgroups, HWE-expected genotype distributions in controls were used for pooling ORs (denoted as HWD-adjusted OR) (**Supplementary Table S4**). Adjusting for HWD in these subgroups confirmed the results of original analyses and showed that rs2292832 is associated with cancer risk in non-RFLP subgroup under the heterozygote model (**Figure 8**, CT vs. CC, HWD-adjusted OR_{RE} [95% CI]: 0.68 [0.48–0.98], P : 0.040) and with colorectal cancer risk under the recessive model (TT vs. CT+CC, HWD-adjusted OR_{FE} [95% CI]: 1.29 [1.11–1.50], P : 0.0007). No association with breast cancer risk was identified after adjusting for HWD (**Supplementary Table S4**).

Heterogeneity, Meta-regression, and Sensitivity Analysis

Heterogeneity was evaluated for both polymorphisms in all genetic models (**Tables 3, 4, 5**). Significant between study heterogeneity was observed in the overall estimation under all genetic models for mir-196a-2 rs11614913 and consequently random effect model was used. Univariate meta-regression using cancer type, country, ethnicity, the quality of study (either high or low), genotyping method, source of controls (PB or HB) or HWE was performed to identify potential sources of heterogeneity. For mir-196a-2, meta-regression showed that at least a part of the observed between study heterogeneity in the heterozygote (R^2 : 24.23%, P : 0.007) and dominant (R^2 : 19.86%, P : 0.028) models could be attributed to the country moderator. However, there was still significant unaccounted heterogeneity even after correcting for the effect of country moderator (Heterozygote I^2 : 63.78 and dominant I^2 : 72.05, P for test of residual heterogeneity < 0.0001). Moreover, Galbraith plot analysis demonstrated three studies (Lv et al., 2013; Wang et al., 2013; Dikeakos et al., 2014) as the most extreme outliers in all genetic models that account for a considerable portion of the observed heterogeneities (**Supplementary Figure S7**). Excluding these studies led to a 11.6% reduction of I^2 in the homozygote model (from 77.1 to 65.5%), a 12.8% reduction in the heterozygote model (from 70.6 to 57.8%), a 12.3%

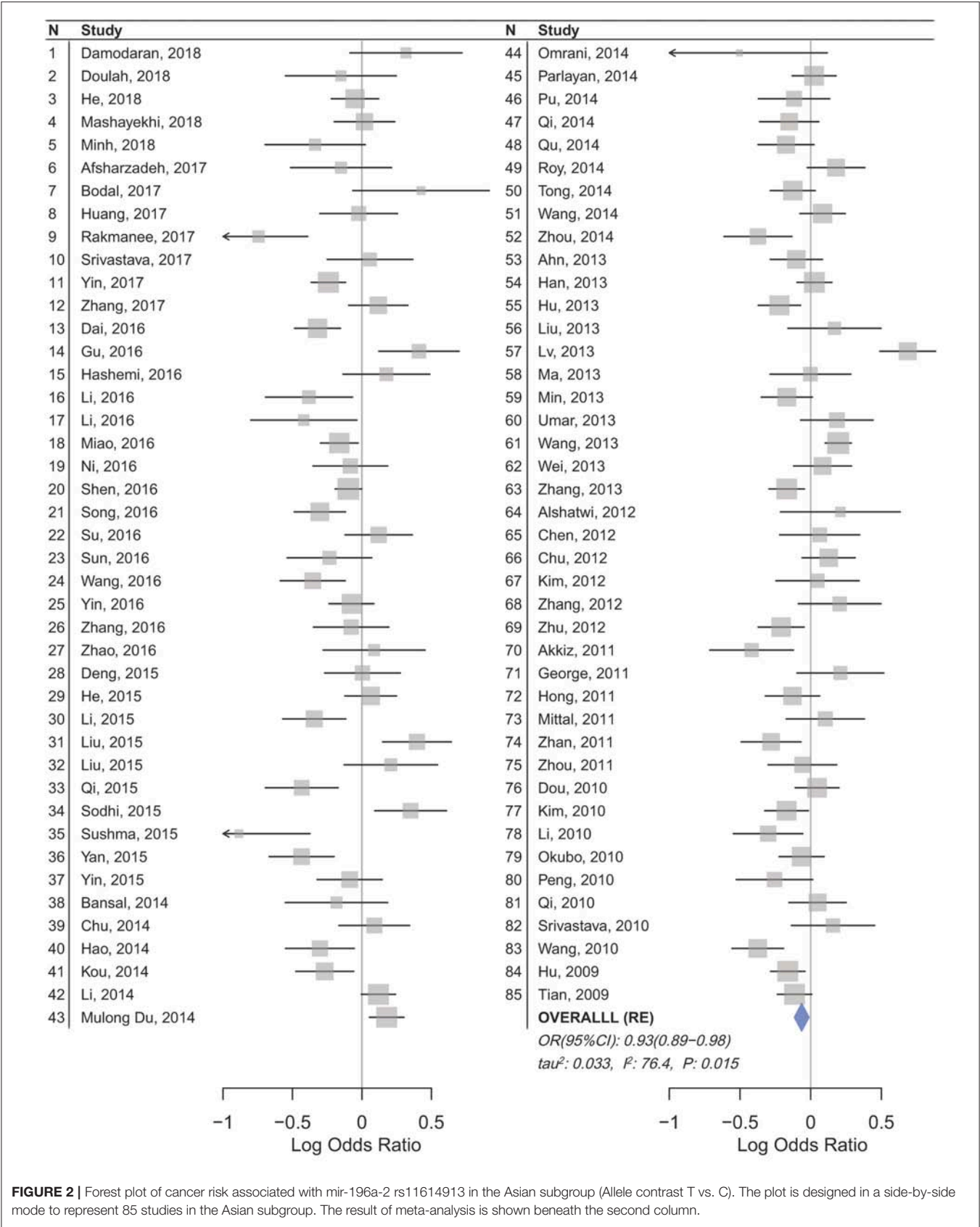
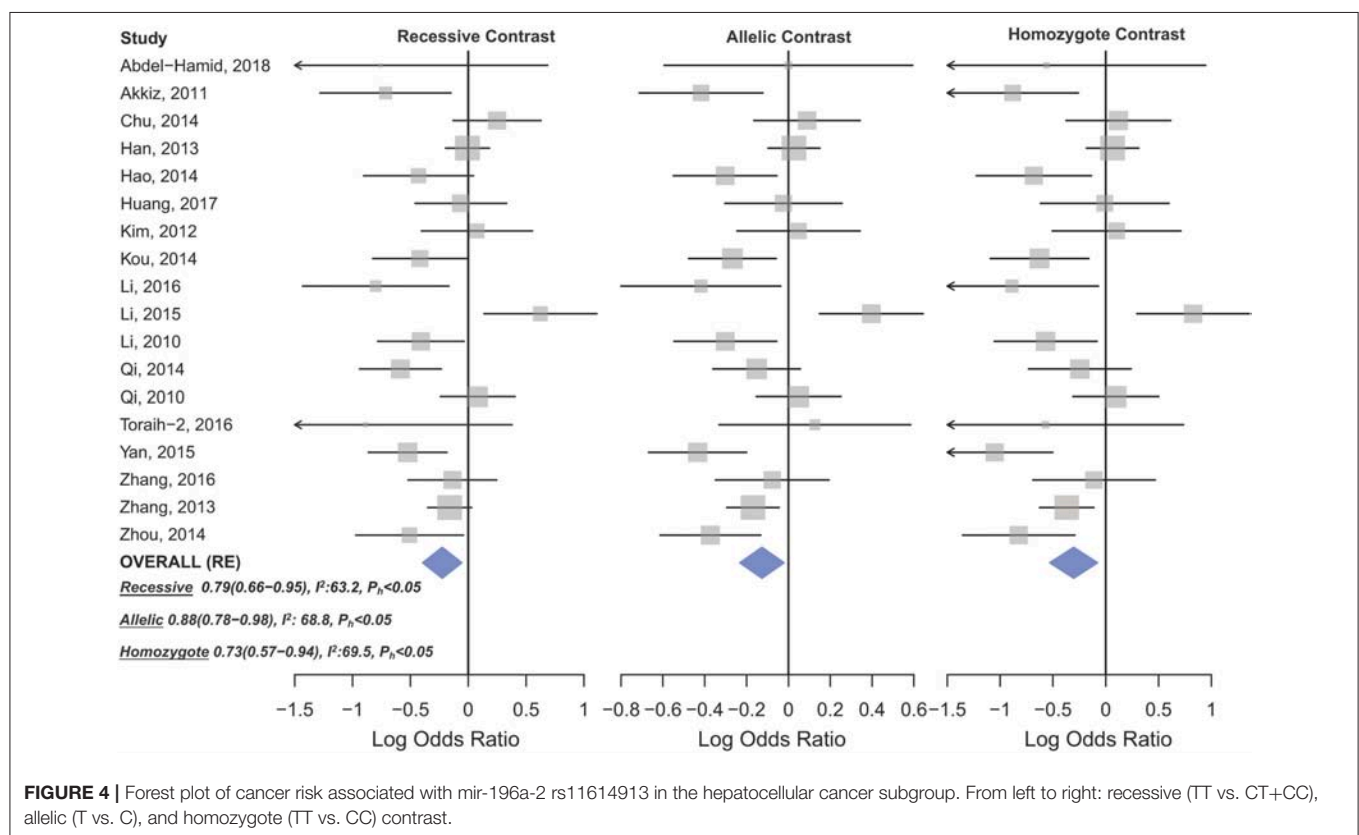
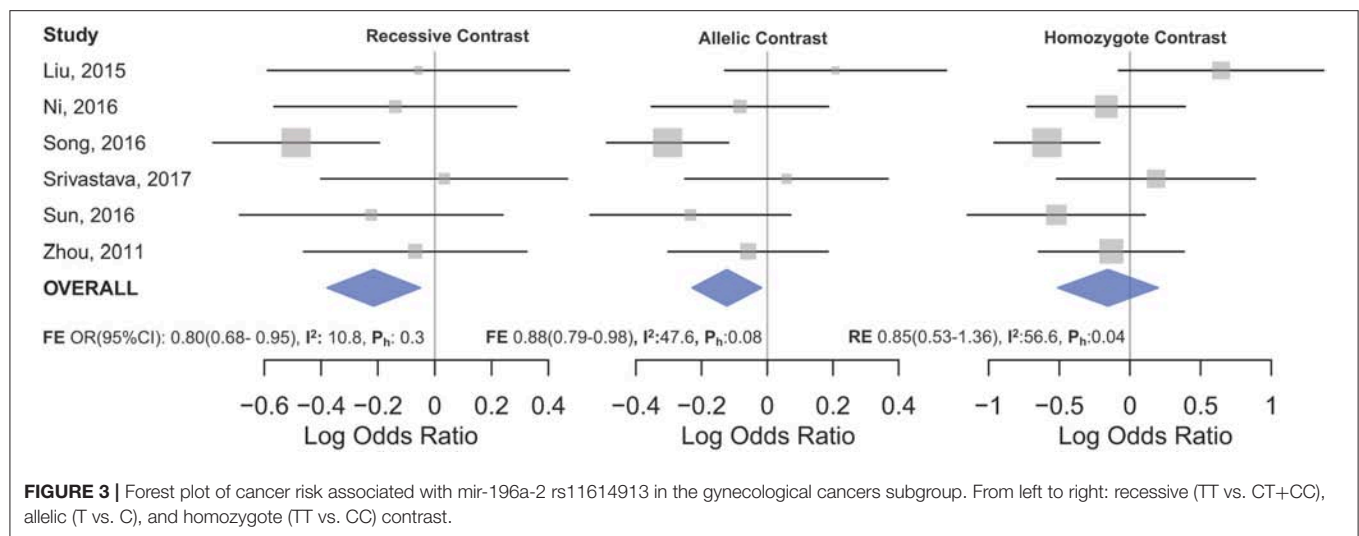


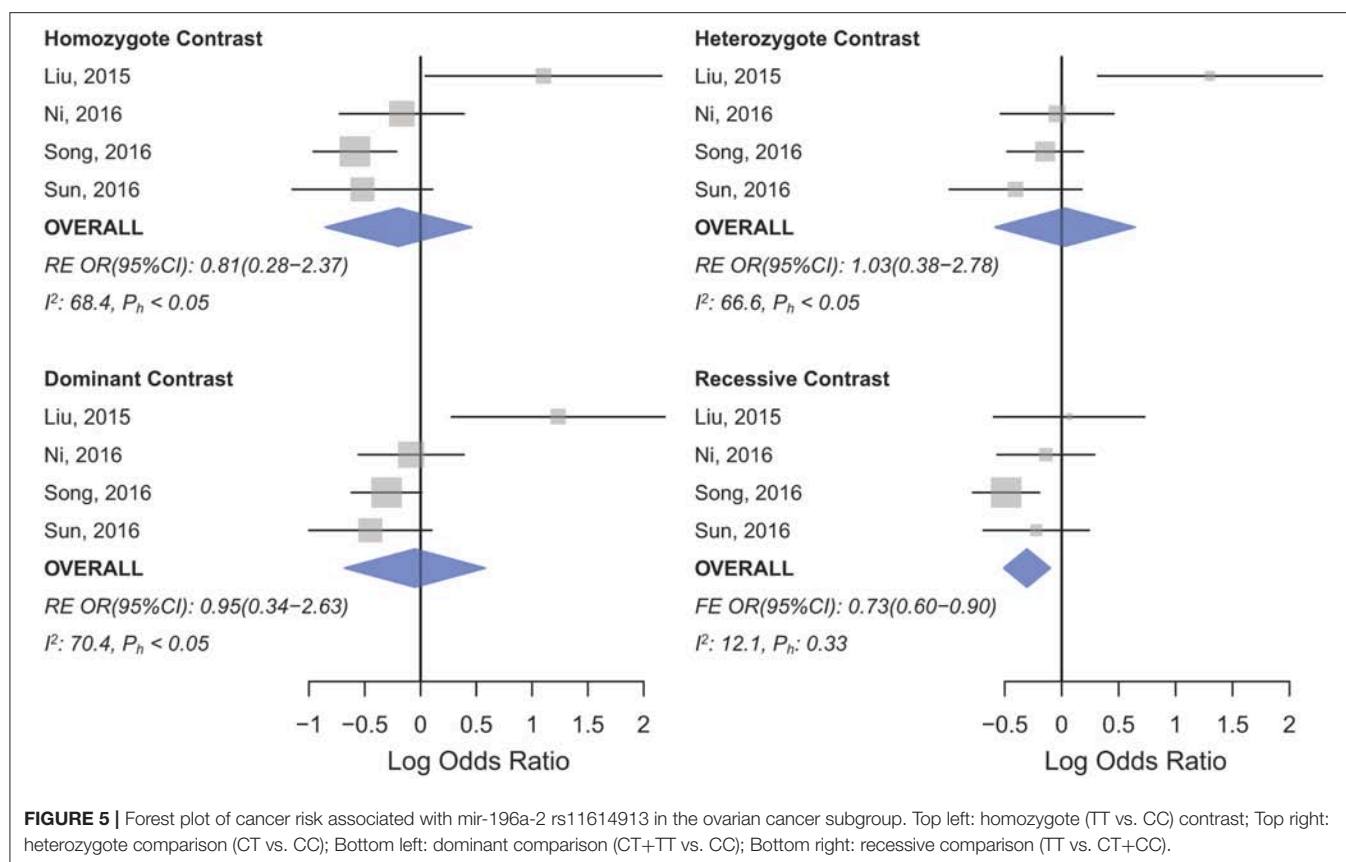
FIGURE 2 | Forest plot of cancer risk associated with mir-196a-2 rs11614913 in the Asian subgroup (Allele contrast T vs. C). The plot is designed in a side-by-side mode to represent 85 studies in the Asian subgroup. The result of meta-analysis is shown beneath the second column.



reduction in the dominant model (from 76.9 to 64.6%), an 9.1% reduction in the recessive model (from 68.8 to 59.7%) and a 11.3% reduction in the allelic model (from 79 to 67.7%). However, excluding these studies did not alter any genotypic contrast and results were comparable to the overall analyses (data not shown). Sensitivity analysis by omitting one study at a time revealed that no individual study significantly influenced the genotype contrasts (**Supplementary Figure S8**).

In the allele contrast, omitting no single study dramatically influenced pooled OR or its 95%CI. However, given that the original 95%CI was borderline (0.90-1.00), omitting some studies lead the upper limit of 95%CI to fall slightly below one (**Supplementary Figure S8-e**).

Statistically significant heterogeneity was also observed in the overall analysis of miR-149 rs2292832 and cancer risk and, consequently, RE model was used to



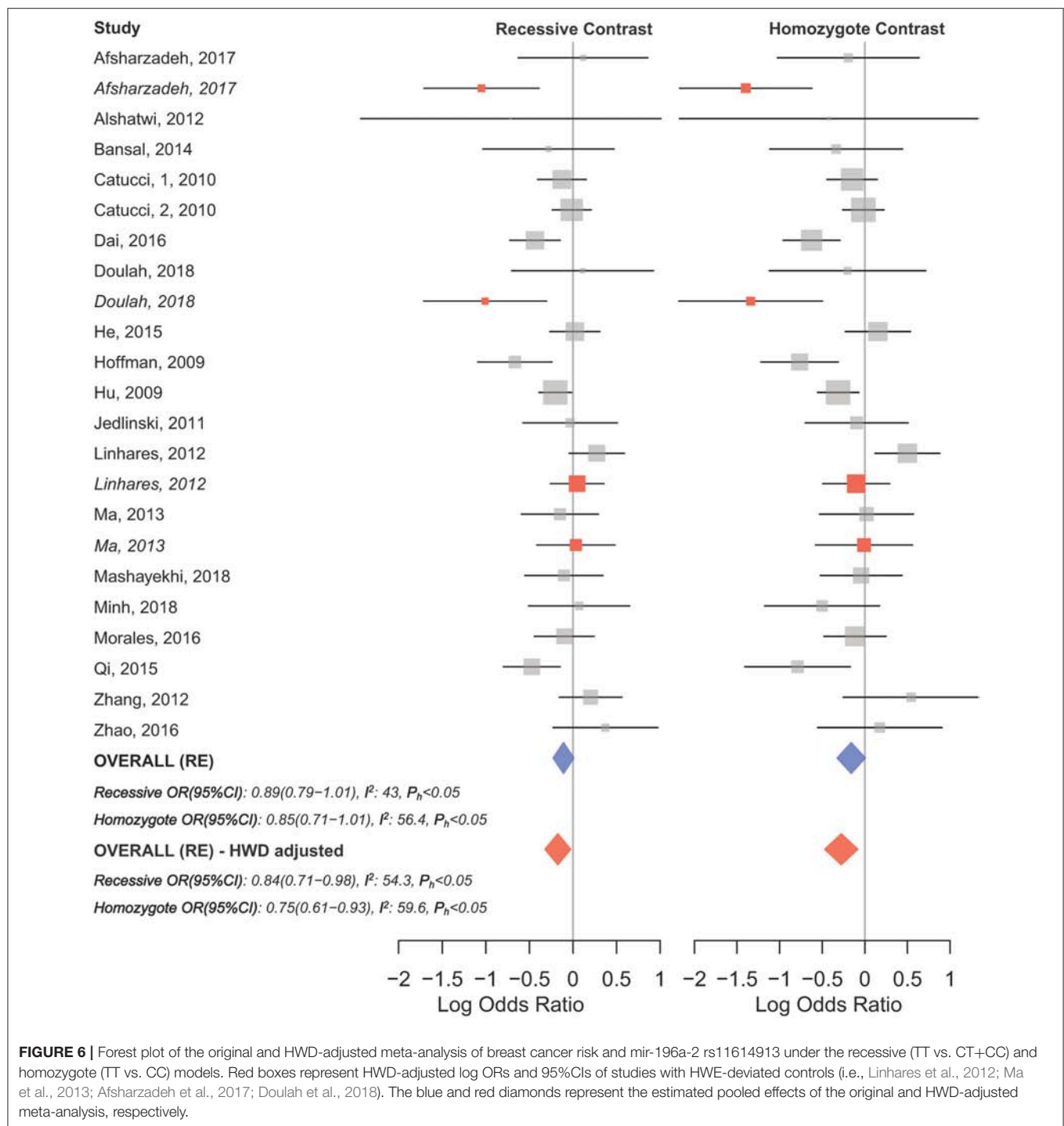
estimate pooled OR (Table 3). Subgrouping by study level moderators led to a reduction in heterogeneity in some subgroups (Table 5). However, univariate meta-regression showed no statistically significant source of heterogeneity (All $P > 0.05$). Sensitivity analysis by omitting one study at a time revealed no single influential study (Supplementary Figure S9).

Publication Bias

Rank correlation test of the mir-196a-2 rs11614913 Begger's funnel plot asymmetry revealed no statistically significant evidence of publication bias in any contrast (Table 3 and Supplementary Figure S10). However, rank correlation test for asymmetry of mir-149 rs2292832 funnel plots showed statistically significant results in the homozygote, recessive and the allelic contrasts (Table 3 and Supplementary Figure S11). Consequently, the "trim and fill" approach (Duval and Tweedie, 2000a,b) employed to correct for funnel plot asymmetry arising from publication bias in these models. The results of overall analysis using original studies or trim and fill method under the three models were comparable (see Supplementary Figure S4 to compare forest plots of the original studies vs. trim-and-fill method and Supplementary Figure S11 for funnel plots). After excluding studies with controls deviating from HWE in sensitivity analysis, rank correlation test was still significant in the mentioned three contrasts.

DISCUSSION

The possible contribution of miRNAs, especially mir-196a-2 and mir-149, to the risk of cancer has stimulated great attention in recent years. Many studies evaluated the functional alterations of these micro-regulators in a wide range of cancers. Accumulating evidence suggests that, at least a part of functional dysregulations of miRNAs in cancers could be attributed to polymorphisms in miRNA sequences (Hu et al., 2008; Hoffman et al., 2009; Tu et al., 2012; Ghaedi et al., 2015; Nariman-Saleh-Fam et al., 2016, 2017). Two mature miRNAs, miR-196a-5p and miR-196a-3p, are generated from the stem-loop structure of hsa-mir-196a-2 (Kozomara and Griffiths-Jones, 2014) with the studied polymorphism, rs11614913, residing in the 3' arm (Figure 9A). This polymorphism, therefore, may potentially alter miRNA processing and also binding to related target mRNAs (Hoffman et al., 2009) (Figure 9B). Previous studies have shown that the expression level of mature miR-196a-3p was higher in CC carriers with lung cancer compared to CT and TT individuals (Hu et al., 2008). More evidences have been provided by Hoffman et al. (2009) who observed elevated expression of mature mir-196a-2 forms in MCF-7 cells transfected with pre-mir-196a-C vector compared with cells transfected with pre-mir-196a-T vector. The potential of rs11614913 in influencing targeting function of mir-196a-2 has also been documented by whole-genome expression microarrays which found different numbers of



dysregulated mRNAs after transfecting cells with pre-mir-196a-C or pre-mir-196a-T vector (Hoffman et al., 2009). Hsa-mir-149 also generates two mature miRNAs (miR-149-5p and miR-149-3p) and the studied polymorphism, rs2292832, does not reside in the mature sequence of neither miR-149-5p or miR-149-3p (Figure 10A). Therefore, it has been hypothesized that

rs2292832 is not a structure-shifting polymorphism for pri-mir-149 or pre-mir-149 (Wei et al., 2014). However, Tu et al. reported that the T allele may disrupt the maturation process compared with the C allele and, consequently, decrease miR-149 expression (Tu et al., 2012) (Figure 10B) in head and neck squamous cell carcinoma patients.

TABLE 5 | Meta-analysis of miR-149 rs2292832 and cancer risk.

Groups	Cases/Controls ^a	Homozygote (TT vs. CC)			Heterozygote (CT vs. CC)			Dominant (TT+CT vs. CC)			Recessive (TT vs. CT+CC)			Allelic (T vs. C)		
		OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²
All studies	15,954/19,594	0.89 (0.77–1.02)	<1e–4	64.2	0.96 (0.87–1.05)	0.008	37.1	1.00 (0.90–1.11)	<1e–4	53.5	1.00 (0.92–1.08)	<1e–4	48.6	0.96 (0.88–1.04)	<1e–4	73.6
Controls in HWE ^d	12,873/15,569	0.88 (0.75–1.04)	<1e–4	67	0.95 (0.89–1.02)	0.054	29.7	0.98 (0.87–1.09)	4e–4	50.8	0.98 (0.89–1.07)	<1e–4	51.3	0.95 (0.87–1.03)	<1e–4	70.8
QUALITY																
High (>8)	15,149/18,939	0.98 (0.87–1.11)	<1e–4	52.8	0.94 (0.88–1.00)	0.060	27.4	0.97 (0.88–1.08)	1e–4	51.3	1.04 (0.96–1.13)	7e–4	47.3	1.02 (0.95–1.10)	<1e–4	66.3
Low (≤8)	805/655	1.32 (1.83–1.34)	0.183	35.7	1.25 (0.55–2.87)	0.043	59.3	1.31 (0.69–2.48)	0.087	50.7	1.15 (0.90–1.46)	0.84	0	1.19 (0.98–1.4)	0.599	0 ^f
GENOTYPING																
PCR-RFLP	9,437/11,117	1.05 (0.90–1.22)	0.003	46.6	1.01 (0.88–1.14)	0.016	39.5	1.06 (0.92–1.22)	3e–4	54.5	1.08 (0.98–1.19)	0.028	36.3	1.08 (0.98–1.18)	<1e–4	64.5
Others ^e	6,517/8,477	0.94 (0.76–1.16)	9e–4	61.6	0.88 (0.79–0.98)	0.178	25	0.911 (0.77–1.06)	0.018	48.3	1.0 (0.88–1.13)	0.011	51.4	0.97 (0.88–1.08)	6e–4	62.8
ETHNICITIES																
Asian	14,279/17,389	1.01 (0.89–1.15)	0.0001	51.9	0.97 (0.88–1.08)	0.006	40	0.97 (0.88–1.08)	0.006	40	1.05 (0.98–1.13)	0.005	40.6	1.05 (0.97–1.13)	<1e–4	65.1
Caucasians	1,675/2,205	0.98 (0.48–1.96)	0.022	64.9	0.93 (0.80–1.07)	0.30	16.5	0.94 (0.82–1.08)	0.13	43.5	1.04 (0.62–1.72)	0.052	57.4	0.95 (0.72–1.27)	0.049	57.9
CANCER CATEGORIES																
GI	6,508/8,150	0.96 (0.83–1.12)	0.043	36.9	0.87 (0.79–0.97)	0.57	0	0.92 (0.84–1.01)	0.206	19.2	1.07 (0.99–1.15)	0.079	31.5	1.01 (0.94–1.10)	0.010	46
HNC	2,885/3,769	1.35 (0.76–2.38)	0.003	68.6	1.34 (0.82–2.19)	0.001	72.2	1.45 (0.86–2.42)	<1e–4	80.3	1.10 (0.97–1.24)	0.096	44.2	1.30 (0.87–1.94)	<1e–4	82.8
Others	6,561/7,675	0.96 (0.78–1.19)	0.001	59.5	0.97 (0.88–1.06)	0.126	30.4	0.98 (0.83–1.15)	0.010	51.9	0.99 (0.87–1.13)	0.011	51.5	0.99 (0.89–1.11)	2e–4	65.5
CANCER TYPES																
BC ^e	1,871/1,894	1.12 (0.53–2.36)	0.014	71.6	1.02 (0.87–1.19)	0.192	36.7	1.07 (0.59–1.93)	0.031	65.9	1.10 (0.68–1.77)	0.063	58.8	1.05 (0.69–1.60)	0.005	76.4
HCC	2,512/3,107	0.95 (0.68–1.34)	0.008	61	0.91 (0.78–1.06)	0.285	17.7	0.93 (0.72–1.20)	0.039	50.6	0.97 (0.86–1.09)	0.049	48.5	0.99 (0.83–1.17)	0.003	65.6
GC	2,527/3,399	0.95 (0.79–1.14)	0.323	13.6	0.85 (0.72–1.00)	0.493	0	0.92 (0.79–1.07)	0.397	4.2	1.08 (0.96–1.22)	0.319	14.2	1.01 (0.93–1.10)	0.194	29.2

(Continued)

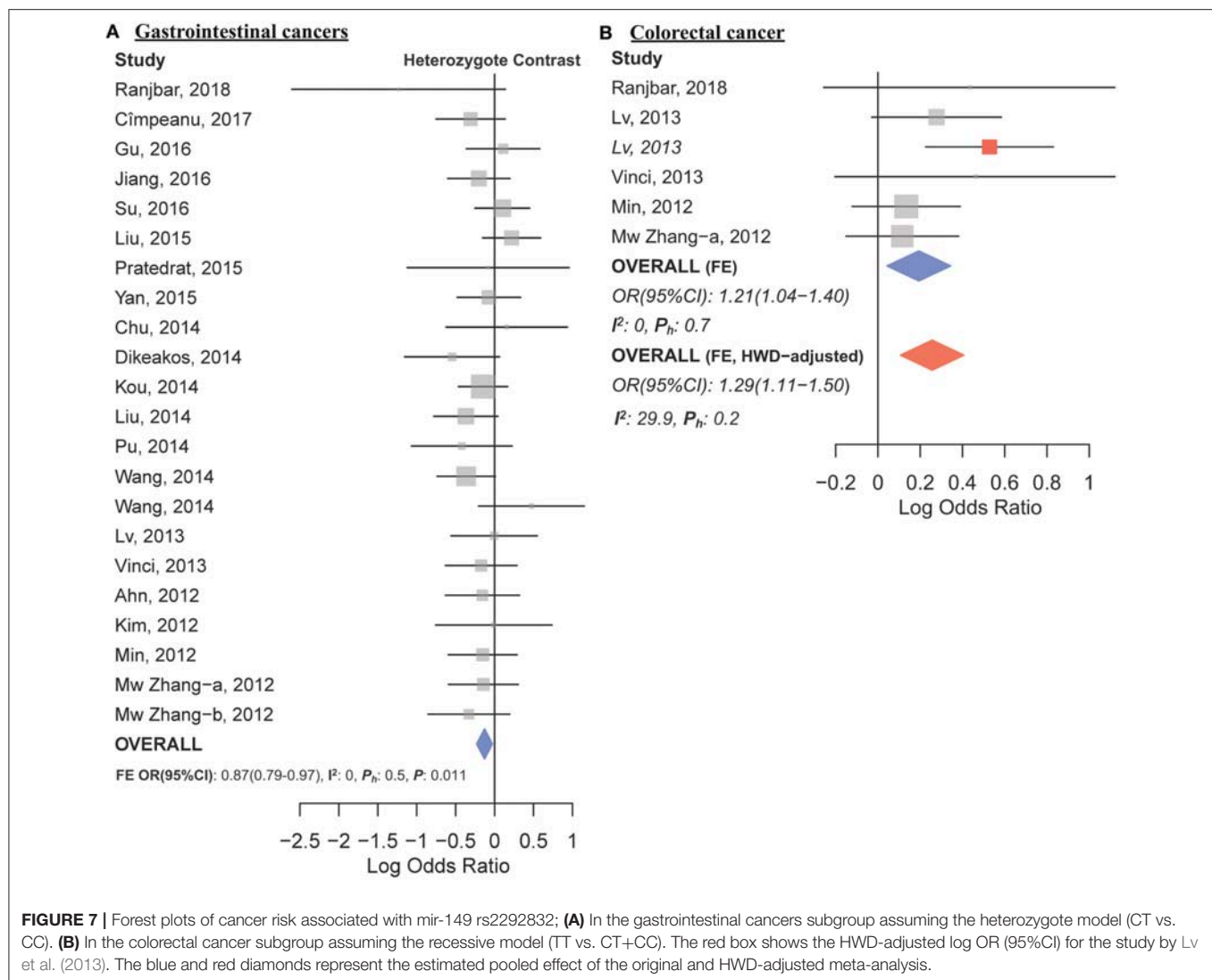
TABLE 5 | Continued

Groups	Cases/Controls ^a	Homozygote (TT vs. CC)			Heterozygote (CT vs. CC)			Dominant (TT+CT vs. CC)			Recessive (TT vs. CT+CC)			Allelic (T vs. C)		
		OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²	OR ^b (95% CI)	P ^c	I ²
CRC ^e	1,469/1,644	1.10 (0.86–1.40)	0.671	0	0.85 (0.67–1.07)	0.615	0	0.97 (0.78–1.20)	0.704	0	1.21 (1.04–1.40)	0.748	0	1.10 (0.98–1.23)	0.801	0
LC	1,714/1,559	1.04 (0.8246–1.31)	0.116	53.5	1.03 (0.83–1.29)	0.602	0	1.04 (0.84–1.29)	0.351	4.3	0.98 (0.85–1.13)	0.175	42.4	1.04 (0.70–1.52)	0.157	45.8
Others	5,861/7,991	1.02 (0.77–1.34)	2e–4	65.2	1.071 (0.84–1.36)	5e–4	63.5	1.10 (0.85–1.44)	<1e–4	73.5	1.00 (0.86–1.16)	0.012	50.7	1.07 (0.89–1.29)	<1e–4	77.9

The overall, HWD-sensitivity and subgroup analyses. ^an represents number of cases and controls in each group.
^bPooled ORs and 95% confidence intervals.
^cP-value of the heterogeneity test.
^dMeta-analysis of all studies excluding those with the control group not in HWE.
^eThese subgroups were found to be influenced by departure from HWE. Please refer to the **Supplementary Table S4** for details on HWD sensitivity and adjustment analysis for these subgroups.
^fAlthough the point estimate of I² was zero for the allele contrast in the low quality subgroup, the random effect model was used based on the 95%CI of I² (0–69.8%) and small number of studies.
GI, Gastrointestinal tract cancers; HNC, Head and neck cancers; BC, Breast cancer; HCC, Hepatocellular cancers; GC, Gastric cancer; CRC, Colorectal cancer; LC, Lung cancer.
Significant associations are shown in boldface.

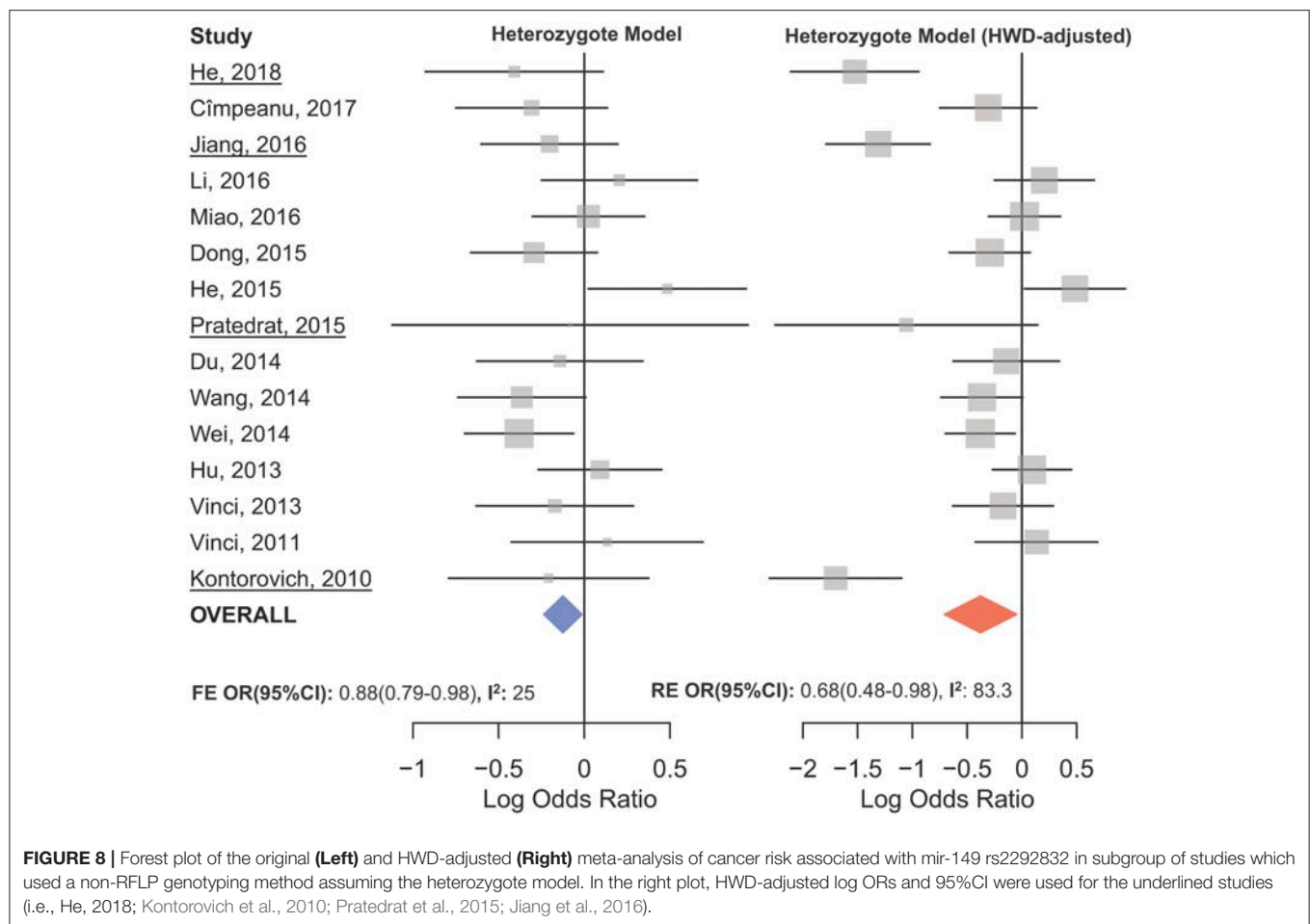
Increasing number of association studies evaluating miRNA polymorphisms and cancer risk with contradictory results merits the need for comprehensive systematic reviews and meta-analyses. Several meta-analyses have evaluated the risk of cancer associated with mir-196a-2 rs11614913 or mir-149 rs2292832 (Chu et al., 2011; Zhang H. et al., 2012; Feng et al., 2016; Yan et al., 2017; Liu et al., 2018). However, the conclusion of these studies with regards to the subgroup analysis and the significant genetic model varies due, at least in part, to differences in the number of studies included or in the methodology. Moreover, several recent genetic association studies have not been included in previously published meta-analyses. Therefore, it was necessary to perform an updated meta-analysis with larger number of studies to clarify the association of mir-196a-2 rs11614913 or mir-149 rs2292832 with cancer risk. Therefore, compared to the previous meta-analysis, we included more studies in the analyses. The present meta-analysis also evaluated and corrected for the possible influence of departure of the control group of association studies from HWE. Although checking for departure from HWE has been recommended, currently there is no consensus about how HWD studies should be handled in meta-analysis (Minelli et al., 2008). Result of simulations suggests no advantage for excluding these studies (Minelli et al., 2008). However, sensitivity analysis to detect any possible bias imposed by such studies and/or using HWE-expected counts instead of the observed genotype frequencies have been recommended and implemented in several studies (Attia et al., 2003; Thakkestian et al., 2005; Trikalinos et al., 2006; Zintzaras et al., 2006; Zintzaras, 2008; Zintzaras and Lau, 2008; Srivastava and Srivastava, 2012; Wang X. B. et al., 2014). The current study noticed that most analyses, especially those with sufficiently large number of studies, were not influenced by excluding HWD studies. However, as it is rationally expected, excluding HWD studies from some subgroup with relatively small number of studies may influence the analysis, and therefore in such situations adjusted analyses were preferred.

For mir-196a-2 rs11614913, the previous largest meta-analysis, conducted by Liu et al. (2018), included 84 studies compared to 111 studies in the present meta-analysis. By including 41,673 patients and 49,570 control subjects, the present meta-analysis showed a decreased risk of cancer in the homozygote and the recessive models (Table 3). Although, the association was not significant in allele contrast, the OR and 95%CI of the allele contrast were borderline and influenced by excluding some individual studies. Excluding HWE-deviated or low quality studies yielded significant associations under allelic model. As there is, currently, no way to adjust allele frequencies for departure from HWE, the possibility that HWD studies may bias the allele contrast cannot be rolled out and a definite conclusion cannot be drawn under allelic model. Apart from the allele contrast, the results of other genetic models were statistically stable and not influenced by removing any single study, HWE deviated or low quality studies. The results also suggest that mir-196a-2 rs11614913 may pose an ethnic dependent effect on cancer risk as associations with cancer were only observed in Asians. However, it should be noted that most



studies enrolled Asian patients, mainly Chinese patients, and the number of studies involving other ethnicities were relatively small. Moreover, different minor allele frequencies (MAF) may partly contribute to the observed differences among ethnicities (Average MAF in Asians T: 0.501 ± 0.127 , Caucasians T: 0.410 ± 0.1 , Others T: 0.338 ± 0.066). In-line with previous studies, the current meta-analysis also confirmed that rs11614913 is associated with decreased risks of hepatocellular cancer under three genetic models (Liu et al., 2018) and that it may not modulate risk of urogenital cancers (Wang et al., 2017). The results of meta-analysis of all studies, subgroup analysis by ethnicity and hepatocellular cancer are in agreement with findings of the previous largest meta-analysis (Liu et al., 2018). However, the increase in the number of analyzed studies led to discrepancies with regards to conclusions in some subgroup analysis. (i) In contrast to the studies by Liu and Pan (Pan et al., 2017; Liu et al., 2018), the present meta-analysis did not find a significant association with head and neck carcinoma in any genetic model. This discrepancy may be attributed to

the number of studies included in meta-analyses [nine studies in the present meta-analysis compared to four studies in the meta-analysis by Liu et al. (2018)]. Furthermore, differences in defining head and neck cancer may also explain different conclusions drawn from the present study and the study by Pan and colleagues (Pan et al., 2017). They included esophageal cancer as a type of head and neck cancer, whereas we considered it as a type of gastrointestinal tract cancers (according to the ICD-10-CM C15-C26). (ii) Additionally, the present meta-analysis found significant associations between mir-196a-2 rs11614913 and decreased risks of gynecologic cancers (especially ovarian cancer), which have not been reported in any previous meta-analysis. Interestingly, low heterogeneity was observed in the gynecological cancers subgroup assuming the two significant contrasts (i.e., recessive and allelic). (iii) Although previous meta-analyses (Yan et al., 2017; Zhang H. et al., 2017; Liu et al., 2018) failed to find a significant association between mir-196a-2 rs11614913 and breast cancer, the current study showed, by incorporating more association studies and performing



HWD sensitivity analysis, that adjusting for departure from HWE may reveal significant associations under the homozygote and recessive contrasts (Figure 6 and Supplementary Table S2). (iv) Moreover, contradictory to previous meta-analyses, no association with gastric (Yan et al., 2017), colorectal (Xie et al., 2015; Yan et al., 2017) or lung cancer (Ren et al., 2016; Yan et al., 2017; Liu et al., 2018), was found. Apart from larger sample sizes and correcting for HWD, sometimes this discrepancy in results may also be related to methodological differences in the design, specifically the inclusion criteria, of meta-analyses. As a case in point, studies by Hu et al. (2008) and Yoon et al. (2012) did not meet the inclusion criteria of our study, as they deal with the survival or recurrence risk of lung cancer patients with approaches that differed from routine case-control genetic association studies. However, we noticed that these studies were included in a previous meta-analysis (Liu et al., 2018).

The current meta-analysis also showed that mir-149 rs2292832 is not associated with risk of cancer in any genetic model and the results were statistically reliable, as summary effects were not influenced by excluding any single study, HWE-deviated or low quality studies. No differences in cancer risk was observed between ethnicities. Similar to miR-196a-2

polymorphism, the Asian subgroup comprised a large proportion of studies and relatively few studies with limited sample sizes were performed in Caucasians. Therefore, a definite conclusion cannot be drawn in Caucasians and more studies are needed further clarify the association of this SNP with cancer risk in Caucasians. The results of overall analysis were comparable to previous meta-analyses (Li L. et al., 2015; Feng et al., 2016). By pooling the results of 22 studies, this meta-analysis found a decreased risk of gastrointestinal tract (GI) cancers for individuals who carry the CT genotype compared to those with the CC genotype in a heterozygote model. Interestingly, there was no significant heterogeneity in the GI subgroup assuming the heterozygote model indicating the reliability of meta-analysis in this subgroup. Previous meta-analyses yielded different results with regard to GI cancers. A previous meta-analysis of seven studies on GI cancers suggested a marginally elevated risk under the recessive model (TT vs. CT+CC), while another pooling of 10 studies found a borderline decreased risk for the CT vs. TT contrast. Although no significant association was identified in the head and neck cancers subgroup, it should be noted that significant heterogeneity was present in all models except the recessive contrast and number of samples were relatively small. For colorectal cancer the association was in reverse direction

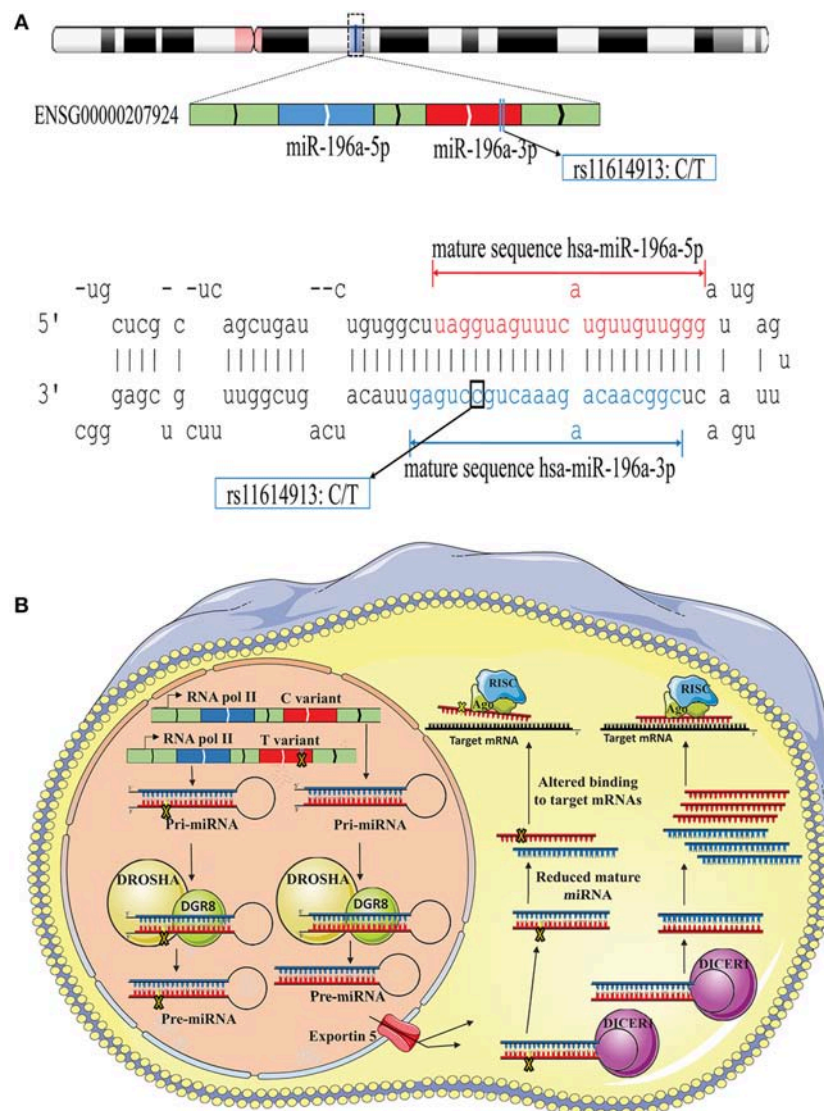


FIGURE 9 | Schematic illustration of hsa-mir-196a-2 locus and effect of rs11614913 on its processing and targeting. **(A)** Top: Hsa-mir-196a-2 generates two mature miRNAs, miR-196a-5p and miR-196a-3p, and rs11614913 lies in miR-196a-3p at GRCh37 chr12: 54,385,599. Bottom: Stem-loop structure of hsa-mir-196, including mature miR-196-5p (Red) and miR-196-3p (Blue) sequences. **(B)** Rs11614913 alters miRNA processing and/or binding to related target mRNAs.

and an increased risk was observed in individuals who carry the TT genotype compared with subject who carry at-least one C allele (Table 5). A similar association based on three studies was previously reported (Rong et al., 2017), but not reproduced in other meta-analyses (Li et al., 2013; Feng et al., 2016). Taken together, the current results based on five studies suggest an increased risk for colorectal cancer that was stable after correcting for departure from HWE. Although no significant heterogeneity was detected in the colorectal cancer subgroup under any genetic model, it should be noted that the limited number of studies may influence heterogeneity evaluation and more definite conclusion may be drawn by analyzing larger sample sizes. In the case of breast cancer, a previous meta-analysis of three studies found a significant association

(Feng et al., 2016). We found no significant association in the original and HWD-adjusted analysis. However, number of studies in the colorectal and breast cancer subgroups are relatively limited and results should be interpreted with caution. More studies with large sample sizes are needed for a definite conclusion.

However, the present study has some limitations. First, significant heterogeneity was present in most analyses especially for mir-196a-2 polymorphism. We, therefore, used random effect model and performed meta-regression; but no significant source of heterogeneity was observed for most analyses, suggesting that other unknown study level moderators may contribute to the heterogeneity. Second: The molecular mechanisms underlying association of these miRNAs-SNP with risk of cancer are complex

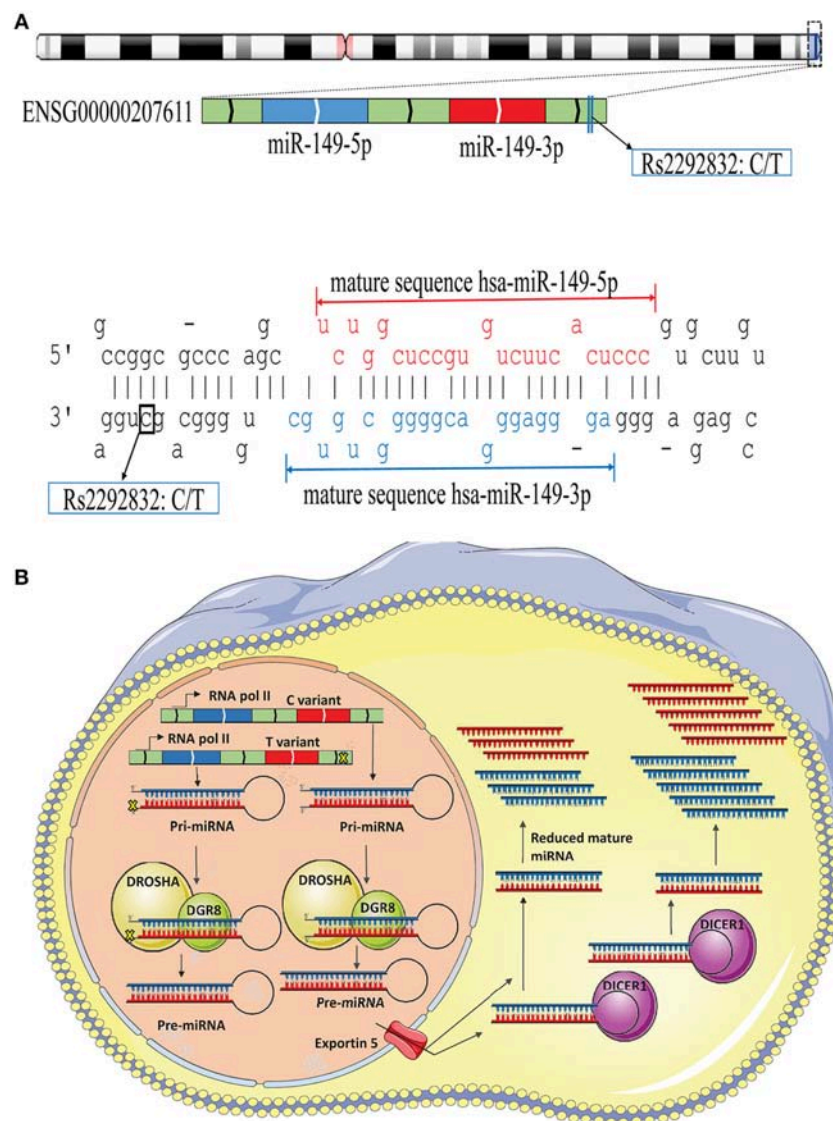


FIGURE 10 | Schematic illustration of hsa-mir-149 locus and effect of rs2292832 on its processing. **(A)** Top: Hsa-mir-149 generates two mature miRNAs (miR-149-5p and miR-149-3p), and rs2292832 resides in pre-miRNA sequence, but not in either mature miRNAs, at GRCh37 chr2: 241,395,593. Bottom: Stem-loop structure of hsa-mir-149, including mature miR-149-5p (Red), and miR-149-3p (Blue) sequences. **(B)** Rs12292832 may alter miRNA processing and decrease mature miR-149 (–5p and –3p forms) expression.

and might be strongly affected by different genetic background as well as other masked variables. This, in turn, may limit the efficacy of the overall analysis especially in the case of miR-196a-2 rs11614913. Stratified analyses based on a specific cancer category or a cancer type may help to reduce this heterogeneity and, therefore, are considered to be more reliable. Third, this study was based on unadjusted ORs of the original studies and no adjustment for covariates like age and gender or interaction with environmental factors were done and this fact may also potentially contribute to the between study heterogeneity. Fourth, some limitations such as language restriction or lack of access to the genotype counts of mir-196a-2 rs11614913 in four studies with insufficiently reported data may bring in publication

bias. The trim and fill method has been shown to reduce the bias in estimates in the presence of publication bias and heterogeneity (Peters et al., 2007). However, it has been recommended that this method should be considered as sensitivity analysis as we cannot be sure that asymmetry in funnel plot is truly caused by publication bias (Peters et al., 2007). Although rank correlation test of funnel plots of mir-149 rs2292832 was significant in three genetic models raising the possibility of publication bias, adjusting for such a bias using trim-and-fill method did not afford any change in analysis of overall studies in these models (**Supplementary Figure S4**). Fifth, number of studies in some subgroup analyses was limited and, consequently, results of such analysis should be interpreted with caution. Most studies

were performed enrolling Asian patients and the number of studies on Caucasians or Africans was limited. Therefore, more association studies with larger sample sizes on Africans and Caucasians are needed to make precise estimations of cancer risk associated with the studied polymorphisms. Assigning ethnicity to each study population could be another limitation of meta-analysis of association studies as each ethnicity may regroup several sub-populations with somewhat different genetic background. Sixth, the control groups of association studies were not uniformly defined and non-differential misclassification bias may have occurred.

In conclusion, this meta-analysis showed that mir-196a-2 rs11614913T allele is associated with decreased cancer risk in overall population, high quality studies and studies on Asian populations. It is also associated with a decreased risk of gynecological cancers, ovarian, breast and hepatocellular cancer. Mir-149 rs2292832 was not associated with cancer risk in overall population, high quality studies, Asians or Caucasians. However, the T allele was associated with a decrease risk of gastrointestinal tract cancers under the heterozygote model and an increased risk of colorectal cancer under the recessive model.

REFERENCES

- Abdel-Hamid, M., Elshaer, S., and Darwish, A. (2018). Association of MicroRNA related single nucleotide polymorphisms 196A-2 and 499 with the risk of hepatocellular carcinoma in Egyptian patients. *Meta Gene*. 16, 139–142. doi: 10.1016/j.mgene.2018.02.007
- Afsharzadeh, S. M., Mohaddes Ardebili, S. M., Seyedi, S. M., Karimian Fathi, N., and Mojarad, M. (2017). Association between rs11614913, rs3746444, rs2910164 and occurrence of breast cancer in Iranian population. *Meta Gene*. 11, 20–25. doi: 10.1016/j.mgene.2016.11.004
- Ahn, D. H., Rah, H., Choi, Y. K., Jeon, Y. J., Min, K. T., Kwack, K., et al. (2013). Association of the miR-146aC>G, miR-149T>C, miR-196a2T>C, and miR-499A>G polymorphisms with gastric cancer risk and survival in the Korean population. *Mol. Carcinogen*. 52, 39–51. doi: 10.1002/mc.21962
- Akkiz, H., Bayram, S., Bekar, A., Akgöllü, E., and Ülger, Y. (2011). A functional polymorphism in pre-microRNA-196a-2 contributes to the susceptibility of hepatocellular carcinoma in a Turkish population: a case-control study. *J. Viral Hepatitis*. 18, e399–e407. doi: 10.1111/j.1365-2893.2010.01414.x
- Alshatwi, A. A., Shafi, G., Hasan, T. N., Syed, N. A., Al-Hazzani, A. A., Alsaif, M. A., et al. (2012). Differential expression profile and genetic variants of MicroRNAs sequences in breast cancer patients. *PLoS ONE* 7:030049. doi: 10.1371/journal.pone.0030049
- Attia, J., Thakkinian, A., and D'Este, C. (2003). Meta-analyses of molecular association studies: methodologic lessons for genetic epidemiology. *J. Clin. Epidemiol.* 56, 297–303. doi: 10.1016/S0895-4356(03)00011-8
- Bansal, C., Sharma, K. L., Misra, S., Srivastava, A. N., Mittal, B., and Singh, U. S. (2014). Common genetic variants in pre-microRNAs and risk of breast cancer in the North Indian population. *Ecancermedicalscience* 8:473. doi: 10.3332/ecancer.2014.473
- Begg, C. B., and Mazumdar, M. (1994). Operating characteristics of a rank correlation test for publication bias. *Biometrics* 50, 1088–1101. doi: 10.2307/2533446
- Bodal, V. K., Sangwan, S., Bal, M. S., Kaur, M., Sharma, S., and Kaur, B. (2017). Association between microRNA 146a and microRNA 196a2 genes polymorphism and breast cancer risk in north Indian women. *Asian Pac. J. Cancer Prev.* 18, 2345–2348. doi: 10.22034/APJCP.2017.18.9.2345
- Bray, F., Jemal, A., Grey, N., Ferlay, J., and Forman, D. (2012). Global cancer transitions according to the Human Development Index

AUTHOR CONTRIBUTIONS

MB and AM conceived the original idea and supervised the project. JC, ZN-S-F, and ZS contributed to the literature search and data management. MB, JC, and ZN-S-F wrote the manuscript with support from all authors. MB contributed to the data analysis, interpretation of results, and data visualization with inputs from AM. EO and ZS assisted with data visualization. All authors provided critical feedback, discussed the results, and contributed to the final manuscript.

ACKNOWLEDGMENTS

We would like to thank Dr. Karl Kelsey and Dr. Brock Christensen for providing the genotype counts of mir-196a-2 rs11614913 for their study (Christensen et al., 2010).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00186/full#supplementary-material>

- (2008–2030): a population-based study. *Lancet Oncol.* 13, 790–801. doi: 10.1016/S1470-2045(12)70211-5
- Cîmpeanu, R. A., Popescu, D. M., Burada, F., Cucu, M. G., Gheonea, D. I., Ioana, M., et al. (2017). miR-149 rs2292832 C>T polymorphism and risk of gastric cancer. *Roman. J. Morphol. Embryol.* 58, 125–129.
- Catucci, I., Yang, R., Verderio, P., Pizzamiglio, S., Heesen, L., Hemminki, K., et al. (2010). Evaluation of SNPs in miR-146a, miR196a2 and miR-499 as low-penetrance alleles in German and Italian familial breast cancer cases. *Hum. Mutat.* 31, E1052–E1057. doi: 10.1002/humu.21141
- Chen, H., Sun, L. Y., Chen, L. L., Zheng, H. Q., and Zhang, Q. F. (2012). A variant in microRNA-196a2 is not associated with susceptibility to and progression of colorectal cancer in Chinese. *Inter. Med. J.* 42, e115–e119. doi: 10.1111/j.1445-5994.2011.02434.x
- Christensen, B. C., Avissar-Whiting, M., Ouellet, L. G., Butler, R. A., Nelson, H. H., McClean, M. D., et al. (2010). Mature microRNA sequence polymorphism in MIR196A2 is associated with risk and prognosis of head and neck cancer. *Clin. Cancer Res.* 16, 3713–3720. doi: 10.1158/1078-0432.CCR-10-0657
- Chu, H., Wang, M., Shi, D., Ma, L., Zhang, Z., Tong, N., et al. (2011). Hsa-miR-196a2 Rs11614913 polymorphism contributes to cancer susceptibility: evidence from 15 case-control studies. *PLoS ONE* 6:e18108. doi: 10.1371/journal.pone.0018108
- Chu, Y. H., Hsieh, M. J., Chiou, H. L., Liou, Y. S., Yang, C. C., Yang, S. F., et al. (2014). MicroRNA gene polymorphisms and environmental factors increase patient susceptibility to hepatocellular carcinoma. *PLoS ONE* 9:089930. doi: 10.1371/journal.pone.0089930
- Chu, Y. H., Tzeng, S. L., Lin, C. W., Chien, M. H., Chen, M. K., and Yang, S. F. (2012). Impacts of microRNA gene polymorphisms on the susceptibility of environmental factors leading to carcinogenesis in oral cancer. *PLoS ONE* 7:39777. doi: 10.1371/journal.pone.0039777
- Dai, Z. M., Kang, H. F., Zhang, W. G., Li, H. B., Zhang, S. Q., Ma, X. B., et al. (2016). The associations of single nucleotide polymorphisms in miR196a2, miR-499, and miR-608 with breast cancer susceptibility: a STROBE-compliant observational study. *Medicine* 95:e2826. doi: 10.1097/MD.0000000000000286
- Damodaran, M., Paul, S. F. D., and Venkatesan, V. (2018). Genetic polymorphisms in miR-146a, miR-196a2 and miR-125a genes and its association in prostate cancer. *Pathol. Oncol. Res.* 1–8. doi: 10.1007/s12253-018-0412-x. [Epub ahead of print].

- Deng, S., Wang, W., Li, X., and Zhang, P. (2015). Common genetic polymorphisms in pre-microRNAs and risk of bladder cancer. *World J. Surg. Oncol.* 13:297. doi: 10.1186/s12957-015-0683-6
- DerSimonian, R., and Laird, N. (1986). Meta-analysis in clinical trials. *Control Clin. Trials* 7, 177–188. doi: 10.1016/0197-2456(86)90046-2
- Dikaikakos, P., Gazouli, M., Rizos, S., Zografos, G., and Theodoropoulos, G. E. (2015). Evaluation of genetic variants in miRNAs in patients with colorectal cancer. *Cancer Biomarkers* 15, 163–168. doi: 10.3233/CBM-140449
- Dikeakos, P., Theodoropoulos, G., Rizos, S., Tzanakis, N., Zografos, G., and Gazouli, M. (2014). Association of the miR-146aC>G, miR-149T>C, and miR-196a2T>C polymorphisms with gastric cancer risk and survival in the Greek population. *Mol. Biol. Rep.* 41, 1075–1080. doi: 10.1007/s11033-013-2953-0
- Dong, G., Zhang, R., Xu, J., and Guo, Y. (2015). Association between microRNA polymorphisms and papillary thyroid cancer susceptibility. *Int. J. Clin. Exp. Pathol.* 8, 13450–13457.
- Dou, T., Wu, Q., Chen, X., Ribas, J., Ni, X., Tang, C., et al. (2010). A polymorphism of microRNA196a genome region was associated with decreased risk of glioma in Chinese population. *J. Cancer Res. Clin. Oncol.* 136, 1853–1859. doi: 10.1007/s00432-010-0844-5
- Doulah, A., Salehzadeh, A., and Mojarad, M. (2018). Association of single nucleotide polymorphisms in miR-499 and miR-196a with susceptibility to breast cancer. *Trop. J. Pharmaceut. Res.* 17, 319–323. doi: 10.4314/tjpr.v17i2.17
- Du, M., Lu, D., Wang, Q., Chu, H., Tong, N., Pan, X., et al. (2014). Genetic variations in microRNAs and the risk and survival of renal cell cancer. *Carcinogenesis* 35, 1629–1635. doi: 10.1093/carcin/bgu082
- Duval, S., and Tweedie, R. (2000a). A Nonparametric “Trim and Fill” Method of Accounting for Publication Bias in Meta-Analysis. *J. Am. Stat. Assoc.* 95, 89–98. doi: 10.2307/2669529
- Duval, S., and Tweedie, R. (2000b). Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 56, 455–463. doi: 10.1111/j.0006-341X.2000.00455.x
- Feng, Y., Duan, F., Song, C., Zhao, X., Dai, L., and Cui, S. (2016). Systematic evaluation of cancer risk associated with rs2292832 in miR-149 and rs895819 in miR-27a: a comprehensive and updated meta-analysis. *Oncotarget* 7, 22368–22384. doi: 10.18632/oncotarget.8082
- Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., and Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127, 2893–2917. doi: 10.1002/ijc.25516
- George, G. P., Gangwar, R., Mandal, R. K., Sankhwar, S. N., and Mittal, R. D. (2011). Genetic variation in microRNA genes and prostate cancer risk in North Indian population. *Mol. Biol. Rep.* 38, 1609–1615. doi: 10.1007/s11033-010-0270-4
- Ghaedi, H., Bastami, M., Zare-Abdollahi, D., Alipoor, B., Movafagh, A., Mirfakhraie, R., et al. (2015). Bioinformatics prioritization of SNPs perturbing microRNA regulation of hematological malignancy-implicated genes. *Genomics* 106, 360–366. doi: 10.1016/j.ygeno.2015.10.004
- Gu, J. Y., and Tu, L. (2016). Investigating the role of polymorphisms in miR-146a, –149, and –196a2 in the development of gastric cancer. *Genetics Mol. Res.* 15:gm15027839. doi: 10.4238/gmr.15027839
- Ha, M., and Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* 15:509. doi: 10.1038/nrm3838
- Han, Y., Pu, R., Han, X., Zhao, J., Zhang, Y., Zhang, Q., et al. (2013). Associations of pri-miR-34b/c and pre-miR-196a2 polymorphisms and their multiplicative interactions with Hepatitis B virus mutations with hepatocellular carcinoma risk. *PLoS ONE* 8:e58564. doi: 10.1371/journal.pone.0058564
- Hao, Y. X., Wang, J. P., and Zhao, L. F. (2014). Associations between three common MicroRNA polymorphisms and hepatocellular carcinoma risk in Chinese. *Asian Pac. J. Cancer Prevent.* 14, 6601–6604. doi: 10.7314/APJCP.2013.14.11.6601
- Hashemi, M., Moradi, N., Ziaee, S. A., Narouie, B., Soltani, M. H., Rezaei, M., et al. (2016). Association between single nucleotide polymorphism in miR-499, miR-196a2, miR-146a and miR-149 and prostate cancer risk in a sample of Iranian population. *J. Adv. Res.* 7, 491–498. doi: 10.1016/j.jare.2016.03.008
- He, B., Pan, Y., Xu, Y., Deng, Q., Sun, H., Gao, T., et al. (2015). Associations of polymorphisms in microRNAs with female breast cancer risk in Chinese population. *Tumor Biol.* 36, 4575–4582. doi: 10.1007/s13277-015-3102-2
- He, J., Zou, Y., Liu, X., Zhu, J., Zhang, J., Zhang, R., et al. (2018). Association of common genetic variants in pre-microRNAs and neuroblastoma susceptibility: a two-center study in Chinese children. *Mol. Ther. Nucleic Acids* 11, 1–8. doi: 10.1016/j.omtn.2018.01.003
- He, Y., Yu, D., Zhu, L., Zhong, S., Zhao, J., and Tang, J. (2018). miR-149 in human cancer: a systemic review. *J. Cancer* 9, 375–388. doi: 10.7150/jca.21044
- Hezova, R., Kovarikova, A., Bienertova-Vasku, J., Sachlova, M., Redova, M., Vasku, A., et al. (2012). Evaluation of SNPs in miR-196-a2, miR-27a and miR-146a as risk factors of colorectal cancer. *World J. Gastroenterol.* 18, 2827–2831. doi: 10.3748/wjg.v18.i22.2827
- Hoffman, A. E., Zheng, T., Yi, C., Leaderer, D., Weidhaas, J., Slack, F., et al. (2009). microRNA miR-196a-2 and breast cancer: a genetic and epigenetic association study and functional analysis. *Cancer Res.* 69, 5970–5977. doi: 10.1158/0008-5472.CAN-09-0236
- Hong, Y. S., Kang, H. J., Kwak, J. Y., Park, B. L., You, C. H., Kim, Y. M., et al. (2011). Association between MicroRNA196a2 rs11614913 genotypes and the risk of non-small cell lung cancer in Korean population. *J. Prevent. Med. Public Health* 44, 125–130. doi: 10.3961/jpmph.2011.44.3.125
- Horikawa, Y., Wood, C. G., Yang, H., Zhao, H., Ye, Y., Gu, J., et al. (2008). Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma. *Clin. Cancer Res.* 14, 7956–7962. doi: 10.1158/1078-0432.CCR-08-1199
- Hu, E., Wang, D., Zhang, X., Li, J., Hu, Y., Gong, H., et al. (2013). Four common polymorphisms in microRNAs and the risk of adult glioma in a Chinese case-control study. *J. Mol. Neurosci.* 51, 933–940. doi: 10.1007/s12031-013-9980-0
- Hu, Z., Chen, J., Tian, T., Zhou, X., Gu, H., Xu, L., et al. (2008). Genetic variants of miRNA sequences and non-small cell lung cancer survival. *J. Clin. Invest.* 118, 2600–2608. doi: 10.1172/JCI34934
- Hu, Z., Liang, J., Wang, Z., Tian, T., Zhou, X., Chen, J., et al. (2009). Common genetic variants in pre-microRNAs were associated with increased risk of breast cancer in Chinese women. *Hum. Mutat.* 30, 79–84. doi: 10.1002/humu.20837
- Huang, G. L., Lu, Y., Pu, X. X., He, Y. X., Chen, M. L., Li, Y. Z., et al. (2013). Association study between miR-149 gene polymorphism and nasopharyngeal carcinoma. *Biomed. Rep.* 1, 599–603. doi: 10.3892/br.2013.97
- Huang, Y., Sheng, S., Chen, B., Lin, R., Yang, J., and Hao, B. (2017). MiR-146a genetic polymorphism contributes to the susceptibility to hepatocellular carcinoma in a Chinese population. *Int. J. Clin. Exp. Pathol.* 10, 1833–1839.
- Jedlinski, D. J., Gabrovská, P. N., Weinstein, S. R., Smith, R. A., and Griffiths, L. R. (2011). Single nucleotide polymorphism in hsa-mir-196a-2 and breast cancer risk: a case control study. *Twin Res. Hum. Genetics* 14, 417–421. doi: 10.1375/twin.14.5.417
- Jiang, J., Jia, Z. F., Cao, D. H., Wu, Y. H., Sun, Z. W., and Cao, X. Y. (2016). Association of the MIR-146a rs2910164 polymorphism with gastric cancer susceptibility and prognosis. *Future Oncol.* 12, 2215–2226. doi: 10.2217/fon-2016-0224
- Kim, M. J., Yoo, S. S., Choi, Y. Y., and Park, J. Y. (2010). A functional polymorphism in the pre-microRNA-196a2 and the risk of lung cancer in a Korean population. *Lung Cancer* 69, 127–129. doi: 10.1016/j.lungcan.2010.04.015
- Kim, W. H., Min, K. T., Jeon, Y. J., Kwon, C.-I., Ko, K. H., Park, P. W., et al. (2012). Association study of microRNA polymorphisms with hepatocellular carcinoma in Korean population. *Gene* 504, 92–97. doi: 10.1016/j.gene.2012.05.014
- Kontorovich, T., Levy, A., Korostishevsky, M., Nir, U., and Friedman, E. (2010). Single nucleotide polymorphisms in miRNA binding sites and miRNA genes as breast/ovarian cancer risk modifiers in Jewish high-risk women. *Int. J. Cancer* 127, 589–597. doi: 10.1002/ijc.25065
- Kou, J. T., Fan, H., Han, D., Li, L., Li, P., Zhu, J., et al. (2014). Association between four common microRNA polymorphisms and the risk of hepatocellular carcinoma and HBV infection. *Oncol. Lett.* 8, 1255–1260. doi: 10.3892/ol.2014.2257
- Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42, D68–D73. doi: 10.1093/nar/gkt1181
- Kupcinskas, J., Bruzaite, I., Juzenas, S., Gvytyte, U., Jonaitis, L., Kiudelis, G., et al. (2014a). Lack of association between miR-27a, miR-146a, miR-196a-2, miR-492 and miR-608 gene polymorphisms and colorectal cancer. *Sci. Rep.* 4:5993. doi: 10.1038/srep05993

- Kupcinkas, J., Wex, T., Link, A., Leja, M., Bruzaite, I., Steponaitiene, R., et al. (2014b). Gene polymorphisms of micrornas in helicobacter pylori-induced high risk atrophic gastritis and gastric cancer. *PLoS ONE* 9:087467. doi: 10.1371/journal.pone.0087467
- Lau, J., Ioannidis, J. P., and Schmid, C. H. (1997). Quantitative synthesis in systematic reviews. *Ann. Intern. Med.* 127, 820–826. doi: 10.7326/0003-4819-127-9-199711010-00008
- Li, H., Ren, Y., Xia, L., Qu, R., Kong, L., Yin, Z., et al. (2016). Association of microRNA-149 polymorphism with lung cancer risk in Chinese non-smoking female: a case-control study. *PLoS ONE* 11:163626. doi: 10.1371/journal.pone.0163626
- Li, J., Cheng, G., and Wang, S. (2016). A single-nucleotide polymorphism of miR-196a2T>C rs11614913 is associated with hepatocellular carcinoma in the Chinese population. *Genetic Test. Mol. Biomarkers* 20, 213–215. doi: 10.1089/gtmb.2015.0271
- Li, L., Liu, T., Li, Z., Zhang, L., and Zhang, Z. (2015). The miR-149 rs2292832 T/C polymorphism may decrease digestive cancer susceptibility: an updated meta-analysis. *Int. J. Clin. Exp. Med.* 8, 15351–15361.
- Li, L., Sheng, Y., Lv, L., and Gao, J. (2013). The association between two MicroRNA variants (miR-499, miR-149) and gastrointestinal cancer risk: a meta-analysis. *PLOS ONE* 8:e81967. doi: 10.1371/journal.pone.0081967
- Li, M., Li, R. J., Bai, H., Xiao, P., Liu, G. J., Guo, Y. W., et al. (2016). Association between the pre-miR-196a2 rs11614913 polymorphism and gastric cancer susceptibility in a Chinese population. *Genetics Mol. Res.* 15:gm15027516. doi: 10.4238/gmr.15027516
- Li, M., Marin-Muller, C., Bharadwaj, U., Chow, K.-H., Yao, Q., and Chen, C. (2009). MicroRNAs: control and loss of control in human physiology and disease. *World J. Surg.* 33, 667–684. doi: 10.1007/s00268-008-9836-x
- Li, P., Yan, H., Zhang, H., Yu, L., Wang, Z., Zhai, Y., et al. (2014). A functional polymorphism in MIR196A2 is associated with risk and progression of nasopharyngeal carcinoma in the Chinese population. *Genetic Test. Mol. Biomarkers* 18, 149–155. doi: 10.1089/gtmb.2013.0400
- Li, T., Niu, L., Wu, L., Gao, X., Li, M., Liu, W., et al. (2015). A functional polymorphism in microRNA-196a2 is associated with increased susceptibility to non-Hodgkin lymphoma. *Tumor Biol.* 36, 3279–3284. doi: 10.1007/s13277-014-2957-y
- Li, X., Li, K., and Wu, Z. (2015). Association of four common SNPs in microRNA polymorphisms with the risk of hepatocellular carcinoma. *Int. J. Clin. Exp. Pathol.* 8, 9560–9566.
- Li, X.-D., Li, Z.-G., Song, X.-X., and Liu, C.-F. (2010). A variant in microRNA-196a2 is associated with susceptibility to hepatocellular carcinoma in Chinese patients with cirrhosis. *Pathology* 42, 669–673. doi: 10.3109/00313025.2010.522175
- Liberati, A., Altman, D. G., Tetzlaff, J., Mulrow, C., Gøtzsche, P. C., Ioannidis, J. P. A., et al. (2009). The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ* 339:b2700. doi: 10.1136/bmj.b2700
- Linhares, J. J., Azevedo Jr, M., Siufi, A. A., de Carvalho, C. V., Wolgast, M. D. C. G. M., Noronha, E. C., et al. (2012). Evaluation of single nucleotide polymorphisms in microRNAs (hsa-miR-196a2 rs11614913 C/T) from Brazilian women with breast cancer. *BMC Med. Genetics* 13:119. doi: 10.1186/1471-2350-13-119
- Liu, C. J., Tsai, M. M., Tu, H. F., Lui, M. T., Cheng, H. W., and Lin, S. C. (2013). MiR-196a overexpression and mir-196a2 gene polymorphism are prognostic predictors of oral carcinomas. *Ann. Surg. Oncol.* 20, S406–S414. doi: 10.1245/s10434-012-2618-6
- Liu, M. F., Chen, W. Q., He, Y. Z., and Gu, Y. L. (2014). Role of miR-149C>T polymorphisms on the risk of hepatocellular carcinoma in a Chinese population. *Genetics Mol. Res.* 13, 7184–7189. doi: 10.4238/2014.September.5.4
- Liu, X. Y. (2015). Association of SNPs in miR-146a, miR-196a2 and miR-499 with risk of endometrial/ovarian cancer. *Clin. Chem. Lab. Med.* 53:S400. doi: 10.1093/abbs/gmv042
- Liu, Y., He, A., Liu, B., Zhong, Y., Liao, X., Yang, J., et al. (2018). rs11614913 polymorphism in miRNA-196a2 and cancer risk: an updated meta-analysis. *OncoTargets Ther.* 11, 1121–1139. doi: 10.2147/OTT.S154211
- Liu, Z., Li, G., Wei, S., Niu, J., El-Naggar, A. K., Sturgis, E. M., et al. (2010). Genetic variants in selected pre-microRNA genes and the risk of squamous cell carcinoma of the head and neck. *Cancer* 116, 4753–4760. doi: 10.1002/cncr.25323
- Lu, Y.-C., Chang, J. T., Chan, E.-C., Chao, Y.-K., Yeh, T.-S., Chen, J.-S., et al. (2016). miR-196, an emerging cancer biomarker for digestive tract cancers. *J. Cancer* 7, 650–655. doi: 10.7150/jca.13460
- Lv, M., Dong, W., Li, L., Zhang, L., Su, X., Wang, L., et al. (2013). Association between genetic variants in pre-miRNA and colorectal cancer risk in a Chinese population. *J. Cancer Res. Clin. Oncol.* 139, 1405–1410. doi: 10.1007/s00432-013-1456-7
- Ma, F., Zhang, P., Lin, D., Yu, D., Yuan, P., Wang, J., et al. (2013). There is no association between microRNA gene polymorphisms and risk of triple negative breast cancer in a Chinese Han population. *PLoS ONE* 8:e60195. doi: 10.1371/journal.pone.0060195
- Mantel, N., and Haenszel, W. (1959). Statistical aspects of the analysis of data from retrospective studies of disease. *J. Natl. Cancer Inst.* 22, 719–748.
- Martin-Guerrero, I., Gutierrez-Camino, A., Lopez-Lopez, E., Bilbao-Aldaiturriaga, N., Pombar-Gomez, M., Ardanaz, M., et al. (2015). Genetic variants in MiRNA processing genes and Pre-MiRNAs are associated with the risk of chronic lymphocytic leukemia. *PLoS ONE* 10:0118905. doi: 10.1371/journal.pone.0118905
- Mashayekhi, S., Saeidi Saedi, H., Salehi, Z., Soltanipour, S., and Mirzajani, E. (2018). Effects of miR-27a, miR-196a2 and miR-146a polymorphisms on the risk of breast cancer. *Br. J. Biomed. Sci.* 75, 76–81. doi: 10.1080/09674845.2017.1399572
- Miao, L., Wang, L., Zhu, L., Du, J., Zhu, X., Niu, Y., et al. (2016). Association of microRNA polymorphisms with the risk of head and neck squamous cell carcinoma in a Chinese population: a case-control study. *Chin. J. Cancer* 35:77. doi: 10.1186/s40880-016-0136-9
- Min, K. T., Kim, J. W., Jeon, Y. J., Jang, M. J., Chong, S. Y., Oh, D., et al. (2012). Association of the miR-146aC>G, 149C>T, 196a2C>T, and 499A>G polymorphisms with colorectal cancer in the Korean population. *Mol. Carcinogenesis* 51, E65–E73. doi: 10.1002/mc.21849
- Minelli, C., Thompson, J. R., Abrams, K. R., Thakkestian, A., and Attia, J. (2008). How should we use information about HWE in the meta-analyses of genetic association studies? *Int. J. Epidemiol.* 37, 136–146. doi: 10.1093/ije/dym234
- Minh, T. T. H., Thanh, N. T. N., Van Thiep, T., and Hue, N. T. (2018). “Association between single nucleotide polymorphism Rs11614913 (C>T) on Mir-196a2 and breast cancer in vietnamese population,” in *6th International Conference on the Development of Biomedical Engineering in Vietnam (BME6)*, Vol. 63, eds T. Vo Van, T. Nguyen Le, and T. Nguyen Duc, BME 2017. IFMBE Proceedings (Singapore: Springer).
- Mittal, R. D., Gangwar, R., George, G. P., Mittal, T., and Kapoor, R. (2011). Investigative role of Pre-MicroRNAs in bladder cancer patients: a case-control study in North India. *DNA Cell Biol.* 30, 401–406. doi: 10.1089/dna.2010.1159
- Morales, S., Gulppi, F., Gonzalez-Hormazabal, P., Fernandez-Ramires, R., Bravo, T., Reyes, J. M., et al. (2016). Association of single nucleotide polymorphisms in Pre-miR-27a, Pre-miR-196a2, Pre-miR-423, miR-608 and Pre-miR-618 with breast cancer susceptibility in a South American population. *BMC Genetics* 17:109. doi: 10.1186/s12863-016-0415-0
- Nariman-Saleh-Fam, Z., Bastami, M., Somi, M. H., Behjati, F., Mansoori, Y., Daraei, A., et al. (2017). miRNA-related polymorphisms in miR-423 (rs6505162) and PEX6 (rs1129186) and risk of esophageal squamous cell carcinoma in an Iranian cohort. *Genet. Test. Mol. Biomarkers* 21, 382–390. doi: 10.1089/gtmb.2016.0346
- Nariman-Saleh-Fam, Z., Bastami, M., Somi, M. H., Samadi, N., Abbaszadegan, M. R., Behjati, F., et al. (2016). In silico dissection of miRNA targetome polymorphisms and their role in regulating miRNA-mediated gene expression in esophageal cancer. *Cell Biochem. Biophys.* 74, 483–497. doi: 10.1007/s12013-016-0754-5
- Ni, J., and Huang, Y. (2016). Role of polymorphisms in miR-146a, miR-149, miR-196a2 and miR-499 in the development of ovarian cancer in a Chinese population. *Int. J. Clin. Exp. Pathol.* 9, 5706–5711.
- Nikolić, Z., Savić Pavićević, D., Vucić, N., Cidilko, S., Filipovic, N., Cerovic, S., et al. (2015). Assessment of association between genetic variants in microRNA genes hsa-miR-499, hsa-miR-196a2 and hsa-miR-27a and prostate cancer risk in Serbian population. *Exp. Mol. Pathol.* 99, 145–150. doi: 10.1016/j.yexmp.2015.06.009
- Okubo, M., Tahara, T., Shibata, T., Yamashita, H., Nakamura, M., Yoshioka, D., et al. (2010). Association between common genetic variants in Pre-microRNAs

- and gastric cancer risk in Japanese population. *Helicobacter* 15, 524–531. doi: 10.1111/j.1523-5378.2010.00806.x
- Omrani, M., Hashemi, M., Eskandari-Nasab, E., Hasani, S. S., Mashhadi, M. A., Arbabi, F., et al. (2014). Hsa-mir-499 rs3746444 gene polymorphism is associated with susceptibility to breast cancer in an Iranian population. *Biomarkers Med.* 8, 259–267. doi: 10.2217/bmm.13.118
- Osada, H., and Takahashi, T. (2007). MicroRNAs in biological processes and carcinogenesis. *Carcinogenesis* 28, 2–12. doi: 10.1093/carcin/bgl185
- Ow, S. H., Chua, P. J., and Bay, B. H. (2018). miR-149 as a Potential Molecular Target for Cancer. *Curr. Med. Chem.* 25, 1046–1054. doi: 10.2174/0929867324666170718102738
- Pan, W., Wu, C., Su, Z., Duan, Z., Li, L., Mi, F., et al. (2017). Genetic polymorphisms of non-coding RNAs associated with increased head and neck cancer susceptibility: a systematic review and meta-analysis. *Oncotarget* 8, 62508–62523. doi: 10.18632/oncotarget.20096
- Parlayan, C., Ikeda, S., Sato, N., Sawabe, M., Muramatsu, M., and Arai, T. (2014). Association analysis of single nucleotide polymorphisms in miR-146a and miR-196a2 on the prevalence of cancer in elderly Japanese: a case-control study. *Asian Pac. J. Cancer Prevent.* 15, 2101–2107. doi: 10.7314/APJCP.2014.15.5.2101
- Pavakis, E., Papaconstantinou, I., Gazouli, M., Theodosopoulos, T., Karamanolis, G., Genatas, K., et al. (2013). MicroRNA gene polymorphisms in pancreatic cancer. *Pancreatol.* 13, 273–278. doi: 10.1016/j.pan.2013.02.005
- Peckham-Gregory, E. C., Thapa, D. R., Martinson, J., Duggal, P., Penugonda, S., Bream, J. H., et al. (2016). MicroRNA-related polymorphisms and non-Hodgkin lymphoma susceptibility in the Multicenter AIDS Cohort Study. *Cancer Epidemiol.* 45, 47–57. doi: 10.1016/j.canep.2016.09.007
- Peng, S., Kuang, Z., Sheng, C., Zhang, Y., Xu, H., and Cheng, Q. (2010). Association of MicroRNA-196a-2 gene polymorphism with gastric cancer risk in a Chinese population. *Digest. Dis. Sci.* 55, 2288–2293. doi: 10.1007/s10620-009-1007-x
- Peng, Y., and Croce, C. M. (2016). The role of MicroRNAs in human cancer. *Signal Transduct. Targeted Ther.* 1:15004. doi: 10.1038/sigtrans.2015.4
- Peters, J. L., Sutton, A. J., Jones, D. R., Abrams, K. R., and Rushton, L. (2007). Performance of the trim and fill method in the presence of publication bias and between-study heterogeneity. *Stat. Med.* 26, 4544–4562. doi: 10.1002/sim.2889
- Poltronieri-Oliveira, A. B., Madeira, F. F., Nunes, D. B. S. M., Rodrigues, G. H., Lopes, B. C., Manoel-Caetano, F. S., et al. (2017). Polymorphisms of miR-196a2 (rs11614913) and miR-605 (rs2043556) confer susceptibility to gastric cancer. *Gene Rep.* 7, 154–163. doi: 10.1016/j.genrep.2017.04.006
- Pratedrat, P., Sopipong, W., Makkoch, J., Praianantathavorn, K., Chuaypen, N., Tangkijvanich, P., et al. (2015). Single nucleotide polymorphisms in miR-149 (rs2292832) and miR-101-1 (rs7536540) are not associated with hepatocellular carcinoma in Thai patients with Hepatitis B virus infection. *Asian Pac. J. Cancer Prevent.* 16, 6457–6461. doi: 10.7314/APJCP.2015.16.15.6457
- Pu, J. Y., Dong, W., Zhang, L., Liang, W. B., Yang, Y., and Lv, M. L. (2014). No association between single nucleotide polymorphisms in pre-miRNAs and the risk of gastric cancer in Chinese population. *Iran. J. Basic Med. Sci.* 17, 128–133. doi: 10.22038/IJBMS.2014.2246
- Qi, J. H., Wang, J., Chen, J., Shen, F., Huang, J. T., Sen, S., et al. (2014). High-resolution melting analysis reveals genetic polymorphisms in MicroRNAs confer hepatocellular carcinoma risk in Chinese patients. *BMC Cancer* 14:643. doi: 10.1186/1471-2407-14-643
- Qi, P., Dou, T.-h., Geng, L., Zhou, F.-g., Gu, X., Wang, H., et al. (2010). Association of a variant in MIR 196A2 with susceptibility to hepatocellular carcinoma in male Chinese patients with chronic hepatitis B virus infection. *Hum. Immunol.* 71, 621–626. doi: 10.1016/j.humimm.2010.02.017
- Qi, P., Wang, L., Zhou, B., Yao, W. J., Xu, S., Zhou, Y., et al. (2015). Associations of miRNA polymorphisms and expression levels with breast cancer risk in the Chinese population. *Genet. Mol. Res.* 14, 6289–6296. doi: 10.4238/2015.June.11.2
- Qu, Y., Qu, H., Luo, M., Wang, P., Song, C., Wang, K., et al. (2014). MicroRNAs related polymorphisms and genetic susceptibility to esophageal squamous cell carcinoma. *Mol. Genet. Genom.* 289, 1123–1130. doi: 10.1007/s00438-014-0873-x
- Rakmanee, S., Pakakasama, S., Hongeng, S., Sanguansin, S., Thongmee, A., and Pongstaporn, W. (2017). Increased risk of Thai childhood acute lymphoblastic leukemia with the MiR196a2 T > G; C polymorphism. *Asian Pac. J. Cancer Prevent.* 18, 1117–1120. doi: 10.22034/APJCP.2017.18.4.1117
- Ranjbar, R., Chaleshi, V., Aghdaei, H. A., and Morovvati, S. (2018). Investigating the association between miR-608 rs4919510 and miR-149 rs2292832 with Colorectal Cancer in Iranian Population. *Microna* 7, 100–106. doi: 10.2174/2211536607666180206145540
- Ren, Y.-G., Zhou, X.-M., Cui, Z.-G., and Hou, G. (2016). Effects of common polymorphisms in miR-146a and miR-196a2 on lung cancer susceptibility: a meta-analysis. *J. Thoracic Dis.* 8, 1297–1305. doi: 10.21037/jtd.2016.05.02
- Rogoveanu, I., Burada, F., Cucu, M. G., Vere, C. C., Ioana, M., and Cîmpeanu, R. A. (2017). Association of microRNA polymorphisms with the risk of gastric cancer in a Romanian population. *J. Gastrointest. Liver Dis.* 26, 231–238. doi: 10.15403/jgld.2014.1121.263.rog
- Rong, G.-Q., Zhang, X.-M., Chen, B., Yang, X.-D., Wu, H.-R., and Gong, W. (2017). MicroRNA gene polymorphisms and the risk of colorectal cancer. *Oncol. Lett.* 13, 3617–3623. doi: 10.3892/ol.2017.5885
- Roy, R., De Sarkar, N., Ghose, S., Paul, R. R., Pal, M., Bhattacharya, C., et al. (2014). Genetic variations at microRNA and processing genes and risk of oral cancer. *Tumour Biol.* 35, 3409–3414. doi: 10.1007/s13277-013-1450-3
- Schwarzer, G. (2007). meta: An R package for meta-analysis. *R. News* 3, 40–45.
- Shen, F., Chen, J., Guo, S., Zhou, Y., Zheng, Y., Yang, Y., et al. (2016). Genetic variants in miR-196a2 and miR-499 are associated with susceptibility to esophageal squamous cell carcinoma in Chinese Han population. *Tumor Biol.* 37, 4777–4784. doi: 10.1007/s13277-015-4268-3
- Sodhi, K. K., Bahl, C., Singh, N., Behera, D., and Sharma, S. (2015). Functional genetic variants in pre-miR-146a and 196a2 genes are associated with risk of lung cancer in North Indians. *Future Oncol.* 11, 2159–2173. doi: 10.2217/fon.15.143
- Song, Z. S., Wu, Y., Zhao, H. G., Liu, C. X., Cai, H. Y., Guo, B. Z., et al. (2016). Association between the rs11614913 variant of miRNA-196a-2 and the risk of epithelial ovarian cancer. *Oncol. Lett.* 11, 194–200. doi: 10.3892/ol.2015.3877
- Srivastava, K., and Srivastava, A. (2012). Comprehensive review of genetic association studies and meta-analyses on miRNA polymorphisms and cancer risk. *PLoS ONE* 7:e50966. doi: 10.1371/journal.pone.0050966
- Srivastava, K., Srivastava, A., and Mittal, B. (2010). Common genetic variants in pre-microRNAs and risk of gallbladder cancer in North Indian population. *J. Hum. Genet.* 55, 495–499. doi: 10.1038/jhg.2010.54
- Srivastava, S., Singh, S., Fatima, N., Mittal, B., and Srivastava, A. N. (2017). Pre-microRNA gene polymorphisms and risk of cervical squamous cell carcinoma. *J. Clin. Diagnostic Res.* 11, GC01–GC04. doi: 10.7860/JCDR/2017/25361.10543
- Su, R., Li, W., and Luo, R. (2016). Association between miR-146a, miR-149, miR-196a2 and miR-499 gene polymorphisms and the susceptibility to gastric cancer in a Chinese population. *Int. J. Clin. Exp. Pathol.* 9, 2192–2199.
- Sun, X. C., Zhang, A. C., Tong, L. L., Wang, K., Wang, X., Sun, Z. Q., et al. (2016). miR-146a and miR-196a2 polymorphisms in ovarian cancer risk. *Genet. Mol. Res.* 15:gm.15038468. doi: 10.4238/gmr.15038468
- Sushma, P. S., Jamil, K., Uday Kumar, P., Satyanarayana, U., Ramakrishna, M., and Triveni, B. (2015). Genetic variation in microRNAs and risk of oral squamous cell carcinoma in South Indian population. *Asian Pac. J. Cancer Prevent.* 16, 7589–7594. doi: 10.7314/APJCP.2015.16.17.7589
- Tandon, D., Dewangan, J., Srivastava, S., Garg, V. K., and Rath, S. K. (2017). MiRNA genetic variants: as potential diagnostic biomarkers for oral cancer. *Pathol. Res. Pract.* 214, 281–289. doi: 10.1016/j.prp.2017.10.002
- Thakkinstant, A., McElduff, P., D'Este, C., Duffy, D., and Attia, J. (2005). A method for meta-analysis of molecular association studies. *Stat. Med.* 24, 1291–1306. doi: 10.1002/sim.2010
- Thakkinstant, A., McKay, G. J., McEvoy, M., Chakravarthy, U., Chakrabarti, S., Silvestri, G., et al. (2011). Systematic review and meta-analysis of the association between complement component 3 and age-related macular degeneration: a HuGE review and meta-analysis. *Am. J. Epidemiol.* 173, 1365–1379. doi: 10.1093/aje/kwr025
- Tian, T., Shu, Y., Chen, J., Hu, Z., Xu, L., Jin, G., et al. (2009). A functional genetic variant in microRNA-196a2 is associated with increased susceptibility of lung cancer in Chinese. *Cancer Epidemiol. Biomarkers Prevent.* 18, 1183–1187. doi: 10.1158/1055-9965.EPI-08-0814
- Tong, N., Xu, B., Shi, D., Du, M., Li, X., Sheng, X., et al. (2014). Hsa-miR-196a2 polymorphism increases the risk of acute lymphoblastic leukemia in Chinese children. *Mutat. Res.* 759, 16–21. doi: 10.1016/j.mrfmmm.2013.11.004
- Toraihi, E. A., Fawz, M. S., Elgazzaz, M. G., Hussein, M. H., Shehata, R. H., and Daoud, H. G. (2016a). Combined genotype analyses of precursor miRNA196a2

- and 499a variants with hepatic and renal cancer susceptibility a preliminary study. *Asian Pac. J. Cancer Prevent.* 17, 3369–3375.
- Toraih, E. A., Fawzy, M. S., Mohammed, E. A., Hussein, M. H., and EL-Labban, M. M. (2016b). MicroRNA-196a2 biomarker and targetome network analysis in solid tumors. *Mol. Diagn. Ther.* 20, 559–577. doi: 10.1007/s40291-016-0223-2
- Trikalinos, T. A., Salanti, G., Khoury, M. J., and Ioannidis, J. P. (2006). Impact of violations and deviations in Hardy-Weinberg equilibrium on postulated gene-disease associations. *Am. J. Epidemiol.* 163, 300–309. doi: 10.1093/aje/kwj046
- Tu, H. F., Liu, C. J., Chang, C. L., Wang, P. W., Kao, S. Y., Yang, C. C., et al. (2012). The association between genetic polymorphism and the processing efficiency of miR-149 affects the prognosis of patients with head and neck squamous cell carcinoma. *PLoS ONE* 7:051606. doi: 10.1371/journal.pone.0051606
- Umar, M., Upadhyay, R., Prakash, G., Kumar, S., Ghoshal, U. C., and Mittal, B. (2013). Evaluation of common genetic variants in pre-microRNA in susceptibility and prognosis of esophageal cancer. *Mol. Carcinogenesis* 52, 10–18. doi: 10.1002/mc.21931
- Vinci, S., Gelmini, S., Mancini, I., Malentacchi, F., Pazzagli, M., Beltrami, C., et al. (2013). Genetic and epigenetic factors in regulation of microRNA in colorectal cancers. *Methods* 59, 138–146. doi: 10.1016/j.jymeth.2012.09.002
- Vinci, S., Gelmini, S., Pratesi, N., Conti, S., Malentacchi, F., Simi, L., et al. (2011). Genetic variants in miR-146a, miR-149, miR-196a2, miR-499 and their influence on relative expression in lung cancers. *Clin. Chem. Lab. Med.* 49, 2073–2080. doi: 10.1515/CCLM.2011.708
- Wang, J., Zhang, Y., Zhang, Y., and Chen, L. (2016). Correlation between miRNA-196a2 and miRNA-499 polymorphisms and bladder cancer. *Int. J. Clin. Exp. Med.* 9, 20484–20488.
- Wang, K., Guo, H., Hu, H., Xiong, G., Guan, X., Li, J., et al. (2010). A functional variation in pre-microRNA-196a is associated with susceptibility of esophageal squamous cell carcinoma risk in Chinese Han. *Biomarkers* 15, 614–618. doi: 10.3109/1354750X.2010.505299
- Wang, N., Li, Y., Zhou, R. M., Wang, G. Y., Wang, C. M., Chen, Z. F., et al. (2014). Hsa-miR-196a2 functional SNP is associated with the risk of ESCC in individuals under 60 years old. *Biomarkers* 19, 43–48. doi: 10.3109/1354750X.2013.866164
- Wang, R., Zhang, J., Ma, Y., Chen, L., Guo, S., Zhang, X., et al. (2014). Association study of miR149 rs2292832 and miR608 rs4919510 and the risk of hepatocellular carcinoma in a largescale population. *Mol. Med. Rep.* 10, 2736–2744. doi: 10.3892/mmr.2014.2536
- Wang, S., Tao, G., Wu, D., Zhu, H., Gao, Y., Tan, Y., et al. (2013). A functional polymorphism in MIR196A2 is associated with risk and prognosis of gastric cancer. *Mol. Carcinogenesis* 52, 87–95. doi: 10.1002/mc.22017
- Wang, X. B., Cui, N. H., Yang, J., Qiu, X. P., Gao, J. J., Yang, N., et al. (2014). Angiotensin-converting enzyme insertion/deletion polymorphism is not a major determining factor in the development of sporadic alzheimer disease: evidence from an updated meta-analysis. *PLoS ONE* 9:e111406. doi: 10.1371/journal.pone.0111406
- Wang, X. H., Wang, F. R., Tang, Y. F., Zou, H. Z., and Zhao, Y. Q. (2014). Association of miR-149C>T and miR-499A>G polymorphisms with the risk of hepatocellular carcinoma in the Chinese population. *Genetics Mol. Res.* 13, 5048–5054. doi: 10.4238/2014.July.4.20
- Wang, Y.-H., Hu, H.-N., Weng, H., Chen, H., Luo, C.-L., Ji, J., et al. (2017). Association between polymorphisms in MicroRNAs and risk of urological cancer: a meta-analysis based on 17,019 subjects. *Front. Physiol.* 8:325. doi: 10.3389/fphys.2017.00975
- Wei, J., Zheng, L., Liu, S., Yin, J., Wang, L., Wang, X., et al. (2013). MiR-196a2 rs11614913 T & C polymorphism and risk of esophageal cancer in a Chinese population. *Hum. Immunol.* 74, 1199–1205. doi: 10.1016/j.humimm.2013.06.012
- Wei, W. J., Lu, Z. W., Li, D. S., Wang, Y., Zhu, Y. X., Wang, Z. Y., et al. (2014). Association of the miR-149 Rs2292832 polymorphism with papillary thyroid cancer risk and clinicopathologic characteristics in a Chinese population. *Int. J. Mol. Sci.* 15, 20968–20981. doi: 10.3390/ijms151120968
- Xie, M., Li, Y., Wu, J., and Wu, J. (2015). A risk of digestive tract neoplasms susceptibility in miR-146a and miR-196a2. *Famil. Cancer* 14, 229–239. doi: 10.1007/s10689-014-9776-6
- Xue, W., Zhu, M., Wang, Y., He, J., and Zheng, L. (2015). Association between PLCE1 rs2274223 A > G polymorphism and cancer risk: proof from a meta-analysis. *Sci. Rep.* 5:7986. doi: 10.1038/srep07986
- Yan, P., Xia, M., Gao, F., Tang, G., Zeng, H., Yang, S., et al. (2015). Predictive role of miR-146a rs2910164 (C>G), miR-149 rs2292832 (T>C), miR-196a2 rs11614913 (T>C) and miR-499 rs3746444 (T>C) in the development of hepatocellular carcinoma. *Int. J. Clin. Exp. Pathol.* 8, 15177–15183.
- Yan, W., Gao, X., and Zhang, S. (2017). Association of miR-196a2 rs11614913 and miR-499 rs3746444 polymorphisms with cancer risk: a meta-analysis. *Oncotarget* 8, 114344–114359. doi: 10.18632/oncotarget.22547
- Yang, H., Dinney, C. P., Ye, Y., Zhu, Y., Grossman, H. B., and Wu, X. (2008). Evaluation of genetic variants in microRNA-related genes and risk of bladder cancer. *Cancer Res.* 68, 2530–2537. doi: 10.1158/0008-5472.CAN-07-5991
- Yang, H., Lu, Q. L., Wu, X. J., Ma, H. Y., Qu, Y. Y., Zhang, D. Z., et al. (2016). Association of genetic variations in miR-146a rs2910164 and mir-149 rs11614913 with the development of classic Kaposi sarcoma. *Genetics Mol. Res.* 15:gm15048855. doi: 10.4238/gmr15048855
- Yin, Z., Cui, Z., Guan, P., Li, X., Wu, W., Ren, Y., et al. (2015). Interaction between polymorphisms in pre-miRNA genes and cooking oil fume exposure on the risk of lung cancer in Chinese non-smoking female population. *PLoS ONE* 10:0128572. doi: 10.1371/journal.pone.0128572
- Yin, Z., Cui, Z., Ren, Y., Xia, L., Li, H., and Zhou, B. (2017). MiR-196a2 and lung cancer in Chinese non-smoking females: a genetic association study and expression analysis. *Oncotarget* 8, 70890–70898. doi: 10.18632/oncotarget.20174
- Yin, Z., Cui, Z., Ren, Y., Xia, L., Wang, Q., Zhang, Y., et al. (2016). Association between polymorphisms in pre-miRNA genes and risk of lung cancer in a Chinese non-smoking female population. *Lung Cancer* 94, 15–21. doi: 10.1016/j.lungcan.2016.01.013
- Yoon, K.-A., Yoon, H., Park, S., Jang, H.-J., Zo, J. I., Lee, H.-S., et al. (2012). The prognostic impact of microRNA sequence polymorphisms on the recurrence of patients with completely resected non-small cell lung cancer. *J. Thoracic Cardiovasc. Surg.* 144, 794–807. doi: 10.1016/j.jtcvs.2012.06.030
- Zhan, J.-f., Chen, L.-h., Chen, Z.-x., Yuan, Y.-w., Xie, G.-z., Sun, A.-m., et al. (2011). A Functional Variant in MicroRNA-196a2 Is Associated with Susceptibility of Colorectal Cancer in a Chinese Population. *Arch. Med. Res.* 42, 144–148. doi: 10.1016/j.arcmed.2011.04.001
- Zhang, E., Xu, Z., Duan, W., Huang, S., and Lu, L. (2017). Association between polymorphisms in premiRNA genes & risk of oral squamous cell cancer in a Chinese population. *PLoS ONE* 12:0176044. doi: 10.1371/journal.pone.0176044
- Zhang, H., Su, Y.-L., Yu, H., and Qian, B.-Y. (2012). Meta-Analysis of the Association between Mir-196a-2 Polymorphism and Cancer Susceptibility. *Cancer Biol. Med.* 9, 63–72. doi: 10.3969/j.issn.2095-3941.2012.01.012
- Zhang, H., Zhang, Y., Yan, W., Wang, W., Zhao, X., Ma, X., et al. (2017). Association between three functional microRNA polymorphisms (miR-499 rs3746444, miR-196a rs11614913 and miR-146a rs2910164) and breast cancer risk: a meta-analysis. *Oncotarget* 8, 393–407. doi: 10.18632/oncotarget.13426
- Zhang, J., Wang, R., Ma, Y. Y., Chen, L. Q., Jin, B. H., Yu, H., et al. (2013). Association between single nucleotide polymorphisms in miRNA196a-2 and miRNA146a and susceptibility to hepatocellular carcinoma in a Chinese population. *Asian Pac. J. Cancer Prevent.* 14, 6427–6431. doi: 10.7314/APJCP.2013.14.11.6427
- Zhang, L. H., Hao, B. B., Zhang, C. Y., Dai, X. Z., and Zhang, F. (2016). Contributions of polymorphisms in mir146a, mir196a, and mir499 to the development of hepatocellular carcinoma. *Genet. Mol. Res.* 15:gm15038582. doi: 10.4238/gmr15038582
- Zhang, M., Jin, M., Yu, Y., Zhang, S., Wu, Y., Liu, H., et al. (2012). Associations of miRNA polymorphisms and female physiological characteristics with breast cancer risk in Chinese population. *Eur. J. Cancer Care* 21, 274–280. doi: 10.1111/j.1365-2354.2011.01308.x
- Zhang, M. W., Jin, M. J., Yu, Y. X., Zhang, S. C., Liu, B., Jiang, X., et al. (2012). Associations of lifestyle-related factors, hsa-miR-149 and hsa-miR-605 gene polymorphisms with gastrointestinal cancer risk. *Mol. Carcinog* 51(Suppl. 1), E21–31. doi: 10.1002/mc.20863
- Zhao, H., Xu, J., Zhao, D., Geng, M., Ge, H., Fu, L., et al. (2016). Somatic Mutation of the SNP rs11614913 and Its Association with Increased

- MIR 196A2 Expression in Breast Cancer. *DNA Cell Biol.* 35, 81–87. doi: 10.1089/dna.2014.2785
- Zhou, B., Dong, L. P., Jing, X. Y., Li, J. S., Yang, S. J., Wang, J. P., et al. (2014). Association between miR-146aG>C and miR-196a2C>T polymorphisms and the risk of hepatocellular carcinoma in a Chinese population. *Tumor Biol.* 35, 7775–7780. doi: 10.1007/s13277-014-2020-z
- Zhou, B., Wang, K., Wang, Y., Xi, M., Zhang, Z., Song, Y., et al. (2011). Common genetic polymorphisms in pre-microRNAs and risk of cervical squamous cell carcinoma. *Mol. Carcinogenesis* 50, 499–505. doi: 10.1002/mc.20740
- Zhu, L., Chu, H., Gu, D., Ma, L., Shi, D., Zhong, D., et al. (2012). A functional polymorphism in miRNA-196a2 is associated with colorectal cancer risk in a Chinese population. *DNA Cell Biol.* 31, 349–353. doi: 10.1089/dna.2011.1348
- Zintzaras, E. (2008). Variance estimation of allele-based odds ratio in the absence of Hardy-Weinberg equilibrium. *Eur. J. Epidemiol.* 23, 323–326. doi: 10.1007/s10654-008-9242-6
- Zintzaras, E., Koufakis, T., Ziakas, P. D., Rodopoulou, P., Giannouli, S., and Voulgarelis, M. (2006). A meta-analysis of genotypes and haplotypes of methylenetetrahydrofolate reductase gene polymorphisms in acute lymphoblastic leukemia. *Eur. J. Epidemiol.* 21, 501–510. doi: 10.1007/s10654-006-9027-8
- Zintzaras, E., and Lau, J. (2008). Synthesis of genetic association studies for pertinent gene-disease associations requires appropriate methodological and statistical approaches. *J. Clin. Epidemiol.* 61, 634–645. doi: 10.1016/j.jclinepi.2007.12.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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miRNome Reveals New Insights Into the Molecular Biology of Field Cancerization in Gastric Cancer

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OPEN ACCESS

Edited by:

Ge Shan,
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equally to this work

Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 05 June 2018

Accepted: 04 June 2019

Published: 19 June 2019

Citation:

Pereira A, Moreira F,
Vinasco-Sandoval T, Cunha A,
Vidal A, Ribeiro-dos-Santos AM,
Pinto P, Magalhães L, Assumpção M,
Demachki S, Santos S, Assumpção P
and Ribeiro-dos-Santos Â (2019)
miRNome Reveals New Insights Into
the Molecular Biology of Field
Cancerization in Gastric Cancer.
Front. Genet. 10:592.
doi: 10.3389/fgene.2019.00592

Background: MicroRNAs (miRNAs) play an important role in gastric carcinogenesis and have been associated with gastric field cancerization; however, their role is not fully understood in this process. We performed the miRNome sequencing of non-cancerous, adjacent to tumor and gastric cancer samples to understand the involvement of these small RNAs in gastric field cancerization.

Methods: We analyzed samples of patients without cancer as control (non-cancerous gastric samples) and adjacent to cancer and gastric cancer paired samples, and considered miRNAs with $|\log_2(\text{fold change})| > 2$ and $P_{adj} < 0.05$ to be statistically significant. The identification of target genes, functional analysis and enrichment in KEGG pathways were realized in the TargetCompare, miRTargetLink, and DAVID tools. We also performed receiver operating characteristic (ROC) curves and miRNAs that had an AUC > 0.85 were considered to be potential biomarkers.

Results: We found 14 miRNAs exclusively deregulated in gastric cancer, of which six have potential diagnostic value for advanced disease. Nine miRNAs with known tumor suppressor activities (TS-miRs) were deregulated exclusively in adjacent tissue. Of these, five have potential diagnostic value for the early stages of gastric cancer. Functional analysis of these TS-miRs revealed that they regulate important cellular signaling pathways (PI3K-Akt, HIF-1, Ras, Rap1, ErbB, and MAPK signaling pathways), that are involved in gastric carcinogenesis. Seven miRNAs were differentially expressed in both gastric cancer and adjacent regarding to non-cancerous tissues; among them, *hsa-miR-200a-3p* and *hsa-miR-873-5p* have potential diagnostic value for early and advanced stages of the disease. Only *hsa-miR-196a-5p* was differentially expressed between adjacent to cancer and gastric cancer tissues. In addition, the other miRNAs identified in this study were not differentially expressed between adjacent to cancer and gastric cancer, suggesting that these tissues are very similar and that share these molecular changes.

Conclusion: Our results show that gastric cancer and adjacent tissues have a similar miRNA expression profile, indicating that studied miRNAs are intimately associated with field cancerization in gastric cancer. The overexpression of TS-miRs in adjacent tissues may be a barrier against tumorigenesis within these pre-cancerous conditions prior to the eventual formation or relapse of a tumor. Additionally, these miRNAs have a great accuracy in discriminating non-cancerous from adjacent to tumor and cancer tissues and can be potentially useful as biomarkers for gastric cancer.

Keywords: miRNome, miRNAs, gastric cancer, field cancerization, biomarkers

INTRODUCTION

Gastric cancer (GC) is an aggressive disease that is considered the third leading cause of cancer death worldwide (Ferlay et al., 2015). GC is usually diagnosed in late stages, due in part to the limited efficiency of existing biomarkers, and the 5-year survival rates of these patients do not exceed 30% (Correa, 2013; Yakirevich and Resnick, 2013). Studies have shown that microRNAs (miRNAs) have excellent sensitivity/specificity and a high discriminatory capacity, which make them potentially useful as molecular biomarkers for this type of cancer (Wu et al., 2014; Shin et al., 2015; Vidal et al., 2016).

MicroRNAs are small non-coding RNAs (~18–30 nt) that regulate gene expression post-transcriptionally, disrupting the expression of target mRNAs (Bartel, 2004; Wu et al., 2014). These molecules play an important role in multiple pathways and the processes responsible for the maintenance of healthy tissue homeostasis (Schneider, 2012; Runtsch et al., 2014). In humans, studies suggest that miRNAs are part of the complex regulatory network of the healthy stomach (Ribeiro-dos-Santos et al., 2010; Moreira et al., 2014a). Therefore, when miRNAs are deregulated in the human stomach, they compromise important pathways that regulate the normal functions of this organ (Zhang et al., 2014), contributing to the onset and progression of gastric carcinogenesis (Wu et al., 2014).

In recent years, our research group joined efforts and used different technologies to understand the relationship between the deregulation of these small RNAs and gastric carcinogenesis (Assumpção et al., 2015; Darnet et al., 2015; Vidal et al., 2016; Magalhães et al., 2018; Pereira et al., 2019). More recently, we have demonstrated the deregulation of miRNAs during the evolution of Correa's cascade (Vidal et al., 2016) and in field cancerization in GC (Assumpção et al., 2015; Pereira et al., 2019).

Field cancerization assumes that the tissues adjacent to the tumor have molecular changes (e.g., genetic and/or epigenetic) that make them susceptible to the onset of tumors or recurrences (Slaughter et al., 1953; Chai and Brown, 2009; Curtius et al., 2018). The preexisting molecular changes in this tissue (which may be caused by an insult to the healthy epithelium) are precursors of carcinogenesis and serve as background for the establishment and progression of the tumor (Hattori and Ushijima, 2016; Padmanabhan et al., 2017; Curtius et al., 2018). In fact, studies have shown that adjacent to the tumor tissues have similar molecular changes to those found in the tumor as well as unique and exclusive changes, which distinguish them

from non-cancerous tissues (Assumpção et al., 2015; Takeshima et al., 2015; Aran et al., 2017; Vidal et al., 2017; Yoshida et al., 2017; Pereira et al., 2019). Recently, miRNAs were related to field cancerization in GC (Assumpção et al., 2015; Pereira et al., 2019), however, little is known about the true role played by these small non-coding RNAs in this process. The use of robust large-scale sequencing technologies is an excellent strategy in both the discovery of new biomarkers and in providing an overview of the complex relationship between miRNAs and field cancerization.

In this study, we used deep sequencing to evaluate the overall expression profile of miRNAs in non-cancerous gastric tissues, adjacent to cancer and with cancer tissues in order to identify miRNAs involved in the field cancerization. In addition, we evaluated the discriminatory performance of miRNAs as biomarkers of gastric carcinogenesis. Our results show new deregulated miRNAs, which are potentially useful as biomarkers for this cancer and suggest a new molecular mechanism involved in the biology of field cancerization.

MATERIALS AND METHODS

Biological Material

A total of 45 fresh samples of stomach antrum tissues were included in the present study. The non-cancerous control (NC) samples were collected from 15 patients without cancer (chronic gastritis; $\pm$ *H. pylori*; mean age = 59.2) during an upper digestive endoscopy (tissue fragments of approximately 4 millimeters). In addition, 15 tumor-adjacent tissues (histopathologically without cancer) and 15 gastric adenocarcinoma samples were collected from patients with gastric cancer. The tumor-adjacent (ADJ) and gastric adenocarcinoma (GC) samples were all paired ($\pm$ *H. pylori*; mean age = 59.9).

The tissues were obtained from patients treated at the Hospital Universitário João de Barros Barreto (HUIBB), Belém, Pará, Brazil. Samples were collected prior to antibiotic, chemotherapeutic, and/or radiotherapeutic treatment. Immediately after collection, all samples were frozen and stored in liquid nitrogen until analysis.

Histopathological characterization of the samples, such as tumor subtype, degree of differentiation, depth of invasion, involvement of lymph nodes, and/or distant metastases were extracted from pathological reports performed by the HUIBB Department of Pathology. Histopathological analysis

of the tumor fragments was performed according to Lauren's classification (Lauren, 1965).

Ethics Statement

This study was reviewed and approved by the Ethics Committee of the Center of Oncology Research of the Federal University of Pará (Protocol No. 1.081.340). All study participants or their legal guardian provided informed written consent in accordance with the Helsinki Declaration of 1964, the Nuremberg Code, in compliance with the National Health Council's Research Guidelines Involving Human Beings (Res. CNS 466/12).

RNA Extraction, Small RNA Library Construction, and Sequencing

Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific). After isolation, total RNA was stored at -80°C until further analysis. The total RNA amount was determined using a Qubit®2.0 (Life Technologies, Foster City, CA, United States), and an Agilent RNA ScreenTape assay and 2200 TapeStation Instrument (Agilent Technologies, United States) were used to detect RNA integrity. Samples with an RNA integrity number (RIN) ≥ 5 were sequenced.

For small RNA-seq, 1 μg of total RNA per sample was used for library preparation utilizing TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, United States). A DNA ScreenTape assay in a 2200 TapeStation Instrument (Agilent Technologies, United States) and real-time PCR with a KAPA Library Quantification Kit (KAPABIOSYSTEM, United States) were used to validate and quantify each library. A 4-nM library pool comprising all samples was sequenced using a MiSeq reagent kit v3 150 cycle on a MiSeq System (Illumina, San Diego, CA, United States).

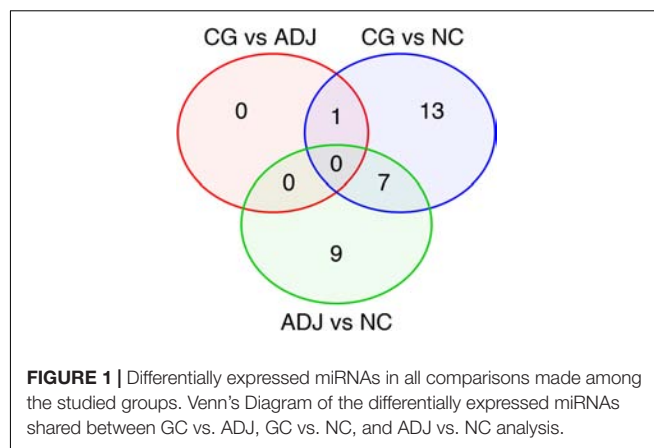
The raw sequencing reads of all libraries have been deposited at EBI-ENA (PRJEB27213).

Bioinformatics Analysis

The resulting reads were pre-processed and quality filtered ($qv > 25$). We used STAR (Dobin et al., 2013) aligner to map the reads to the human genome reference (GRCH37). We quantified mature miRNA sequencing using miRBase human annotation (v20). Counting expression data was performed with HTSeq (Anders et al., 2015).

Differential expression analysis of all processed data was performed using the bioconductor-DESeq2 package (Love et al., 2014) in R software, with a detection threshold of 10 counts per miRNA (present at least 10 read counts in at least of the libraries). Comparison between (i) gastric cancer (GC) vs. non-cancerous (NC) samples; (ii) adjacent to gastric cancer (ADJ) vs. NC samples; and (iii) GC vs. ADJ samples were made separately. Adjusted P -values ≤ 0.05 and $|\log_2(\text{fold change})| > 2$ were considered statistically significant.

For graphical analysis of miRNAs, expression data was normalized to RPKM. Heatmaps were used for hierarchical clustering of differentially expressed miRNAs. The area under the curve (AUC > 0.85) from the receiver operating characteristic (ROC) curves was used to identify biomarkers with the best



sensitivity/specificity relation, and a discriminant analysis of principal components (DAPC) was constructed to infer the number of clusters of epigenetically related samples. All graphical analyses were performed using the R statistical platform.

Identification of Differentially Expressed miRNA Target Genes

We used two online tools to identify the differentially expressed (D. E.) miRNA target genes: (i) TargetCompare (Moreira et al., 2014b) and (ii) miRTargetLink Human (Hamberg et al., 2016). TargetCompare database allows the user to filter miRNAs and its targets genes that are associated with determined diseases, such as GC. miRTargetLink Human is a tool that allows the search for interactions between target genes and miRNAs that have been experimentally validated by molecular biology techniques.

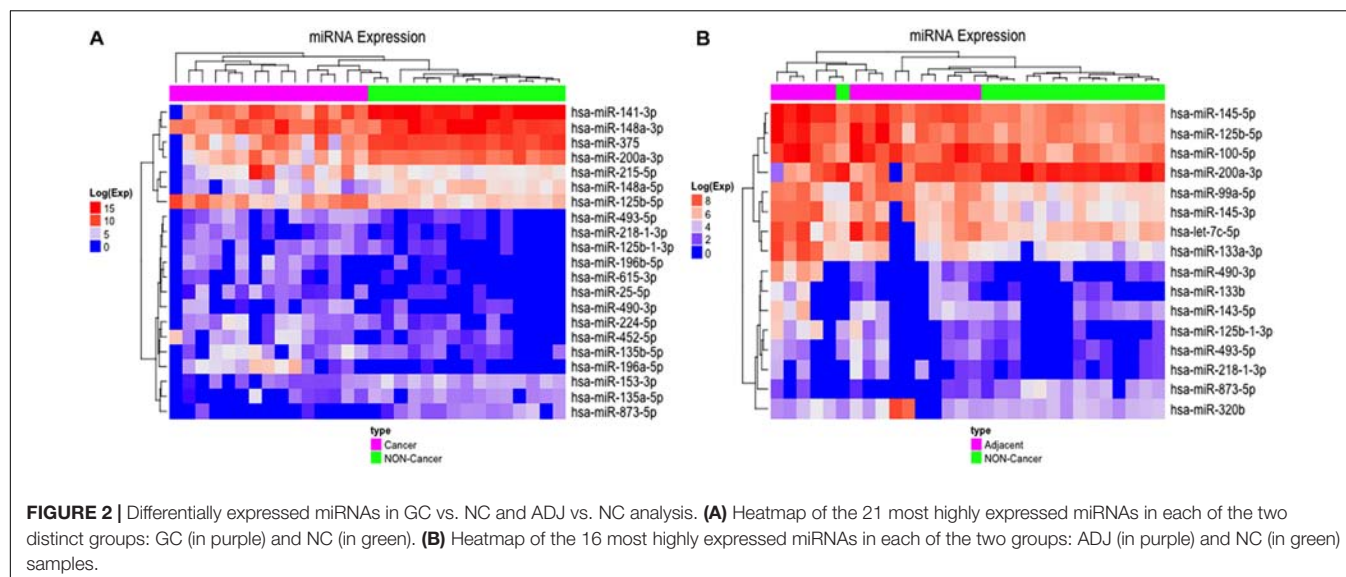
The identified target genes were submitted to functional annotation and enrichment in KEGG pathways using DAVID Bioinformatics Resources v.6.8 online tool (Huang et al., 2009a,b).

RESULTS

After quality control (Supplementary Figure S1), alignment and transcript quantitation, several small non-coding RNAs (sncRNAs) and other transcript fragments were identified. From them, $\sim 30\%$ (~ 9.5 million reads) were recognized as microRNAs reads, identifying 1,144 mature miRNAs. Approximately 90% of the miRNA reads (~ 8.5 million reads) were concentrated on the 35 most expressed miRNAs. The number and representativity of D. E. expressed miRNAs identified in this study is similar to other studies that performed miRNome analysis (Hou et al., 2011; Maltseva et al., 2014; Liu et al., 2015; Castro-Magdonel et al., 2017; Liang et al., 2017).

Differentially Expressed miRNAs in GC vs. NC Analysis

We found 21 differentially expressed (D. E.) miRNAs when we compared gastric cancer samples (GC) with non-cancerous gastric samples (NC) (Figure 1 and Supplementary Table S1),



of which eight were down-regulated and 13 were up-regulated in GC (**Supplementary Table S1**). The heatmap obtained from the normalized expression of the D. E. miRNAs perfectly clustered the GC and NC samples together (**Figure 2A**).

Fourteen miRNAs were exclusively D. E. in the GC vs. NC analysis: nine were up-regulated (six oncomiRs, one TS-miR and two with unknown functions in GC) and five were down-regulated (all TS-miRs) in GC (**Figure 1** and **Table 1**). Six miRNAs, of which five were down-regulated (*hsa-miR-141-3p*, *-miR-148a-3p*, *-miR-148a-5p*, *-miR-153-3p*, and *-miR-375*) and one were up-regulated (*hsa-miR-196a-5p*), presented the best sensitivity/specificity relation ($AUC > 0.85$) and were considered as potential biomarkers to identify GC (**Figure 3**).

Differentially Expressed miRNAs in ADJ vs. NC Analysis

Comparing adjacent to gastric cancer samples (ADJ) with NC revealed 16 D. E. miRNAs (**Figure 1** and **Supplementary Table S2**), of which two were down-regulated and 14 were up-regulated (**Supplementary Table S2**). The heatmap obtained from the normalized expression of the D. E. miRNAs clustered ADJ and NC samples together (**Figure 2B**). Furthermore, nine miRNAs (eight TS-miRs and one with unknown function in GC) were exclusively D. E. in the ADJ vs. NC analysis: they were all up-regulated in ADJ (**Figure 1** and **Table 2**).

Five D. E. miRNAs (*hsa-miR-99a*, *-miR-100-5p*, *-miR-125b-5p*, *-miR-145-3p*, and *-miR-145b-5p*, all up-regulated in ADJ) presented the best sensitivity/specificity relation ($AUC > 0.85$) and were considered as potential early biomarkers for GC (**Figure 4**).

Functional Analysis of the Up-Regulated TS-miRs in ADJ Tissue

To evaluate the biological and functional role of the eight up-regulated TS-miRs and *hsa-miR-99a-5p* in ADJ tissue (**Table 2**),

TABLE 1 | Deregulated miRNAs only in gastric cancer.

miRNA	Role in GC*	GC vs. NC	ADJ vs. NC	GC vs. ADJ
<i>miR-135b-5p</i>	OncomiR ^{1,2,3}	Up	–	–
<i>miR-196a-5p</i>	OncomiR ^{4,5}	Up	–	Up
<i>miR-196b-5p</i>	OncomiR ⁶	Up	–	–
<i>miR-215-5p</i>	OncomiR ⁷	Up	–	–
<i>miR-224-5p</i>	OncomiR ⁸	Up	–	–
<i>miR-615-3p</i>	OncomiR ⁹	Up	–	–
<i>miR-25-5p</i>	OncomiR ^{10,11}	Up	–	–
<i>miR-141-3p</i>	TS-miR ^{12,13}	Up	–	–
<i>miR-452-5p</i>	TS-miR ¹⁴	Up	–	–
<i>miR-135a-5p</i>	TS-miR ^{15,16}	Down	–	–
<i>miR-148a-3p</i>	TS-miR ¹⁷	Down	–	–
<i>miR-148a-5p</i>	TS-miR ¹⁷	Down	–	–
<i>miR-153-3p</i>	TS-miR ^{18,19}	Down	–	–
<i>miR-375</i>	TS-miR ^{20,21,22}	Down	–	–

GC, gastric cancer; ADJ, adjacent to cancer; NC, non-cancerous; Up, up-regulated; Down, down-regulated; TS-miR, tumor suppressor miRNA; OncomiR, oncogenic miRNA. (–) Not differentially expressed. (*) Putative role in GC. (1) Wang et al., 2012; (2) Lu et al., 2018; (3) Shao et al., 2019; (4) Sun et al., 2012; (5) Pan et al., 2017; (6) Liao et al., 2012; (7) Deng et al., 2014; (8) He et al., 2017; (9) Wang et al., 2018; (10) Li B.S. et al., 2015; (11) Larki et al., 2018; (12) Zuo et al., 2015; (13) Zhou et al., 2019; (14) Gao et al., 2016; (15) Zhang et al., 2016; (16) Xie et al., 2019; (17) Zheng et al., 2011; (18) Wang and Liu, 2016; (19) Ouyang et al., 2018; (20) Ding et al., 2010; (21) Chen et al., 2017; (22) Hwang et al., 2018.

we performed enrichment and functional annotation of their experimentally validated common target genes. We used nine miRNAs as input in the online tools TargetCompare (Moreira et al., 2014b) and miRTargetLink Human (Hamberg et al., 2016), which output five miRNAs (*hsa-miR-let7-c-5p*, *-miR-99a-5p*, *-miR-100-5p*, *-miR-133a-3p*, and *-miR-145-5p*) that regulate 18 common target genes (**Figure 5A**). The enrichment and functional annotation analysis of the 18 target genes performed in DAVID v.6.8 (Huang et al., 2009a,b) revealed that 13 genes participate in 23 different biological pathways

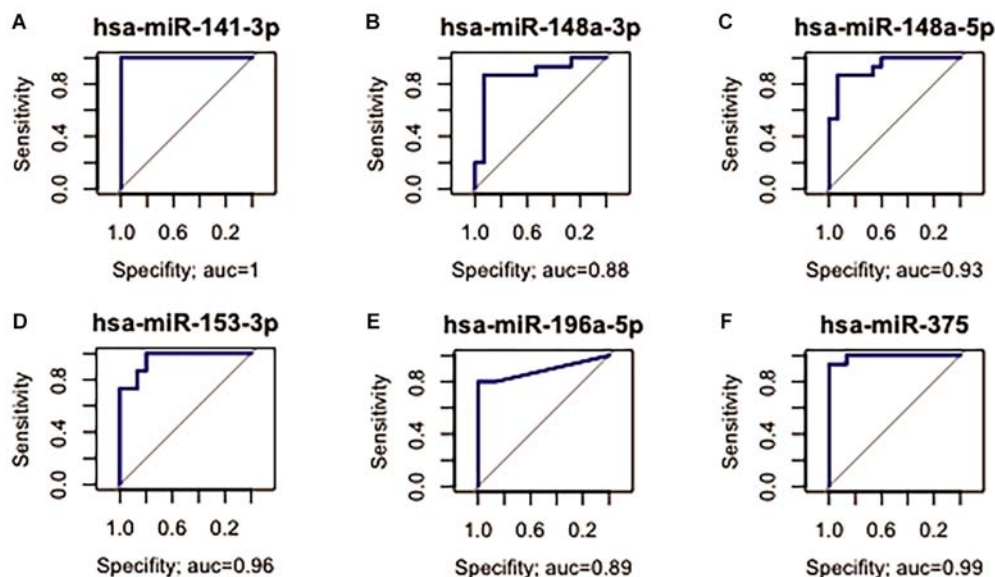


FIGURE 3 | Receiver operating characteristic (ROC) curve analysis identified miRNAs with potential to identify GC. **(A)** *hsa-miR-141-3p* (AUC = 1; 95% CI: 1.000–1.000*); **(B)** *hsa-miR-148a-3p* (AUC = 0.88; 95% CI: 0.733–1.000); **(C)** *hsa-miR-148a-5p* (AUC = 0.93; 95% CI: 0.840–1.000); **(D)** *hsa-miR-153-3p* (AUC = 0.96; 95% CI: 0.893–1.000); **(E)** *hsa-miR-196a-5p* (AUC = 0.89; 95% CI: 0.767–1.000); **(F)** *hsa-miR-375* (AUC = 0.99; 95% CI: 0.970–1.000).

TABLE 2 | Deregulated miRNAs only in adjacent to tumor tissue.

miRNA	Role in GC*	GC vs. NC	ADJ vs. NC	GC vs. ADJ
<i>miR-let7-c-5p</i>	TS-miR ¹	–	Up	–
<i>miR-133a-3p</i>	TS-miR ^{2,3}	–	Up	–
<i>miR-133b</i>	TS-miR ^{2,4}	–	Up	–
<i>miR-143-5p</i>	TS-miR ^{5,6}	–	Up	–
<i>miR-145-3p</i>	TS-miR ^{5,6}	–	Up	–
<i>miR-145-5p</i>	TS-miR ^{5,6}	–	Up	–
<i>miR-99a-5p</i>	?	–	Up	–
<i>miR-100-5p</i>	TS-miR ⁷	–	Up	–
<i>miR-320b</i>	TS-miR ⁸	–	Up	–

GC, gastric cancer; ADJ, adjacent to cancer; NC, non-cancerous; Up, up-regulated; TS-miR, tumor suppressor miRNA; OncomiR, oncogenic miRNA. (–) Not differentially expressed. (*) Putative role in GC. (?) Unknown role in GC. (1) Tsai et al., 2015; (2) Qiu et al., 2014; (3) Zhang et al., 2018; (4) Yang et al., 2017; (5) Wu et al., 2013; (6) Lei et al., 2017; (7) Shi et al., 2015; (8) Zhao et al., 2017.

(Supplementary Table S3), and 11 genes are involved in nine biological pathways that are important for the development and progression of GC (Figure 5B).

Differentially Expressed miRNAs Common to GC vs. ADJ vs. NC Analysis

hsa-miR-125b-5p, *miR-125b-1-3p*, *-miR-200a-3p*, *miR-218-1-3p*, *-miR-490-3p*, *-miR-493-5p* and *-miR-873-5p* were D. E. in both the GC vs. NC (Table 3 and Supplementary Table S1) and ADJ vs. NC (Table 3 and Supplementary Table S2) analyses. These miRNAs were not D. E. in the GC vs. ADJ analysis (Table 3), suggesting that these two tissues are similar regarding the expression of these miRNAs. In addition, the DAPC plot

(Figure 6) generated by all D. E. miRNAs indicates that GC and ADJ, despite generating distinct clusters, have much more similarity in its expression profiles when compared with NC tissue, which clustered apart. Thus, we assembled the GC and ADJ samples in a single group to compare to the NC samples during the ROC curve analysis. Two down-regulated miRNAs (*hsa-miR-200a-3p* and *hsa-miR-873-5p*) had the best sensitivity/specificity relation and were considered as potential biomarkers to identify gastric carcinogenesis (Figures 7A,B).

Comparing GC with ADJ, only one miRNA (*hsa-miR-196a-5p*) was differentially expressed in GC (Figure 1 and Table 1). *hsa-miR-196a-5p* was significantly up-regulated in GC ($P = 0.029$) but had a low sensitivity/specificity relation in the ROC curve analysis (AUC < 0.85). *hsa-miR-196a-5p* was also up-regulated in the GC vs. NC comparison (Table 1 and Supplementary Table S1). The difference in the number of miRNAs D. E. in GC vs. ADJ compared with the previous analysis corroborates to premise of that these two tissues have similar miRNA expression profiles.

DISCUSSION

miRNA deregulation is closely related to gastric cancer development (Wu et al., 2014; Zhang et al., 2014) and its relationship to field cancerization becomes evident (Assumpção et al., 2015; Pereira et al., 2019). In the search for new biomarkers and a better understanding of epigenetic field cancerization in GC, we evaluated the global expression profile of miRNAs in NC, ADJ, and GC gastric samples. Our data showed that all three types of tissue share many differentially expressed miRNAs and present miRNAs that occur exclusively in either

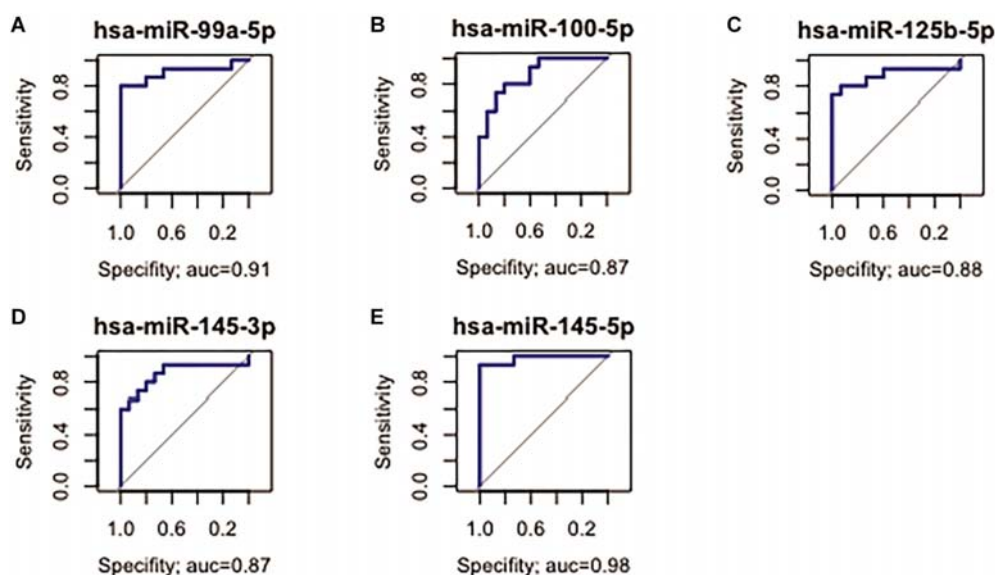


FIGURE 4 | Receiver operating characteristic curve analysis of the five selected miRNAs with potential to predict alterations in ADJ tissue. **(A)** *hsa-miR-99a-5p* (AUC = 0.91; 95% CI: 0.783–1.000); **(B)** *hsa-miR-100-5p* (AUC = 0.87, 95% CI: 0.747–0.995); **(C)** *hsa-miR-125b-5p* (AUC = 0.88; 95% CI: 0.742–1.000); **(D)** *hsa-miR-145-3p* (AUC = 0.87; 95% CI: 0.721–1.000); **(E)** *hsa-miR-145-5p* (AUC = 0.98; 95% CI: 0.944–1.000).

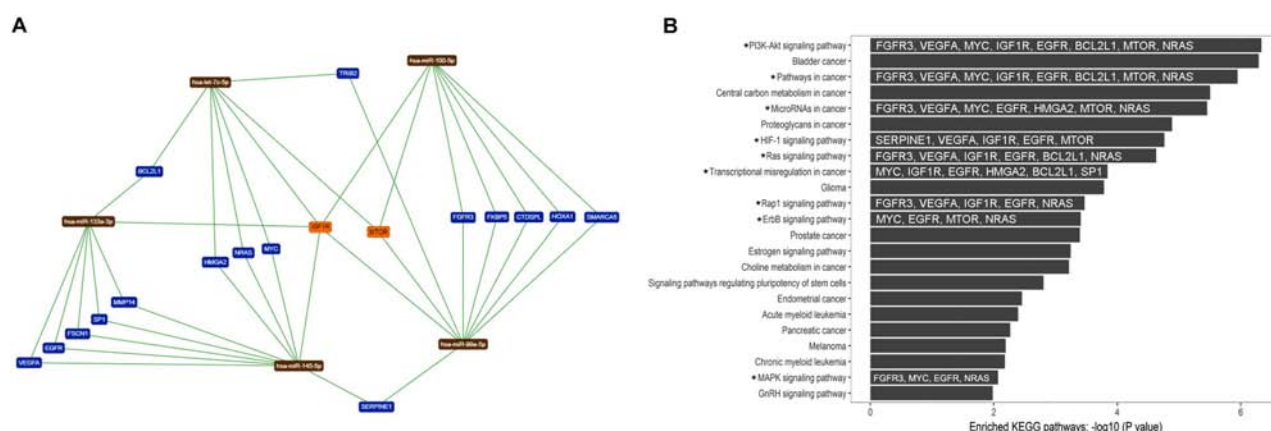


FIGURE 5 | Target genes common to the five up-regulated TS-miRNAs in adjacent tissue and their relationship to important biological pathways. **(A)** Network of interaction among miRNAs and target genes generated by miRTargetLink Human (Hamberg et al., 2016). **(B)** Enhanced KEGG biological pathways in which the target genes of the studied miRNAs participate (by DAVID v.6.8). (*) Important pathway in GC. The target genes involved in important pathways in GC are in white inside the dark bars.

ADJ or GC tissues, behaving as molecular signatures for those conditions (Tables 1, 2).

Among the deregulated miRNAs only in GC (Table 1), seven up-regulated (*hsa-miR-135b-5p*, *-miR-196a-5p*, *-miR-196b-5p*, *-miR-215-5p*, *-miR-224-5p*, *-miR-615-3p*, and *-miR-25-5p*) and five down-regulated (*hsa-miR-135a-5p*, *-miR-148a-3p*, *-miR-148a-5p*, *-miR-153-3p*, and *-miR-375*) miRNAs are likely oncomiRs and TS-miRNAs, respectively. In fact, studies demonstrate that *miR-135b* (Wang et al., 2012; Lu et al., 2018; Shao et al., 2019), *miR-196a* (Pan et al., 2017), *miR-196b* (Liao et al., 2012), *miR-215* (Deng et al., 2014), *miR-224* (He et al., 2017), *miR-615-3p* (Wang et al., 2018), and *miR-25* (Li B.S. et al., 2015;

Larki et al., 2018) were reported as oncomiRs, while *miR-135a* (Zhang et al., 2016; Xie et al., 2019), *miR-148a* (Zheng et al., 2011), *miR-153* (Wang and Liu, 2016; Ouyang et al., 2018) and *miR-375* (Ding et al., 2010; Chen et al., 2017; Hwang et al., 2018) were reported as TS-miRNAs in GC. These results suggest that the joint deregulation of both oncomiRs and TS-miRNAs is required for the support and progression of GC.

hsa-miR-196a-5p was up-regulated in GC when comparing to ADJ and NC tissues; at the same time, it was not differentially expressed between the ADJ and NC tissues. Deregulation of this miRNA is important for GC progression because it promotes cell proliferation by down-regulating the expression of *CDKN1B*

TABLE 3 | Deregulated miRNAs considering both adjacent to tumor and gastric cancer tissues.

miRNA	Role in GC*	GC vs. NC	ADJ vs. NC	GC vs. ADJ
<i>miR-125b-5p</i>	OncomiR ^{1,2}	Up	Up	–
<i>miR-125b-1-3p</i>	OncomiR ^{1,2}	Up	Up	–
<i>miR-218-1-3p</i>	TS-miR ^{3,4,5,6}	Up	Up	–
<i>miR-490-3p</i>	TS-miR ^{7,8,9}	Up	Up	–
<i>miR-200a-3p</i>	TS-miR ¹⁰	Down	Down	–
<i>miR-873-5p</i>	TS-miR ^{11,12}	Down	Down	–
<i>miR-493-5p</i>	TS-miR ¹³	Down	Up	–

GC, gastric cancer; ADJ, adjacent to cancer; NC, non-cancerous; Up, up-regulated; Down, down-regulated; TS-miR, tumor suppressor miRNA; OncomiR, oncogenic miRNA. (–) Not differentially expressed. (*) Putative role in GC. (1) Wu et al., 2015; (2) Zhang et al., 2017b; (3) Deng et al., 2017; (4) Tang et al., 2016; (5) Zhang et al., 2017a; (6) Wang et al., 2017; (7) Shen et al., 2015; (8) Qu et al., 2017; (9) Yu et al., 2019; (10) Cong et al., 2013; (11) Chen et al., 2016; (12) Cao et al., 2016; (13) Zhou et al., 2015.

(p27^{kip1}) tumor suppressor (Sun et al., 2012) and invasion and epithelial to mesenchymal transition of cancer stem cells by down-regulating the expression of *SMAD4* in GC (Pan et al., 2017). In addition, *hsa-miR-196a* has been associated with metastasis in lymph nodes and the clinical stage of GC (Li H.L. et al., 2015).

Nine miRNAs were found to be up-regulated only in ADJ tissue (*hsa-miR-let7-c-5p*, *-miR-99a-5p*, *-miR-100-5p*, *-miR-133a-3p*, *-miR-133b-3p*, *-miR-143-5p*, *-miR-145-3p*, *-miR-145-5p*, and *-miR-320b*) and may act as TS-miRs in this tissue. Studies have shown that *miR-let7-c* (Tsai et al., 2015), *miR-133a* (Qiu et al., 2014; Zhang et al., 2018), *miR-133b* (Qiu et al., 2014; Yang et al., 2017), *miR-143-5p* (Wu et al., 2013; Lei et al., 2017), *miR-145* (Qiu et al., 2014; Lei et al., 2017), *miR-100-5p* (Shi et al., 2015), and *miR-320b* (Zhao et al., 2017) are down-regulated in GC and play the role of TS-miRs because they inhibit proliferation, migration, invasion, and cell cycle progression.

Gene enrichment analysis in KEGG pathways revealed that these miRNAs regulate genes involved in important pathways that contribute to gastric carcinogenesis, such as the PI3K-Akt, HIF-1, Ras, Rap1, ErbB, and MAPK signaling pathways (Figures 5A,B). These pathways control important cell functions such as proliferation, migration, invasion, and progression of the cell cycle. The overexpression of TS-miRs in ADJ tissue may be a mechanism to compensate for preexisting molecular alterations in an attempt to contain the tumorigenesis process. However, we believe that during the progression of carcinogenesis in ADJ tissue, the up-regulated TS-miRs identified herein are inversely deregulated (become down-regulated), contributing to the eventual onset, establishment and progression of GC. In a previous study, we reported the overexpression of the TS-miR *hsa-miR-29c* in adjacent to the gastric cancer tissues and its down-regulation in GC and NC tissues (Pereira et al., 2019).

The intense inflammatory process to which the adjacent tissue is subjected may be one of the causes of over representation of these TS-miRs, since inflammation can alter the local microenvironment by stimulating the expression

of some miRNAs. Studies have shown that IL-6 and IL-17 pro-inflammatory interleukins activity may stimulate the expression of miRNAs with known oncogenic activity (*miR-21* and *miR-135b*) in tissues submitted to the intense inflammatory process (Löffler et al., 2007; Iliopoulos et al., 2010; Matsuyama et al., 2011; Rozovski et al., 2013; Singh et al., 2015). Another possibility would be the existence of pre-cancerous lesions in adjacent tissues (e.g., atrophic and non-atrophic gastritis and intestinal metaplasia) and/or *Helicobacter pylori* infection, since the expression levels of many miRNAs (e.g., *miR-21*, *miR-29c*, *miR-135b*, *miR-155*, *miR-204*, and *miR-223*) may be change under these conditions (Link et al., 2015; Vidal et al., 2016; Pereira et al., 2019). Therefore, the local microenvironment and the histopathological characteristics of these tissues can directly influence the expression of many miRNAs, making them susceptible to eventual molecular alterations and carcinogenesis.

Our data (overexpression of nine TS-miRs only in adjacent to the gastric cancer tissues) corroborate with the findings of Aran et al. (2017), who used the transcriptomic profile of normal tissue adjacent to the tumor (NAT) to demonstrate that this tissue has an intermediate and unique expression profile when compared to truly normal and cancerous tissues. Therefore, despite the similarity between adjacent and tumor tissues, the former is not a malignant tissue, but it is not a molecularly normal tissue either (De Assumpção et al., 2016; Aran et al., 2017).

Many genetic and epigenetic changes identified in GC are shared by ADJ tissue (Assumpção et al., 2015; Takeshima et al., 2015; Aran et al., 2017; Vidal et al., 2017; Yoshida et al., 2017; Pereira et al., 2019). Probably some of these shared alterations were already present in the adjacent tissue before the onset of the tumor *in situ* and contributed to its establishment, making this tissue still susceptible to carcinogenesis even after surgical removal of the tumor.

We found that both ADJ and GC tissues share the up-regulation of *hsa-miR-125b-5p* and *hsa-miR-125b-1-3p* oncomiRs and the down-regulation of *hsa-miR-200a-3p* and *hsa-miR-873-5p* TS-miRs, suggesting that the joint deregulation of both oncomiRs and TS-miRs is required for the progression of gastric carcinogenesis. In addition, seven miRNAs (*hsa-miR-125b-5p*, *-miR-125b-1-3p*, *-miR-200a-3p*, *-miR-218-1-3p*, *-miR-490-3p*, *-miR-873-5p*, and *-miR-493-5p*) are deregulated in both ADJ and GC tissues when compared to NC tissue; however, these miRNAs are not D. E. between ADJ and GC tissues. Our results suggest that these two tissues share molecular alterations and that ADJ is an epigenetically altered tissue. Five miRNAs (*hsa-miR-125b-5p*, *-miR-125b-1-3p*, *-miR-493-5p*, *-miR-200a-3p*, and *-miR-873-5p*) can promote tumor onset and progression, as studies have shown that the deregulation of *miR-125b* (Wu et al., 2015; Zhang et al., 2017b), *miR-493-5p* (Zhou et al., 2015), *miR-200a* (Cong et al., 2013), and *miR-873* (Cao et al., 2016; Chen et al., 2016) contributes to cell proliferation, migration, invasion, and cell growth in GC.

Although we have not performed further experimental assays to confirm the deregulation and the functional role of the identified miRNAs in our study, the literature consistently

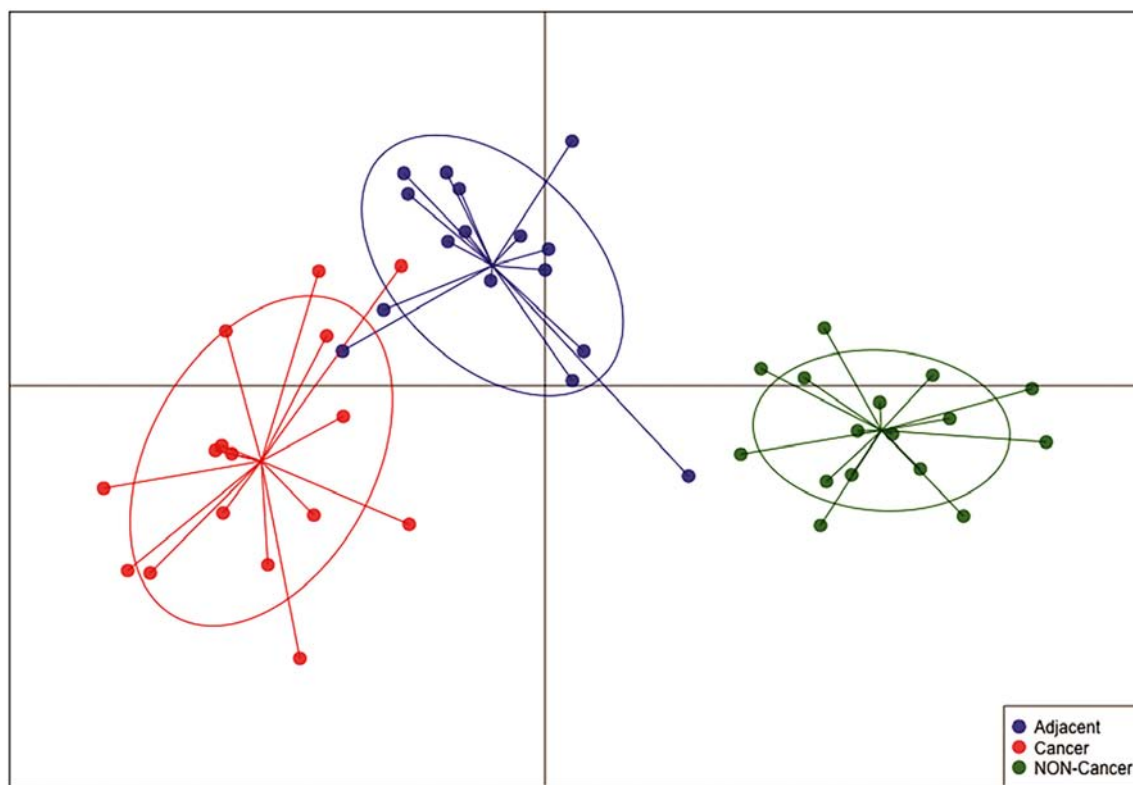


FIGURE 6 | Discriminant analysis of principal component (DAPC) plot shows that NC, ADJ, and GC samples form distinct clusters. The DAPC analysis showed great similarity between GC and ADJ tissues.

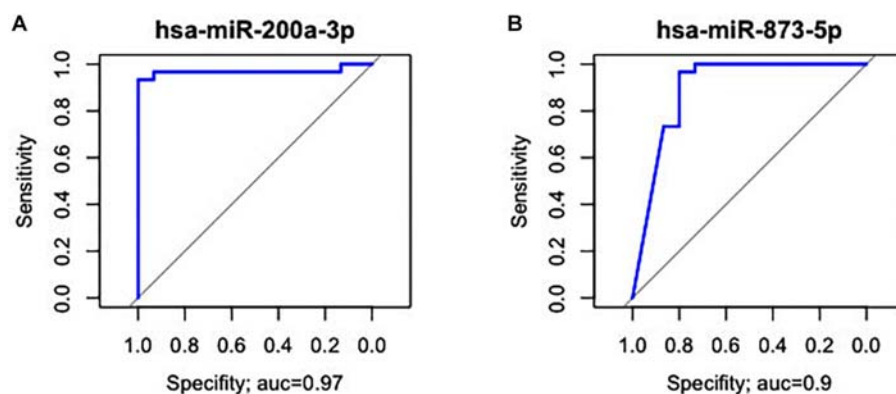


FIGURE 7 | Receiver operating characteristic curve analysis of the two miRNAs with the potential to predict changes in both ADJ and GC tissues. **(A)** *hsa-miR-200a-3p* (AUC = 0.97; 95% CI: 0.983–1.000). **(B)** *hsa-miR-873-5p* (AUC = 0.90; 95% CI: 0.807–1.000).

supports and corroborates our findings and hypothesis through studies that have applied safe, sensitive and reliable techniques and methods (e.g., RT-qPCR, Western Blot, Cell and/or Reporter Assays) that provides strong evidences of these miRNAs' deregulation in GC (Ding et al., 2010; Zheng et al., 2011; Liao et al., 2012; Sun et al., 2012; Wang et al., 2012, 2017, 2018; Cong et al., 2013; Wu et al., 2013, 2015; Deng et al., 2014, 2017; Qiu et al., 2014; Li B.S. et al., 2015; Shen et al., 2015; Shi et al., 2015;

Tsai et al., 2015; Zhou et al., 2015, 2019; Zuo et al., 2015; Cao et al., 2016; Chen et al., 2016, 2017; Gao et al., 2016; Tang et al., 2016; Vidal et al., 2016; Wang and Liu, 2016; Zhang et al., 2016, 2017a,b, 2018; He et al., 2017; Lei et al., 2017; Pan et al., 2017; Qu et al., 2017; Yang et al., 2017; Zhao et al., 2017; Hwang et al., 2018; Larki et al., 2018; Lu et al., 2018; Magalhães et al., 2018; Ouyang et al., 2018; Pereira et al., 2019; Shao et al., 2019; Xie et al., 2019; Yu et al., 2019).

For the evaluation of the studied miRNAs as potential biomarkers, we selected three different groups of markers with $AUC > 85\%$. Among the D. E. miRNAs only in GC, six miRNAs (*hsa-miR-141-3p*, *-miR-148a-3p*, *-miR-148b-5p*, *-miR-153-3p*, *-miR-196a-5p*, and *-miR-375*) are potentially useful in identifying patients with advanced disease (Figure 3). Among the deregulated miRNAs only in ADJ tissue, five (*hsa-miR-99a-5p*, *-miR-100-5p*, *-miR-125b-5p*, *-miR-145-3p*, and *-miR-145-5p*) are potentially useful in identifying patients susceptible to tumor development or the early stages of gastric cancer (Figure 4). Two miRNAs (*hsa-miR-200a-3p* and *hsa-miR-873-5p*) are potentially useful in identifying both patients with established disease and patients susceptible to developing it (Figure 7). Thus, these miRNAs may be a potential diagnostic alternative for this type of cancer because the currently available biomarkers do not have a good sensitivity/specificity relationship, which makes it difficult to diagnose the disease early and to start curative treatment.

Studies that analyzed the data from miRNA sequencing in gastric cancer demonstrated their deregulation in this type of tumor (Assumpção et al., 2015; Darnet et al., 2015; Liu et al., 2015; Liang et al., 2017). Among the miRNAs found in these studies, *hsa-miR-99a*, *-miR-100*, *-miR-133a/b-3p*, *-miR-135b*, *-miR-141*, *-miR-143*, *-miR-145*, *-miR-148a*, *-miR-196a/b*, *-miR-200a*, *-miR-218-1*, *-miR-215*, and *-miR-490* were also observed in the present study. In addition to those miRNAs, we also found 16 novel differentially expressed miRNAs in the gastric field cancerization.

Liu et al. (2015) analyzed miRNome in gastric cancer downloaded from the TCGA; however, this database basically has samples from European and Asian populations. These authors identified 54 D. E. miRNAs (using the $P_{adj} < 0.05$ and $|\log_2(\text{fold change})| > 3$) and two miRNAs (*hsa-miR-133a/b*) were the most D. E. Liang et al. (2017) also analyzed miRNomes in GC using samples from the same database (TCGA) and identified 43 D. E. miRNAs (using the $FDR < 0.001$ and $|\log_2(\text{fold change})| > 1.5$); of these, 5 miRNAs (*hsa-miR-30a*, *-miR-135b*, *-miR-133b*, *-miR-143*, and *-miR-145*) were associated with patient survival time. We emphasize that the “normal” or “healthy” tissues used in these two studies are of patients with the disease (adjacent to the gastric cancer tissues), since the TCGA does not have expression data of gastric tissues of patients without the history of GC. By using samples from the Brazilian population (that has genetic admixture), we identified 30 miRNAs differentially expressed when comparing patients with GC and individuals without the history of GC (we considered $P_{adj} < 0.05$ and $|\log_2(\text{fold change})| > 2$). In addition, we found that the *hsa-miR-196a-5p* was D. E. between the GC and ADJ tissues. Many D. E. miRNAs identified by Liu et al. (2015) and Liang et al. (2017) were also identified in this study; however, there are differences in the number of D. E. miRNAs found among these studies (mainly in GC and ADJ analysis), which may be a consequence of: (i) the statistical criteria used, (ii) the number of samples used, and/or (iii) the ethnical and genetic characteristics of the studied populations. Our data are important because we analyzed the miRNA sequencing of a larger number of samples from the Brazilian population. This population has a genetic contribution from different parental populations, such as the European, African, Asian, and Amerindian ones (Santos et al., 2010; Andrade et al., 2018). The strong genetic substructure and

admixture (Santos et al., 2010) of our population may interfere in the expression profile of some genes (Pinto et al., 2015; Dluzen et al., 2016). Therefore, this study provides important and relevant information about the expression profile of miRNAs associated to gastric field cancerization in populations with genetic substructure and admixture, such as the Brazilian one.

Overall, our study was able to demonstrate that the tissue adjacent to gastric cancer shares some epigenetic changes (miRNAs deregulation) present in the tumor and also has unique and exclusive alterations; therefore, it should not be used as a healthy and/or normal tissue as a benchmark for gastric cancer. Thus, we recommend the use of gastric samples from patients with no history of GC as a control to exclude any biases that the adjacent tissue may provide in the miRNAs' expression profile in GC.

In this study, we also observed that the tissues adjacent to GC have an over representation of microRNAs with known tumor suppressor activities, suggesting that these microRNAs may represent a barrier against tumorigenesis within these pre-cancerous tissues prior to the eventual formation of a tumor. The excellent performance of the studied miRNAs in identifying with good sensitivity and specificity, both early and advanced stages of the disease, make them potentially useful as biomarkers and therapeutic targets for GC.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Health Council's Research Guidelines Involving Human Beings (Res. CNS 466/12), Research Ethics Committees and National Research Ethics Commission. The protocol was approved by the Ethics Committee of the Center of Oncology Research of the Federal University of Pará (Protocol No. 1.081.340). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AP and FM study design, formal analyses, interpretation of data, and drafting of manuscript. ÂRS, PA, and SS study concept and design and obtaining funding. PA, MA, and AC provided clinical materials. SD histopathological evaluation. TV-S, AV, PP, LM, and AMRS prepared nucleic acids, sequencing libraries, and performed the experiments. All authors reviewed the manuscript.

FUNDING

This study was supported by Rede de Pesquisa em Genômica Populacional Humana (CAPES/Biologia Computacional: No. 3381/2013/CAPES) and PROPESP/UFGA. AP was supported by CAPES Ph.D. fellowship (PROEX – 0487) in Genetics and Molecular Biology (UFGA) and is supported actually by CAPES/BRASIL Post-Doctoral fellowship (Edital No. 51/2013/Biologia Computacional; Proc. No. 88887.313819/2019-00) in Oncology and Medical Sciences

(UFPA). ÂRS was supported by CNPq/Productivity: 304413/2015-1 and SS was supported by CNPq/Productivity: 305 256/2013-3.

ACKNOWLEDGMENTS

We thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento

Científico e Tecnológico (CNPq), and PROPESP/UFPA for the financial support and fellowships.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00592/full#supplementary-material>

REFERENCES

- Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638
- Andrade, R. B., Amador, M. A. T., Cavalcante, G. C., Leitão, L. P. C., Fernandes, M. R., Modesto, A. A. C., et al. (2018). Estimating Asian contribution to the Brazilian population: a new application of a validated set of 61 ancestry informative markers. *G3* 8, 3577–3582. doi: 10.1534/g3.118.200650
- Aran, D., Camarda, R., Odegaard, J., Paik, H., Oskotsky, B., Krings, G., et al. (2017). Comprehensive analysis of normal adjacent to tumor transcriptomes. *Nat. Commun.* 8:1077. doi: 10.1038/s41467-017-01027-z
- Assumpção, M. B., Moreira, F. C., Hamoy, I. G., Magalhães, L., Vidal, A., Pereira, A., et al. (2015). High-throughput miRNA sequencing reveals a field effect in gastric cancer and suggests an epigenetic network mechanism. *Bioinform. Biol. Insights* 9, 111–117. doi: 10.4137/BBI.S24066
- Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism and function. *Cell* 116, 281–297.
- Cao, D., Yu, T., and Ou, X. (2016). MiR-873-5P controls gastric cancer progression by targeting hedgehog-Gli signaling. *Pharmazie* 71, 603–606. doi: 10.1691/ph.2016.6618
- Castro-Magdonel, B. E., Orjuela, M., Camacho, J., García-Chéquer, A. J., Cabrera-Muñoz, L., Sadowinski-Pine, S., et al. (2017). miRNome landscape analysis reveals a 30 miRNA core in retinoblastoma. *BMC Cancer* 17:458. doi: 10.1186/s12885-017-3421-3
- Chai, H., and Brown, R. E. (2009). Field effect in cancer—an update. *Ann. Clin. Lab. Sci.* 39, 331–337.
- Chen, B., Guo, S., Yu, Z., Feng, Y., and Hui, L. (2017). Downregulation of microRNA-375, combined with upregulation of its target gene Janus kinase 2, predicts unfavorable prognosis in patients with gastric cancer. *Int. J. Clin. Exp. Pathol.* 10, 11106–11113.
- Chen, X., Chen, R., Wu, W., and Huang, Z. (2016). MicroRNA-873 inhibits proliferation and induces apoptosis by targeting CXCL1 in gastric cancer. *Int. J. Clin. Exp. Pathol.* 9, 10011–10019.
- Cong, N., Du, P., Zhang, A., Shen, F., Su, J., Pu, P., et al. (2013). Downregulated microRNA-200a promotes EMT and tumor growth through the wnt/ β -catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma. *Oncol. Rep.* 29, 1579–1587. doi: 10.3892/or.2013.2267
- Correa, P. (2013). Gastric cancer: overview. *Gastroenterol. Clin. N. Am.* 42, 211–217. doi: 10.1016/j.gtc.2013.01.002
- Curtius, K., Wright, N. A., and Graham, T. A. (2018). An evolutionary perspective on field cancerization. *Nat. Rev. Cancer* 18, 19–32. doi: 10.1038/nrc.2017.102
- Darnet, S., Moreira, F. C., Hamoy, I. G., Burbano, R., Khayat, A., Cruz, A., et al. (2015). High-throughput sequencing of miRNAs reveals a tissue signature in gastric cancer and suggests novel potential biomarkers. *Bioinform. Biol. Insights* 9(Suppl. 1), 1–8. doi: 10.4137/BBI.S23773
- De Assumpção, P. P., Dos Santos, S. E., Dos Santos, ÂK., Demachki, S., Khayat, A. S., Ishak, G., et al. (2016). The adjacent to tumor sample trap. *Gastric Cancer* 19, 1024–1025. doi: 10.1007/s10120-015-0539-3
- Deng, M., Zeng, C., Lu, X., He, X., Zhang, R., Qiu, Q., et al. (2017). miR-218 suppresses gastric cancer cell cycle progression through the CDK6/Cyclin D1/E2F1 axis in a feedback loop. *Cancer Lett.* 403, 175–185. doi: 10.1016/j.canlet.2017.06.006
- Deng, Y., Huang, Z., Xu, Y., Jin, J., Zhuo, W., Zhang, C., et al. (2014). MiR-215 modulates gastric cancer cell proliferation by targeting RB1. *Cancer Lett.* 342, 27–35. doi: 10.1016/j.canlet.2013.08.033
- Ding, L., Xu, Y., Zhang, W., Deng, Y., Si, M., Du, Y., et al. (2010). MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. *Cell Res.* 20, 784–793. doi: 10.1038/cr.2010.79
- Diuzen, D. F., Noren, H. N., Zhang, Y., Kim, Y., Glover, F. E., Tajuddin, S. M., et al. (2016). Racial differences in microRNA and gene expression in hypertensive women. *Sci. Rep.* 6:35815. doi: 10.1038/srep35815
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635
- Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., et al. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* 136, E359–E386. doi: 10.1002/ijc.29210
- Gao, H., Liu, P., Yang, Y., and Gao, F. (2016). Decreased miR-452 expression and its tumor suppressive function in human gastric cancer. *Int. J. Clin. Exp. Med.* 9, 16078–16085.
- Hamberg, M., Backes, C., Fehlmann, T., Hart, M., Meder, B., Meese, E., et al. (2016). MiRTargetLink—miRNAs, Genes and Interaction Networks. *Int. J. Mol. Sci.* 17:564. doi: 10.3390/ijms17040564
- Hattori, N., and Ushijima, T. (2016). Epigenetic impact of infection on carcinogenesis: mechanisms and applications. *Genome Med.* 8:10. doi: 10.1186/s13073-016-0267-2
- He, C., Wang, L., Zhang, J., and Xu, H. (2017). Hypoxia-inducible microRNA-224 promotes the cell growth, migration and invasion by directly targeting RASSF8 in gastric cancer. *Mol. Cancer* 16:35. doi: 10.1186/s12943-017-0603-1
- Hou, J., Lin, L., Zhou, W., Wang, Z., Ding, G., Dong, Q., et al. (2011). Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. *Cancer Cell* 19, 232–243. doi: 10.1016/j.ccr.2011.01.001
- Huang, W. D., Sherman, B. T., and Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids. Res.* 37, 1–13. doi: 10.1093/nar/gkn923
- Huang, W. D., Sherman, B. T., and Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57. doi: 10.1038/nprot.2008.211
- Hwang, J., Min, B.-H., Jang, J., Kang, S. Y., Bae, H., Jang, S. S., et al. (2018). MicroRNA expression profiles in gastric carcinogenesis. *Sci. Rep.* 8:14393.
- Iliopoulos, D., Jaeger, S. A., Hirsch, H. A., Bulyk, M. L., and Struhl, K. (2010). STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol. Cell* 39, 493–506. doi: 10.1016/j.molcel.2010.07.023
- LArki, P., Ahadi, A., Zare, A., Tarighi, S., Zaheri, M., Souri, M., et al. (2018). Up-Regulation of miR-21, miR-25, miR-93, and miR-106b in Gastric Cancer. *Iran Biomed. J.* 22, 367–373.
- Lauren, P. (1965). The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol. Microbiol. Scand.* 64, 31–49. doi: 10.1111/apm.1965.64.1.31
- Lei, C., Du, F., Sun, L., Li, T., Li, T., Min, Y., et al. (2017). miR-143 and miR-145 inhibit gastric cancer cell migration and metastasis by suppressing MYO6. *Cell Death Dis.* 8:e3101. doi: 10.1038/cddis.2017.493
- Li, B. S., Zuo, Q. F., Zhao, Y. L., Xiao, B., Zhuang, Y., Mao, X. H., et al. (2015). MicroRNA-25 promotes gastric cancer migration, invasion and proliferation

- by directly targeting transducer of ERBB2, 1 and correlates with poor survival. *Oncogene* 34, 2556–2565. doi: 10.1038/ncr.2014.214
- Li, H. L., Xie, S. P., Yang, Y. L., Cheng, Y. X., Zhang, Y., Wang, J., et al. (2015). Clinical significance of upregulation of miR-196a-5p in gastric cancer and enriched KEGG pathway analysis of target genes. *Asian Pac. J. Cancer Prev.* 16, 1781–1787. doi: 10.7314/apjcp.2015.16.5.1781
- Liang, L., Zhang, L., Cui, D., and Yang, D. (2017). Identification of the key miRNAs associated with survival time in stomach adenocarcinoma. *Oncol. Lett.* 14, 4563–4572. doi: 10.3892/ol.2017.6792
- Liao, Y. L., Hu, L. Y., Tsai, K. W., Wu, C. W., and Chan, W. C. (2012). Transcriptional regulation of miR-196b by ETS2 in gastric cancer cells. *Carcinogenesis* 33, 760–769. doi: 10.1093/carcin/bgs023
- Link, A., Schirrmeister, W., Langner, C., Varbanova, M., Bornschein, J., Wex, T., et al. (2015). Differential expression of microRNAs in preneoplastic gastric mucosa. *Sci. Rep.* 5:8270. doi: 10.1038/srep08270
- Liu, Y., Zhang, X., Zhang, Y., Hu, Z., Yang, D., Wang, C., et al. (2015). Identification of miRNomes in human stomach and gastric carcinoma reveals miR-133b/a-3p as therapeutic target for gastric cancer. *Cancer Lett.* 369, 58–66. doi: 10.1016/j.canlet.2015.06.028
- Löffler, D., Brocke-Heidrich, K., Pfeifer, G., Stocsits, C., Hackermüller, J., Kretschmar, A. K., et al. (2007). Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* 110, 1330–1333. doi: 10.1182/blood-2007-03-081133
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8
- Lu, M., Huang, Y., Sun, W., Li, P., Li, L., and Li, L. (2018). miR-135b-5p promotes gastric cancer progression by targeting CMTM3. *Int. J. Oncol.* 52, 589–598. doi: 10.3892/ijo.2017.4222
- Magalhães, L., Quintana, L. G., Lopes, D. C. F., Vidal, A. F., Pereira, A. L., D'Araujo, P. L. C., et al. (2018). APC gene is modulated by hsa-miR-135b-5p in both diffuse and intestinal gastric cancer subtypes. *BMC Cancer* 18:1055. doi: 10.1186/s12885-018-4980-7
- Maltseva, D. V., Galatenko, V. V., Samatov, T. R., Zhikrivetskaya, S. O., Khaustova, N. A., Nechaev, I. N., et al. (2014). miRNome of inflammatory breast cancer. *BMC Res. Notes* 7:871. doi: 10.1186/1756-0500-7-871
- Matsuyama, H., Suzuki, H. I., Nishimori, H., Noguchi, M., Yao, T., Komatsu, N., et al. (2011). miR-135b mediates NPM-ALK-driven oncogenicity and renders IL-17-producing immunophenotype to anaplastic large cell lymphoma. *Blood* 118, 6881–6892. doi: 10.1182/blood-2011-05-354654
- Moreira, F. C., Assumpção, M., Hamoy, I. G., Darnet, S., Burbano, R., Khayat, A., et al. (2014a). MiRNA expression profile for the human gastric antrum region using ultra-deep sequencing. *PLoS One* 9:e92300. doi: 10.1371/journal.pone.0092300
- Moreira, F. C., Dustan, B., Hamoy, I. G., Ribeiro-dos-Santos, A. M., and Ribeiro-dos-Santos, A. (2014b). TargetCompare: a web interface to compare simultaneous miRNAs targets. *Bioinformatics* 10, 602–605. doi: 10.6026/97320630010602
- Ouyang, Y., Yuan, W., and Qiu, S. (2018). MicroRNA-153 functions as a tumor suppressor in gastric cancer via targeting Kruppel-like factor 5. *Exp. Ther. Med.* 16, 473–482. doi: 10.3892/etm.2018.6226
- Padmanabhan, N., Ushijima, T., and Tan, P. (2017). How to stomach an epigenetic insult: the gastric cancer epigenome. *Nat. Rev. Gastroenterol. Hepatol.* 14, 467–478. doi: 10.1038/nrgastro.2017.53
- Pan, Y., Shu, X., Sun, L., Yu, L., Sun, L., Yang, Z., et al. (2017). miR-196a-5p modulates gastric cancer stem cell characteristics by targeting Smad4. *Int. J. Oncol.* 50, 1965–1976. doi: 10.3892/ijo.2017.3965
- Pereira, A. L., Magalhães, L., Moreira, F. C., Reis-das-Mercês, L., Vidal, A. F., Ribeiro-dos-Santos, A. M., et al. (2019). Epigenetic field cancerization in gastric cancer: microRNAs as promising biomarkers. *J. Cancer* 10, 1560–1569. doi: 10.7150/jca.27457
- Pinto, P. S. C., Santos, N. P., Santos, S., and Ribeiro-dos-Santos, A. (2015). Influence of genetic ancestry on INDEL markers of NFKB1, CASP8, PAR1, IL4 and CYP19A1 genes in leprosy patients. *PLoS Negl. Trop. Dis.* 9:e0004050. doi: 10.1371/journal.pntd.0004050
- Qiu, T., Zhou, X., Wang, J., Du, Y., Xu, J., Huang, Z., et al. (2014). MiR-145, miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. *FEBS Lett.* 588, 1168–1177. doi: 10.1016/j.febslet.2014.02.054
- Qu, M., Li, L., and Zheng, W. C. (2017). Reduced miR-490-3p expression is associated with poor prognosis of Helicobacter pylori induced gastric cancer. *Eur. Rev. Med. Pharmacol. Sci.* 21, 3384–3388.
- Ribeiro-dos-Santos, A., Khayat, A. S., Silva, A., Alencar, D. O., Lobato, J., Luz, L., et al. (2010). Ultra-deep sequencing reveals the microRNA expression pattern of the human stomach. *PLoS One* 5:e13205. doi: 10.1371/journal.pone.0013205
- Rozovski, U., Calin, G. A., Setoyama, T., D'Abundo, L., Harris, D. M., Li, P., et al. (2013). Signal transducer and activator of transcription (STAT)-3 regulates microRNA gene expression in chronic lymphocytic leukemia cells. *Mol. Cancer* 12:50. doi: 10.1186/1476-4598-12-50
- Runtsch, M. C., Round, J. L., and O'Connell, R. M. (2014). MicroRNAs and the regulation of intestinal homeostasis. *Front. Genet.* 5:347. doi: 10.3389/fgene.2014.00347
- Santos, N. P., Ribeiro-Rodrigues, E. M., Ribeiro-Dos-Santos, A. K., Pereira, R., Gusmão, L., Amorim, A., et al. (2010). Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INSEL) ancestry-informative marker (AIM) panel. *Hum. Mutat.* 31, 184–190. doi: 10.1002/humu.21159
- Schneider, M. R. (2012). MicroRNAs as novel players in skin development, homeostasis and disease. *Br. J. Dermatol.* 166, 22–28. doi: 10.1111/j.1365-2133.2011.10568.x
- Shao, L., Chen, Z., Soutto, M., Zhu, S., Lu, H., Romero-Gallo, J., et al. (2019). Helicobacter pylori-induced miR-135b-5p promotes cisplatin resistance in gastric cancer. *FASEB J.* 33, 264–274. doi: 10.1096/fj.201701456RR
- Shen, J., Xiao, Z., Wu, W. K., Wang, M. H., To, K. F., Chen, Y., et al. (2015). Epigenetic silencing of miR-490-3p reactivates the chromatin remodeler SMARCD1 to promote Helicobacter pylori-induced gastric carcinogenesis. *Cancer Res.* 75, 754–765. doi: 10.1158/0008-5472.CAN-14-1301
- Shi, D. B., Wang, Y. W., Xing, A. Y., Gao, J. W., Zhang, H., Guo, X. Y., et al. (2015). C/EBP α -induced miR-100 expression suppresses tumor metastasis and growth by targeting ZBTB7A in gastric cancer. *Cancer Lett.* 369, 376–385. doi: 10.1016/j.canlet.2015.08.029
- Shin, Y. Y., Ng, E. K., Chan, V. W., Kwong, A., and Chu, K. M. (2015). A three-miRNA signature as promising non-invasive diagnostic marker for gastric cancer. *Mol. Cancer* 14:202. doi: 10.1186/s12943-015-0473-3
- Singh, M., Garg, N., Venugopal, C., Hallett, R., Tokar, T., McFarlane, N., et al. (2015). STAT3 pathway regulates lung-derived brain metastasis initiating cell capacity through miR-21 activation. *Oncotarget* 6, 27461–27477. doi: 10.18632/oncotarget.4742
- Slaughter, D. P., Southwick, H. W., and Smejkal, W. (1953). Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 6, 963–968. doi: 10.1002/1097-0142(195309)6:5<963::aid-cnrcr2820060515>3.0.co;2-q
- Sun, M., Liu, X. H., Li, J. H., Yang, J. S., Zhang, E. B., Yin, D. D., et al. (2012). MiR-196a is upregulated in gastric cancer and promotes cell proliferation by downregulating p27(kip1). *Mol. Cancer Ther.* 11, 842–852. doi: 10.1158/1535-7163.MCT-11-1015
- Takeshima, H., Niwa, T., Takahashi, T., Wakabayashi, M., Yamashita, S., Ando, T., et al. (2015). Frequent involvement of chromatin remodeler alterations in gastric field cancerization. *Cancer Lett.* 357, 328–338. doi: 10.1016/j.canlet.2014.11.038
- Tang, S., Wang, D., Zhang, Q., and Li, L. (2016). miR-218 suppresses gastric cancer cell proliferation and invasion via regulation of angiopoietin-2. *Exp. Ther. Med.* 12, 3837–3842. doi: 10.3892/etm.2016.3893
- Tsai, K. W., Hu, L. Y., Chen, T. W., Li, S. C., Ho, M. R., Yu, S. Y., et al. (2015). Emerging role of microRNAs in modulating endothelin-1 expression in gastric cancer. *Oncol. Rep.* 33, 485–493. doi: 10.3892/or.2014.3598
- Vidal, A. F., Cruz, A. M. P., Magalhães, L., Pereira, A. L., Anaissi, A. K. M., Alves, N. C. F., et al. (2016). hsa-miR-29c and hsa-miR-135b differential expression as potential biomarker of gastric carcinogenesis. *World J. Gastroenterol.* 22, 2060–2070. doi: 10.3748/wjg.v22.i6.2060
- Vidal, A. F., Ribeiro-Dos-Santos, A. M., Vinasco-Sandoval, T., Magalhães, L., Pinto, P. L., Anaissi, A. K. M., et al. (2017). The comprehensive expression analysis of circular RNAs in gastric cancer and its association with field cancerization. *Sci Rep.* 7:14551. doi: 10.1038/s41598-017-15061-w

- Wang, G., Fu, Y., Liu, G., Ye, Y., and Zhang, X. (2017). miR-218 inhibits proliferation, migration, and EMT of gastric cancer cells by targeting WASF3. *Oncol. Res.* 25, 355–364. doi: 10.3727/096504016X14738114257367
- Wang, J., Liu, L., Sun, Y., Xue, Y., Qu, J., Pan, S., et al. (2018). miR-615-3p promotes proliferation and migration and inhibits apoptosis through its potential target CELF2 in gastric cancer. *Biomed. Pharmacother.* 101, 406–413. doi: 10.1016/j.biopha.2018.02.104
- Wang, L. P., Ma, X. Q., and Cai, J. C. (2012). Clinicopathological significance and function of miR-135b in the occurrence and development of gastric cancer. *Zhonghua Yi Xue Za Zhi* 92, 3269–3273.
- Wang, Z., and Liu, C. (2016). MiR-153 regulates metastases of gastric cancer through Snail. *Tumor Biol.* 37, 15509–15515. doi: 10.1007/s13277-015-3846-8
- Wu, H. H., Lin, W. C., and Tsai, K. W. (2014). Advances in molecular biomarkers for gastric cancer: miRNAs as emerging novel cancer markers. *Expert. Rev. Mol. Med.* 16, e1. doi: 10.1017/erm.2013.16
- Wu, J. G., Wang, J. J., Jiang, X., Lan, J. P., He, X. J., Wang, H. J., et al. (2015). MiR-125b promotes cell migration and invasion by targeting PPP1CA-Rb signal pathways in gastric cancer, resulting in a poor prognosis. *Gastric Cancer* 18, 729–739. doi: 10.1007/s10120-014-0421-8
- Wu, X. L., Cheng, B., Li, P. Y., Huang, H. J., Zhao, Q., Dan, Z. L., et al. (2013). MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2. *World J. Gastroenterol.* 19, 7758–7765. doi: 10.3748/wjg.v19.i43.7758
- Xie, Y., Li, F., Li, Z., and Shi, Z. (2019). miR-135a suppresses migration of gastric cancer cells by targeting TRAF5-mediated NF- κ B activation. *Onco Targets Ther.* 12, 975–984. doi: 10.2147/OTT.S189976
- Yakirevich, E., and Resnick, M. B. (2013). Pathology of gastric cancer and its precursor lesions. *Gastroenterol. Clin. N. Am.* 42, 261–284. doi: 10.1016/j.gtc.2013.01.004
- Yang, D., Zhao, D., and Chen, X. (2017). MiR-133b inhibits proliferation and invasion of gastric cancer cells by up-regulating FBN1 expression. *Cancer Biomark.* 19, 425–436. doi: 10.3233/CBM-160421
- Yoshida, S., Yamashita, S., Niwa, T., Mori, A., Ito, S., Ichinose, M., et al. (2017). Epigenetic inactivation of FAT4 contributes to gastric field cancerization. *Gastric Cancer* 20, 136–145. doi: 10.1007/s10120-016-0593-5
- Yu, H., Sun, J., Jiang, S., and Xu, Y. (2019). MicroRNA-490-3p regulates cell proliferation and apoptosis in gastric cancer via direct targeting of AKT1. *Exp. Ther. Med.* 17, 1330–1336. doi: 10.3892/etm.2018.7042
- Zhang, C., Chen, X., Chen, X., Wang, X., Ji, A., Jiang, L., et al. (2016). miR-135a acts as a tumor suppressor in gastric cancer in part by targeting KIFC1. *Onco Targets Ther.* 9, 3555–3563. doi: 10.2147/OTT.S105736
- Zhang, X., Li, Z., Xuan, Z., Xu, P., Wang, W., Chen, Z., et al. (2018). Novel role of miR-133a-3p in repressing gastric cancer growth and metastasis via blocking autophagy-mediated glutaminolysis. *J. Exp. Clin. Cancer Res.* 37:320. doi: 10.1186/s13046-018-0993-y
- Zhang, X., Dong, J., He, Y., Zhao, M., Liu, Z., Wang, N., et al. (2017a). miR-218 inhibited tumor angiogenesis by targeting ROBO1 in gastric cancer. *Gene* 615, 42–49. doi: 10.1016/j.gene.2017.03.022
- Zhang, X., Yao, J., Guo, K., Huang, H., Huai, S., Ye, R., et al. (2017b). The functional mechanism of miR-125b in gastric cancer and its effect on the chemosensitivity of cisplatin. *Oncotargets* 9, 2105–2119. doi: 10.18632/oncotarget.23249
- Zhang, Z., Li, Z., Li, Y., and Zang, A. (2014). MicroRNA and signaling pathways in gastric cancer. *Cancer Gene Ther.* 21, 305–316. doi: 10.1038/cgt.2014.37
- Zhao, Y., Dong, Q., and Wang, E. (2017). MicroRNA-320 inhibits invasion and induces apoptosis by targeting CRKL and inhibiting ERK and AKT signaling in gastric cancer cells. *Onco Targets Ther.* 10, 1049–1058. doi: 10.2147/OTT.S123324
- Zheng, B., Liang, L., Wang, C., Huang, S., Cao, X., Zha, R., et al. (2011). MicroRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer. *Clin. Cancer Res.* 17, 7574–7583. doi: 10.1158/1078-0432.CCR-11-1714
- Zhou, W., Zhang, C., Jiang, H., Zhang, Z., Xie, L., and He, X. (2015). MiR-493 suppresses the proliferation and invasion of gastric cancer cells by targeting RhoC. *Iran J. Basic Med. Sci.* 18, 1027–1033.
- Zhou, Y., Zhong, J. H., Gong, F. S., and Xiao, J. (2019). MiR-141-3p suppresses gastric cancer induced transition of normal fibroblast and BMSC to cancer-associated fibroblasts via targeting STAT4. *Exp. Mol. Pathol.* 107, 85–94. doi: 10.1016/j.yexmp.2018.11.014
- Zuo, Q. F., Zhang, R., Li, B. S., Zhao, Y. L., Zhuang, Y., Yu, T., et al. (2015). MicroRNA-141 inhibits tumor growth and metastasis in gastric cancer by directly targeting transcriptional co-activator with PDZ-binding motif, TAZ. *Cell Death Dis.* 6, e1623. doi: 10.1038/cddis.2014.573

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The Epigenetics of Alzheimer's Disease: Factors and Therapeutic Implications

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OPEN ACCESS

Edited by:

Yujing Li,
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Reviewed by:

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Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 04 June 2018

Accepted: 08 November 2018

Published: 30 November 2018

Citation:

Liu X, Jiao B and Shen L (2018)
The Epigenetics of Alzheimer's
Disease: Factors and Therapeutic
Implications. *Front. Genet.* 9:579.
doi: 10.3389/fgene.2018.00579

Alzheimer's disease (AD) is a well-known neurodegenerative disorder that imposes a great burden on the world. The mechanisms of AD are not yet fully understood. Current insight into the role of epigenetics in the mechanism of AD focuses on DNA methylation, remodeling of chromatin, histone modifications and non-coding RNA regulation. This review summarizes the current state of knowledge regarding the role of epigenetics in AD and the possibilities for epigenetically based therapeutics. The general conclusion is that epigenetic mechanisms play a variety of crucial roles in the development of AD, and there are a number of viable possibilities for treatments based on modulating these effects, but significant advances in knowledge and technology will be needed to move these treatments from the bench to the bedside.

Keywords: Alzheimer's disease (AD), epigenetics, non-coding RNA (ncRNA), DNA methylation, histone modifications and chromatin structure

INTRODUCTION

Alzheimer's disease (AD), an irreversible and progressive neurological disease, is the most common form of neurodegenerative dementia (Mckhann et al., 2011; Rocca et al., 2011; Lansdall et al., 2017). The most prominent early symptom is short-term memory loss. As the disease progresses, other symptoms such as personality changes, apathy and language problems emerge. 95 percent of patients who are hospitalized with AD have the sporadic form, which is late-onset Alzheimer's disease (LOAD) (Diniz et al., 2017; Zhao et al., 2017). The pathology of LOAD is multi-factorial with biological, genetic and environmental factors interacting with each other to aggravate the process of AD. Many genetic risk factors for LOAD have been identified. Among them, the $\epsilon 4$ isoform of apolipoprotein E (ApoE4) is well-known as the strongest genetic risk factor for LOAD (Lane-Donovan and Herz, 2017). However, less than 1 percent of patients have autosomal dominant inherited Alzheimer's disease (DIAD) which has a much earlier age of onset, around 45 years old. Genetic mutations in the genes encoding APP, PS1, and PS2 that cause overproduction or formation of an aberrant form of A β are found in this group (Levy et al., 1990; Masters et al., 2015).

At the cellular level, AD can be characterized by the appearance of extracellular plaques from accumulations of insoluble amyloid beta (A β) filaments, intracellular neurofibrillary tangles of hyperphosphorylated tau and neuroinflammation (Selkoe, 2012). It appears that AD is not only

one or two types of diseases, but is rather a group of diseases with similar APP and Tau pathologies that are triggered by different mechanisms (Sery et al., 2013). Among them, the most popular theory is the amyloid cascade hypothesis and detection of the accumulation of A β is now available in MCI and AD patients using cerebrospinal fluid biomarkers and PET. However, it is still difficult to find stable biomarkers which could help to discover this disease much earlier, and the exact mechanisms of AD are still unknown. Now the number of studies examining epigenetic mechanisms playing in the etiology of AD has risen dramatically. With this increased focus, the epigenetic modification in AD has become a highly popular topic. This review summarizes the literature on the role of epigenetic mechanisms in the development of AD and the possibilities for treating AD by epigenetically based interventions.

EPIGENETICS FACTORS IN AD

Epigenetic factors include DNA methylation, histone modifications, chromatin remodeling, and regulation by non-coding RNA (**Figure 1**) (Morange, 2002; Holliday, 2006; Santana et al., 2017). The functions of epigenetics have been studied in various areas of biology, cancer biology and so on (Jones and Laird, 1999; Champagne, 2013). Now, many researchers are focusing on investigating the potential roles of epigenetics in AD pathogenesis.

DNA METHYLATION

DNA methylation modifies cytosine residues by adding methyl groups in cytosine/guanine-rich regions such as CpG islands (Mehler, 2008). The process is initiated by DNA methyltransferases (DNMT), including DNMT1, DNMT2, DNMT3a, and DNMT3b (Kemmer et al., 2017). In this section we discuss the possibility that AD could be caused by aberrations in DNA methylation in some certain genes and the potential role of methylation in being a biomarker in AD.

For example, it was found that some cytosines, particularly those at -207 to approximately -182, in the promoter region of the APP gene are mostly methylated and that their demethylation with age may lead to A β deposition in the aged brain (Tohgi et al., 1999a,b). Methylation of the microtubule-related protein tau (MAPT) gene could also suppress the MAPT expression, which could affect the levels of tau protein (Zhang et al., 2016). Mano et al. (2017) used postmortem brain samples from AD patients and found that the expression of BRCA1 was significantly upregulated, consisting of its hypomethylation. Additionally, BRCA1 protein levels were increased in response to A β and became mislocalized to the cytoplasm, in both *in vitro* cellular and *in vivo* mouse models (Mano et al., 2017). Recently, it was found that reduced DNA methylation at the triggering receptor expressed on myeloid cells 2 (TREM2) gene intron 1 caused higher TREM2 mRNA expression in the leukocytes of AD subjects than in the controls (Ozaki et al., 2017). Furthermore, DNA methylation in some other genes also plays a role in

the mechanisms of AD and might be a potential biomarker. A study found that DNA methylation (CpG5) of the BDNF gene promoter and a tag SNP (rs6265) have a significant role in the etiology of amnesic mild cognitive impairment (aMCI) and its progression to AD (Xie et al., 2017a). In addition, a 5-year longitudinal study using multivariate Cox regression analysis revealed that elevated methylation of CpG5 of BDNF promoters was a significant independent predictor of AD conversion. This suggests that increased levels of peripheral BDNF promoter methylation may be an epigenetic biomarker indicating the transformation of aMCI to AD (Xie et al., 2017b). Kobayashi et al. (2016) examined the DNA methylation levels of the COASY and SPINT gene promoter regions and found that DNA methylation in the two regions was significantly increased in AD and aMCI as compared to controls. Di Francesco et al. (2015) found that global DNA methylation in the peripheral blood mononuclear cells of LOAD patients was higher compared to healthy controls, and higher DNA methylation levels were associated with the presence of APOE ϵ 4 allele ($p = 0.0043$) and APOE ϵ 3 carriers ($p = 0.05$) in the global population. This indicated global DNA methylation in peripheral samples is a useful marker for screening individuals at risk of developing AD (Di Francesco et al., 2015). Moreover, DNA methylation levels in the NCAPH2/LMF2 promoter region were found to be a useful biomarker for the diagnosis of AD and aMCI (Shinagawa et al., 2016). One study has found that APOE CGI holds lower DNA methylation levels in AD compared to control in the frontal lobe, mostly in the non-neuronal cells of the AD brain (Tulloch et al., 2018). Additionally, PICALM gene methylation was found to be associated with cognitive decline in blood cells of AD patients (Mercurio et al., 2018). Recently, it was discovered that elevated DNA methylation across a 48-kb region spanning the HOXA gene cluster is associated with AD neuropathology (Smith et al., 2018). Moreover, a differentially methylated region was identified within the alternative promoter of the PLD3 gene showing higher DNA methylation levels in the AD hippocampus compared to controls (Blanco-Luquin et al., 2018). All of these indicated that the methylation levels of some genes could be potential biomarkers in AD.

In some postmortem brains or neuronal cells of AD individuals, a lot of work has been done to find more specific evidence. In cortical neurons of a postmortem AD brain, immunoreactivity for 5-methylcytosine (5-mC) was decreased compared to the control (Mastroeni et al., 2010). Levels of 5mC were reduced in the hippocampus, entorhinal cortex and cerebellum of patients with AD (Chouliaras et al., 2013; Condliffe et al., 2014). In hippocampal tissue, Mastroeni et al. (2010) found weak staining for antibodies against DNA methylation maintenance factors in AD cases, contrasting with normal brains. More compellingly, in a comparison of cortical neurons of monozygotic twins, one of whom had AD and the other not, they found extensive co-localization of 5 mC, with three cell-specific markers in the non-AD twin yet an absence of co-localization in the AD twin (Mastroeni et al., 2009). Furthermore, a study identified that numerous AD-related genes, such as MCF2L, ANK1, MAP2, LRRC8B, STK32C, and S100B, have cell-type-specific methylation signatures and document

conditions of stress and injury, the level of HDAC2 increases, and this causes decreased expression of genes related to memory and cognition (Levenson et al., 2004; Graff et al., 2012). HDAC3 also plays a vital role in regulating long-term memory formation. Deletion of HDAC3 in the dorsal hippocampus leads to enhanced long-term memory for object location (McQuown et al., 2011).

In addition to class I HDACs, there is some evidence that class II HDACs are involved to some degree in AD. Class II HDACs are subdivided into class IIa and class IIb (Haggarty and Tsai, 2011). HDAC6, a class IIb HDAC, is elevated by 52% and 91% in the cortex and hippocampus of patients with AD. HDAC6 co-localizes with tau protein in the AD hippocampus, and tau phosphorylation can be decreased by reducing HDAC6 levels (Ding et al., 2008). Govindarajan et al. (2013) using a mouse model of AD, found that decreasing HDAC6 levels could improve cognitive impairment. HDAC4, a member of class IIa, is reported to be associated with synaptic plasticity and memory formation, and loss of it is detrimental to learning and memory (Kim et al., 2012). D'Addario et al. (2017) found that in the peripheral blood cells of monozygotic twins discordant for AD, higher levels of gene expression of HDAC2 and HDAC9 were found in the AD twin. Recently, it was reported that HDAC2 dysregulation could contribute to cholinergic nucleus basalis of Meynert (nbM) neuronal dysfunction, NFT pathology, and cognitive decline during clinical progression of AD (Mahady et al., 2018).

The class III HDACs are called sirtuins, SIRT1-7 (Gray and Ekstrom, 2001). The level of SIRT1 is reduced in the AD cortex, and SIRT1 levels are negatively correlated with the accumulation of paired helical tau filaments (Julien et al., 2009). Tau acetylation can promote pathological tau aggregation, while SIRT1 causes tau deacetylation. There is extensive evidence that SIRT1 reduction is involved in AD tau pathology, and tau acetylation of lysine 28 inhibits tau function via impaired tau-microtubule interactions, promoting tau aggregation (Cohen et al., 2011). Moreover, a significant positive correlation between SIRT1 levels and dementia was found whereby dementia risk increases by a factor of 1.16 due to an increase in the SIRT1 level and a factor of 24.23 due to a decrease in the TLR4 level (Kilic et al., 2018).

Histone acetyltransferases are also involved in the regulation of AD neurogenesis. For example, Tip60, a type of histone acetyltransferase, epigenetically regulates genes enriched in neurons, and its function in axonal transport is mediated by APP. More importantly, Tip60 plays a neuroprotective role in APP-induced axonal transport and functional locomotion defects (Johnson et al., 2013).

In conclusion, a range of studies indicates that histone modifications play a vital role in the development of AD. HDACs can both promote and impair memory formation and cognition.

NON-CODING RNA REGULATION

Non-coding RNAs (ncRNAs) can cause changes in gene expression and the production of proteins and they are involved in the pathology of many neurodegenerative diseases. A recent

study used microarray analysis to characterize the expression patterns of circular RNAs (circRNAs), miRNAs and mRNAs in hippocampal tissue from an A β 1-42-induced AD rat model, and found that a total of 555 circRNAs, 183 miRNAs, and 319 mRNAs were significantly dysregulated (change ≥ 2 -fold and p -value < 0.05) in the hippocampus (Wang et al., 2018). In this section, we discuss three aspects of the relationship between ncRNAs and AD.

miRNAS IN AD

In AD, miRNAs are expressed differently in the postmortem brain, blood monocytes and cerebrospinal fluid (CSF). The expression of miRNAs in the hippocampus, medial frontal gyrus and CSF is regionally and stage-specifically altered in AD patient brains using a sensitive qRT-PCR platform, and miRNAs are involved in pathways related to amyloid processing, neurogenesis, insulin resistance, and innate immunity in AD pathogenesis (Cogswell et al., 2008). Levels of members of the miR-29 family, including miR-29a, miR-29b, and miR-29c, are decreased in the brains of patients with AD, and this is related to high BACE1 protein expression (Hebert et al., 2008). It has been reported that miRNA-7, miRNA-9-1, miRNA-23a/miRNA-27a, miRNA-34a, miRNA-125b-1, miRNA-146a, and miRNA-155 are significantly increased in abundance in AD-affected superior temporal lobe neocortex (Brodmann area 22). These seven upregulated miRNAs are involved in the coordination of amyloid production and clearance (Pogue and Lukiw, 2018). Higaki et al. (2018) suggested that miR-200b/c reduces A β secretion and A β -induced cognitive impairment by promoting insulin signaling. Moreover, exosome miR-135a and miR-384 were found to be upregulated, while miR-193b was downregulated, in the serum of AD patients compared to that of normal controls (Yang et al., 2018). A study using Tg2576 AD transgenic mice and human AD brain samples found that miR-206 could regulate brain-derived neurotrophic factor (BDNF), which regulates synaptic plasticity and memory (Lee et al., 2012). Moreover, of 8098 individually measured miRNAs in blood cells, six of these miRNAs such as miR-107, miR-125b, miR-146a, miR-181c, miR-29b, and miR-342 were found to be significantly downregulated in individuals with AD compared to controls (Fransquet and Ryan, 2018). These findings indicate that miRNAs are potential biomarkers for AD, and that miRNAs might be involved in the pathophysiology of AD.

LONG NON-CODING RNAs IN AD

Long non-coding RNAs (lncRNA) also participate in the pathology of AD. Using a microarray analysis, it was found that in the hippocampal tissue of a rat model of AD, a total of 315 lncRNAs and 311 mRNAs were significantly dysregulated (≥ 2 -fold, $p < 0.05$) (Yang et al., 2017). A type of ncRNA called 51A has been found to regulate the SORL1 gene, which encodes a sorting receptor for the APP holoprotein (Rogaeva et al., 2007). Another

study concluded that 51A might increase AD susceptibility by increasing APP formation (Ciarlo et al., 2013). Other lncRNAs are also related to AD mechanisms. For example, RNA 17A was found to be increased in AD. This lncRNA regulates GABAB receptor alternative splicing and signaling in response to inflammatory stimuli (Massone et al., 2011). NDM29 RNA upregulation is accompanied by altered APP modulation and can promote the cleavage activities of BACE (Massone et al., 2012). BC200 RNA is reported to be highly increased in the AD brain in parallel with disease progression (Wu et al., 2013). BC1, another type of lncRNA, is found to induce APP mRNA translation via association with fragile X syndrome protein (FMRP) in mouse model AD brains (Zhang T. et al., 2017). It was recently reported that EBF3 knockdown can reverse A β 25-35-induced apoptosis in SH-SY5Y cells, and that lncRNA EBF3-AS promotes neuron apoptosis in AD through regulation of EBF3 expression (Gu et al., 2018). Furthermore, the overexpression of A β and A β -42 in AD could increase BACE1 antisense transcript lncRNA levels, leading to amyloid protein aggregation (Faghihi et al., 2008).

OTHER ncRNAs IN AD

In addition to miRNA and lncRNA, there are other classes of ncRNAs that play roles in AD that are not as well understood. Recently, it was reported that circRNA-associated-ceRNA networks in an AD mouse model are involved in A β clearance and myelin function (Zhang S. et al., 2017). For example, circRNA CIRS-7 significantly decreases in brains with AD. Its effect is to reduce the protein levels of APP and BACE1 (Lukiw, 2013). Y RNA is reported to play an essential pathological role in the altered mRNA landscape of AD (Champagne et al., 2001). Another interesting ncRNA is small NRSE dsRNA, which is a 20bp double-stranded RNA. NRSE dsRNA has been found to bind to the REST complex and activate it. The REST complex protects neurons from oxidative stress and amyloid β -protein toxicity (Rougeulle et al., 1998). The U1 snRNP is reported to show very high expression in neurofibrillary tangles in sporadic AD and early-onset AD (Hales et al., 2014a,b). All these findings point to ways that ncRNAs may take part in the development of AD.

APPLICATIONS OF EPIGENETICS TO AD THERAPEUTICS

DNA Methylation-Based Therapy

It has been shown that hypomethylation of AD risk genes such as APP, PSEN1, and PSEN2 has effects on learning and memory. Studies have shown that increases in methyl donor S-adenosyl-L-methionine (SAM) can decrease APP and PSEN1 expression by promoter hypermethylation (Scarpa et al., 2003; Fusco et al., 2005). It may also be relevant that the impaired learning ability induced by lead can be improved significantly by SAM, perhaps by ameliorating the impairments in the long-term potentiation of excitatory postsynaptic potentials that are caused by lead (Cao et al., 2008). Moreover, increasing levels of B12, folate and

other methionine sources in the diet can promote methionine bioavailability and reverse elevated expression of APP and PSEN1 (Chan and Shea, 2007; Fusco et al., 2008).

Several studies have focused on finding DNMT inhibitors that could modulate the methylation of AD risk genes. DNMT inhibitors such as azacitidine and decitabine have been approved by the FDA for used in tumors and leukemia (Mompalmer et al., 1997; Issa et al., 2004). This approach has also been used in some other neurodegenerative diseases, including Friedreich's ataxia and fragile X syndrome (Chiurazzi et al., 1998; Sandi et al., 2013).

It has also been found that DNA methylation is related to the state of NSC differentiation. As a result, the use of the DNA methylation inhibitor 5-aza-cytidine to treat NSCs could alter DNA methylation, thereby disrupting migration and differentiation (Singh et al., 2009). Loss of Dnmt3a, a type of DNA methyltransferase, was found to impair hematopoietic stem cell differentiation (Challen et al., 2011). Another study showed that intrinsic epigenetic mechanisms play critical roles in the regulation of adult NSC functions. Using methylated CpG binding protein 1 (Mbd1)-deficient mice, it was found that both acute knockdown of Mbd1 or overexpression of fibroblast growth factor 2 (Fgf-2) in adult neural stem and progenitor cells inhibited neuronal differentiation (Li et al., 2008).

Histone Modification-Based Therapy

Histone deacetylase inhibitors, including valproic acid (VPA), sodium 4-phenylbutyrate (4-PBA), vorinostat (SAHA), trichostatin A (TSA) and nicotinamide, have been shown to produce good results in AD mouse models. We discuss these five inhibitors separately.

Valproic acid has been shown to inhibit A β production in a transgenic AD mouse model (Su et al., 2004). In neuroblastoma and glioma cell lines, VPA could prevent amyloid- β aggregation by increasing clusterin expression (Nuutinen et al., 2010). VPA was also found to decrease A β production in transgenic mice, improving their behavioral deficits (Qing et al., 2008). In an AD mouse model it relieved contextual memory deficits via histone H4 acetylation (Kilgore et al., 2010).

4-PBA is reported to decrease β -amyloid production and reverse spatial learning and memory deficits. In the Tg2576 AD mouse model, administration of 4-PBA led to a decrease in tau phosphorylation by increasing GSK-3 β (Ricobaraza et al., 2009) as well as increasing intraneuronal A β clearance and restoration of dendritic spine densities in hippocampal CA1 pyramidal neurons (Ricobaraza et al., 2012).

SAHA is found to increase the H4K12 acetylation level, thus restoring learning ability in a mouse model (Peleg et al., 2010). In an astrocyte cell line, it induced the expression of clusterin, which might prevent the progress of AD pathogenesis (Nuutinen et al., 2010). In the APP^{swe}/PS1^{dE9} AD mouse model, SAHA was reported to restore contextual memory by inhibiting HDAC6 in the early stage of AD (Kilgore et al., 2010).

Trichostatin A is found to induce brain-derived neurotrophic factor (BDNF) exon I-IX mRNA in Neuro-2a cells (Ishimaru et al., 2010). Another study of hippocampal neurons also suggested that TSA treatment could be useful at promoter 1 by acetylating histones (H3AcK9/K14) (Tian et al., 2010).

Nicotinamide was found to restore cognitive deficits. Nicotinamide can also increase the acetylation of alpha-tubulin and MAP2c, thereby increasing microtubule stability. These observations suggest a possibility that nicotinamide could be useful in treating AD (Green et al., 2008).

ncRNA Regulation-Based Therapy

Non-coding RNAs are widely expressed in the brain and show a range of differences between AD and healthy controls. Numerous studies have focused on finding potential treatments using ncRNAs. Studies have found that overexpression of miR-124 and miR-195 could down-regulate A β levels by controlling BACE1 gene expression (Fang et al., 2012; Zhu et al., 2012). It is reported that DNA antisense oligonucleotides can hasten the degradation of UBE3A-ATS, which might be an avenue of treatment for AD (Meng et al., 2015). In Tg-19959 mice, knockdown of BACE1 or BACE1-AS transcripts causes reductions in BACE1 protein and insoluble A β (Modarresi et al., 2011). In an *in vitro* preparation, MiR-16 was found to be an effective inhibitor of amyloid precursor protein (APP) and BACE1 expression, A β peptide production, and tau phosphorylation (Parsi et al., 2015).

miRNA regulators have also been evaluated for use in treating AD. For example, a type of neutralizing inhibitor of miR-206 called AM206 was injected into the cerebral ventricles of an AD mouse model and found to improve memory function (Lee et al., 2012) as well as improving hippocampal neurogenesis and synaptic density. It was reported that RNA interference-mediated knockdown of the long-form phosphodiesterase-4D (PDE4D) enzyme could reverse memory deficits caused by accumulation of amyloid-beta (42) (Zhang et al., 2014). Another study found that miR-34c levels were increased in the hippocampus of AD patients, leading to a suggestion that it might be a biomarker for AD. In an AD mouse model, targeting miR-34 was found to lead to the recovery of learning ability (Zovoilis et al., 2011).

DISCUSSION AND FUTURE PERSPECTIVES

Alzheimer's disease is a progressive neurodegenerative disease which is already a substantial burden on the world. The mechanisms of AD are complex and have been the subject of many theories. We have gained insight into the role of epigenetic modulation, which comprises DNA methylation, histone modification and the effects of ncRNAs. While many differences in these three kinds of epigenetic regulation have been observed between AD patients or models and controls, much work will still be needed to understand this topic and to find potential targets for the treatment of AD.

One of the main challenges for epigenetic therapy is to find molecules that can pass through the blood-brain barrier (BBB). In general, only molecules less than 500 daltons, with fewer than 8 pairs of hydrogen bonds, can move through the BBB. There is a variety of ways of working around this limitation, including BBB disruption, intracerebral implantation and intracerebroventricular infusion (Pardridge, 2005). As discussed above, DNMT inhibitors and HDAC inhibitors are

potential therapeutic drugs, and these two kinds of molecules are able to cross the BBB (Hockly et al., 2003; Rai et al., 2008; Kwa et al., 2011). However, several additional factors need to be considered, including efficacy, toxicity, delivery and patient factors. Even though DNMT inhibitors are widely used in treating tumors and leukemia, they are not yet suitable for continuous treatment of AD. Because of their genotoxicity and low stability, they remain at the preclinical stage of development (Cuadrado-Tejedor et al., 2013; Erdmann et al., 2015). However, the clinical side effects of HDAC inhibitors are well known (Subramanian et al., 2010). As an example, the HDAC inhibitor SAHA can induce reactive oxygen species and DNA damage in acute myeloid leukemia cells (Petrucelli et al., 2011). Therefore, more detailed studies are needed to develop safe and effective treatments using DNMT inhibitors and HDAC inhibitors.

Other approaches to ncRNA-based therapy has been examined in recent years. A study showed that a short peptide derived from rabies virus glycoprotein (RVG) assists the transvascular delivery of small interfering RNA (siRNA) to the brain, leading to specific gene silencing inside the brain. Intravenous treatment with RVG-9R-bound antiviral siRNA afforded robust protection against fatal viral encephalitis in mice (Kumar et al., 2007). Other studies have shown that nanoparticle carriers may be a useful method of moving drugs through the BBB (Kreuter, 2001). All of these are methods that may be useful for implementing small interfering RNA therapy.

CONCLUSION

In conclusion, we have discussed various epigenetic approaches to the treatment of AD, and new methods have emerged to aid in validating epigenetic targets. However, careful characterization of chemical probes is essential to ensure accurate biological conclusions. A new generation of selective chemical probes needs to be developed to reduce side effects. Studies on epigenetics may lead researchers to new methods of AD diagnosis and therapy. Even though the transition from bench to bedside may be challenging in the near future, current research strongly suggests that epigenetic regulation will become an important tool in AD treatment.

AUTHOR CONTRIBUTIONS

XL contributed to the central idea and wrote the initial draft of the manuscript. BJ and LS contributed to refining the ideas, carrying out additional modifications, and finalizing the manuscript. All authors contributed to writing and revising the manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (No. 81701134 to BJ) and Youth Research Foundation of Xiangya Hospital, Central South University (No. 2016Q01 to BJ).

REFERENCES

- Blanco-Luquin, I., Altuna, M., Sanchez-Ruiz, D. G. J., Urdániz-Casado, A., Roldán, M., Cámara, M. et al. (2018). PLD3 epigenetic changes in the hippocampus of Alzheimer's disease. *Clin. Epigenet.* 10:116. doi: 10.1186/s13148-018-0547-3
- Broide, R. S., Redwine, J. M., Aftahi, N., Young, W., Bloom, F.E., and Winrow, C.J. (2007). Distribution of histone deacetylases 1-11 in the rat brain. *J. Mol. Neurosci.* 31, 47–58. doi: 10.1007/BF02686117
- Cao, X. J., Huang, S. H., Wang, M., Chen J. T. and Ruan, D. Y. (2008). S-adenosyl-L-methionine improves impaired hippocampal long-term potentiation and water maze performance induced by developmental lead exposure in rats. *Eur. J. Pharmacol.* 595, 30–34. doi: 10.1016/j.ejphar.2008.07.061
- Challen, G. A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg J. S. et al. (2011). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat. Genet.* 44, 23–31. doi: 10.1038/ng.1009
- Champagne, F., Diorio, J., Sharma, S., and Meaney M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12736–12741. doi: 10.1073/pnas.221224598
- Champagne, F. A. (2013). Epigenetics and developmental plasticity across species. *Dev. Psychobiol.* 55, 33–41. doi: 10.1002/dev.21036
- Chan, A. and Shea, T. B. (2007). Folate deprivation increases presenilin expression, gamma-secretase activity, and Abeta levels in murine brain: potentiation by ApoE deficiency and alleviation by dietary S-adenosyl methionine. *J. Neurochem.* 102, 753–760. doi: 10.1111/j.1471-4159.2007.04589.x
- Chirazzi, P., Pomponi, M. G., Willemsen, R., Oostra BA, and Neri G. (1998). In vitro reactivation of the FMR1 gene involved in fragile X syndrome. *Hum. Mol. Genet.* 7, 109–113. doi: 10.1093/hmg/7.1.109
- Chouliaras, L., Mastroeni, D., Delvaux, E., Grover, A., Kenis, G., Hof, P. R., et al. (2013). Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. *Neurobiol. Aging* 34, 2091–2099. doi: 10.1016/j.neurobiolaging.2013.02.021
- Ciarlo, E., Massone, S., Penna, I., Nizzari, M., Gigoni, A., Dieci, G., et al. (2013). An intronic ncRNA-dependent regulation of SORL1 expression affecting Abeta formation is upregulated in post-mortem Alzheimer's disease brain samples. *Dis. Model. Mech.* 6, 424–433. doi: 10.1242/dmm.009761
- Cogswell, J. P., Ward, J., Taylor, I. A., Waters, M., Shi, Y., Cannon, B., et al. (2008). Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *J. Alzheimers Dis.* 14, 27–41. doi: 10.3233/JAD-2008-14103
- Cohen, T. J., Guo, J. L., Hurtado, D. E., Kwong, L. K., Mills, I. P., Trojanowski, J. Q., et al. (2011). The acetylation of tau inhibits its function and promotes pathological tau aggregation. *Nat. Commun.* 2:252. doi: 10.1038/ncomms1255
- Condliffe, D., Wong, A., Troakes, C., Proitsi, P., Patel, Y., Chouliaras, L., et al. (2014). Cross-region reduction in 5-hydroxymethylcytosine in Alzheimer's disease brain. *Neurobiol. Aging* 35, 1850–1854. doi: 10.1016/j.neurobiolaging.2014.02.002
- Cuadrado-Tejedor, M., Oyarzabal, J., and Lucas, M. P. and García-Osta. (2013). Epigenetic drugs in Alzheimer's disease. *Biomol. Concepts* 4, 433–445. doi: 10.1515/bmc-2013-0012
- D'Addario, C., Candia, S. B., Arosio, B., Di Bartolomeo, M., Abbate, C., Casè A., et al. (2017). Transcriptional and epigenetic phenomena in peripheral blood cells of monozygotic twins discordant for alzheimer's disease, a case report. *J. Neurol. Sci.* 372, 211–216. doi: 10.1016/j.jns.2016.11.052
- Datta, M., Staszewski, O., Raschi, E., Frosch, M., Hagemeyer, N., Tay, T. L., et al. (2018). Histone deacetylases 1 and 2 regulate microglia function during development, homeostasis, and neurodegeneration in a context-dependent manner. *Immunity* 48, 514–529. doi: 10.1016/j.immuni.2018.02.016
- Di Francesco, A., Arosio, B., Falconi, A., Micioni Di Bonaventura, M. V., Karimi, M., Mari, D., et al. (2015). Global changes in DNA methylation in Alzheimer's disease peripheral blood mononuclear cells. *Brain Behav. Immun.* 45, 139–144. doi: 10.1016/j.bbi.2014.11.002
- Ding, H., Dolan, P. J., and Johnson, G. V. (2008). Histone deacetylase 6 interacts with the microtubule-associated protein tau. *J. Neurochem.* 106, 2119–2130. doi: 10.1111/j.1471-4159.2008.05564.x
- Diniz, L. P., Tortelli, V., Matias, I., Morgado, J., Bérnago Araujo, A. P., Melo, H. M., et al. (2017). Astrocyte transforming growth factor beta 1 protects synapses against Abeta oligomers in Alzheimer's disease model. *J. Neurosci.* 37, 6797–6809. doi: 10.1523/JNEUROSCI.3351-16.2017
- Erdmann, A., Halby, L., Fahy, J., and Arimondo, P. B. (2015). Targeting DNA methylation with small molecules: what's next? *J. Med. Chem.* 58, 2569–2583. doi: 10.1021/jm500843d
- Faghihi, M. A., Modarresi, F., Khalil, A. M., Wood, D. E., Sahagan, B. G., Morgan, T. E., et al. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat. Med.* 14, 723–730. doi: 10.1038/nm1784
- Fang, M., Wang, J., Zhang, X., Geng, Y., Hu, Z., Rudd, J. A., et al. (2012). The miR-124 regulates the expression of BACE1/beta-secretase correlated with cell death in Alzheimer's disease. *Toxicol. Lett.* 209, 94–105. doi: 10.1016/j.toxlet.2011.11.032
- Fransquet, P. D. and Ryan, J. (2018). Micro RNA as a potential blood-based epigenetic biomarker for Alzheimer's disease. *Clin. Biochem.* 58, 5–14. doi: 10.1016/j.clinbiochem.2018.05.020
- Fuso, A., Nicolai, V., Cavallaro, R. A., Ricceri, L., D'Anselmi, F., Coluccia, P., et al. (2008). B-vitamin deprivation induces hyperhomocysteinemia and brain S-adenosylhomocysteine, depletes brain S-adenosylmethionine, and enhances PS1 and BACE expression and amyloid-beta deposition in mice. *Mol. Cell Neurosci.* 37, 731–746. doi: 10.1016/j.mcn.2007.12.018
- Fuso, A., Seminara, L., Cavallaro, R. A., D'Anselmi, F., and Scarpa, S. (2005). S-adenosylmethionine/homocysteine cycle alterations modify DNA methylation status with consequent deregulation of PS1 and BACE and beta-amyloid production. *Mol. Cell Neurosci.* 28, 195–204. doi: 10.1016/j.mcn.2004.09.007
- Gasparoni, G., Bultmann, S., Lutsik, P., Kraus, T. F. J., Sordon, S., Vlcek, J., et al. (2018). DNA methylation analysis on purified neurons and glia dissects age and Alzheimer's disease-specific changes in the human cortex. *Epigenet. Chromatin.* 11:41. doi: 10.1186/s13072-018-0211-3
- Govindarajan, N., Rao, P., Burkhardt, S., Sananbenesi, F., Schlüter, O. M., Bradke, F., et al. (2013). Reducing HDAC6 ameliorates cognitive deficits in a mouse model for Alzheimer's disease. *EMBO Mol. Med.* 5, 52–63. doi: 10.1002/emmm.201201923
- Graff, J., Rei, D., Guan, J. S., Wang, W. Y., Seo, J., Hennig, K. M., et al. (2012). An epigenetic blockade of cognitive functions in the neurodegenerating brain. *Nature* 483, 222–226. doi: 10.1038/nature10849
- Gray, S. G. and Ekstrom, T. J. (2001). The human histone deacetylase family. *Exp. Cell Res.* 262, 75–83. doi: 10.1006/excr.2000.5080
- Green, K. N., Steffan, J. S., Martinez-Coria, H., Sun, X., Schreiber, S. S., and Thompson, L. M. (2008). Nicotinamide restores cognition in Alzheimer's disease transgenic mice via a mechanism involving sirtuin inhibition and selective reduction of Thr231-phosphotau. *J. Neurosci.* 28, 11500–11510. doi: 10.1523/JNEUROSCI.3203-08.2008
- Gu, C., Chen, C., Wu, R., Dong, T., Hu, X., Yao, Y., et al. (2018). Long noncoding RNA EBF3-as promotes neuron apoptosis in Alzheimer's Disease. *DNA Cell Biol.* 37, 220–226. doi: 10.1089/dna.2017.4012
- Guan, J. S., Haggarty, S. J., Giacometti, E., Dannenberg, J. H., Joseph, N., Gao, J., et al. (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459, 55–60. doi: 10.1038/nature07925
- Haggarty, S. J., and Tsai, L. H. (2011). Probing the role of HDACs and mechanisms of chromatin-mediated neuroplasticity. *Neurobiol. Learn. Mem.* 96, 41–52. doi: 10.1016/j.nlm.2011.04.009
- Hales, C. M., Dammer, E. B., Diner, I., Yi, H., Seyfried, N. T., Gearing, M., et al. (2014a). Aggregates of small nuclear ribonucleic acids (snRNAs) in Alzheimer's disease. *Brain Pathol.* 24, 344–351. doi: 10.1111/bpa.12133
- Hales, C. M., Seyfried, N. T., Dammer, E. B., Duong, D., Yi, H., Gearing, M., et al. (2014b). U1 small nuclear ribonucleoproteins (snRNPs) aggregate in Alzheimer's disease due to autosomal dominant genetic mutations and trisomy 21. *Mol. Neurodegener.* 9:15. doi: 10.1186/1750-1326-9-15
- Hebert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahtaroglu, A. N., et al. (2008). Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6415–6420. doi: 10.1073/pnas.0710263105

- Higaki, S., Muramatsu, M., Matsuda, A., Matsumoto, K., Satoh, J. I., Michikawa, M., et al. (2018). Defensive effect of microRNA-200b/c against amyloid-beta peptide-induced toxicity in Alzheimer's disease models. *PLoS One* 13:e196929. doi: 10.1371/journal.pone.0196929
- Hockly, E., Richon, V. M., Woodman, B., Smith, D. L., Zhou, X., Rosa, E., et al. (2003). Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2041–2046. doi: 10.1073/pnas.0437870100
- Holliday, R. (2006). Epigenetics: a historical overview. *Epigenetics* 1, 76–80. doi: 10.4161/epi.1.2.2762
- Ishimaru, N., Fukuchi, M., Hirai, A., Chiba, Y., Tamura, T., Takahashi, N., et al. (2010). Differential epigenetic regulation of BDNF and NT-3 genes by trichostatin A and 5-aza-2'-deoxycytidine in Neuro-2a cells. *Biochem. Biophys. Res. Commun.* 394, 173–177. doi: 10.1016/j.bbrc.2010.02.139
- Issa, J. P., Garcia-Manero, G., Giles, F. J., Mannari, R., Thomas, D., Faderl, S., et al. (2004). Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* 103, 1635–1640. doi: 10.1182/blood-2003-03-0687
- Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. *Science* 293, 1074–1080. doi: 10.1126/science.1063127
- Johnson, A. A., Sarthi, J., Pirooznia, S. K., Reube, W., and Elefant, F. (2013). Increasing Tip60 HAT levels rescues axonal transport defects and associated behavioral phenotypes in a Drosophila Alzheimer's disease model. *J. Neurosci.* 33, 7535–7547. doi: 10.1523/JNEUROSCI.3739-12.2013
- Jones, P. A., and Laird, P. W. (1999). Cancer epigenetics comes of age. *Nat. Genet.* 21, 163–167. doi: 10.1038/5947
- Julien, C., Tremblay, C., Emond, V., Lebbadi, M., Salem, N. Jr., Bennett, D. A., et al. (2009). Sirtuin 1 reduction parallels the accumulation of tau in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 68, 48–58. doi: 10.1097/NEN.0b013e3181922348
- Kemme, C. A., Marquez, R., Luu, R. H., and Iwahara, J. (2017). Potential role of DNA methylation as a facilitator of target search processes for transcription factors through interplay with methyl-CpG-binding proteins. *Nucleic Acids Res.* 45:7751–7759. doi: 10.1093/nar/gkx387
- Kilgore, M., Miller, C. A., Fass, D. M., Hennig, K. M., Haggarty, S. J., Sweatt, J. D., et al. (2010). Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease. *Neuropsychopharmacology* 35, 870–880. doi: 10.1038/npp.2009.197
- Kilic, U., Elibol, B., Uysal, O., Kilic E., Yulug, B., Sayin Sakul A., et al. (2018). Specific alterations in the circulating levels of the SIRT1, TLR4, and IL7 proteins in patients with dementia. *Exp. Gerontol.* 111, 203–209. doi: 10.1016/j.exger.2018.07.018
- Kim, M. S., Akhtar, M. W., Adachi, M., Mahgoub M., Bassel-Duby R, Kavalali ET., et al. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. *J. Neurosci.* 32, 10879–10886. doi: 10.1523/JNEUROSCI.2089-12.2012
- Kobayashi, N., Shinagawa, S., Nagata, T., Shimada, K., Shibata, N., Ohnuma, T., et al. (2016). Usefulness of DNA methylation levels in COASY and SPINT1 gene promoter regions as biomarkers in diagnosis of Alzheimer's disease and amnesic mild cognitive impairment. *PLoS One* 11:e168816. doi: 10.1371/journal.pone.0168816
- Kreuter, J. (2001). Nanoparticulate systems for brain delivery of drugs. *Adv. Drug Deliv. Rev.* 47, 65–81. doi: 10.1016/S0169-409X(00)00122-8
- Kumar, P., Wu, H., McBride, J. L., Jung, K. E., Kim, M. H., Davidson, B. L., et al. (2007). Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 448, 39–43. doi: 10.1038/nature05901
- Kwa, F. A., Balcerzyk, A., Licciardi, P., El-Osta, A., and Karagiannis, T. C. (2011). Chromatin modifying agents – the cutting edge of anticancer therapy. *Drug Discov. Today* 16, 543–547. doi: 10.1016/j.drudis.2011.05.012
- Lane-Donovan, C., and Herz, J. (2017). ApoE, ApoE receptors, and the synapse in Alzheimer's disease. *Trends Endocrinol. Metab.* 28, 273–284. doi: 10.1016/j.tem.2016.12.001
- Lansdall, C. J., Coyle-Gilchrist, I., Jones, P. S., Vázquez Rodríguez, P., Wilcox, A., Wehmann, E., et al. (2017). Apathy and impulsivity in frontotemporal lobar degeneration syndromes. *Brain* 140, 1792–1807. doi: 10.1093/brain/awx101
- Lardenoije, R., Iatrou, A., Kenis, G., Kompotis, K., Steinbusch, H. W., Mastroeni, D., et al. (2015). The epigenetics of aging and neurodegeneration. *Prog. Neurobiol.* 131, 21–64. doi: 10.1016/j.pneurobio.2015.05.002
- Lee, S. T., Chu, K., Jung, K. H., Kim, J. H., Huh, J. Y., Yoon, H., et al. (2012). miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann. Neurol.* 72, 269–277. doi: 10.1002/ana.23588
- Levenson, J. M., O'Riordan, K. J., Brown, K. D., Trinh, M. A., Molfese, D. L., and Sweatt, J. D. (2004). Regulation of histone acetylation during memory formation in the hippocampus. *J. Biol. Chem.* 279, 40545–40559. doi: 10.1074/jbc.M402229200
- Levy, E., Carman, M. D., Fernandez-Madrid, I. J., Power, M. D., Lieberburg, I., van Duinen, S. G., et al. (1990). Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* 248, 1124–1126. doi: 10.1126/science.2111584
- Li, X., Barkho, B. Z., Luo, Y., Smrt, R. D., Santistevan, N. J., Liu, C., et al. (2008). Epigenetic regulation of the stem cell mitogen Fgf-2 by Mbd1 in adult neural stem/progenitor cells. *J. Biol. Chem.* 283, 27644–27652. doi: 10.1074/jbc.M804899200
- Lukiw, W. J. (2013). Circular RNA (circRNA) in Alzheimer's disease (AD). *Front. Genet.* 4:307. doi: 10.3389/fgenet.2013.00307
- Mahady, L., Nadeem, M., Malek-Ahmadi, M., Chen, K., Perez, S. E., and Mufson, E. J. (2018). HDAC2 dysregulation in the nucleus basalis of Meynert during the progression of Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* doi: 10.1111/nan.12518. [Epub ahead of print].
- Mano, T., Nagata, K., Nonaka, T., Tarutani, A., Imamura, T., Hashimoto, T., et al. (2017). Neuron-specific methylome analysis reveals epigenetic regulation and tau-related dysfunction of BRCA1 in Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 114, E9645–E9654. doi: 10.1073/pnas.1707151114
- Massone, S., Ciarlo, E., Vella, S., Nizzari, M., Florio, T., Russo, C., et al. (2012). NDM29, a RNA polymerase III-dependent non coding RNA, promotes amyloidogenic processing of APP and amyloid beta secretion. *Biochim. Biophys. Acta* 1823, 1170–1177. doi: 10.1016/j.bbamcr.2012.05.001
- Massone, S., Vassallo, I., Fiorino, G., Castelnovo, M., Barbieri, F., Borghi, R., et al. (2011). 17A, a novel non-coding RNA, regulates GABA B alternative splicing and signaling in response to inflammatory stimuli and in Alzheimer disease. *Neurobiol. Dis.* 41, 308–317. doi: 10.1016/j.nbd.2010.09.019
- Masters, C. L., Bateman, R., Blennow, K., Rowe, C. C., Sperling, R. A., and Cummings, J. L. (2015). Alzheimer's disease. *Nat. Rev. Dis. Primers* 1:15056. doi: 10.1038/nrdp.2015.56
- Mastroeni, D., Grover, A., Delvaux, E., Whiteside, C., Coleman, P. D., and Rogers, J. (2010). Epigenetic changes in Alzheimer's disease: decrements in DNA methylation. *Neurobiol. Aging* 31, 2025–2037. doi: 10.1016/j.neurobiolaging.2008.12.005
- Mastroeni, D., Mckee, A., Grover, A., Rogers, J., and Coleman, P. D. (2009). Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. *PLoS One* 4:e6617. doi: 10.1371/journal.pone.0006617
- Mckhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R Jr., Kawas, C. H., et al. (2011). The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* 7, 263–269. doi: 10.1016/j.jalz.2011.03.005
- McQuown, S. C., Barrett, R. M., Matheos, D. P., Post, R. J., Rogge, G. A., Alenghat, T., et al. (2011). HDAC3 is a critical negative regulator of long-term memory formation. *J. Neurosci.* 31, 764–774. doi: 10.1523/JNEUROSCI.5052-10.2011
- Mehler, M. F. (2008). Epigenetic principles and mechanisms underlying nervous system functions in health and disease. *Prog. Neurobiol.* 86, 305–341. doi: 10.1016/j.pneurobio.2008.10.001
- Meng, L., Ward, A. J and Chun, S., Bennett, C. F., Beaudet, A. L., and Rigo, F. (2015). Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature* 518, 409–412. doi: 10.1038/nature13975
- Mercorio, R., Pergoli, L., Galimberti, D., Favero, C., Carugno, M., Dalla Valle, E., et al. (2018). PICALM Gene Methylation in Blood of Alzheimer's Disease Patients Is Associated with Cognitive Decline. *J. Alzheimers Dis.* 65, 283–292. doi: 10.3233/JAD-180242

- Modarresi, F., Faghihi, M. A., Patel, N. S., Sahagan, B. G., and Wahlestedt, C. R. (2011). Knockdown of BACE1-AS nonprotein-coding transcript modulates beta-amyloid-related hippocampal neurogenesis. *Int. J. Alzheimers Dis.* 2011:929042. doi: 10.4061/2011/929042
- Momparler, R. L., Bouffard, D. Y., Momparler, L. F., Dionne J., Belanger K, and Ayoub J. (1997). Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* 8, 358–368. doi: 10.1097/00001813-199704000-00008
- Morange, M. (2002). The relations between genetics and epigenetics: a historical point of view. *Ann. N.Y. Acad. Sci.* 981, 50–60. doi: 10.1111/j.1749-6632.2002.tb04911.x
- Nuutinen, T., Suuronen, T., Kauppinen, A., and Salminen, A. (2010). Valproic acid stimulates clusterin expression in human astrocytes: implications for Alzheimer's disease. *Neurosci. Lett.* 475, 64–68. doi: 10.1016/j.neulet.2010.03.041
- Ozaki, Y., Yoshino, Y., Yamazaki, K., Sao, T., Mori, Y., Ochi, S., et al. (2017). DNA methylation changes at TREM2 intron 1 and TREM2 mRNA expression in patients with Alzheimer's disease. *J. Psychiatr. Res.* 92, 74–80. doi: 10.1016/j.jpsychires.2017.04.003
- Pardridge, W. M. (2005). The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* 2, 3–14. doi: 10.1602/neurorx.2.1.3
- Parsi, S., Smith, P. Y., Goupil, C., Dorval, V., and Hébert, S. S. (2015). Preclinical evaluation of miR-15/107 family members as multifactorial drug targets for Alzheimer's disease. *Mol. Ther. Nucleic Acids* 4:e256. doi: 10.1038/mtna.2015.33
- Peleg, S., Sananbenesi, F., Zovoilis, A., Burkhardt, S., Bahari-Javan, S., Agis-Balboa, R. C. et al. (2010). Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* 328, 753–756. doi: 10.1126/science.1186088
- Petrucelli, L. A., Dupere-Richer, D., Pettersson, F., Retrouvey, H., and Skoulidakis, S., Miller, W. H. Jr. (2011). Vorinostat induces reactive oxygen species and DNA damage in acute myeloid leukemia cells. *PLoS One* 6:e20987. doi: 10.1371/journal.pone.0020987
- Pogue, A. I. and Lukiw, W. J. (2018). Up-regulated Pro-inflammatory MicroRNAs (miRNAs) in Alzheimer's disease (AD) and Age-Related Macular Degeneration (AMD). *Cell Mol. Neurobiol.* 38, 1021–1031. doi: 10.1007/s10571-017-0572-3
- Qing, H., He, G., Ly, P. T., Fox, C. J., Staufenbiel, M., Cai, F., et al. (2008). Valproic acid inhibits Abeta production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models. *J. Exp. Med.* 205, 2781–2789. doi: 10.1084/jem.20081588
- Qureshi I A, and Mehler, M. F. (2010). Impact of nuclear organization and dynamics on epigenetic regulation in the central nervous system: implications for neurological disease states. *Ann. N.Y. Acad. Sci.* 1204(Suppl.), E20–E37. doi: 10.1111/j.1749-6632.2010.05718.x
- Rai, M., Soragni, E., Jenssen, K., Burnett, R., Herman, D., Coppola, G., et al. (2008). HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. *PLoS One* 3:e1958. doi: 10.1371/journal.pone.0001958
- Ricobaraza, A., Cuadrado-Tejedor, M., Marco, S., Pérez-Otaño, I., and García-Osta, A. (2012). Phenylbutyrate rescues dendritic spine loss associated with memory deficits in a mouse model of Alzheimer disease. *Hippocampus* 22, 1040–1050. doi: 10.1002/hipo.20883
- Ricobaraza, A., Cuadrado-Tejedor, M., Perez-Mediavilla, A., Frechilla, D., Del Río, J., and García-Osta, A. (2009). Phenylbutyrate ameliorates cognitive deficit and reduces tau pathology in an Alzheimer's disease mouse model. *Neuropsychopharmacology* 34, 1721–1732. doi: 10.1038/npp.2008.229
- Rocca, W. A., Petersen, R. C., Knopman, D. S., Hebert, L. E., Evans, D. A., Hall, K. S., et al. (2011). Trends in the incidence and prevalence of Alzheimer's disease, dementia, and cognitive impairment in the United States. *Alzheimers Dement.* 7, 80–93. doi: 10.1016/j.jalz.2010.11.002
- Rogaeva, E., Meng, Y., Lee, J. H., Gu, Y., Kawarai, T., Zou, F., et al. (2007). The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. *Nat. Genet.* 39, 168–177. doi: 10.1038/ng1943
- Rougeulle, C., Cardoso, C., Fontes, M., Colleaux, L., and Lalonde M. (1998). An imprinted antisense RNA overlaps UBE3A and a second maternally expressed transcript. *Nat. Genet.* 19, 15–16. doi: 10.1038/ng0598-15
- Sandi, C., Al-Mahdawi, S., and Pook, M. A. (2013). Epigenetics in Friedreich's ataxia: challenges and opportunities for therapy. *Genet. Res. Int.* 2013:852080. doi: 10.1155/2013/852080
- Santana, V. P., Miranda-Furtado, C. L., de Oliveira-Gennaro, F. G., and Dos Reis, R. M. (2017). Genetics and epigenetics of varicocele pathophysiology: an overview. *J. Assist. Reprod. Genet.* 34, 839–847. doi: 10.1007/s10815-017-0931-5
- Scarpa, S., Fuso, A., D'Anselmi, F., and Cavallaro, R. A. (2003). Presenilin 1 gene silencing by S-adenosylmethionine: a treatment for Alzheimer disease? *FEBS Lett.* 541, 145–148. doi: 10.1016/S0014-5793(03)00277-1
- Selkoe, D. J. (2012). Preventing Alzheimer's disease. *Science* 337, 1488–1492. doi: 10.1126/science.1228541
- Sery, O., Povova, J., Misek, I., Pešák, L., and Janout, V. (2013). Molecular mechanisms of neuropathological changes in Alzheimer's disease: a review. *Folia Neuropathol.* 51, 1–9. doi: 10.5114/fn.2013.34190
- Shinagawa, S., Kobayashi, N., Nagata, T., Kusaka, A., Yamada, H., Kondo K., et al. (2016). DNA methylation in the NCAPH2/LMF2 promoter region is associated with hippocampal atrophy in Alzheimer's disease and amnesic mild cognitive impairment patients. *Neurosci. Lett.* 2016, 629, 33–37. doi: 10.1016/j.neulet.2016.06.055
- Singh, R. P., Shiue, K., Schomberg, D., and Zhou, F. C. (2009). Cellular epigenetic modifications of neural stem cell differentiation. *Cell Transplant.* 18, 1197–1211. doi: 10.3727/096368909X12483162197204
- Smith, R. G., Hannon, E., De Jager, P. L., Chibnik, L., Lott, S. J., Condliffe, D., et al. (2018). Elevated DNA methylation across a 48-kb region spanning the HOXA gene cluster is associated with Alzheimer's disease neuropathology. *Alzheimers Dement.* doi: 10.1016/j.jalz.2018.01.017. [Epub ahead of print].
- Su, Y., Ryder, J., Li, B., Wu, X., Fox, N., Solenberg, P., et al. (2004). Lithium, a common drug for bipolar disorder treatment, regulates amyloid-beta precursor protein processing. *Biochemistry* 43, 6899–6908. doi: 10.1021/bi035627j
- Subramanian, S., Bates, S. E., Wright, J. J., Espinoza-Delgado, I., and Piekarz, R. L. (2010). Clinical toxicities of histone deacetylase inhibitors. *Pharmaceuticals* 3, 2751–2767. doi: 10.3390/ph3092751
- Taher, N., McKenzie, C., Garrett, R., Baker, M., Fox, N., and Isaacs, G. D. (2014). Amyloid-beta alters the DNA methylation status of cell-fate genes in an Alzheimer's disease model. *J. Alzheimers Dis.* 38, 831–844. doi: 10.3233/JAD-131061
- Tian, F., Marini, A. M., and Lipsky, R. H. (2010). Effects of histone deacetylase inhibitor Trichostatin A on epigenetic changes and transcriptional activation of Bdnf promoter 1 by rat hippocampal neurons. *Ann. N.Y. Acad. Sci.* 1199, 186–193. doi: 10.1111/j.1749-6632.2009.05175.x
- Tohgi, H., Utsugisawa, K., Nagane, Y., Yoshimura, M., Genda, Y., and Ukitsu, M. (1999a). Reduction with age in methylcytosine in the promoter region -224 approximately -101 of the amyloid precursor protein gene in autopsy human cortex. *Brain Res. Mol. Brain Res.* 70, 288–292.
- Tohgi, H., Utsugisawa, K., Nagane, Y., Yoshimura, M., Ukitsu, M., and Genda, Y. (1999b). The methylation status of cytosines in a tau gene promoter region alters with age to downregulate transcriptional activity in human cerebral cortex. *Neurosci. Lett.* 275, 89–92.
- Tulloch, J., Leong, L., Thomson, Z., Chen, S., Lee, E. G., Keene, C. D., et al. (2018). Glia-specific APOE epigenetic changes in the Alzheimer's disease brain. *Brain Res.* 1698, 179–186. doi: 10.1016/j.brainres.2018.08.006
- Wang, Z., Xu, P., Chen, B., Zhang Z, Zhang C, Zhan Q, et al. (2018). Identifying circRNA-associated-ceRNA networks in the hippocampus of Abeta1-42-induced Alzheimer's disease-like rats using microarray analysis. *Aging* 10, 775–788. doi: 10.18632/aging.101427
- Wu, P., Zuo, X., Deng, H., Liu, X., Liu, L., and Ji, A. (2013). Roles of long noncoding RNAs in brain development, functional diversification and neurodegenerative diseases. *Brain Res. Bull.* 97, 69–80. doi: 10.1016/j.brainresbull.2013.06.001
- Xie, B., Liu, Z., Liu, W., Jiang, L., Zhang, R., Cui, D., et al. (2017a). DNA Methylation and Tag SNPs of the BDNF gene in conversion of amnesic mild cognitive impairment into Alzheimer's disease: a cross-sectional cohort study. *J. Alzheimers Dis.* 58, 263–274. doi: 10.3233/JAD-170007
- Xie, B., Xu, Y., Liu, Z., Liu, W., Jiang, L., Zhang, R., et al. (2017b). Elevation of peripheral BDNF promoter methylation predicts conversion from amnesic mild cognitive impairment to Alzheimer's disease: a 5-year longitudinal study. *J. Alzheimers Dis.* 56, 391–401. doi: 10.3233/JAD-160954

- Yang, B., Xia, Z. A., Zhong, B., Xiong X., Sheng C., Wang Y., et al. (2017). Distinct hippocampal expression profiles of long non-coding RNAs in an Alzheimer's disease model. *Mol. Neurobiol.* 54, 4833–4846. doi: 10.1007/s12035-016-0038-5
- Yang, T. T., Liu, C. G., and Gao, S. C. (2018). The serum exosome derived microRNA-135a, -193b, and -384 Were potential Alzheimer's disease biomarkers. *Biomed. Environ. Sci.* 31, 87–96. doi: 10.3967/bes2018.011
- Zhang, C., Cheng, Y., Wang, H., Xu, J., Zhang, C., and Zhang, H. (2014). RNA interference-mediated knockdown of long-form phosphodiesterase-4D (PDE4D) enzyme reverses amyloid-beta42-induced memory deficits in mice. *J. Alzheimers Dis.* 38, 269–280. doi: 10.3233/JAD-122236
- Zhang, C. C., Xing, A., Tan, M. S., Tan, L., and Yu, J. T. (2016). The role of MAPT in neurodegenerative diseases: genetics, mechanisms and therapy. *Mol. Neurobiol.* 53, 4893–4904. doi: 10.1007/s12035-015-9415-8
- Zhang, S., Zhu, D., Li, H., Li, H., Feng, C., and Zhang, W. (2017). Characterization of circRNA-associated-ceRNA networks in a senescence-accelerated mouse prone 8 brain. *Mol. Ther.* 25, 2053–2061. doi: 10.1016/j.ymthe.2017.06.009
- Zhang, T., Pang, P., Fang, Z., Guo Y, Li H, Li X., et al. (2017). Expression of BC1 impairs spatial learning and memory in Alzheimer's disease via APP translation. *Mol. Neurobiol.* 55, 6007–6020. doi: 10.1007/s12035-017-0820-z
- Zhao, R., Hu, W., Tsai, J., Li, W., and Gan, W. B. (2017). Microglia limit the expansion of beta-amyloid plaques in a mouse model of Alzheimer's disease. *Mol. Neurodegener.* 12(1): 47. doi: 10.1186/s13024-017-0188-6
- Zhu, H. C., Wang, L. M., Wang, M., Song, B., Tan, S., Teng, J. F., et al. (2012). MicroRNA-195 downregulates Alzheimer's disease amyloid-beta production by targeting BACE1. *Brain Res. Bull.* 88, 596–601. doi: 10.1016/j.brainresbull.2012.05.018
- Zovoilis, A., Agbemenyah, H. Y, Agis-Balboa, R. C., Stilling, R. M., Edbauer, D., Rao, P., et al. (2011). microRNA-34c is a novel target to treat dementias. *EMBO J.* 30, 4299–4308. doi: 10.1038/emboj.2011.327

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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miR-205-5p Mediated Downregulation of PTEN Contributes to Cisplatin Resistance in C13K Human Ovarian Cancer Cells

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OPEN ACCESS

Edited by:

Yujing Li,
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Reviewed by:

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equally to this work

Specialty section:

This article was submitted to
RNA,
a section of the journal
Frontiers in Genetics

Received: 02 May 2018

Accepted: 31 October 2018

Published: 19 November 2018

Citation:

Shi X, Xiao L, Mao X, He J,
Ding Y, Huang J, Peng C and Xu Z
(2018) miR-205-5p Mediated
Downregulation of PTEN Contributes
to Cisplatin Resistance in C13K
Human Ovarian Cancer Cells.
Front. Genet. 9:555.
doi: 10.3389/fgene.2018.00555

Cisplatin resistance is a major cause of treatment failure in advanced ovarian cancer. The limited evidence shows the paradoxical regulation of miR-205 on chemotherapy resistance in cancer. Herein, we found that miR-205-5p was enormously increased in cisplatin-resistant C13K ovarian cancer cells compared with its cisplatin-sensitive OV2008 parental cells using miRNA microarrays, which was further verified by quantitative PCR. Furthermore, we confirmed that inhibition of miR-205-5p upregulated PTEN and subsequently attenuated its downstream target p-AKT, which inversed C13K cells from cisplatin resistance to sensitivity. Our data suggest that miR-205-5p contributes to cisplatin resistance in C13K ovarian cancer cells may via targeting PTEN/AKT pathway.

Keywords: miR-205-5p, PTEN, cisplatin resistance, ovarian cancer, AKT

INTRODUCTION

Cisplatin is an anti-cancer chemotherapy drug which used to treat ovarian cancer in women who have already received surgery and/or radiation treatment. However, either resistance to cisplatin or multi-drug resistance (MDR) to cisplatin-centered chemotherapy is a major cause of treatment failure in human ovarian cancer. As we know, four well-characterized mechanisms have been suggested to account for cisplatin-resistance in cancer: (1) pre-target resistance – involving steps preceding the binding of cisplatin to DNA, (2) on-target resistance – directly related to DNA-cisplatin adducts, (3) post-target resistance – concerning the lethal signaling pathways elicited by cisplatin-mediated DNA damage, and (4) off-target resistance – affecting molecular circuitries that do not present obvious links with cisplatin-elicited signals (Galluzzi et al., 2012, 2014). Besides lots of genes involved, emerging evidences demonstrate that miRNAs such as miR-141 (Seki, 2011), miR-184 (Tung et al., 2016), miR-199a (Wang et al., 2013), miR-214 (Yang et al., 2008), and miR-421 (Ge et al., 2016) contribute to cisplatin-resistance in cancer. miR-205, a frequently silenced microRNA in cancer, has recently been implicated in chemotherapy resistance. De Cola et al. (2015) showed that upregulation of miR-205 led to lapatinib resistance in breast cancer stem cells via targeting ErbB/HER receptors. On the contrary, however, results from

Puhr et al. (2012), Bhatnagar et al. (2010), and Alla et al. (2012) suggested that downregulation of miR-205 contributed to docetaxel or cisplatin resistance in prostate cancer, and cisplatin resistance in melanoma cells. Therefore, more extensive and detailed studies are required to explore the role of miR-205 in drug resistance associated with cancer chemotherapy.

In present study, we measured the miRNA expression profiles of cisplatin-resistant C13K ovarian cancer cell line compared with its cisplatin-sensitive OV2008 parent cell line using miRNA microarrays, and found that miR-205-5p was enormously increased in cisplatin-resistant C13K ovarian cancer cells, which was further verified by quantitative real-time PCR (qPCR). Furthermore, we investigated the effects of miR-205-5p on cisplatin resistance in ovarian cancer cells and the underlying mechanism, and confirmed that inhibition of miR-205-5p upregulated PTEN and subsequently attenuated its downstream target p-AKT, which inversed C13K cells from cisplatin resistance to sensitivity. Our data suggest that miR-205-5p contributes to cisplatin resistance in C13K ovarian cancer cells via targeting PTEN/AKT pathway.

MATERIALS AND METHODS

Cell Culture and Transfection

The cisplatin-sensitive human ovarian cancer cell line OV2008 and its cisplatin-resistant clone C13K were supplied by Dr Wencheng Ding (Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology) (Asselin et al., 2001; Su et al., 2011). OV2008 cells were maintained in complete RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. C13K cells were cultured in RPMI1640 supplemented with 10% fetal bovine-serum medium containing 1 μmol/L cisplatin to maintain resistance. The cells were transfected with 100 pmol hsa-miR-205-5p mimics or hsa-miR-205-5p inhibitor using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, United States). The sequences for hsa-miR-205-5p are as follow: hsa-miR-205-5p mimics (5'-UCCUUAUCCACCGGAGUCUG-3', 5'-GACUCCGGUGGAAUGAAGGAUU-3'); hsa-miR-205-5p mimics negative control (NC) (5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUGACACGUUCGGAGAATT-3'); hsa-miR-205-5p inhibitor (5'-CAGACUCCGGUGGAAUGAAGGA-3'); mircoRNA inhibitor NC (5'-CAGUACUUUUGUGUAGUACAA-3').

Cytotoxicity Analysis

Cell cytotoxicity was analyzed with Cell Counting Kit-8 (CCK-8) assay kit (Sigma-Aldrich, St. Louis, MO, United States). Briefly, cells were seeded into flat-bottomed 96-well plates at a density of 8000 per well and incubated overnight. Cisplatin (Sigma-Aldrich, St. Louis, MO, United States) with concentrations of 0, 20, 40, 60, 80, 100 and 120 μM was added after adherence. Then cells were continuously cultured for 24 or 48 h followed by treatment with 10 ul of CCK-8 solution for additional 1h at 37°C, and

the absorbance (A) was measured at 450 nm by an Enspire microplate reader (Perkin Elmer, United States). Cell viability (%) = experimental group A value/control group A value × 100. IC50 values (50% inhibition of surviving fraction) were then estimated using the fitted dose-response curves for cell viability.

RNA Extraction and miRNA Microarray Analysis

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, United States). The RNA sample was purified with an RNeasy Mini Column (Qiagen, CA, United States) and the RNA quality was assessed by 1.0% agarose gel electrophoresis. miRNA-profiling was carried out by miRNA microarray using Affymetrix miRNA 4.0 and Affymetrix GeneChip (Affymetrix, United States). The random-variance model (RVM) F test was applied to filter differentially expressed genes for the two ovarian cancer cell lines. After the significance analysis and false discovery rate (FDR) analysis, differentially expressed genes were selected according to their *p*-value threshold.

qPCR

miRNAs expression was measured using TaqMan miRNA reverse transcription kit and TaqMan miRNA assay kits (Applied Biosystems, United States). Primer sequences for hsa-miR-205-5p: (5'-CCTTCATTCCACCGGAGT-3'; 5'-GTCCAGTTTTTTTTTTTTTCAGACT-3'). PTEN mRNA expression was measured using TaqMan mRNA reverse transcription kit and SYBR Green Supermix (BioRad, United States), primer sequences for PTEN: (5'-TGGATTGACTTAGACTTGACCT; 5'-TTTGGCGGTGTCATA ATGTCTT).

Cell Apoptosis Assay

Cell apoptosis was measured by Flow Cytometry. All samples were washed in phosphate-buffered saline and resuspended in 200 μl binding buffer. Next, 5 μl Annexin-V-fluorescein isothiocyanate and 10 μl propidium iodide (PI; 1 μg/ml) were added and the cell suspension was incubated in a dark chamber for 1h at room temperature. Cell apoptosis was then determined using a FACSCalibur flow cytometer (BD Biosciences, United States) and data were analyzed using CellQuest software (BD Biosciences, United States).

Western Blotting Analysis

Cells were harvested with ice-cold PBS and lysed in lysis buffer containing a protease inhibitor cocktail. A total of 60 μg protein was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Following blocking with Tris-buffered saline containing 5% skimmed milk for 1 h at room temperature, the membranes were incubated with the primary antibodies (anti-PTEN, anti-actin, anti-AKT, anti-phospho-AKT, Santa Cruz or Cell Signaling, United States) in blocking buffer overnight at 4°C. The membranes were washed three times in TBST and then incubated with horseradish peroxidase-conjugated anti-mouse/rabbit antibodies at a dilution of 1:3,000 for 1 h at room temperature. Signals were detected on X-ray film using an enhanced chemiluminescent

detection system (Pierce Biotechnology, Inc., Rockford, IL, United States).

Statistical Analysis

All experiments were repeated at least three times and the data were expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed with SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL, United States). Statistical significance was determined by the Student *t*-test or one-way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Differentially Expressed miRNAs in Cisplatin-Resistant Variant C13K Cells Versus Its Cisplatin-Sensitive OV2008 Parental Cells

C13K cells are cisplatin-resistant variant originating from its cisplatin-sensitive OV2008 parental cells (Su et al., 2011). We used miRNA microarray to determine miRNA expression profiles in both C13K and OV2008 cell lines. We have identified 113 miRNAs that were significantly differentially expressed (GEO accession number: GSE120256) in C13K cells, including 32 upregulated miRNAs (such as hsa-miR-205-5p, hsa-miR-200c-3p, hsa-miR-100-5p, hsa-miR-155-5p, and hsa-miR-125b-5p) and 81 down-regulated miRNAs (such as hsa-miR-214-3p, hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-199a-5p), compared to OV2008 cells (Figure 1A). Surprisingly, miR-205-5p was around 9000 fold changes among 32 up-regulated miRNAs, which was further verified by qPCR (Figure 1B).

To investigate the role of miR-205, target prediction using softwares such as miRanda, TargetScan and PicTar was performed (data in **Supplementary Data Sheets 1,2**). Several of the predicted targets are known to be involved in cancer progression (E2F1, E2F5, ERBB, PTEN), invasion

and metastasis (ZEB1/2, LRP-1). mirTarBase analysis was performed to further identify the experimentally validated targets of miR-205. These indicated that some but not all of these genes are aberrantly expressed in C13K cisplatin-resistant cells.

miR-205-5p Attenuated Cisplatin-Induced Cytotoxicity

We confirmed that the viability of C13K cells was significantly increased following treatment with cisplatin for 48 h compared to OV2008 cells, and the IC₅₀ values of cisplatin in C13K cells (107 μ mol/L) were higher than the corresponding values in OV2008 (37 μ mol/L), which indicated that C13K cells exhibited cisplatin resistance (Figures 2A–C).

To investigate whether miR-205-5p is associated with cisplatin-induced cytotoxicity in ovarian cancer cells, we examined the cell viability of C13K and OV2008 cells transfected with miR-205-5p inhibitor or mimics following cisplatin treatment for 48 h. We observed that both cell viability and IC₅₀ in C13K decreased upon downregulation of miR-205-5p (Figures 2D–F), whereas both of them in OV2008 elevated upon upregulation of miR-205-5p (Figures 2G–I). These data suggested that miR-205-5p attenuated cisplatin-induced cytotoxicity and enhanced cisplatin resistance in ovarian cancer cells.

miR-205-5p Inhibits Cisplatin-Induced Apoptosis

To explore whether miR-205-5p accounts for cisplatin-induced apoptosis in ovarian cancer cells, we measured cell apoptosis in both C13K and OV2008 cells transfected with miR-205-5p inhibitor or mimics following cisplatin treatment for 48 h. We found that C13K cells apoptosis increased upon downregulation of miR-205-5p (Figures 3A,B), whereas OV2008

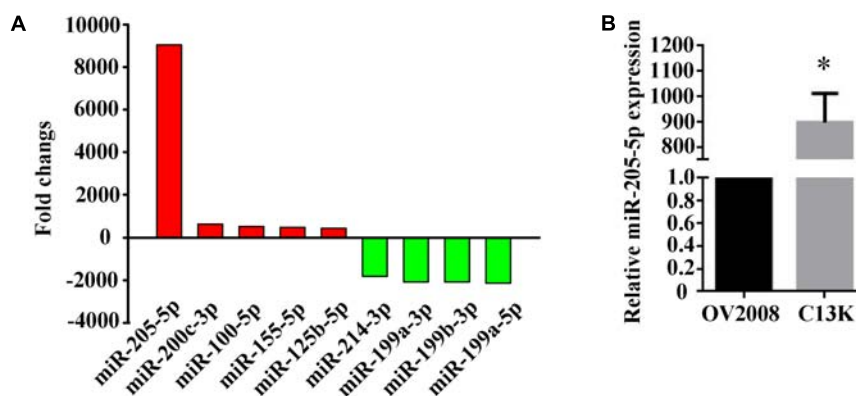


FIGURE 1 | Elevated expression of miR-205-5p in cisplatin-resistant C13K cells compared with its cisplatin-sensitive OV2008 parental cells. **(A)** 9 representative differentially expressed miRNAs with statistically significant fold changes in C13K cells vs. OV2008 cells. Upregulated miRNAs (red bars) are shown above the x-axis, whereas downregulated miRNAs (green bars) below the x-axis. **(B)** qPCR validation of the differential expression of miR-205-5p in C13K cells vs. OV2008 cells. The data represent mean $\pm$ SD of three independent experiments (* $P < 0.01$).

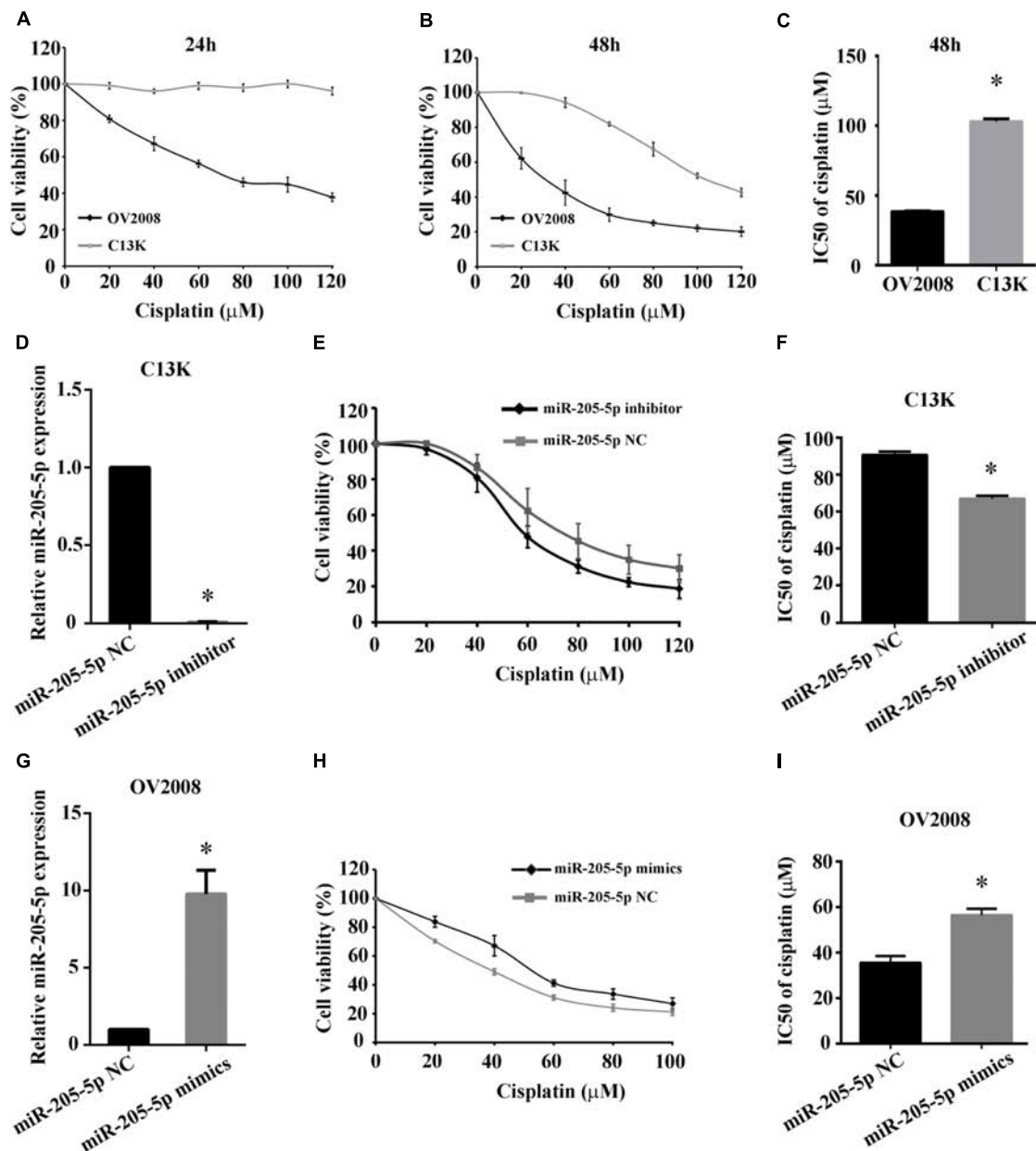


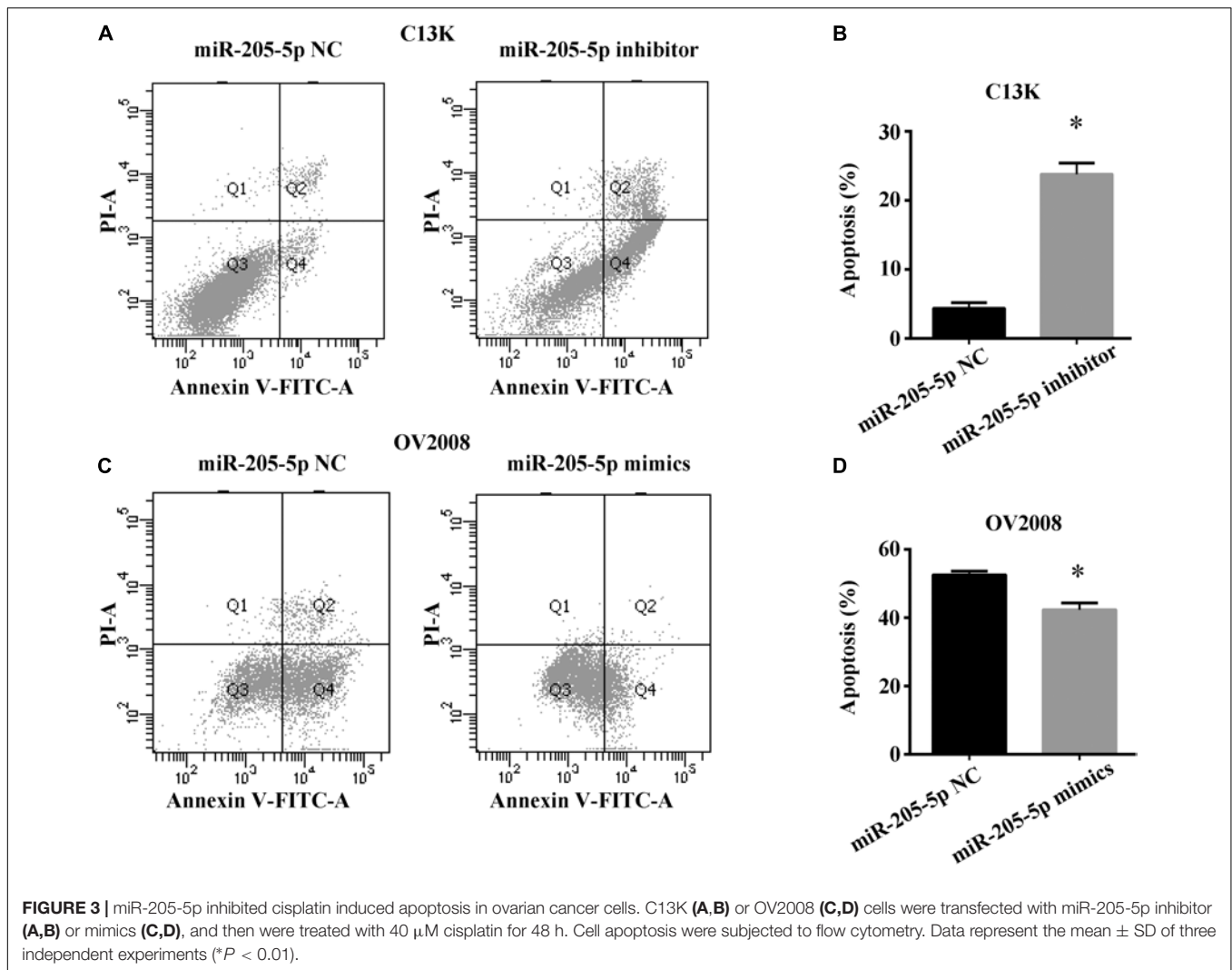
FIGURE 2 | Overexpression of miR-205-5p reduced cisplatin-induced cytotoxicity in ovarian cancer cells. C13K and OV2008 cells were treated with cisplatin for 24 h (A) or 48 h (B) and then were assessed for cell viability by CCK-8 assay. IC50 values at 48 h were estimated using the fitted dose-response curves for cell viability (C). Cells transfected with miR-205-5p inhibitor (D–F) or mimics (G–I) were treated with 40 μM cisplatin for 48 h. Cell viability was assessed by CCK-8 assay. miR-205-5p expression was measured by qPCR in C13K cells (D–F) or OV2008 cells (G–I). The data represent mean ± SD of three independent experiments (* $P < 0.01$).

cells apoptosis reduced upon upregulation of miR-205-5p (Figures 3C,D).

miR-205-5p Contributes to Cisplatin-Resistance in Ovarian Cancer Cells via Targeting PTEN/AKT Pathway

Given the involvement of PTEN in chemotherapy resistance including cisplatin in cancer (Lee et al., 2005; Juric et al., 2015),

it is important to reveal whether PTEN, one of miR-205 target genes, is linked to miR-205 upregulation in cisplatin-resistant ovarian cancer cells. We examined PTEN mRNA and protein expression by using qPCR and Western blotting. Our results showed remarkable lower expression of PTEN mRNA and protein in C13K cells compared to that in OV2008 (Figures 4A,B). We further measured the expression of PTEN and AKT in C13K and OV2008 cells transfected with miR-205-5p inhibitor



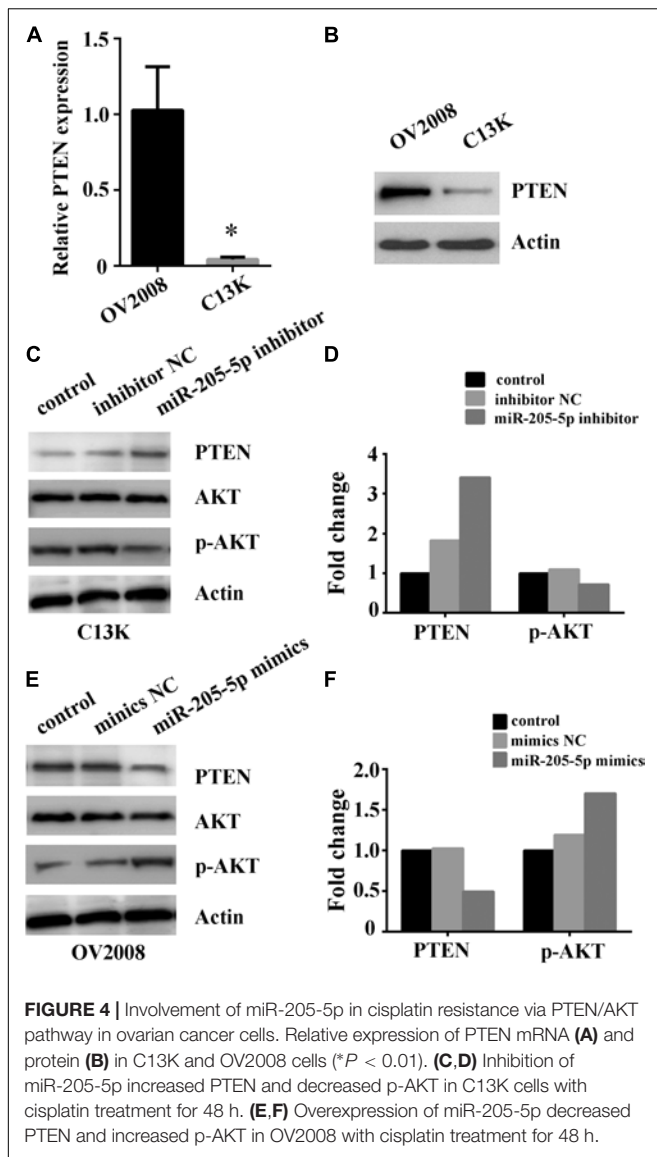
or mimics following cisplatin treatment for 48 h. We found that PTEN increased and phospho-AKT (p-AKT) decreased in C13K upon downregulation of miR-205-5p (Figures 4C,D). On the contrary, PTEN decreased and p-AKT increased in OV208 upon upregulation of miR-205-5p (Figures 4E,F).

DISCUSSION

In the last few years, drug resistance in various cancers has been linked with aberrant expression of miRNAs, suggesting that miRNAs might play important roles in chemoresistance and chemosensitivity (Koster et al., 2013). Although resistance to current chemotherapeutics represents a significant barrier to the improvement of the long-term overcome of patients with ovarian cancer, chemotherapy is still one of the most effective therapies for ovarian cancer patients. Although altered miRNAs are differentially expressed in ovarian cancer, the potential role of miRNAs in the induction of drug resistance, particularly in

platinum resistance, has not been fully investigated. Growing evidences demonstrate that more and more miRNAs contribute to cisplatin-resistance in cancer (Yang et al., 2008; Wang et al., 2013; Ge et al., 2016; Tung et al., 2016). In this study, we identified 113 miRNAs which were significantly differentially expressed, including 32 upregulated miRNAs and 81 down-regulated miRNAs. Most of them (such as miR-205, miR-200c, miR-100, miR-155, miR-125b, miR-214, miR-199a, miR-199b and miR-199a) are involved in cell differentiation, proliferation and apoptosis, and cancer development, progression and metastasis.

In present report, we suggests that upregulation of miR-205-5p contributes to cisplatin resistance in C13K ovarian cancer cells. However, evidences showed the paradoxical regulation of miR-205 on chemotherapy resistance in various cancers. Specifically, increased miR-205 level and paclitaxel resistance were noted in CD133+ ovarian cancer stem cells compared to adherent OVCAR3 cells (Nam et al., 2012). Additionally, increased miR-205 level was correlated with high proliferation, invasion and migration rates, enhanced resistance



to carboplatin both in lung cancer A549 cells and H1975 cells by inhibiting cellular apoptosis and augmenting cancer cell survival (Zarogoulidis et al., 2015). However, downregulation of miR-205 was noted in pancreatic cancer cell lines resistant to gemcitabine (Bera et al., 2014) and prostate cancer cell lines resistant to docetaxel (Puhr et al., 2012). Overexpression of miR-205 resensitized drug-resistant cancer cells and acted as a tumor suppressor miRNA (Puhr et al., 2012; Mittal et al., 2014; Ippolito et al., 2016; Chaudhary et al., 2017). As we know, miR-205 is involved in both physiological and pathological processes including cell proliferation, apoptosis, angiogenesis and mesenchymal transition by targeting either oncogenes or tumor suppressor genes such as PHLPP-2, CTGF, CYR61, ESRRGr, PTEN, ErbB3, E2F1, E2F5, Zeb1&2, HBx, PCNA, Her 2&3, VEGF-A, Bcl2, etc (Vosgha et al., 2014), which results in “dual roles” of cell regulation. Nevertheless, more extensive and detailed studies are required to explore the

dual role of miR-205 in drug resistance associated with cancer chemotherapy.

The tumor suppressor PTEN, one of miR-205 target genes, plays an essential role in cancer development via modulating cell cycle progression (Qu et al., 2012; Cai et al., 2013). It was found that PTEN deletion in malignant gliomas, endometrial cancer, melanomas, breast cancer and other tumors, indicating that the PTEN gene has an important function in suppressing transformation to a malignant tumor (Habib et al., 2011). PTEN levels were significantly decreased in progesterone-resistant endometrial cancer Ishikawa cells and that the miR-205 inhibitor increased PTEN expression in these cells (Zhuo and Yu, 2017). Besides, PTEN acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which results in inhibition of AKT signaling pathway (Cantley and Neel, 1999). It has been implicated that PTEN is involved in chemotherapy resistance including cisplatin in breast cancer, prostate cancer, melanoma, and EMT (Lee et al., 2005; Juric et al., 2015). Therefore, we speculated that miR-205 confers cisplatin resistance upon ovarian cancer cells through targeting PTEN. In this study, we confirmed that inhibition of miR-205-5p upregulated PTEN expression and subsequently attenuated its downstream target p-AKT, which inverted C13K cells from cisplatin resistance to sensitivity. Contrarily, overexpression of miR-205-5p downregulated PTEN expression and subsequently increased p-AKT level, which turned OV2008 cells from cisplatin sensitivity into resistance.

Our findings suggest that miR-205 plays an important role in the loss of PTEN expression and the development of cisplatin-resistant in ovarian cancer cells. Nevertheless, this research has limitation. All the data was based on cisplatin-resistant C13K ovarian cancer cells and its cisplatin-sensitive OV2008 parental cells. Therefore, we should note that differential expression patterns of miRNAs in C13K and OV2008 cells in this study are not applicable for ovarian cancer in general. More ovarian cancer cell lines need be checked by modulating miR-205 level and drug resistance. However, it was found that miR-205 regulated the proliferation and invasion of ovarian cancer OVCAR-3 cell line via suppressing PTEN/SMAD4 expression (Chu et al., 2018). miR-205 level was increased in ovarian cancer and it promoted the invasive behavior of ovarian cancer cell lines (OVCAR-5, OVCAR-8, and SKOV-3) (Wei et al., 2017). These studies support our conclusion, although the roles of miR-205 in the induction of drug resistance has not been investigated.

CONCLUSION

Our data suggest that miR-205-5p mediates downregulation of PTEN and upregulation of p-AKT, which contributes to cisplatin-resistance in C13K human ovarian cancer cells. Biological or pharmacological intervention based on miR-205-5p may be a new promising strategy to inverse the cisplatin resistance in human ovarian cancer cell.

AUTHOR CONTRIBUTIONS

ZX and CP conceived and designed the experiments and explained the data. XS, LX, and XM performed the experiment. ZX, CP, XS, LX, and XM analyzed the content of the data with the help of JH, YD, and JH. XS, LX, and XM wrote the manuscript with the help of ZX and CP.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (81301084 and 31400916),

the Doctoral Program of Higher Education of China (20130142120057) and the Bureau of Science and Technology of Wuhan Municipality (20150601010 10058).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00555/full#supplementary-material>

DATAT SHEET S2 | miR-205 targets prediction using softwares such as miRanda, TargetScan, PicTar and mirTarBase analysis.

REFERENCES

- Alla, V., Kowtharapu, B. S., Engelmann, D., Emmrich, S., Schmitz, U., Steder, M., et al. (2012). E2F1 confers anticancer drug resistance by targeting ABC transporter family members and Bcl-2 via the p73/DNp73-miR-205 circuitry. *Cell Cycle* 11, 3067–3078. doi: 10.4161/cc.21476
- Asselin, E., Mills, G. B., and Tsang, B. K. (2001). XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res.* 61, 1862–1868.
- Bera, A., VenkataSubbaRao, K., Manoharan, M. S., Hill, P., and Freeman, J. W. (2014). A miRNA signature of chemoresistant mesenchymal phenotype identifies novel molecular targets associated with advanced pancreatic cancer. *PLoS One* 9:e106343. doi: 10.1371/journal.pone.0106343
- Bhatnagar, N., Li, X., Padi, S. K., Zhang, Q., Tang, M. S., and Guo, B. (2010). Downregulation of miR-205 and miR-31 confers resistance to chemotherapy-induced apoptosis in prostate cancer cells. *Cell Death Dis.* 1:e105. doi: 10.1038/cddis.2010.85
- Cai, J., Fang, L., Huang, Y., Li, R., Yuan, J., Yang, Y., et al. (2013). miR-205 targets PTEN and PHLPP2 to augment AKT signaling and drive malignant phenotypes in non-small cell lung cancer. *Cancer Res.* 73, 5402–5415. doi: 10.1158/0008-5472.can-13-0297
- Cantley, L. C., and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4240–4245.
- Chaudhary, A. K., Mondal, G., Kumar, V., Kattel, K., and Mahato, R. I. (2017). Chemosensitization and inhibition of pancreatic cancer stem cell proliferation by overexpression of microRNA-205. *Cancer Lett.* 402, 1–8. doi: 10.1016/j.canlet.2017.05.007
- Chu, P., Liang, A., Jiang, A., and Zong, L. (2018). miR-205 regulates the proliferation and invasion of ovarian cancer cells via suppressing PTEN/SMAD4 expression. *Oncol. Lett.* 15, 7571–7578. doi: 10.3892/ol.2018.8313
- De Cola, A., Volpe, S., Budani, M. C., Ferracin, M., Lattanzio, R., Turdo, A., et al. (2015). miR-205-5p-mediated downregulation of ErbB/HER receptors in breast cancer stem cells results in targeted therapy resistance. *Cell Death Dis.* 6:e1823. doi: 10.1038/cddis.2015.192
- Galluzzi, L., Senovilla, L., Vitale, I., Michels, J., Martins, I., Kepp, O., et al. (2012). Molecular mechanisms of cisplatin resistance. *Oncogene* 31, 1869–1883. doi: 10.1038/onc.2011.384
- Galluzzi, L., Vitale, I., Michels, J., Brenner, C., Szabadkai, G., Harel-Bellan, A., et al. (2014). Systems biology of cisplatin resistance: past, present and future. *Cell Death Dis.* 5:e1257. doi: 10.1038/cddis.2013.428
- Ge, X., Liu, X., Lin, F., Li, P., Liu, K., Geng, R., et al. (2016). MicroRNA-421 regulated by HIF-1 α promotes metastasis, inhibits apoptosis, and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer. *Oncotarget* 7, 24466–24482. doi: 10.18632/oncotarget.8228
- Habib, S. L., Yadav, A., Mahimainathan, L., and Valente, A. J. (2011). Regulation of PI 3-K, PTEN, p53, and mTOR in malignant and benign tumors deficient in tuberlin. *Genes Cancer* 2, 1051–1060. doi: 10.1177/1947601912445376
- Ippolito, L., Marini, A., Cavallini, L., Morandi, A., Pietrovito, L., Pintus, G., et al. (2016). Metabolic shift toward oxidative phosphorylation in docetaxel resistant prostate cancer cells. *Oncotarget* 7, 61890–61904. doi: 10.18632/oncotarget.11301
- Juric, D., Castel, P., Griffith, M., Griffith, O. L., Won, H. H., Ellis, H., et al. (2015). Convergent loss of PTEN leads to clinical resistance to a PI(3)K α inhibitor. *Nature* 518, 240–244. doi: 10.1038/nature13948
- Koster, R., van Vugt, M. A., Timmer-Bosscha, H., Gietema, J. A., and de Jong, S. (2013). Unravelling mechanisms of cisplatin sensitivity and resistance in testicular cancer. *Expert Rev. Mol. Med.* 15:e12. doi: 10.1017/erm.2013.13
- Lee, S., Choi, E. J., Jin, C., and Kim, D. H. (2005). Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. *Gynecol. Oncol.* 97, 26–34. doi: 10.1016/j.ygyno.2004.11.051
- Mittal, A., Chitkara, D., Behrman, S. W., and Mahato, R. I. (2014). Efficacy of gemcitabine conjugated and miRNA-205 complexed micelles for treatment of advanced pancreatic cancer. *Biomaterials* 35, 7077–7087. doi: 10.1016/j.biomaterials.2014.04.053
- Nam, E. J., Lee, M., Yim, G. W., Kim, J. H., Kim, S., Kim, S. W., et al. (2012). MicroRNA profiling of a CD133(+) spheroid-forming subpopulation of the OVCAR3 human ovarian cancer cell line. *BMC Med. Genomics* 5:18. doi: 10.1186/1755-8794-5-18
- Puhr, M., Hoefer, J., Schafer, G., Erb, H. H., Oh, S. J., Klocker, H., et al. (2012). Epithelial-to-mesenchymal transition leads to docetaxel resistance in prostate cancer and is mediated by reduced expression of miR-200c and miR-205. *Am. J. Pathol.* 181, 2188–2201. doi: 10.1016/j.ajpath.2012.08.011
- Qu, C., Liang, Z., Huang, J., Zhao, R., Su, C., Wang, S., et al. (2012). MiR-205 determines the radioresistance of human nasopharyngeal carcinoma by directly targeting PTEN. *Cell Cycle* 11, 785–796. doi: 10.4161/cc.11.4.19228
- Seki, N. (2011). A commentary on MicroRNA-141 confers resistance to cisplatin-induced apoptosis by targeting YAP1 in human esophageal squamous cell carcinoma. *J. Hum. Genet.* 56, 339–340. doi: 10.1038/jhg.2011.26
- Su, W., Huang, L., Ao, Q., Zhang, Q., Tian, X., Fang, Y., et al. (2011). Noscapine sensitizes chemoresistant ovarian cancer cells to cisplatin through inhibition of HIF-1 α . *Cancer Lett.* 305, 94–99. doi: 10.1016/j.canlet.2011.02.031
- Tung, M. C., Lin, P. L., Cheng, Y. W., Wu, D. W., Yeh, S. D., Chen, C. Y., et al. (2016). Reduction of microRNA-184 by E6 oncoprotein confers cisplatin resistance in lung cancer via increasing Bcl-2. *Oncotarget* 7, 32362–32374. doi: 10.18632/oncotarget.8708
- Vosgha, H., Salajegheh, A., Smith, R. A., and Lam, A. K. (2014). The important roles of miR-205 in normal physiology, cancers and as a potential therapeutic target. *Curr. Cancer Drug Targets* 14, 621–637.
- Wang, Z., Ting, Z., Li, Y., Chen, G., Lu, Y., and Hao, X. (2013). microRNA-199a is able to reverse cisplatin resistance in human ovarian cancer cells through the inhibition of mammalian target of rapamycin. *Oncol. Lett.* 6, 789–794. doi: 10.3892/ol.2013.1448
- Wei, J., Zhang, L., Li, J., Zhu, S., Tai, M., Mason, C. W., et al. (2017). MicroRNA-205 promotes cell invasion by repressing TCF21 in human ovarian cancer. *J. Ovarian Res.* 10:33. doi: 10.1186/s13048-017-0328-1

- Yang, H., Kong, W., He, L., Zhao, J. J., O'Donnell, J. D., Wang, J., et al. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res.* 68, 425–433. doi: 10.1158/0008-5472.can-07-2488
- Zarogoulidis, P., Petanidis, S., Kioseoglou, E., Domvri, K., Anastakis, D., and Zarogoulidis, K. (2015). MiR-205 and miR-218 expression is associated with carboplatin chemoresistance and regulation of apoptosis via Mcl-1 and survivin in lung cancer cells. *Cell. Signal.* 27, 1576–1588. doi: 10.1016/j.cellsig.2015.04.009
- Zhuo, Z., and Yu, H. (2017). miR-205 inhibits cell growth by targeting AKT-mTOR signaling in progesterone-resistant endometrial cancer ishikawa cells. *Oncotarget* 8, 28042–28051. doi: 10.18632/oncotarget.15886

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewers SK, FW, and handling Editor declared their shared affiliation at the time of review.

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