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lncRNAs in cancer metastasis and therapy resistance Volume II

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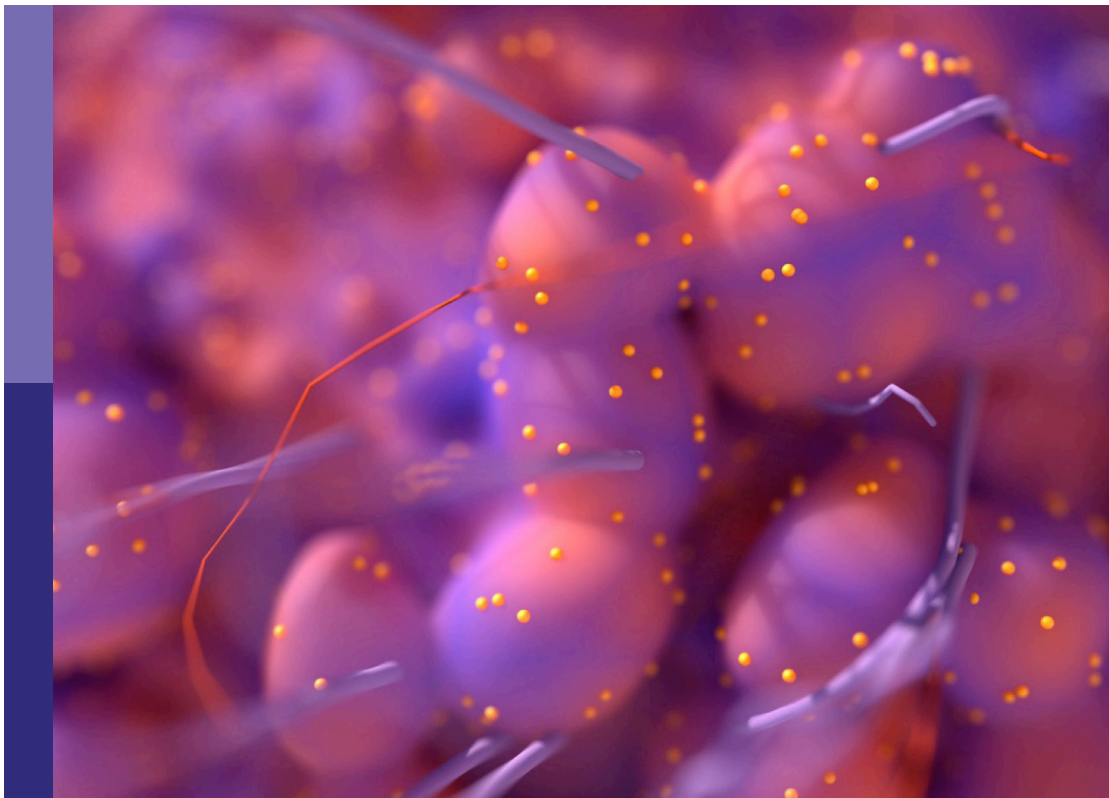

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lncRNAs in cancer metastasis and therapy resistance Volume II

Topic editors

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Table of contents

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HOTTIP Mediated Therapy Resistance in Glioma Cells Involves Regulation of EMT-Related miR-10b

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The advanced grade glioblastomas are characterized by dismal five-year survival rates and are associated with worse outcomes. Additionally, resistance to therapies is an additional burden responsible for glioma associated mortality. We studied the resistance against temozolomide (TMZ) as a surrogate to understand the mechanism of therapy resistance in glioma cancer cells. Screening of three glioma cells lines, A172, LN229 and SF268 revealed that SF268 glioma cells were particularly resistant to TMZ with the IC-50 of this cell line for TMZ ten times higher than for the other two cell lines. A role of lncRNAs in glioma progression has been identified in recent years and, therefore, we focused on lncRNAs for their role in regulating TMZ resistance in glioma cancer cells. lncRNA HOTTIP was found to be particularly elevated in SF268 cells and over-expression of HOTTIP in both A172 and LN229 remarkably increased their TMZ IC-50s, along with increased cell proliferation, migration, clonogenicity and markers of angiogenesis and metastasis. As a mechanism we observed increased expression of miRNA-10b and mesenchymal markers Zeb1/Zeb2 and reduced expression of E-cadherin in SF268 cells indicating a role of EMT in TMZ resistance. A172 and LN229 cells with overexpressed HOTTIP also had similarly induced EMT and the elevated miR-10b levels. Further, silencing of miR-10b in HOTTIP overexpressing cells as well as the SF268 cells reversed EMT with associated sensitization of all the tested cells to TMZ. Our results thus present a case for HOTTIP in native as well as acquired resistance of glioma cells against chemotherapy, with a key mechanistic role of EMT and the miR-10b. Thus, HOTTIP as well as miR-10b are critical targets for glioma therapy, and need to be tested further.

Keywords: glioma, HOTTIP, miR-10b, temozolomide, EMT

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INTRODUCTION

Glioma is a brain tumor which is fairly common and represents about one-thirds of all brain tumors. Less than a quarter of glioma patients survive for more than five years and the median survival is less than 2 years (1). It is an aggressive cancer in adults and largely considered incurable (2). The clinical management of glioma patients involves surgical resection, if possible, followed by temozolomide (TMZ) together with radiotherapy and finally adjuvant TMZ (1). This underlines the importance of TMZ in clinical management of glioma patients, particularly in view of the use of

TMZ for almost two decades (3). Despite this importance of TMZ in glioma, the resistance against this drug is fairly common with almost half of the patients failing to respond to it (4). Even with the relative wealth of information on possible mechanisms that can lead to TMZ resistance, including epigenetic ones that include aberrations in DNA methyltransferase, the topic remains poorly understood (5). In view of the high mortality cause by therapy resistance, it is important to better understand the overall mechanism of TMZ resistance in gliomas.

LncRNAs are increasingly being investigated for their role in diagnosis, prognosis and treatment of cancers (6), including gliomas (7). They are also being investigated for possible role in determining resistance against therapies (8, 9), including in gliomas (8, 10). In view of this knowledge, we focused our study to understand the lncRNA mediated TMZ resistance in glioma. For the model system, we hypothesized that the glioma cells with endogenous resistance against TMZ might be the best models, relative to TMZ-sensitive glioma cells. Thus, we screened a number of cell lines in order to find the appropriate working model. Our study identified lncRNA HOTTIP as the lncRNA of interest. Previously, lncRNA HOTTIP has been shown to mediate hypoxia-induced EMT (epithelial-mesenchymal transition) in glioma cells U87 and U251 (11), thus increasing confidence in our work. We further elucidated the mechanism of TMZ resistance by looking at underlying mechanism and confirmed EMT induction that also involved microRNA-10b (miR-10b).

MATERIALS AND METHODS

Cell Lines and Culture

A172 (ATCC: CRL-1620), LN229 (ATCC: CRL-2611) and SF268 glioma cell lines were all purchased from ATCC (Virginia, USA) and regularly screed for mycoplasma in the laboratory. All of these cell lines were cultured in DMEM culture medium with added FBS at 10% final concentration and added antibiotics penicillin and streptomycin at 1% final concentrations (Life Technologies, China). Cells were cultured in certified incubators at 37^0C under humidified conditions and 5% CO_2.

TMZ and Dilutions

TMZ was purchased from Sigma Chemical Company (China). Stock of TMZ was prepared in DMSO at a concentration of 20mg/ml and further dissolved in culture medium, as needed for individual assays. TMZ treatment was done for a 3 day cycle as also described earlier by Perazzoli and co-workers (12).

HOTTIP Transfections

We transfected full-length HOTTIP into pcDNA3.1 vector (GenePharma, Shanghai, China) and the control plasmid without HOTTIP was used as a negative control, similar to the method described elsewhere (13). HOTTIP was transfected into the glioma cells, using Lipofectamine 3000 (Life Technologies, China), as per the instructions supplied by the company for the transfection reagent.

MTT Assay

For proliferation assay, we conducted MTT assays. Cells were seeded in 96-well plates at a density of 3500 cells/well. Once the indicated assays were done, 20 μL reconstituted MTT reagent (5 mg/mL) was added to all wells for 2 hours. Then the wells were emptied and filled with 100 μL DMSO. The optical density (OD) was measured at 570 nm using a Shimadzu colorimeter (Japan).

Migration Assay

Migration assays were performed using QCM cell migration assay kits (Millipore, China), which assess the potential of cells to migrate colorimetrically, and as described in an earlier publication (14). Glioma cancer cells were seeded into the upper chamber with media that did not contain FBS. This initiated migration towards the lower chamber which was filled with complete media that contained 10% FBS. The cells that migrated were stained with crystal violet and absorbance was read using a Shimadzu colorimeter (Japan).

ELISA Assays

Angiogenesis and metastasis potentials were assessed by quantitating VEGF and MMP-9, respectively, in the supernatants of the glioma cell cultures, using quantitative ELISA kits (Sigma, China). Exact protocol recommended by the manufacturer was followed and the absorbance at 450nm was read using a Shimadzu colorimeter (Japan).

lncRNA and miRNA Detection

Total RNA was extracted using Trizol reagent (Life Technologies, China). lncRNAs as well as miRNAs (using Taqman primer-probes) were quantitated using commercially available reagents from ThermoFisher Scientific (USA). This included reagents needed for all lncRNAs, miRNAs and the controls.

Anti-Pre-miRNA Transfections

Anti-miR-10b was purchased from ThermoFisher Scientific (USA) and transfected in cells using G-fectin (Genolution, South Korea). The protocol of manufacturer was used without any modification to accomplish successful transfection of miRNA.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the cells using Trizol reagent (Life technologies, China). cDNA was prepared by reverse transcription using 1 μg RNA. We sued Prime Script™ RT Master Mix (Takara Bio, Japan) for cDNA preparation. The mRNAs of EMT related genes were amplified using SYBR® Premix Kits (Takara Bio, Japan) and quantitated using CFX96™ real-time machine (Bio-Rad, China). GAPDH was used as internal control for mRNA quantitation.

Statistical Analysis

All of the statistical evaluations were carried out using SPSS statistical software (SPSS Inc., Chicago, IL, USA). The values of $p < 0.05$ were considered significant. The experiments were

repeated at least three time with atleast duplicate samples in each run. Student's t-test was used for sample comparisons.

RESULTS

TMZ Resistance Glioma Models

We started our study by looking for the most appropriate model system to investigate TMZ resistance. We screened a number of glioma cell lines and finally shortlisted three, viz. A172, LN229 and SF268 glioma cell lines. These three cell lines were selected because of their sensitivity/resistance to TMZ. MTT assays revealed that the two cell lines A172 and LN229 were relatively sensitive to TMZ (**Figure 1A**) while the third cell line SF268 was relatively resistant to TMZ (**Figure 1B**). The IC-50 values of the three cell lines were 13.7 ± 1.2 μM, 14.4 ± 1.0 μM and 155.1 ± 1.7 μM for A172, LN229 and SF268 respectively (**Table 1**). Thus, SF268 cells were quite resistant to TMZ as their IC-50 for TMZ was more than ten-times that of both A172 and LN229 cells.

Next, we confirmed the TMZ resistance nature of SF268 cells by carrying out a number of assays that are determinants of cancer aggressiveness. We chose a dose of TMZ that was little higher than the IC-50 of these cells i.e. 160 μM and then tested a few different parameters, viz. migration, angiogenesis and metastasis. Migration was performed using boyden chamber assay and we found that the migration was reduced by more than half in SF268 cells by the 160 μM dose (**Figure 1C**). Angiogenesis was measured by quantitating the release of biomarker VEGF by ELISA and we found reduction in release of VEGF by more than half when SF268 cells were treated with 160 μM TMZ (**Figure 1D**). Metastasis was measured by quantitating the release of MMP-9 by ELISA and our assay revealed that the used TMZ dose resulted in significantly reduced MMP-9 secretion (reduced by more than half) (**Figure 1E**). Thus, these experiments confirmed that a dose of 160 μM TMZ

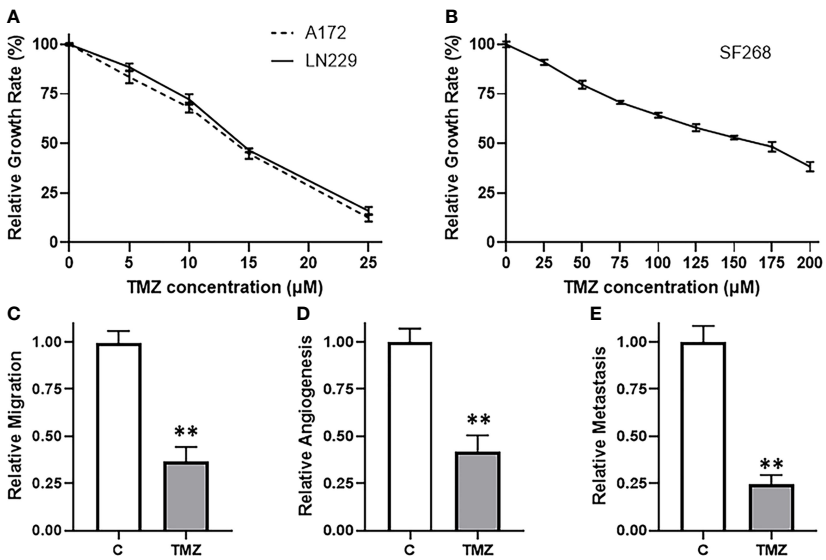

FIGURE 1 | SF268 are resistant to TMZ. MTT assays were performed to test the sensitivity of **(A)** A172 and LN229 and **(B)** SF268 glioma cells against TMZ after 3 day cycle. Vehicle treated control vs. 160μM TMZ treated SF268 cells were subjected to assays for migration **(C)**, VEGF secretion **(D)** and MMP-9 secretion **(E)**. VEGF and MMP-9 were detected by ELISA as surrogates for angiogenesis and metastasis, respectively. The values of controls were regarded as '1' and relative values of TMZ-treated SF268 cells are reported. C: control, TMZ: TMZ-treated SF268 cells **p < 0.01.

TABLE 1 | IC-50 values of glioma cells against Temozolomide.

Cell Line	Condition	IC-50 (μM)
SF268	Native	155.1 ± 1.7
	+i10b	100.1 ± 1.2
A172	Native	13.7 ± 1.2
	+HOTTIP	54.6 ± 1.5
	+H+i10b	26.3 ± 0.7
LN229	Native	14.4 ± 1.0
	+HOTTIP	63.8 ± 1.8
	+H+i10b	25.0 ± 0.9

+ i10b: silenced for miR-10b, +HOTTIP: transfected with HOTTIP, +H+ i10b: Transfected with HOTTIP and silenced for miR-10b.

was more than IC-50 and significantly impacted the various parameters that lead to TMZ-resistance associated mortality.

HOTTIP Is Elevated in TMZ Resistant Cells

Since our aim was to find a potential lncRNA that can mediate TMZ resistance in glioma cells, we compared TMZ-sensitive A172 and TMZ-resistant SF268 cells for their expression of several lncRNAs. Our screening revealed that a number of lncRNAs, such as HOTTIP, H19, LINC00152, SUMO1P3, LINC01116 and AGAP2-AS1 were significantly overexpressed in SF268 cells, relative to A172 cells (**Figure 2**), indicating their role in mediating TMZ resistance. While AGAP2-AS1, LINC01116 and SUMO1P3 were less than doubled, lncRNAs LINC00152, H19 and HOTTIP were elevated many folds. Of these, HOTTIP was found to be particularly elevated with its levels increased more than nine-folds in TMZ-resistant SF268 cells.

HOTTIP Induced Changes in TMZ Sensitive Cells

With the observation that lncRNA HOTTIP was relatively highly expressed in TMZ resistant SF268 cells, we hypothesized that HOTTIP was involved in determining the resistance of SF268 against TMZ. To test this hypothesis, we transfected HOTTIP in the otherwise TMZ sensitive glioma cells and tested the various cancer parameters that are connected with therapy resistance. First, we transfected A172 cells with HOTTIP and evaluated the resulting effect on cell growth/proliferation, migration, angiogenesis and metastasis. Transfection of HOTTIP into A172 cells, significantly increased their proliferation, migration as well as the secretion of VEGF and MMP-9 (**Figure 3A**). To further confirm our results, we transfected the other TMZ sensitive cells LN229 with HOTTIP as well and evaluated the same parameters. As shown in **Figure 3B**, HOTTIP transfection significantly increased the proliferation, migration and the release of VEGF and MMP-9 from LN229 cells as well. Thus, our results established a role of HOTTIP in inducing several parameters in glioma cells that can impact resistance against therapy.

miR-10b Is Also Elevated in TMZ Resistant Cells

The wealth of literature on lncRNAs in cancer has taught us that their functions involve regulation of miRNAs (15, 16). Therefore, our next task was to find a miRNA that is relevant to TMZ resistance in our glioma models. For this, we again compared TMZ-sensitive A172 and TMZ-resistant SF268 cells, this time for their expression of miRNAs. We found a number of miRNAs that were significantly different between the two cell lines and the top ones are presented in **Figure 4**. miR-10b stood out as the most overexpressed miRNA in the TMZ resistant SF268 cells with its expression more than ten-folds, compared to A172 cells. A few other miRNAs were also increased in SF268 cells which included miR-21 and miR-221. A number of other miRNAs, on the other hand, were significantly reduced in TMZ resistant SF268 cells and these included miR-125b, miR148a, miR-216a, miR-615 and miR-744. No other miRNAs was significantly changed in resistant cells as the miR-10b and, therefore, we chose this miRNA for further involvement and mechanism-based studies.

EMT Is Induced in TMZ Resistant Cells

Among many potential mechanisms that can play a part in resistance against therapy, EMT is a promising one. Therefore, we next checked for the possible involvement of EMT in the induction of TMZ resistance in glioma cells. When we compared the gene expression of various EMT markers, viz. ZEB1, ZEB2 and e-cadherin in resistant vs sensitive cells, we found that mesenchymal markers ZEB1 and ZEB2 were significantly elevated whereas the epithelial marker E-cadherin was significantly decreased in resistant SF268 cells (**Figure 5A**). We also checked if HOTTIP overexpression in sensitive cells could impact miR-10b levels, and found that overexpression of HOTTIP in both A172 and

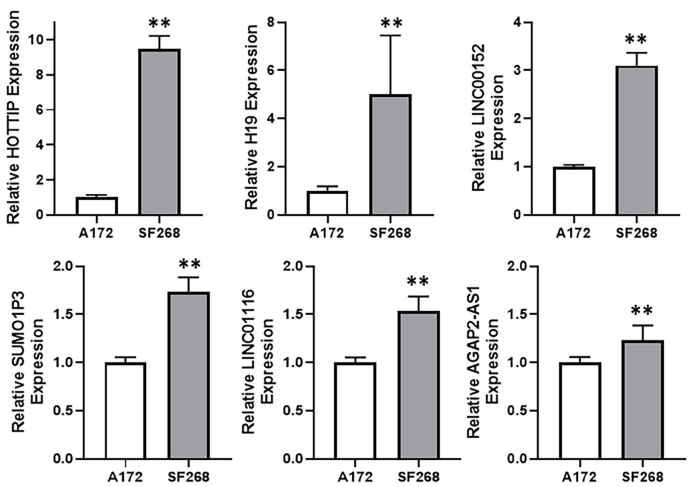

FIGURE 2 | HOTTIP is elevated in TMZ resistant cells. Expression levels of different lncRNAs were evaluated in A172 vs. SF268 cells, by RT-PCR. The values in A172 cells were regarded as '1' and relative values in SF268 cells are reported. **p < 0.01.

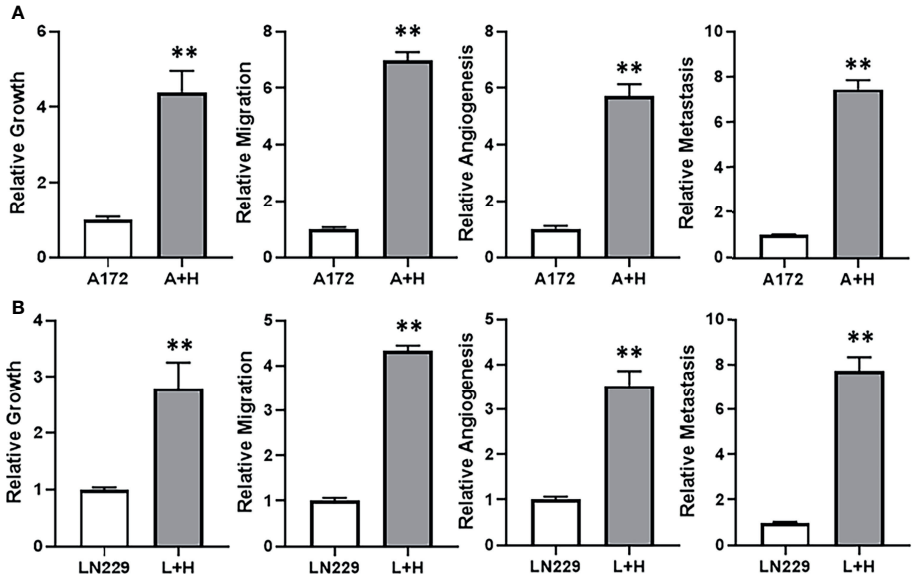

FIGURE 3 | HOTTIP impacts cellular proliferation, migration and markers of angiogenesis and metastasis. A172 **(A)** and LN229 **(B)** cells were transfected with lncRNA HOTTIP and cell growth (by MTT), migration, angiogenesis (VEGF secretion by ELISA) and metastasis (MMP-9 secretion by ELISA) were measured. A+H: A172 cells with HOTTIP, L+H:LN229 cells with HOTTIP. **p < 0.01.

LN229 cells significantly increased miR-10b levels (**Figure 5B**). This meant that the relationship between HOTTIP and miR-10b is valid in all glioma cells that turn resistant against TMZ. In A172 cells with overexpressed HOTTIP, we also found evidence of EMT as evidenced by increased mesenchymal markers and decreased epithelial marker (**Figure 5C**). In LN229 cells as well, overexpression of HOTTIP led to increased mesenchymal markers and decreased epithelial marker (**Figure 5D**). Similar induction of EMT was also evident in A172 as well as LN229 cells when, instead of HOTTIP overexpression, they were subjected to miR-10b overexpression (**Figures 5E, F**). Again, mesenchymal markers ZEB1 and ZEB2 were increased while the epithelial marker E-cadherin was decreased. In summary, these observations increased our confidence in the finding that HOTTIP increases miR-10b and over expression of both leads to EMT in glioma cells.

Role of miR-10b in HOTTIP Mediated TMZ Resistance

We also further experimentally confirmed our hypothesis for the involvement of miR-10b in HOTTIP mediated TMZ resistance of glioma cells. Firstly, in the comparison between resistant SF268 and sensitive A172 cells where EMT markers were found elevated in

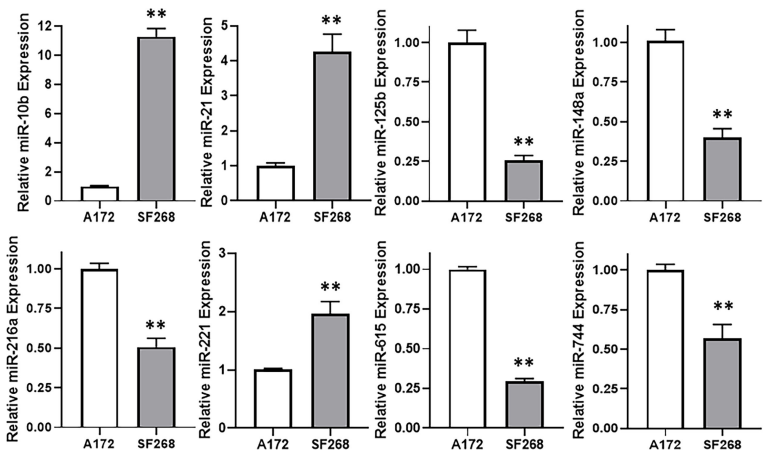

FIGURE 4 | miR-10b is elevated in TMZ resistant cells. Expression levels of different miRNAs were evaluated in A172 vs. SF268 cells, by RT-PCR. The values in A172 cells were regarded as '1' and relative values in SF268 cells are reported. RNU6B was used as the endogenous control miRNA. **p < 0.01.

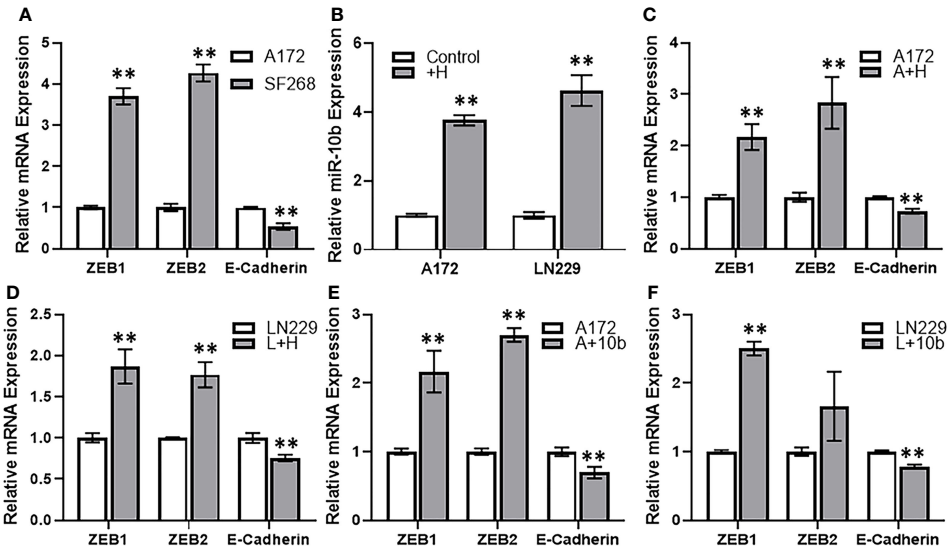

FIGURE 5 | HOTTIP induces miR-10b and EMT in glioma cells. **(A)** Expression levels of EMT markers were evaluated in A172 vs. SF268 cells, by RT-PCR. The values in A172 cells were regarded as '1' and relative values in SF268 cells are reported. GAPDH was evaluated as the endogenous control mRNA. **(B)** A172 and LN229 cells were transfected with lncRNA HOTTIP and expression levels of miR-10b were evaluated in control vs. HOTTIP transfected cells were evaluated by RT-PCR. The levels of miR-10b in control cells were regarded as '1' and relative levels in HOTTIP transfected cells are reported. RNU6B was used as the endogenous control miRNA. Expression levels of EMT markers were evaluated in A172 cells **(C)** or LN229 cells **(D)** transfected with HOTTIP or the A172 cells **(E)** or LN229 cells **(F)** transfected with miR-10b **(D)** by RT-PCR. The values in control cells were regarded as '1' and relative values in transfected cells are reported. GAPDH was evaluated as the endogenous control mRNA. +H: HOTTIP transfected cells, A+H: A172 cells with HOTTIP, A+10b: A172 cells with miR-10b. **$p < 0.01$.

SF268 cells, we added an additional group i.e. SF268 cells with silenced miR-10b. We checked for the efficiency of miR-10b silencing and found reduction in miR-10b levels of anywhere between 62% and 45% of the non-specific controls (results not shown). We found that antagonizing miR-10b significantly reversed EMT as evidenced by significantly reduced ZEB1 and ZEB2 while significantly increased E-cadherin (**Figure 6A**). Similar observations were made when A172 cells and the LN229 cells with overexpressed HOTTIP were subjected to silencing of miR-10b. Again, EMT was reversed by silencing of miR-10b (**Figures 6B, C**). As a final experiment to firmly link miR-10b with HOTTIP mediated TMZ resistance, we compared SF268 and miR-10b silenced SF268 cells for their sensitivity to TMZ by exposing the cells to increasing concentrations of TMZ. We found that silencing of miR-10b significantly reduced the resistance of SF268 cells against TMZ (**Figure 6D**) with IC-50 value dropping to 100.1 ± 1.2 μM (**Table 1**). On similar lines, when A172 or the LN229 cells with HOTTIP overexpression were silenced for miR-10b, their IC-50 also significantly reduced (**Figures 6E, F**) dropping from 54.6 ± 1.5 and 63.8 ± 1.8 μM, respectively for the A172 and LN229 HOTTIP overexpressed cells to 26.3 ± 0.7 and 25.0 ± 0.9 μM, respectively, for the A172 and LN229 HOTTIP overexpressed cells with silenced miR-10b.

DISCUSSION

Glioma is an aggressive cancer with poor prognosis and outcomes, thus making it important to find novel targets of therapy. In particular, resistance against therapy, both inherent as well as acquired, such as resistance against TMZ is a major clinical challenge making the condition of patients worse and increasing the mortality. LncRNAs are quickly emerging as the molecules of interest, particularly according to reports in recent years (17, 18). This prompted us to investigate TMZ resistance of glioma as regulated by lncRNAs.

The IC-50 values of glioma cell lines that we tested and reported in this manuscript have been reported by other researchers as well. In a report published by Perazzoli and co-workers, A172 cells had an IC-50 of 14.1 μM, LN229 cells had an IC-50 of 14.5 μM and SF268 had an IC-50 of 147.2 μM (12). Our results, as reported here are in general agreement with those previously reported values as we also report A172 and LN229 cells as the cell lines sensitive to TMZ with IC-50 values with IC-50 values 13.7 μM and 14.4 μM respectively, which are very close to what was reported by Perazzoli et al. (12). Moreover, we also show that SF268 cells are comparatively resistant to TMZ with IC-50 value of 155.1 μM. Interestingly, our analysis reveal a little more than ten-times higher IC-50 value for SF268 cells, compared to A172 and LN229 cells, which is also in general agreement with the results from study by Perazzoli et al. (12).

For the lncRNAs that can positively impact glioma cells resistance against TMZ, in addition to playing a role in glioma cells' proliferation, invasion and metastasis, we tested a total of thirty lncRNAs, based on the reported literature. The top six lncRNAs have been proven to exhibit multiple effects against glioma cells. H19 lncRNA has been shown to promote the proliferation, migration and invasion of glioma cells (19)

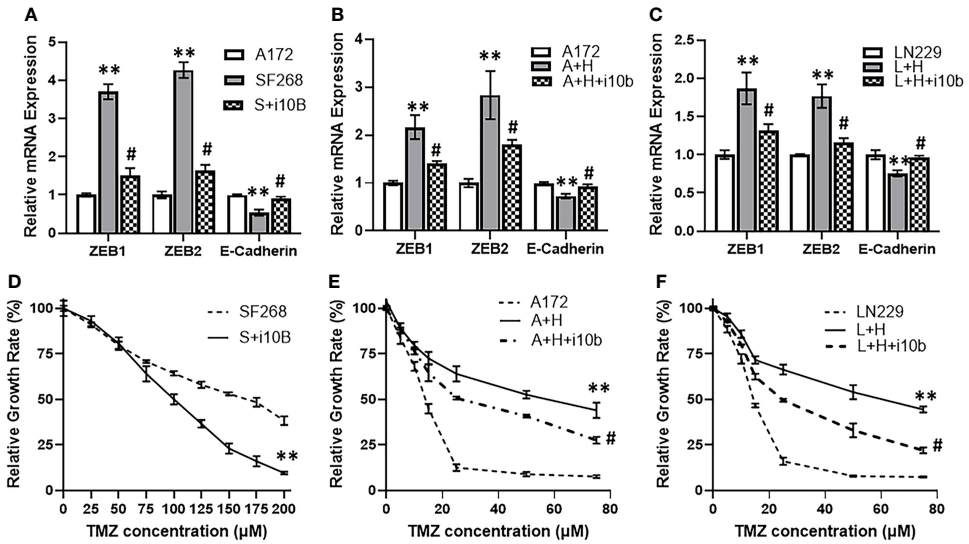

FIGURE 6 | Silencing of miR-10b reverses HOTTIP effects. **(A)** Expression levels of EMT markers were evaluated in A172 vs. SF268 cells (with and without miR-10b inhibition), by RT-PCR. The values in A172 cells were regarded as '1' and relative values in SF268 cells are reported. GAPDH was evaluated as the endogenous control mRNA. **(B, C)** Expression levels of EMT markers were also evaluated in A172 and LN229 cells transfected with HOTTIP (with and without miR-10b inhibition), by RT-PCR. The values in control cells were regarded as '1' and relative values in transfected cells are reported. GAPDH was evaluated as the endogenous control mRNA. MTT assays were performed to test the sensitivity of **(D)** SF268, **(E)** A172 and **(F)** LN229 glioma cells against TMZ after 3 day cycle, under different conditions. S+i10b: SF268 + anti-miR-10b, A+H: A172 cells with HOTTIP, A+H+i10b: A172 cells transfected with HOTTIP and anti-miR-10b. $^{**}p < 0.01$ vs control, $^{\#}p < 0.01$ in miR-10b silenced cells vs. unsilenced (transfected with control anti-miRNA oligos) cells.

through targeting of miR-200a. LINC00152 is similarly expressed at higher levels in gliomas where it increases proliferation and invasion (20). SUMO1P3 is also elevated in gliomas and associates with poor survival of glioma patients (21). Its knock down negatively affects proliferation and invasion of cells. LINC01116 is highly expressed in gliomas and promotes proliferation and invasiveness of glioma cells by targeting miR-744 (22). Finally, the last lncRNA, AGAP2-AS1, regulates proliferation and metastasis of glioma cells (23).

We show effect of HOTTIP on EMT. In glioma, there is one published report on the role of HOTTIP in EMT. This study (11) focused on hypoxia mediated EMT and found an important role of lncRNA in the process. HOTTIP was identified based on lncRNA array analysis between U87 glioma cells with and without hypoxia. Hypoxia was found to promote HOTTIP expression and metastasis, which also correlated with poor patient survival. However, a different mechanism was identified as this published study reported an involvement of miR-101 in HOTTIP action while we report an involvement of miR-10b. It is important to note that whereas miR-101 is sponged by HOTTIP as reported in the hypoxia report (11), we found elevated miR-10b in cells that also had increased HOTTIP levels. Additionally, the hypoxia report used U87 and U251 cells whereas we report our findings in three completely different cells, viz, A172, LN229 and SF268. Thus, a cell line effect can also not be ruled out. It might be important to conduct a study that uses all of these five cell lines. In addition to the one report on a connection between HOTTIP and EMT in glioma, there are a few other reports connecting HOTTIP with EMT in some other cancers. Examples include the effect of HOTTIP on EMT in breast cancer (24), gastric cancer (25), osteosarcoma (26), ovarian cancer (27). Such EMT induction by HOTTIP has been linked to cisplatin resistance in gastric cancer (28), thus further validating HOTTIP mediated EMT in resistance against therapies.

Interestingly, one of the other shown lncRNA, H19 also seems to affect EMT in glioma cells (19). The very indication that this H19 affects MET comes through the miRNA it targets as this miRNA is very well known to be involved in regulation of EMT (29), thus making this miRNA an attractive cancer biomarker (30). Similar to our findings reported here, H9 was found to regulate EMT marker ZEB1 (19) which thus appears to be important EMT gene regulated by lncRNAs. Similarly, lncRNA SUMO1P3 also seems to affect EMT as it regulates another EMT biomarker e-cadherin (21). Finally, lncRNA AGAP2-AS1 also affects EMT (23). This is one of the top lncRNAs with possible role in TMZ resistance, based on its elevated levels in resistant cells, as observed in this study. AGAP2-AS1 regulates EMT genes that can explain the observed effects of its downregulation on cellular behaviors.

Elucidation of a miRNA, downstream of HOTTIP in glioma cells, particularly those resistant to therapy, was another important goal of this study and for this we screened thirty potential miRNAs, based on available literature. Of these, we presented here the data we obtained on the top eight. One of the criteria for screening was proven targeting of miRNA in question by HOTTIP in addition to screening of some promising miRNAs based on their relevance to therapy resistance. miR-125b belongs to the category of miRNAs that have earlier been shown to be regulated by HOTTIP (31). Other miRNAs shown here that were reported to be regulated by HOTTIP in earlier studies are miR-

216a (32), miR-615 (33), miR-148a (34) and miR-744 (35). All these five miRNAs that have earlier been reported to be regulated by HOTTIP are inversely associated with HOTTIP expression i.e. their expression is negatively regulated by HOTTIP, similar to the general reports in sponging of miRNAs by lncRNAs. However, for over study we also evaluated a few miRNAs that are positively correlated with therapy resistance. These are miR-10b, miR-21 and miR-221. There is a lot of published literature on involvement of these miRNAs in therapy resistance in different cancers (36–38), however, we are the first to provide a mechanism of miR-10b mediated EMT in the HOTTIP-regulated TMZ resistance of glioma cells.

miR-10b is a well-studied miRNA in terms for its role in therapy resistance. A report published a decade back suggested the role of miR-10b in conferring resistance against 5-fluorouracil in colorectal cancer cells (39). In an agreement with our findings, Zhang and co-workers found that miR-10b regulates EMT to participate in resistance against therapy (40). Their focus was on nasopharyngeal cancer and their study found a role of miR-10b in cisplatin resistance of nasopharyngeal carcinoma cells (40). In other reports, miR-10b was shown to regulate tamoxifen resistance in breast cancer cells (41) and cisplatin resistance of ovarian cancer cells (36).

Based on the results that we presented in this study, it is reasonable to conclude that lncRNA HOTTIP confers resistance against TMZ in glioma cells. Further, it induces miR-10b and EMT which finally converge in determining TMZ resistance. This work has a lot of clinical potential but first *in vivo* and clinical studies need to further verify these findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ZLi and ML performed experiments. ZLi, ML, and PX analyzed results and performed statistical evaluations. ZLi and ZLu drafted manuscript. ZLu provided support and guidance. All authors proofread the manuscript. All authors contributed to the article and approved the submitted version.

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Circulating miRNAs as Diagnostic and Prognostic Biomarkers in High-Grade Gliomas

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Objectives: miR-181a/b and miR-410 downregulation and miR-155 upregulation has been shown to play important roles in the oncogenesis and progression of gliomas including high-grade gliomas. However, the potential role of plasma miR-181a/b, miR-410 and miR-155 in the diagnosis and prognosis of high-grade gliomas remains poorly known.

Methods: We retrieved published articles from the PubMed, the Cochrane Central Register of Controlled Trials, and Web of Science database and obtained different sets of data on microRNAs (miRNAs) expression profiling in glioma and highlighted the most frequently dysregulated miRNAs and their gene-targets (PDCD4, WNT5A, MET, and EGFR) in high-grade gliomas. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out to measure the pre- and postoperative plasma levels of miR-181a/b, miR-410 and miR-155 in 114 Grade 3-4 glioma patients, 77 Grade 1-2 glioma patients and 85 healthy volunteers as control group. The diagnostic and prognostic value of circulating miR-181a/b, miR-410 and miR-155 as biomarker was estimated by the Receiver Operating Characteristic (ROC) curve and the area under the curve (AUC) and Kaplan–Meier analysis.

Results: We found a plasma miRNA signature including three downexpressed miRNAs and one overexpressed (miR-181a, miR-181b and miR-410; miR-155) in high-grade glioma patients in comparison with low-grade glioma patients control group. The ROC curve AUC of these four circulating miRNAs were ≥ 0.75 for high-grade glioma patients in before and after surgery. Higher circulating miR-155 and lower miR-181a/b and miR-410 expression is associated with clinical data, clinic pathological variables, worse overall survival (OS) of patients and negative correlated with potential gene-targets expression. Moreover, Kaplan–Meier analysis showed that miR-181a/b, miR-410 and miR-155 were independent predictors of OS in high-grade glioma patients.

Conclusions: Our data, for the first time, demonstrated that circulating miR-181a/b, miR-410 and miR-155 could be a useful diagnostic and prognostic non-invasive biomarkers in high-grade gliomas.

Keywords: high-grade glioma, circulating, diagnosis, prognosis, biomarker, miRNA

1 INTRODUCTION

In recent decades, there has been a steady trend towards an increase in both the general oncological incidence in general and the general incidence of central nervous system (CNS tumors) tumors in particular. Gliomas are the most common and aggressive type of primary tumor in adults. The largest proportion among CNS tumors are gliomas (50–55%), which are malignant in 50–70% of cases (1). According to the 2021 World Health Organization (WHO) classification of CNS tumors, gliomas were classified into four main histological groups (Grades 1-4) according to their microscopic characteristics (such as cytological atypia, anaplasia, mitotic activity, microvascular proliferation and necrosis) and clinical manifestations (2). Glioblastoma (WHO Grade 4) and anaplastic astrocytoma (WHO Grade 3), a type of high-grade gliomas, are the most common primary brain tumors, affecting patients of all ages. Despite all the achievements of modern medicine, the prognosis for patients with high-grade gliomas remains unsatisfactory. So, with anaplastic astrocytomas, the average life expectancy is 2-3 years, and with glioblastomas - from 8 to 15 months (3). In this regard, in order to improve the diagnosis and optimization of ongoing therapy, as well as predict the course of the disease and understand the mechanisms of oncogenesis, an active search is being carried out for biomarkers that are informative and public accessibility. The possibility of accurate diagnosis and prognosis of the course of the disease, along with the improvement of the treatment of patients with high-grade gliomas is an urgent problem. Despite significant recent advances in the diagnosis of gliomas using various modifications of imaging techniques followed by histopathological examination, tumor detection is still limited by its size and location, as well as by the heterogeneity of its tissue (4). In this regard, it is necessary to develop new diagnostic approaches that, together with the available methods, will improve the accuracy of diagnosis. A promising approach for diagnosis in CNS tumors is fluid biopsy, which involves finding and measuring the level of various circulating biomolecules in human body fluids, such as blood or cerebrospinal fluid (CSF).

MicroRNAs (miRNAs) are small non-coding RNAs consisting of 18-20 nucleotides that play an important role in the regulation of gene expression at the post-transcriptional level by interacting with 3'-untranslated regions (3'-UTR) of messenger RNAs (mRNA)-targets (5). MiRNAs are involved in the regulation of such physiological processes as cell proliferation, differentiation, apoptosis, angiogenesis, etc. MiRNAs play an important role in the regulation of both physiological processes (5). On the other hand, miRNAs have been found to be involved in the oncogenesis of many human tumors, acting as tumor suppressors or oncogenes (6). MiRNAs are capable of influencing all events considered in terms of tumor progression, including tumor growth, invasion, metastasis, and angiogenesis. The significance of miRNAs in high-grade gliomas has been proven both by changes in their expression and by dysregulation of the expression of mRNA-targets (7). MiR-155is one of the most well-known oncogenic miRNA, miR-155 overexpression has been documented in gliomas, extraordinarily in high-grade glioma cells and tissue. Wu et al. in their research found that the high level of miR-155 in U87-MG cell line can promote the proliferation, invasion and migration of tumor cells, inhibit their senescence and apoptosis, and activate the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (8). In other study results showed that the level of miR-155 was up-regulated in glioma patients, accompanied by high pathological grade (9). In addition, miR-155 may activates the growth of U87 glioma cells and increases the sensitivity of glioblastoma to temozolomide (TMZ) by targeting Six1 *in vivo* (10).

Some number of studies about miR-181a/b and miR-410 therapeutics have been carried out, and verified its tumor-suppressive role in gliomas. For instance, the expression of miR-181 family members has been decreased in glioma (11). While miR-181c has been the most down-regulated one in the WHO Grade I gliomas, miR-181a/b exhibited the fastest decrease rate, with a significant decrease in the glioblastoma (11, 12). Forced up-regulation of miR-181a/b remarkably suppressed high-grade glioma cell lines (U87, TJ905, and U251) tumor growth, proliferation, invasion and promote tumor cells apoptosis (13). In the other research, Chen et al. demonstrated that high-grade glioma expressed comparatively higher MET expression and lower miR-410 while low-grade glioma with lower MET and higher miR-410 (14). The authors concluded that miR-410 directly targets 3'-UTR of MET mRNA and may affect high-grade glioma cell proliferation and invasion through MET regulated AKT signaling.

It is known that circulating miRNAs are in a stable form and are detected in human biological fluids such as blood, urine, cerebrospinal fluid (CSF) and saliva. In this regard, circulating miRNAs are considered as new biomarkers of interest in a number of diseases, including tumors (15). Circulating miR-181a/b, miR-410 and miR-155 can be released into the biological fluids in response to activation of process oncogenesis. Thus, the present study examined the expression profile of these circulating miRNAs in the plasma of patients with high-grade glioma. Furthermore, we evaluated the association among gene-targets expression, clinical data, clinic pathological variables, and diagnostic or prognosis value.

2 MATERIALS AND METHODS

2.1 Patients and Clinical Samples

We enrolled 301 subjects in this study from January 2019 to August 2020 in Federal Center of Neurosurgery (Tyumen, Russia), including 85 healthy volunteers as control group and 191 newly diagnosed glioma patients with various stages. Glioma patients were diagnosed by brain magnetic resonance imaging (MRI), computed tomography (CT) perfusion imaging (Canon Aquilion One, Iomeron 400 mg – 50 ml), dynamic susceptibility contrast (DSC) perfusion (General Electric Discovery W750 3T, Gadovist 7.5 ml) (**Figures 1A, B**, **2A, B**) and histological examination based on the WHO categories, and all patients were classified according to 2021 WHO classification system, including 16 cases of pilocytic astrocytoma (Grade 1), 61 diffuse

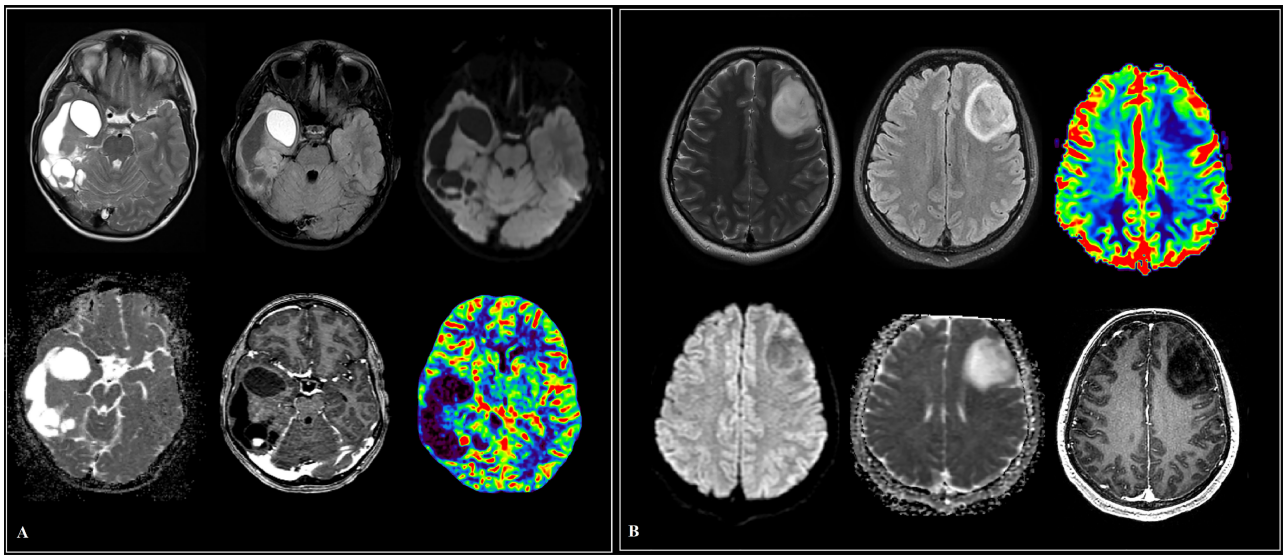

FIGURE 1 | Cases of low-grade glioma patients **(A, B)**. **(A)** A large intracerebral tumor of the right temporo-occipital areas with a heterogeneous cystic-solid structure, contrasted as a group of nodules and small closed rings in the solid part. The contents of the cysts are similar to the cerebrospinal fluid in terms of signal characteristics, with the exception of a cyst in the pole of the temporal lobe, where a protein admixture and traces of hemorrhage are detected, the walls of the cysts do not increase. Diffusion restriction in the structure of education is not determined. In the posterior sections, a node with elevated median cerebral blood volume (CBV) values according to multi-slice computed tomography (MSCT) perfusion. **(B)** Predominantly in the middle frontal gyrus of the left frontal lobe, an intracerebral mass with diffuse distribution, clear contours and a sign of T2/fluid attenuation inversion recovery (FLAIR) mismatch. The tumor involves white and gray matter with "swelling" of the cortical plate. There is no restriction of diffusion, contrasting in the formation. Reliable areas of hyperperfusion according to dynamic susceptibility contrast (DSC) MR perfusion are not determined.

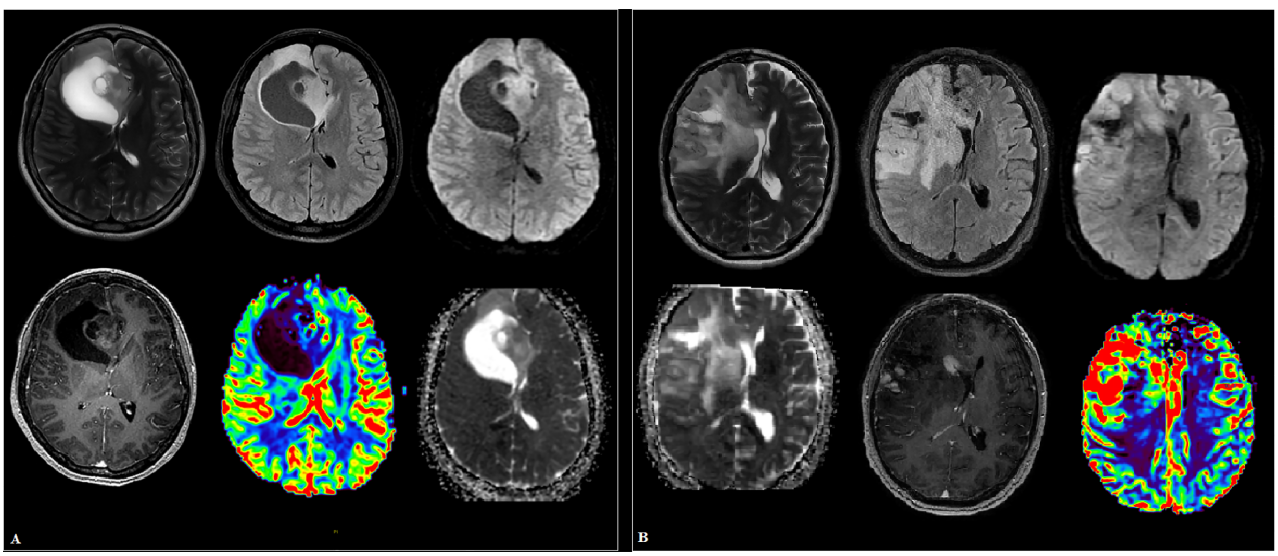

FIGURE 2 | Cases of high-grade glioma patients **(A, B)**. **(A)** Intracranial tumor of the right frontal lobe and genu corpus callosum, in addition to the white matter, involving the cortical plate with a mass effect on the anterior horn of the right lateral ventricle. The parasagittal solid component has small foci of hyperperfusion on dynamic susceptibility contrast (DSC) MR perfusion and areas of increased cellularity in apparent diffusion coefficient (ADC), is heterogeneous in signal in T2 weighted image (T2WI), and shows heterogeneous contrast enhancement. **(B)** A large "intra-axial" area of in homogeneously elevated signal in T2 and fluid attenuation inversion recovery (FLAIR) in the right frontal lobe and basal ganglia, extending into the gray matter, the opposite hemisphere along the genu corpus callosum, with a group of subpial (and one subependial) nodules of homogeneous intense contrast enhancement. Against the background of uneven diffusion, zones of increased relative cerebral blood volume (rCBV) are revealed along the lateral contour of the right frontal lobe.

astrocytoma (Grade 2), 76 cases of anaplastic astrocytoma (Grade 3), and 38 cases of glioblastoma (Grade 3) (2). All patients were grouped into low-grade (WHO Grade 1–2, 77/191) or high-grade (Grade 3–4, 114/191). Plasma samples of patients with low-grade gliomas were collected 3 days before medical preparation for the surgical intervention. Plasma samples of patients with high-grade gliomas were collected twice: 1) 3 days before medical preparation for the surgical intervention; and 2) 10 days after the surgery, usually on the day of discharge from the hospital. None of the patients had received chemotherapy or radiotherapy prior to surgery. In addition, we excluded patients with other tumors, cardiovascular diseases, immune diseases, injuries, organ failure, and infections in their past medical history since these diseases may influence the levels of circulating miRNAs in our patients. All patients with high-grade were followed up at intervals of 1 month in the initial 1–2 years and every 3 months thereafter. Clinical follow-up of 114 patients was finished by August 2021 (44 months). Overall survival time was defined as the period between the initial operation and death, and disease-free survival was the period between the initial operation and tumor recurrence or death. This study was approved by the Ethics Committee of Federal Center of Neurosurgery (Tyumen, Russia) and implemented in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from all subjects. Patient characteristics are summarized in **Table 1**.

2.2 Study Design

The study was separated into four steps. We performed a comprehensive search for original articles demonstrating the dysregulated miRNAs and their gene-targets in high-grade gliomas. Databases including PubMed, the Cochrane Central Register of Controlled Trials, and Web of Science were used to obtain all relevant studies up to February 2021. Keywords including "glioma" or "high-grade glioma" or "anaplastic astrocytoma" or "glioblastoma" or "malignant brain tumors" or "primary", "microRNA" or "miRNA" or "miR" or "non-coding RNAs" or "cell-free" or "circulating" or "diagnosis" or "prognosis" or "biomarker" and "gene-targets" or "epigenetic regulation" or "oncogenesis" were used. However, our current study did not find statistically significant identified 4 from 12 circulating miRNAs expression in plasma samples of glioma patients and control group. The flow diagram of the study design is shown in **Figure 3**.

2.3 Plasma Preparation

All 10 ml venous blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged, where hemolyzed blood samples were excluded. After the first centrifugation at 1600 x g for 10 min at 4°C, the supernatants were carefully removed and transferred to a new tube follow by centrifugation again at 16,000 x g for 10 min at 4°C to remove residual blood cells. Plasma was then stored at −80°C until further processing.

2.4 Total RNA Extraction

Total RNA was extracted from 200 μL plasma samples of all glioma patients and healthy controls using the miRNeasy Serum/Plasma Kit for purification of total RNA, including miRNA (Qiagen, Germany) and QIAzol Lysis Reagent (Qiagen,

TABLE 1 | Clinicopathological characteristics of glioma patients for the study of circulating miRNAs.

Clinical variables (n = 191)	No. of cases	P value
Age		0.002
<50	83	
≥50	108	
Sex		0.09
Male	141	
Female	50	
Tumor size (cm)		0.013
≤ 3	132	
> 3	59	
WHO grade		<0.001
Low-grade (I-II)	77	
High-grade (III-IV)	114	
Extent of resection for high-grade gliomas patients		<0.05
Total	71	
Partial	43	
Tumor location		0.40
Supratentorial	171	
Infratentorial	20	
KPS score		0.008
<90	68	
≥90	123	
Recurrence for high-grade gliomas patients		0.039
Yes	77	
No	37	

WHO, World Health Organization; KPS score, Karnofsky Performance Scale.

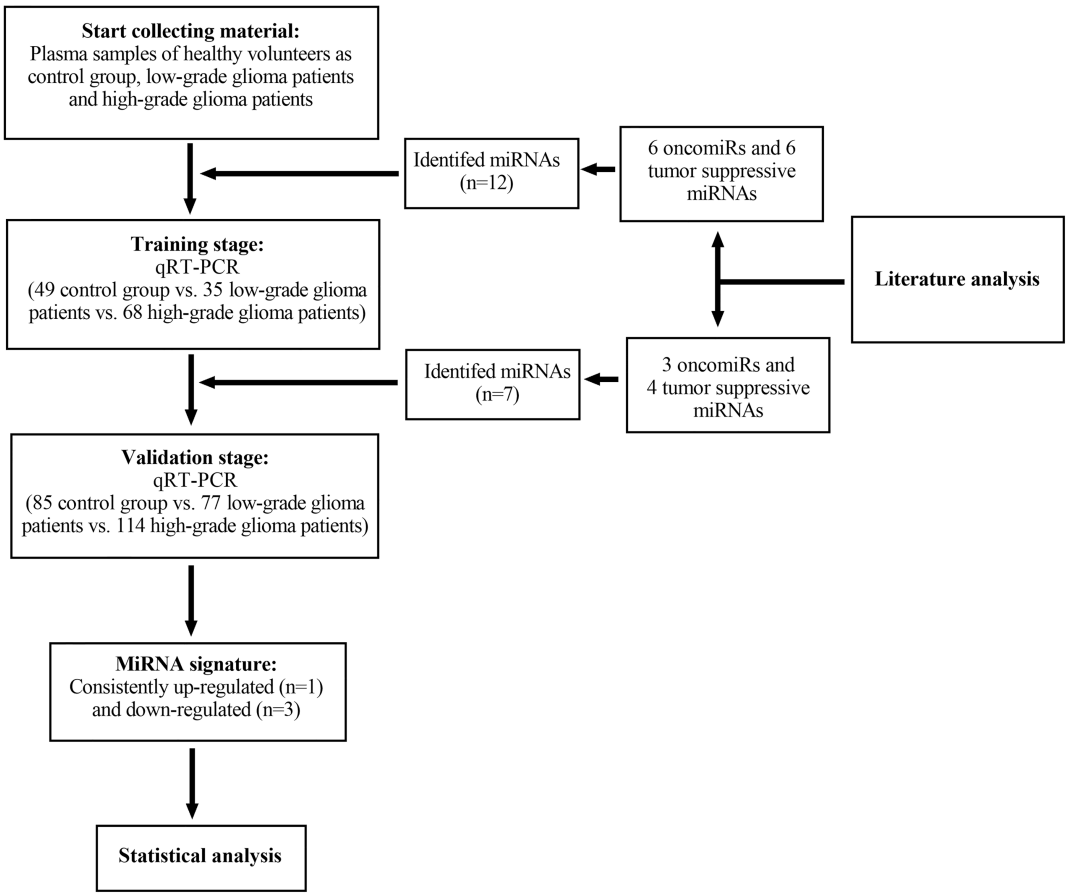

FIGURE 3 | Flow diagram illustrating the steps for microRNAs (miRNAs) selection, expression profiling and differential expression analysis. qRT-PCR, quantitative reverse transcription polymerase chain reaction; miRNAs, microRNAs.

Germany) according to the manufacturer's instructions. Total RNA purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and consistently yielded A260:A280 and A260:A230 ratios close to 2.0. All isolated total RNA was stored at a −80°C freezer until use.

2.5 Synthesis of Complementary DNA

cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) by reverse transcription according to the manufacturer's instructions. Reverse transcription was carried out in 20 μl solution that contained 0.5 μl 20 U/μl Transcriptor reverse transcriptase, 4μl Transcriptor RT Reaction Buffer (5x concentrated), 2 μl Deoxynucleotide Mix, 0.5 μl 40 U/μl protector RNase inhibitor, 2 μl 10 mmol/ml stem-loop RT primers (Invitrogen), 7 μl DEPC water (Invitrogen, USA), and 4 μl total RNA template. After being mixed gently, the reaction mixtures were incubated at 25°C for 10 min, 55°C for 30 min and then 85°C for 5 min. The final cDNA products were stored at −20°C until use. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the reference genes. The sequence of all primers used in the present study is provided in **Table 2**.

2.6 Quantitative Reverse Transcription Polymerase Chain Reaction

qRT-PCR was carried out following the manufacturer's protocol of FastStart Universal SYBR Green Master (Rox) (Roche, Germany) with 2 μL cDNA template. The PCR mixture (18 μL) contains 10 μL SYBR Green (Rox) (Roche, Germany), 1 μL 10 mmol/mL forward primer (Invitrogen, USA), 1 μL 10 mmol/mL reverse primer (Invitrogen, USA) and 6 μL DEPC water (Invitrogen, USA). PCR reaction was performed in duplicates. All PCR reactions were carried out on an ABI 7500 Real-Time PCR machine (Thermo Fishers, USA). Reaction conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 s and 60°C for 10 seconds. U6 and GAPDH were used as the reference genes. The sequence of all primers used in the present study is provided in **Table 2**.

TABLE 2 | Sequence of all primers.

miRNA/Gene-target/Reference gene	Primer Sequence (5'-3')
miR-181a	RT:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACCGA
	Forward: GCCCGAACATTCAACGCTGT
	Reverse: GTGCAGGGTCCGAGGT
miR-181b	RT:CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGACCCACC;
	Forward: CGCCGAACATTCATTCATTGCTGTC
	Reverse: CAGCCACAAAAGAGCACAAT
miR-410	RT:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGCCA
	Forward: GTCAGCGCAATATAACACAG
	Reverse: GTGCAGGGTCCGAGGT
miR-155	RT:CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGACCCCTA
	Forward: CGCCGTTAATGCTAATCGTGA
	Reverse: CAGCCACAAAAGAGCACAAT
PDCD4	Forward: GAAGGTTGCTGGATAGGC
	Reverse: ATAAACACAGTTCTCCTGGTCATCA
WNT5A	Forward: GTGCAATGTCTTCCAAGTTCTTC
MET	Reverse: GGCACAGTTTCTTCTGTCCTTG
EGFR	Forward: AACACCCTGGTCTGGAAGTACG
	Reverse: TCGTTGGACAGCCTTCAAGACC
GAPDH	Forward: GTCTCCTCTGACTTCAACAGCG
	Reverse: ACCACCCTGTTGCTGTAGCCAA
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT

miRNA, microRNA; RT, reverse transcription; miR, microRNA; WNT5A, Wnt family member 5A; PDCD4, programmed cell death 4; DFFA, DNA fragmentation factor subunit alpha; EGFR, epidermal growth factor receptor; GAPDH, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.7 Statistical Analysis

Relative levels of the circulating miR-181a/b, miR-410 and miR-155 were quantified using the 2-ΔΔCq method. ROC curves and the area under the curve (AUC) was applied to analysis the diagnostic values of the circulating miR-181a/b, miR-410 and miR-155. Kaplan–Meier analysis was used to generate and analyze survival time data. The univariate Cox proportional hazards regression was used for univariate and multivariate analyses. The Student t-test, ANOVA, chi-square analysis, or Mann-Whitney test was applied, where appropriate. A probability of $p<0.05$ (*) or $p<0.001$ (**) or $p<0.0001$ (***) was considered statistically significant. The statistical analyses were carried out with the IBM SPSS 13.0 software and the graphs were generated by using Graphpad Prism 7.0.

3 RESULTS

3.1 Detection of Circulating miRNAs in Plasma Samples From Glioma Patients Before Surgery

To verify the expression profiles of circulating miR-181a/b, miR-410 and miR-155 in presurgery plasma samples of glioma patients, we detected the expression levels of circulating miR-181a/b, miR-410 and miR-155 in 114 high-grade glioma patients with compare 77 low-grade glioma patients and 85 subjects as control group using qRT-PCR. The results of qRT-PCR assay in **Figures 4A–D** show that the expression levels of circulating miR-155 in plasma of high-grade glioma patients was significantly higher than in low-grade glioma patients and control group ($p < 0.05$, $p<0.001$). However, the expression levels of circulating miR-181a/b and miR-410 in plasma of high-grade glioma patients was significantly lower than in low-grade glioma patients and control group ($p < 0.05$, $p<0.001$). Our results indicated that increased circulating miR-155 and decreased miR-181a/b and miR-410 levels might play a role in glioma oncogenesis particularly high-grade gliomas. In this case, miR-155 may acts as a potential oncomiR were miR-181a/b and miR-410 may play as a potential tumor suppressive role.

3.2 Circulating miRNAs Expression in Before and After Surgery Plasma Samples From Patients With High-Grade Glioma

The results of the circulating miRNA analysis of plasma samples high-grade glioma patients acquired pre- and post-operatively (10 days after surgery) indicated significant upregulation of circulating miR-181a ($p=0.045$), miR-181b ($p<0.001$) and miR-410 ($p=0.017$), and insignificant downregulation of miR-155 ($p<0.001$) with compare control group (**Figures 5A–D**).

3.3 Circulating miRNA Expression in Paired Before vs. After Surgery Plasma Samples From Patients With High-Grade Glioma

We next asked if these circulating miRNAs were different in the plasma of the high-grade glioma patients before and after surgery. We identified that the expression levels of miR-155

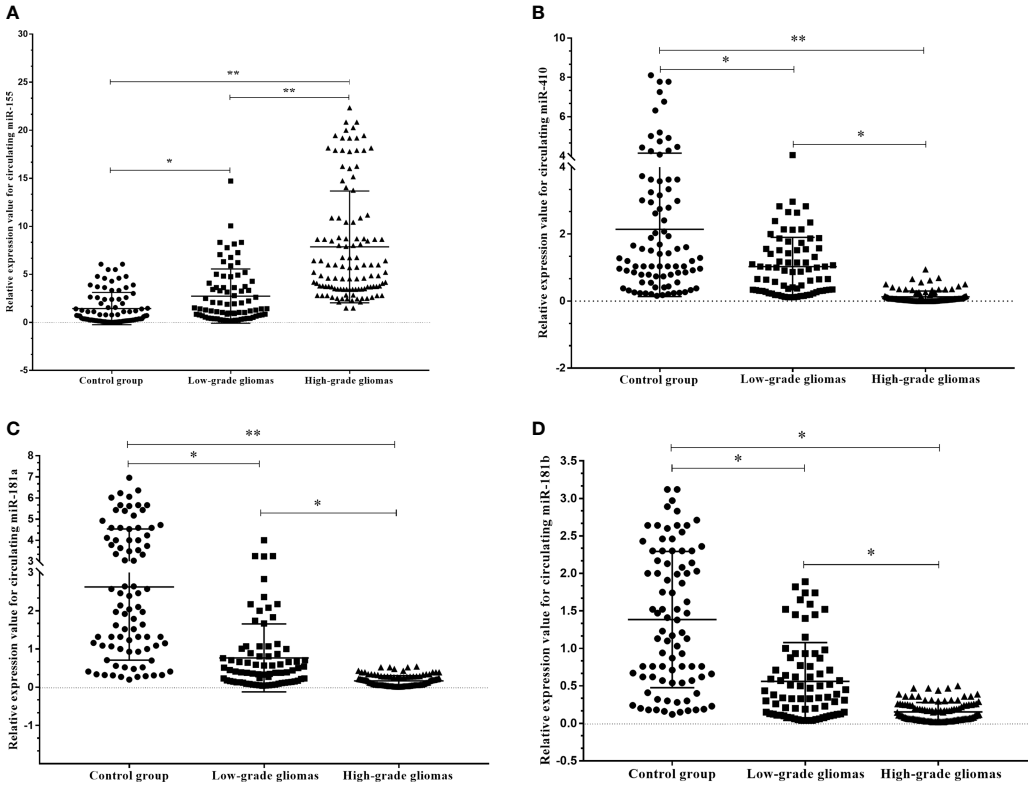

FIGURE 4 | Circulating miR-181a/b, miR-410 and miR-155 expression levels within different grade gliomas and control group **(A–D)**. **(A)** The expression levels of circulating miR-155 in the plasma of high-grade glioma patients were significantly higher than in low-grade glioma patients and control group. In the same time, **(B–D)** the expression levels of circulating miR-181a, miR-181b and miR-410 in the plasma of high-grade glioma patients were significantly lower than in low-grade glioma patients and control group. A probability of $p < 0.05$ (*) or $p < 0.001$ (**) was considered statistically significant.

(p=0.001) were found to be decreased in the plasma 10 days after surgery compared to that before operation, while miR-181a (p<0.001), miR-181b (p<0.001) and miR-410 (p<0.001) were increased more than ten-fold in the plasma after surgery (**Figures 6A–D**).

3.4 Diagnostic Value of Circulating miRNAs in Glioma Patients

The diagnostic value in before and after surgery of circulating miR-181a/b, miR-410 and miR-155 in differentiating the high-grade glioma patients group from the low-grade glioma patients and control group was also examined (**Figures 7A–D**, **8A–D**). Our circulating miRNA-based signature could perform well in distinguishing high-grade glioma patients in before and after surgery time from control group as evidenced by a high AUC (diagnostic value AUC≥0.75) (16). These findings suggest that circulating miR-181a/b, miR-410 and miR-155 had high power to distinguish high-grade glioma patients in before and after surgery time from low-grade glioma patients and control group. More detailed information about the ROC curves for diagnostic value of circulating miRNAs are presented in **Tables 3**, **4**.

3.5 Expression Levels of Circulating miR-181a/b, miR-410 and miR-155 Predicts a poor Prognosis of High-Grade Glioma Patients

The prognostic value of circulating miR-181a/b, miR-410 and miR-155-based signature in overall survival (OS) was detectable through the Kaplan–Meier curve of two cohorts (total tumor resection or partial tumor resection) of high-grade glioma patients as shown in (**Figures 9A–H**). The relative expression of these circulating miRNAs in high-grade glioma patients were divided into a higher-expression group and a lower-expression group. Our analysis showed that high-grade glioma patients in the higher-expression group of circulating miR-155 had a poorer OS (**Figure 9A**; p= 0.001) particularly in high-grade glioma patients with partial tumor resection (**Figure 9E**; p=0.05). On the other hand, high-grade glioma patients in the lower-expression group of circulating miR-181a, miR-181b, miR-410 had a poorer OS (**Figures 9B–D**; p=0.001) particularly in high-grade glioma patients with partial tumor resection (**Figures 9F–H**; p=0.05). Based on these findings, we suggest that circulating miR-181a/b, miR-410 and miR-155 expression can be used as an independent factor to predict the survival of high-grade glioma patients.

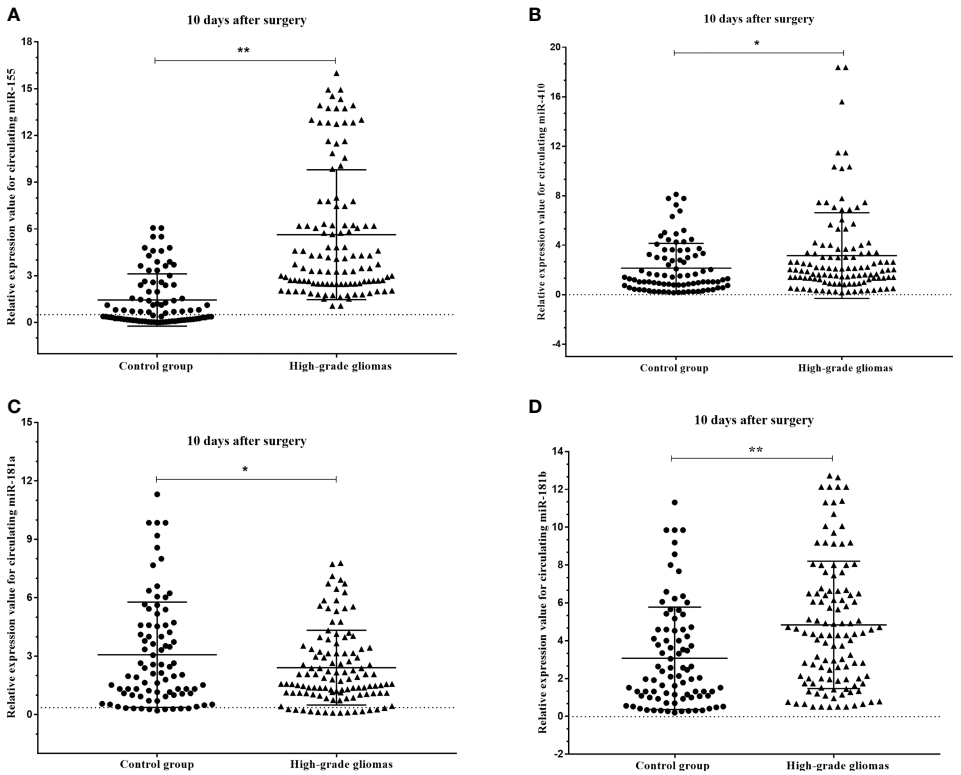

FIGURE 5 | Box plots showing the expression levels of **(A)** miR-155, **(B)** miR-410, **(C)** miR-181a and **(D)** miR-181b before and after 10 days surgery of patients with high-grade glioma with compare control group. A probability of $p < 0.05$ (*) or $p < 0.001$ (**) was considered statistically significant.

3.6 Interaction Between Potential Gene-Targets and miRNAs

We screened out the possible gene-targets of the aforementioned circulating miRNAs by using common miRNA target predicting datasets: DIANA-microT, mirSVR, PicTar5, RNA22, RNAhybrid, TargetScan, PITA, MirTarget2, TargetMiner, miRanda, and TmiRWalk2.0. In order to improve the reliability of the predicted target genes, we extracted only the corresponding target regulations that emerged from at least five of the datasets listed above. The selected eight genes, targeted by the miRNAs panel, were MET, phosphatidylinositol 3-kinase (PI3KCA), Wnt family member 5A (WNT5A), programmed cell death 4 (PDCD4), DNA fragmentation factor subunit alpha (DFFA), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 1/2 (FGFR1/2). Primer sequences for each gene are provided in **Table 2**.

3.6.1 Relationship Between Expression Circulating miRNAs and Expression Gene-Targets of High-Grade Gliomas Patients

The expression level of circulating miR-155 correlated positively with oncogene PDCD4 ($p < 0.0001$, $r = 0.753$) in plasma samples of high-grade glioma patients (**Figure 10A**). However, circulating miR-155, miR-410, and miR-181a/b showed a negative correlation with tumor suppressive gene WNT5A ($p < 0.0001$, $r = -0.671$; **Figure 10B**) and with oncogenes MET ($p < 0.0001$, $r = -0.4778$; **Figure 10C**), EGFR ($p < 0.0001$, $r = -0.5235$, $r = -0.5217$; **Figures 10D, E**). Consequently, the expression of circulating miR-155, miR-410, and miR-181a/b and its putative gene-targets PDCD4, WNT5A, MET, and EGFR had a strong inverse relation in high-grade glioma, thereby suggesting that the potential roles of miR-155, miR-410, and miR-181a/b in oncogenesis of glioma tumors.

4 DISCUSSION

Gliomas are the most common and aggressive type of primary brain tumor in adults. It should be noted that for gliomas, the progression of the disease to a higher class of anaplasia is not excluded (e.g. anaplastic astrocytoma and glioblastoma) (3). Despite all the achievements of modern medicine, the prognosis for patients with high-grade gliomas remains unsatisfactory. Current treatment strategies of high-grade gliomas are based on open surgery, radio- and chemotherapy (1, 3). However, none of these treatments, alone or in combination, is considered effective in controlling the disease, resulting in an average life expectancy after diagnosis of about than 12-15 months. Thus, the issues of timely diagnosis and prognosis of the outcomes of this type of

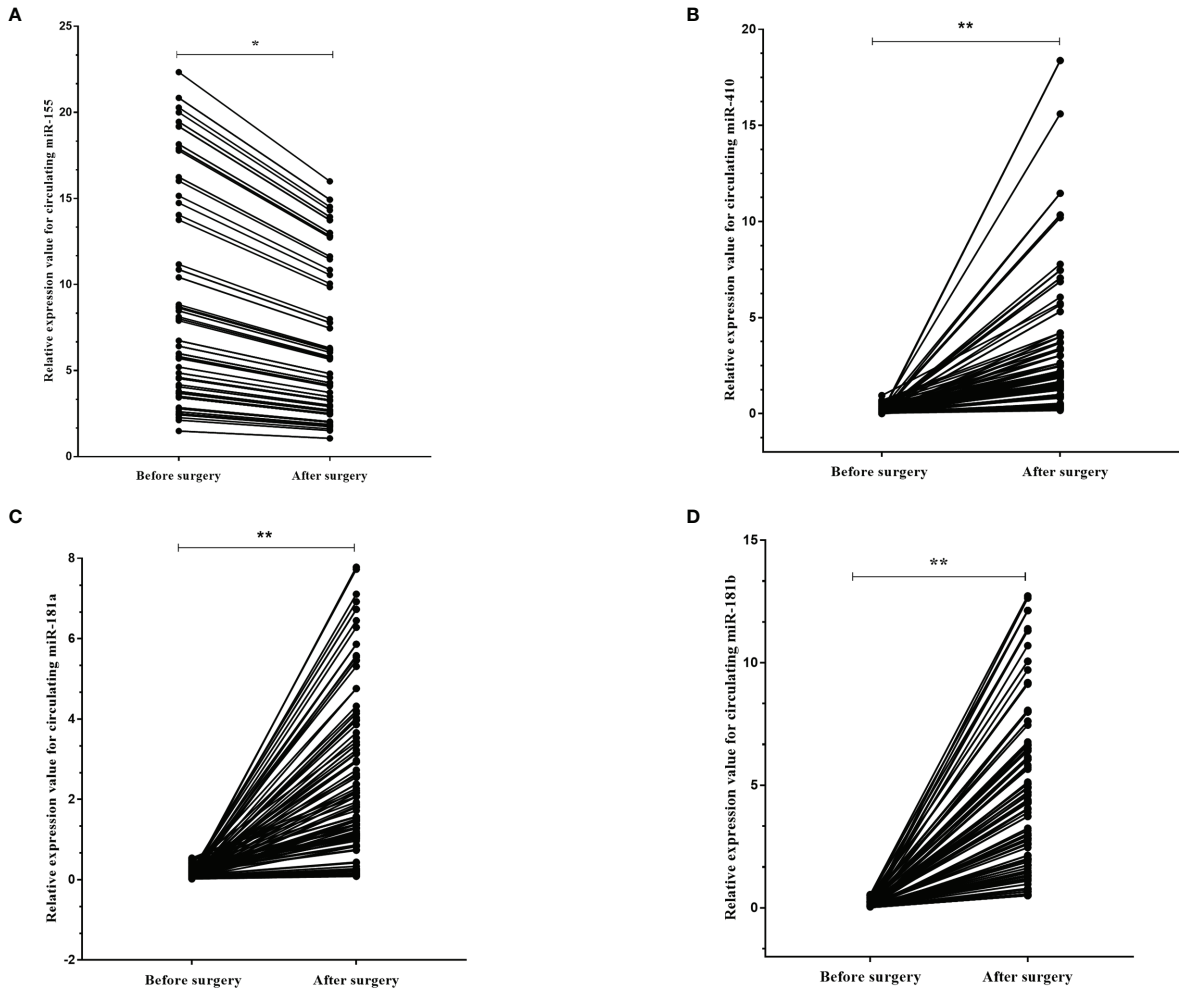

FIGURE 6 | The dynamic change of circulating miR-181a/b, miR-410 and miR-155 in plasma samples of high-grade glioma patients before and after surgery. The expression levels of the 4 circulating miRNAs in the patients before and after surgery **(A–D)**. Each point represents the mean of the triplicate samples. A probability of $p < 0.05$ (*) or $p < 0.001$ (**) was considered statistically significant.

tumors do not lose their relevance. The concept of molecular biomarkers has been widely developed in the last decade. The use of biomarkers contributes to the understanding of the pathogenetic mechanisms of gliomas, promotes early detection of tumors, stratification of the risk of recurrence, control and timely correction of the treatment strategy, and, consequently, a more favorable prognosis. Circulating miRNAs are one of the widely studied biomarkers, and although they are not currently used in clinical practice, advances in this field indicate that the effectiveness of circulating miRNAs in the diagnosis and prognosis of high-grade gliomas can be critical and replace specific steps in modern diagnostic practice (15).

In this study, expression levels of circulating miR-155, miR-410, and miR-181a/b in 114 high-grade glioma patients, 77 low-grade glioma patients and 85 healthy volunteers as control group were first screened using qRT-PCR arrays. We found that the expression levels of circulating miR-155 were significantly higher in high-grade glioma patients than in low-grade glioma patients in the before surgery period and control group. Wherein, the expression levels of circulating miR-410 and miR-181a/b were significantly lower in high-grade glioma patients than in low-grade glioma patients in the before surgery period and control group. In addition, using ROC curve analysis, we demonstrated that the circulating miR-155, miR-410, and miR-181a/b had high accuracy in glioma diagnosis, especially in patients with high-grade gliomas in before surgery where AUC ≥0.75, respectively (see **Table 3**) (16). Furthermore, we saw that in the 10 days after surgery the expression levels of circulating miR-155 were insignificantly lower in high-grade glioma patients while and in the same patients group there was a significantly decrease in the expression levels of circulating miR-410, and miR-181a/b than in the control group. The AUC for the diagnosis value of circulating miR-155, miR-410, and miR-181a/b after surgery was 0.87, 0.76, 0.84, and 0.87, respectively (see **Table 4**). In addition, to analyze the association of these circulating miRNAs expression with prognosis, the Kaplan-Meier analysis showed that higher

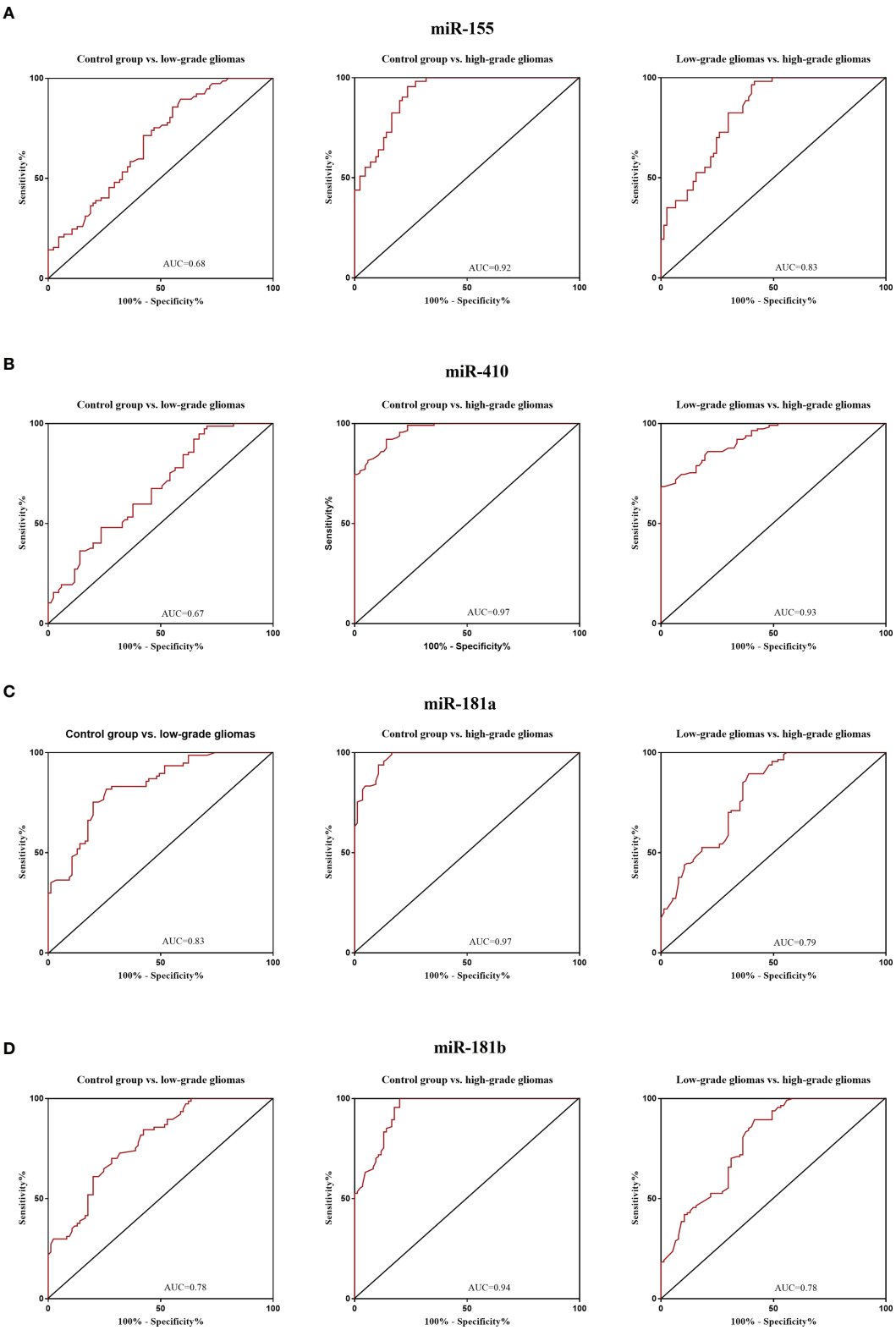

FIGURE 7 | Receiver Operating Characteristic (ROC) curves for circulating miR-181a/b, miR-410 and miR-155 for glioma patients before surgery **(A–D)**. In panel **(A, B)** of the figure the area under the curves (AUCs) for circulating miR-155 and miR-410 were 0.68, 0.92, 0.83, 0.67, 0.97, 0.93 and in panel **(C, D)** for circulating miR-155 and miR-410 were 0.83, 0.97, 0.79, 0.78, 0.94, 0.78 respectively.

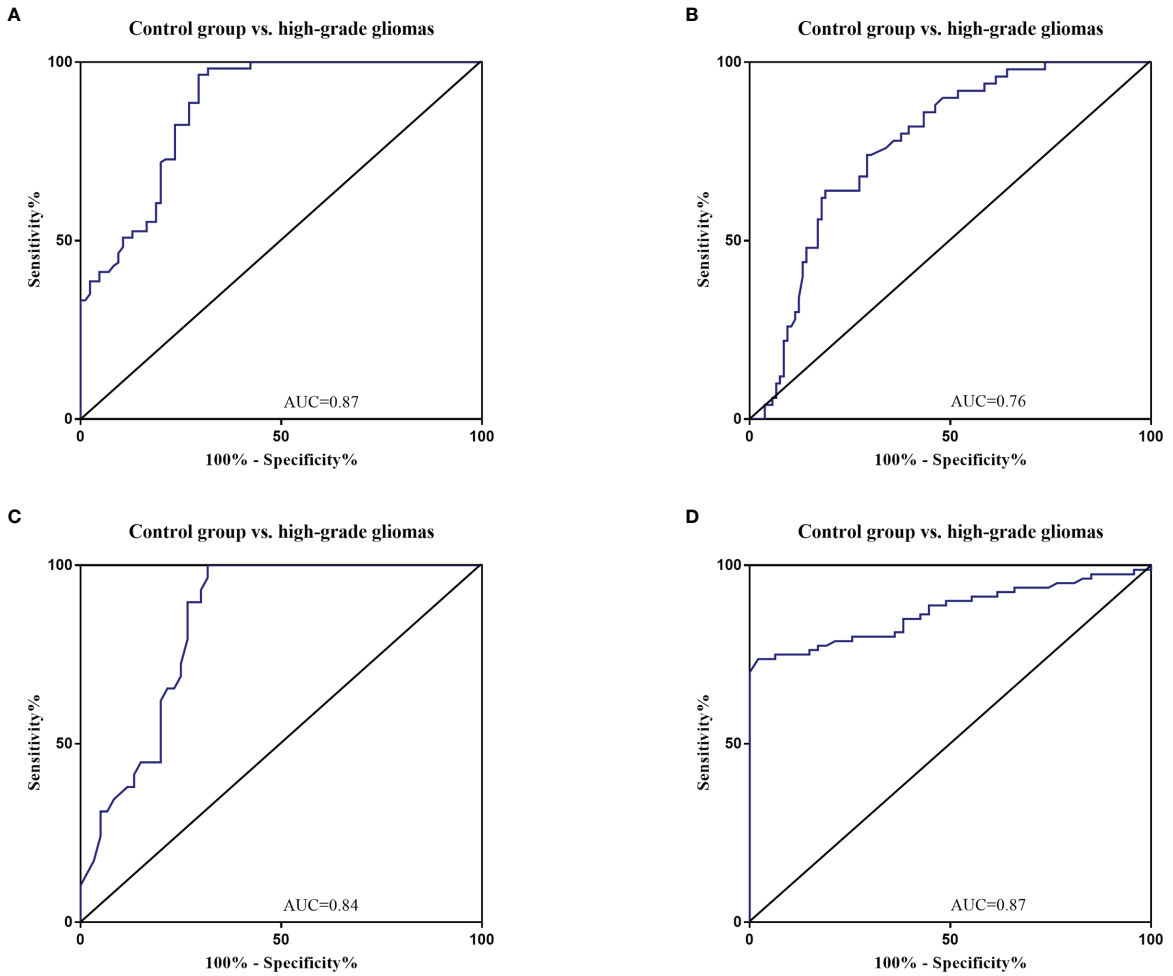

FIGURE 8 | Receiver Operating Characteristic (ROC) curves for circulating miR-181a/b, miR-410 and miR-155 for high-grade glioma patients after surgery **(A–D)**. The area under the curves (AUCs) for circulating miR-155 **(A)**, miR-410 **(B)**, miR-181a **(C)**, and miR-181b **(D)** were 0.87, 0.76, 0.84, and 0.87, respectively, suggesting that these circulating miRNAs can distinguish high-grade glioma patients after surgery from control group.

TABLE 3 | Receiver Operating Characteristic (ROC) curves for diagnostic value of circulating miR-181a/b, miR-410 and miR-155 for glioma patients in before surgery time.

miRNAs	Area Under the ROC Curve (95% CI)	Sensitivity (%)	Specificity (%)	p value
Control group vs. low-grade				
miR-155	0.68 (95% CI: 0.6019 to 0.7636)	66.7	76.9	p<0.0001
miR-410	0.67 (95% CI: 0.5898 to 0.7533)	65.7	74.1	p<0.0001
miR-181a	0.83 (95% CI: 0.7671 to 0.8902)	73.34	86.00	p<0.0001
miR-181b	0.78 (95% CI: 0.7122 to 0.8499)	68.21	82.75	p<0.0001
Control group vs. high-grade				
miR-155	0.92 (95% CI: 0.882 to 0.9573)	82.3	84.1	p<0.0001
miR-410	0.97 (95% CI: 0.9467 to 0.9858)	86.8	94.21	p<0.0001
miR-181a	0.97 (95% CI: 0.9602 to 0.9927)	87.5	96.7	p<0.0001
miR-181b	0.94 (95% CI: 0.9154 to 0.975)	93.1	88.7	p<0.0001
Low-grade vs. high-grade				
miR-155	0.83 (95% CI: 0.7688 to 0.89)	73.24	85.95	p<0.0001
miR-410	0.93 (95% CI: 0,8885 to 0,9573)	90.2	82.4	p<0.0001
miR-181a	0.79 (95% CI: 0.7303 to 0.8618)	70.21	83.75	p<0.0001
miR-181b	0.78 (95% CI: 0.719 to 0.8534)	68.51	83.11	p<0.0001

ROC, Receiver Operating Characteristic; CI, Confidence interval.

TABLE 4 | Receiver Operating Characteristic (ROC) curves for diagnostic value of circulating miR-181a/b, miR-410 and miR-155 for high-grade glioma patients in after surgery time.

miRNAs	Area Under the ROC Curve (95% CI)	Sensitivity (%)	Specificity (%)	p value
Control group vs. high-grade				
miR-155	0.87(95% CI: 0.8211 to 0.9225)	66.7	76.9	p<0.0001
miR-410	0.76 (95% CI: 0.6956 to 0.8436)	65.7	74.1	p<0.0001
miR-181a	0.84 (95% CI: 0.7617 to 0.9205)	73.34	86.00	p<0.0001
miR-181b	0.87 (95% CI: 0.8087 to 0.9312)	68.21	82.75	p<0.0001

ROC, Receiver Operating Characteristic; CI, Confidence interval.

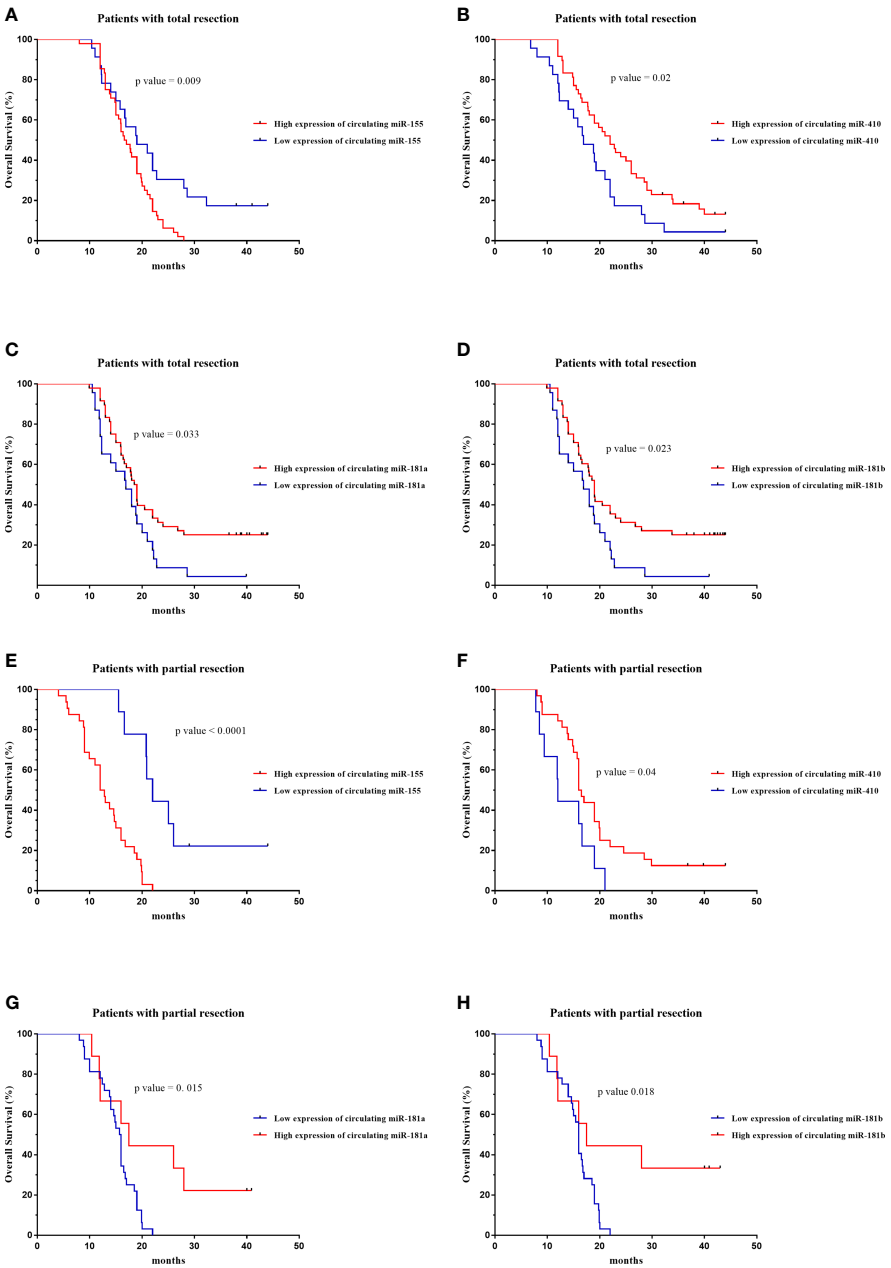

FIGURE 9 | Kaplan–Meier curves of overall survival (OS) for high-grade glioma patients based on the circulating miR-155, miR-410, miR-181a and miR-181b signature in patients with total (n = 71) **(A–D)** or partial tumor resection (n = 43) **(E–H)**.

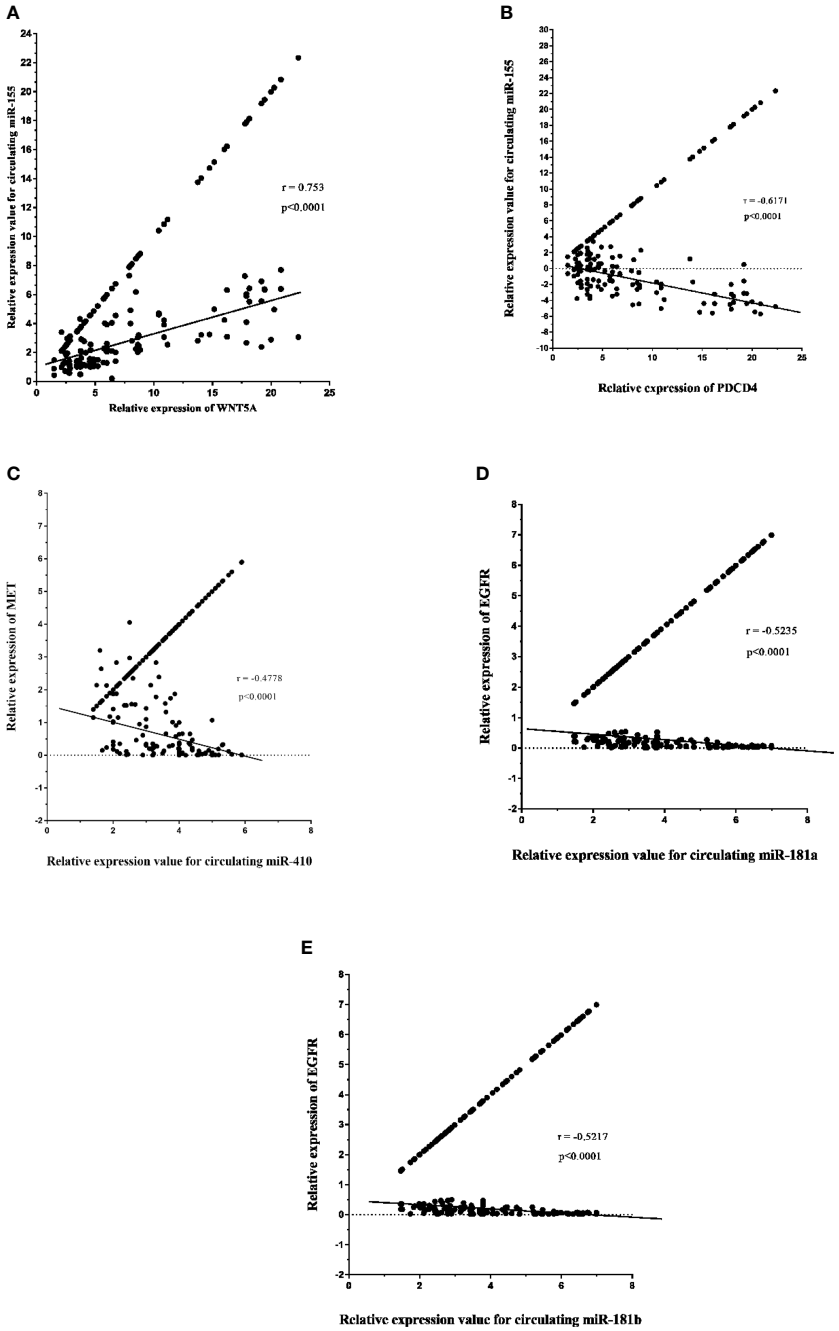

FIGURE 10 | Relative expression of circulating miR-181a/b, miR-410 and miR-155 and its potential gene-targets, programmed cell death 4 (PDCD4), Wnt family member 5A (WNT5A), MET, and epidermal growth factor receptor (EGFR), in plasma samples from high-grade glioma patients **(A–E)**. **(A, B)** Correlation between circulating miR-155 expression and PDCD4 and WNT5A levels in plasma samples ($p < 0.0001$; Spearman rank correlation test: $r = 0.753$, $r = -0.671$). **(C)** Correlation between miR-410 expression and MET levels in in plasma samples ($p < 0.0001$; Spearman rank correlation test: $r = -0.4778$). **(D, E)** Correlation between circulating miR-181a/b expression and EGFR levels in plasma samples ($p < 0.0001$; Spearman rank correlation test: $r = -0.5235$, $r = -0.5217$).

circulating miR-155 the expression levels and lower circulating miR-410 and miR-181a/b the expression levels showed a shorter OS in high-grade glioma patients. Wherein in the patients group with partial tumor resection with higher-expression of circulating miR-155 and lower-expression miR-410 and miR-181a/b had a poorer OS than in the patients with total tumor resection. To the best of our knowledge, the present study was the first comprehensive study on the expression and clinical significance of the expression levels of panel circulating miR-155, miR-410, and miR-181a/b in high-grade glioma patients.

Previous studies *in vitro* and *in vivo* have identified that miR-155, miR-410, and miR-181a/b is important miRNAs in regulating the oncogenesis in glioma, including high-grade gliomas. For instance, Wu et al. demonstrated that miR-155 could effectively accelerate proliferation, migration and invasion of U-87 MG cell line through targeting the PI3K/AKT signaling pathway (8). In other study, using data from The Cancer Genome Atlas (TCGA) dataset for clinical information of 480 glioblastoma samples had demonstrates that miR-155 could serve as prognostic and predictive biomarkers for survival of glioblastoma patients (17). Moreover, miR-155 highly expressed in glioma cells and demonstrated to regulate caspase-3 gene expression, were the target of PNA-based induction of apoptosis in the TMZ-resistant T98G glioma cell line (10). MiR-410, and miR-181a/b has tumor suppressive roles in glioma, and their reduced expression leads to abnormalities in cellular processes, such as an increase in apoptosis, enhanced cell growth, invasion and decreased sensitivity to radio- and chemotherapy through negative suppression of oncogene function (18–22). Wang et al., showed that miR-410 was significantly down-regulated in glioma tissues and in glioma cell lines (U87MG, SF126, LN229, and U251MG). These results indicate that decreased expression of miR-410 correlates with poor prognosis of glioma patients. In additional, the authors indicated that miR-410 exerts tumor-suppressing functions (inhibitory effects on tumor cell proliferation, migration, and invasion) in glioma by directly targeting mRNA 3′-untranslated regions (3′UTR) Ras-related protein 1A (RAP1A) (23). Wang et al. demonstrated that long non-coding RNA (lncRNA) colon cancer-associated transcript-1 (CCAT1) promoted U251 cell line proliferation and colony formation, induced the cell cycle arrest in G0/G1 phase and promoted the tumor cells apoptosis *via* inhibiting miR-410. In other words, these results indicated that miR-410 mediated the tumor-suppressive effects of CCAT1 knockdown on glioblastoma (24). Zhang et al. indicated that upregulation of miR-181b targets B-cell lymphoma 2 (Bcl-2) directly and may function as an important modifier to sensitize U87MG and U251 cells line to TMZ (25). Shi et al. demonstrated that miR-181a and miR-181b are low expression in human gliomas (WHO Grade 1-4) and glioma cell lines, U87, TJ905, and U251 (13). Moreover, miR-181a and miR-181b has great biological effect on tumor cells growth, proliferation, invasion and apoptosis, and may function as tumor suppressors in high-grade gliomas.

Given the roles of miR-155, miR-410, and miR-181a/b in the glioma oncogenesis, we used the online tools DIANA-microT, mirSVR, PicTar5, RNA22, RNAhybrid, TargetScan, PITA, MirTarget2, TargetMiner, miRanda, and TmiRWalk2.0 to predict the possible genes candidate as targets for these miRNAs. The selected eight genes, targeted by the miRNAs panel, were MET, PI3KCA, WNT5A, PDCD4, DFFA, EGFR, and FGFR1/2. As result, the current study showed that circulating miR-155 correlated positively with PDCD4 in plasma of high-grade glioma patients. In the same time, miR-155, miR-410, and miR-181a/b correlated negatively with WNT5A, MET, and EGFR in plasma of high-grade glioma patients. Latest reports have shown that these miRNAs by regulation PDCD4, WNT5A, MET, and EGFR also serves certain biological function in the progression of various human tumors including glioma. For instance,

These results further suggest that miR-155, miR-410, and miR-181a/b may play a significant role in the development and progression of glioma by regulation PDCD4, WNT5A, MET, and EGFR. Future studies *in vitro* and *in vivo* may address whether targeting miR-155, miR-410, and miR-181a/b and these potential gene-targets may provide a novel therapeutic strategy to suppress the progression of high-grade gliomas.

In summary, ours is the first study to systematically interrogate the clinical significance of panel circulating miR-155, miR-410, and miR-181a/b in glioma, and we provide comprehensive evidence that miR-155, miR-410, miR-181a/b may act as an oncomiR and tumor suppressive miRNAs, as well as a non-invasive diagnostic and prognostic biomarkers in gliomas, particularly high-grade gliomas.

5 CONCLUSIONS

We verified that increased the expression levels of circulating miR-155 and reduced of circulating miR-181a/b and miR-410 are a prospective candidate non-invasive biomarker for high-grade glioma diagnosis and prognosis. Moreover, miR-155, miR-181a/b and miR-410 may participate in the molecular mechanism of high-grade glioma by interaction PDCD4, WNT5A, MET, and EGFR. Confirming the potential role of miR-155, miR-181a/b and miR-410 in the oncogenesis of glioma and confirming it as non-invasive biomarkers in diagnosis and prognosis of high-grade glioma requires more clinical trials and *in vitro* and *in vivo* studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The approval was provided by the ethics committee of Federal Center of Neurosurgery (Tyumen, Russia). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization, Writing - original draft and Project administration: JW, AA-Z and OB. Writing - review and editing, Investigation and Resources: RS and IG. Formal

analysis and Methodology: SM and GS. Data curation: AS. Validation and Visualization: RT. Funding acquisition: JW. Supervision: AS. All authors have read and agreed to the published version of the manuscript.

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Overall Survival Signature of 5-Methylcytosine Regulators Related Long Non-Coding RNA in Hepatocellular Carcinoma

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Purpose: Studies reported that 5-methylcytosine (m5C) RNA transferase alters tumor progression; however, studies of m5C-related lncRNA remain lacking. This article intends to study the lncRNA modified by m5C RNA transferase in hepatocellular carcinoma using a combination of computational biology and basic experiments.

Method: We identified 13 m5C RNA transferase-related genes and selected long non-coding RNAs with a Pearson correlation coefficient greater than 0.4. Univariate Cox regression analysis was used to screen m5C RNA transferase lncRNA related to survival phenotype. We divided TCGA-LIHC into two types of m5C RNA using non-negative matrix decomposition. According to WGCNA, the co-expression models of two lncRNA regulation modes were constructed to analyze the characteristic biological processes of the two m5C RNA transferase-related lncRNA gene models. Then, a predictive model of m5C RNA transferase lncRNA was using LASSO regression. Finally, we used cell experiments, transwell experiments, and clone formation experiments to test the relationship between SNHG4 and tumor cell proliferation in Hep-G2 and Hep-3b cells line.

Results: We identified 436 m5C RNA transferase-related lncRNAs. Using univariate Cox regression analysis, 43 prognostic-related lncRNAs were determined according to $P < 0.001$. We divided TCGA-LIHC into two regulation modes of m5C RNA transferase using non-negative matrix factorization. The two regulation modes showed significant differences in overall and disease-free survival. We used LASSO to construct m5c-related lncRNA prognostic signature. Thus, a predictive m5C-lncRNA model was established using four lncRNAs: AC026412.3, AC010969.2, SNHG4, and AP003392.5. The score calculated by the m5C-lncRNA model significantly correlated with the overall survival of hepatocellular carcinoma. The receiver operating characteristic curve and

decision curve analysis verified the accuracy of the predictive model. We observed a more robust immune response in the high-risk score group. The transwell experiments and clone formation experiments suggested that m5C RNA transferase-related lncRNA SNHG4 promotes the proliferation and migration of Hep-G2 and Hep-3b cells line.

Conclusion: Two lncRNA expression patterns regulated by m5C RNA transferase were identified. The difference between the two expression patterns and the survival phenotype in the biological process was pointed out. A 5-methylcytosine RNA methyltransferases-related lncRNA overall survival signature was constructed. These results provide some understanding of the influence of m5C transferase on hepatocellular carcinoma. The prediction model of m5C transferase lncRNA has potential clinical value in managing hepatocellular carcinoma.

Keywords: 5-methylcytosine RNA methyltransferases, long non-coding RNA, weighted gene co-expression network analysis (WGCNA), liver hepatocellular carcinoma, prognosis model

INTRODUCTION

Hepatocellular cancer (HCC) is the sixth most common cause of malignant tumors. In 2020, there will be 900,000 new cases of stem cell cancer worldwide, making HCC the third leading cause of tumor-related death worldwide (1). HCC accounts for nearly 90% of primary liver cancers (2). Because the initial symptoms of HCC are not apparent, many patients are diagnosed with advanced liver cancer, hampering the success of treatment. In recent years, chemoradiotherapy for HCC has benefited patients with progressive disease; however, some patients remain with poor outcomes. Therefore, predicting the outcome of HCC patients by gene sequencing technology can assist clinicians in diagnosis and treatment strategies.

High heterogeneity is a significant feature of HCC. The primary characteristics of high heterogeneity are multiple genomic alterations and epigenetic modifications. Of these, epigenetic modifications are closely associated with tumor progression and metastasis and can be used as targets for cancer treatment. Epigenetics consists of the modification of DNA, RNA, and protein levels. Compared with the relatively limited spectrum of DNA modifications (six types), the abundance of RNA modifications is much higher. Post-transcriptional modification of RNA is an area of intense study. Of the 170 post-transcriptional modifications of RNA discovered to date, 2/3 are methylation modifications, including m1A, m6A, m5C, and m7G (3). Methylation of RNA 5-methylcytosine (m5C) is methylation at the fifth carbon atom of an RNA cytosine. This modification was discovered in rRNA in the 1970s and then successively in transport RNA, messenger RNA, and long non-coding RNA (lncRNA). M5C modification of RNA exists widely in cells and plays an essential role in regulating gene expression and RNA stability. In addition, m5C methylation is associated with proto-oncogene activation, and m5C modified methyltransferase NSUN2 is differentially expressed in tumor and para cancer tissues.

LncRNA is defined as a DNA transcript with no coding protein action over 200 bp in length (4), first proposed in a study of mouse cDNA sequencing (5). LncRNA is classified as lncRNA, antisense lncRNA, bidirectional lncRNA, intragenic lncRNA, and intergenic lncRNA, depending on its location in the genome (6). RNA methylation of lncRNA has been demonstrated in cancer progression. For example, in HCC, the m6A "writer" METTL3 increases the stability of LINC00958 and promotes cancer progression (7). Similarly, m6A "eraser" ALKBH5 increases the invasion and metastasis of gastric cancer tumor cells by inhibiting the methylation of NEAT1 (8).

In the present study, we analyze 5mC RNA methyltransferase-related lncRNA using computational biology and basic experiments to provide a basis for studying the heterogeneity of HCC.

METHODS

Expression Collection

The gene transcripts and clinical features of the tumor tissues of patients with HCC were obtained from TCGA (https://cancergenome.nih.gov/), including 374 samples of HCC tissues and 50 samples of normal adjacent tissues. The clinical characteristics of patients included gender, survival status, survival time, tumor stage, and TNM stage.

Screening for Differential m5C-Related lncRNA

The "EdgeR" program package in RStudio software used applied, and "FDR <0. 1, | log2FC |> 2" was the standard initially to screen the differentially expressed m5C related lncRNA. The "DEseq2" program package was used to identify differentially expressed m5C-related lncRNA according to "Padj < 0.05 and |log2FC| > 2."

Abbreviations: LIHC, Liver hepatocellular carcinoma; M5C, 5-Methylcytosine RNA methyltransferases; NMF, non-negative matrix factorization; WGCNA, Weighted correlation network analysis; LASSO, Least absolute shrinkage and selection operator; GSEA, Gene Set Enrichment Analysis; ESTIMATE, Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data; SSGSEA, single sample gene set enrichment analysis.

Negative Matrix Factorization (NMF) Clustering of m5C Related lncRNAs Gene Sets

Thirteen m5C-related genes were collected from literature mining (9–20). Based on Pearson coefficient >0.4 and cox coefficient P<0.001. The m5C related lncRNAs were uploaded as **Supplementary Table 1**—the 43 m5C related lncRNA genes for non-negative matrix dimensionality reduction clustering NMF. The non-negative matrix dimensionality reduction method was implemented using the "NMF" R package (21).

Weighted Correlation Network Analysis

A weighted standard expression network was constructed using the R language WGCNA package (22). The pickSoftThreshold function was used to obtain the optimal value of weighting parameters of adjacent parts, which was used as a soft threshold for subsequent network construction. Then, the weighted adjacency matrix was then constructed, and the related gene modules were built using hierarchical clustering based on the dissimilarity measure (1-Tom) of the topological overlap matrix (23). To determine the biological significance of each module, the potential correlation between genes and clinical traits was calculated using the characteristic genes of each module as the main component, and the expression patterns of genes of each module were summarized. Then, the correlation between the module significance and the average gene significance within the module was calculated. Finally, the correlation between the co-expression module and the expression pattern of NMF clustering subtypes was calculated.

LASSO Regression

The LASSO (24) regression algorithm was used to identify genes related to the outcome and survival of hepatocellular cancer patients and construct a risk-scoring model. The model's predictive performance was evaluated by the time-dependent receiver operating characteristic curve (ROC). Kaplan-Meier survival curves were used to compare survival differences of HCC patients between the two groups using the log-rank test.

GSEA

We used GSEA 4.1.0 software with the c2.cp.kegg.v7.0.symbols.gmt dataset in the Molecular Signature Database as the functional gene set to perform GSEA for patients in different risk groups (25). The iterative operations were set to 1000, and other parameters were set to default values.

The Proportion of Infiltrating Immune Cells in HCC

We used six methods to evaluate the relative proportion of immune infiltrating cells in the immune microenvironment, namely CIBERSORT (26, 27), EPIC (28), quanTIseq (29), MCPcounter (30), XCELL (31), and TIMER (32) algorithms to evaluate the immune response of different risk scores. We used Heatmap to analyze the differences in immune responses using the various algorithms.

The Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE) is an algorithmic tool. The detailed algorithm is shown in **Supplementary File 2**.

The Correlation Between Risk Score and Immune Inflammation Response

We selected several classic immune-related sub-gene sets, including primary histocompatibility complex class II, lymphocyte-specific kinase, hematopoietic cell kinase, immunoglobulin G, signal transduction, and activation transcription 1, costimulatory molecule, interferon, and TNF gene sets (33). Genes with concentrations are displayed in **Supplementary Table 3**. We analyzed the association between risk scores and the genes associated with immune responses.

Cell Culture

The Hep-G2 and Hep-3b cell line was provided by the Shanghai Cell Bank of the Chinese Academy of Sciences. Cells were cultured in a complete DMEM medium containing 10% fetal bovine serum and placed in an incubator at 37°C and 5% CO_2. Cells were seeded in 6-well plates at 4×10^5 cells per well, and we observed cell fusion after culturing overnight for subsequent experiments.

Cell Transfection Experiment

We selected Hep-G2 and Hep-3b cells in the logarithmic growth phase, trypsinized them, and seeded them in 6-well plates. After adherence, according to the lentivirus packaging manual, we transfected the cells with a multiplicity of infection of 10. After 24 hours, we added two μl of polybrene at a final concentration of 5 μg/ml for screening for 1–2 weeks, incubated at 37°C, and changed the medium once according to cell status 8–12 hours. We transferred the successfully transfected cells from each group to the cell flask and continued culturing to obtain stable cells. Cells were grouped as follows: Si-NC group, si1-SNHG4 group, and si2-SNHG4 group. Si-SNHG4F: GTCAGCGAGCGAA CCCAATTGGC; R: CCGATCGGCAGCCGCGCGCGA.

RT-PCR Detection of SNHG4 Gene Expression

We extracted the total RNA from each group of cells after transfection and reverse transcribed the RNA into cDNA according to kit instructions. We designed the primer sequence and used the cDNA containing the amplified sequence as a template for PCR reaction. After the response, the results of each group were recorded, and GAPDH was used as an internal control to compare and analyze the expression of SNHG4 in each group. SNHG4 F: CCGCCGATAGGAGCGACACCCC AAC, R: AACCATCGAGCGGGGGCTCTCGCAAA.

Clone Formation Experiment to Observe the Effect of SNHG4 Gene on the Proliferation of Hep-G2 and Hep-3b Tumor Cell Line

After the cells were transfected, we transferred cell suspensions to 1.5 mL Eppendorf tubes, mixed and diluted, and inoculated 6-well plates at 20,000/well. We changed the medium once every three days and cloned for about ten days to observe the

formation of cloning groups. The medium was then aspirated, and the cells were washed and fixed in 4% paraformaldehyde for 15 min, followed by staining with 0.1% crystal violet for 15 min. Finally, cells were washed, dried, and photographed to count clonal cell clusters and perform statistical analysis.

Transwell Method to Observe the Effect of SNHG4 Gene on the Migration of Hep-G2 and Hep-3b Cells

We added 200 μL of HepG2 cell line suspension (1×10^4 cells) to the upper chamber of the Transwell chambers. The experiment was divided into regular cell group (si-NC), SNHG4 gene knockdown 1 group (si1-SNHG4), and SNHG4 gene knockdown 2 Group (si2-SNHG4). Cells were placed in a 37 °C incubator for 24 hours, after which the upper chamber was removed and washed with PBS three times. Cells were then fixed with paraformaldehyde for 20 minutes, stained with 0.1% crystal violet for 30 minutes after air-drying, and we randomly selected five fields under the microscope for counting. The number of cells and the ratio of the number of penetrating cells between the experimental and control groups represent cell migration changes. The Hep-3b cells line was tested using the HepG2 cell line.

Western Blotting

First, we used precooled RIPA buffer containing protease inhibitor (Thermo Scientific, USA) (Beyotime, Shanghai, China) to extract total protein from cells. Equivalent amounts of protein samples were isolated with 4-12% SDS-Page (GenScript, Nanjing, China) and then transferred to 0.45μm PVDF membrane (Millipore, USA). The membrane was sealed with TBST containing 5% skim milk for two h and incubated with primary antibody at four °C overnight. After washing with TBST 3 times, the antibody was coupled with HRP and incubated for one h at room temperature. Immunoblots were detected by an imaging system (Bio-Rad, USA) using an enhanced chemiluminescence detection kit (Servicebio, Wuhan, China). Western blots were performed using an imaging system (Bio-RAD, USA) using an enhanced chemiluminescence assay kit (Servicebio, Wuhan, China). The primary antibodies consisted of beta-catenin (Proteintech, 51067-2-AP), cyclin D1 (Cell Signaling Technology, 55506S), and GAPDH (Cell Signaling Technology, 5174S). The above antibodies are used in accordance with manufacturer's agreement and instructions

RESULTS

The Molecular Subtypes of lncRNA Regulated by m5C in HCC Based on the NMF Classification Method

The flow chart of the article is shown in **Figure 1A**. The coxph function in R was used to evaluate the predictive value of m5C-regulated lncRNA. According to the Pearson correlation coefficient greater than 0.4, we identified 436 m5C-related lncRNAs (**Figures 1B, C**). Then, according to the standard of single-factor Cox regression $P < 0.01$, we obtained 436 cancer outcome-related m5C-regulated lncRNA genes. The significance and risk ratio of m5C-regulated lncRNA significant genes are shown in **Figure 2A**. We then performed non-NMF on these prognostic-related hepatocellular cancer lncRNA-related genes using 50 iterations. We conducted nine clusters; the number of collections k was 2-10, and the minimum sample of each group was set to 10 using the 'NMF' R package. According to three parameters (cophenetic, dispersion, and silhouette), we choose the ideal cluster group to be 2 (**Figures 2B, C**). We found that patients with different lncRNA gene expression patterns showed differences in overall survival and disease survival rates (**Figure 2D**; log-rank $p = 0.01$).

WGCNA Gene Co-Expression Network Analysis to Identify the Biological Characteristics of Different lncRNA Groups

We included the protein-coding genes and clinical samples in HCC into a WGCNA input file. In the subsequent investigation, we followed the omics cluster analysis to include samples with similar expression patterns. According to the cut-off value of 10000 and the β-value setting at 5, the gene in the smallest module is set to 30, and 18 co-expression modules are finally obtained (**Figures 3A, B**). The C1 lncRNA feature group strongly correlates with the brown module (**Figure 3C**; Cor = 0.58). We enriched the genes in the brown module that were associated with greater than 0.4 with the C1 group and found that the genes in the brown module were involved in the biological processes of oxidative phosphorylation and ATP metabolic process (**Figure 3D**). The lncRNA feature group of the C2 group had the strongest correlation with the yellow module (**Figure 3C**; Cor = 0.28). We enriched the genes in the yellow module that correlate greater than 0.4 with the C2 group. We found that yellow genes in the module were involved in the small molecule catabolic process, carboxylic acid catabolic process, and cellular amino acid metabolic process (**Figure 3D**).

Construction of HCC Outcome Model Based on lncRNA-Related Prognostic Genes

First, we randomly divided the entire TCGA-LIHC queue into training and validation sets. We arranged them in ascending order according to the ID of the sample and used SPSS to assign a random number to each sample for classification. The classification results satisfy the following criteria: 1) the two groups were similar in age distribution, clinical staging, follow-up time, and patient mortality; and 2) the gene expression profiles of the two randomized data sets were similar. Then we used LASSO regression to construct the lncRNA-related outcome model. First, we used the 43 prognostic-related m5C-related lncRNAs obtained above as input data and regression based on the overall survival rate as clinical follow-up data. This number of genes is not conducive to clinical detection. Therefore, to reduce the range of m5C-related lncRNAs while maintaining high accuracy, the R package glmnet was used to perform LASSO regression analysis with the trajectory of each independent

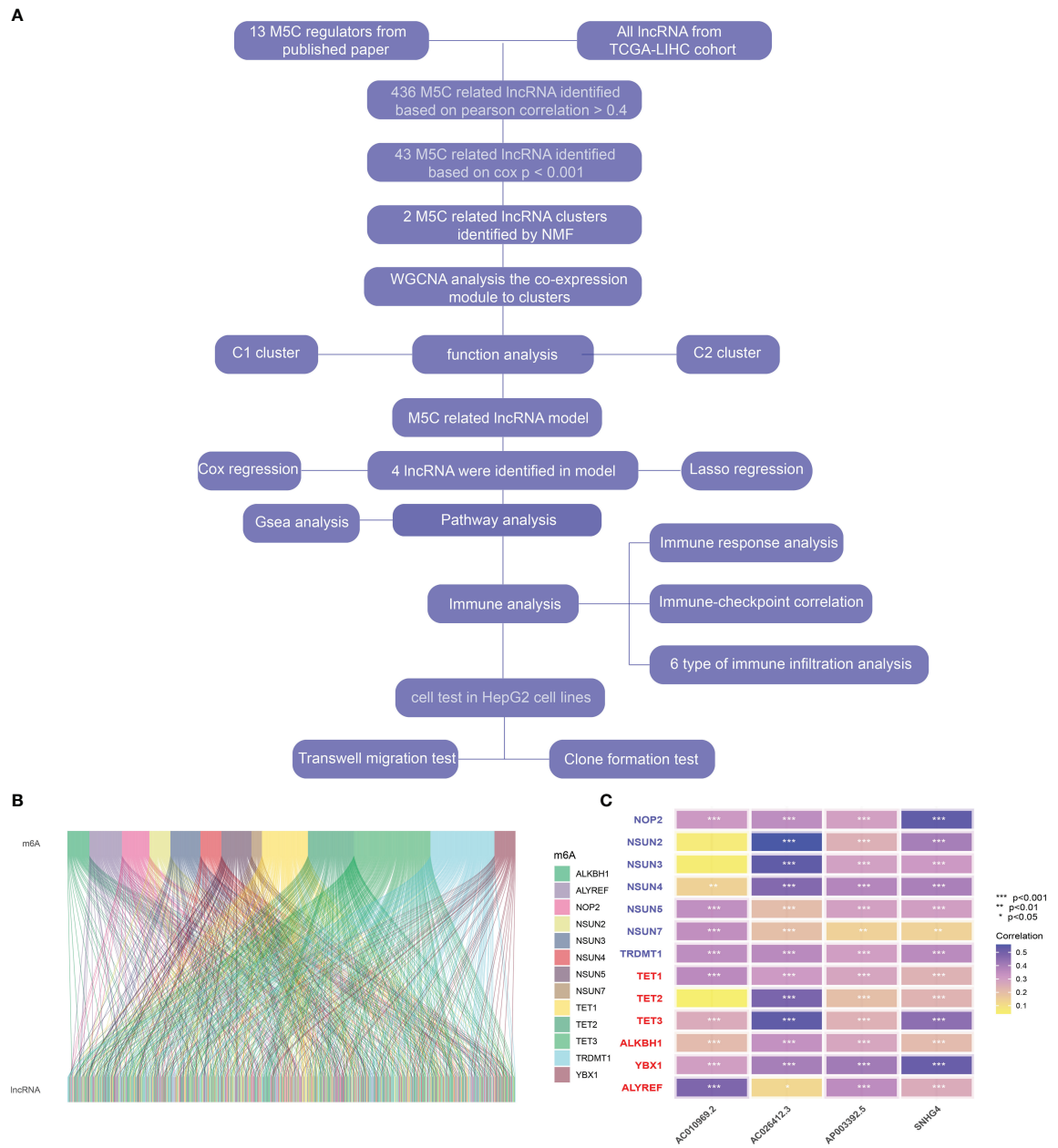

FIGURE 1 | **(A)** Flow chart of this study. **(B)** Correlation between m5C correlated genes and lncRNA in hepatocellular carcinoma. **(C)** Heatmap of correlation between m5C-related genes and four prognostic m5C-related lncRNAs * represents p value < 0.05, ** represents p value < 0.01, *** represents p value < 0.001, ns means not statistically significant.

variable (**Figure 4A**). As the lambda increased, the independent coefficients also gradually increased, and the same was obtained for the independent coefficients. Three-fold cross-validation was used to build the model and analyze the confidence interval under each lambda. Finally, we constructed a predictive risk model containing the four genes. RiskScore = 0.75 * expAC026412.3 + 0.13 * expAC010969.2 + 0.15 * expSNHG4 + 0.33 * expAP003392.5 We calculated the RiskScore according to the expression level of the gene, and obtained the RiskScore distribution of the sample (**Figure 4B**). The death rate of the high-risk samples was significantly higher than that of the samples with a low-risk score, indicating that the samples with high RiskScore had a worse outcome. We divided the RiskScore into high- and low-risk groups and drew Kaplan-Meier curves; there was a significant difference between the two (**Figure 4C**). We used ROC to classify RiskScore. We analyzed the 1.3 and 5-year forecast classification efficiency. The 5-year AUC area was 0.612, the 3-year AUC area was 0.636, and the 1-year AUC area was 0.746. Finally,

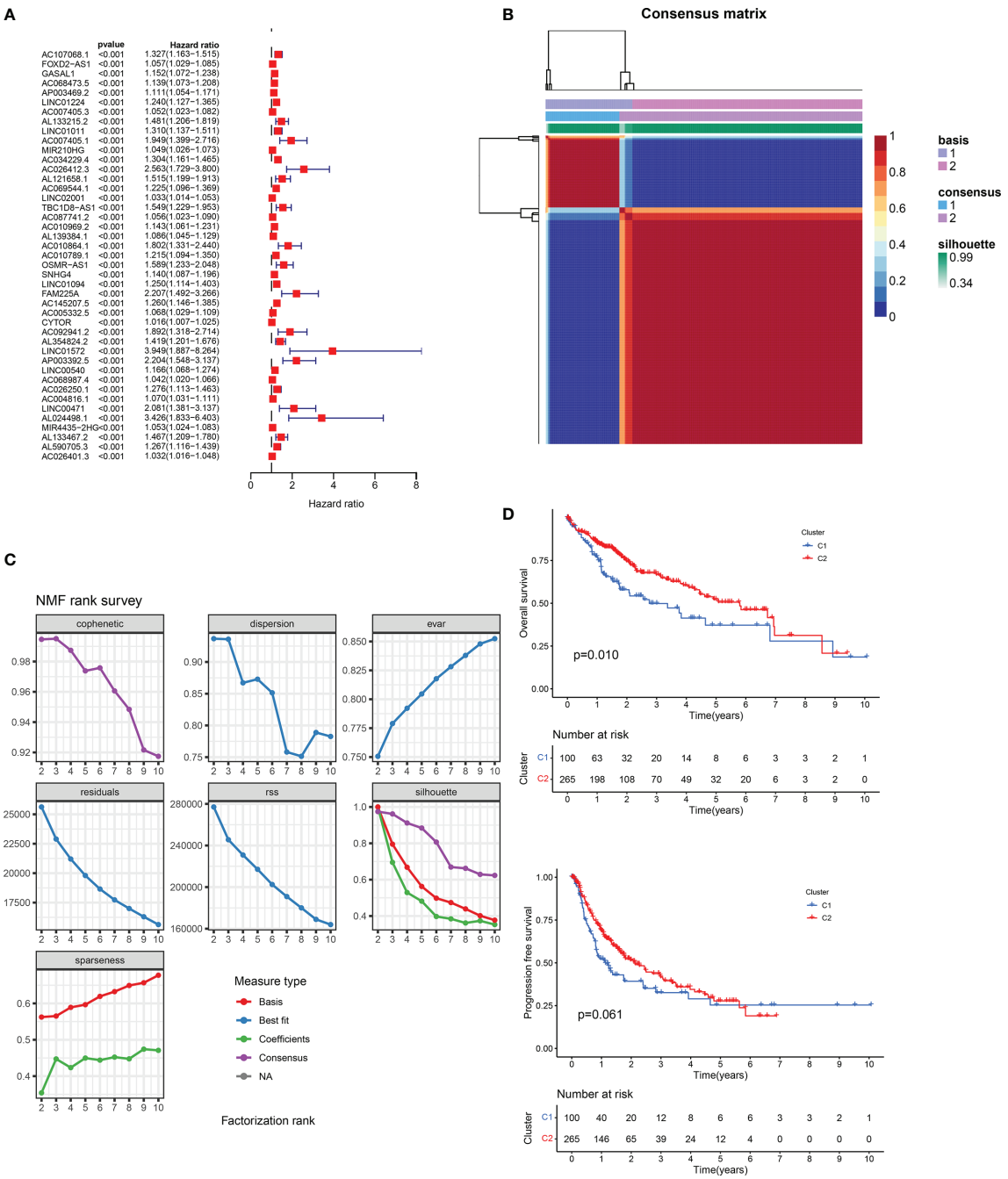

FIGURE 2 | **(A)** Univariate Cox regression analysis of prognostic m5C-related lncRNAs. **(B)** Consensus map of non-negative matrix factorization clustering. **(C)** Consensus clustering parameter. **(D)** Overall survival and disease-free survival prognostic survival curves of two molecular subtypes. NA, Not application.

the variables in the model were used as independent prognostic factors to assess patient risk (**Figure 4D**).

Evaluation of Model Results

We drew Kaplan-Meier curves for risk scores in the training and validation sets and found a significant difference between the high- and low-risk groups in the training and validation sets (**Figure 5**). We analyzed the prediction classification efficiency of risk scores 1, 3, and 5 years in the training and validation sets (**Figure 5**). The 5-year AUC area in the training set was 0.629, the 3-year AUC area was 0.658, and the 1-year AUC area was 0.771. In the verification set, the 5-year AUC area was 0.578, the 3-year AUC area was 0.608, and the 1-year AUC area was 0.692. We included risk scores into different subgroups, such as age, stage, and others. We grouped

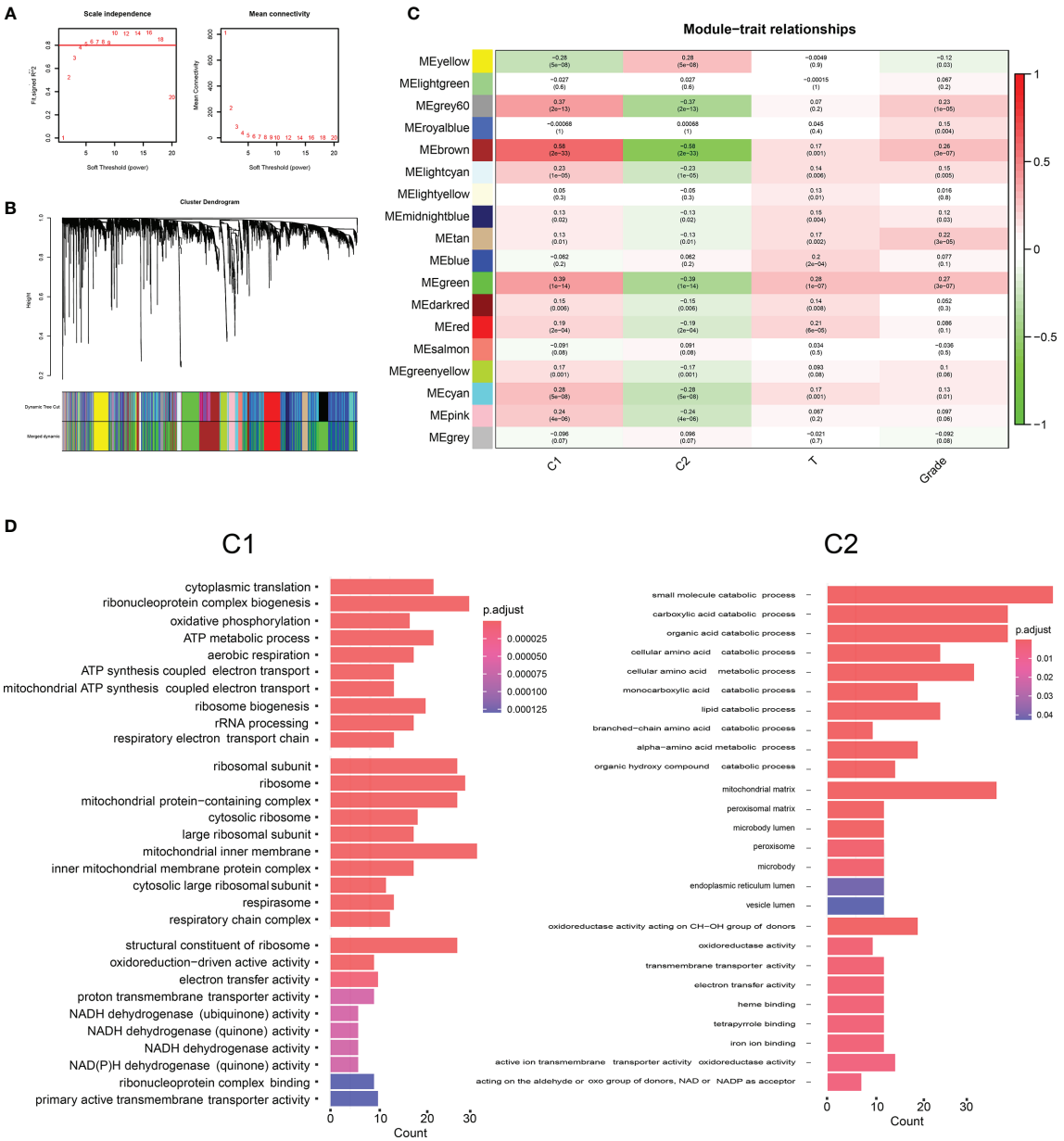

FIGURE 3 | **(A)** Network topology analysis for different soft threshold powers. **(B)** A hierarchical clustering tree was constructed, with each leaf representing a gene and each branch representing a co-expression module. A total of 18 co-expression modules were generated. **(C)** The correlation coefficients between two molecular phenotypes, T stages, grade, and co-expression modules. **(D)** The primary enrichment biological pathways of co-expression modules of two molecular types.

them according to subgroup indicators to evaluate the prognostic assessment ability of risk scores in the various subgroups (**Figure 6A**). The risk score distinguished patients with different outcomes in the whole cohort and patients in groups with characteristics such as age, stage, and others (**Figure 6A**). We then compared the area under the AUC curve of the nomogram, RiskScore, age, and staging and found that the area under the curve of the risk score in the training set was the largest, with the AUC area in the training set 0.749 (**Figure 6B**).

GSEA Analysis

We performed GSEA analysis in high- and low-risk patients to determine pathways related to the patient's prognostic risk. As shown in **Figure 6C**, in patients with high-risk scores of HCC, cell cycle, cytokine-cytokine-receptor interaction, ECM receptor interaction, and other tumor-related pathways were enriched. In patients with low-risk scores of HCC, butanoate metabolism, fatty-acid metabolism, and tryptophan metabolism were enriched in several tumor metabolism-related pathways.

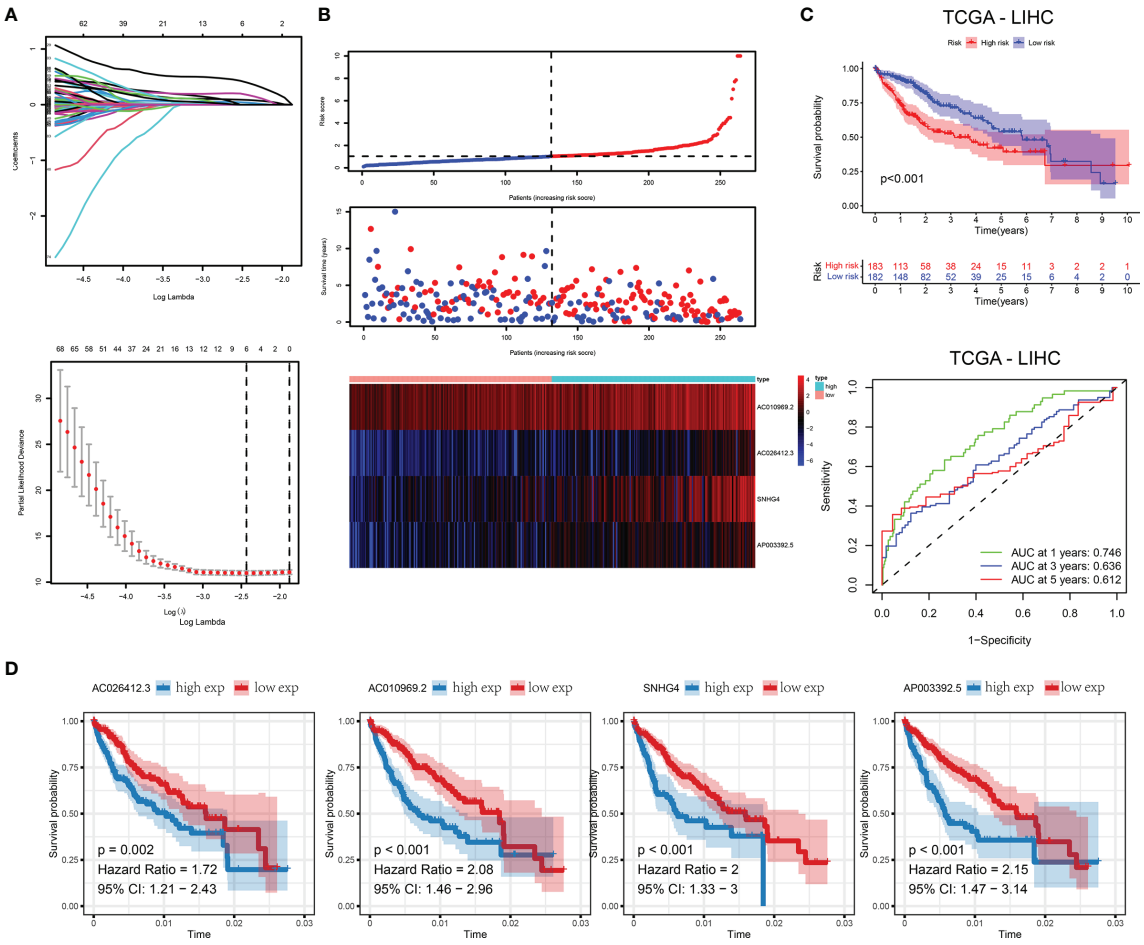

FIGURE 4 | **(A)** Trajectories for each independent variable and confidence intervals for different values. **(B)** Distribution of RiskScore and survival status of 4-gene signature. **(C)** In the training set, the survival curve of the four-gene signature and the ROC curves of 1, 3, and 5 years. **(D)** Kaplan-Meier survival curves of four genes.

The Relationship Between Risk Score and Immune Microenvironment

The risk score positively correlated with inflammatory and immune responses. These reactions were induced by hematopoietic cell kinases, immunoglobulin G, interferon, lymphocyte-specific kinase, primary histocompatibility complex class I, major histocompatibility complex class II, and activator of transcription 1. Patients with higher risk scores had more clustered immune-inflammatory responses **(Figure 7A).**

The Relationship Between Risk Score and Immune Infiltration

The relationship between the level of immune cell infiltration and risk score evaluated based on the six methods of CIBERSORT, EPIC, quanTIseq, MCPcounter, XCELL, and TIMER is shown in **Figure 7B**. We found significant differences in the level of infiltration of macrophages and $CD8^+$ T cells in different RiskScore groups.

The Effect of Knocking Out the SNHG4 Gene on the Clone Formation Ability of Hep G2 and Hep-3b Cells

The above paper constructed a predictive scoring model based on M5C methylation-related long non-coding RNA. The predictive scoring gene model contained four long non-coding RNAs: AC026412.3, AC010969.2, SNHG4, and AP003392.5. SNHG4 has been extensively studied in several cancers. Long Non-Coding RNA SNHG4 was a biomarker in Non-Small Cell Lung Cancer in colorectal cancer (34, 35). However, there are not enough studies on the effect of SNHG4 on liver cancer, and we used a cell assay to analyze the impact of SNHG4 in liver cell carcinoma. Clone formation experiments showed that the number of clones formed by Hep G2 and Hep-3b cell lines in the si1-SNHG4 and si2-SNHG4 groups after culture and staining was significantly lower than that of the si-NC group ($P < 0.05$) **(Figures 8A, B).**

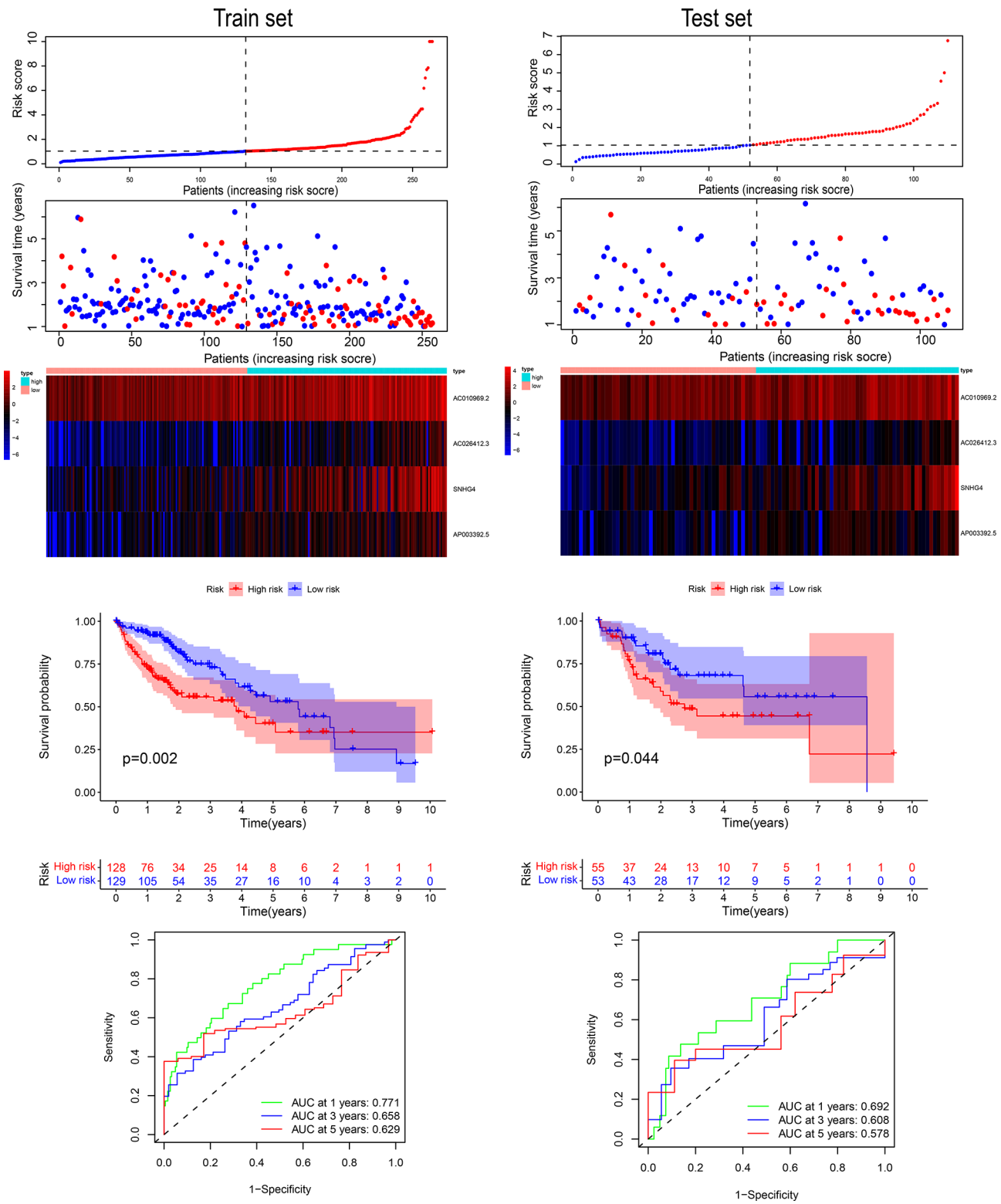

FIGURE 5 | The four-gene signature's risk score distribution and survival status in the training and validation sets. ROC curves of 1, 3, and 5 years, and survival curves between the two risk groups based on the four-gene signature classification.

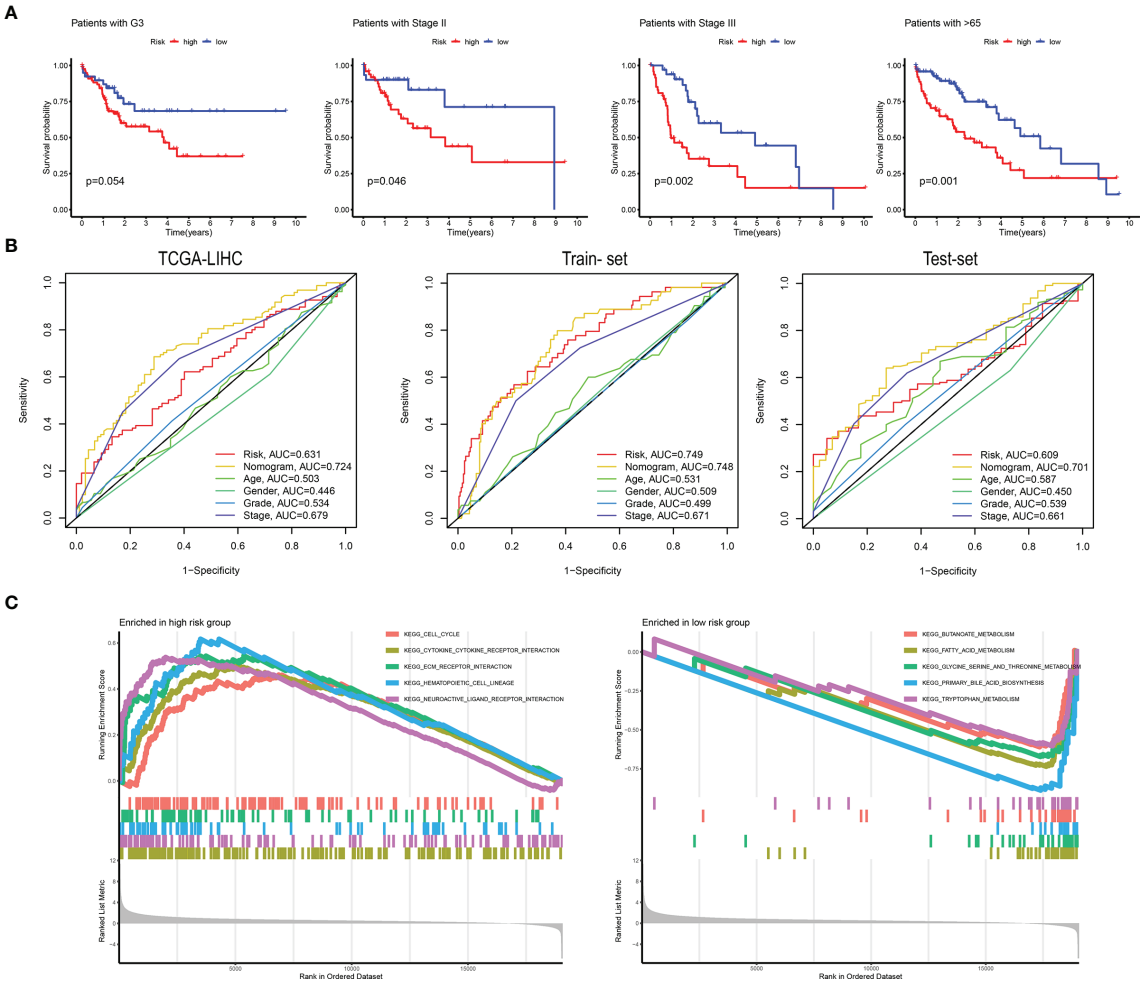

FIGURE 6 | **(A)** The high-risk and low-risk groups were divided according to G3, Stage II, Stage III, and 65 years. Survival curves were plotted. **(B)** ROC curves are grouped by risk, nomogram, age, sex, grade, and stage in TCGA-LIHC, training set, and validation set. **(C)** The main KEGG pathways are enriched in the high- and low-risk groups.

Transwell Assay

Compared with the si-NC control group, the number of Hep-G2 and Hep-3b cells passing through the Transwell chamber in the Si1-SNHG4 and si2-SNHG4 groups was significantly lower, suggesting SNHG4 promotes the migration of Hep-G2 and Hep-3b cells ($P < 0.05$) **(Figures 8C, D)**.

Knocking SNHG4 Affects Wnt Signaling Pathways

After pathway enrichment analysis in the TCGA-LIHC cohort, SNHG4 was found to be closely associated with liver cancer progression. To verify the specific effect mechanism of SNHG4 on liver cancer, hepatocyte carcinoma cell lines of HEP-G2 and HEP-3B cells with SNHG4 knockdown cell lines were used for Western blotting analysis. The results showed that the expression level of cyclin D1 and β -catenin protein in the SNHG4 knockout group was significantly lower than that in the negative control group (NC) **(Figures 9A, B).** Three repeated experiments demonstrated that knocking down SNHG4 down-regulated the WNT signaling pathway and affected the expression of cyclin D1.

DISCUSSION

The mechanism of m5C methylation modification of lncRNA is unclear; therefore, we attempted to comprehensively analyze lncRNA related to m5C methylation modification using computational biology. The m5C methylation modification of RNA is dynamically regulated by methyltransferase and demethyltransferase. Under the action of methyltransferase, RNA undergoes m5C modification and then combines with recognition protein to exert specific biological functions (3). The methyltransferases modified by m5C include NSUN1, NSUN2, NSUN3, NSUN4, NSUN5, NSUN7, and DNMT2. The point is a structurally conserved cysteine residue that catalyzes m5C methylation in various types of RNA with the

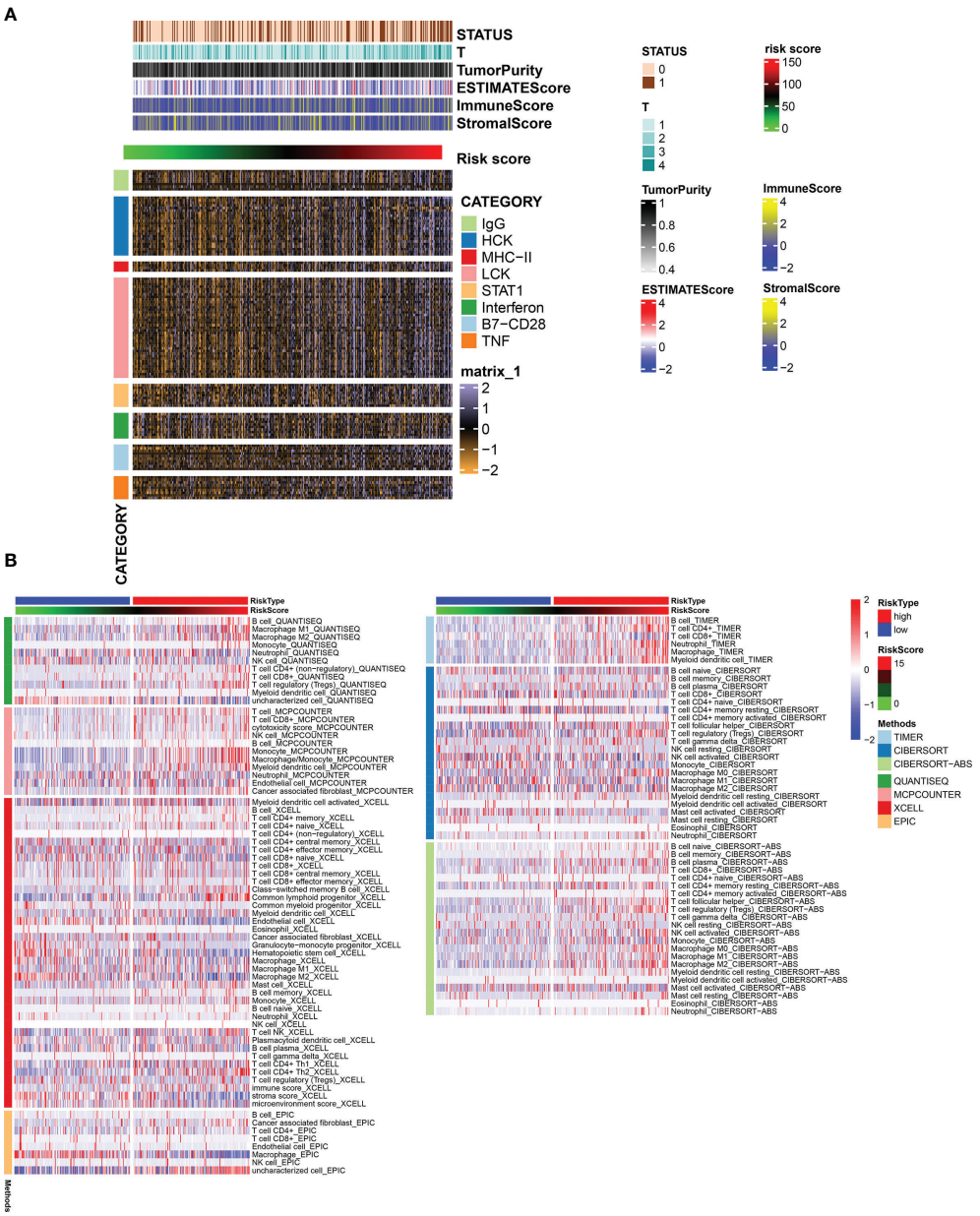

FIGURE 7 | **(A)** Heatmap for genesets associated with immune and inflammation. **(B)** Heatmap for immune responses based on TIMER, CIBERSORT, CIBERSORTE-ABS, QUANTISEQ, MCPCOUNTER, XCELL, and EPIC algorithms among high- and low-risk groups.

help of the methylated donor S-adenosine-L-methionine. Therefore, we focused on the lncRNA, regulated by the aforementioned transferases.

It is believed that m5C methyltransferase regulates lncRNA in liver cancer. The role of m5C methylation in the occurrence and progression of cancer has been identified in liver cancer, including mRNA, microRNA, lncRNA, and other types of RNA. The m5C modification of RNA plays an essential regulatory role in the occurrence and progression of tumors (13). The m5C methyltransferase NSUN4 recognizes protein ALYREF associated with liver cancer outcomes. A study found that expression levels of H19 lncRNA in cancer tissues were significantly higher than those in adjacent tissues. Other studies found that this effect was due to the m5C modification on H19 lncRNA mediated by NSUN2, which increases the stability of H19 lncRNA. H19 lncRNA with m5C change specifically binds to G3BP1 protein, further leading to the accumulation of oncoprotein and promoting the occurrence and progression of liver cancer (36).

We identified SNHG4 as an m5C methylation modification lncRNA in the present study. SNHG4 encodes small nucleolar RNA host gene 4. Some lncRNAs encode small nuclear RNA host

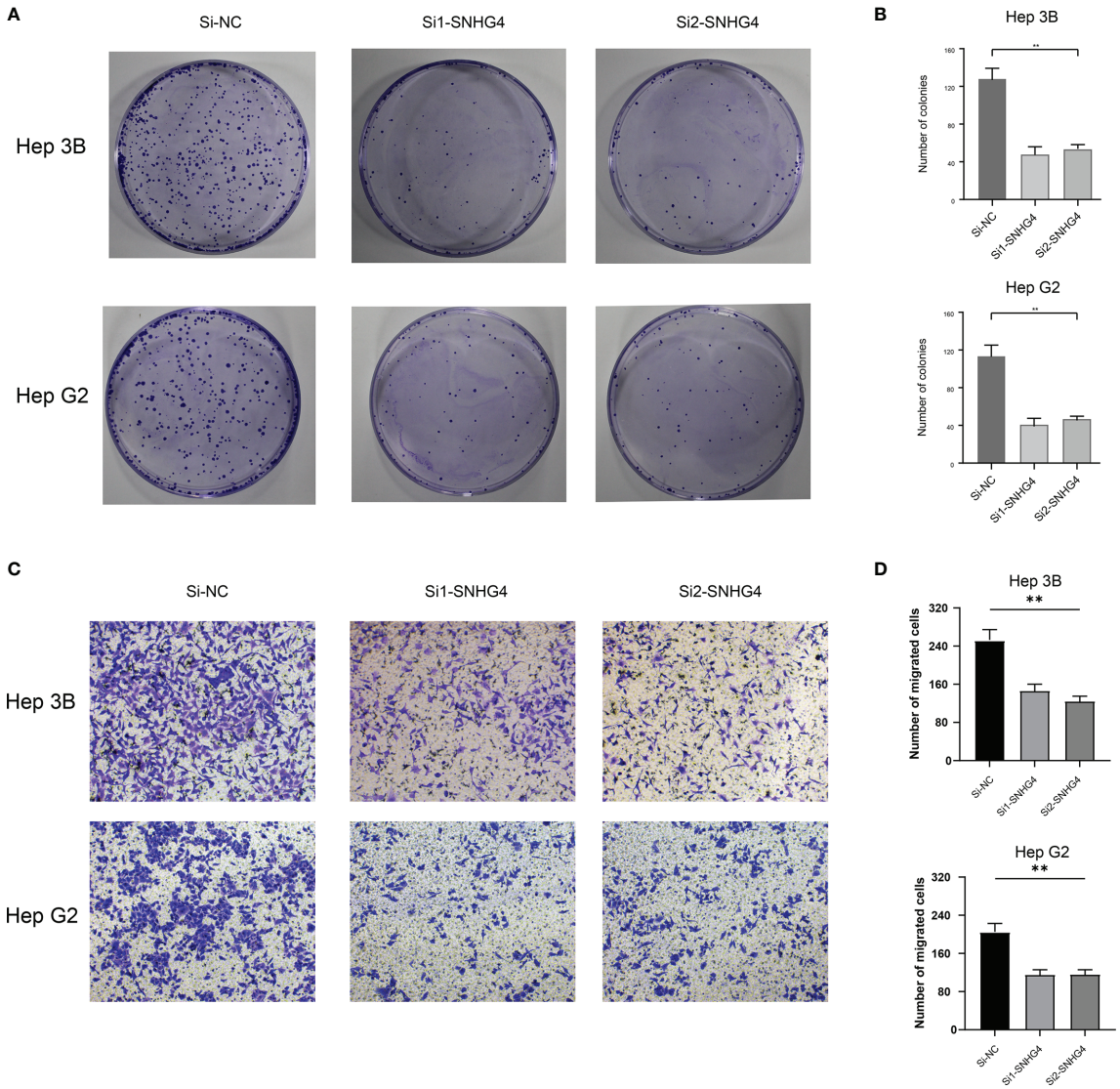

FIGURE 8 | **(A, B)** Clone formation assay. **(C, D)** Transwell assay. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.

genes. In recent years, many studies have found that the abnormal expression of snRNAs may play the role of oncogenes in the development of tumors. For example, Chen found that SNHG8 was upregulated in non-small cell lung cancer (37). Other investigators found that the expression trend of SNHG8 in glioma and liver cancer was consistent with these studies (38–40).

There are few in-depth studies discussing the predictive value of SNHGs. SNHG1, SNHG3, and SNHG20 are predictive biomarkers for neuroblastoma (41), ovarian cancer (42), and colorectal cancer (43), respectively. Zhu et al. conducted a bioinformatics analysis of lncRNA and found that SNHG4 may be a valuable prognostic marker in HCC (40). In the present study, we reached the same conclusion that the expression of SNHG4 was an independent predictor of poor outcomes in HCC. We further studied the predictive value of SNHG4 in the subgroups and found its limitations in women and young patients, which may help direct precision therapy.

There are some limitations to our paper. We only analyzed lncRNAs associated with m5C transferase in TCGA; more sequencing cohorts are needed to validate our findings. This paper only conducted a comprehensive analysis of m5C related lncRNA and did not include a complete regulatory mechanism study.

CONCLUSION

We immediately identified 436 m5C transferase-related long non-coding RNAs and 43 prognostic-related lncRNAs related to m5C transferase. Four lncRNA were determined by LASSO regression to reduce the screening range further. Finally, we found that SNHG4

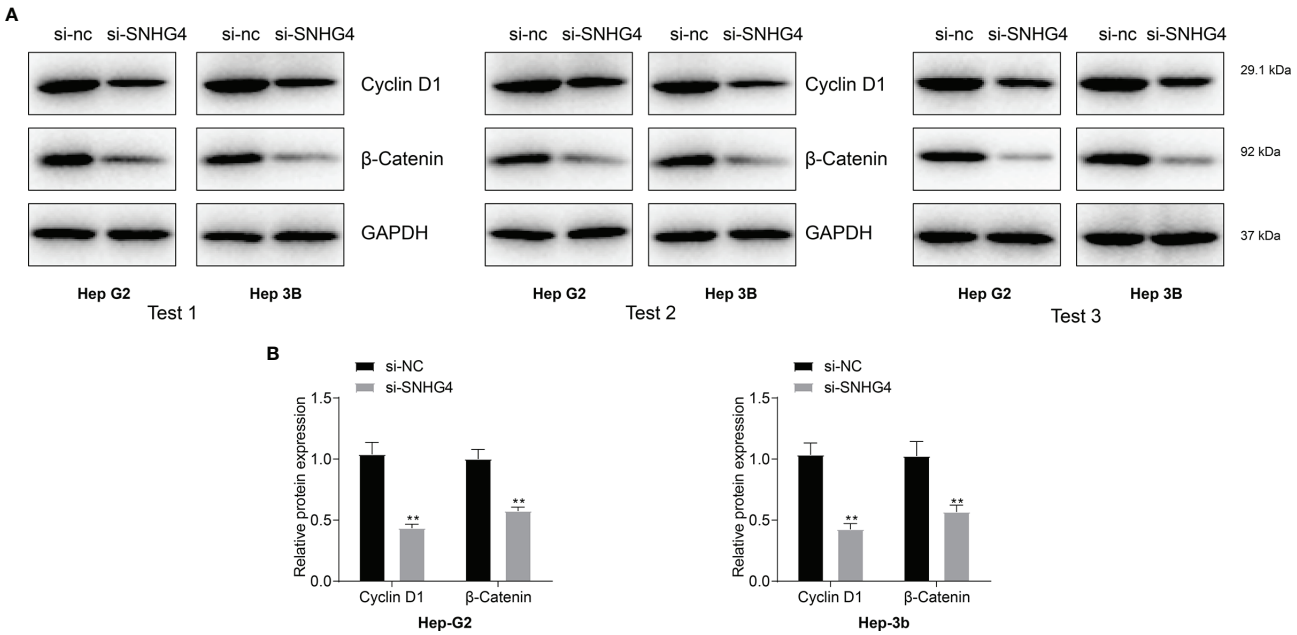

FIGURE 9 | **(A)** Protein levels of WNT signaling pathway-related molecules (β-Catenin and cyclin D1) were measured using western blotting in HEP-G2 and HEP-3B cell lines. **(B)** Knocking down SNHG4 down-regulated the WNT signaling pathway and affected the expression of cyclin D1. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.

was significantly associated with the protein-coding gene of m5C methyltransferase. Cell experiments showed that knocking down SNHG4 affected the proliferation and migration of HCC. This comprehensive analysis of lncRNA regulated by m5C transferase provides a basis for future research on the methylation regulation of long-chain non-coding RNA.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The datasets TCGA-LIHC for this study can be found at http://cancergenome.nih.gov/.

AUTHOR CONTRIBUTIONS

QP wrote the manuscript. CY and YZ revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.884377/full#supplementary-material

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Long Intergenic Noncoding RNA00265 Enhances Cell Viability and Metastasis *via* Targeting miR-485-5p/USP22 Axis in Osteosarcoma

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Osteosarcoma is one of the bone malignancies in children and adolescents. Long noncoding RNAs (lncRNAs) have been demonstrated to participate in osteosarcoma development and progression. Linc00265 has been shown to involve in osteosarcoma oncogenesis; however, the underlying mechanism is largely unclear. In this study, we investigated the function of linc00265 in osteosarcoma cells, including cell viability, migration and invasion. Moreover, we elucidated mechanistically the involvement of linc00265 in osteosarcoma. We found that linc00265 overexpression promoted viability, migration and invasion of osteosarcoma cells. Notably, linc00265 sponged miR-485-5p and increased the expression of USP22, one target of miR-485-5p, in osteosarcoma cells. Strikingly, linc00265 exerted its oncogenic function *via* regulating miR-485-5p and USP22 in osteosarcoma. Taken together, targeting linc00265 is a promising approach for treating osteosarcoma patients.

Keywords: Linc00265, osteosarcoma, miR-485-5p, USP22, viability

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INTRODUCTION

Osteosarcoma is one of the common bone malignancies, which often happens in children and adolescents (1, 2). The standard therapeutic strategy is surgery in combination with chemotherapy for osteosarcoma patients (3–5). Notwithstanding the treatment and diagnostic approaches have been improved, some osteosarcoma patients still have worse prognosis due to resistance to chemotherapy and distant metastasis (6–8). Hence, more investigations need to determine the mechanism of osteosarcoma carcinogenesis and discover the new promising agents for improving the efficiency of treatment outcome in osteosarcoma patients.

LncRNA is one type of noncoding RNA without protein encoding functions, which has more than 200 nucleotides (9). LncRNAs affect the cellular functions *via* modulating gene expression at multiple regulatory levels, such as transcriptional and post-transcriptional levels (10, 11). LncRNAs have been demonstrated to play a necessary role in a number of types of cancers, including osteosarcoma (12–15). Evidence has revealed that linc00265 is critically involved in carcinogenesis and tumor progression in multiple type cancers (16). For example, studies have shown that the

expression of linc00265 was increased in bone marrow and serum of acute myeloid leukemia (AML) patients, which was associated with poor overall survival (16, 17). Linc00265 regulated proliferation, migration and invasion *via* activation of phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway in AML cell lines (17). Moreover, linc00265 overexpression increased autophagy and attenuated apoptosis of AML cells *via* sponging miR-485-5p and subsequent upregulation of interferon-regulatory factor 2 (IRF2) (16). Similarly, another study also showed that linc00265 expression was elevated in peripheral blood and bone marrow in leukemia patients (18).

Studies have demonstrated that linc00265 downregulation blocked colorectal cancer (CRC) oncogenesis *via* enhancement of zinc finger MIZ-type containing 2 (ZMIZ2) expression due to sponging several miRNAs, including miR-375, miR-30c-2-3p, miR-324-3p and miR-130-3p, leading to upregulation of ubiquitin specific peptidase 7 (USP7) and stabilization of β-catenin (19). Moreover, Ge et al. showed that linc00265 knockdown attenuated the expression of epidermal growth factor receptor (EGFR) in CRC cells, resulting in suppression of proliferation, invasion and induction of apoptosis in CRC cells (20). Furthermore, linc00265 enhanced glycolysis and lactate production *via* binding to miR-216b-5p and elevating the expression of tripartite motif containing 44 (TRIM44) in CRC (21). Clinically, higher expression of linc00265 was correlated with poorer prognosis in CRC patients, indicating that linc00265 might be an independent prognostic marker (21, 22). In lung adenocarcinoma, linc00265 was uncovered to interact with miR-7 and subsequently upregulate fibroblast growth factor 2 (FGF2), contributing to lung cancer tumorigenesis (23). The role of linc00265 in osteosarcoma has not been fully investigated. Thus, we aimed to determine the function and molecular insight of linc00265 in osteosarcoma progression.

Ubiquitin-specific protease 22 (USP22) can act as a deubiquitinating enzyme and exhibit its implication in oncogenesis due to regulation of proliferation, cell cycle, apoptosis, cancer stemness and chemoresistance (24). USP22 is abnormally expressed in several cancer types and facilitates tumor malignant progression. For example, USP22 can stabilize the E2F6 stability and activate Akt pathway in hepatocellular carcinoma (HCC), leading to aggressive progression of HCC (25). USP22 inhibits HER2-mediated breast cancer aggressiveness *via* reducing the unfolded protein response (26). Moreover, USP22 regulates necroptosis in tumor cells *via* governing receptor-interacting protein kinase 3 (RIPK3) stability (27). USP22 expression was increased in osteosarcoma tissues and linked to osteosarcoma progression (28). The detailed mechanism of USP22-mediated osteosarcoma is still elusive.

In the present study, we investigated the expression and biological functions of linc00265 in osteosarcoma cells. Moreover, we further explored the molecular mechanism of linc00265-mediated carcinogenesis in osteosarcoma. Our study demonstrated that linc00265 promoted cell viability, migration and invasion *via* targeting miR-485-5p/USP22 axis in osteosarcoma, suggesting that linc00265 might be a useful target for osteosarcoma therapy.

MATERIALS AND METHODS

Cell Culture

The osteosarcoma cell lines, MG63 and U2OS, were cultured in Dulbecco's modified eagle medium (DMEM) medium with 10% fetal bovine serum (FBS). SAOS-2 cell line was cultured in McCoy's 5A modified medium with 10% FBS. The human osteoblast (HOB) cells were cultured in MEM-F12 medium with 10% FBS. The cells were maintained in the presence of 5% CO_2 at 37°C.

CCK-8 Cell Viability Assay

The cell counting kit-8 (CCK-8) kit (Beyotime, Shanghai, China) was used to measure viability of osteosarcoma cells after different treatments. Osteosarcoma cells were seeded on 96-cell plates and incubated in medium for 24, 48, and 72 hours, respectively. Then. 10 μl CCK-8 reagent was added and incubated for 3 hours at 37°C. The OD values were measured at 450 nm by a microplate reader (Sunnyvale, CA, USA).

Colony Formation Assay

The treated osteosarcoma cells were seeded into 6-well plates and maintained with 5% CO_2 at 37°C in a humidified incubator for 14 days. The culture media was removed and the cells were fixed with 4% paraformaldehyde for half hour after the cells were washed by PBS. Then, 0.1% crystal violet was added to stain the cell colony for 15 minutes. Finally, we counted the number of cell colony.

Quantitative Real-Time Reverse Transcription-PCR Analysis

The treated osteosarcoma cells were harvested and total RNA was extracted using TRIzol agent. The mRNA was reverse-transcribed by the cDNA Reverse Transcription Kit (Thermo Fishes, USA) following the manufacturer's instructions. Then, qRT-PCR was conducted using SYBR Green PCR Master Mix Kit as described previously (REF). The primers are: USP22, forward primer, 5'-AGC CAA GGG TGT TGG TCG CG-3', and reverse primer, 5'-ACT GCC ACC ACG CCC GAA AG-3'. GAPDH, forward primer, 5′- ACC CAG AAG ACT GTG GAT GG -3′; reverse primer, 5′- CAG TGA GCT TCC CGT TCA G- 3′.

Western Blotting Analysis

The treated osteosarcoma cells were harvested and lysed in a lysis buffer containing protease inhibitor cocktail. The protein concentration was measured by the bicinchoninic acid (BCA) assay. The protein was determined by SDS-PAGE and probed with antibodies against USP22 (SC-390585, Santa Cruz, USA, 1:1000) and tubulin (#T9028, Sigma-Aldrich, St. Louis, MO, USA, 1:5000) as described previously (29). The quantitative results were analyzed by ImageJ software (NIH, USA).

Transfection Assay

Osteosarcoma cells were seeded into 6-well plates and transfected with different plasmids by Lipofectamine 2000 as

described previously (29). USP22-specific shRNA, USP22 lentiviral particles, miR-485-5p mimics, miR-485-5p inhibitors and control vectors were provided by GenePharma Company (Shanghai, China). After cells were transfected for 48 hours, the cells were subjected to analysis for cell viability, migration and invasion, which were described under the result sections.

Wound Healing Assay

The treated osteosarcoma cells were seeded on 6-well plates for overnight. Then, we created a wound *via* scratch approach by a 100 µl pipette tip after cell confluence reached to higher than 90%. The wound area was photographed at 0 hour and 20 hours using an inverted microscope, respectively. The distance of wound closure was analyzed by ImageJ software.

Transwell Invasion Assay

The treated osteosarcoma cells were seeded on the upper layer of 24-well inserts (Corning Incorporated, NY, USA) with 200 µl serum-free medium. The membrane of the upper layer was precoated with Matrigel. In addition, 500 µl complete medium with 10% FBS were added in the under layer. After 20 hours incubation, the invaded cells through the membrane were stained by 4 µg/ml Calcein AM. The invaded cells were photographed by a microscope.

Luciferase Report Assay

The wild-type or mutant binding sequence of miR-485-5p in linc00265 or USP22 3'UTR was sub-cloned into pmirGLO dual-luciferase vector. The dual luciferase report assay system (Promega, Madison, WI, USA) was utilized to measure luciferase activity as described previously (30).

Statistical Analysis

Data are presented as mean ± SEM. Two-way ANOVA followed by Tukey's test was conducted for comparison among multiple groups. Student's *t*-test was used for comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Linc00265 Overexpression Promotes Viability of Osteosarcoma Cells

Several studies have uncovered that linc00265 exerts tumor promotive functions in a number of cancer types. We are wondering whether linc00265 upregulation could inhibit viability of osteosarcoma cells. First, we used real-time RT-PCR analysis to detect the expression of linc00265 in normal human osteoblasts (HOB) and osteosarcoma cells (U2OS, MG63 and SW1353). The results showed that linc00265 was highly expressed in osteosarcoma cell lines compared with HOB cells (**Figure 1A**). To investigate the biological function of linc00265 in osteosarcoma cells, we transfected linc00265 plasmid and shR-linc00265 into MG63 and U2OS cells. As shown in **Figure 1B**, MG63 and U2OS cells after transfection with linc00265 plasmid exhibited higher expression of linc00265 compared with pcDNA3 transfection (**Figure 1B**). Osteosarcoma cells treated with shR-linc00265 plasmid displayed the lower expression of linc00265 compared with PSilencer transfection in both MG63 and U2OS cell lines (**Figure 1B**). Next, we aimed to explore the cell viability in MG63 and U2OS cells after linc00265 modulation by CCK-8 assay. The results from CCK-8 assay clearly demonstrated that downregulation of linc00265 attenuated cell viability in U2OS cells and MG63 cells (**Figure 1C**). In line with this finding, upregulation of linc00265 facilitated viability of osteosarcoma cells in both osteosarcoma cell lines (**Figure 1D**). Moreover, colony formation assay was performed to examine the ability of colony formation in both MG63 cells and U2OS cells after linc00265 changes. We observed that downregulation of linc00265 suppressed colony formation capacity in U2OS cells, while overexpression of linc00265 stimulated colony formation ability of MG63 cells (**Figure 2A**). Taken together, linc00265 governs cell viability and colony formation in osteosarcoma cells.

Linc00265 Overexpression Enhances Invasion and Migration of Osteosarcoma Cells

Studies have reported that linc00625 regulates migratory and invasive capacities in a spectrum of cancers. Here, we explored the invasiveness and migration of osteosarcoma cells after linc00625 overexpression and depletion. We observed that linc00265 plasmid transfection facilitated cell invasion in MG63 and U2OS cells (**Figure 2B**). Consistently, shR-linc00265 transfection reduced the cell invaded numbers through Matrigel membrane in both osteosarcoma cell lines (**Figure 2B**). Next, wound healing assays were conducted to examine the motility of osteosarcoma cells after linc00265 upregulation or downregulation. As demonstrated in **Figure 3**, increased linc00265 promoted closure in wound area in osteosarcoma cells. In contrast, decreased linc00265 retarded closure of wound area in MG63 and U2OS cells (**Figure 3**). The wound healing data indicated that linc00265 could control migratory and invasive capacity in osteosarcoma cells.

Linc00265 Sponges miR-485-5p in Osteosarcoma Cells

It has been known that linc00265 often sponges specific miRNAs to regulate its downstream genes. The potential miRNAs that might bind with linc00265 were predicted using starBasev2.0. From this database, we predicted that miR-485-5p could be a sponging miRNA of linc00265 because this lncRNA has a position interacting with miR-485-5p (**Figure 4A**). We transfected miR-485-5p mimics and inhibitors into osteosarcoma cells and observed that miR-485-5p expression was elevated in mimics-treated group, while miR-485-5p levels were downregulated in inhibitor-treated group (**Figure 4B**). To validate whether the miR-485-5p is the downstream target of linc00265, the luciferase reporter assay was performed using a wild-type or mutant target site from linc00265. We found that miR-485-5p mimic decreased luciferase activity in the wild-type linc00265 reporter, but not in mutant linc00265 reporter (**Figure 4C**). At the same time, we uncovered that the

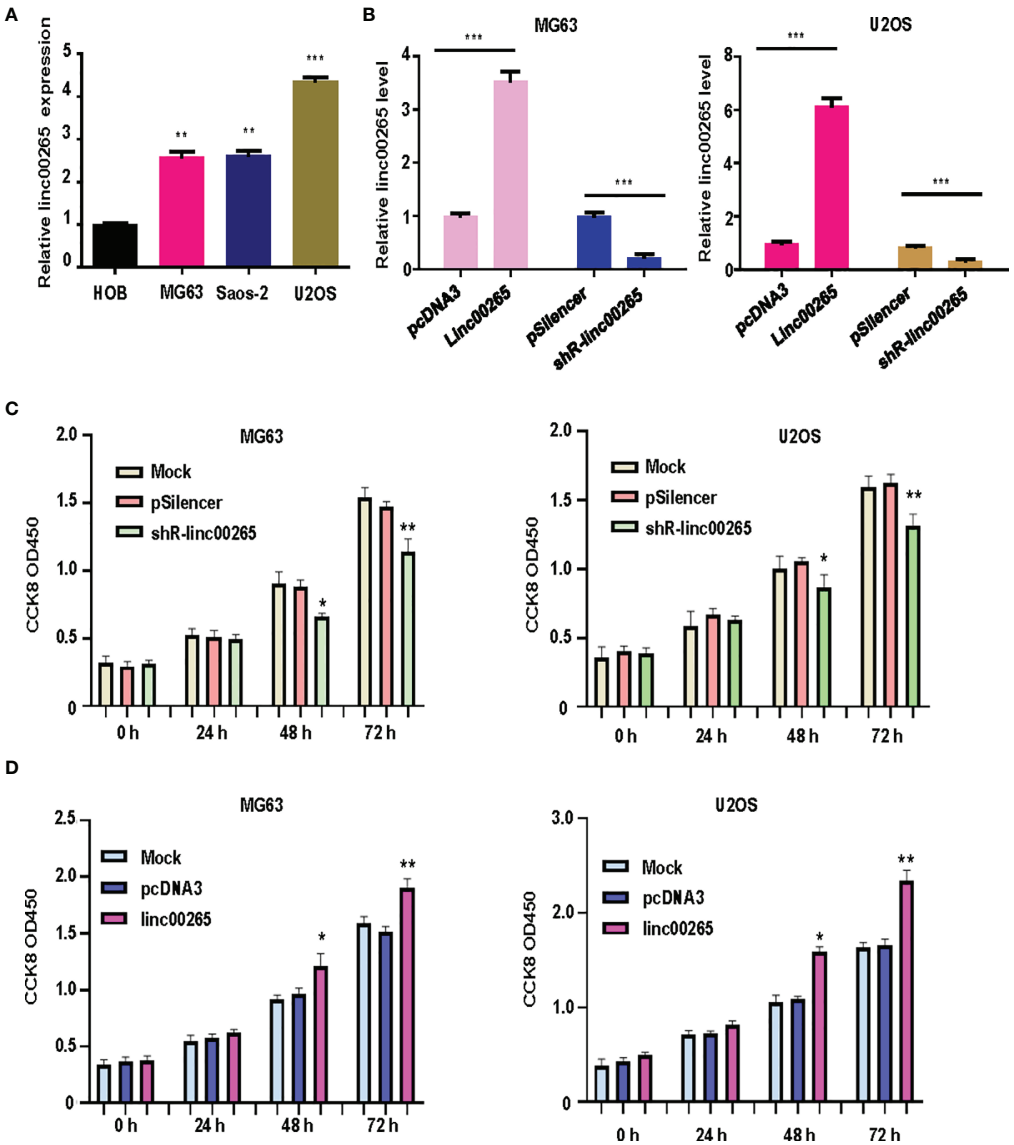

FIGURE 1 | Linc00265 overexpression promotes viability of osteosarcoma cells. **(A)** Real-time RT-PCR was utilized to examine the expression levels of linc00265 in HOB cells and three osteosarcoma cell lines, including MG63, SaOS-2 and U2OS. **(B)** Real-time RT-PCR was performed to examine linc00265 expression levels in MG63 and U2OS cells after various plasmid transfections. **(C)** CCK-8 assay was conducted to detect the viability of osteosarcoma cells after linc00265 knockdown for 24 h, 48 h, and 72 h. **(D)** CCK-8 assay was utilized to detect the cell viability in linc00265-overexpressing osteosarcoma cells for 24 h, 48 h, and 72 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

expression levels of miR-485-5p were decreased in osteosarcoma cells after linc00265 overexpression (**Figure 4C**). Meanwhile, the levels of miR-485-5p were elevated in osteosarcoma cells after linc00265 depletion. Therefore, linc00265 might target miR-485-5p in osteosarcoma cells.

MiR-485-5p Mimics Inhibits Cell Viability, Which Is Abrogated by linc00265

To evaluate the association between miR-485-5p and linc00265 in osteosarcoma cells, MG63 and U2OS cells were transfected with miR-485-5p mimic, miR-485-5p inhibitor and matched controls. The data from CCK-8 assay suggested that miR-485-5p mimics repressed viability of osteosarcoma cells (**Figure 4D**). Meanwhile, miR-485-5p inhibitors facilitated cell viability in both MG63 and U2OS cells. Moreover, miR-485-59-mediated effect on cell viability was abrogated by linc00265 overexpression in osteosarcoma cells (**Figure 4D**). To further confirm this phenotype, colony formation assay was utilized in osteosarcoma cells after co-transfection of miR-485-5p mimics and linc00265 plasmid. The results indicated that miR-485-5p mimics attenuated colony formation ability of MG63 cells, which was rescued by linc00265 overexpression (**Figure 4E**).

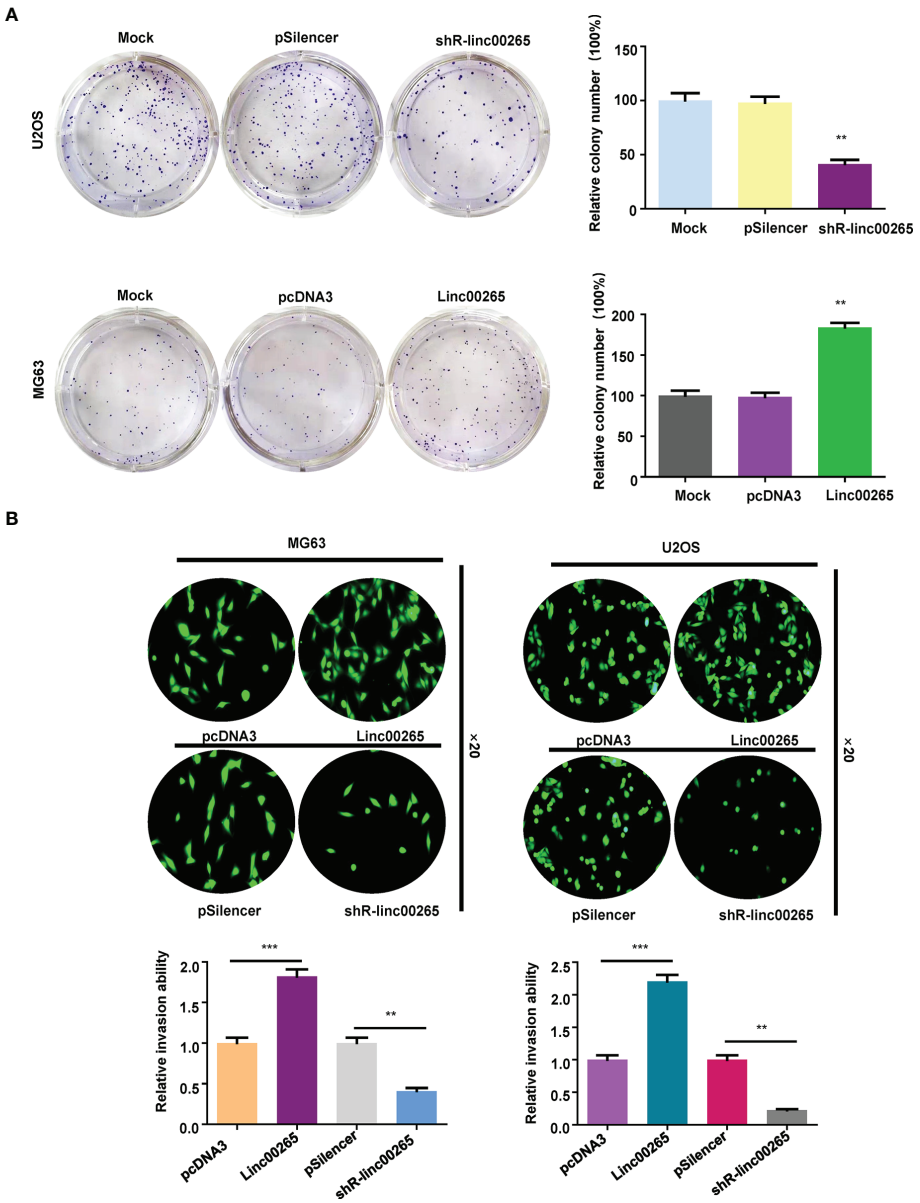

FIGURE 2 | Linc00265 overexpression promotes colony formation and invasion of osteosarcoma cells. **(A)** Colony formation assay was used to detect the ability of colony formation in osteosarcoma cells after linc00265 downregulation (U2OS cells, Top) and upregulation (MG63 cells, Bottom). The quantitative data were illustrated (Right panel). **(B)** Transwell invasion assays were conducted in MG63 and U2OS osteosarcoma cells after linc00265 overexpression or downregulation. The quantitative data of invasion ability were shown (Bottom panel). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

Consistently, miR-485-5p inhibitor treatment enhanced colony formation capacity of U2OS cells, which was abolished by shR-linc00265 infection (**Figure 4E**).

USP22 Is a Direct Target of miR-485-5p

Evidence has demonstrated that miRNAs regulate the expression of targets due to that miRNAs contain special sequences that are complementary to downstream targets. The TargetScan database was utilized to predict the downstream targets of miR-485-5p. We found that USP22 3'UTR has the complementary binding sites with miR-485-5p (**Figure 5A**). Luciferase reporter results revealed that miR-485-5p mimic decreased luciferase activity for the WT USP22 reporter, but not for mutant USP22 reporter. Meanwhile, miR-485-5p inhibitor increased luciferase activity for the WT USP22 reporter (**Figure 5B**). RT-PCR was performed to examine the expression of USP22 mRNA in osteosarcoma cells after miR-485-5p mimic treatment or inhibitor exposure. As demonstrated in **Figure 5C**, miR-485-5p mimic treatment reduced the USP22 mRNA expression in U2OS cells, while

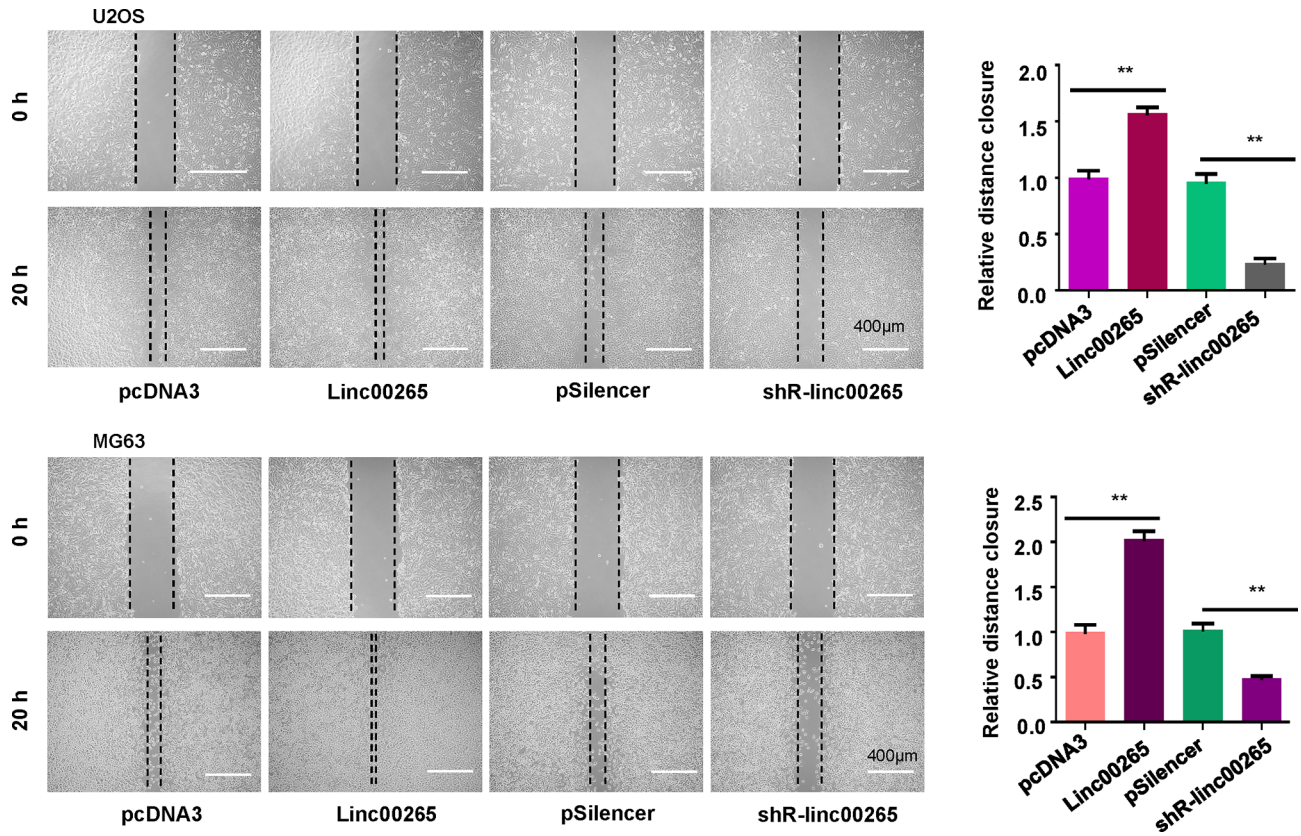

FIGURE 3 | Linc00265 overexpression promotes migration of osteosarcoma cells. Wound healing assay was utilized to examine the cell migratory ability in U2OS and MG63 osteosarcoma cells after linc00265 modification (Left panel). The quantitative data of wound healing assays were demonstrated (Right panel). $^{**}P < 0.01$ vs. control.

miR-485-5p inhibitor enhanced the USP22 mRNA levels in MG63 cells. Western blotting data further validated that miR-485-5p overexpression reduced the expression of USP22 protein, whereas miR-485-5p downregulation increased the USP22 protein levels in osteosarcoma cells (**Figure 5D**). To check whether linc00265 could regulate the expression of USP22, RT-PCR and western blotting analysis were utilized to test the expression of USP22 in osteosarcoma cells after linc00265 changes. Our results suggested that linc00265 upregulation increased the expression of USP22 at mRNA and protein levels, and linc00265 downregulation attenuated the USP22 expression levels in osteosarcoma cells (**Figures 5E, F**).

Linc00265 Exerts Its Functions *via* miR-485-5p/USP22 Axis

Lastly, we examined whether miR-485 and USP22 were involved in linc00265-mediated oncogenic function in osteosarcoma cells. MG63 cells were transfected with miR-485-5p or USP22 plasmid or linc00265 plasmid or shR-USP22 or combination treatments. Then, CCK-8 assay, colony formation, wound healing assay, and Transwell invasion assays were done to determine cell viability, colony formation ability, migratory capacity and invasive ability in osteosarcoma cells, respectively. CCK-8 data showed that USP22 overexpression accelerated viability of osteosarcoma cells, which was rescued by miR-485-5p upregulation (**Figure 6A**). Overexpression of linc00265 increased cell viability in MG63 cells, which was abolished by downregulation of USP22 (**Figure 6A**). In line with this result, USP22-mediated promotion of colony formation was abrogated by overexpression of miR-485-5p. Moreover, linc00265-induced colony formation was rescued by inhibition of USP22 in osteosarcoma cells (**Figure 6B**). Furthermore, USP22-induced cell migration and invasion were abolished by miR-485-5p overexpression in osteosarcoma cells (**Figures 7A, B**). Linc00265-triggered motility of osteosarcoma cells was blocked by upregulation of miR-485-5p in osteosarcoma cells (**Figures 7A, B**). Altogether, linc00265 exerted its functions in part *via* regulation of miR-485-5p/USP22 axis.

DISCUSSION

A line of evidence uncovered that linc00265 plays an essential role in tumorigenesis. Yang et al. revealed that linc00265

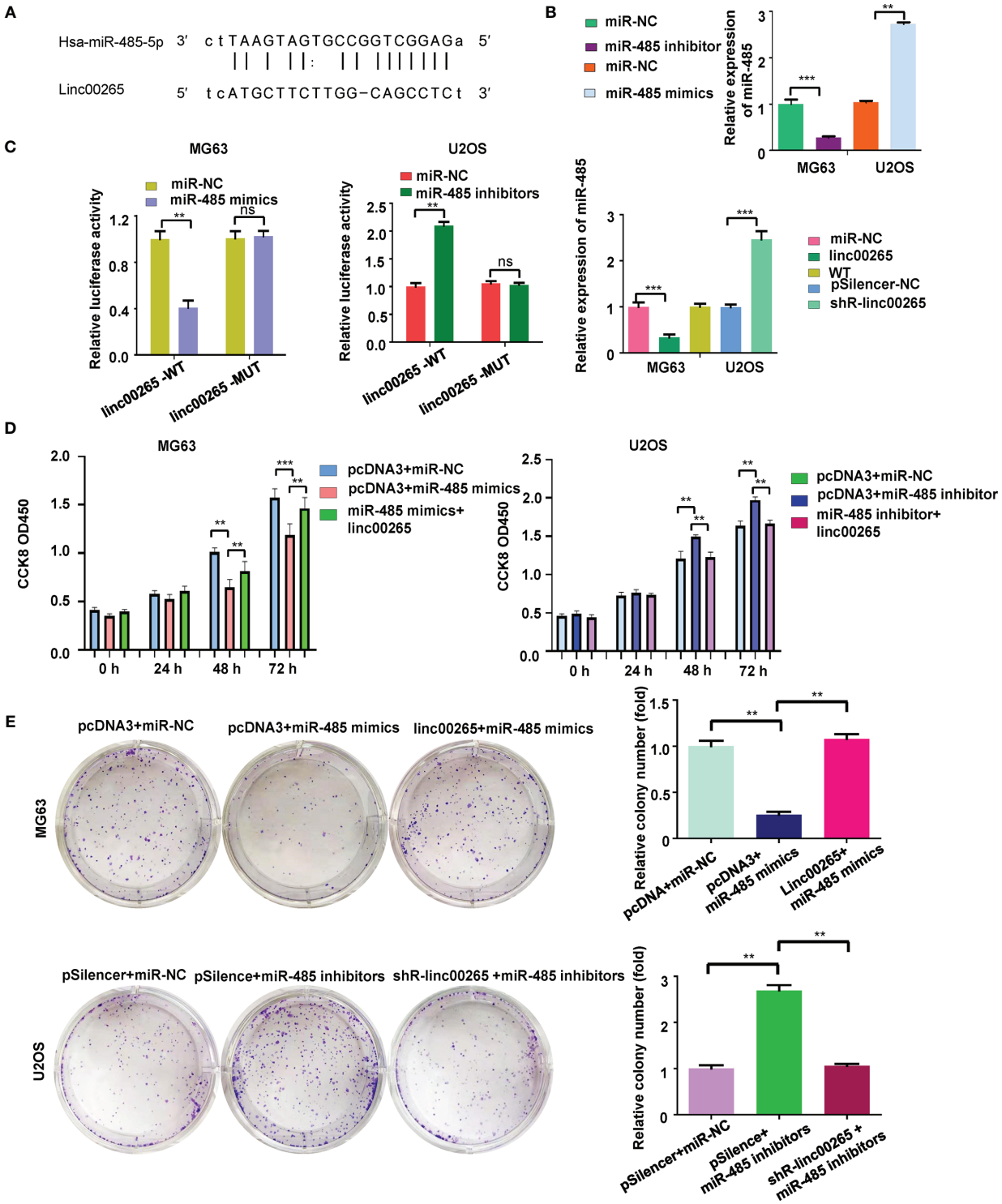

FIGURE 4 | Linc00265 sponges miR-485-5p in osteosarcoma cells. **(A)** The target gene of linc00265 was predicted by starbase database. **(B)** The efficacy of transfection was confirmed by qRT-PCR in MG63 and U2OS osteosarcoma cells. **(C)** Luciferase reporter assay was used to confirm miR-485-5p as a target of linc00265 (Left and middle panels). The expression of miR-485 was detected by qRT-PCR in MG63 and U2OS osteosarcoma cells after linc00265 changes (Right panel). **(D)** Cell viability was examined by CCK-8 assay in MG63 and U2OS osteosarcoma cells after linc00265 modification and miR-485-5p changes. **(E)** The colony formation ability was measured in MG63 and U2OS osteosarcoma cells after linc00265 modification and miR-485-5p changes (Left panel). The quantitative data of colony formation ability were demonstrated for colony formation (Right panel). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. ns, no statistic difference..

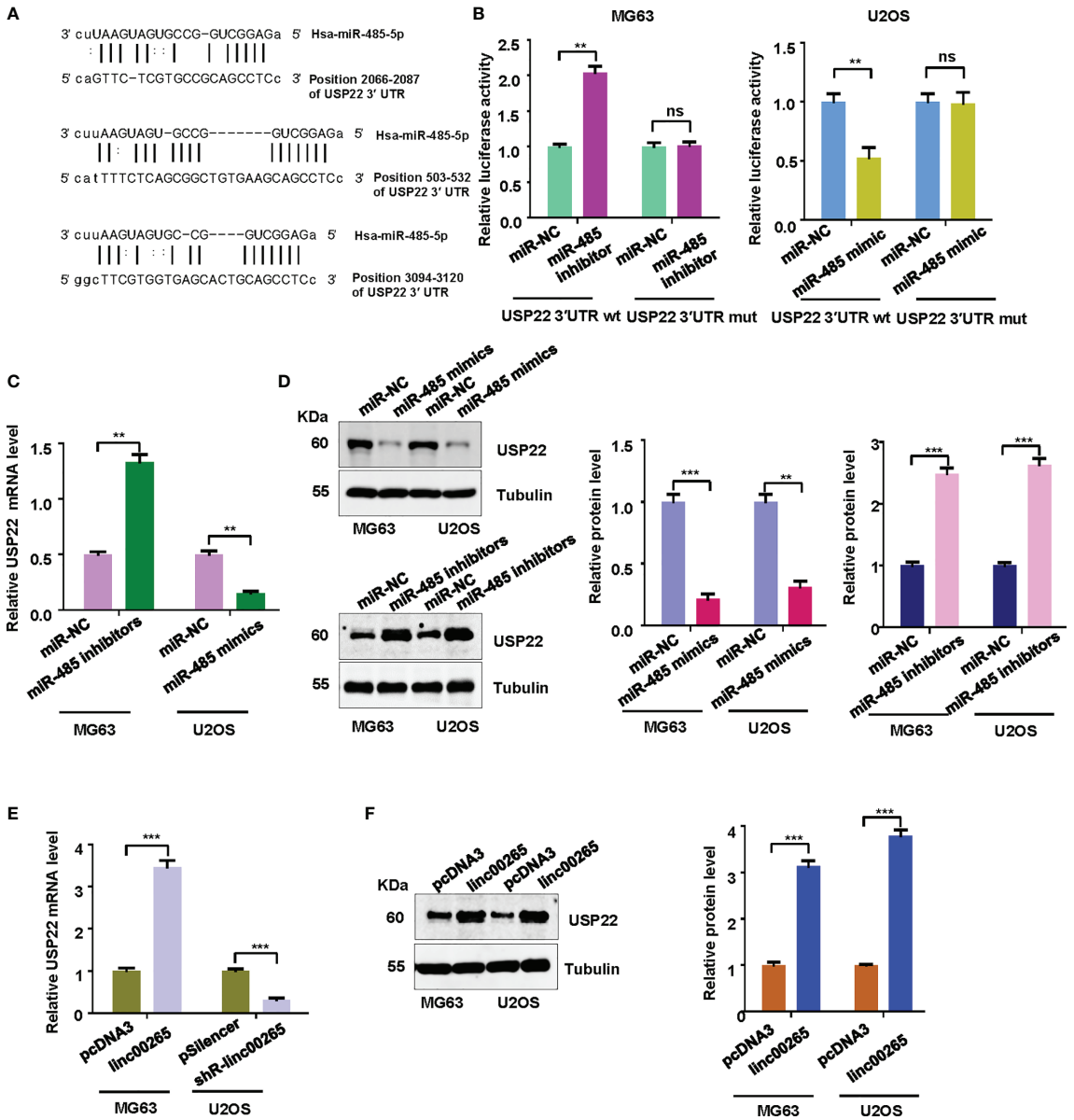

FIGURE 5 | USP22 is a downstream target of miR-485-5p. **(A)** The TargetScan database was utilized to predict the downstream target of miR-485-5p. USP22 3'UTR has the complementary binding sites with miR-485-5p. **(B)** Luciferase reporter assay was used to confirm USP22 as a target of miR-485-5p in MG63 and U2OS cells. **(C)** The expression of USP22 mRNA levels was detected by qRT-PCR in MG63 and U2OS osteosarcoma cells after miR-485 changes. **(D)** The expression of USP22 protein levels was detected by Western blotting in osteosarcoma cells after miR-485 changes (Left panel). The quantitative data were demonstrated for USP22 protein levels (Right panel). **(E)** The expression of USP22 mRNA levels was detected by qRT-PCR in osteosarcoma cells after linc00265 changes. **(F)** The expression of USP22 protein levels was detected by Western blotting in osteosarcoma cells after linc00265 changes (Left panel). The quantitative data were demonstrated for USP22 protein levels (Right panel). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. ns, no statistic difference.

stimulated cell proliferation *via* interaction with miR-144-3p and increasing chromobox 4 (CBX4) in gastric cancer (31). In bladder cancer cells, linc00265 was found to facilitate cell viability, proliferation and migratory ability *via* inhibition of miR-4677-3p and promotion of fibroblast growth factor 6 (FGF6) expression (32). Xiao and colleagues reported that linc00265 facilitated cell angiogenesis *via* sponging miR-382-5p, leading to upregulation of Spermidine/spermine acetyltransferase-1 (SAT1) and vavguanine nucleotide exchange factor 3 (VAV3) in osteosarcoma, which caused suppression of proliferation, motility and angiogenesis in osteosarcoma cells (33). Moreover, linc00265 was highly

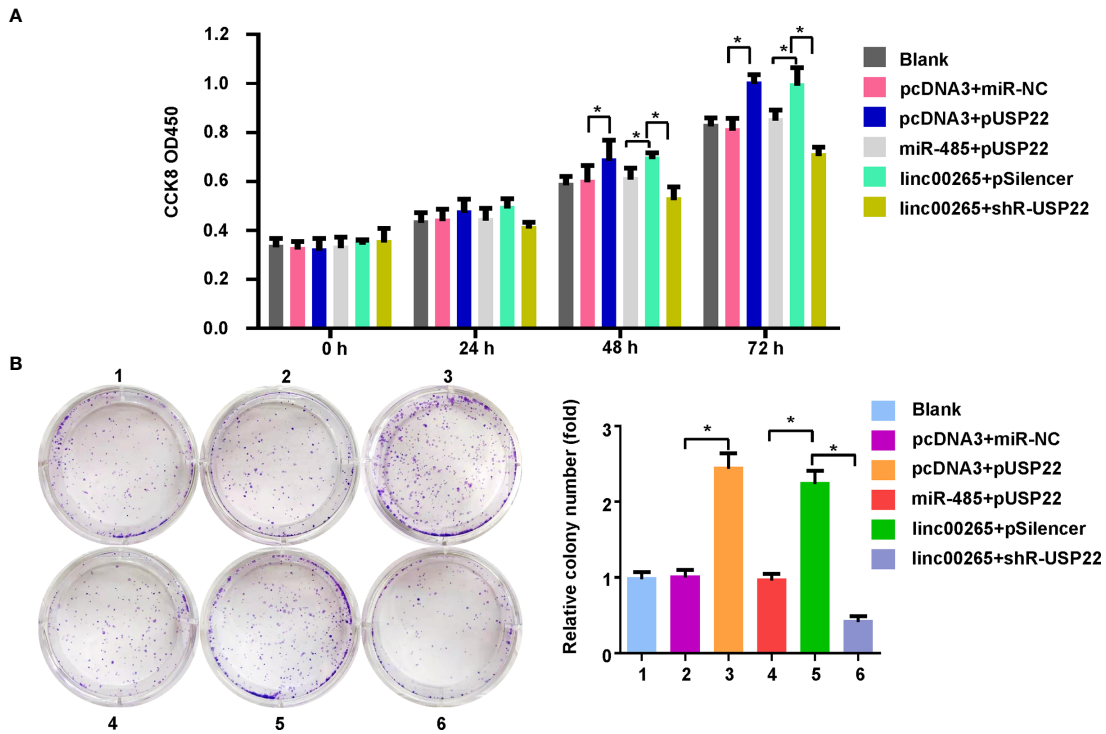

FIGURE 6 | Linc00265 promoted cell viability and colony formation *via* miR-485-5p/USP22 axis. **(A)** Cell viability was examined by CCK-8 assay in osteosarcoma cells after linc00265 modification, miR-485-5p changes, or USP22 modification or combination. **(B)** The colony formation ability was measured in osteosarcoma cells after linc00265 modification, miR-485-5p changes, or USP22 modification or combination. The quantitative data were demonstrated for colony formation (Right panel). 1: Blank. 2: pcDNA3 + miR-NC. 3: pcDNA3 + pUSP22. 4: miR-485 + pUSP22. 5. Linc00265 + pSilencer. 6. Linc00265 + shR-USP22. *$P < 0.05$ vs. control.

expressed in osteosarcoma patients and correlated with a poor prognosis (33). Here, we reported that linc00265 facilitated osteosarcoma progression *via* targeting miR-485-5p/USP22 axis.

Evidence has shown that miRNAs critically participate in oncogenesis of numerous types of cancer, including osteosarcoma. For example, miR-485-5p retarded cell proliferation and metastasis *via* inhibition of CX3CL1 in osteosarcoma cells (34). Similarly, one study reported that miR-495-5p targeted heat shock protein (Hsp90) expression and inactivated Akt1 phosphorylation and blocked PI3K/Akt pathway, resulting in suppression of cell proliferation in osteosarcoma (35). Another study showed that miR-485-5p can downregulate the expression of baculoviral IAP repeat containing 5 (BIRC5) and block the malignant phenotype of osteosarcoma (36). In our study, we found that miR-485-5p inhibited the expression of USP22 in osteosarcoma cells, leading to suppression of viability and motility of osteosarcoma cells.

USP22 promoted tumor development and progression in certain cancer types. For instance, USP22 enhanced cell proliferation *via* increasing surviving stability in renal cell carcinoma (RCC) cells (37). In pancreatic ductal adenocarcinoma (PDAC) cells, USP22 stimulated cell growth *via* targeting dual-specificity tyrosine regulated kinase 1A (DYRK1A) (38). In breast cancer cells, USP22 positively regulated ERα expression *via* maintaining its stability (39). Moreover, USP22 downregulation repressed cell proliferation, invasion and epithelial-mesenchymal transition (EMT) *via* inactivation of PI3K/Akt signaling pathway in osteosarcoma cells (28). Moreover, inhibition of USP22 reduced osteosarcoma tumor growth and metastasis in mice (28). Liu et al. also found that miR-140 attenuated osteosarcoma progression *via* interference of USP22-involved lysine-specific demethylase 1 (LSD1) stabilization and elevating p21 expression (40). We also confirmed that USP22 enhanced viability and motility of osteosarcoma cells.

CONCLUSIONS

In summary, linc00265 promoted cell viability, migration and invasion in osteosarcoma cells, indicating that linc00265 is an oncogene in osteosarcoma. Moreover, linc00265 sponged miR-485-5p and suppressed its expression in osteosarcoma cells. Furthermore, we identified that USP22 is a direct target

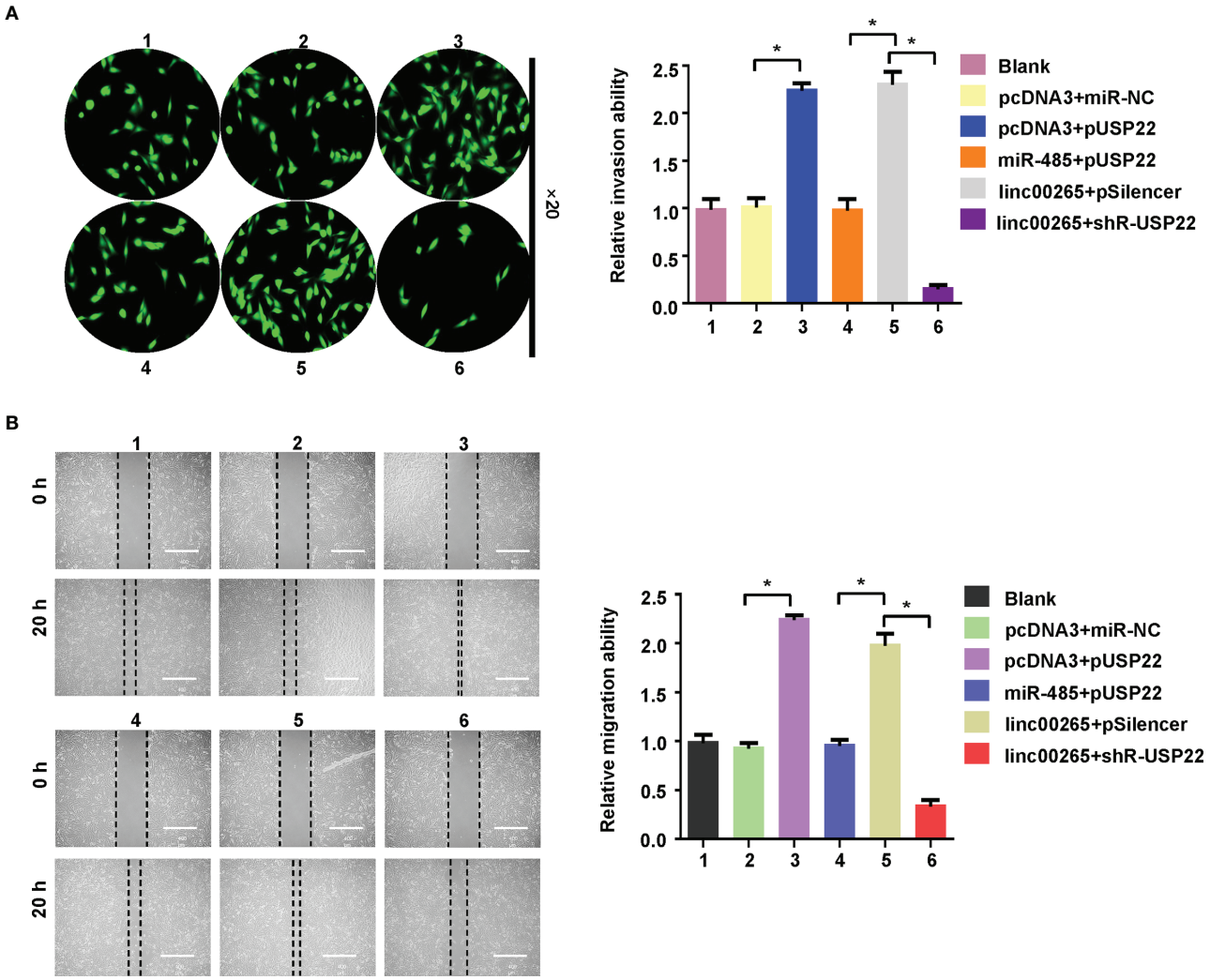

FIGURE 7 | Linc00265 enhanced migration and invasion *via* miR-485-5p/USP22 axis. **(A)** Cell invasion was examined by Transwell invasion assay in osteosarcoma cells after linc00265 modification, miR-485-5p changes, or USP22 modification or combination. **(B)** Cell migration was measured by wound healing assay in osteosarcoma cells after linc00265 modification, miR-485-5p changes, or USP22 modification or combination (Left panel). The quantitative data were demonstrated for wound healing assay (Right panel). 1: Blank. 2: pcDNA3 + miR-NC. 3: pcDNA3 + pUSP22. 4: miR-485 + pUSP22. 5. Linc00265 + pSilencer. 6. Linc00265 + shR-USP22. *$P < 0.05$ vs. control.

of miR-485-5p in osteosarcoma cells. Thus, miR-485-5p/USP22 axis was critically involved in linc00265-induced oncogenesis. Together, inhibition of linc00265 could be a potential strategy for osteosarcoma therapy. It is necessary to mention that *in vivo* mouse study will further validate the function of linc00265 in osteosarcoma development and progression. Moreover, it is required to explore the association between linc00265 levels and prognosis in osteosarcoma patients. In addition, linc00265 could have multiple miRNAs to sponge downstream targets. USP22 and miR-485-5p could have several downstream targets, which need to be further explored in osteosarcoma cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TC and GS designed this study. TC, JXL, HZ, and JTL performed the experiments and analyzed the data. TC and GS wrote the manuscript. All authors have read and approved the final version of the manuscript.

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LncRNA OIP5-AS1 Knockdown Targets miR-183-5p/GLUL Axis and Inhibits Cell Proliferation, Migration and Metastasis in Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is often associated with the infection of Epstein-Barr virus in nasopharynx and is mainly happened in South China and Southeast Asia. Recently, noncoding RNAs have been reported to regulate NPC carcinogenesis. LncRNA OIP5-AS1 participates in tumorigenesis and progression; however, the inherent mechanism of OIP5-AS1-mediated progression of NPC is unclear. In the current study, we aimed to explore the role of OIP5-AS1 in NPC progression. We measured the cell viability, apoptosis, migration, and invasion in NPC cells after OIP5-AS1 modulation. Moreover, we determined whether OIP5-AS1 exerts its oncogenic functions *via* sponging miR-183-5p in NPC. Furthermore, we determined whether glutamate ammonia ligase (GLUL) was a downstream target of miR-183-5p. We found that OIP5-AS1 downregulation inhibited the viability, migration and invasion of NPC *via* targeting miR-183-5p. We also identified that GLUL might be a potential downstream target of miR-183-5p in NPC cells. Mechanistically, OIP5-AS1 promotes cell motility *via* regulating miR-183-5p and GLUL in NPC cells. We concluded that OIP5-AS1 performed its biological functions *via* targeting miR-183-5p and GLUL in NPC cells.

Keywords: nasopharyngeal carcinoma, OIP5-AS1, miR-183-5p, GLUL, viability

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is often associated with the infection of Epstein-Barr virus in nasopharynx (1). This disease generally has ethnic and geographic features, such as in South China and Southeast Asia (2). Many factors have been believed to contribute to NPC development, including EBV infection, genetic susceptibility, dietary habits, smoking, epigenetic alterations. The current therapy for NPC patients includes chemotherapy and radiotherapy (3–5). NPC patients

often have recurrence, leading to poor overall survival (6). Therefore, it is important to determine the molecular mechanism of NPC development and progression, which is still not thoroughly elucidated.

Recently, noncoding RNAs have been reported to regulate NPC carcinogenesis (7, 8). Several studies have revealed that lncRNA OIP5-AS1 participates in tumorigenesis and progression (9–11). Higher expression of OIP5-AS1 could be associated with an advanced stage and a poor survival in multiple cancer types (12). Zhang et al. reported that depletion of OIP5-AS1 suppressed cell proliferation, EMT and metastasis *via* upregulation of miR-186a-5p and inhibition of ZEB1 in hepatoblastoma cells (13). Wang et al. found that OIP5-AS1 increased cell proliferation *via* sponging miR-378a-3p in lung cancer (14). Similarly, OIP5-AS1 enhanced lung cancer stemness *via* promotion of Oct4 mRNA stability (15). One group identified that OIP5-AS1 facilitated cell proliferation and invasive activity *via* interacting with miR-143-3p and increasing integrin alpha6 expression in cervical cancer (16). Similarly, OIP5-AS1 promoted viability, migration and invasion of cervical cancer cells *via* binding with miR-143-3p and upregulating SMAD3 expression in cervical cancer cells (17). OIP5-AS1 increased invasion, migration and EMT *via* targeting miR-147a and IGF1R in cervical cancer (18). OIP5-AS1 inhibited miR-92a and increased cell growth and metastasis *via* upregulation of ITGA6 in ovarian cancer (19). OIP5-AS1 interacted with miR-34a and increased the PD-L1 expression in NSCLC cells (20). Depletion of OIP5-AS1 retarded cell growth, migration and stimulated apoptosis *via* targeting miR-129-5p and SOX2 in breast cancer (21). Knockdown of OIP5-AS1 expression attenuated cell viability, triggered cell cycle arrest and activated apoptosis in bladder cancer (22).

Multiple researches demonstrated that OIP5-AS1 exerts anti-tumor functions in various types of cancers. For example, one study showed that OIP5-AS1 reduced clonogenic survival and induced apoptosis in colorectal cancer cells after irradiation *via* targeting miR-369-3p and DYRK1A, suggesting that OIP5-AS1 enhances radio-sensitivity in colorectal cancer (23). One study revealed that OIP5-AS1 enhanced NPC progression *via* sponging miR-203 (24). The inherent mechanism of OIP5-AS1-mediated progression of NPC is unclear. In the current study, we investigated the role of OIP5-AS1 in NPC progression. To achieve this goal, we measured the cell viability, apoptosis, migration, and invasion in NPC cells after OIP5-AS1 modulation. Moreover, we determined whether OIP5-AS1 exerts its oncogenic functions *via* sponging miR-183-5p in NPC. Furthermore, we explored whether glutamate ammonia ligase (GLUL) was a downstream target of miR-183-5p. Our results showed that OIP5-AS1 performed its biological functions *via* targeting miR-183-5p and GLUL in NPC cells.

MATERIALS AND METHODS

Cell Culture and Reagents

Human NPC cells(CNE1, CNE2 and HNE1), which are Epstein-Barr virus (EBV)-negative cells, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). MTT [3-(4,5-dimethythi-azol-2-yl)-2,5-diphenyl tetrazolium bromide] was obtained from Sigma Company. Matrigel was bought from BD Biosciences Company. Lipofectamine 3000 was obtained from Invitrogen Company. The anti-GLUL and anti-tubulin antibodies were purchased from Cell Signaling Technology Company.

Cell Viability Assay

The transfected CNE1 and CNE2 cells were seeded in 96-well plates for 72 hours. MTT assay was performed to measure cell viability by a spectrophotometer at 570 nm as described previously (25).

Transfection

The NPC cells were seeded in 60 mm dishes overnight and then transfected with different plasmids (GenePharma, Shanghai, China) using Lipofectamine 3000 following the manufacture's instruments (25, 26).

Real-Time Quantitative RT-PCR

Total RNAs were extracted from the transfected NPC cells and then reversely transcribed into cDNA. The RT-1PCR was performed as described before (26). The mRNA levels were calculated using $^{\Delta\Delta}$Ct methods.

Wound Healing Assay

The transfected NPC cells were seeded in 6-well plate. After the cells grew to around 100% confluence, the scratch wound was created using a pipette tip and the cells were washed by PBS. The cells were cultured for 20 hours. The photographs were taken at 0 hour and 20 hours, respectively.

Transwell Matrigel Invasion Assay

The invasive activity of NPC cells was determined by Transwell Matrigel invasion assay using 24-well Transwell inserts with precoated Matrigel. The transfected NPC cells were seeded in upper level of the inserts with serum-free medium. The bottom level of the insert was added completed medium. After 24 hours, the cells that invaded on the bottom level were stained and photographed by a microscope.

Western Blotting Analysis

The transfected cells were washed and harvested and then lysed by protein lysis buffer. The concentrations of proteins were measured using BCA assay. After SDS-PAGE was used to separate the protein samples, the proteins were transferred onto PVDF membranes. The membranes were incubated with 5% milk for 1 hour and then immunoblotted with anti-GLUL antibody overnight at cold room. Then, TBST was used to wash the membranes for three times and subsequently probed with the proper secondary antibody for 1 hour. The expression level of proteins was measured by ECL assay. Tubulin expression was used to act as a control.

Dual Luciferase Reporter Gene Analysis

The GLUL wild-type, GLUL mutant, OIP5-AS1 wild-type, and OIP5-AS1 mutant were amplified and cloned in pmirGLO vector

with luciferase. Cells were treated with different plasmids. OIP5-AS1 mutant has the mutated binding sites of miR-183-5p. GLUL mutant has the mutated binding sites of miR-183-5p. After 48 hours, the dual luciferase reporter gene analysis was detected following the manufacturer's protocols (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was measured by GraphPad Prism 5.0 (CA, USA). The significance was analyzed using the two-tailed Student's *t-test* for comparing with two different groups. ANOVA was used for comparing with multiple groups. Data are shown with means ± SEM.

RESULTS

Inhibition of OIP5-AS1 Suppressed Cell Viability in NPC Cells

We examined the expression of lncRNA OIP5-AS1 in NP69, CNE1, CNE2 and HNE1 cell lines by real-time RT-PCR analysis. We found that the expression of OIP5-AS1 was highly expressed in NPC cells compared with NP69 nasopharyngeal normal cell line (**Figure 1A**). Next, we used shRNA to knockdown the expression of OIP5-AS1 in CNE1 and CNE2 cells, which had the high expression of OIP5-AS1. We found that sh-OIP5-AS1 transfection led to downregulation of OIP5-AS1 in both CNE1 and CNE2 cells (**Figure 1B**). In the following study, we used sh-OIP5-AS1#2 to investigate its function in CNE1 and CNE2 cells. To measure the effect of OIP5-AS1 downregulation in NPC cells, we examined the cell viability in CNE1 and CNE2 cells after OIP5-AS1 knockdown. We observed that knockdown of OIP5-AS1 attenuated the viability of NPC cells (**Figure 1C**). Moreover, we performed the colony formation study to further determine the function of OIP5-AS1 in NPC cells. Our data showed that depletion of OIP5-AS1 reduced the colony formation activity in CNE1 and CNE2 cells (**Figure 1D**). Altogether, knockdown of OIP5-AS1 inhibited viability of NPC cells.

Inhibition of OIP5-AS1 Reduced Migration and Invasion in NPC Cells

It is known that OIP5-AS1 participates in cell migration and invasion in cancer. Therefore, we measured the migratory and invasive capacity of NPC cells after OIP5-AS1 knockdown. Our wound healing assay data showed that knockdown of OIP5-AS1 retarded would closure in both CNE1 and CNE2 cells (**Figure 2A**). Moreover, transwell invasion assay data demonstrated that knockdown of OIP5-AS1 reduced the invasiveness activity in CNE1 and CNE2 cells (**Figure 2B**). Taken together, OIP5-AS1 knockdown retarded the migrative and invasive capacity in NPC cells.

OIP5-AS1 Targets miR-183-5p in NPC Cells

According to the database from website RAID v2.0, OIP5-AS1 could bind to hsa-miR-183-5p. Moreover, the data from mircode.org also showed that OIP5-AS1 can interact with hsa-miR-183-5p. There were binding sites between miR-183-5p and lncRNA OIP5-AS1 (**Figure 3A**). To further validate this concept, we performed the dual luciferase reporter gene analysis. We found that the luciferase activity of OIP5-AS1 was decreased in the miR-183-5p mimics group when compared with miRNA control group (**Figure 3B**). In consistent, the activity of OIP5-AS1 mutation did not change in the both miR-183-5p mimics and miRNA control groups (**Figure 3B**). This result indicated that OIP5-AS1 might bind to miR-183-5p in NPC cells.

GLUL Is a Potential Target of miR-183-5p

We used bioinformatic analysis to predict the downstream target of miR-183-5p. From the several public algorithms, such as TargetScan, miRanda, microRNA.org, PicTar, GLUL was revealed to be a target of miR-183-5p. The GLUL sequence has specific binding regions to interact with miR-183-5p (**Figure 3C**). To demonstrate whether miR-183-5p bound to GLUL, we conducted the dual luciferase reporter gene analysis. We observed that the luciferase activity of the GLUL was inhibited in the miR-183-5p mimic transfection group, whereas its luciferase activity did not change in the miR-183-5p mutation group (**Figure 3D**). Thus, GLUL might be a potential downstream target of miR-183-5p in NPC cells.

OIP5-AS1 and miR-183-5p Target GLUL Expression

Next, we determined whether miR-183-5p could regulate the expression of GLUL in NPC cells. Our western blotting results showed that miR-183-5p mimic transfection inhibited the expression of GLUL in CNE1 and CNE2 cells (**Figure 4A**). Moreover, downregulation of miR-183-5p by its inhibitors increased the expression of GLUL in CNE1 and CNE2 cells (**Figure 4B**). Furthermore, overexpression of OIP5-AS1 rescued the inhibitory effect of miR-183-5p mimics on GLUL expression in NPC cells (**Figure 4A**). Consistently, depletion of OIP5-AS1 abrogated the promotive effect of miR-183-5p inhibitors on GLUL expression level in both NPC cell lines (**Figure 4B**). Therefore, GLUL might be a downstream factor of miR-183-5p.

OIP5-AS1 Promotes Cell Viability *via* Regulating miR-183-5p in NPC Cells

We tested whether OIP5-AS1 governs cell viability *via* regulation of miR-183-5p in NPC cells. Our MTT assay showed that OIP5-AS1 downregulation inhibited cell viability in CNE1 and CNE2 cells (**Figure 5A**). Downregulation of miR-183-5p rescued the inhibitory effects of shRNA OIP5-AS1 transfection on cell viability in NPC cells (**Figure 5A**). Moreover, overexpression of GLUL abolished the suppression of cell viability by shRNA OIP5-AS1 transfection (**Figure 5A**). Consistently, colony formation experiments showed the similar trends in CNE1 and CNE2 cells (**Figure 5B**). Altogether, downregulation of OIP5-AS1 inhibited viability of NPC cells *via* regulation of miR-183-5p and its target GLUL.

OIP5-AS1 Promotes Cell Motility *via* Regulating miR-183-5p and GLUL in NPC Cells

We explored whether OIP5-AS1 regulates cell migration and invasion through targeting miR-183-5p and GLUL in NPC cells. The invasive ability was measured by Transwell invasion assay in

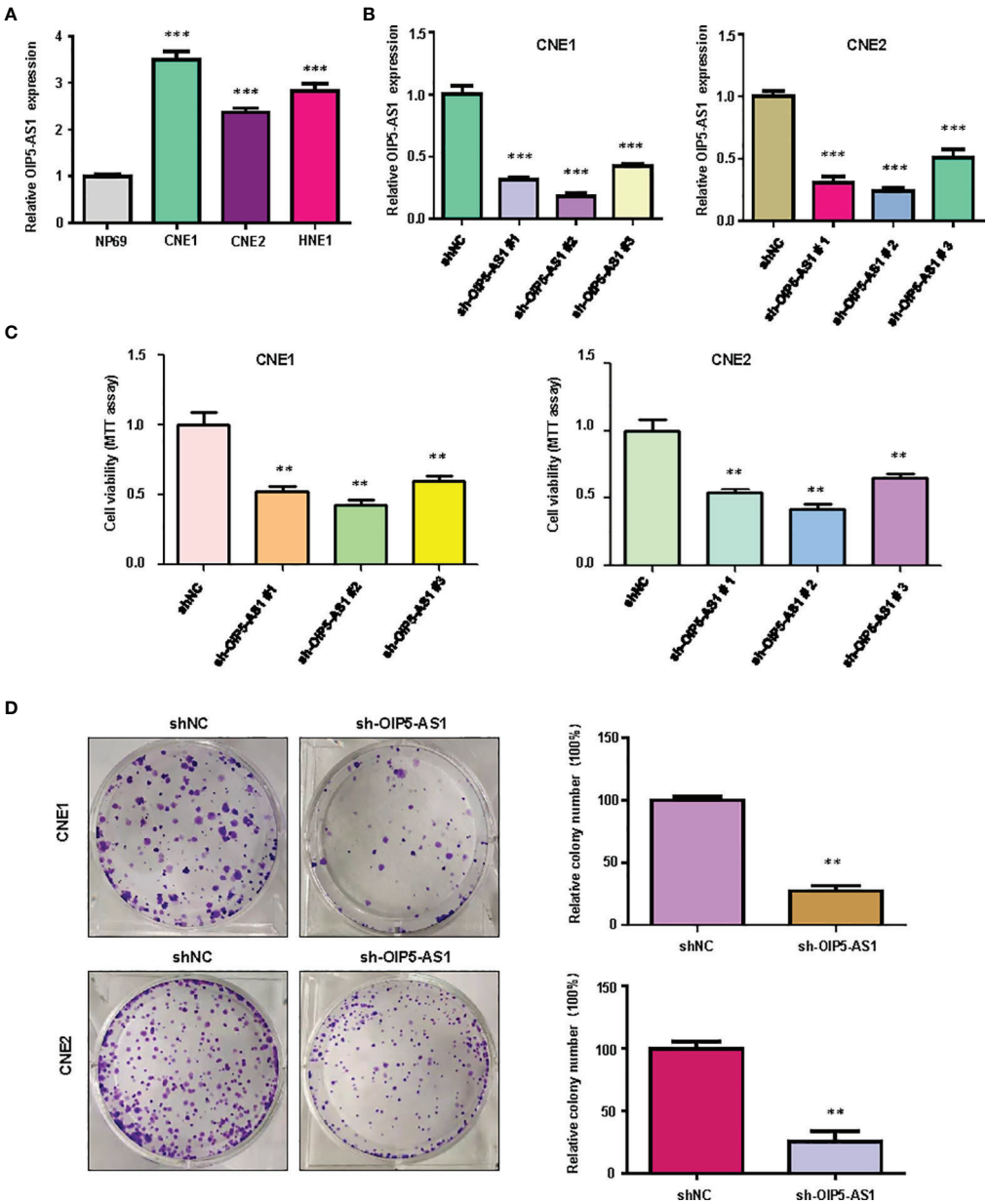

FIGURE 1 | Knockdown of OIP5-AS1 inhibits viability of NPC cells. **(A),** RT-PCR assay was used to measure the expression of OIP5-AS1 in several NPC cell lines. ***P<0.001 vs control. **(B),** RT-PCR was used to detect the efficacy of OIP5-AS1 knockdown in CNE1 and CNE2 cells. ***P<0.001 vs control. **(C),** MTT assay was used to measure the viability of NPC cells after OIP5-AS1 knockdown. **P<0.01 vs control. **(D),** Cell colony formation was performed in NPC cells after OIP5-AS1 knockdown (Left panel). Quantitative data are represented (Right panel). **P<0.01 vs control.

NPC cells after modification of OIP5-AS1, GLUL and miR-183-5p. The results showed that shRNA OIP5-AS1 transfection reduced cell invasion capacity in CNE1 and CNE2 cells (**Figure 6A**). Overexpression of GLUL blocked the inhibitory function of OIP5-AS1 knockdown on cell invasion in NPC cells (**Figure 6A**). Moreover, suppression of miR-183-5p abolished the inhibitory effects on NPC cells that were induced by OIP5-AS1 knockdown (**Figure 6A**). Wound healing assay data demonstrated that knockdown of OIP5-AS1 retarded cell migratory ability, which could be rescued by overexpression of GLUL and inhibition of miR-183-5p in both NPC cell lines (**Figure 6B**). Taken together, OIP5-AS1 regulated cell invasion and migration *via* targeting miR-183-5p and GLUL in NPC cells.

DISCUSSION

LncRNAs have been reported to involve in carcinogenesis and tumor progression in many types of cancers (27–31). Evidence has

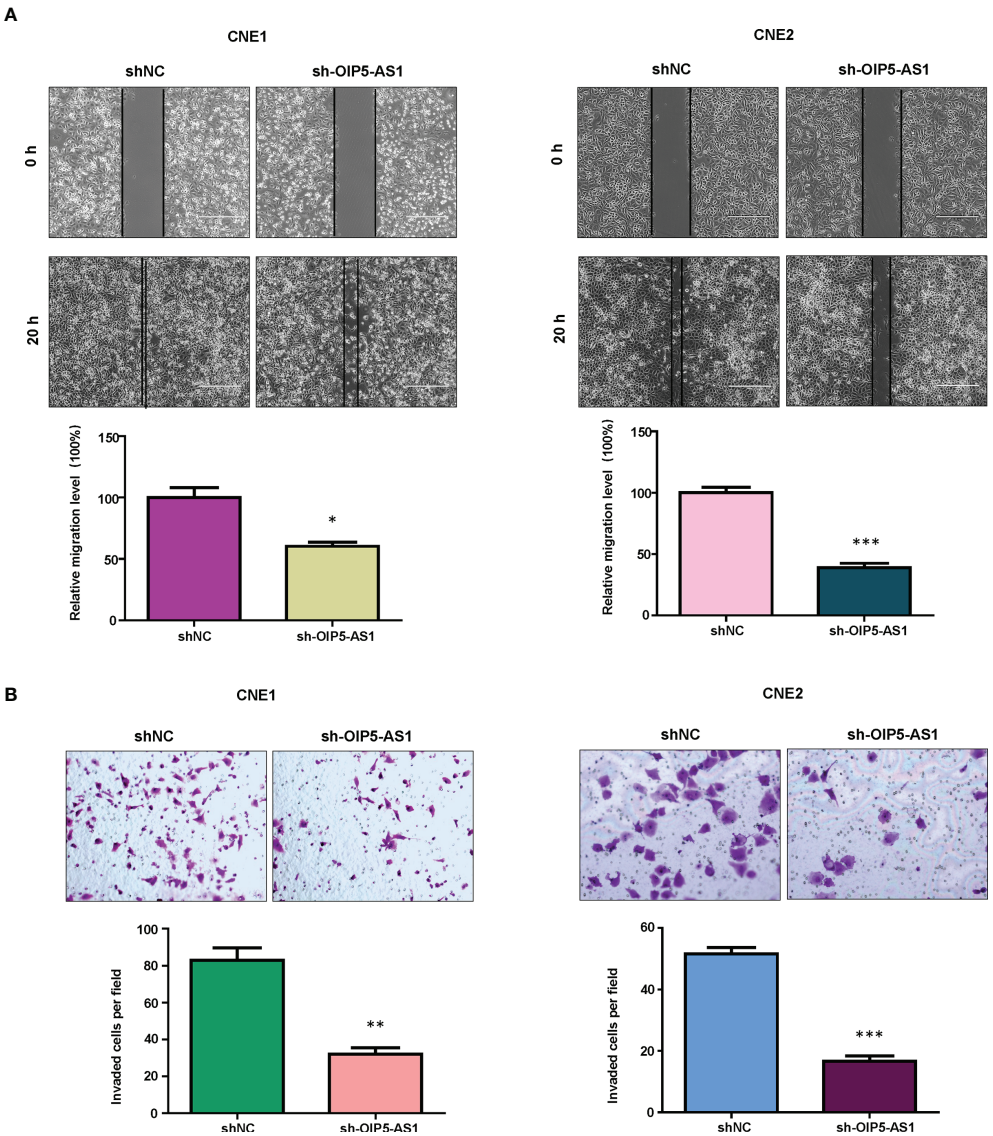

FIGURE 2 | Knockdown of OIP5-AS1 inhibits migration and invasion of NPC cells. **(A),** Wound healing assays were utilized to measure the migratory activity of NPC cells after OIP5-AS1 knockdown (Top panel). Quantitative data are represented (Bottom panel). * P<0.05 vs control; ***P<0.001 vs control. **(B),** Transwell matrigel invasion analysis was utilized to test the invasiveness capacity of NPC cells after OIP5-AS1 knockdown (Top panel). Quantitative data are represented (Bottom panel) ** P<0.01 vs control; ***P<0.001 vs control.

revealed that OIP5-AS1 aggravated cell growth and migratory ability *via* interaction with EZH2 and downregulation of NLRP6 in gastric cancer (32). Wang et al. reported that OIP5-AS1 enhanced cell proliferation and promoted cell cycle *via* sponging miR-641 in gastric cancer (33). Song et al. observed that OIP5-AS1 facilitated cell proliferation and blocked apoptosis *via* targeting miR-143-3p/ROCK1 axis in cervical cancer (34). Tao et al. found that OIP5-AS1 increased cell growth and suppressed apoptosis *via* modulation of the miR-367-3p/HMGA2 pathway in gastric cancer (35). Moreover, OIP5-AS1 accelerated cell growth *via* affecting miR-422a and ANO1 axis in gastric cancer cells (36). Similarly, Zhi et al. found that OIP5-AS1 enhanced gastric cancer progression *via* sponging miR-153-3p and targeting ZBTB2 axis (37). Furthermore, OIP5-AS1 facilitated growth of pancreatic cancer cells *via* decoying miR-342-3p and activation of AKT/ERK pathway (38). OIP5-AS1 silencing led to inhibition of cell proliferation and apoptosis in 5-8F cells and CNE1 cells *via* sponging miR-203 in NPC (24). Herein, our study showed that OIP5-AS1 downregulation inhibited the viability of NPC *via* targeting miR-183-5p.

OIP5-AS1 was reported to enhance the proliferation, motility activity and EMT *via* decoying miR-204-5p and increasing ZEB1 in laryngeal squamous cell carcinoma (39). Knockdown of OIP5-AS1 repressed proliferation and migration *via* regulating miR-3163/VEGFA axis in liver cancer cells (40). Moreover, OIP5-AS1

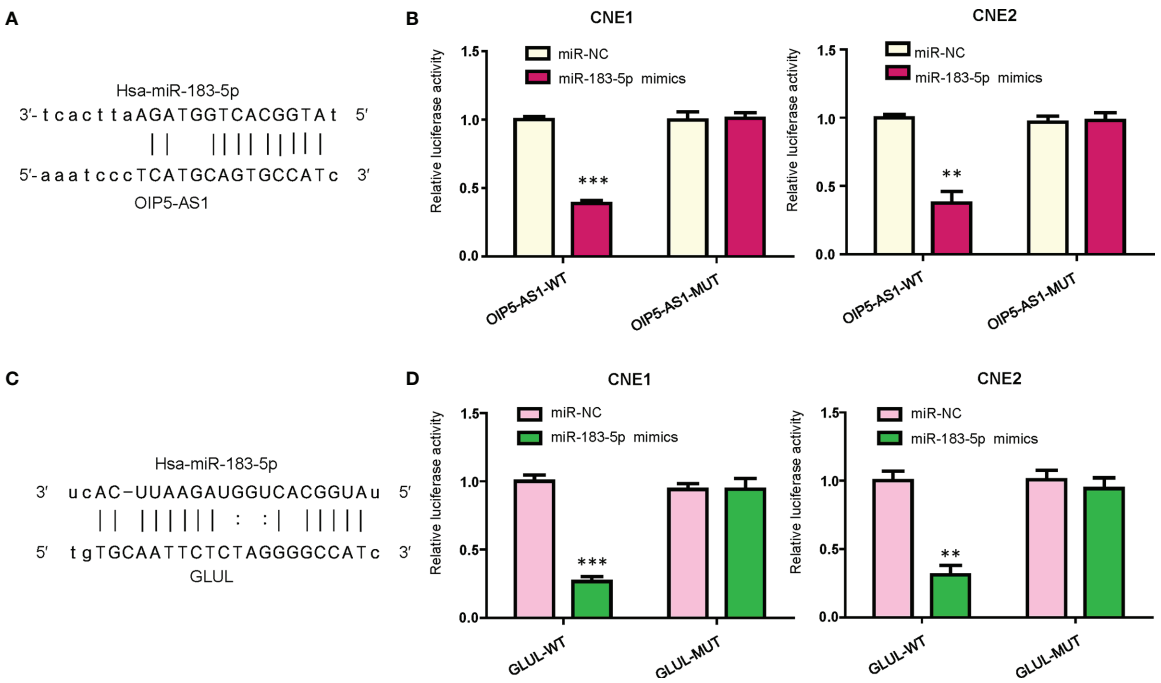

FIGURE 3 | OIP5-AS1 targets miR-183-5p. **(A),** Potential binding sites between OIP5-AS1 and miR-183-5p are shown. **(B),** Dual luciferase reporter assays were used to verify the binding sites of miR-183-5p to OIP5-AS1. **P<0.01; ***P<0.001 vs control. **(C),** Potential binding sites between GLUL and miR-183-5p are shown. **(D),** Dual luciferase reporter assays were used to verify the binding sites of miR-183-5p to GLUL. **P<0.01; ***P<0.001 vs control.

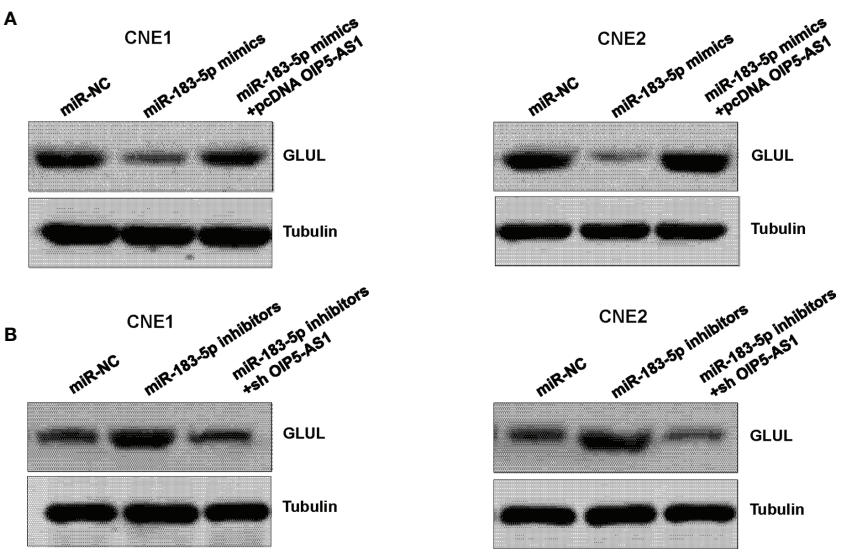

FIGURE 4 | GLUL is a target of miR-183-5p. **(A),** Western blotting assay was performed to measure the expression of GLUL in NPC cells after miR-183-5p mimic and pcDNA OIP5-AS1 co-transfections. **(B),** Western blotting analysis was used to measure the expression of GLUL in NPC cells after miR-183-5p inhibitor and shRNA OIP5-AS1 co-transfections.

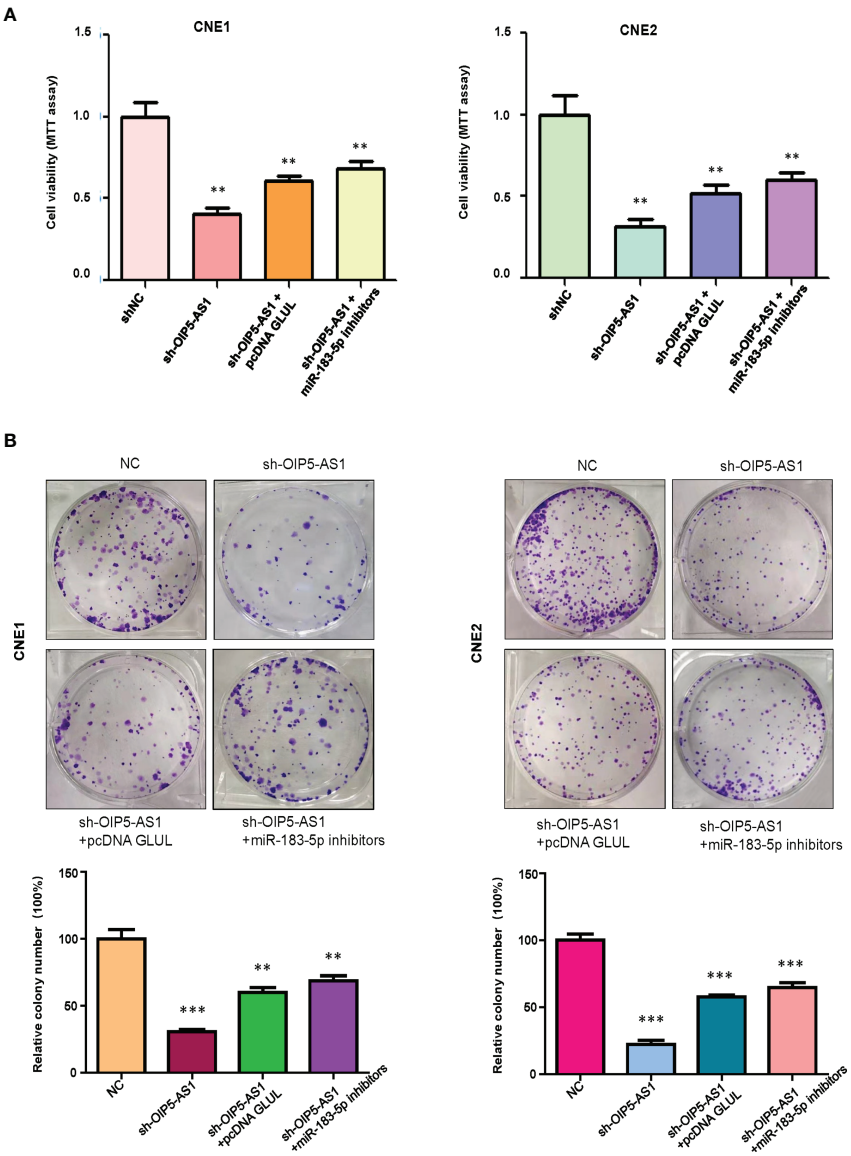

FIGURE 5 | OIP5-AS1 knockdown suppressed cell viability *via* regulating miR-183-5p in NPC cells. **(A),** MTT assay was used to measure the viability of NPC cells after OIP5-AS1 knockdown, GLUL overexpression and miR-183-5p downregulation. **P<0.01 vs control. **(B),** Cell colony formation was performed in NPC cells after OIP5-AS1 knockdown, GLUL overexpression and miR-183-5p downregulation (Top panel). Quantitative data are represented (Bottom panel). **P<0.01; ***P<0.001 vs control.

facilitated the tumor malignant progression *via* targeting miR-429/FOXD1/ERK axis in pancreatic cancer (41). OIP5-AS1 silencing led to inhibition of cell migration and invasion in 5-8F and CNE1 cells *via* sponging miR-203 in NPC (24). Our study demonstrated that OIP5-AS1 knockdown suppressed cell invasion and migration *via* regulating miR-183-5p and GLUL in NPC cells. Tang et al. found that miR-183-5p is a biomarker for patients with NPC (42). This study reported that miR-183-5p expression was negatively associated with lymph node status in NPC patients (42). GLUL has been identified to participate in carcinogenesis and tumor progression (43). GLUL is involved in tumorigenesis in a variety of cancers; however, the function of GLUL in NPC is unclear. Here, we found that GLUL was involved in OIP5-AS1-mediated tumor promotion in NPC cells.

It has been reported that OIP5-AS1 is involved in drug resistance in various malignancies. Song et al. reported that OIP5-AS1 increased cisplatin resistance *via* binding with miR-340-5p and upregulating LPAATβ and PI3K/AKT/mTOR pathway in osteosarcoma (44). Similarly, OIP5-AS1 promoted cisplatin resistance *via* regulating miR-27b-3p/TRIM14 axis in oral squamous cell carcinoma (45). Liang et al. reported that OIP5-AS1 targeted miR-137 and increased L-OHP sensitivity in

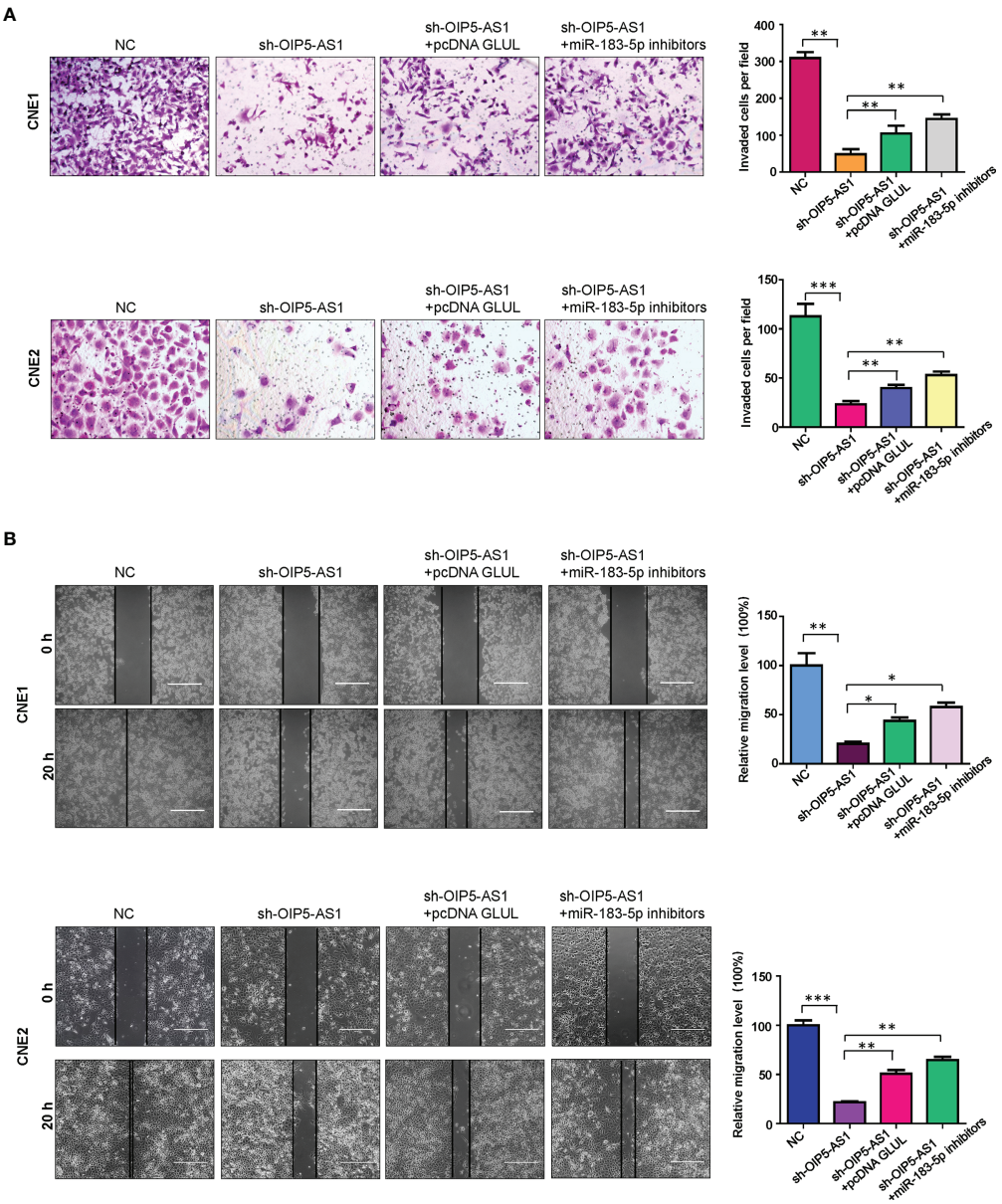

FIGURE 6 | OIP5-AS1 knockdown inhibits cell motility *via* regulating miR-183-5p and GLUL in NPC cells. **(A),** Transwell Matrigel invasion analysis was utilized to test the invasiveness capacity of NPC cells after OIP5-AS1 knockdown, GLUL overexpression and miR-183-5p downregulation. (Left panel). Quantitative data are represented (Right panel) * P<0.05 vs control; ** P<0.01; ***P<0.001. **(B),** Wound healing assays were utilized to measure the migratory activity of NPC cells after OIP5-AS1 knockdown, GLUL overexpression and miR-183-5p downregulation (Left panel). Quantitative data are represented (Right panel). *P<0.05; **P<0.01; ***P<0.001.

colon cancer cells (46). Another group found that OIP5-AS1 increased doxorubicin resistance *via* sponging miR-137-3p and upregulating PTN in osteosarcoma (47). Moreover, exosomal-OIP5-AS1 promoted trastuzumab chemoresistance *via* decoying miR-381-3p and increasing HMGB3 in breast cancer (48). OIP5-AS1 increased cell resistance to imatinib *via* targeting miR-30e-5p and ATG12 in chronic myeloid leukemia cells (49). However, it is unclear whether OIP5-AS1 is involved in drug resistance in NPC, which needs to further investigate.

CONCLUSION

In summary, OIP5-AS1 downregulation suppressed the viability, migration and invasion of NPC *via* targeting miR-183-5p. GLUL might be a potential downstream target of miR-183-5p in NPC cells. Moreover, OIP5-AS1 promotes cell motility *via* regulating miR-183-5p and GLUL in NPC cells. Therefore, OIP5-AS1 exerted its biological functions *via* targeting miR-183-5p and GLUL in NPC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SL performed experiments and wrote the manuscript, MT performed data analysis, NZ, JL, XX, DH, and FL conceived and designed the study, revised the manuscript. XZ supervised the study and edited the manuscript. All authors have approved the final manuscript.

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A Novel Matrisomal-Related LncRNA Signature Associated With Survival Outcome and Immune Evasion in Patients With Gastric Cancer

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Background: Different matrisomal patterns are shared across carcinomas. However, little is known about whether there exists a unique tumor matrisome that modulates GC progression and immune regulation.

Methods: We conducted a genome-wide analysis based on matrisomal-related lncRNAs (MRLs) in 375 patients with GC from the Cancer Genome Atlas (TCGA) database. Patients were split into the training set and validation set at a ratio of 1:1 using the R package cart. Pearson correlation analysis (PCA) was performed to identify lncRNAs that correlated with matrisome based on differential expression genes. Subsequently, we performed univariate Cox regression analyses and lasso Cox analysis on these lncRNAs to construct a risk model. Considering the primary effect of GRASLND on the GC prognosis, we chose it for further validation in an experimental setting.

Results: We identified a 15-MRL signature to predict overall survival and immune cell infiltration of patients with GC. The AUC values to predict 5-year outcome in three sets were 0.89, 0.65, and 0.78, respectively. Further analyses suggested that the high-risk group showed more obvious immune cell infiltration, and demonstrated an immunologically "cold" profile. *In vitro*, knockdown of GRASLND could inhibit the invasion capability of GC cells, and downregulate the protein expression of crucial matrisomal-related gene MMP9.

Conclusions: The 15-MRL gene signature might serve as a relatively good predictive tool to manage patients with GC.

Keywords: matrisomal pattern, gastric cancer, overall survival (OS), immune regulation, risk model

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INTRODUCTION

Gastric cancer (GC) represents one of the most common digestive malignancies worldwide, particularly in East Asia. The number of deaths was 768,793 in 2020 (1). Despite recent advances in GC treatment, a significant proportion of GC patients are diagnosed at the time of later disease progression (2). The outcomes of GC patients still need to be improved. Chemotherapy has been widely applied as a clinical treatment for advanced GC (3). However, these drugs show few satisfactory therapeutic effects due to chemoresistance or other adverse events (4), which limited their further application in some cases. As a promising innovative therapy, immunotherapy provides an effective strategy for the treatment of GC (5). Seeking effective immune therapeutic indicators for GC remains a unique challenge (6). Therefore, we aimed to establish a novel risk model associated with immune infiltration and survival outcome of GC patients based on large cancer datasets.

The extracellular matrix (ECM) consists of a complex network of cross-linked proteins and has been extensively investigated in recent years. Alterations in the composition, ultrastructure, and mechanical properties of ECM elements could have an impact on the phenotype of the cell, thus participating in the tumorigenesis and development of cancers. Long non-coding RNAs (lncRNAs) exert a variety of biological functions through various gene-regulatory mechanisms (7). Aberrant expression of lncRNAs contribute to the progression of cancers, including GC (8). Several lines of evidence have demonstrated that lncRNAs are mainly involved in the regulation of ECM through several crucial ECM-related regulators. Previous studies revealed that TGFBI, as an important player in the ECM, was negatively regulated by lncRNA H19 *via* the lncRNA H19/miR-675 axis (9, 10). Gu et al. indicated that lncRNA CTD-2589M5.4 could inhibit ovarian cancer progression *via* regulation of ECM remodeling (11). LINC01089 is a novel conserved lncRNA, which could function as the inhibitor of ECM invasion in breast cancer (Sas-12). Moreover, a recent study has also found that the miR-150-related regulatory axis was involved in biological processes that relied on the ECM in hepatocellular carcinoma (13). Therefore, ECM-related lncRNAs might play crucial roles in tumor progression and immune response. Nevertheless, direct lines of evidence about matrisomal-related lncRNAs in GC are still lacking and need further study.

In this study, we conducted a genome-wide analysis about matrisomal-related lncRNAs and developed a new and robust gene signature based on the TCGA database. This new classification could accurately predict survival outcome and was associated with immune infiltration. Furthermore, several experiments *in vitro* showed that knockdown of GRASLND could regulate matrisomal-related gene MMP9. This might provide new insights into exploring the regulatory roles of matrisomal-related lncRNAs in GC.

METHODS

Data Source

We downloaded high-throughput sequencing profile data of 375 GC patients from the TCGA public database and removed four samples missing clinical follow-up information. LncRNAs and protein-coding RNAs were annotated based on the GENCODE project (https://www.gencodegenes.org/) (14). A total of 1,068 matrisomal-related genes were obtained from the M.I.T. "Matrisome Project" website (www.matrisomeproject.mit.edu/other-resources/human-matrisome). The workflow is illustrated in **Figure 1**.

Identification of Matrisomal-Related LncRNAs

The Pearson coefficient was used to investigate links between the potential lncRNAs and the matrisomal-related genes. The absolute value of a correlation coefficient of more than 0.4 and a p-value of less than 0.001 were set as threshold values (15–17). The candidate matrisomal-related lncRNAs were considered for subsequent analyses.

Construction of a Matrisomal-Related LncRNA Risk Model

Differentially expressed matrisomal-related lncRNAs (DE-MRLs) between the cancer and normal tissues were firstly obtained based on R package "limma". Our criteria were set as |log2 fold change (FC)| > 1 and FDR < 0.05. Based on the survival information for patients with GC, we screened out prognostic matrisomal-related lncRNAs ($p < 0.001$) using univariate Cox regression analysis. Then, GC patients were randomly split into the training and validation set at a ratio of 1:1 using the R package "caret." We applied lasso analysis in the training set using the R package "glmnet". A coefficient for each prognostic matrisomal-related lncRNAs was generated accordingly. Multivariate Cox regression analysis was performed to propose the following formula:Risk Score = $\sum_{i=1}^{n} bi * Si$. GC patients were classified into high- and low-risk groups using the threshold of median value.

Evaluation of the Prognostic Signature

Kaplan–Meier survival analysis was employed to investigate overall survival discrepancy between the different risk groups. Subsequently, receiver operating characteristic (ROC), decision curve analysis (DCA), and concordance index (C-index) were used to evaluate the performance of this prognostic model. Additionally, we constructed a nomogram to predict 1-, 3-, and 5-year survival status. Meanwhile, the calibration curve was performed to evaluate the predictive power of the nomogram.

Immune Cell Infiltration Analysis

Seven immune infiltration prediction algorithms based on RNA-seq were utilized to compare tumor-infiltrating immune cell (TIC) infiltration between the different risk groups. Subsequently, we examined the relationship between the proportions of TICs and candidate matrisomal-related lncRNAs using the CIBERSORT algorithm. Additionally, we performed ssGSEA analysis to quantify the immune regulatory roles. Finally, several common immune checkpoint genes and TIDE score were selected and evaluated between two groups.

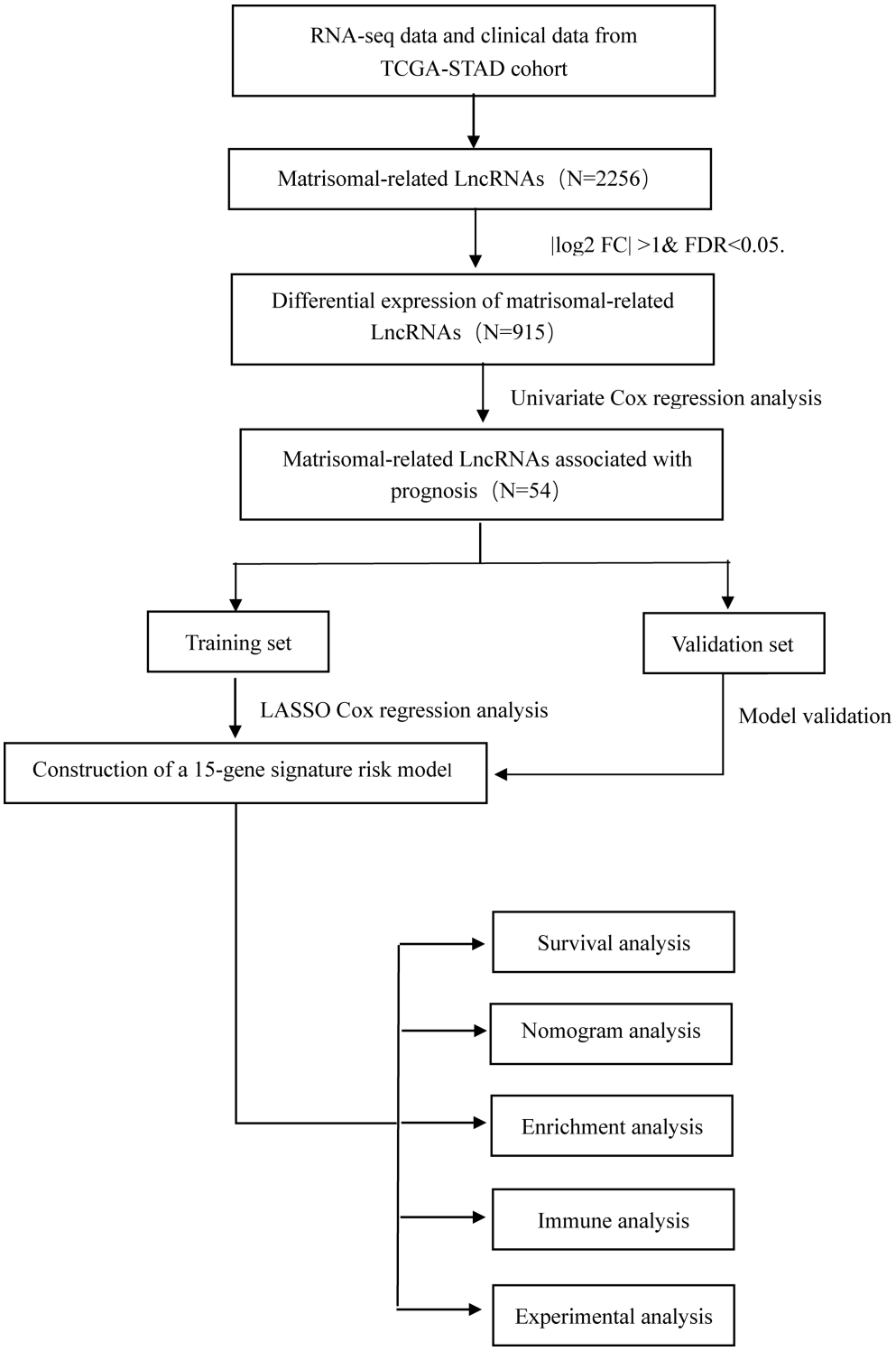

FIGURE 1 | The flowchart of this study.

Cell Lines and Transfection

Human GC cell lines, AGS and MKN45, were obtained from the Cell Bank of the Chinese Academy of Science. Two cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS: Gibco). GenePharma (Shanghai) synthesized small interfering RNA (siRNA) specific for GRASLND. siRNA was diluted into 100 μl of Opti-MEM Medium, and then transfected into AGS and MKN45 cells using 100 μl of Opti-MEM Medium with Lipofectamine 3000 (Invitrogen, CA, USA). Knockdown efficiency of the siRNA was assessed using quantitative PCR. The sequence was as follows (18):
GRASLND sense-1 GCUUUGACUUAGACUUCUAGC
GRASLND antisense-1 UAGAAGUCUAAGUCAAAGCUU
GRASLND sense-2 CUGUGAUGGUUAAUGUUAAGU
GRASLND antisense-2 UUAACAUUAACCAUCACAGGG

Quantitative Real-Time PCR

TransScript One step gDNA kit (TransGen, China) was utilized to achieve complementary DNA (cDNA) synthesis following the manufacturer's instructions. RT-PCR was performed using TransStart Top Green qPCR SuperMix (TransGen, China). The genetic expression level was normalized to the internal control GAPDH. The standard $2^{-\Delta\Delta Ct}$ method was utilized to analyze the relative lncRNA abundances. Specific primers utilized in this study are as follows (18):
GRASLND Forward AGGATTCAGGGGATGCACAG
GRASLND Reverse TGGGCTGAAGATGAGACGTT

Invasion Assays

Cell invasion assays were conducted as previously described (19). Matrigel was diluted at a ratio of 1:8 with PBS. After transfection, 1×10^5 cells were added to the upper chamber with 200 μl of serum-free medium. Complete cell culture medium (600 μl) was added to the lower chamber. Then, they were incubated for 48 h at 37°C. Finally, 4% PFA was used to fix all the inserts for 20 min. The inserts were immersed in 0.5% crystal violet for 20 min and quantified using a microscope.

Western Blot Analysis

The procedures of Western blotting were followed as described in previous reports (20). The primary antibody used was MMP9 (1:1,000, CST). The endogenous protein GAPDH (1:1,000, CST) was utilized for normalization.

RESULTS

Identification of Matrisomal-Related LncRNAs in Gastric Cancer

Firstly, we identified 2,256 matrisomal-related lncRNAs (**Table S1**). Subsequently, we carried out differential gene expression analysis between the tumor and normal tissues, and obtained 912 differential expression non-coding RNAs. Finally, 54 lncRNAs were found to be significantly associated with the OS of GC patients using univariate Cox regression analysis (**Figure 2A**).

Development and Validation of the Matrisomal-Related LncRNA Risk Model

A total of 371 GC patients were randomly segregated into two sets at a ratio of 1:1. Lasso Cox analysis was further performed in the training set, and a coefficient for each prognostic matrisomal-related lncRNA was generated accordingly. A 15 matrisomal-related lncRNAs model was eventually developed using the coefficient value (**Figures 2B, C**). The risk score of each sample was as follows:

$$\begin{aligned}\text{risk score} = {} & \text{AL353693.1} * 0.064 + \text{Z69666.1} * (-0.325) \\ & + \text{GRASLND} * 0.953 + \text{AC009283.1} * \\ & 0.004 + \text{AC245100.5} * 0.242 + \text{AC007277.1} * (-0.157) \\ & + \text{LINC02544} * 0.0221 + \\ & \text{AC068790.7} * 0.253 + \text{AC022509.2} * 0.033 + \text{UBE2R2} \\ & - \text{AS1} * (-0.007) + \text{LINC00460} * \\ & 0.010 + \text{AC005165.1} * 0.050 + \text{LINC00857} * (-0.052) \\ & + \text{AL355574.1} * (-0.178) + \\ & \text{AP000695.2} * 0.0323\end{aligned}$$

GC patients were split into two different risk groups based on the median risk value. Kaplan–Meier analysis was employed to investigate OS discrepancy between two groups. As shown in **Figures 3A–C**, GC patients in the high-risk group exhibit significantly shorter median OS ($p < 0.001$). Furthermore, we found that patients with high grade, metastasis, and immune score were associated with higher risk scores, suggesting that there exists a potential relationship between the risk score and the immune status (**Figures 3D–K**).

Kaplan–Meier Survival Analysis Based on Clinicopathological Characteristics

To assess the prognostic value for established MRL signature, GC patients were split into multiple subgroups based on their clinicopathological characteristics to perform Kaplan–Meier analysis. As shown in **Figures 4A–N**, the results demonstrated that GC patients in the high-risk group were correlated with worse survival in most groups.

Independent Prognostic Value of the Matrisomal-Related LncRNA Signature

Next, we visualized the distribution of risk score, overall survival status, and matrisomal-related lncRNA expression in multiple datasets. We observed that higher risk scores appeared depending on the increasing risk in the training (**Figure 5A**), validation (**Figure 5B**), and total (**Figure 5C**) set. Moreover, the results from two regression analyses indicated that the risk score was closely linked to OS ($p < 0.001$) after adjusting for age, gender, grade, and stage in the training (**Figure 5D**), validation (**Figure 5E**), and total sets (**Figure 5F**), suggesting that the risk score could be used as a potential independent prognostic variable.

Evaluation of the Validity of the Model

The results in **Figure 6** showed the accuracy of this signature based on time-dependent ROC curves in three sets. The AUC value for 1, 3,

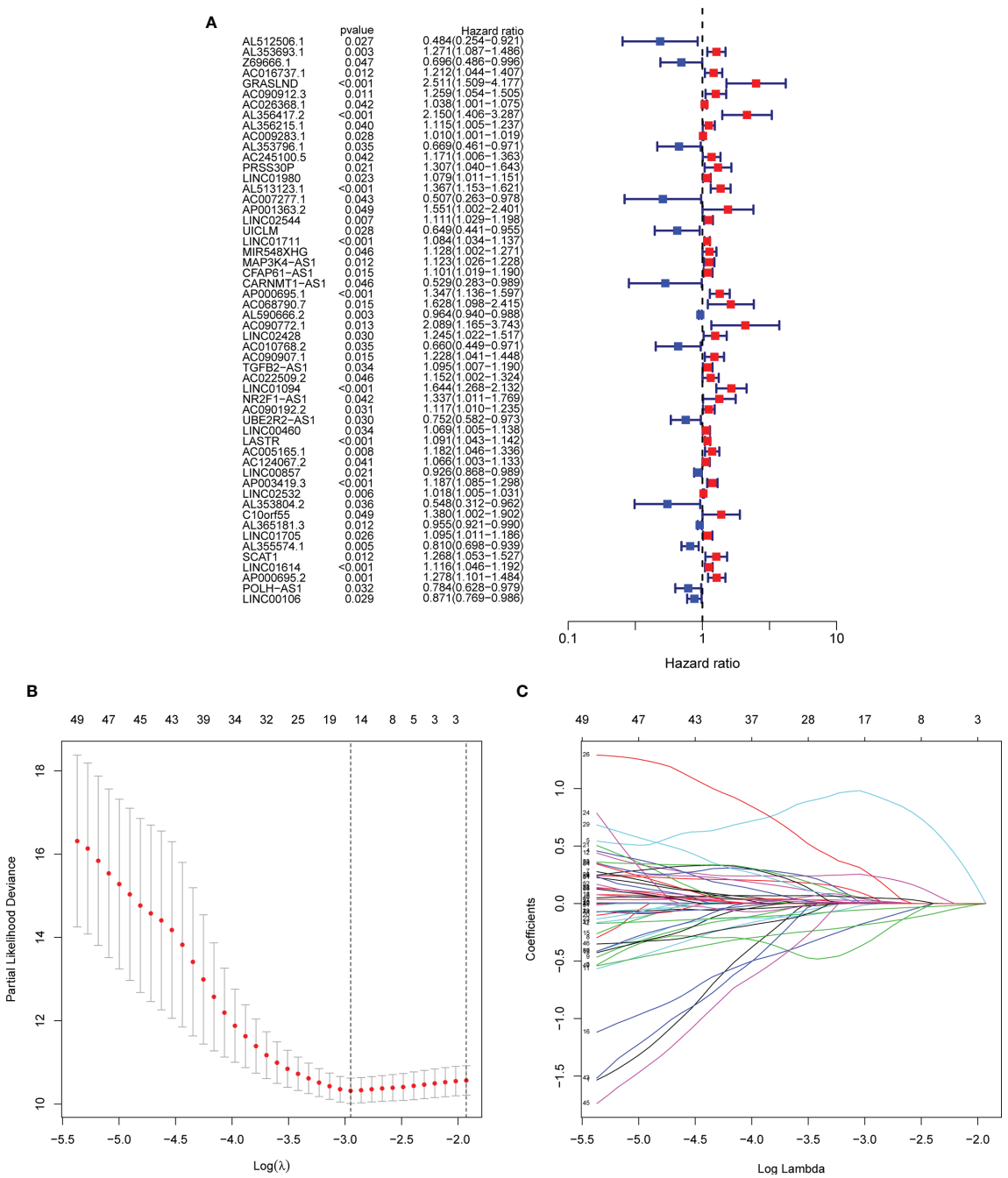

FIGURE 2 | Screening of matrisomal-related lncRNAs and construction of a risk model. **(A)** Forest plot shows the *p*-value and the hazard ratio of 54 matrisomal-related lncRNAs *via* univariate Cox regression analysis. **(B)**. The change trajectory of each independent candidate variable. **(C)**. The optimal model was eventually determined when lambda = 15.

and 5 years was 0.773, 0.794, and 0.893 in the training set (**Figure 6A**), 0.666, 0.624, and 0.648 in the validation set (**Figure 6B**), and 0.718, 0.712, and 0.784 (**Figure 6C**) in the total set, respectively. Moreover, we compared the risk score with multiple clinicopathological features (**Figures 6D–F**). The risk score in three sets was more effective than other factors in predicting GC prognosis. Finally, we employed C-index (**Figure 6G**) and DCA (**Figure 6H**) to assess the performance of the risk model. PCA was further performed to reflect the distribution of the overall samples in different statuses (**Figures 7A–D**). The results showed that GC samples were distributed more obviously in distinct directions, indicating a good discrimination for the risk model.

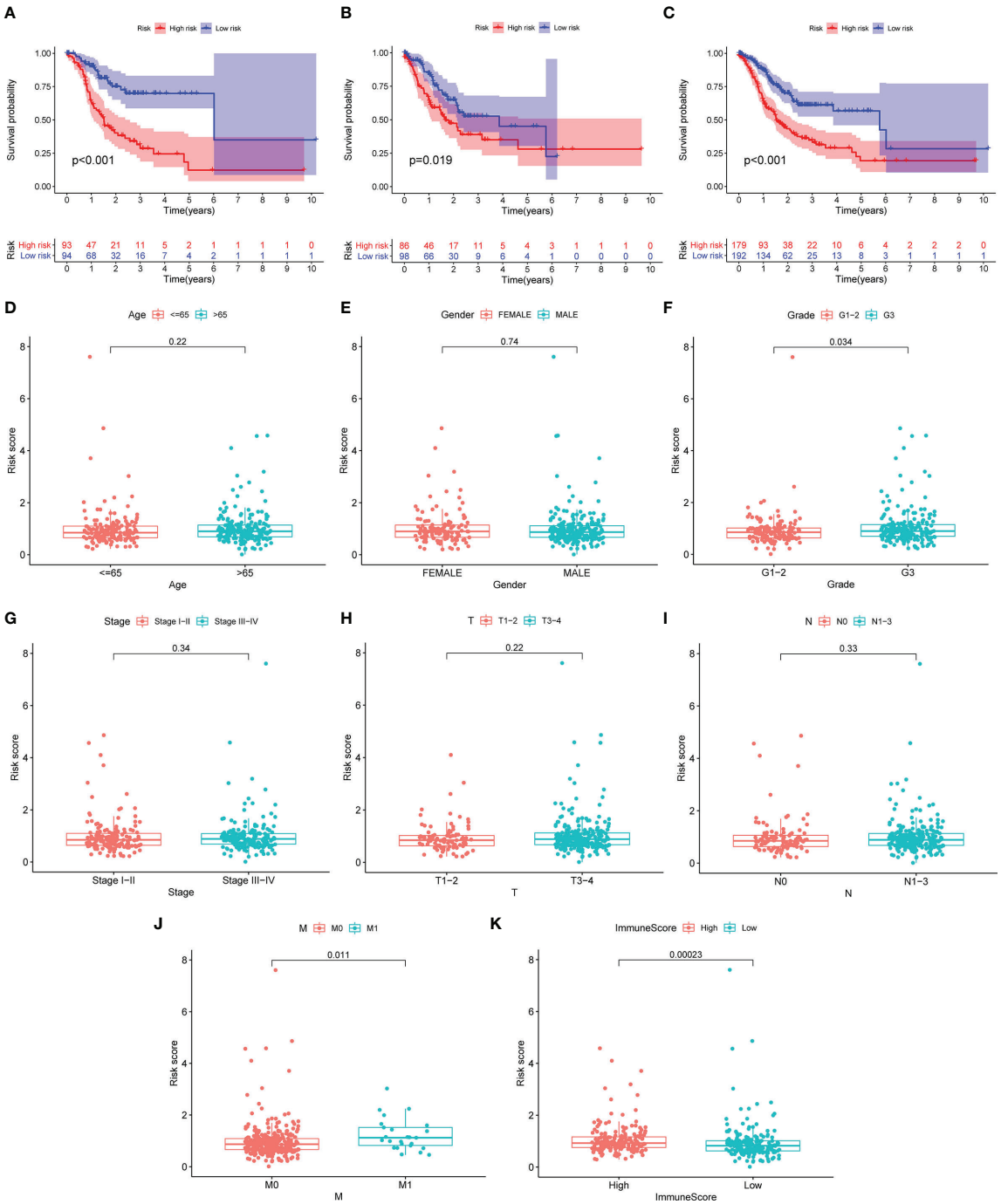

FIGURE 3 | The risk score correlated with overall survival and clinicopathological features. **(A–C)** Kaplan–Meier curves of GC patients between the high- and low-risk group in the training **(A)**, validation **(B)**, and total **(C)** set. **(D–K)** Comparison of risk score distribution between the two groups: age **(D)**, gender **(E)**, G1–2/G3 **(F)**, stage I–II/III–IV **(G)**, T1–2/T3–4 **(H)**, N0/N1–3 **(I)**, M0/M1 **(J)**, and high and low immunescore **(K)**

Construction and Validation of the Nomogram

In order to promote the clinical application of the risk model, the nomogram was eventually built to predict 1-, 3-, and 5-year OS. Several clinical variables, including age, gender, grade, stage, T, N, M, and risk score, were used to establish the nomogram (**Figure 8A**). Moreover, calibration curves were introduced to predict 1-, 3-, and 5-year OS (**Figures 8B–D**). The stability and accuracy of this hybrid nomogram could plausibly function in the areas of GC management.

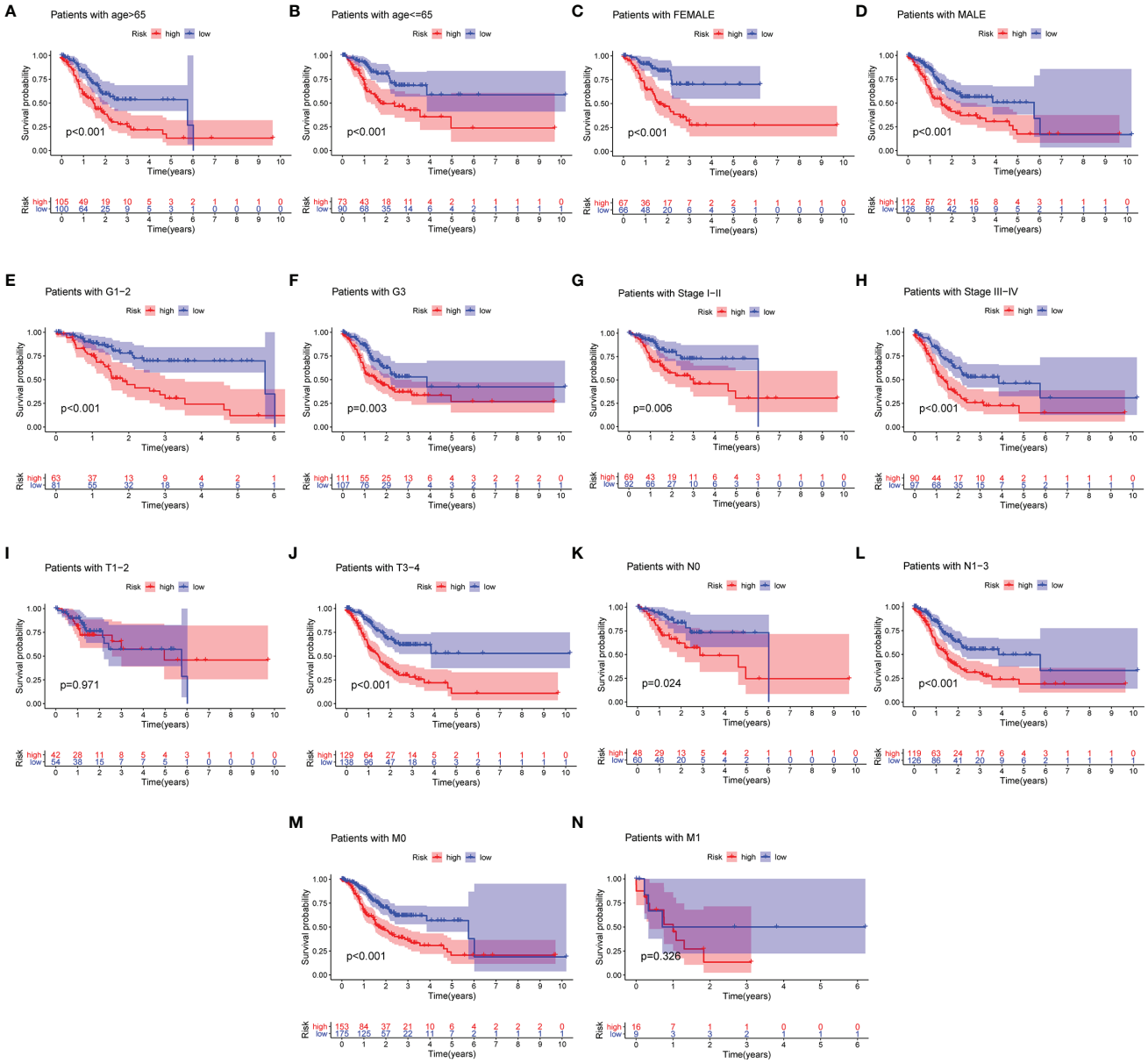

FIGURE 4 | Differential survival outcomes of GC in the clinical subgroup. **(A–N)** Kaplan–Meier curves for age **(A, B)**, gender **(C, D)**, grade **(E, F)**, stage **(G, H)**, T **(I, J)**, N **(K, L)**, and M **(M, N)**.

Functional Enrichment Analysis of the Risk Signature

To determine potential biological roles that were correlated with risk signature, we performed functional enrichment analysis for DEGs between two groups. A total of 241 DEGs were further identified between the low- and high-risk subgroups. Gene Ontology (GO) analysis consisted of cellular component (CC), molecular function (MF), and biological process (BP). The CC GO terms showed that the top 4 enriched pathways were collagen-containing ECM, contractile fiber, contractile fiber part, and myofibril (**Figure 9**). The MF GO terms indicated that the top 4 enriched pathways were ECM structural constituent, glycosaminoglycan binding, heparin binding, and sulfur compound binding (**Figure 9**). The BP GO terms demonstrated that the top 4 enriched pathways were muscle system process, muscle contraction, extracellular structure organization, and ECM organization (**Figure 9**). These results indicated that the risk signature might be functionally implicated in the regulation of ECM networks.

Functional Enrichment Analysis of the Risk Signature

To confirm the association between the risk score and infiltration of immune cells, we conducted further analysis based on seven

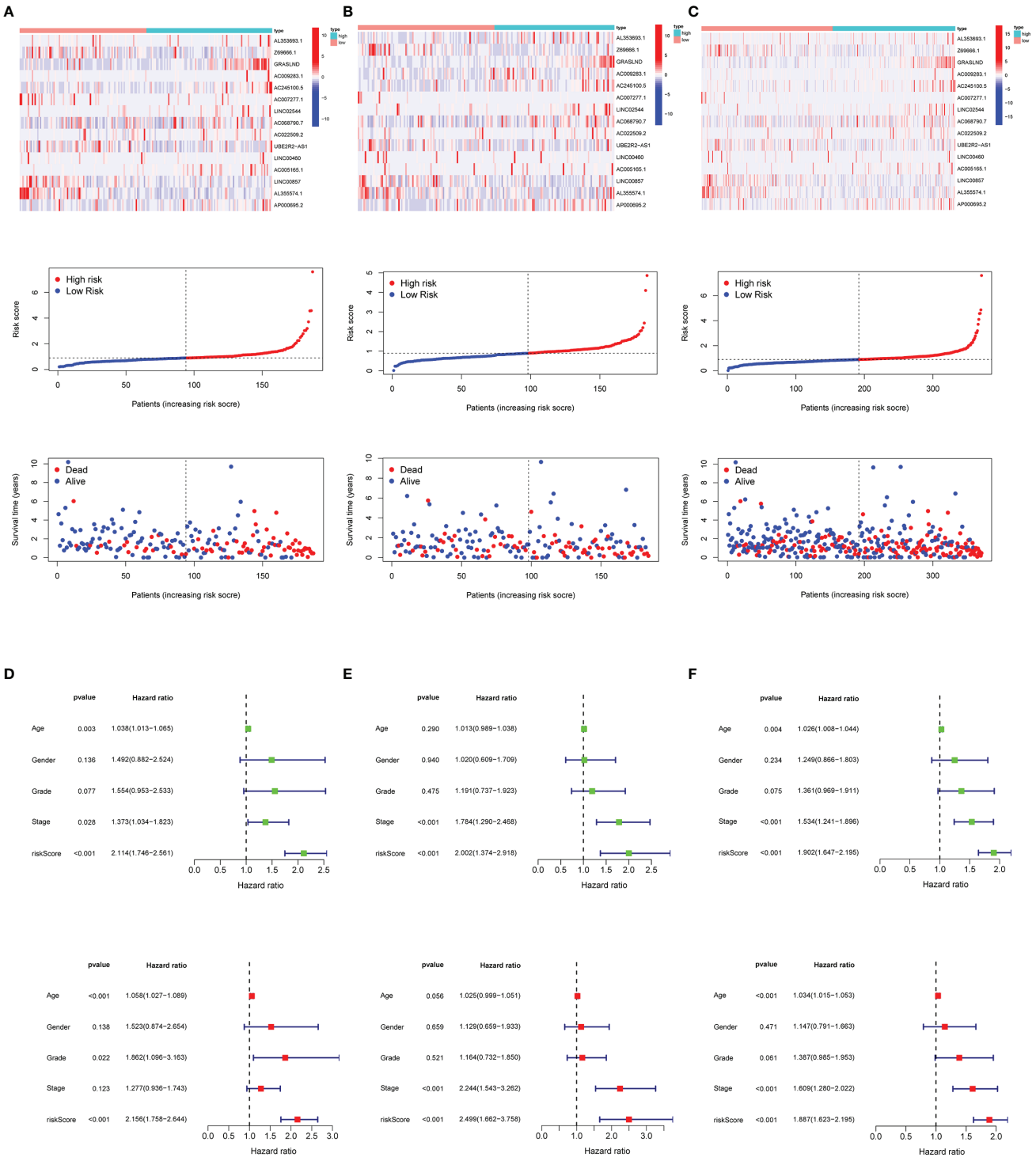

FIGURE 5 | Risk score distribution, survival status, and 15 matrisomal-related lncRNA expression. **(A)** Training set. **(B)** Validation set. **(C)** Total set. Risk score function as an independent prognostic factor. **(D)** Training set. **(E)** Validation set. **(F)** Total set.

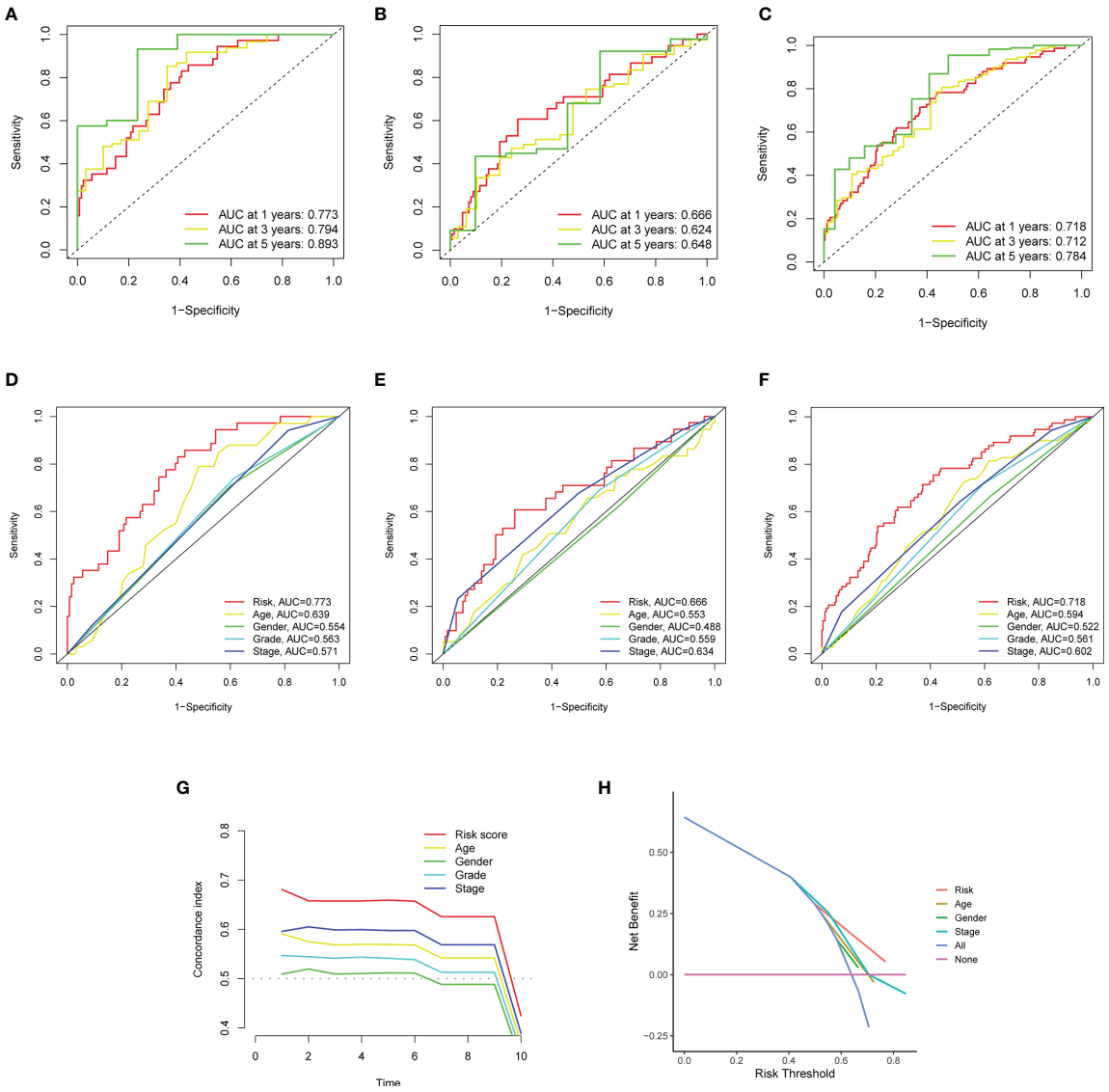

FIGURE 6 | The performance of the matrisomal-related lncRNAs in prognostic prediction. **(A–C)** Time-dependent ROC analysis for evaluating the sensitivity and specificity of this prognostic model in the training **(A)**, validation **(B)**, and total sets **(C)**. **(D–F)** Comparison of clinicopathological features and the risk score in the training **(D)**, validation **(E)**, and total sets **(F)**. **(G, H)** Comparison of clinicopathological features and the risk score in the total sets based on concordance index **(G)** and decision curve analysis **(H)**.

algorithms (**Figure 10A**). The heatmap was used to display the tumor-infiltrating cell proportions in the different groups. As shown in **Figure 10A**, GC patients in the high-risk group showed higher tumor-infiltrating cell proportions. Macrophage infiltration has been mostly observed in the high-risk group using six algorithms. We further showed the results based on CIBERSORT algorithm as an example, M2 macrophage infiltration was easier to detect in the high-risk group (**Figure 10B**). The results indicated that the proportions of M2 macrophage in the high-risk group might contribute to immune evasion or tumor-promoting effects. Afterwards, we analyzed the potential correlation between the risk score and immune function. As is shown **Figures 10C, D**, the contents of the primary antigen presentation process showed higher proportions in the high-risk group. However, lower MHC_class_I was observed in the high-risk group. The results indicated that this might be associated with the immunosuppressive microenvironment. As is seen in **Figure 10E**, most of the immune checkpoint genes (CD86, CD200, LAIR1, CD44, TNFSF9, PDCD1LG2, NRP1CD276, CD48, and HAVCR2) were dramatically upregulated in the high-risk group. The abnormal expression of genes might contribute to the immunosuppressive microenvironment. To confirm this speculation, we further examined the TIDE score in the different groups. As we expected, our high-risk group showed a

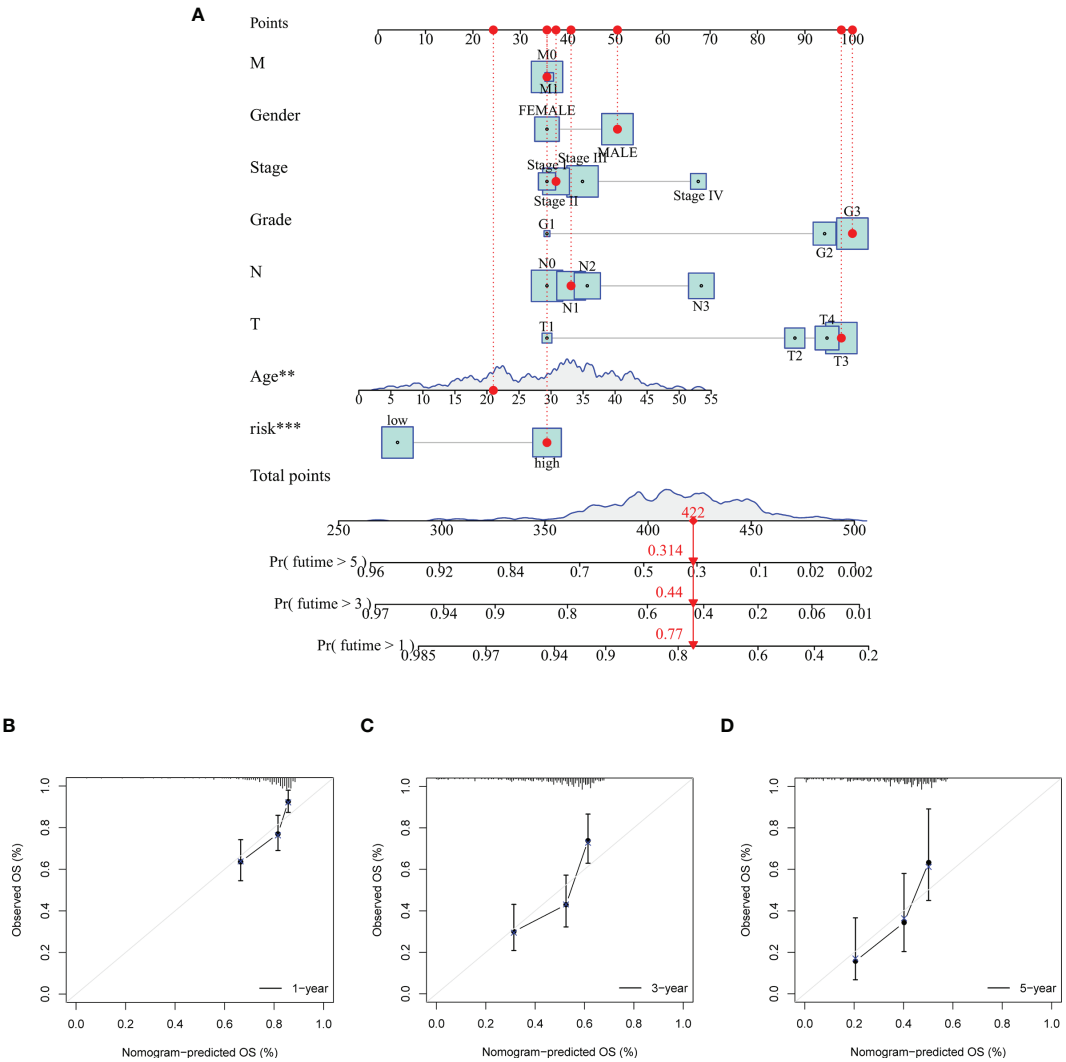

FIGURE 7 | Principal component analysis (PCA) to visualize gene expression patterns in the different groups. The groups defined by all genes **(A)**, matrisomal-related genes **(B)**, matrisomal-related lncRNAs **(C)**, and risk score based on matrisomal-related lncRNAs **(D)**. $^{**}p < 0.01$, $^{***}p < 0.001$

higher TIDE score, suggesting that the risk score could discriminate subgroups of GC patients with different immune responses (**Figure 10F**).

Effects of LncRNA GRASLND on the Regulation of Matrisome in Gastric Cancer

Furthermore, we sought to prove that these lncRNAs in the risk model contribute to the regulation of ECM. Since lncRNA GRASLND showed the highest coefficient and hazard ratio, we chose it to evaluate the potential effects on the regulation of ECM in GC. AGS and MKN45 cell lines were transfected with two si-GRASLNDs, respectively. Subsequently we performed qRT-PCR analysis to confirm the knockdown efficiency (**Figure 11A**). Afterwards, GRASLND depletion significantly decreased the invasive capacity of AGS and MKN45 (**Figures 11B, C**). Furthermore, we analyzed the protein expression of the key enzyme of ECM, MMP9. We found that MMP9 was significantly downregulated after lncRNA GRASLND knockdown in GC (**Figure 11D**). Together, our results suggest that there is a strong correlation between lncRNA GRASLND and the regulation of ECM in GC.

DISCUSSION

Although surgical resection has been proven to improve the curative effect of GC, the therapeutic effect of advanced and metastatic GC is still not fully satisfactory. Several remarkable advances and breakthroughs have been witnessed in the management of advanced cancers, especially immunotherapy (21, 22). In recent years, researchers have pointed out the dominant roles of tumor heterogeneity in drug resistance and

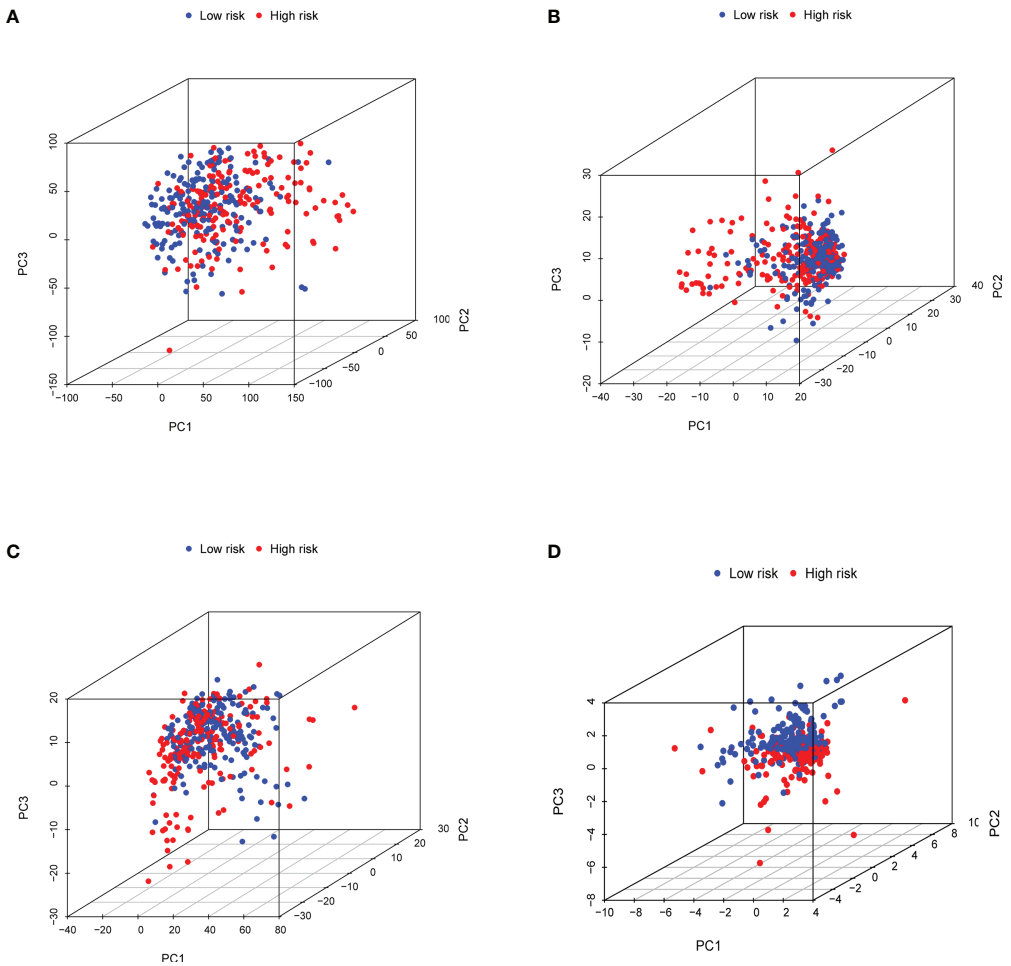

FIGURE 8 | Establishment and validation of a nomogram for predicting the overall survival of GC patients. **(A)** The nomogram was used for predicting the 1-, 3-, and 5-year OS of patients in the total set. Calibration curves of the nomogram predicting OS of 1 year **(B)**, 3 years **(C)**, and 5 years **(D)**.

treatment failure (23). Moreover, the complexity of the tumor microenvironment has also exerted a distinct effect on these processes (24, 25). Considering the roles of ECM during tumor progression and immunotherapy resistance, we sought to explore the potential roles of matrisomal-related genes in prognosis prediction and immune regulation.

In this study, we constructed the risk model with a good performance based on matrisomal-related lncRNAs. This model could achieve the robustness of prognosis prediction and assess the robustness of prognosis prediction. Firstly, we obtained 1,068 matrisomal-related genes from the M.I.T. and identified matrisomal-related lncRNAs by Pearson correlation analysis. Among these candidate matrisomal-related lncRNAs, 54 prognostic-related lncRNAs were firstly selected and introduced to the lasso regression. Ultimately, 15 optimal matrisomal-related lncRNAs were obtained for risk model construction. The 1-, 3-, and 5-year AUCs were 0.718, 0.712, and 0.784, respectively, suggesting that the model showed good predictive power for the prognosis of GC patients. The predictive efficiency of the risk model was better than common clinicopathological variables. Multivariate analysis further demonstrated that the risk score was an independent prognostic factor for GC. In addition, to better guide clinical application, a nomogram model was established to predict the prognosis of GC patients. This nomogram model showed a better performance in the prediction of OS. Moreover, we uncovered a positive correlation between risk score and M2 macrophage infiltration. Finally, the experimental validation presented in this study suggested the strong correlation between lncRNA GRASLND and the regulation of ECM in GC.

Recent studies have demonstrated that specific matrix gene sets were predictors of immunosuppression, and thus might predict the efficacy of anti-PD1 therapy (26). Furthermore, Erkan et al. have also identified several stromal signatures that served as predictive and prognostic indicators for patients with pancreatic cancer (12). These results suggested the potential link between matrisome and anti-tumor immunity. Tumor immune-infiltrating cells not only exert an impact on tumor progression,

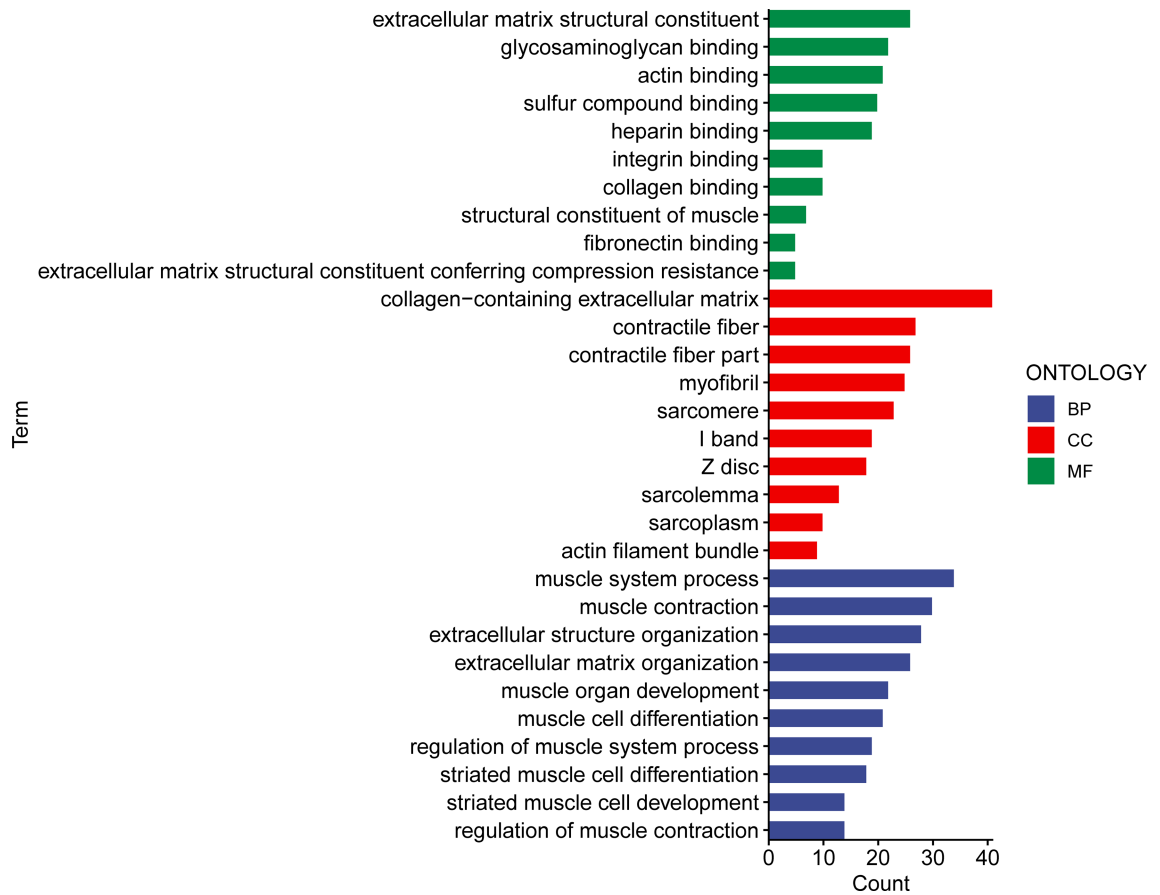

FIGURE 9 | The GO enriched pathways in the different risk subsets obtained from the 15 matrisomal-related lncRNA-based signature.

but also induced immune responses to the anti-tumor therapy. Therefore, we explored tumor immune-infiltrating cells to further reveal the underlying mechanisms of immune evasion. In this study, we explored the correlation between risk score and the immune microenvironment. Our results showed that M2 macrophage infiltration was significantly altered in the high-risk group. Several studies demonstrated that M2 macrophage was associated with poor prognosis and disease processes (27, 28). Increasing lines of evidence suggested that different patterns of tumor-associated macrophages (TAMs) played a crucial role in immune escape. M2 macrophages have been proven to be associated with inhibitory cytokine secretion and immune cell infiltration, further attracting negative regulatory factors to the favorable immunosuppressive TME (29, 30). The mechanisms on how M2 macrophages mediated tumor immune escape was complex according to previous studies. Several cells like Th2, Tregs, and MDSCs could achieve M2 macrophage polarization and enhance its infiltration *via* different pathways (31). Conversely, cross-talk among these cells could also lead to a consequent immunosuppressive effect. These findings highlight the crucial roles of M2 macrophage in immune escape. As such, our risk model shows the close relation to M2 macrophage infiltration. Therefore, this model might offer important and unique advantages in the future of cancer immunotherapy.

Subsequently, we studied the interaction between risk score and immune functions. The results of ssGSEA showed that the contents of the antigen presentation process significantly differed in the high-risk group. The above findings further explained the reasons for the tumor-promoting status in the high-risk group and revealed that the immunosuppressive microenvironment exists in this group. These results supported findings from immune cell infiltration analysis. Due to the significance of ICIs, we further assessed the genetic basis of expression of ICI genes, and found the difference between two groups. Interestingly, we found that CD86, CD200, LAIR1, CD44, TNFSF9, PDCD1LG2, NRP1, CD276, CD48, and HAVCR2 were significantly higher in the high-risk group. Finally, the TIDE score was introduced to predict immune response. We found that the high-risk score was associated with a high TIDE score. Collectively, these observations suggested that these patients demonstrated an immunologically "cold" profile not to gain from immunotherapy.

Considering that GRASLND showed the primary effect on the prognosis of GC patients and had a larger coefficient in the risk model, we finally chose GRASLND to conduct functional

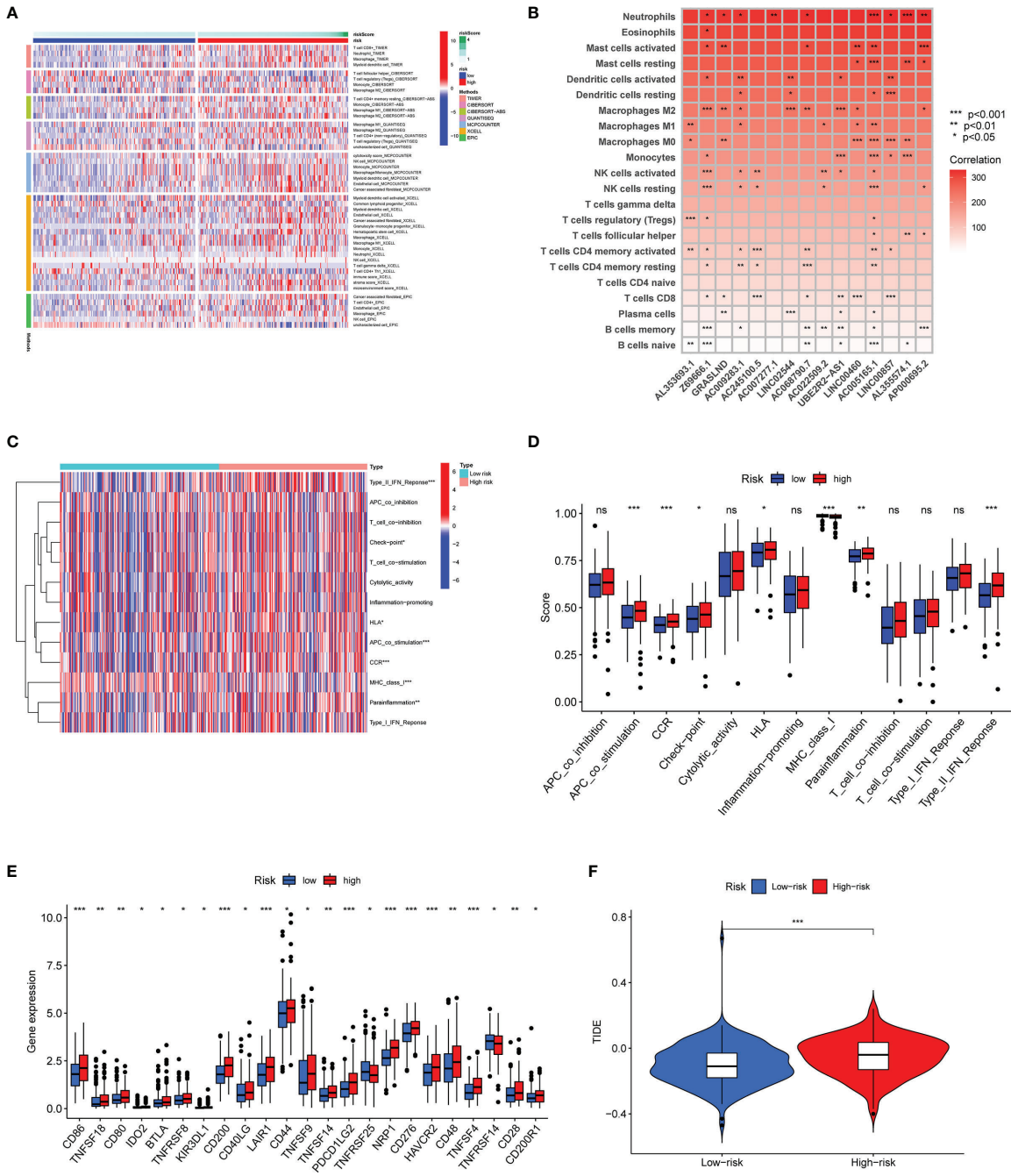

FIGURE 10 | Differential immune features in the high-risk and low-risk subsets. **(A)** The association between infiltrating immune cells and risk scores using seven algorithms. **(B)** The correlation between the expression of 15 matrisomal-related lncRNA-based signature and infiltrating immune cells using the CIBERSORT algorithm. **(C, D)** The heatmap and boxplot show the correlation analysis between immune functions and risk score. **(E)** Box plots revealed the differential expression of the immune checkpoint genes between two groups. **(F)** Correlation of risk score and TIDE score in different risk subgroups. ns no statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

experiments and explore the correlation with matrisome in GC cells. Our results indicated that knockdown of lncRNA GRASLND could decrease the protein expression of the key enzyme of ECM, MMP9. MMP9 plays a crucial role in the degradation of the ECM (32–34). Moreover, the upregulation of MMP9 was related to metastasis in GC (35, 36). Thus, our experiments confirmed that lncRNA GRASLND was the potential contributor towards regulation of ECM. However, there are still several limitations to our study. External validation of the risk model would be beneficial for its wide

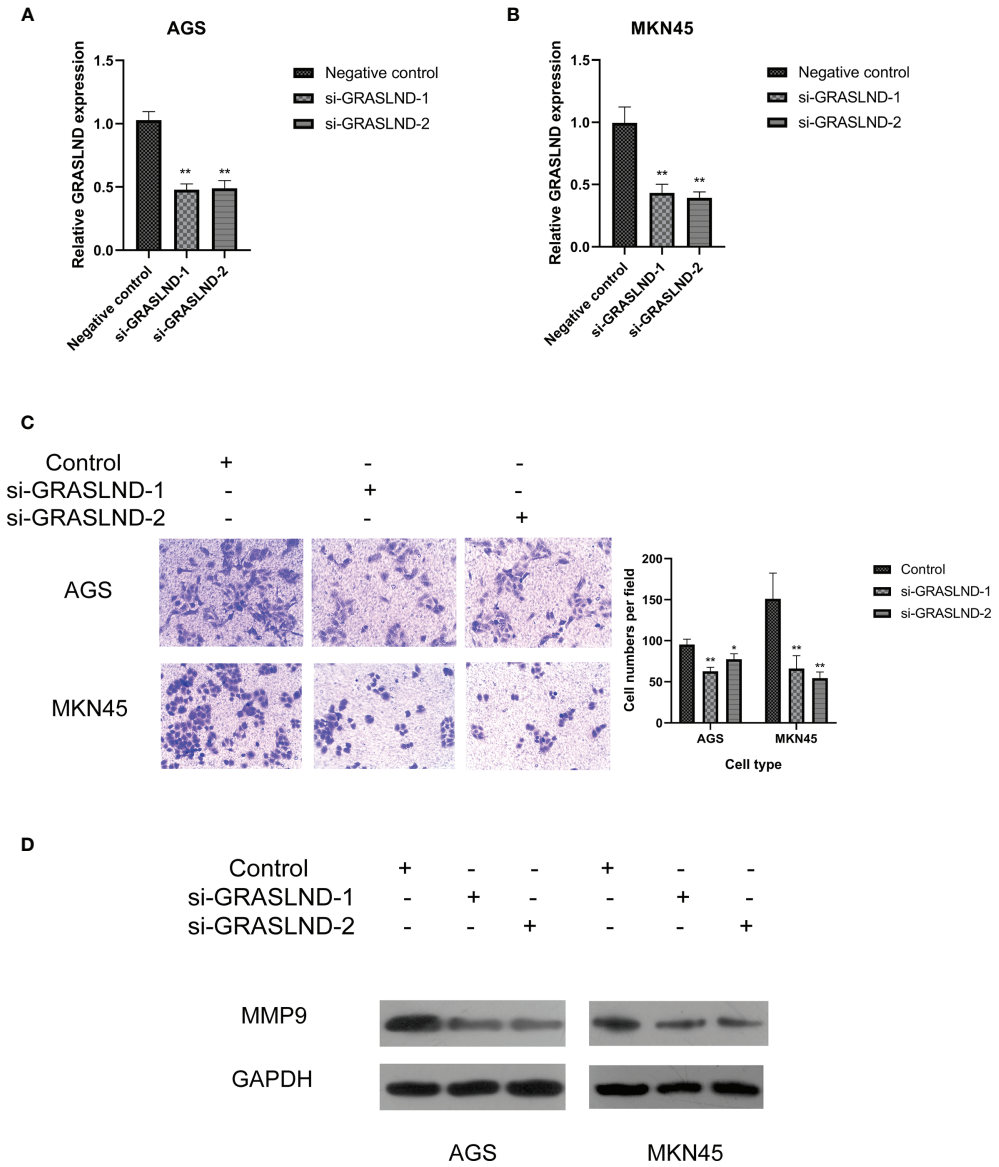

FIGURE 11 | Effects of lncRNA GRASLND on the regulation of extracellular matrix in GC cells. **(A)** AGS and MKN45 cell lines were transfected with si-GRASLND, and qRT-PCR was performed to confirm the knockdown efficiency. **(B)** Knockdown of lncRNA GRASLND decreased the invasive ability of MKN45 cells. **(C)** Knockdown of lncRNA GRASLND decreased the invasive ability of AGS cells. **(D)** Knockdown of lncRNA GRASLND decreased the protein level of MMP9. $^{*}p < 0.05$, $^{**}p < 0.01$.

use. Hence, clinical samples would also be collected for further verification despite being time-consuming. Furthermore, further experiments are also needed to clarify the unknown functions and the potential mechanism of these lncRNAs.

CONCLUSION

In conclusion, we first constructed the 15 matrisomal-related lncRNAs model with a good performance in predicting the prognosis of GC patients. The risk signature might serve as a relatively good predictive tool to manage patients with GC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Conception and design: YY, LS, and JZ. Administrative support: YNZ and QG. Provision of study materials or patients: YZ, GW,

and ML. Collection and assembly of data: JS, ZC, YW, and RJ. Data analysis and interpretation: YY, LS, and JZ. Manuscript writing: All authors. Final approval of manuscript: All authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.926404/full#supplementary-material

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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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A Necroptosis-Related lncRNA to Develop a Signature to Predict the Outcome, Immune Landscape, and Chemotherapeutic Responses in Bladder Urothelial Carcinoma

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Objective: Many studies have drawn their attention to the immunotherapy of bladder urothelial carcinoma in terms of immunologic mechanisms of human body. These include immunogenicity of the tumor cells and involvement of long non-coding RNA (lncRNA). We constructed a necroptosis-related long noncoding RNA (nrlncRNA) risk factor model to predict BLCA outcomes and calculate correlations with chemosensitivity and immune infiltration.

Methods: Transcriptomic data from BLCA specimens were accessed from The Cancer Genome Atlas, and nrlncRNAs were identified by performing co-expression analysis. Univariate analysis was performed to identify differentially expressed nrlncRNA pairs. We constructed least absolute contraction and selector operation regression models and drew receiver operating characteristic curves for 1-, 3-, and 5-year survival rates. Akaike information criterion (AIC) values for survival over 1 year were determined as cutoff values in high- and low-risk subgroups. We reassessed the differences between subgroups in terms of survival, clinicopathological characteristics, chemotherapy efficacy, tumor-infiltrating immune cells, and markers of immunosuppression.

Results: We identified a total of 260 necroptosis-related lncRNA pairs, of which we incorporated 13 into the prognostic model. Areas under the curve of 1-, 3-, and 5- year survival time were 0.763, 0.836, and 0.842, respectively. We confirmed the excellent predictive performance of the risk model. Based on AIC values, we confirmed that the high-risk group was susceptible to unfavorable outcomes. The risk scores correlated with survival were age, clinical stage, grade, and tumor node metastases. The risk model was an independent predictor and demonstrated higher predictive power. The risk model can also be utilized to determine immune cell infiltration status, expression levels of immune checkpoint genes, and the sensitivity to cisplatin, doxorubicin, and methotrexate.

Conclusion: We constructed a novel necroptosis-related signature that predicts BLCA outcomes and performs satisfactorily in the immune landscape and chemotherapeutic responses.

Keywords: bladder urothelial carcinoma, necroptosis-related, lncRNA signature, outcomes, immune checkpoint, chemotherapeutic response

INTRODUCTION

Bladder urothelial carcinoma (BLCA) is the 9th most prevalent malignancy worldwide (1, 2). More than 199,000 people died from the disease in 2018, and more than 549,000 new cases were confirmed in 2018 (3). The number of BLCA events has been increasing worldwide over the past two decades, and the incidence of BLCA has been increasing yearly due to population aging and environmental pollution (4, 5). Treatment options for BLCA include transurethral resection, radical cystectomy, radiotherapy, and chemotherapy; nevertheless, BLCA remains an aggressive neoplasm with a substantial incidence of recurrence, metastasis, rapid progression, and unfavorable outcomes (6–8). One study in the United States estimated 80,470 new diagnoses of BLCA cases and at least 1,767 deaths in 2019 (9). Once the tumor has progressed and metastasized, the combination of systemic chemotherapy and surgery tends to be ineffective (10, 11). At least 30% respond to immunotherapies and immune checkpoint inhibitors (ICIs) (12). There is a need for research to construct reliable prognostic biomarkers through molecular profiling to identify prognostic markers and therapeutic targets for BLCA to improve outcomes.

Necroptosis is programmed cell death triggered by MLKL, RIP1, and RIP3 (13, 14). Several lines of evidence suggest that necroptosis is involved in Parkinson's disease, infectious diseases, cancer, and other diseases (14, 15). Researchers found that necroptosis is a critical factor influencing tumor metastasis and T cell death (16). Interestingly, necroptosis has been related to antitumor immunity (13). As a substitute mode of programmed cell death to control apoptosis resistance, necroptosis plays a role in antitumor immunity in cancer therapy (13). Studies showed that necroptosis is a crucial cellular response that regulates many tumors' onset, progression, and metastasis (17). Studies also found that necroptosis can serve as a biomarker in some diseases, particularly cancer (18, 19). Necroptosis enhances cancer cell migration and invasion in pancreatic carcinoma *via* the production of CXCL5 (20). Nevertheless, the precise functions of necroptosis in BLCA and the molecular mechanisms remain undetermined.

Long non-coding RNAs (lncRNAs) are a family of RNAs with no capacity for protein-coding that are more than two hundred nucleotides in length (21). A body of evidence suggests that necroptosis-related lncRNAs (nrlncRNAs) influence tumor progression and metastasis by triggering immune system processes and immune responses (17, 22). Studies showed that nrlncRNAs are associated with outcomes of various tumors (18, 22, 23). Bioinformatics analysis based on The Cancer Genome Atlas (TCGA) revealed that lncRNAs are associated with the progression of BLCA *via* immune-related pathways (24).

Differentially expressed nrlncRNAs may serve as prognostic indicators and drug targets in BLCA. This study utilized gene expression profiles of high-throughput sequencing data from TCGA to identify lncRNAs targeting necroptosis-related genes and to develop necroptosis-related prognostic signatures for patients with BLCA.

MATERIALS AND METHODS

Datasets and Preprocessing

We queried TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) (level 3 data, FPKM value) to obtain RNA sequencing profiles of 414 BLCA and 19 normal bladder specimens. We set the workflow type to "HTSeq-FPKM" and the data type to "Gene Expression Quantification" in the dataset download. To conduct subsequent analysis, normalization of the expression profiles to transcripts kilobase million values was carried out, and all analyses were conducted using R (version 4.1.1). Gencode (version 26) GTF files were obtained through Ensembl (http://asia.ensembl.org) for annotation and differentiation of lncRNAs and mRNAs (25). Sex, age, clinical stage, and survival rates were obtained from TCGA for clinical data after removing specimens with insufficient clinical data or a survival duration of 0 days. Finally, we included 19 normal bladder specimens and 408 BLCA specimens. **Supplementary Table 1** displays the clinical characteristics.

Identification of Necroptosis-Related lncRNAs

We accessed the Gene Set Enrichment Analysis site (http://www.gsea-msigdb.org/gsea/index.jsp) and obtained the necroptosis gene set M24779, which included eight necroptosis genes. We combined prior reports on necroptosis and obtained 67 necroptosis-related genes, details of which are displayed in **Supplementary Table 2**. We calculated Pearson correlations between necroptosis-related genes and identified lncRNAs. Correlation coefficients > 0.5 and $p < 0.001$ were used to identify lncRNAs linked to necroptosis.

Identification of Differentially Expressed Necroptosis-Related lncRNAs

We acquired 67 nrlncRNAs and used the R language version 4.1.1 "limma" package to identify differentially expressed necroptosis-related lncRNAs (DEnrlncRNAs) between BLCA and normal bladder specimens. The screening conditions were $|\log \text{fold-change}| > 1.0$ and $p < 0.05$ (26). The expression matrix of differentially expressed lncRNAs was visualized using the heatmap package.

Paired DEnrlncRNAs

DEnrlncRNAs were identified using several pairing cycles, assuming that C was equal to the sum of lncRNA A and lncRNA B. A 0 or 1 matrix was then created. If lncRNA A expression level was greater than the level of lncRNA B, C was defined as 1; otherwise, C was defined as 0. The 0-or-1 matrix

Abbreviations: BLCA, bladder urothelial carcinoma; TCGA, The Cancer Genome Atlas; nrlncRNA, necroptosis-related long noncoding RNA; DEnrlncRNA, differentially expressed necroptosis-related long noncoding RNA; AIC, Akaike Information Criterion; ICIs, immune checkpoint inhibitors; FDR, false discovery rate; LASSO, Least Absolute Shrinkage and Selector Operation; ROC, receiver operating characteristic; AUC, area under the curve; IC50, half inhibitory concentration.

was then re-evaluated. The correlation between pairs and patient outcomes was not evaluated if the expression level of lncRNA pairs was 0 or 1 because no pair ranks could accurately anticipate patient survival outcomes. In cases where the number of lncRNA pairs with an expression level of 0 or 1 accounted for more than 20% of the total logarithm, it was considered a valid match; otherwise, re-pairing was required.

Development of a Necroptosis-Related Prognostic Risk Model

According to BLCA data obtained from TCGA, univariate Cox proportional hazards regression analysis was utilized to identify lncRNA pairs associated with outcomes from necroptosis-related lncRNAs ($p < 0.05$). We then performed least absolute contraction and selector operation (LASSO) regression with 10-fold cross-validation and a p-value of 0.05 and ran 1,000 loops. For each cycle, 1000 random stimuli were set to prevent overfitting. We then selected the best-paired combination to obtain nrlncRNA pairs in constructing the Cox risk coefficient model. By creating Cox univariate and multivariate analysis models, the risk coefficient for every necroptosis-related lncRNA pair associated with the outcomes of patients with BLCA was determined, and the risk score for each tumor specimen was determined. The aggregated risk score for every BLCA sample was the sum of the expression levels of each necroptosis-related lncRNA pair in the sample multiplied by the risk factor. The formula is as follows: risk score = $\sum_{i=1}^{n}$risk coefficient $_{i} \times$ nrlncRNA expressioni. The *survminer* and *survival* tools in R software were used to visualize the findings of the Cox analysis.

Assessing the Predictive Power of Prognostic Risk Model

The area under the curve (AUC) was evaluated to ascertain the predictive capacity of the risk model for determining patient outcomes, and receiver operating characteristic (ROC) curves were produced using the survivalROC module in the R software, which included ROCs at 1, 3, and 5 years. To compute the Akaike information criterion (AIC) value at every point on the 1-year ROC curve to obtain threshold values that optimize the aggregate of specificity and sensitivity in separating low-risk from high-risk individuals, we conducted a Kaplan-Meier analysis to identify disparities in survival between individuals in the high- and low-risk groups, which we demonstrated using survival curves to calculate this cutoff value.

Prognostic Risk Model Validation

We performed the chi-square test to examine the correlation between the model and clinical and pathological features to assess the clinical significance of the constructed model. The Wilcoxon signed-rank test was performed for these clinicopathological variables to examine the difference in riskScore between groups. The analysis findings were displayed using box plots. We conducted univariate and multivariate Cox regression analyses between clinicopathological parameters and riskScore to determine whether the model could serve as an independent outcome predictor. To present the results, we generated forest plots. *Survival*, *Heatmap*, and *ggupbr* were the R packages we used.

Analysis of Tumor-Infiltrating Immune Cells

We used CIBERSORT to determine the association between risk scores and immune cell signatures (http://cibersort.stanford.edu/) (27), TIMER (version 2.0; http://timer.cistrome.org/) (28), QUANTISEQ (http://icbi.at/quantiseq) (29), Microenvironmental Cell Population Counter (30), EPIC (http://epic.gfellerlab.org) (31) and XCELL (http://xCell.ucsf.edu/) (32) to determine immune infiltration status in patients with BLCA. The Wilcoxon signed-rank test was performed to determine the differences in the content of immune infiltrating cells between the high- and low-risk groups of the constructed model. The findings were expressed in bubble charts. The *ggplot2* tool in R software was used to visualize the data.

Analysis Between Immune Checkpoints and Risk Models

To investigate the relationship between the expression of immune checkpoint-related genes (TIM-3, PDL1, LAG3 PD1, GAL9, TIGIT, PD1LG2, and CTLA4) and the model, we compared high- and low-risk subgroups and visualized them using the *ggstatsplot* package and violin plots.

The Value of Risk Models in Clinical Management

The half-inhibitory concentration (IC_{50}) of frequently used chemotherapeutic medicines for BLCA was determined in the dataset to assess the model's utility in medical therapy. The Wilcoxon signed-rank test was performed to calculate differences in IC_{50} between high- and low-risk groups. The data were presented using the R software packages *ggplot2* and *pRRophetic* (33).

Statistical Analyses

This study used R software (version 4.1.1) for statistical analysis. Differences between the two subgroups were estimated using the Wilcoxon rank-sum test. All statistical tests were two-way when $p < 0.05$ indicated statistical significance.

RESULTS

Identification of DEnrlncRNAs

Figure 1 demonstrates this study's flowchart. The initial step was to obtain transcriptome data for BLCA from TCGA. Finally, we included 19 normal samples and 414 BLCA samples. In the second step, data were annotated in accordance with the Gene Transfer Format files from Ensembl. We combined prior reports on necroptosis and obtained 67 necroptosis-related genes, according to 67 necroptosis-related genes and DEnrlncRNAs between normal and tumor samples ($|\log$ fold-change$| > 1.0$ and $p < 0.05$), we identified a total of 291 nrlncRNAs (**Supplementary Table 3**), of which 89 were classified as

DEnrlncRNAs (**Figure 2A**); 76 underwent upregulation, and 13 underwent downregulation (**Figure 2B** and **Supplementary Table 4**).

Development of DEnrlncRNAs Pairs and a Risk Model

Using multiple rounds of matching of 89 DEnrlncRNAs, 3101 necroptosis-related lncRNA pairs were identified (**Supplementary Table 5**). Next, univariate Cox regression analysis was performed to extract 260 DEnrlncRNA pairs affecting survival (**Supplementary Table 6**). To create a risk model, LASSO regression analysis was conducted to identify 13 necroptosis-related lncRNA pairs (**Figures 2C, D**). Then, univariate and multivariate Cox regression analyses were conducted on these 13 nrlncRNA pairs (**Figures 2E, F**), and each nrlncRNA pair's risk coefficient was calculated (**Table 1**).

Evaluating the Risk Model's Outcomes Predictive Capability

The 13 nrlncRNA pairs were used to construct the 1-, 3-, and 5-year ROC curves of BLCA patients (**Figure 3A**), and the 1-year AUC was computed as a maximum of 0.763 (**Figure 3B**). The AUC values for three and five years were 0.836 and 0.842, respectively, demonstrating that this risk model can also be used to predict 3- and 5-year outcomes for BLCA. According to best fit, the threshold value for differentiating between high and low-risk groups of BLCA patients was 1.189 (**Figure 3C**).

Clinical Assessment by the Risk Model

We classified the patients into high- and low-risk groups based on the threshold value. We assigned 204 patients to the low-risk and 195 to the high-risk subgroup (**Figure 4A**). **Figure 4B** demonstrates the distribution of survival status in the subgroups. The high-risk subgroup had more deaths than the low-risk subgroup, and the differences in survival time between subgroups were compared. **Figure 4C** illustrates that, compared to high-risk patients, low-risk patients had better outcomes ($p < 0.001$). We then conducted several chi-square tests to determine the relationship between risk scores and clinical and pathological features and generated a heatmap using the Wilcoxon signed-rank test (**Figure 5A**). Scatterplots showed that survival (**Figure 5B**), age (**Figure 5C**), grade (**Figure 5E**), clinical stage (**Figure 5F**), T stage (**Figure 5G**), M stage

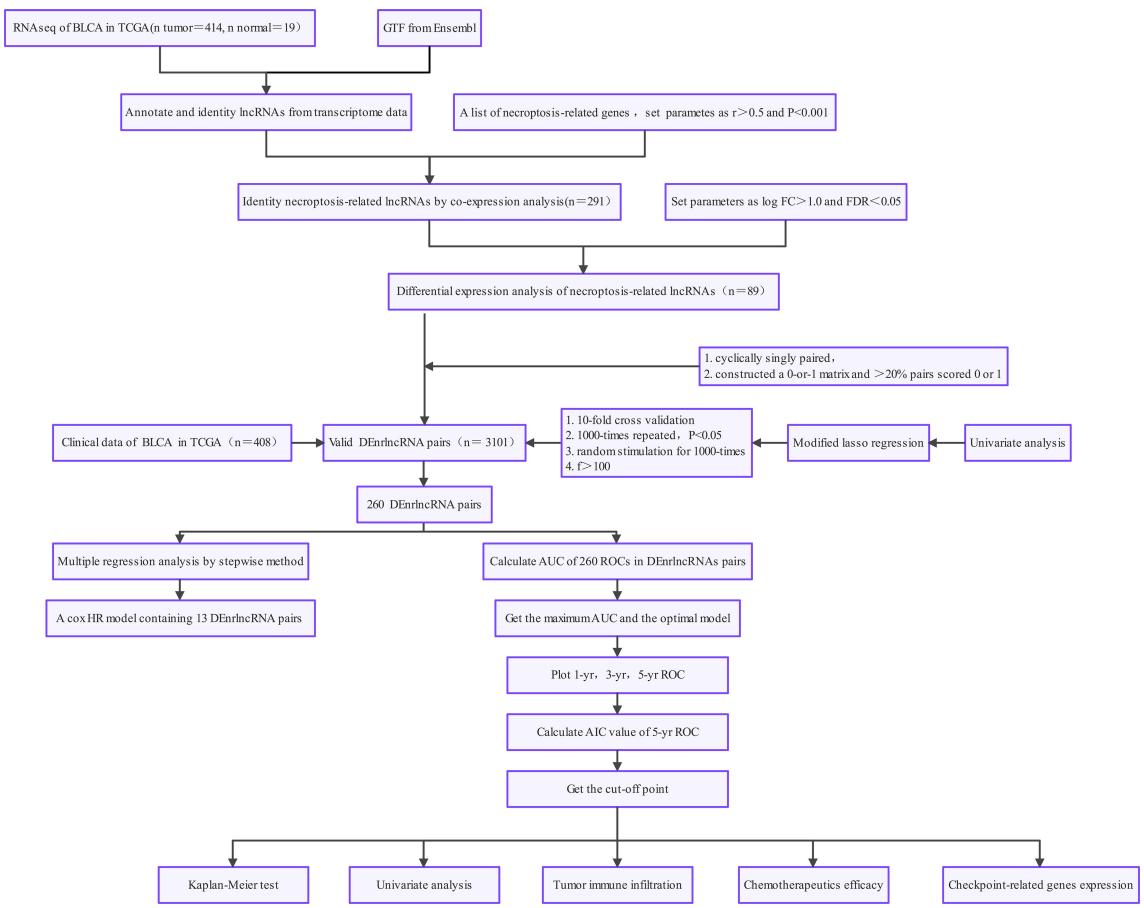

FIGURE 1 | Study workflow.

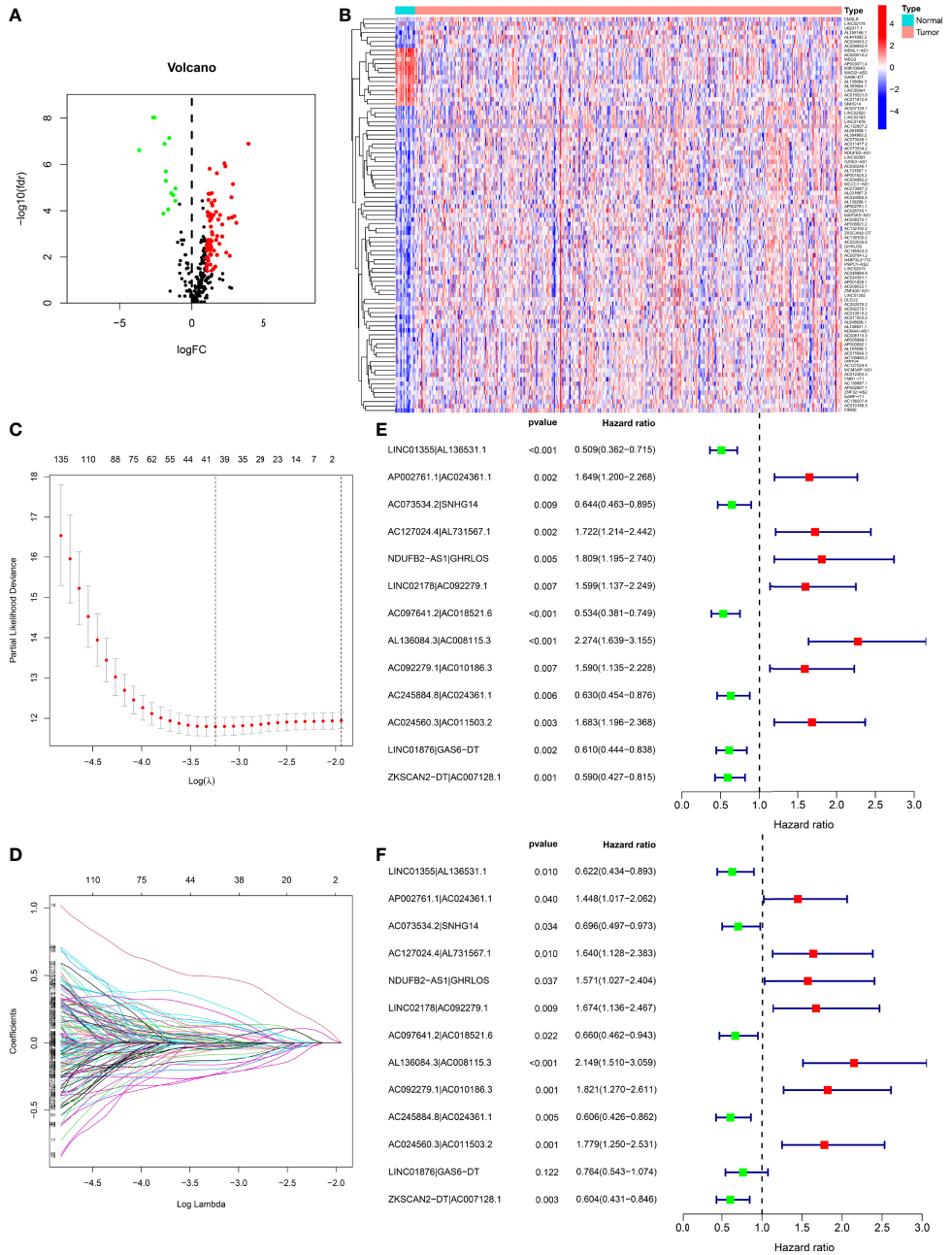

FIGURE 2 | Development of a risk model with DEnrlncRNA pairs. **(A)** The volcano plot of necroptosis-related lncRNAs. **(B)** The heatmap of necroptosis-related lncRNAs between BLCA and normal tissues. **(C)** LASSO coefficient distribution of 13 necroptosis-related lncRNAs. **(D)** Ten-fold cross-validation for variable selection in LASSO models. **(E)** A forest map revealing 13 DEirlncRNA pairs detected using univariate Cox regression analyses. **(F)** A forest map revealing 13 DEirlncRNA pairs detected using multivariate Cox regression analyses.

(**Figure 5H**), and N stage (**Figure 5I**) were significantly associated with risk scores. Gender (**Figure 5D**) was not significantly associated with risk scores. Then, the univariate and multivariate Cox regression analyses were performed on risk scores and clinical correlation factors, and forest maps were drawn (**Figures 6A, B**). In univariate Cox analysis, we found that T stage, N stage, and riskScore were significantly associated with outcomes (**Figure 6A**). Multivariate Cox regression analysis revealed that only riskScore could be utilized as an independent predictor for BLCA (**Figure 6B**). ROCs were used to compare the differences in 1-year survival prediction performance. This risk model had the greatest AUC value (0.763) (**Figure 6C**), suggesting that it has an excellent capacity to predict outcomes.

TABLE 1 | Thirteen pairs of prognostic necroptosis-related lncRNA pairs multivariate Cox regression analysis results.

LncRNAs	Coefficient	HR	HR.95L	HR.95H	P-value
LINC01355|AL136531.1	-0.4742	0.6224	0.4338	0.8929	0.0100
AP002761.1|AC024361.1	0.3702	1.4480	1.0168	2.0621	0.0401
AC073534.2|SNHG14	-0.3631	0.6955	0.4969	0.9735	0.0343
AC127024.4|AL731567.1	0.4944	1.6395	1.1278	2.3834	0.0096
NDUFB2-AS1|GHRLOS	0.4520	1.5714	1.0273	2.4039	0.0372
LINC02178|AC092279.1	0.5151	1.6738	1.1356	2.4670	0.0093
AC097641.2|AC018521.6	-0.4155	0.6600	0.4620	0.9428	0.0224
AL136084.3|AC008115.3	0.7651	2.1492	1.5099	3.0592	2.16E-05
AC092279.1|AC010186.3	0.5996	1.8214	1.2704	2.6113	0.0011
AC245884.8|AC024361.1	-0.5008	0.6060	0.4261	0.8620	0.0053
AC024560.3|AC011503.2	0.5758	1.7785	1.2497	2.5311	0.0014
LINC01876|GAS6-DT	-0.2690	0.7641	0.5434	1.0744	0.1218
ZKSCAN2-DT|AC007128.1	-0.5047	0.6037	0.4306	0.8463	0.0034

HR, hazard ratio; HR.95L, 95% CI lower limit; HR.95H, 95% CI upper limit.

Correlation Analysis Between Risk Models and Tumor-Infiltrating Immune Cells

We used CIBERSORT-ABS, QUANTISEQ, XCELL, EPIC, TIMER, CIBERSORT, and MCPCOUNTER, to study whether the risk model is correlated with the tumor immune microenvironment. The link between the risk model and tumor immune infiltrating cells was investigated by performing the Pearson correlation test. The screening criterion was $P < 0.05$ (**Supplementary Table 7**). Data visualization was performed using R language software (**Figure 7**). The high-risk subgroup positively correlated with tumor-infiltrating immune cells such as cancer-related fibroblasts, T cell CD8+, M2 macrophages, and macrophage and was negatively associated with T cell CD4+ and T cell follicular helper cells (**Supplementary Figure 1**).

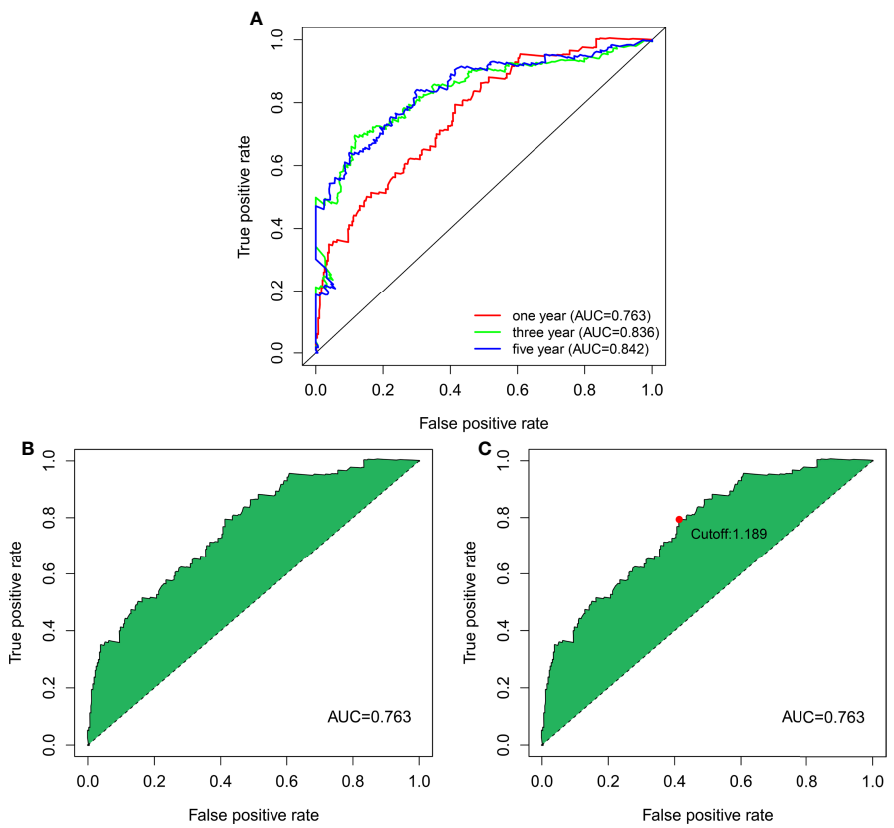

FIGURE 3 | DEnrlncRNA pairs to establish a risk assessment model. **(A)** The 1-, 3-, and 5-year ROC curves derived by optimal model construction showed that all AUC values were above 0.763. **(B)** The 1-year ROC curve with the largest AUC value generated by the model. **(C)** The cutoff value of 1.198 for distinguishing high-risk and low-risk patients was generated using the optimal model.

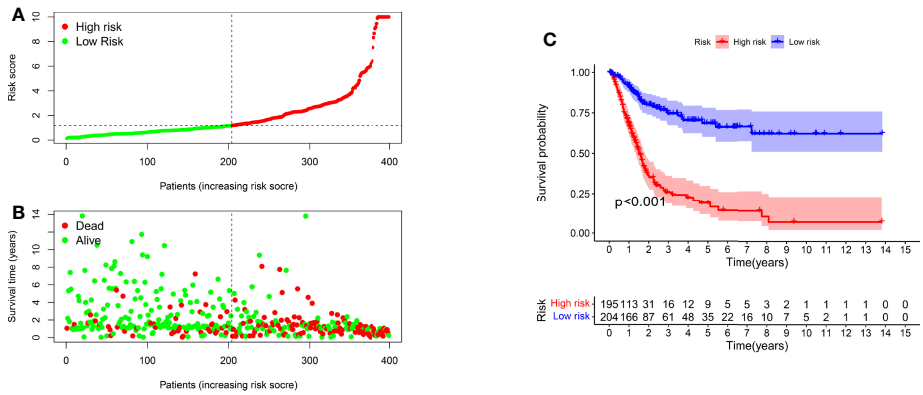

FIGURE 4 | A risk model for outcome prediction. **(A)** Risk score for each patient. **(B)** Survival outcomes for every patient. **(C)** Kaplan-Meier curves based on the status of survival of patients in high- and low-risk groups.

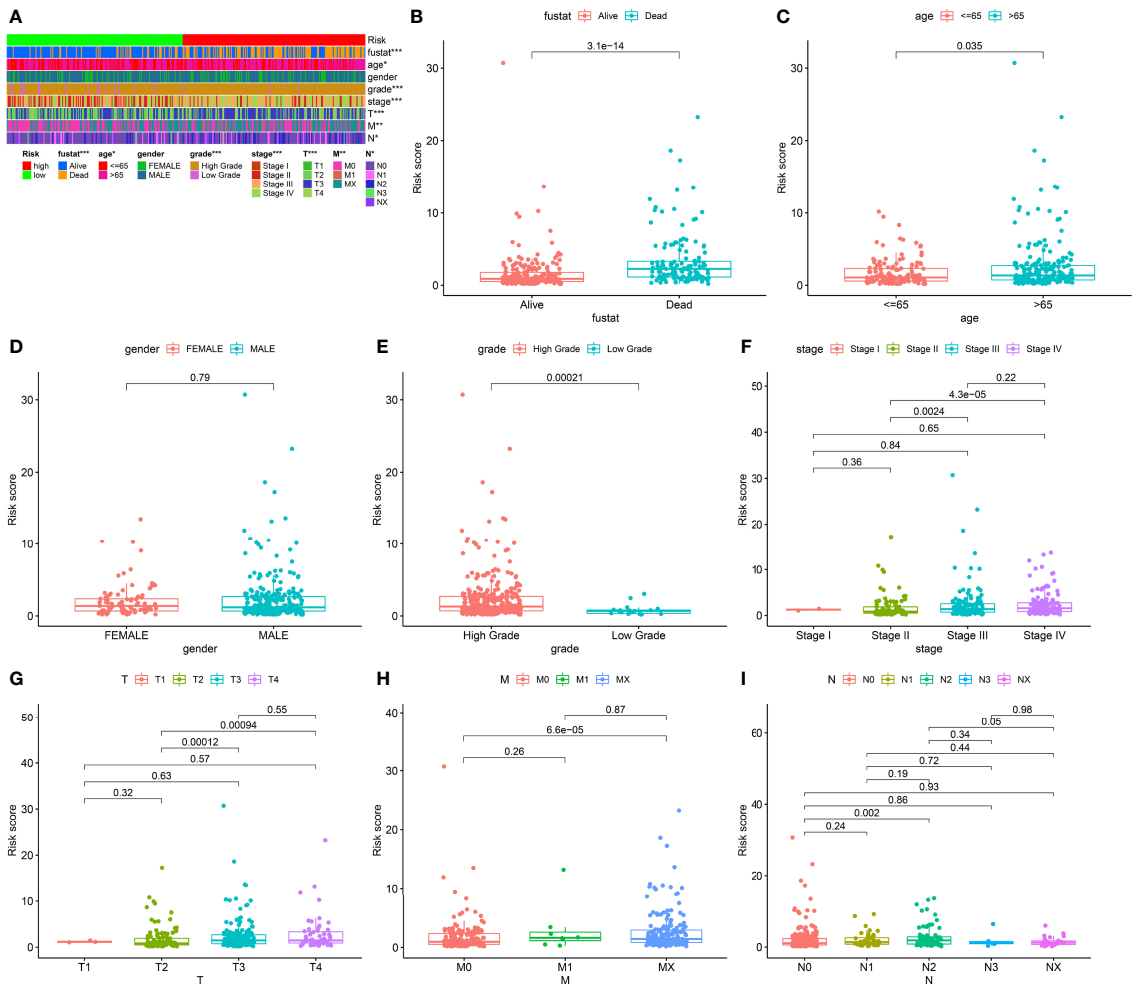

FIGURE 5 | Clinical evaluation of risk models. **(A)** Heatmap of clinicopathological features. **(B)** Survival status. **(C)** Age. **(D)** Gender. **(E)** Grade. **(F)** Clinical stage. **(G)** T stage. **(H)** M stage. **(I)** N stage. *p < 0.05; **p < 0.01; ***p < 0.001.

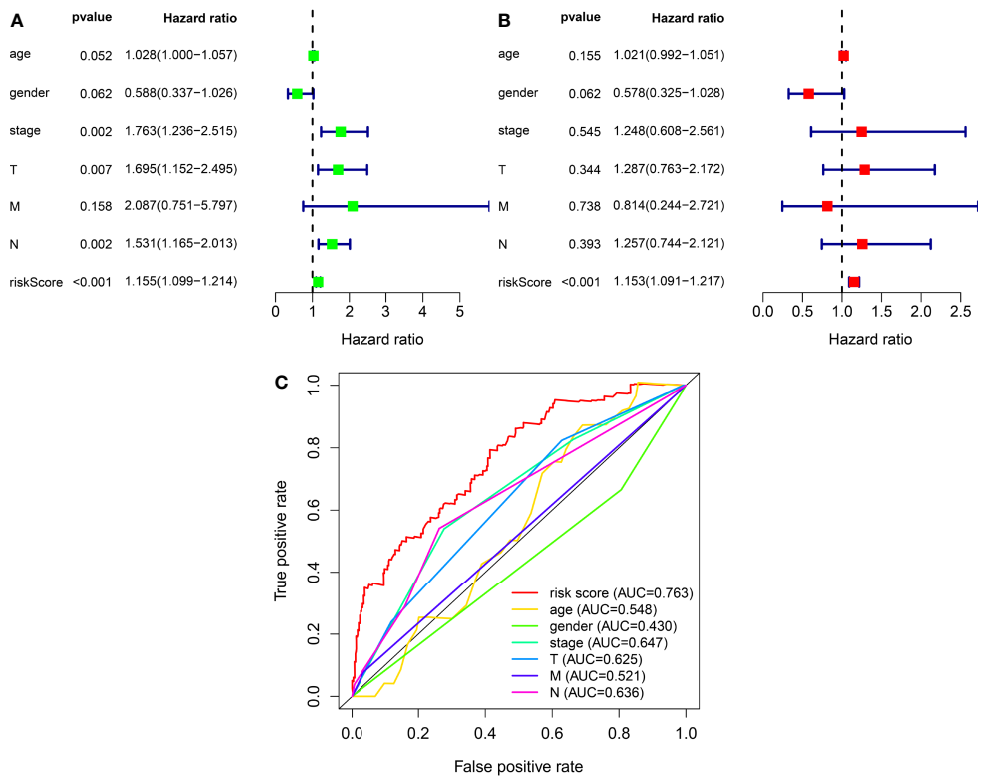

FIGURE 6 | The independence of prognostic models and clinicopathological characteristics for BLCA outcome prediction. **(A)** A forest map of univariate Cox regression analysis of BLCA prognostic associations. **(B)** A forest map of multivariate Cox regression analysis of BLCA prognostic associations. **(C)** Comparison of AUC values for clinicopathological characteristics and risk scores.

Correlation Analysis Between Risk Models and Immune Checkpoints

ICIs are therapeutic agents for management of BLCA. We determined whether the risk model was associated with biomarkers related to ICIs and found that PDL1 ($p < 0.001$; **Figure 8A**), HAVCR2 ($p < 0.001$; **Figure 8C**), LAG3 ($p < 0.001$; **Figure 8D**), PDCD1LG2 ($p < 0.001$; **Figure 8G**), and TIGIT ($p < 0.001$; **Figure 8H**) expression levels were significantly elevated in high-risk patients. Expression levels of CTLA4 ($p > 0.05$; **Figure 8B**) and PD1 ($p > 0.05$; **Figure 8F**) were increased; however, the differences were not significant. GAL9 ($p < 0.001$; **Figure 8E**) expression was attenuated in high-risk patients. These genes could be used as therapeutic targets for BLCA.

Correlation Analysis Between Risk Models and Chemotherapy Drugs

In addition to ICIs, chemotherapy is the first-line treatment for individuals with advanced BLCA. We also explored the correlation between risk models and the efficacy of conventional cancer medicines in BLCA. We found that a higher risk score was related to a reduced IC_{50} for chemotherapy drugs including cisplatin ($p < 0.001$; **Figure 9A**) and doxorubicin ($p < 0.001$; **Figure 9B**), while it was associated with a higher IC_{50} for methotrexate ($p < 0.01$; **Figure 9D**), gemcitabine (**Figure 9C**), and vinblastine (**Figure 9E**). Values were not significantly different between high- and low-risk groups, suggesting that the risk model predicts chemosensitivity.

DISCUSSION

An imbalance between tumor cell death and growth causes tumor formation and progression (34). Excessive cell growth or prevention of natural cell death exacerbates cancer progression. Some investigators argue that immortal cell proliferation and cell death suppression are distinct characteristics of malignant tumors (35). Necroptosis is a recently discovered type of cell death with morphological characteristics similar to necrosis. By contrast, necrosis refers to passive death induced by external physicochemical stress (e.g., inflammation or infection) and is not modulated by signaling pathways, while necroptosis is governed by programmed cell death (36). Many studies linked necroptosis to cancer incidence, progression, and metastasis (37, 38). Necroptosis is also a viable strategy for eliminating cancer cells (39).

Many studies investigated the role of lncRNAs in tumor onset and progression. Abnormally expressed lncRNAs in

FIGURE 7 | Correlation analysis of tumor-infiltrating immune cells and risk models of patients with BLCA.

malignancies can be used as markers for clinical diagnosis, predicting outcomes, and developing targeted therapies (34). In BLCA, lncRNAs have been linked to cancer immunology and the tumor microenvironment (35). The lncRNA urothelial carcinoma-associated 1, the most studied lncRNA in BLCA, participates in several processes in the development of BLCA and is responsible for BLCA resistance (36, 37). Based on the literature, several immune-related lncRNA models have been developed (24, 38). These signature models were created using quantification of necroptosis-related lncRNA expression. To the best of our knowledge, there are no studies exploring the relationship between necroptosis-related lncRNAs and BLCA outcomes and underlying molecular mechanisms. We identified nrlncRNA pairs and developed a robust and independent risk profile of nrlncRNAs to determine the relationship between the model and BLCA outcomes and the potential impact on the BLCA tumor microenvironment and its corresponding treatment responses.

Zhang et al. evaluated the expression levels of ten hypoxia-related lncRNAs to establish a signature predicting survival in BLCA (39). However, there is currently no study of nrlncRNAs in BLCA. Because of the critical role of nrlncRNAs, we developed a risk model with 13 DEnrlncRNAs pairs. This novel model is clinically useful, can distinguish high- or low-risk cases, and determine outcomes. In addition, we determined the correlation between the risk factor score and various clinical markers for each BLCA sample and found that the risk factor score independently predicted outcomes. We constructed ROC curves for clinically relevant indicators and compared the 1-year ROC curves in the same chart. We confirmed that the risk factor score was the best predictor of BLCA outcome at 1 year, suggesting the robustness of the risk model. Then, we evaluated each of the DEnrlncRNAs identified in our model and found that they participate in the malignant phenotype of different cancer types, while lncSNHG14 overexpression promoted breast cancer proliferation and accelerated cell cycle progression (40). Wu et al.

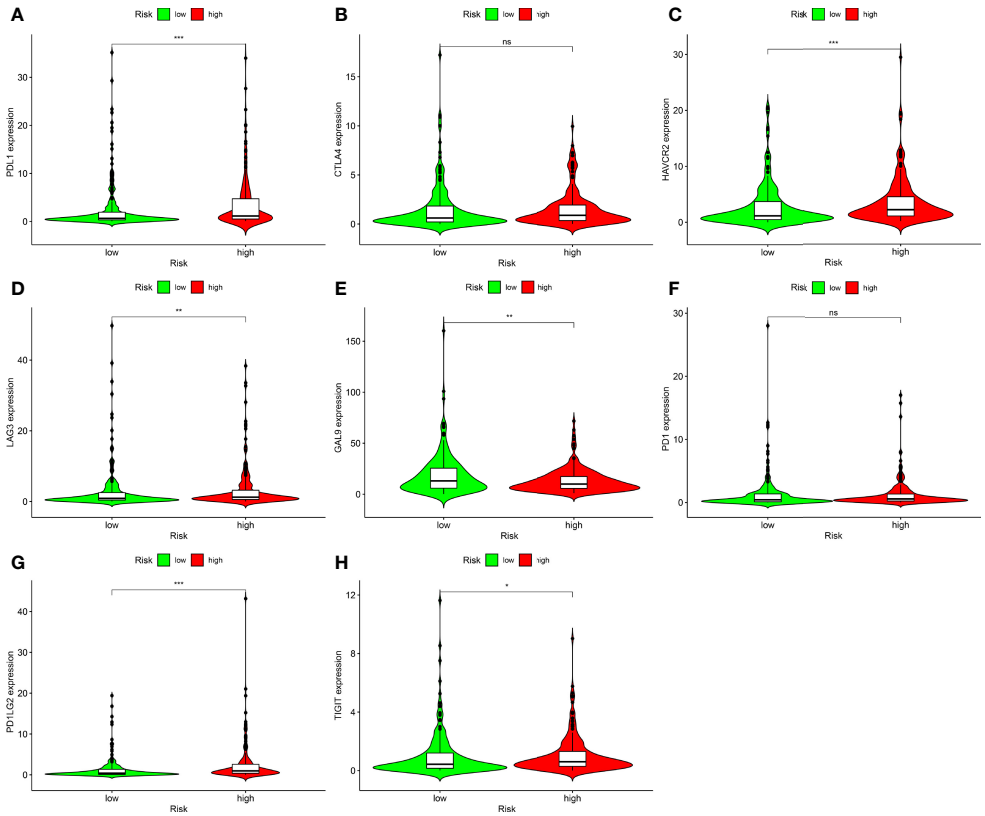

FIGURE 8 | Correlation analysis between risk models and immune checkpoints of BLCA. **(A)** PDL1. **(B)** CTLA4. **(C)** HAVCR2. **(D)** LAG3. **(E)** GAL9. **(F)** PD1. **(G)** PDCD1LG2. **(H)** TIGIT in high- and low-risk BLCA patients. Ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

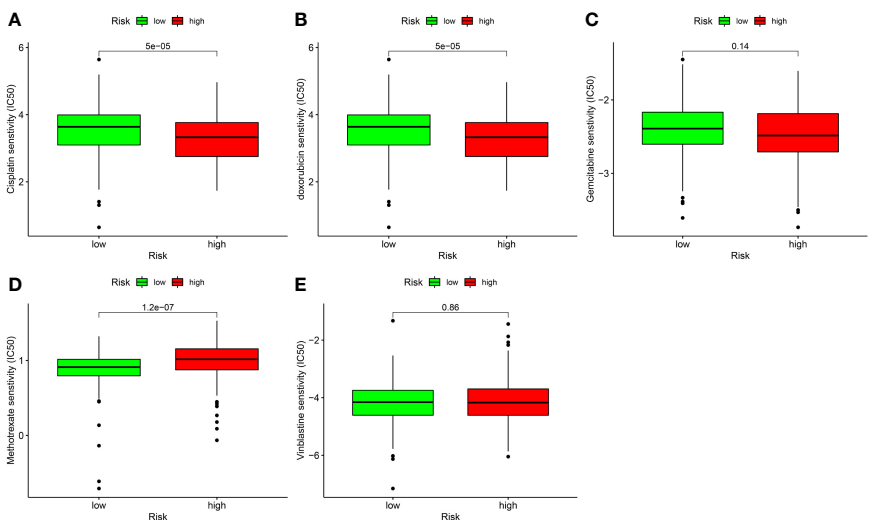

FIGURE 9 | Correlation analysis between risk models and chemotherapy drugs for BLCA. Comparing the IC_{50} values of **(A)** cisplatin, **(B)** doxorubicin, **(C)** gemcitabine, **(D)** methotrexate, and **(E)** vinblastine in high- and low-risk BLCA patients.

found that lncRNA GHRLOS can be a biomarker for colorectal cancer metastasis and outcomes (41). LncRNA AC092279.1 was reported in a thyroid cancer prognostic model (42). LncRNA AC008115.3 was reported in a head and neck squamous cell carcinoma prognostic model (43). LncRNA AC010186.3 was reported in an ovarian Cancer prediction signature (44). LncRNA AC011503.2 and AC007128.1 were reported once in a bladder cancer predictive signature (45, 46). Regarding AL136531.1, AC024361.1, AL731567.1, AC018521.6, AC024361.1, and GAS6-DT, there are no reports yet.

Immune checkpoints and immune cell infiltration in cancerous tissues are critical for enhancing or inhibiting cell growth, invasion, and migration; immunotherapy is a novel therapeutic approach for managing diseases like BLCA (29). To explore the relationship between nrlncRNA signatures and immune cell infiltration, we used XCELL, TIMER, QUANTISEQ, EPIC MCPCOUNTER, CIBERSORT, and CIBERSORT-ABS algorithms to compare the content of immune cells in different risk score groups and found that the high-risk group was positively correlated with tumor-infiltrating immune cells such as cancer-associated fibroblast, T cell CD8+, M2 macrophages, and macrophages. It was negatively associated with T cell CD4+ and T cell follicular helper. Jóźwicki reported that breast cancer patients with reduced CD4+ T cell infiltration had shorter overall survival, and CD4+ T cells are a critical prognostic indicator, consistent with our findings (47). These findings suggest that this risk model could be used to anticipate the response to immunotherapy in patients with BLCA.

We also performed a correlation analysis of immune checkpoint genes and risk models and found that the PDL1, HAVCR2, LAG3, PDCD1LG2, and TIGIT expression levels were substantially higher in high-risk patients, and expression levels of GAL9 lower decreased in high-risk patients. These genes could be used as therapeutic targets for BLCA.

Bladder cancer is a complex malignant tumor, and chemotherapy is one of its essential treatment options. The guidelines recommend neoadjuvant chemotherapy before radical cystectomy of patients with BLCA, and the survival benefit of patients is close to 5–10%; nevertheless, some patients still do not respond to chemotherapy (48). Therefore, identifying predictors can avoid missing the optimal timing of surgery and minimize the adverse effects of chemotherapy. Here, we correlated BLCA chemotherapeutic agents with risk models and found that high-risk patients were more responsive to cisplatin and doxorubicin than low-risk individuals. Conversely, low-risk subjects were more responsive to methotrexate than high-risk subjects. These findings suggest that the risk model may help predict the sensitivity to doxorubicin, methotrexate, and cisplatin in patients with BLCA.

Although we employed rigorous approaches and algorithms to construct the model, this study has a few limitations. This study lacks external data to confirm the robustness of our risk model because the existing public databases do not include valid external data for verification. In a subsequent study, we will gather more clinical data and expand the sample size.

Finally, our findings revealed that a new signature created by nrlncRNAs might predict outcomes of BLCA and describe the immune landscape and chemotherapeutic therapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

The manuscript written was completed by JH, ZL, RD and GW, Experiment performance was done by GQ and YX, Data collection was conducted by HN, GY and CT, All the authors reviewed the manuscript and discussed the results and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.928204/full#supplementary-material

Supplementary Table 1 | The clinical features of BLCA patients.

Supplementary Table 2 | A list of necroptosis-related genes.

Supplementary Table 3 | A list of necroptosis-related lncRNAs in BLCA.

Supplementary Table 4 | The sum of 89 differentially necroptosis-related lncRNAs in BLCA.

Supplementary Table 5 | 3101 effective necroptosis lncRNA pairs.

Supplementary Table 6 | Univariate Cox regression analysis of necroptosis-related lncRNA pairs significantly influencing outcomes in BLCA.

Supplementary Table 7 | The association between risk score and tumor-infiltrating immune cells.

Supplementary Figure 1 | Infiltration of immune cells in high- and low-risk BLCA samples.

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The functions of long noncoding RNAs on regulation of F-box proteins in tumorigenesis and progression

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Accumulated evidence has revealed that F-box protein, a subunit of SCF E3 ubiquitin ligase complexes, participates in carcinogenesis and tumor progression *via* targeting its substrates for ubiquitination and degradation. F-box proteins could be regulated by cellular signaling pathways and noncoding RNAs in tumorigenesis. Long noncoding RNA (lncRNA), one type of noncoding RNAs, has been identified to modulate the expression of F-box proteins and contribute to oncogenesis. In this review, we summarize the role and mechanisms of multiple lncRNAs in regulating F-box proteins in tumorigenesis, including lncRNAs SLC7A11-AS1, MT1JP, TUG1, FER1L4, TTN-AS1, CASC2, MALAT1, TINCR, PCGEM1, linc01436, linc00494, GATA6-AS1, and ODIR1. Moreover, we discuss that targeting these lncRNAs could be helpful for treating cancer *via* modulating F-box protein expression. We hope our review can stimulate the research on exploration of molecular insight into how F-box proteins are governed in carcinogenesis. Therefore, modulation of lncRNAs is a potential therapeutic strategy for cancer therapy *via* regulation of F-box proteins.

KEYWORDS

cancer, lncRNAs, F-box protein, treatment, oncogenesis, noncoding RNA

Introduction

F-box protein is a subunit in Skp1-Cullin1-F-box protein (SCF) E3 ubiquitin ligase complexes (1). It has been well documented that F-box proteins target their substrates *via* ubiquitination and proteasome degradation (2). It has been accepted that F-box proteins have 69 members in human genome (3). According to specific motifs in F-box proteins, these proteins are classified into three types: 10 FBXW proteins (WD40 repeat domains), and 22 FBXL proteins (leucine-rich repeat motifs), 37 FBXO proteins (other motifs) (4). Accumulated evidence demonstrated that F-box proteins participate in cancer initiation and progression *via* regulation of cell proliferation (5–7), apoptosis (8), motility and

metastasis (9), cell cycle (10), EMT (11), cancer stem cells (12, 13), drug resistance (14, 15) and autophagy (16).

Noncoding RNAs have little or no protein coding capacity to encode proteins (17). Based on the lengths of nucleotides, noncoding RNAs are classified multiple types: long noncoding RNA (lncRNA, high than 200 bp), short noncoding RNA (17-30 bp) and mid-size noncoding RNA (31-200 bp) (18). It has been known that microRNA (miRNA) has approximately 22 nucleotides and targets gene expression *via* regulation of post-transcription (19, 20). Now, noncoding RNAs have been validated to critically participate in oncogenesis in various types of cancers (21–25). Not surprisingly, noncoding RNAs regulated numerous cellular biological processes and dysregulated noncoding RNAs lead to various diseases, including cancer (26–29). In recent years, accumulated evidence suggests that noncoding RNAs targets the expression of F-box proteins, leading to carcinogenesis and malignant progression. One review has well summarized the role of noncoding RNAs in regulation of F-box proteins in carcinogenesis (30). However, this review mainly described the role of microRNAs in governing the expression of F-box proteins. Here, we summarized the functions and mechanisms of lncRNAs in controlling F-box protein expression, leading to tumor development and progression.

LncRNAs regulate the expression of F-BOX proteins

Targeting FBXW family by lncRNAs

LncRNA SLC7A11-AS1 regulates β-TrCP1

SLC7A11-AS1 was downregulated in tumor tissues in patients with gastric cancer, and correlated with poor prognosis in these patients (31). Depletion of SLC7A11-AS1 contributed to tumor growth in cells and in mice *via* controlling the ASK1/p38/JNK signaling pathway in gastric cancer (31). In lung cancer cells, SLC7A11-AS1 facilitated tumor progression *via* suppressing miR-4775 and increasing the expression of TRAIP (32). SLC7A11-AS1 has been revealed to play a key role in chemoresistance in various types of cancers (33). Luo et al. found that SLC7A11-AS1 targeted miR-33a-5p and changed the expression of xCT, weakened cell growth, promoted ROS levels, and regulated cisplatin resistance in gastric cancer (34).

SLC7A11-AS1 was found to be significantly increased in PDAC tissues (35). PDAC cells with gemcitabine resistance have a high expression of SLC7A11-AS1, indicating that SLC7A11-AS1 could play an essential role in regulation of drug resistance. In fact, downregulation of SLC7A11-AS1 potentiated gemcitabine sensitivity in resistant PDAC cells and inhibited the PDAC stemness. In line with this, overexpression of SLC7A11-AS1 increased gemcitabine resistance *via* suppressing intracellular ROS levels by maintaining NRF2 stability (35). Mechanically, SLC7A11-AS1 can bind to the F-box motif of β-TrCP1 (also known as FBXW1), which blocks the ubiquitination and degradation of NRF2. Therefore, SLC7A11-AS1 attenuated β-TrCP-mediated degradation of NRF2, reduced ROS levels, and increased cancer stemness, which promoted gemcitabine resistance in PDAC (35). Hence, targeting SLC7A11-AS1 could overcome gemcitabine resistance to improve treatment benefits in PDAC patients.

LncRNA PCGEM1 regulates β-TrcP2

β-TrcP2, also known as FBXW11, has been characterized to take part in tumorigenesis (36). FBXW11 activated the β-catenin/TCF and NF-kappa B pathways and increased cell proliferation in lymphocytic leukemia (37). FBXW11 maintained stem-cell-like characters and enhanced liver metastasis *via* governing SIRT1 transcription in colorectal cancer (38). LncRNA PCGEM1 has been found to participate in the initiation and development of a variety of cancers *via* regulating several signaling pathways (39). LncRNA PCGEM1 expression was remarkably increased in cervical cancer specimens, which was associated with FIGO stage, lymph node metastasis, poor survival and distant metastasis in cervical cancer patients (40). PCGEM1 upregulation stimulated proliferation, invasion, migration, and cell cycle process and reduced apoptosis in cervical cancer cells (40). PCGEM1 can work as a ceRNA to sponge miR-182 and suppress its expression, leading to upregulation of FBXW11. Moreover, PCGEM1 can activate the NF-kappa B and β-catenin/TCF pathways, and this activation by PCGEM1 can be abrogated by knockdown of FBXW11 (40). Altogether, PCGEM1 exerted cervical cancer progression *via* modulation of miR-182 and FBXW11.

Several lncRNAs regulates FBXW7 expression

F-box and WD repeat domain containing 7 (FBXW7) is well studied and acts as one tumor suppressor gene in human carcinogenesis and tumor progression (41–43). One study identified that several lncRNAs are correlated with Fbxw7 deficiency in radiation-mediated thymic lymphoma (44). In

Abbreviations: CASC2, Cancer Susceptibility 2; FER1L4, Fer-1 Like Family Member 4; GATA6-AS1, GATA6 Antisense RNA 1; HCC, Hepatocellular carcinoma; LncRNA, Long noncoding RNA; MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; MT1JP, Metallothionein 1J, pseudogene; NRF2, Nuclear factor erythroid-2-related factor 2; ODIR1, osteogenic differentiation inhibitory regulator 1; PCGEM1, Prostate cancer gene expression marker 1; PDAC, Pancreatic ductal adenocarcinoma; ROS, Reactive oxygen species; SLC7A11-AS1, Solute carrier family 7 member 11 antisense RNA 1; SCF, Skp1-Cullin1-F-box protein; STAT3, Signal transducer and activator of transcription 3; TCPTP, T-cell protein tyrosine phosphatase; TINCR, Terminal differentiation-induced non-coding RNA; TUG1, Taurine up-regulated 1; TTN-AS1, TTN Antisense RNA 1.

mice with Fbxw7 deficiency, microarray dada from radiation-induced thymic lymphomas revealed that 372 lncRNAs are differentially expressed in tumor tissues. Among these lncRNAs, 170 lncRNAs were decreased, while 202 lncRNAs were increased in thymic lymphomas (44). Moreover, these FBXW7-associated lncRNAs were found to participate in DNA repair, cell cycle processes, lymphocyte activation and cell differentiation. Two lncRNAs (lncRNA position: 5119300, 5162836) were observed to be linked to Anxa2, Cecr2, Zeb1 and Zfp438 expressions, whereas one lncRNA (position: 182808654) was decreased and associated with Ampd1, Cd6, Clip1, Dap, Edaradd and Ptk2b (44). Furthermore, lncRNA A_30_P01032978 is correlated with poor disease free survival in patients with breast cancer (44). In this section, we will discuss how the several lncRNAs regulated the expression of FBXW7 in carcinogenesis.

LncRNA MT1JP regulates FBXW7

LncRNA MT1JP has been reported to be a tumor suppressor *via* promotion of the translation of p53 by interaction with TIAR (45). In retinoblastoma, MT1JP plays a tumor suppressive role *via* targeting Wnt/β-catenin signaling pathway (46). In breast cancer cells, MT1JP repressed oncogenesis and reversed cisplatin resistance through sponging miR-24-3p and inhibiting the Wnt/β-catenin (47). Consistently, MT1JP exhibited tumor suppressive functions *via* sponging miR-92-3p and targeting miR-214/RUNX3 axis in breast cancer cells (48, 49). In lung cancer cells, MT1JP blocked cell proliferation, migration and invasion through modulation of miR-423-3p/Bim axis (50). In glioma, MT1JP retarded tumor progression *via* competitively binding with miR-24 (51). In osteosarcoma cells, MT1JP was reported to increase the inhibitory function of miR-646 on FGF2 expression (52). In HCC cells, upregulation of MT1JP modulated cell apoptosis and migratory abilities *via* targeting miR-24-3p and regulating AKT, RUNX3 and p21 (53–55). Furthermore, MT1JP regulated miR-24-3p/Bcl2L2 signaling pathway and reduced lenvatinib sensitivity *via* suppression of apoptosis in HCC (56). Moreover, MT1JP upregulation abrogated the PTEN inactivation *via* miR-32 reduction in HCC cells (57).

In intrahepatic cholangiocarcinoma, MT1JP acted as a protective lncRNA *via* regulation of miR-18a-5p and FBP1 (58). MT1JP regulated miR-214-3p/RUNX3 signaling pathway and subsequently inhibited proliferation and migration of gastric cancer (59). Notably, low expression of MT1JP was related with poor prognosis in patients with gastric cancer (60). LncRNA MT1JP was downregulated in gastric cancer tissues compared with adjacent normal tissues (61). Gastric cancer patients had a better prognosis, who often have higher expression of MT1JP. *In vitro* experiment data showed that lncRNA MT1JP upregulation suppressed proliferation, invasion and migration and enhanced apoptosis of gastric cancer cells (61). *In vivo* data revealed that lncRNA MT1JP reduced tumor sizes and tumor metastasis. Mechanistical analysis demonstrated that lncRNA MT1JP sponged miR-92a-3p and upregulated FBXW7 in gastric cancer (61). Rescue experiments exhibited that downregulation of FBXW7 reversed MT1JP-induced inhibition of proliferation, invasion and migration in gastric cancer (61).

LncRNA TUG1 regulates FBXW7

Numerous studies have demonstrated the critical role of lncRNA Taurine upregulated gene 1 (TUG1) in cancer initiation and progression. LncRNA TUG1 underlined a tumor promotive property *via* impairing miR-421-mediated suppression of KDM2A and activating the ERK signaling in colorectal cancer cells (62). TUG1 suppressed cancer progression *via* targeting Siglec-15-mediated anti-immune activity in HCC (63). Moreover, TUG1 was reported to sponge miR-328-3p and increase the SRSF9 mRNA expression in HCC cells, leading to promotion of proliferation, invasion and migration (64). Xiu et al. found that TUG1 enhanced tumor malignant progression *via* binding with miR-516b-5p and increasing H6PD expression (65). Xia et al. reported that TUG1 stabilization by IGF2BP2 increased cisplatin resistance *via* targeting autophagy in colorectal cancer (66). One group identified that TUG1 governed the miR-320a/FOXQ1 axis and caused promotion of bladder tumor malignant phenotypes (67).

Sun et al. discovered that TUG1 increased chemoresistance and enhanced cancer stem cell behaviors *via* stabilizing GATA6 protein in colorectal cancer (68). TUG1 targeted AKT/mTOR signaling pathway *via* sponging miR-582-3p, which promoted ovarian cancer malignant behaviors (69). TUG1 sponged miR-29c-3p and upregulated the expression of VEGFA, which facilitated malignant phenotypes in stomach cancer (70). In addition, TUG1 competitively interacted with miR-29a and triggered the expression of IFITM3 in HCC cells (71). TUG1 promoted tumor progression and metastasis *via* modulating miR-140-3p and Annexin A8 axis in bladder cancer cells (72). Zhang et al. reported that TUG1 targeted miR-187-3p and TESC and modulated the NF-kappa B signaling pathway, which governed progression of pituitary adenoma (73). Li group reported that miR-199a-3p/MSI2 signaling pathway was involved in TUG1-mediated promotion of cell migration, invasion and proliferation in Ewing's sarcoma (74). TUG1 upregulated the expression of XBP1 by sponging miR-498 in ESCC cells, which enhanced tumor metastasis and growth (75). One study revealed that TUG1 upregulated the expression of FBXW7 and induced FBXW7-triggered SIRT1 ubiquitination and degradation (76). Moreover, TUG1 compromised neuronal mitophagy *via* targeting TUG1/FBXW7 axis in cerebral ischemia and reperfusion injury (76). It is necessary to explore whether TUG1 regulates the expression of FBXW7 in carcinogenesis.

LncRNA FER1L4 regulates FBXW7

LncRNA Fer-1-like protein 4 (FER1L4) has been discovered to be involved in development of human cancer (77). Xia and

colleagues found that FER1L4 knockdown suppressed cell growth and cell cycle progression *via* interacting with miR-372 and upregulating E2F1 expression in gliomas (78). In lung cancer cells, FER1L4 suppressed metastasis and growth and enhanced apoptosis *via* control of PI3K/AKT and p53 signaling pathways (79, 80). In osteosarcoma cells, FER1L4 regulated cell apoptosis and EMT *via* suppression of miR-18a-5p and promotion of SOCS5 and activation of PI3K/AKT pathway (81). In clear cell renal cell carcinoma (ccRCC) tissues, FER1L4 expression is higher than that in adjacent normal tissues (82). High expression of FER1L4 was linked to tumor grade, stage, metastasis and tumor aggressiveness and patient survival (82). In oral squamous cell carcinoma, FER1L4 facilitated tumor progression through regulation of miR-133a-5p/Prx1 axis (83). In colorectal cancer patients, FER1L4 expression levels were downregulated, while RB1 expression was upregulated. FER1L4 expression was associated with RB1 expression in colorectal cancer patients (84).

FER1L4 sponged miR-1273g-3p and increased the expression of PTEN and led to cell cycle arrest and metastasis suppression in colorectal cancer (85). One study showed that downregulation of FER1L4 inhibited the mRNA levels of RB1 in gastric cancer (86). Moreover, FER1L4 reduced cell growth *via* binding with miR-106a-5p and increased the expression of PTEN at both mRNA and protein levels in gastric cancer (87). Similarly, FER1L4 reduced growth, invasion, migration and metastasis by suppressing the Hippo-YAP signaling pathway in gastric cancer (88). Qiao et al. found that FER1L4 repressed cell proliferation and blocked cell cycle at G0/G1 phase as well as enhanced apoptosis *via* upregulation of PTEN in endometrial carcinoma (89). Furthermore, FER1L4 overexpression was correlated with favorable survival outcome in endometrial carcinoma patients (90). Ma et al. reported that FER1L4 decreased cell invasion and growth and promoted cell apoptosis and cell cycle arrest at G0/G1 phase in ESCC cells (91). In HCC cells, overexpression of FER1L4 attenuated cell migration and proliferation, increased apoptosis through targeting PI3K/AKT signaling pathway (92).

In ovarian cancer cells, FER1L4 upregulation reduced paclitaxel tolerance *via* modulation of the MAPK signaling pathway (93). The lower expression of lncRNA FER1L4 was observed in prostate cancer samples compared with normal prostate tissues (94). Early stage of prostate cancer patients had the higher expression of FER1L4 in prostate cancer specimens. Upregulation of FER1L4 decreased proliferation and increased apoptosis in prostate cancer cells *via* sponging miR-92a-3p and upregulating FBXW7 (94). Depletion of FBXW7 abrogated inhibition of cell proliferation caused by upregulation of FER1L4 in prostate cancer cells, indicating that FER1L4 exerted antitumor activities *via* miR-92a-3p/FBXW7 axis (94).

LncRNA TTN-AS1 targets FBXW7

LncRNA Titin-antisense RNA1 (TTN-AS1) has been reported to be involved in tumorigenesis in various type cancers, including esophageal squamous cell carcinoma (ESCC), cervical cancer, gastric cancer and lung cancer (95–98). Lin et al. reported that TTN-AS1 worked as an oncogene and was highly expressed in ESCC cells and tumor specimens, and overexpression of TTN-AS1 enhanced ESCC proliferation and metastasis (95). Mechanistically, TTN-AS1 competitively interacted with miR-133b and increased the expression of Snail1, leading to EMT cascade in ESCC cells (95). In addition, TTN-AS1 sponged miR-133b and increased the expression level of FSCN1 and resulted in invasion cascades in ESCC cells (95). Chen et al. reported that TTN-AS1 enhanced growth and metastasis of cervical cancer cells *via* regulation of miR-573/E2F3 axis (96). Dong et al. revealed that TTN-AS1 stimulated gastric cancer development *via* interacting with miR-376b-3p and KLF12 (97). Luo et al. observed that TTN-AS1 contributed to tumor progression *via* modulating PTEN/PI3K/AKT signaling pathway in lung adenocarcinoma (98). Similarly, TTN-AS1 activated cell invasion and migration *via* governing miR-4677-3p/ZEB1 axis in lung adenocarcinoma (99). In prostate cancer cells, TTN-AS1 reduced cell apoptosis and facilitated cell proliferation *via* binding with miR-193a-5p (100).

LncRNA TTN-AS1 sponged miR-134-5p and increased the expression of malignant brain tumor domain containing 1 (MBTD1), contributing to promotion of viability and drug resistance, inhibition of apoptosis in osteosarcoma cells (101). TTN-AS1 interacted with miR-376a-3p and subsequently upregulated KLF15, resulting in promotion of colorectal cancer progression (102). Cui et al. also reported that TTN-AS1 facilitated the cell invasion and growth through activation of miR-497-induced PI3K/AKT/mTOR pathway in colorectal cancer (103). Fang et al. found that TTN-AS1 enhanced invasion, EMT and cell growth *via* governing miR-139-5p/ZEB1 axis and miR-524-5p/RRM2 axis in breast cancer cells (104, 105). One group studied the role of TTN-AS1 in clear cell renal cell carcinoma and found that TTN-AS1 acted as a sponging RNA of miR-195 to increase the expression of cyclin D1 and promote tumor progression (106). It has been reported that lncRNA TTN-AS1 can sponge miR-15b-5p and regulate the expression of FBXW7 in ovarian cancer (107). The low expression of TTN-AS1 was found in ovarian cancer cells and tumor tissues. Upregulation of TTN-AS1 reduced proliferation and colony formation and stimulated apoptosis in ovarian cancer cells (107). Moreover, knockdown of FBXW7 attenuated the functions of TTN-AS1 upregulation on cell behaviors, suggesting that TTN-AS1 exerts its biological behaviors *via* upregulating FBXW7 in ovarian cancer cells (107).

LncRNA CASC2 targets FBXW7

LncRNA cancer susceptibility candidate 2 (CASC2) has been reported to serve as a tumor suppressor in carcinogenesis by sponging several miRNAs (108, 109). Upregulation of lncRNA CASC2 attenuated cell viability, induced apoptosis and affected autophagy *via* regulation of miR-19a and NF-kappa B signaling

pathway in colon cancer (108). In line with this finding, lncRNA CASC2 enhance apoptosis and autophagy through targeting TRIM16 expression in colon cancer cells (110). CASC2 promoted berberine-mediated cytotoxicity *via* inhibition of Bcl2 in colorectal cancer (111). One group showed that CASC2 overexpression exhibited antitumor activities through sponging miR-24-3p in thyroid cancer (109). Another group reported that CASC2 increased radiotherapy sensitivity *via* sponging miR-155 in papillary thyroid cancer (112). Similarly, lncRNA CASC2 increased irradiation-triggered endoplasmic reticulum stress *via* regulation of PERK signaling pathway in NSCLC cells (113). In pancreatic cancer cells, lncRNA CASC2 increased the expression of PTEN and retarded cell metastasis *via* sponging miR-21 (114).

LncRNA CASC2 overexpression suppressed cell proliferation and tumor growth in mice in hepatocellular carcinoma (HCC) (115, 116). LncRNA CASC2 enhanced apoptosis and suppressed viability *via* targeting miR-24-3p in HCC cells (116). In TNF-related apoptosis-inducing ligand (TRAIL)-resistant HCC cells, CASC2 targeted miR-18a/receptor-interacting serine/threonine-protein kinase 1 (RIPK1) axis and the NF-kappa B pathway, whereas in TRAIL-sensitive cells, CASC2 affected miR-221/caspase-3 and miR-24/caspase-8 (115). In clinical tissues, HCC patients have lower expression of CASC2, which is associated with a poor overall survival rate (115, 117). Sun et al. observed that lncRNA CAS2 reduced cell viability, invasion and migratory activities *via* directly inhibiting miR-183 in HCC cells (118). Wang et al. reported that lncRNA CASC2 inhibited epithelial-mesenchymal transition (EMT) *via* targeting miR-367 and FBXW7 in HCC cells (117). Overexpression of lncRNA CASC2 repressed invasion and migration of HCC cells and suppressed EMT and blocked metastasis *via* sponging miR-367. In addition, FBXW7 was found to be a downstream target of miR-367 in HCC cells (117). Therefore, CASC2 regulates the expression of FBXW7 *via* regulation of miR-367 in HCC cells.

LncRNA MALAT1 targets FBXW7

LncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) has been known to be correlated with tumor metastasis in human cancer (119). MALAT1 expression was linked to the WHO grade, tumor size and poor survival in glioma patients (120). MALAT1 depletion increased proliferation of glioma stem cells and inhibited the expression of Nestin and Sox2, two stemness markers (121). MALAT1-mediated cell proliferation promotion was due to activation of ERK/MAPK signaling pathway in glioma cells (121). Han et al. found that MALAT1 downregulated MMP2 and blocked ERK/MAPK signaling pathway as well as exhibited tumor suppressive behaviors in glioma cells (122). Xiang et al. reported that knockdown of MALAT1 induced apoptosis *via* reduction of Cyclin D1 and Myc in U87 and U251 glioma cells (123). Studies showed that MALAT1 inhibited cell apoptosis and increased cell growth and activated autophagy *via* targeting miR-101 and derepressing Rap1B, RAB5A, ATG4D and STMN1 expression in glioma (124, 125).

MALAT1 was identified to recruit FBXW7 to stimulate the degradation of CRY2 and regulate trophoblast invasion and migration (126). MALAT1 was significantly downregulated in glioma samples and associated with tumor grade, tumor size and Karnofsky Performance status in glioma patients (127). MALAT1 repressed viability of glioma cells *via* suppressing miR-155 *in vitro*. Moreover, FBXW7 was identified as a key downstream molecule of miR-155 in glioma cells. Notably, FBXW7 mediated miR-155-triggered oncogenesis in U87 and SHG139 glioma cells. Strikingly, MALAT1 reduced cell viability by upregulation of FBXW7 expression due to downregulation of miR-155 (127). Hence, MALAT1 might be a potential therapeutic target for glioma.

LncRNA TINCR targets FBXW7

LncRNA terminal differentiation-induced lncRNA (TINCR) have been implicated in carcinogenesis and tumor progression (128). TINCR can reduce cell invasion and growth, and induce apoptosis through controlling the expression of miR-424-5p and LATS1 in cutaneous malignant melanoma (129). TINCR attenuated cell invasion and growth *via* targeting miR-210 and BTG in laryngeal squamous cell carcinoma (130). In HCC cells, TINCR enhanced cell invasion and growth *via* regulation of STAT3 pathway by binding to TCPTP (131). In breast cancer, TINCR governed cell metastatic ability and cell growth *via* regulating miR-761 and targeting OAS1 and EGFR (132–134).

In lung cancer tissues, TINCR expression levels were downregulated (135). In lung cancer cells, TINCR upregulation retarded cell invasion and proliferation *via* acting as a sponge of miR-544a. Moreover, FBXW7 was validated as a downstream target of miR-544a in lung cancer cells. In a rescue experiment, depletion of FBXW7 abrogated the suppression of TINCR on invasion and proliferation (135). Altogether, lncRNA TINCR performed anti-proliferative and invasive abilities in lung cancer cells through modulating miR-544a/FBXW7 axis. However, one study found that TINCR promoted tumor progression by BRAF-induced MAPK pathway in NSCLC (136). Therefore, further investigation is essential to determine the role of TINCR in lung cancer progression.

LncRNA MALAT1 targets FBXW8

MALAT1 has been validated to have a role in cancer diagnosis, prognosis and therapy (137). Emerging study has shown that MALAT1 can regulate the expression of FBXW8 in human cancer (138). FBXW8 has been reported to involve in cell growth and cell cycle progression in choriocarcinoma (139). Depletion of FBXW8 by siRNA transfection suppressed cell growth and induced cell cycle arrest at G2/M phase in

choriocarcinoma JEG-3 cells (139). Overexpression of FBXW8 exhibited the opposite functions on cell growth and cell cycle. FBXW8 can regulate the expression of CDK1, CDK2, p27, Cyclin A and Cyclin B1 in choriocarcinoma cells (139). One study reported that miR-218 suppressed the cell proliferation *via* inhibition of FBXW8 in choriocarcinoma JEG-3 cells (140).

In choriocarcinoma cells, MALAT1 upregulation increased cell proliferation, while depletion of MALAT1 hindered cell growth (138). Moreover, MALAT1 exerted its biological behaviors *via* targeting miR-218 in choriocarcinoma cells. Depletion of MALAT1 reduced the tumor growth *in vivo*. What is more, FBXW8 was found to be a direct target of miR-218 and was involved in MALAT1-meidiated promotion of cell proliferation in choriocarcinoma (138). Hence, MALAT1 promoted cell proliferation *via* interaction with miR-218 and upregulation of FBXW8 in choriocarcinoma.

Targeting FBXO family by lncRNAs

Linc01436 regulates FBXO11

Linc01436 was reported to be controlled by E2F6 and served as a tumor promoter in NSCLC cells (141). Linc01436 worked as a miR-30a-3p sponge to increase the expression of EPAS1 in NSCLC, resulting in promotion of cell growth, invasion and migration *in vitro* and enhancement of tumor growth and tumor metastasis in mice (141).

Emerging evidence has revealed that linc01436 plays an oncogenic role in gastric cancer progression (142–144). Linc01436 repressed the expression of miR-585-3p and increased mitogen-activated protein kinase 1 (MAPK1) expression, which contributed to gastric cancer development (143). Similarly, linc01436 triggered gastric cancer progression through modulation of miR-513a-5p and apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1) (144). The higher expression of linc01436 was observed in tumor tissues of gastric cancer patients and was associated with a poor survival in gastric cancer cases (142). Moreover, using *in vitro* experiments, knockdown of linc01436 retarded metastasis and blocked proliferation in BGC823 gastric cancer cells, while increased linc01436 promoted metastasis and proliferative activity in AGS gastric cancer cells (142). Mechanistically, miR-585 can bind to linc01463 and FBXO11, suggesting that linc01436 sponges miR-585 and inhibit it, leading to indirect promotion of FBXO11 expression in gastric cancer (142). Taken together, linc01463 targets miR-585/FBXO11 axis and subsequently promotes progression of gastric cancer.

LincRNA GATA6-AS1 regulates FBXO11

Xu et al. reported that lincRNA GATA6-AS1 regulated invasive and migratory capacities and viability *via* binding to miR-19a-5p and increasing TET2 in ovarian cancer cells (145). LincRNA GATA6-AS1 promoted GATA6 expression and controlled the behaviors of lung cancer cells (146). In lung cancer cells, lincRNA GATA6-AS1 suppressed cell proliferation and invasive ability (147). Using several approaches, including RNA sequencing dataset, RT-qPCR and TCGA data, one group found that GATA6-AS1 expression levels were downregulated in lung cancer tissues (147). Moreover, GATA6-AS1 overexpression increased the expression of FBXO11 and SP1 *via* sponging miR-324-5p, contributing to enhancement of invasion and proliferation in lung cancer cells. Furthermore, miR-324-5p overexpression abolished the effects of GATA6-AS1 upregulation in lung cancer (147). In a word, lincRNA GATA6-AS1 might regulate miR-324-5p/FBXO11 axis and facilitated lung cancer development.

LncRNA ODIR1 regulates FBXO25

FBXO25 has been reported to participate in cancer development and malignant behaviors (148, 149). Impairing PRKCD-FBXO25-HAX-1 signaling pathway led to lymphomagenesis and reduced the apoptotic reaction (150). FBXO25 facilitated cell invasion, migration and proliferation *via* regulation of YAP, cyclins, MMPs and β-catenin in NSCLC cells (148). Clinically, FBXO25 had the higher expression in the nucleus and cytoplasm of tumor tissues in lung cancer patients, and was associated with lymph node metastasis and TNM stage and overall survival (148). In cutaneous squamous cell carcinoma cells, FBXO25 increased cell growth and metastasis *via* binding with Oct-1, a Cyclin D1 repressor, and stabilization of Cyclin D1 (149). One study showed that lncRNA RP11-527N22.2, also known as osteogenic differentiation inhibitory lncRNA 1 (ODIR1), interacted with FBXO25 and promoted the destruction of FBXO25 protein by recruiting Cullin 3 (151). FBXO25 promoted H2BK120 ubiquitination and increased the trimethylation of H3K4 (H3K4me3), which increased osterix transcription and the expression of osteocalcin, osteopontin and ALP (151). In human umbilical cord-derived mesenchymal stem cells, downregulation of ODIR1 contributed to osteogenic differentiation, while upregulation of ODIR1 suppressed osteogenic differentiation (151). It is required to investigate the role of ODIR1-mediated FBXO25 disruption in oncogenesis and progression.

Linc00494 regulates FBXO32

FBXO32 promoter hypermethylation has been revealed to be linked to poor prognosis in patients with ovarian cancer (152). FBXO32 has been involved in carcinogenesis and tumor malignant behaviors. FBXO32 worked as an E3 ligase for PHPT1 ubiquitination, leading to reduction of PHPT1 accumulation, inactivation of the ERK/MAPK axis, which inhibited the proliferation of lung cancer cells (153). FBXO32 repressed tumorigenesis by targeting KLF4 for ubiquitination and proteasomal degradation in breast cancer (154). Linc00494 was predicted to bind with NF-kappa B1 by bioinformatics

analysis in ovarian cancer cells (155). Dual-luciferase reporter assay, RIP and RNA pull-down confirmed the interaction between linc00494 and NF-kappa B1. Linc00494 increased the activity of NF-kappa B1 after their interaction. Moreover, NF-kappa B1 suppressed the transcription of FBXO32 *via* binding with the promoter region of FBXO32. Linc00494 upregulation accelerated the expression of NF-kappa B1 and caused invasion, migration and tumorigenesis in ovarian cancer cells. In consistent, upregulation of FBXO22 reversed the linc00494-mediated tumorgenicity in ovarian cancer (155). Strikingly, linc00494 expression levels were highly upregulated in ovarian cancer tissues, while FBXO32 has a lower expression in ovarian tumor specimens (155). In summary, linc00494 modulated NF-kappa B1 and FBXO32 and enhanced progression of ovarian cancer.

Conclusions and future perspectives

In conclusion, multiple lncRNAs have been reported to regulate the expression of several F-box proteins in tumorigenesis, including lncRNAs SLC7A11-AS1, MT1JP, TUG1, FER1L4, TTN-AS1, CASC2, MALAT1, TINCR, PCGEM1, linc01436, linc00494, GATA6-AS1, and ODIR1 (Figures 1 and 2). Modulation of these lncRNA expressions is a potential therapeutic strategy for cancer therapy *via* regulation of F-box proteins. Besides lncRNAs, miRNAs and circRNAs have also participated in modulation of F-box protein in carcinogenesis. It is necessary to note that several issues need to be addressed for clarifying the functions of lncRNAs in oncogenesis *via* targeting F-box proteins. For example, there are thousands of lncRNAs. However, only about a dozen lncRNAs were identified to regulate the expression of F-box proteins. More lncRNAs should be discovered, which modulate the F-box protein expression in cancer. Among the 69 F-box proteins, no lncRNA was discovered to target FBXL proteins in tumorigenesis. In addition, one lncRNA can target several F-box proteins. For example, MALAT1 targets both FBXW7 and FBXW8 in cancer cells. It is unclear whether MALAT1 targets two F-box proteins at the same time in carcinogenesis. Hence, further in-depth investigation is pivotal to determine whether regulation of F-box proteins by related lncRNAs is a therapeutic strategy for cancer treatment.

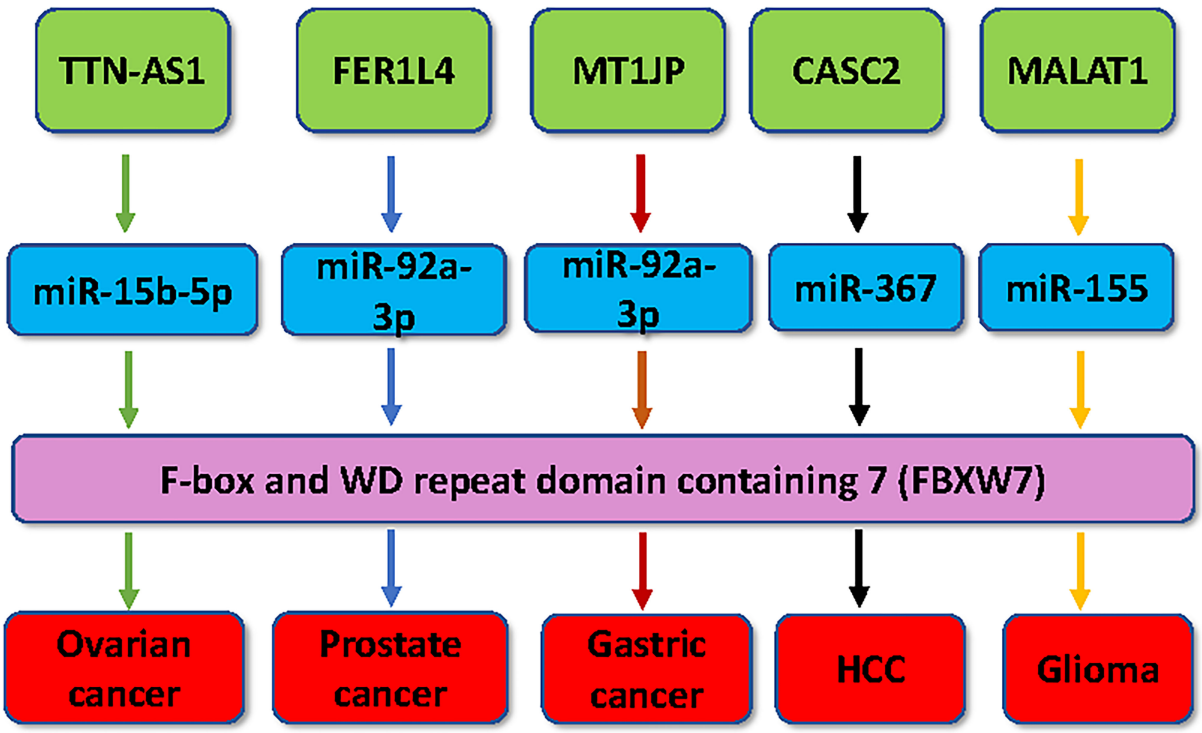

FIGURE 1
Multiple lncRNAs regulate the expression of FBXW7 in human cancer. Multiple lncRNAs, including MT1JP, FER1L4, TTN-AS1, CASC2 and MALAT1, have been demonstrated to regulate the expression of FBXW7 in tumorigenesis.

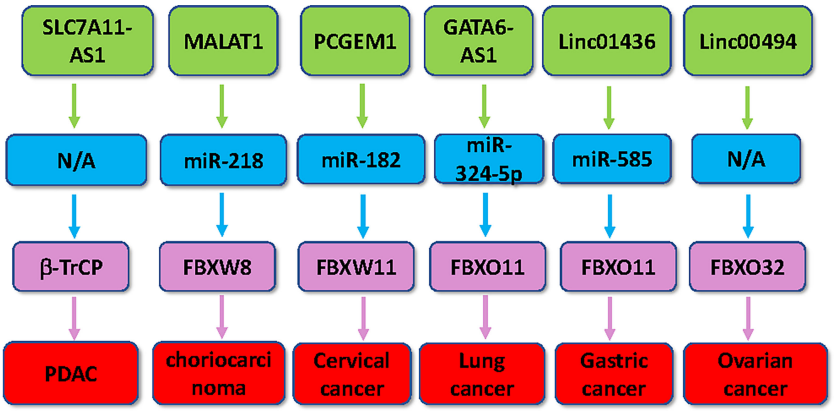

FIGURE 2
Multiple lncRNAs regulate the expression of F-box proteins in human cancer. Multiple lncRNAs, including SLC7A11-AS1, MALAT1, PCGEM1, linc01436, linc00494 and GATA6-AS1, regulate the expression of several F-box proteins in tumorigenesis.

Author contributions

LX drafted the original manuscript and made the figure. JC, MH, and JM edited the manuscript. ML edited this manuscript and supervised this study. All authors have read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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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 non-coding RNA PVT1: A promising chemotherapy and radiotherapy sensitizer

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The long non-coding RNA (lncRNA) PVT1 was first found to activate variant translocations in the plasmacytoma of mice. Human lncPVT1 is located on chromosome 8q24.21, at the same locus as the well-known MYC oncogene. LncPVT1 has been found to promote the progression of various malignancies. Chemoresistance and radioresistance seriously affect tumor treatment efficacy and are associated with the dysregulation of physiological processes in cancer cells, including apoptosis, autophagy, stemness (for cancer stem cells, CSC), hypoxia, epithelial–mesenchymal transition (EMT), and DNA damage repair. Previous studies have also implicated lncPVT1 in the regulation of these physiological mechanisms. In recent years, lncPVT1 was found to modulate chemoresistance and radioresistance in some cancers. In this review, we discuss the mechanisms of lncPVT1-mediated regulation of cellular chemoresistance and radioresistance. Due to its high expression in malignant tumors and sensitization effect in chemotherapy and radiotherapy, lncPVT1 is expected to become an effective antitumor target and chemotherapy and radiotherapy sensitizer, which requires further study.

KEYWORDS

PVT1, lncRNA, chemoresistance, radioresistance, cancer

Introduction

Long non-coding RNAs (lncRNAs), a class of functional RNA molecules larger than 200 nucleotides that cannot be translated into proteins, play an important regulatory role in epigenetics (1). LncPVT1 is an important member of the lncRNA family and was first found to activate variant translocations in the plasmacytoma of mice (2). Human lncPVT1 is located on chromosome 8q24.21, at the same locus as the well-known

oncogene MYC (3) (Figure 1). It not only interacts with MYC to promote cancer progression but also performs different oncogenic functions independent of MYC (4). LncPVT1 has been reported to be overexpressed in many types of malignant tumors, promoting cancer progression (5–9) (Figure 2). Moreover, lncPVT1 can regulate proliferation, invasion, autophagy, apoptosis, epithelial–mesenchymal transition (EMT), hypoxia, stemness (for cancer stem cells, CSC), exosomes, and other important physiological mechanisms (10–14).

Chemotherapy is an important cancer treatment regimen that kills cancer cells primarily through the systemic or local use of chemosynthetic drugs and has a significant clinical benefit for patients (15). Chemoresistance leads to cancer recurrence and metastasis, hindering patient survival; hence, it remains the main obstacle in cancer treatment. Therefore, we need to understand the detailed regulatory mechanisms underlying chemoresistance to improve tumor cell sensitivity to chemotherapy (16). The currently reported molecular mechanisms associated with chemoresistance include the action of oncogenes, tumor suppressor genes, mitochondrial changes, DNA repair, autophagy, EMT, CSC, and exosomes (17). These pathways often intersect to increase the tolerance of tumors to cytotoxic drugs; therefore, the inhibition of these pathways will significantly improve the sensitivity of tumors to chemotherapy.

Radiotherapy is also an important form of cancer treatment. It can be used alone or in combination with other forms of treatment, either curative or palliative, for all stages of cancer. More than 50% of patients with cancer receive radiotherapy (18). Unfortunately, radioresistance reduces the efficacy of radiotherapy and seriously affects the quality of life of patients with cancer (19). Radiation therapy kills cancer cells directly or indirectly by causing DNA damage (20). However, ionizing radiation can also activate multiple prosurvival signaling pathways to promote DNA damage checkpoint activation, DNA repair, autophagy, apoptosis inhibition, and CSC. These signaling pathways conjointly protect cancer cells from radiation injury and promote radioresistance (21). Current literature indicates that the tumor microenvironment is closely related to radioresistance. Under hypoxic conditions, it has been shown that the radiosensitivity of tumor cells decreased significantly (22, 23).

LncPVT1 has gradually become a research hotspot regarding the regulation of chemoresistance and radioresistance, as studies have found that the regulatory mechanism of lncPVT1 in tumors is also associated with these processes. In recent years, lncPVT1 has been reported to regulate the chemoresistance of tumor cells through various pathways (Table 1). LncPVT1 silencing has also been shown to enhance radiosensitivity in nasopharyngeal carcinoma and lung cancer (Table 2). In this review, we summarize the various mechanisms through which lncPVT1 regulates chemosensitivity and radiosensitivity. We further emphasized that lncPVT1 is expected to be a new sensitizer of chemotherapy and radiotherapy and that it can be

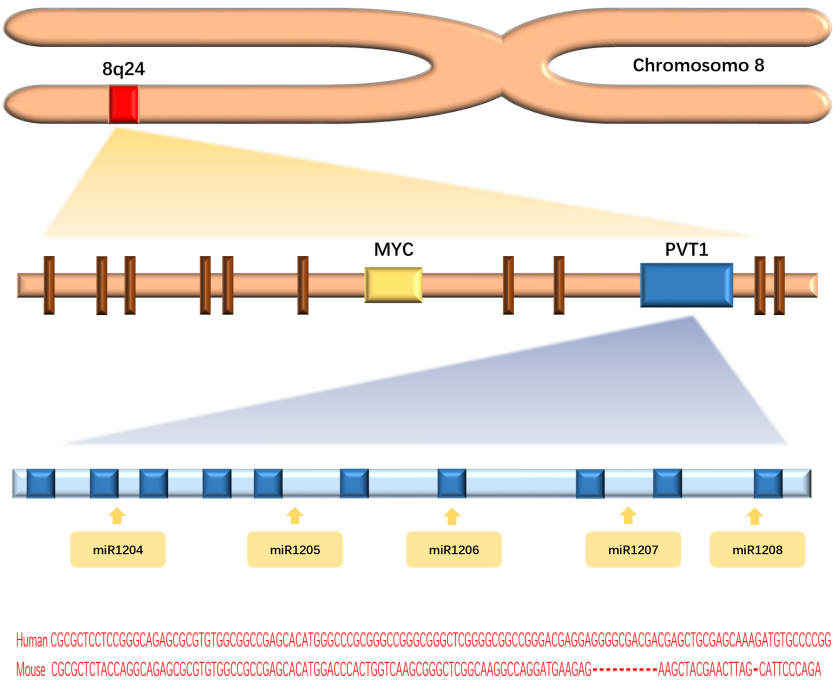

Figure 1 Graphical representation of the lncPVT1 genomic locus.

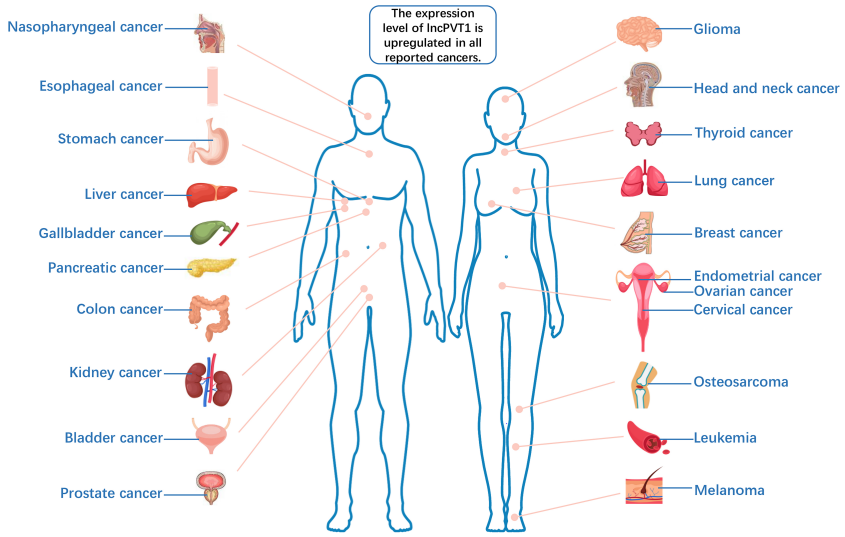

FIGURE 2
The expression level of lncPVT1 is upregulated in all reported cancers.

used to develop better clinical therapeutic strategies for patients with cancer.

PVT1 and Chemoresistance

Pancreatic cancer

Pancreatic cancer is a highly malignant tumor with an extremely poor prognosis. The 1- and 5-year survival rates of this disease are only 24% and 9%, respectively (55). Gemcitabine is a first-line chemotherapeutic agent for advanced pancreatic cancer, and resistance to gemcitabine and other chemotherapeutic drugs is an important factor in the poor prognosis of this malignancy (56).

The expression level of lncPVT1 is significantly increased in pancreatic cancer. Moreover, lncPVT1 has been reported to promote the proliferation and migration of pancreatic cancer cells (13). Previous reports have suggested that decreased lncPVT1 levels can increase sensitivity of pancreatic cancer cells to gemcitabine chemotherapy (57, 58). Studies have shown that the activation of the Wnt/β-catenin pathway can induce chemoresistance in cancer cells (59). Autophagy is an important mechanism for tumor cell survival and has been proven to improve the tolerance of tumor cells to radiotherapy and chemotherapy (60). LncPVT1 competitively binds to microRNA-619-5p (miR-619-5p) to regulate the expression of Pygo2, mediating the Wnt/β-catenin pathway to increase the chemoresistance of pancreatic cancer cells to gemcitabine. At the same time, the autophagy-related protein ATG14 is also regulated by lncPVT1/miR-619-5p to increase autophagy, thereby promoting gemcitabine resistance in pancreatic cancer (24).

O6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme regulated by Wnt/β-catenin that protects cells from physicochemical damage by repairing damaged DNA (61). In addition, the Hedgehog (Hh) signaling pathway has been shown to be closely associated with tumor resistance and regulates autophagy (62, 63). Its terminal transcription factor Gli is responsible for transmitting signals into the nucleus and promoting transcriptional activation to upregulate the expression level of downstream target genes, MGMT (64, 65). Based on these studies, Yu Shi et al. found that lncPVT1 acts as a competing endogenous RNA (ceRNA) of miR-409 in pancreatic cancer cells. At the same time, miR-409 directly targets The Sonic Hedgehog (SHH) and regulates apoptosis and autophagy through the SHH/GLI/MGMT pathway, thus mediating the resistance of pancreatic cancer to gemcitabine (25). Furthermore, the hypoxia-inducible factor 1-alpha (HIF-1α)/vacuole membrane protein 1 (VMP1) axis mediates therapeutic resistance in colon cancer cells (66). Researchers recently found that lncPVT1 competitively binds to miR-143 and that decreased lncPVT1 levels and upregulated miR-143 levels increase the chemosensitivity of pancreatic cancer cells to gemcitabine through the HIF-1α/VMP1 axis (26).

In addition, lncPVT1 regulates the sensitivity of pancreatic cancer cells to gemcitabine by inhibiting enhancer of zeste homolog-2 (EZH2) at polycomb repressive complex 2 (PRC2), where EZH2 upregulates the expression of lncPVT1. LncPVT1, as an enhancer of MYC, promotes the expression of c-Myc, thus stimulating the activity of CSCs. The dysregulation of CSC in pancreatic cancer can induce chemoresistance (67). Another

TABLE 1 Molecular mechanisms underlying the lncPVT1-induced regulation of drug resistance.

Cancer	Pathway	Drug	References
Pancreatic cancer	miR-619-5p/Pygo2/Wnt/β-catenin axis; miR-619-5p/ATG14 axis	Gemcitabine resistance	(24)
	miR-409/SHH/GLI/MGMT axis	Gemcitabine resistance	(25)
	miR-143/HIF-1α/VMP1 axis	Gemcitabine resistance	(26)
	Curcumin regulates the PRC2/PVT1/C-MYC axis and then targets CSC	Gemcitabine resistance	(27)
	HAT1 binds EZH2 to prevent its degradation and promote lncPVT1 expression	Gemcitabine resistance	(28)
	Drosha and DGCR8 promote lncPVT1 to encode miR-1207-5p and miR-1207-3p, thereby regulating the expression of SRC and RhoA, respectively	Gemcitabine resistance	(29)
Gastric Cancer	mTOR/HIF-1α/P-gp and MRP1 pathway	Cisplatin (DPP) resistance	(30)
	Kanglaite (KLT) inhibits the expression of MDR1 and MRP1 by suppressing the expression of lncPVT1	Cisplatin (DPP) resistance	(31)
	miR-3619-5p/TBL1XR1 axis	Cisplatin (DPP) resistance	(32)
	Bcl2 activation to inhibit apoptosis	5-Fluorouracil resistance	(33)
Ovarian cancer	TGF-β1/p-Smad4/caspase-3 axis	Cisplatin (DPP) resistance	(34)
	miR-370/FOXM1 axis	Cisplatin (DPP) resistance	(35)
	JAK2/STAT3/PD-L1 axis	Cisplatin (DPP) resistance	(36)
Colorectal cancer	Inhibiting apoptosis and upregulating the expression of MDR1 and MRP1	Cisplatin (DPP) resistance	(37)
	Inhibiting apoptosis and upregulating the expression of MRP1, P-gp, mTOR, and Bcl-2	5-Fu resistance	(38)
Bladder cancer	Wnt/β-catenin axis	Doxorubicin (DOX) and Cisplatin (DDP) resistance	(39)
	MDM2/AURKB/p53 axis	Doxorubicin (ADM) resistance	(40)
Lung cancer	miR-216b/Beclin-1 axis	Cisplatin (DPP) resistance	(41)
	HIF-1α/miR-140-3p/ATG5/autophagy	Cisplatin (DPP) resistance	(42)
Breast Cancer	Preventing Nrf2 protein degradation by inhibiting the binding of Keap1 and Nrf2	Doxorubicin resistance	(43)
Osteosarcoma	miR152/c-MET/p-PI3K/p-AKT axis	Gemcitabine resistance	(44)
Glioma	Apoptosis pathways	Paclitaxel resistance	(45)
	miR-365/ELF4/SOX2 axis	Temozolomide (TMZ) resistance	(46)
Cervical cancer	Decreasing miR-195 expression by enhancing histone H3K27me3 in the miR-195 promoter region and also *via* direct sponging of miR-195	Paclitaxel (PTX) resistance	(47)
Head and neck squamous cell carcinoma (HNSCC)	By sponging miR-124-3p	Cetuximab resistance	(48)*
Prostate Cancer	Not mentioned	Castration resistance	(49)*

*LncPVT1 regulates resistance to immunotherapy and castration therapy.

study has concluded that curcumin downregulates cancer stemness by inhibiting the PRC2/lncPVT1/c-MYC pathway, increasing the sensitivity of pancreatic cancer cells to gemcitabine (27). Sun et al. found that histone acetyltransferase 1 (HAT1) promotes the binding of bromodomain-containing 4 (BRD4) to the lncPVT1 promoter to enhance lncPVT1 expression. Simultaneously, HAT1 competitively binds to the N-terminus of EZH2 with the ubiquitin protein ligase E3 component n-recognin 4 (UBR4), preventing the ubiquitination of EZH2. Thus, HAT1 promotes gemcitabine resistance in pancreatic cancer cells (28). Based on this mechanism, tripolyphosphate (TPP)-siHAT1 nanoparticles have been developed to inhibit HAT1 expression and overcome gemcitabine resistance in pancreatic cancer cells. However, the efficacy and safety of TPP-siHAT1 nanoparticles require further validation.

TABLE 2 Molecular mechanisms underlying PVT1-induced radioresistance.

Cancer	Pathway	Mechanism	References
Nasopharyngeal carcinoma (NPC)	ATM/Chk2/p53 axis; caspase-9/caspase-7/PARP axis	DNA repair, apoptosis	(50)
	KAT2A/H3K9ac/TIF1β/NF90/HIF-1α axis	Hypoxic	(51)
	miR-515-5p/PIK3CA/p-AKT axis	Apoptosis	(52)
Non-small-cell lung cancer (NSCLC)	By sponging miR-195	Apoptosis	(53)
	miR-424-5p/CARM1 axis	Apoptosis	(54)

You et al. have also discussed the mechanism of gemcitabine resistance in pancreatic cancer cells. They found that Drosha ribonuclease III (Drosha) and DGCR8 promote the lncPVT1-mediated processing of miR-1207-5p and miR-1207-3p. The generated products miR-1207-5p and miR-1207-3p inhibit sarcoma gene (SRC) and ras homolog family member A (RhoA), respectively, to increase the sensitivity of pancreatic cancer cells to gemcitabine chemotherapy (29). These studies highlight lncPVT1 as a promising target for improving the sensitivity of pancreatic cancer cells to gemcitabine.

Gastric cancer

Gastric cancer remains the most common malignancy of the digestive system, ranking fifth in incidence and third in causing cancer-related deaths worldwide (68). Although radical resection is the standard treatment for early gastric cancer, patients are often diagnosed with gastric cancer at the advanced stage, making chemotherapy the main treatment regimen after diagnosis. Cisplatin, 5-FU, and other chemotherapeutic drugs are common first-line treatments for gastric cancer (69). However, the emergence of multi-drug resistance (MDR) reduces the sensitivity of chemotherapy and makes the survival benefit of patients with advanced gastric cancer worse (70).

Many studies have suggested that lncPVT1 can be used as a biomarker for the diagnosis and prognosis of gastric cancer (71). In addition, lncPVT1 can promote the growth and invasion of gastric cancer and induce angiogenesis (11, 72). The MDR-associated proteins include MDR1, mTOR, HIF-1α, and MRP. A decrease in lncPVT1 expression was found to increase the apoptosis rate of cisplatin-treated gastric cancer cell lines. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blotting (WB) showed that MDR1, mTOR, HIF-1α, and multi-drug resistance-associated protein 1 (MRP1) expression were all increased when lncPVT1 expression increased. The expression of these MDR-related genes promotes the expression of P-glycoprotein (P-gp). P-gp transports chemotherapeutic drugs out of the cell and helps the cell develop resistance to these drugs (73). Hence, lncPVT1 could be an effective target for reversing MDR in gastric cancer (30). Kanglaite (KLT) is a Chinese herbal formulation with antitumor effects (74), which is often combined with chemotherapy drugs to reduce the side effects of chemotherapy and increase chemosensitivity (75). Researchers found that KLT inhibited the expression of lncPVT1, reducing the expression of MDR1 and MRP1 in cisplatin-resistant gastric cancer cell lines (31).

Recently, the mechanism by which lncPVT1 regulates chemoresistance in gastric cancer has been further elaborated. Wu et al. found that lncPVT1 competes with miR-3619-5p to regulate the downstream target transducin beta-like 1 X-linked receptor 1 (TBL1XR1), regulating the sensitivity of gastric cancer cells to cisplatin (32). Moreover, 5-FU is also one of the main chemotherapeutic drugs for gastric cancer (76), so chemoresistance to 5-FU in gastric cancer deserves more attention. Du et al. found that lncPVT1 silencing could inhibit the expression of the anti-apoptotic protein Bcl-2, promoting apoptosis and inducing 5-FU resistance in gastric cancer cells (33). Therefore, lncPVT1 might play an important role in regulating cisplatin resistance in gastric cancer cells.

Ovarian cancer

Ovarian cancer is the deadliest gynecological cancer and is a serious threat to women's health worldwide. Approximately 70% of patients with this disease are already at an advanced stage upon diagnosis. Platinum-based chemotherapy and cytoreductive operations are standard treatments for ovarian cancer (77). Similarly, the clinical emergence of chemoresistance also poses great challenges for the survival of patients with this disease. Therefore, determining the target of chemoresistance in ovarian cancer is urgently needed.

LncPVT1 plays an oncogene role in ovarian cancer and promotes the proliferation and invasion of ovarian cancer cell (78, 79). Researchers found that lncPVT1 expression levels were significantly elevated in cisplatin-resistant ovarian cancer tissues (80). Liu et al. silenced lncPVT1 expression in cisplatin-resistant ovarian cancer cell lines and measured their cell viability and apoptosis rate after treatment with cisplatin. The results showed that the cell viability was significantly decreased and the apoptosis rate of tumor cells was significantly higher in downregulation of the lncPVT1 group than that of the control group after treatment with cisplatin. They also overexpressed lncPVT1 in cisplatin-sensitive ovarian cancer cell lines, which were then treated with cisplatin. Compared to the control group, cell viability in these lncPVT1-overexpressing cell lines was significantly increased, and the apoptosis rate was significantly reduced. RT-qPCR and WB results showed that the mRNA levels and protein expression of apoptosis-related genes TGF-β1, p-Smad4, and caspase-3 were significantly increased with a decrease in lncPVT1 expression level. Hence, lncPVT1 overexpression induces cisplatin resistance in ovarian cancer cells by inhibiting the apoptotic pathway (34).

Researchers further investigated how lncPVT1 regulates cisplatin resistance in ovarian cancer. Forkhead box M1 (FOXM1) has been reported to be involved in a variety of malignant behaviors of ovarian cancer cells, including growth, proliferation, invasion, and metastasis (72, 81). Yi et al. found that lncPVT1 acts as a molecular sponge for miR370 to inhibit miR370 and promote FOXM1 expression. Furthermore, lncPVT1 directly binds to FOXM1 and increases its protein expression level. Moreover, lncPVT1 promoted cisplatin resistance in ovarian cancer by increasing FOXM1 expression (35). Chen et al. found that lncPVT1 silencing can inhibit the

growth and proliferation and promote apoptosis in cisplatin-resistant ovarian cancer cells by downregulating the JAK2/STAT3/PD-1 signaling pathway (36). The combination of lncPVT1-targeted therapy and PD-L1 immunotherapy is expected to improve the efficacy in patients with cisplatin-resistant ovarian cancer (82).

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide. Cisplatin and 5-FU are the first-line chemotherapy agents for colorectal cancer (83). However, MDR is the main cause of treatment failure in patients with this disease. Researchers have found that lncPVT1 acts as a molecular sponge targeting miR-16-5p, thereby promoting proliferation, invasion, and migration of CRC cells by regulating VEGFA/VEGFR1/AKT signaling pathway (84). Moreover, lncPVT1/VEGFA axis promotes colon cancer metastasis and stemness by downregulation of miR-152-3p (10). LncPVT1 has also been found to promote proliferation, invasion, and migration of CRC cells by regulating the miR-106b-5p/FJX1 axis (85). Furthermore, lncPVT1 knockdown increased the apoptosis rate of cisplatin-resistant colorectal cancer cells, whereas the overexpression of lncPVT1 significantly enhanced the resistance of colorectal cancer cells to cisplatin. Moreover, silencing lncPVT1 increased the expression levels of pro-apoptotic proteins Bax and cleaved caspase-3 in cisplatin-resistant colorectal cancer cells while at the same time decreasing the expression levels of MDR1, MRP1, and the anti-apoptotic protein Bcl-2. We concluded that lncPVT1 regulates the sensitivity of colorectal cancer cells to cisplatin through the apoptotic pathway (37).

In addition, increased lncPVT1 expression promoted colorectal cancer cell tolerance to 5-FU. RT-qPCR and WB showed that lncPVT1 upregulated the expression levels of MRP1, mTOR, P-gp, and Bcl-2 (38). Therefore, lncPVT1 is an effective target for treating chemoresistance in colorectal cancer.

Bladder cancer

Bladder cancer is a fatal malignancy of the urinary system that mainly affects men over 65 years of age. Surgical treatment, radiotherapy, chemotherapy, immunotherapy, and bladder perfusion therapy are used for patients with different stages of bladder cancer (86). As an oncogene of bladder cancer cells, lncPVT1 acts as a ceRNA targeting miR-194-5p to regulate the expression level of BCLAF1, thereby promoting the proliferation, migration, and anti-apoptosis of bladder cancer cells (87). A previous study showed that lncPVT1 promotes growth, migration, and invasion of bladder cancer by miR-31/CDK1 (88).

Researchers found a significant increase in lncPVT1 expression in bladder cancer cells resistant to doxorubicin (DOX) and cisplatin (DDP). Moreover, lncPVT1 silencing not only attenuated the growth, proliferation, and other malignant behaviors of drug-resistant bladder cancer cell lines but also increased their cell apoptosis rate and sensitivity to DOX and DDP. WB results showed that the expression levels of the drug-resistant related proteins MDR1 and MRP1 decreased with a decrease in lncPVT1 expression. Further studies on the regulatory mechanism of lncPVT1 revealed that lncPVT1 regulates the expression levels of drug-resistant related proteins MDR1 and MRP1 through positive regulation of the Wnt/β-catenin pathway, thus affecting the sensitivity of bladder cancer cells to DOX and DDP (39). In another study, Jiang et al. found that increased lncPVT1 expression promoted mouse double minute 2 (MDM2) and MDM2-mediated aurora kinase B (AURKB) expression. Subsequently, p53 ubiquitination is enhanced, increasing resistance to doxorubicin (ADM) in bladder cancer cells (40).

Lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide, and smoking remains a major risk factor for this disease. Platinum-based chemotherapy is one of the main treatments for inoperable lung cancer (89); however, chemoresistance limits the application of platinum-based drugs in patients with lung cancer patients, increasing their mortality (90). Therefore, there is a need to study the mechanism of cisplatin tolerance in lung cancer and identify an effective target to solve this clinical problem.

LncPVT1 is highly expressed in non-small cell lung cancer (NSCLC) and is associated with poor prognosis (91). Wang et al. found that lncPVT1 facilitates the proliferation, migration, and invasion of NSCLC cells by indirectly mediating FGFR1 *via* targeting miR-551b (92). In another study, lncPVT1 activates the Wnt/β-catenin signaling pathway by miR-361-3p/SOX9 axis, thereby promoting the proliferation, migration, invasion, and anti-apoptosis of NSCLC cells (93). A previous study showed that lncPVT1 promotes angiogenesis by regulating the miR-29c/VEGF signaling axis in NSCLC (94). Beclin-1 is a biomarker for autophagy that has been reported to be involved in the malignant biological behavior of lung cancer cells (95). Chen et al. found that lncPVT1 sponges miR216b to inhibit Beclin-1 expression and induce cisplatin tolerance in NSCLC cells by regulating apoptosis and autophagy (41). Further studies have found that HIF-1α and other related pathways are activated to induce lncPVT1 expression under hypoxic conditions. The induction of lncPVT1 expression increases the expression level of ATG5 through competitive binding with miR140-3p and reduces the sensitivity of lung cancer cells to cisplatin by influencing the autophagy pathway (42). These studies indicate

that lncPVT1 is expected to become a new target to solve the problem of chemoresistance in lung cancer and improve the efficacy of treatment for this disease.

Breast Cancer

Breast cancer has replaced lung cancer as the most common malignant tumor worldwide. Triple-negative breast cancer (TNBC) is a subtype of breast cancer with negative estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression. It is characterized by its invasiveness, aggressive metastasis, and recurrence (96). Chemotherapy is the standard treatment for TNBC; unfortunately, chemoresistance is a major obstacle in treating patients with breast cancer.

LncPVT1 promotes breast cancer cell proliferation, migration, invasion, and anti-apoptosis *via* regulating miR-543/TRPS1 axis (97). In TNBC cells cultured with mature adipogenic medium (MAM), lncPVT1 facilitates EMT, cell proliferation, and cell migration by regulating p21 expression (98). A recent study showed that lncPVT1 promotes cell migration and invasion by regulating miR-148a-3p/ROCK1 axis in breast cancer (99). The Keap1/Nrf2/ARE pathway is an important antioxidant stress signaling pathway in the body, which has been observed to regulate drug resistance in tumor cells (100, 101). Using bioinformatics analysis, Luo et al. concluded that lncPVT1 could interact with Keap1. They then conducted experiments to verify that lncPVT1 blocked the Keap1-mediated degradation of Nrf2 through competitive binding with Keap1. In turn, this binding increased the expression of Nrf2 and increased the expression of downstream drug-resistance-related molecules. Moreover, lncPVT1 increased adriamycin resistance in TNBC through this mechanism (43).

Osteosarcoma

Osteosarcoma (OS) is a malignant tumor that usually occurs in children and adolescents. Patients with this disease often develop lung metastases. Surgical treatment, chemotherapy, and radiotherapy are the main therapeutic methods for OS (102, 103). In recent years, there have been breakthroughs in the application of immunotherapy in patients with OS (104). Chemotherapy treatments for OS often involve multi-drug combination therapy, usually including adriamycin, cisplatin, bleomycin, cyclophosphamide, and gemcitabine, among other drugs. Chemoresistance often leads to recurrence and metastasis in patients with osteosarcoma.

LncPVT1 plays a carcinogenic role in OS, promoting OS cell glucose metabolism, growth, proliferation, and invasion through regulating miR-497/HK2 axis (105). In a previous study, exosomes secreted by bone marrow mesenchymal stem cells (BMSCs) transfer lncPVT1 into osteosarcoma cells; the upregulation of lncPVT1 can promote ERG expression by inhibiting ERG ubiquitination and sponging miR-183-5p. LncPVT1 promotes the growth and metastasis of OS by regulating ERG expression (106). Moreover, lncPVT1 plays a potential role in regulating CSCs in OS (107). LncPVT1 is a ceRNA of miR-152. It activates the c-MET/PI3K/AKT pathway to enhance the resistance of OS cells to gemcitabine (44). Moreover, c-MET is a hepatocyte growth factor (HGF) receptor that has been reported to promote the progression of various cancers (108). The inhibition of c-MET has been shown to enhance the sensitivity of OS cells to cisplatin by inhibiting the PI3K/AKT pathway (109).

Glioma

Glioma is the most common primary malignant brain tumor in adults. The treatment for this disease includes a combination of surgery, radiotherapy, and chemotherapy. Due to the blood–brain barrier and tumor heterogeneity, gliomas have strong drug resistance, leading to a high degree of malignancy and poor prognosis (110); hence, understanding the mechanisms of drug resistance in glioma is expected to improve patient prognosis.

LncPVT1 is highly expressed in gliomas, indicating poor prognosis. In addition, lncPVT1 can promote angiogenesis by regulating miR-1207-3p/hepatocyte nuclear factor 1β (HNF1B)/EMT axis in glioma (111). In another study, lncPVT1 influence bone morphogenetic protein (BMP) signaling pathway by regulating miR-128-3p/GREM1 axis, thereby promoting glioma cell proliferation, invasion, migration, and anti-apoptosis (112). A recent study showed that tumor suppressor gene p53 inhibits glioma cell proliferation, migration, and invasion while inducing apoptosis by blocking lncPVT1/TGF-β/Smad signaling pathway (113). Researchers knocked down lncPVT1 expression levels in SHG-44 glioma cells, added different concentrations of paclitaxel, and then measured their apoptosis rate. The results showed that the apoptosis rate of glioma cells was significantly higher in the lncPVT1-silenced group compared to the control group. These results suggest that decreased lncPVT1 expression increases the sensitivity of glioma cells to paclitaxel (45). Further research found that lncPVT1 acts as ceRNA to target miR-365 and positively regulate the expression level of E74-like ETS transcription factor 4 (ELF4). ELF4 has been reported to be highly expressed in glioma and can upregulate SOX2 expression to promote stemness (114). In conclusion, lncPVT1 facilitates stemness and TMZ resistance through regulating miR-365/ELF4/SOX2 signal axis in glioma (46).

Cervical Cancer

Cervical cancer is the fourth most common cancer in women worldwide. Human papillomavirus (HPV) infection has been confirmed to be closely related to the occurrence and development of cervical cancer. HPV16, 18, 31, and 45 are the main causes of cervical cancer (115). Chemotherapy is the main treatment for advanced and recurrent cervical cancer, and platinum-based chemotherapy, such as cisplatin combined with paclitaxel, is often used. However, the emergence of chemoresistance significantly reduces the efficacy of this regimen in patients with cervical cancer.

It was observed that serum lncPVT1 levels in cervical cancer patients is generally elevated and correlated with cervical cancer tumor size, lymph node metastasis, and clinical stage. This suggests that lncPVT1 may be a novel serum biomarker for early diagnosis of cervical cancer (116). LncPVT1 acts as a ceRNA or sponge of miR-424 to promote the proliferation, migration, and invasion of cervical cancer cells (117). In addition, lncPVT1 has been found to regulate the sensitivity of cervical cancer cells to paclitaxel. Specifically, lncPVT1 directly and competitively binds to miR-195 and recruits EZH2 to induce histone H3 lysine 27 trimethylation (H3K27me3) in the miR-195 promoter region, thereby inhibiting the expression of miR-195. Downregulation of miR-195 promotes EMT in cervical cancer cells, thereby enhancing their chemoresistance to paclitaxel (47).

PVT1 and Radioresistance

Nasopharyngeal Cancer

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the epithelial cells of the nasopharynx. This disease often occurs in southern China, with a significant regional concentration and ethnic susceptibility (118). Although the incidence of NPC is not high, NPC cells easily invade other tissues and metastasize, showing a strong degree of malignancy. NPC is closely related to Epstein–Barr virus infections (119). Radiotherapy has been established as the cornerstone of NPC treatment since 1965. However, in recent years, the development of intensity-modulated radiotherapy (IMRT) and proton radiotherapy (PRT) has brought significant survival benefits to patients with NPC (120). However, 10%–20% of NPC patients develop recurrence after radiotherapy due to radioresistance (121). Therefore, it is important to explore the molecular mechanism underlying radioresistance in NPC and search for new clues for radiotherapy sensitization in this disease (122).

In NPC, lncPVT1 has been found to promote cell proliferation and induce stemness by targeting miR-1207 (123). It was previously reported that the expression level of lncPVT1 was significantly upregulated in a radiation-induced mouse model, wherein RT-qPCR analysis showed that the expression level of lncPVT1 was significantly increased in the irradiated mice (124). Yi He et al. found that lncPVT1 silencing led to a decrease in phosphorylation of ATM/Chk2/p53 signaling pathway in NPC cells, which further led to the inhibition of the ATM-mediated homologous recombination (HR) repair pathway. Hence, more cells became apoptotic due to DNA damage that cannot be repaired in time (125). Meanwhile, decreased lncPVT1 expression levels after radiotherapy activated caspase, which is a core pro-apoptotic caspase, resulting in a cascade reaction that successively activates caspase-9, caspase-7, and PARP. PARP is believed to be the receptor for DNA damage and the cleavage substrate of caspase-7. Apoptosis is induced when activated caspase-7 binds to PARP (126). Moreover, the presence of lncPVT1 and MYC on the same chromosome band forms a positive feedback loop to promote tumor progression synergistically (3). In conclusion, lncPVT1 can promote DNA repair by phosphorylation of ATM/Chk2/p53 signaling pathway in NPC cells. LncPVT1 can also significantly inhibit apoptosis by blocking caspase-9/caspase-7/PARP axis, thereby leading to radioresistance (50).

Hypoxia induces radioresistance in tumor cells. HIF-1α is a major regulator of the hypoxia response and can be stably expressed under hypoxic conditions (127). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that lncPVT1 was associated with the hypoxic phenotype. Because tumor hypoxia is common in NPC and it is associated with disease progression and resistance to therapy, Hong et al. suggested that targeting tumor hypoxia could be an effective approach for NPC treatment (128). Further studies revealed that lncPVT1 acts as a molecular scaffold for KAT2A and WDR5, coordinating their localization, and enabling KAT2A to acetylate H3K9 effectively. Acetylated H3K9 then recruits TIF1β to bind to chromatin, forming an H3K9ac/TIF1β complex that induces the transcription of NF90 and stabilizes HIF-1α expression (51). LncPVT1 induces radioresistance in NPC by activating the above-mentioned pathways and generating a hypoxic tumor microenvironment.

In addition, Han et al. found that lncPVT1 silencing inhibited cell proliferation, promoted cell apoptosis, and increased radiosensitivity in NPC cells. Moreover, lncPVT1 binds to miR-515-5p and negatively regulates its expression. An increase in lncPVT1 expression can reverse the inhibitory effect of miR-515-5p overexpression on the growth and proliferation of cancer cells and the promotive effect of miR-515-5p on the apoptosis and radiosensitivity of cancer cells. Meanwhile, miR-515-5p overexpression also reversed the inhibition of cyclin D1 expression and the elevation of Bax and cleaved caspase-3 levels. Further studies have shown that PIK3CA promotes growth and proliferation, inhibits apoptosis, and increases the radioresistance of NPC cells, whereas the overexpression of miR-515-5p reversed these effects. Moreover,

miR-515-5p inhibition reversed the inhibitory effect of lncPVT1 silencing on the expression levels of PIK3CA and p-AKT. It has previously been reported that the p-AKT pathway is involved in regulating radioresistance in various cancers, and that PIK3CA can activate this pathway (129, 130). In conclusion, increased lncPVT1 expression activates the P-AKT pathway through the miR-515-5p/PIK3CA axis, thereby promoting cell proliferation, inhibiting apoptosis, and inducing radioresistance (52).

Lung cancer

Patients with lung cancer typically receive a combination of treatments, but radiotherapy is the only treatment used for all types and stages of lung cancer. According to evidence-based models, 77% of patients with lung cancer are eligible for radiotherapy (131). With advancements in technology, radiotherapy has effectively improved treatment efficacy in patients with lung cancer while reducing the incidence of adverse reactions (132). However, radioresistance in lung cancer usually leads to recurrence and metastasis, which significantly reduces patient survival. Therefore, there is an urgent need to identify new targets for lung cancer radiotherapy sensitization to improve patient prognosis (133).

It has been reported that reduced lncPVT1 expression significantly inhibits the growth and proliferation of NSCLC cells. The apoptosis rate of NSCLC cells treated with a certain radiation dose was determined. The results showed that the apoptosis rate of the lncPVT1-silenced group was significantly higher than that of the control group, indicating that lncPVT1 silencing enhances the radiosensitivity of NSCLC cells. Luciferase reporter assays showed that lncPVT1 could directly interact with miR-195 and negatively regulate its expression. miR-195 inhibitors reversed the inhibitory effect of lncPVT1 silencing on cell proliferation and promoted apoptosis *in vitro*. In addition, miR-195 inhibitors also reversed the radiosensitization effect of lncPVT1 silencing in NSCLC cells. In conclusion, lncPVT1 acts as a molecular sponge of miR-195 in NSCLC, and the decreased expression level of lncPVT1 inhibits cell proliferation and promotes cell apoptosis, thus increasing the radiosensitivity of NSCLC cells (53).

Other related studies have shown that coactivator-associated arginine methyltransferase 1 (CARM1) can co-activate transcriptional regulation with PRMT1 and is overexpressed in NSCLC (134). CARM1 has been reported to be involved in regulating radiosensitivity in cervical cancer cells and colorectal cancer cells (135, 136). Wang et al. found that lncPVT1, as a ceRNA of miR-424-5p, promoted the expression of CARM1. The decrease in lncPVT1 levels or the increase in miR-424-5p levels inhibited the growth, proliferation, invasion, metastasis, and other malignant behaviors of NSCLC cells, promoted the apoptosis of NSCLC cells, and upregulated their radiosensitivity. RT-qPCR and WB showed that lncPVT1 silencing or miR-424-5p overexpression regulated the expression levels of MMP-2, MMP-9, Bcl-2, and Bax, which are related to tumor progression and apoptosis. Therefore, lncPVT1, as a ceRNA of miR-424-5p, regulates the radiosensitivity of NSCLC by regulating CARM1 (54).

LncPVT1 and Resistance to other drugs

Head and neck squamous cell carcinoma (HNSCC) is the most common type of head and neck cancer and originates from the mucosal surface of the head and neck. Alcohol, tobacco, and HPV infection are risk factors for the induction of squamous cell carcinoma of the head and neck (137). EGFR is also usually highly expressed in HNSCC and is involved in regulating tumor occurrence and progression. Therefore, EGFR-targeted therapies are becoming increasingly popular for this disease. Cetuximab, a monoclonal antibody targeting EGFR, is often used in combination with radiotherapy to treat locally advanced HNSCC and has achieved good efficacy (138). LncPVT1 promotes laryngeal squamous cell carcinoma cell proliferation, migration, and anti-apoptosis by acting as a molecular sponge to regulate miR-519d-3p (139). In addition, lncPVT1 competitively combined with miR-150-5p to regulate GLUT1, thereby promoting the proliferation, migration, invasion, and anti-apoptosis of oral squamous cell carcinoma (140). LncPVT1 overexpression promoted HNSCC growth, significantly reduced the apoptosis rate of HNSCC cells treated with cetuximab, and reduced the drug sensitivity of HNSCC cells. As a tumor suppressor, miR-124-3p can reverse HNSCC tolerance to cetuximab. Furthermore, lncPVT1 can promote the resistance of HNSCC to cetuximab by inducing the methylation of the miR-124-3p promoter and downregulating miR-124-3p expression (48).

Prostate cancer is one of the most common malignant tumors in men worldwide, seriously affecting their health. Since androgens regulate the occurrence and progression of tumors, androgen-stripping therapy, specifically castration therapy, has become an important part of the prostate cancer treatment regimen. The clinical use of androgen inhibitors has significantly benefited patients with metastatic prostate cancer; however, the use of these drugs in metastatic castration-resistant prostate cancer (mCRPC), which occurs in some patients, remains a challenge (141). LncPVT1 could be used as a prognostic factor in patients with prostate cancer, representing poor survival (142). In prostate cancer, lncPVT1 has been found to promote EMT by regulating miR-186/Twist1 (143). LncPVT1 competitively binds to miR-15b-5p, miR-143-3p, miR-27a-3p, and miR-627-5p, then promotes prostate cancer invasion and metastasis by upregulating NOP2 (144). In another study, lncPVT1 promoted the viability of prostate cancer cells and inhibited their apoptosis by targeting miR-146a (145).

Abiraterone is a second-generation androgen inhibitor used clinically for prostate cancer treatment. Abiraterone was used to treat lncPVT1-overexpressing prostate cancer cell lines and untreated pancreatic cancer cell lines *in vitro*. It was shown that lncPVT1 overexpression retained higher cell viability than the control group, suggesting that lncPVT1 induces castration resistance in prostate cancer cells (49).

Conclusion

Cancer treatment should be diversified to improve the survival of patients with various malignancies. As two powerful tools of cancer treatment, chemotherapy and radiotherapy have been shown to improve the survival of patients with cancer, which is of great significance (20, 146). However, the consequent chemoresistance and radioresistance have become the main obstacles to maintain the efficacies of these treatments. Hence, there is an urgent need to find new targets to overcome this barrier. The physiological mechanisms involved in the regulatory activities of lncPVT1 have many similarities with those involved in chemoresistance and radioresistance, including DNA damage repair, apoptosis, autophagy, EMT, stem cells, and hypoxia (Figure 3). This has led to extensive research on the relationship between lncPVT1, chemoresistance, and radioresistance (147, 148).

In recent years, several preclinical studies have explored the mechanisms underlying lncPVT1-induced chemoresistance and radioresistance in malignant tumor models (Figures 4, 5). Among them, lncPVT1 can act as a molecular sponge of ceRNAs to inhibit miRNA expression, as a skeleton to bind proteins to specific targeted sequences, or bind proteins to form specific modules. In addition, lncPVT1 can encode miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208 (149). Subsequently, downstream apoptosis and DNA damage repair pathways can be regulated to induce chemoresistance and radioresistance in cancer. Previous reviews have shown that lncPVT1 regulated various signaling pathways through miRNAs to influence cancer progression and chemoresistance (150). However, the types of miRNAs involved in the current study were limited. The interaction between lncPVT1 and other miRNAs needs to be revealed in a more comprehensive study of mechanisms related to chemoresistance and radioresistance. Many studies have only identified phenotypes in which lncPVT1 regulates chemoresistance or radioresistance in certain cancer cells, but a detailed analysis of the underlying regulatory mechanisms still needs to be performed. Hence, further research is required to address these gaps. Most therapeutic agents involved in the studies regarding lncPVT1 and

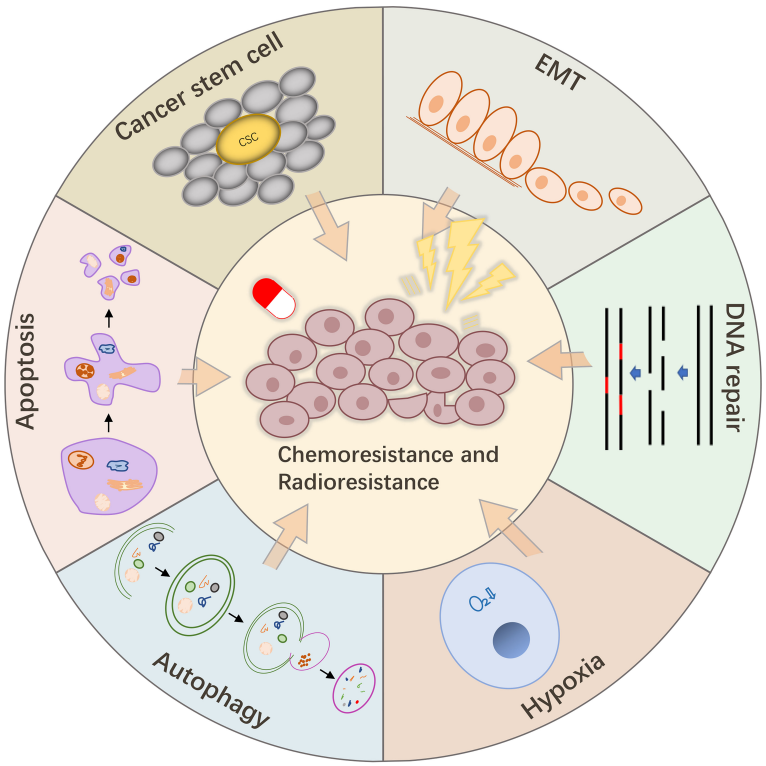

FIGURE 3
LncPVT1 regulates chemoresistance and radioresistance through autophagy, apoptosis, EMT, CSC, DNA damage repair, hypoxia, and other mechanisms.

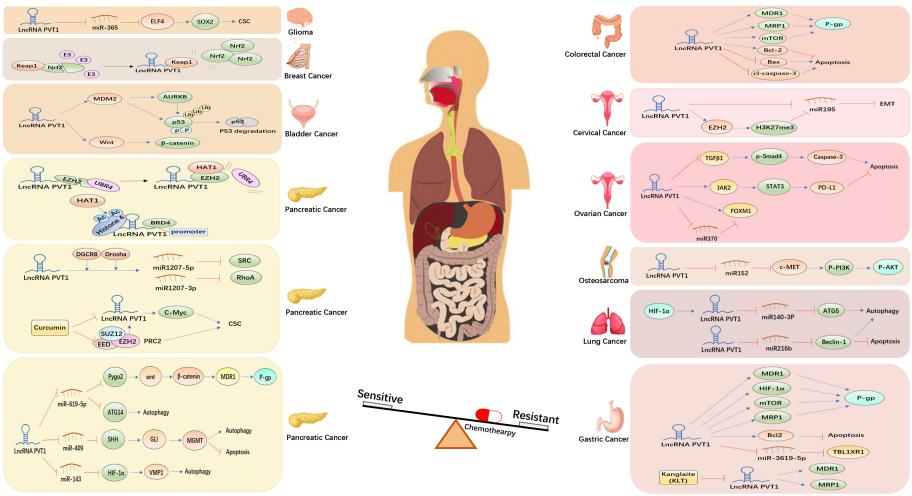

FIGURE 4
Molecular mechanisms underlying the lncPVT1-induced regulation of cancer chemoresistance.

chemoresistance are first-line treatments. Second- or third-line therapies are also commonly used in clinical oncology; therefore, it is necessary to investigate the effect of lncPVT1 expression on the efficacy of these therapeutic agents (151). So far, compared with chemoresistance, there have been few studies associating lncPVT1 and radioresistance, and the types of tumors involved are relatively few. Therefore, further research is needed on lncPVT1 and radiotherapy resistance.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as a new human coronavirus, has caused the coronavirus disease 2019 (COVID-19) pandemic, bringing significant harm and challenges to all parts of the world (152). In recent years, research on the link between COVID-19 and cancer has also increased. Patients with cancer, especially hematological malignancies, are at higher risk for breakthrough infections and severe consequences. The COVID-19 mRNA vaccine significantly reduces the risk of breakthrough infection in cancer patients (153). In addition, lncPVT1 has been reported to be involved in regulating SARS-CoV-2 infection (154). Therefore, the potential roles of lncPVT1 in cancer patients with COVID-19 is expected to be further investigated.

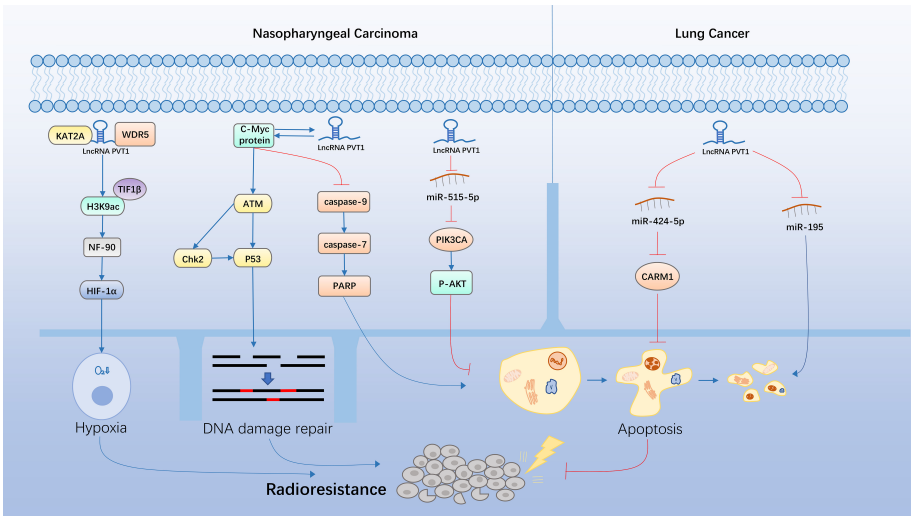

FIGURE 5
Molecular mechanisms underlying the lncPVT1-induced regulation of cancer radioresistance.

In conclusion, targeting lncPVT1 has broad therapeutic prospects in oncology. LncPVT1 inhibitors are expected to be developed in the future, which can be used in combination with radiotherapy and chemotherapy to reduce the incidence of chemoresistance and radioresistance and further improve the efficacy of these treatments in patients with malignancies. The detailed molecular mechanisms underlying the lncPVT1-induced regulation of chemoresistance and radioresistance require further study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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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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Emerging role of lncRNAs in drug resistance mechanisms in head and neck squamous cell carcinoma

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Head and neck squamous cell carcinoma (HNSCC) originates in the squamous cell lining the mucosal surfaces of the head and neck region, including the oral cavity, nasopharynx, tonsils, oropharynx, larynx, and hypopharynx. The heterogeneity, anatomical, and functional characteristics of the patient make the HNSCC a complex and difficult-to-treat disease, leading to a poor survival rate and a decreased quality of life due to the loss of important physiologic functions and aggressive surgical injury. Alteration of driver-oncogenic and tumor-suppressing lncRNAs has recently been recently in HNSCC to obtain possible biomarkers for diagnostic, prognostic, and therapeutic approaches. This review provides current knowledge about the implication of lncRNAs in drug resistance mechanisms in HNSCC. Chemotherapy resistance is a major therapeutic challenge in HNSCC in which lncRNAs are implicated. Lately, it has been shown that lncRNAs involved in autophagy induced by chemotherapy and epithelial–mesenchymal transition (EMT) can act as mechanisms of resistance to anticancer drugs. Conversely, lncRNAs involved in mesenchymal–epithelial transition (MET) are related to chemosensitivity and inhibition of invasiveness of drug-resistant cells. In this regard, long non-coding RNAs (lncRNAs) play a pivotal role in both processes and are important for cancer detection, progression, diagnosis, therapy response, and prognostic values. As the involvement of more lncRNAs is elucidated in chemoresistance mechanisms, an improvement in diagnostic and prognostic tools could promote an advance in targeted and specific therapies in precision oncology.

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KEYWORDS

lncRNA, autophagy, cancer, EMT, stemness, HNSCC (head and neck squamous cell carcinoma), drug resistance, chemoresistance

Introduction

Cancer is a group of multifactorial diseases with an estimated 9.9 million deaths globally in 2020 (1). HNSCC is the sixth most common cancer in the world, accounting for more than 850,000 cases and 400,000 deaths every year (1). HNSCC originates in the squamous cell lining the mucosal surfaces of the head and neck region, involving the oral cavity, nasopharynx, tonsils, oropharynx, larynx, and hypopharynx (2, 3). The main risk factors related to them are smoking, alcohol consumption, betel nuts, smokeless tobacco, and viral infections, including Epstein–Barr and human papillomavirus (4). Nowadays, the treatment for advanced HNSCC includes chemotherapeutic agents, radiotherapy, and surgical resection, leading to mutilation of essential tissues that affect functions such as breathing, feeding, and speaking, thus decreasing the quality of life of patients (5). The heterogeneous nature of HNSCC leads to a poor 5-year overall survival rate due to its local invasion, chemoresistance, metastasis, and late diagnosis (2, 6).

Chemotherapy has been widely used in recent decades for cancer treatment. The combination of platinum-based 5-fluorouracil (5-FU) and DNA synthesis inhibitor cisplatin (CDDP) is still the main regimen for HNSCC (5). However, combinations like paclitaxel (PTX), carboplatin (CDBCA), and cetuximab have been proposed, with unpredictable results (7, 8). Recently, immune checkpoint blockade (ICB) treatment is gaining importance as an immunologic approach for cancer control. HNSCC has a high tumor mutational burden and a relatively high expression of programmed cell death-1-ligand 1 (PD-L1), making it eligible for ICB (9, 10). Nevertheless, drug resistance (DR) is still a key factor for HNSCC progression and poor prognosis (11). The detailed mechanisms of DR are not fully understood, but recent studies suggest that autophagy (12–14), epithelial–mesenchymal transition (EMT) (13, 15, 16), and cancer cell stemness (17–19) play a pivotal role in this major problem. Other mechanisms implied in DR are inactivation of the drug, multi-drug resistance, apoptosis suppression, alterations in the drug metabolism, epigenetic changes, changes in the drug targets, enhanced DNA-repair, and target gene amplification (20). Besides, there are also biological determinants of drug resistance such as tumor heterogeneity, physical barriers, immune system and tumor microenvironment, undruggable cancer drivers, and selective therapeutic pressure that induces changes in the tumor and its ecosystem, modifying the response of the cells to different drugs (21).

Recent studies indicate that non-coding RNAs (ncRNAs) comprise 98% of the total transcribed RNAs in the human genome, and although at first they were classified as "junk" transcriptional products, nowadays they play crucial roles in many biological processes modulating gene expression (22, 23). ncRNAs dysregulation contributes to an increasing number of human diseases, including cancer (2). Long non-coding RNAs (lncRNAs) are a class of functional RNA composed of at least 200 nucleotides (24). LncRNAs have a high transcriptional rate as they are involved in gene regulation at the transcriptional level in the nucleus and posttranscriptional level in the cytoplasm (25, 26). Moreover, lncRNAs are implicated in various cancer progression mechanisms, including proliferation, differentiation, autophagy, EMT, invasion, and metastasis (27–29). Increasing evidence suggests that lncRNAs are implicated in DR in different types of cancer, including HNSCC (30–33). In this regard, this review provides current knowledge about lncRNAs and their implication in DR through known processes in HNSCC, emphasizing in autophagy, EMT, and cancer cell stemness mechanisms. A systematic search was performed in PubMed, Web of Science, Google Scholar, Cochrane Library, and Embase from 2017 to May 2022 for articles matching the following criteria: (long non-coding RNA (lncRNA) and (head and neck squamous cell carcinoma (HNSCC), or oral cancer, or oral squamous cell carcinoma (OSCC), or buccal cancer, or lip cancer or tongue cancer (TSCC) or pharyngeal carcinoma, or nasopharyngeal carcinoma (NPC) or laryngeal squamous cell carcinoma (LSCC)), and (chemoresistance or drug resistance or cisplatin resistance or CDDP resistance), and (autophagy or epithelial-mesenchymal transition or EMT or stemness or cancer stem cells (CSCs)). The titles and abstracts were screened and acquired relevant full-text manuscripts were for further analysis.

Long non-coding RNAS

Biogenesis, classification, and function

Approximately 93% of the human genome can be transcribed into RNAs, but only 2% of these transcripts are translated into

proteins; the remaining 98% are ncRNAs (24). LncRNAs, also referred to as competing endogenous RNAs (ceRNAs) (34), include different kinds of RNA polymerase II (Pol II)-transcribed molecules, mostly 5'-capped, polyadenylated, and spliced (35), and studies suggest there could be more than 10,000 lncRNA transcripts in humans (36, 37). LncRNAs are engaged in multiple functions, including the modulation of crucial functions of other ncRNAs such as micro-RNAs (miRNAs), small nucleolar RNAs (snoRNAs), etc. (38).

Advances in RNA sequencing and other techniques have allowed the discovery of an increasing number of lncRNA classes based on diverse parameters such as transcript length, mRNA resemblance, biogenesis, and unique regulatory mechanisms, among others (39, 40). According to Schmitz et al., one of the most used categorizations is related to the position of the lncRNAs in the genome relative to protein-coding genes (Figure 1) (35). The lncRNA can be divergent (pancRNA) when the lncRNA and neighboring protein-coding gene are transcribed in opposite strands (41), convergent when the lncRNA and protein-coding gene neighbor are transcribed to the same point (42), intergenic when a lncRNA sequence belongs to two genes as a distinct unit (43), overlapping when a protein-coding gene is included in the intron of the same lncRNA in sense or antisense orientation (44), enhancer RNAs expressed as uni- or bidirectional transcripts (45), intronic when the sequence of the lncRNA belongs to the intron of a protein-coding gene (46). Some lncRNAs are generated by back splicing from introns of mRNAs or other lncRNAs and are thus circular (circRNAs) (47).

An increasing number of lncRNAs have been associated with both important biological functions and pathological conditions such as diabetes, neurodegenerative diseases, rheumatoid arthritis, cardiovascular diseases, and cancer (48–50). Dahariya et al. suggest that activation and inhibition of gene expression are promoted by lncRNAs through diverse molecular mechanisms comprising of four basic mechanisms: signal, decoy, guide, and scaffold. In this regard, recent evidence suggests that signaling mediators like kinases, receptors, and transcription factors are strongly associated with lncRNAs *via* numerous signaling pathways, such as PI3K/AKT/mechanistic target of rapamycin (mTOR), Wnt, and the MAPK signaling pathways (51–53). On the other hand, decoy lncRNAs can diminish the availability of

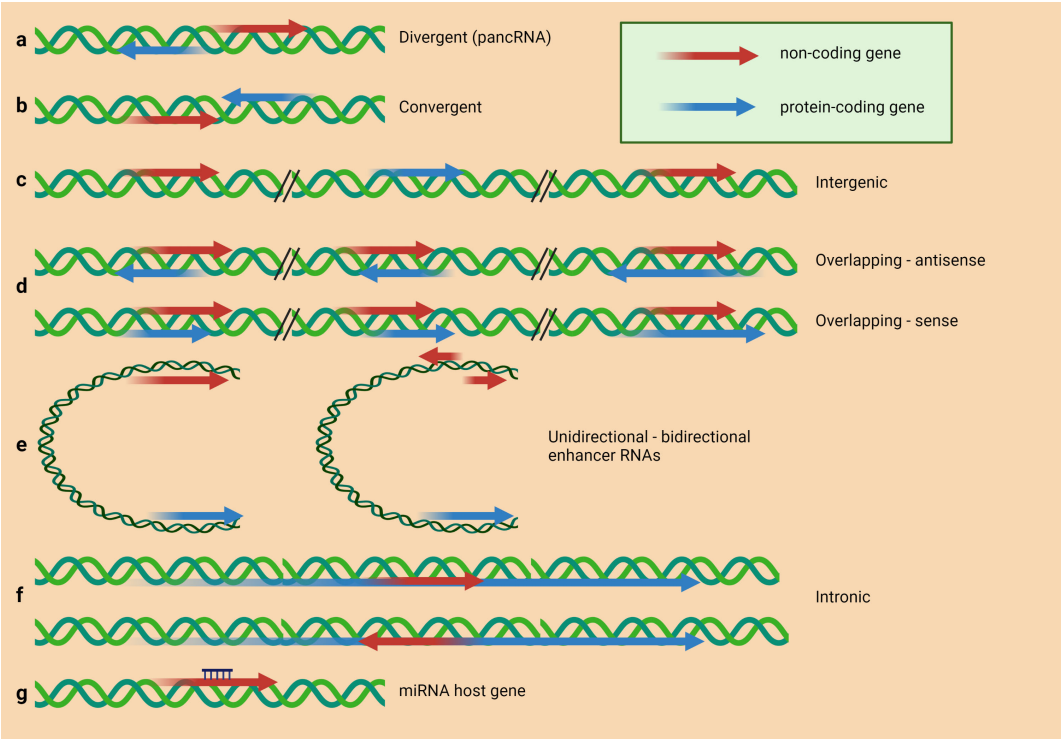

FIGURE 1
LncRNAs classification based on their structural origin. According to Schmitz et al. (35), lncRNAs can be classified in **(A)** divergently transcribed lncRNA originating from the same promoter region as the adjacent protein-coding gene, but from the opposite strand; **(B)** genes encoded on opposite strands, facing each other and convergently transcribed; **(C)** intergenic lncRNA (lincRNA) located distant from other genes; **(D)** lncRNAs overlapping with other genes on the same or opposite strand; **(E)** enhancer RNAs expressed as uni- or bidirectional transcripts; **(F)** lncRNA transcribed from an intron of another gene; **(G)** lncRNA hosting miRNA.

regulatory factors by presenting binding sites (54). For instance, Zhang et al. (55) demonstrated that LINC00160 functions as a decoy of miRNA-132 targeting PIK3R3 to mediate DR in hepatocellular carcinoma, whereas lncRNA GAS5 can also act as a molecular sponge that blocks their downstream functions by targeting RNA or proteins (56). Besides, lncRNAs can interact with ribonucleoproteins (RNPs) in the genome to guide their precise localization, such as HOTAIR, which directs PRC2 to the HOXD locus, leading to silencing genes involved in metastasis suppression (54, 57). Also, CASC9 acts as a guide for EZH2 and CREB-binding protein (CBP) to the promoter regions of target genes (58). In the case of scaffold lncRNAs, they can act as the central platform for assembling complexes, for example, by binding to RNP K and EZH2 to induce the formation of a complex to repress SOX2 (51, 54, 59). Overall, the diverse functions of lncRNAs depend on their subcellular location (60). Genome sequencing has shown that a large proportion of lncRNAs are localized in the nucleus or associated with chromatin, whereas the remaining fraction is localized in the cytoplasm (61).

Many studies have demonstrated the crosstalk of lncRNAs with many epigenetic factors to regulate gene expression and modulate nuclear structure by facilitating the architecture of nuclear speckles, paraspeckles, and interchromatin granules (36, 62). Some lncRNAs play a role as regulators to initiate, elongate or terminate actions of transcription factors (38). Other types of lncRNAs act as decoys by binding to transcription factors or proteins and deviating from protein factors in their action on target DNA (37). They also act as sponges or molecular sinks for miRNAs, mediating changes in gene expression by acting on transcription factors, cell receptors, growth factors, and splicing regulators (63). The main functions of lncRNAs are depicted in Figure 2.

Role of lncRNAs in cancer

Recently, an increasing number of studies of high RNA-sequencing have provided resources for the identification of many lncRNAs that are dysregulated in solid tumors (65–67).

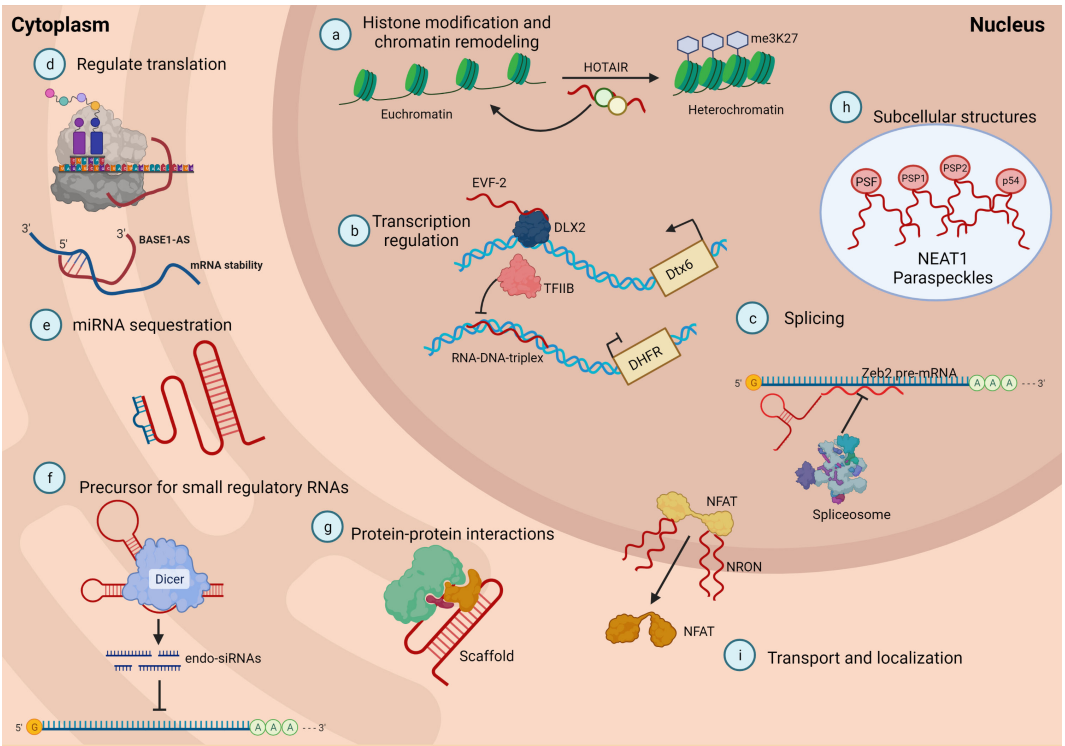

FIGURE 2
LncRNAs can be classified based on their functions. **(A)** lncRNA can guide chromatin complexes controlling between transcriptionally active euchromatin and silent heterochromatin; **(B)** the recruitment of polymerase II and transcription factors can be inhibited or facilitated by lncRNAs; **(C)** lncRNAs contribute to transcriptome complexity by regulating alternative splicing of pre-mRNAs; **(D)** lncRNAs affect the stability and translation of mRNA by base pairing with mRNA molecules; **(E)** they influence in the expression of miRNAs by binding to them and preventing their function; **(F)** lncRNAs can act as siRNAs and target other RNAs, which subsequently could result in target degradation; **(G)** lncRNAs can join multiple protein factors as flexible scaffolds to interact or cooperate on protein-protein interactions; **(H), (I)** the scaffold function is also important for protein activity and localization as well as subcellular structures. Adapted from Meng et al. (64).

LncRNAs are often found as regulators in tumorigenesis, progression, and metastasis of cancer by modulating signaling cascades at the epigenetic, transcriptional, posttranscriptional, translational, or posttranslational levels (65). Cancer-controlling lncRNAs are categorized as proto-oncogenic or tumor suppressors based on their function, being the tumorigenic lncRNAs expressed in tumors as cancer drivers that activate the cell cycle, promote proliferation, and/or exert anti-apoptosis effects (65, 68). Moreover, cancer-progressing lncRNAs have been related to EMT, cell migration, and cell invasion (65, 68). Approximately 100 lncRNAs have been identified recently as regulators of the development and progression of multiple cancer types, including prostate (69–72), breast (73–75), lung (76–79), colorectal (80–82), liver (83–85), and leukemia (86–88), among others.

On the other hand, many lncRNAs have been documented as tumor suppressors and they are generally downregulated in tumor biopsies compared with their normal counterparts (65). When these tumor-suppressing lncRNAs are downregulated or suppressed, they can lead to increased proliferation and tumor growth (65). Although many tumor-suppressing lncRNAs are under investigation, the most documented are the growth arrest-specific transcript 5 (GAS5) (89), the maternally expressed gene 3 (MEG3) (90), and the NF-kB interacting lncRNA (NKILA) (91).

LncRNAs in HNSCC

HNSCC comprises a group of cancers that originate in the squamous-cell layer of the mucosa lining in the head and neck region. The heterogeneity, anatomical, and functional features make the HNSCC a complex and difficult-to-treat disease, leading patients to a poor survival rate and a decreased life quality due to the loss of important physiologic functions and aggressive surgical mutilation (2, 24, 25). Alteration of driver-oncogenic and tumor-suppressing lncRNAs has been recently studied in HNSCC to obtain possible biomarkers for diagnostic, prognostic, and therapeutic approaches (2, 24, 25). Table 1 summarizes oncogenic and tumor suppressor lncRNAs commonly found in HNSCC.

Several lncRNAs have been assessed for their involvement at different stages in HNSCC. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is localized in the nuclear speckle periphery and it is widely studied in cancer development and progression (136). This lncRNA has been strongly associated with squamous cell carcinomas (SCC), including oral SCC (100) and laryngeal SCC (132), demonstrating poorer 5-year survival when highly expressed in tumor tissues.

A novel identified homeobox A11 antisense lncRNA (HOXA11-AS) has also been categorized as a facilitator in the process of malignant tumor proliferation and metastasis (137). Wang et al. demonstrated the proliferation of OSCC cells when HOXA11-AS was upregulated, whereas its downregulation increased apoptosis and caspase 3 activity in CDDP-resistant OSCC cells (98). Moreover, HOXA11-AS knockdown inhibited viability, migration, and invasion in LSCC and enhanced cisplatin sensitivity, thus promoting cell apoptosis in NPC tumor tissues (116, 117, 134).

In a tongue SCC study, the upregulation of the lncRNA KCNQ1 opposite strand/antisense transcript 1 (KCNQ1OT1) demonstrated a strong correlation with the survival rate, proliferation, migration, invasion, and EMT of tongue cancer cells (28). Another study by Zhang et al. found that KCNQ1OT1 facilitated tumor growth and chemoresistance by acting as a modulating ceRNA of miR-211-5p (103). Also, in NPC cell lines, the knockdown of KCNQ1OT1 promoted chemosensitivity and decreased cell proliferation, migration, and invasion by interfering with the miR-454/USP47 axis (118).

Some lncRNAs act as tumor suppressors when upregulated. In this regard, MEG3 downregulation is associated with poor survival of most cancer patients (138) since its upregulation enables the expression of tumor suppressor genes p53 and Rb, induces inhibition of angiogenesis-related factors, and can sponge miRNAs (138, 139). In HNSCC, Lin et al. demonstrated that MEG3 expression was downregulated in NPC cells, inhibiting autophagy and apoptosis ability by acting as a ceRNA to miR-21 (113). Another previously identified tumor-suppressing lncRNA related to HNSCC is the nuclear paraspeckle assembly transcript 1 (NEAT1), strongly associated with suppressing cisplatin resistance by modulating several signaling pathways like the Ras-MAPK and the miR-129/Bcl-2 axis in NPC cells (122, 126). Other lncRNAs identified as tumor suppressors in HNSCC are LINC00460 (33), GAS5 (130), MRVI1-AS1 (128), and MPRL (107). Further identification of these transcripts remains to be elucidated.

Several efforts have been made to identify valuable prognostic lncRNA signatures in different head and neck cancers. Jian et al. associated eight different lncRNAs with OSCC/OPSCC (oropharyngeal squamous cell carcinoma) prognosis, indicating a significantly lower overall survival in the high-risk group (13). Moreover, 493 HNSCC patients were screened for 363 prognostic-related lncRNAs, finding 17 lncRNAs related to the progression and prognosis of HNSCC. These differentially expressed genes (DEGs) between high- and low-risk groups are mainly enriched in immune-related pathways and regulated by a prognostic-lncRNA-directed ceRNA network (12). In a study by Li et al., 501 HNSCC cases were obtained from the National Cancer Institute GDC Data Portal and analyzed by gene set enrichment analysis (GSEA) and gene ontology (GO) functional annotation, proving that the autophagy-related lncRNA signature (LINC00958, PSMA3−AS1 UBAC2−AS1, AC008115.3, AL139 9158.2, AC136475.2, AL160006.1, AL3 57033.4, AC007991.2, AC104083.1, A L139287.1, and AL450992.2) could be considered to predict the prognosis of patients with HNSCC (140).

TABLE 1 Overview of proto-oncogene and tumor-suppressor lncRNAs involved in head and neck cancers.

LNCRNA	TARGET	FUNCTION	REFERENCE
OSCC			
CASC9	AKT/mTOR pathway	Enhances cell proliferation and suppresses autophagy-mediated cell apoptosis.	(92)
GALAT1	miRNA-149	It Promotes proliferation and migration, and inhibits apoptosis and autophagy.	(93)
LINC01207	miR-1301-3p	It Promotes cell proliferation, migration, and inhibits apoptosis and autophagy.	(94)
HOTAIR	MAP1L3B, Beclin1, ATG3, and ATG7	Its silencing promoted proliferation, migration, and invasion.	(95)
LINC00958	miR-4306	Its silencing suppressed cell proliferation, induced cell death, and reduced autophagy.	(96)
PTCSC3	ND	Its overexpression caused a significant decrease in invasion.	(29)
UCA1	miR-184	Accelerates proliferation, increases cisplatin (CDDP) chemoresistance, and restrains apoptosis.	(97)
HOXA11-AS	miR214-3p/PIM1 axis	Promotes proliferation and inhibits cisplatin-induced cytotoxicity.	(98)
XIST	miR-27b-3p	Promotes proliferation, CDDP resistance, and inhibits apoptosis.	(99)
MALAT1	PI3K/AKT/m-TOR pathway	Induces EMT and CDDP resistance.	(100)
ANRIL	ND	Increases anti-apoptotic protein Bcl-2 expression.	(101)
OIP5-AS1	miR-27b-3p	Its knockdown enhanced CDDP sensitivity.	(102)
KCNQ1OT1	miR-124-3p miR-211-5p	Its knockdown inhibited survival rate, proliferation, migration, invasion, and EMT. Facilitates tumor growth and chemoresistance.	(28) (103)
SNHG26	AKT/m-TOR pathway	Promotes proliferation, EMT, migration, invasion, and CDDP resistance.	(104)
CYTOR	miR-1252-5p and miR-3148	Promotes EMT and chemoresistance	(15)
LHFLP3-AS1	miR-194-5p	Its knockdown suppresses proliferation, migration, and invasion.	(105)
CEBPA-DT	ND	Its downregulation enhances cisplatin sensitivity.	(106)
MPRL	Pre-miR-483	High expression is associated with chemosensitivity and a better prognosis.	(107)
PVT1	miR-194-5p	Correlated with worse overall survival and CDDP resistance.	(108)
HEIH	miR-3619-5p	Promotes CDDP resistance.	(109)
CILA1	ND	Promotes EMT, invasiveness, and chemoresistance.	(110)
APCDD1L-AS1	miR-1224-5p/NSD2 axis	Confers resistance to 5-FU.	(111)
TUG1	miR-133-b and CXCR4	Its downregulation impeded cisplatin resistance.	(112)
LINC00953	ABCB5	Its downregulation inhibited CSC hallmarks.	(17)
NPC			
MEG3	miR-21	Promotes autophagy and apoptosis.	(113)
CASC19	AMPK/m-TOR pathway and PARP1 pathway	Contributes to radioresistance and promotes apoptosis.	(114)
ZFAS1	miR-100-3p	Promotes cell proliferation, migration, and tumor growth.	(115)
HOXA11-AS1	miR-98/PBX3 axis miR-454-3p	Enhances CDDP resistance. Promotes cell apoptosis and CDDP sensitivity.	(116) (117)
KCNQ1OT1	miR-454/USP47 axis	Enhances CDDP resistance.	(118)
TINCR	INCR-ACLY-PADI1-MAPK-MMP2/9 axis	Acts as a driver of progression and chemoresistance.	(119)
AFAP1-AS1	miR-320a	Its silencing promoted chemoresistance.	(120)
MIAT	HMB1	It correlates with poor clinical outcome.	(121)
NEAT1	Let-7p-5p	Its inhibition represses CDDP resistance.	(122)
LINC00346	miR-342-5p	Its over-expression promotes CDDP resistance.	(123)
MAGI2-AS3	miR-218-5p/GDPD5/SEC61A1 axis	Promotes cell proliferation, migration, and EMT.	(124)
n375709	ND	Its inhibition increased paclitaxel sensitivity.	(125)
NEAT1	miR-129/Bcl-2 axis	Its depletion enhances SAHA-induced apoptosis.	(126)
CCAT1	miR-181a/CPEB2 axis	Enhances paclitaxel resistance.	(127)
MRVI1-AS1	Hippo-TAZ pathway	Increases paclitaxel chemosensitivity.	(128)
DLEU1	miR-381-3p	Promotes CDDP resistance.	(129)
LSCC			
GAS5	miR-26a-5p	Activates autophagy and induces apoptosis.	(130)

(Continued)

TABLE 1 Continued

LNCRNA	TARGET	FUNCTION	REFERENCE
H19	miR-107	Inhibits autophagy and drug resistance.	(131)
MALAT1	ND	Enhances chemoresistance and poorer 5-year survival.	(132)
FOXD2-AS1	STAT3 and PRMT5	Predicts poor prognosis, maintains cancer stemness and promotes chemotherapeutic resistance.	(18)
FGD5-AS1	miR-497-5p/SEPT2 axis	Its overexpression increases CDDP resistance.	(133)
HOXA11-AS1	miR-518a/SPATS2L axis	Enhances CDDP resistance.	(134)
LINC-PINT	miR-425-5p	Its downregulation increases cancer stemness and chemoresistance to cisplatin.	(19)
BANCR	ND	Its downregulation reverses CDDP resistance.	(30)
HNSCC			
LINC00460	miR-206	Facilitates apoptosis and autophagy.	(33)
PVT1	miR-124-3p	Decreases sensitivity to cetuximab.	(135)
LINC00461	miR-195	Promotes EMT and chemoresistance.	(16)
Lnc-POP-1	VN1R5	Its upregulation promotes DNA repair.	(32)
LINC00958	ND	Facilitates cancer development and resistance to chemo- and radiotherapy.	(31)

LncRNAs in drug resistance mechanisms

Three of the most important mechanisms of DR in HNSCC, autophagy, EMT, and stemness, are regulated by multiple lncRNAs (Figure 3). Chemotherapy remains a very common treatment option for cancer patients, although it has been established that DR is responsible for around 90% of deaths in cancer patients receiving chemotherapeutics or targeted drugs (141). After the drug is administered, the therapeutic agents pass

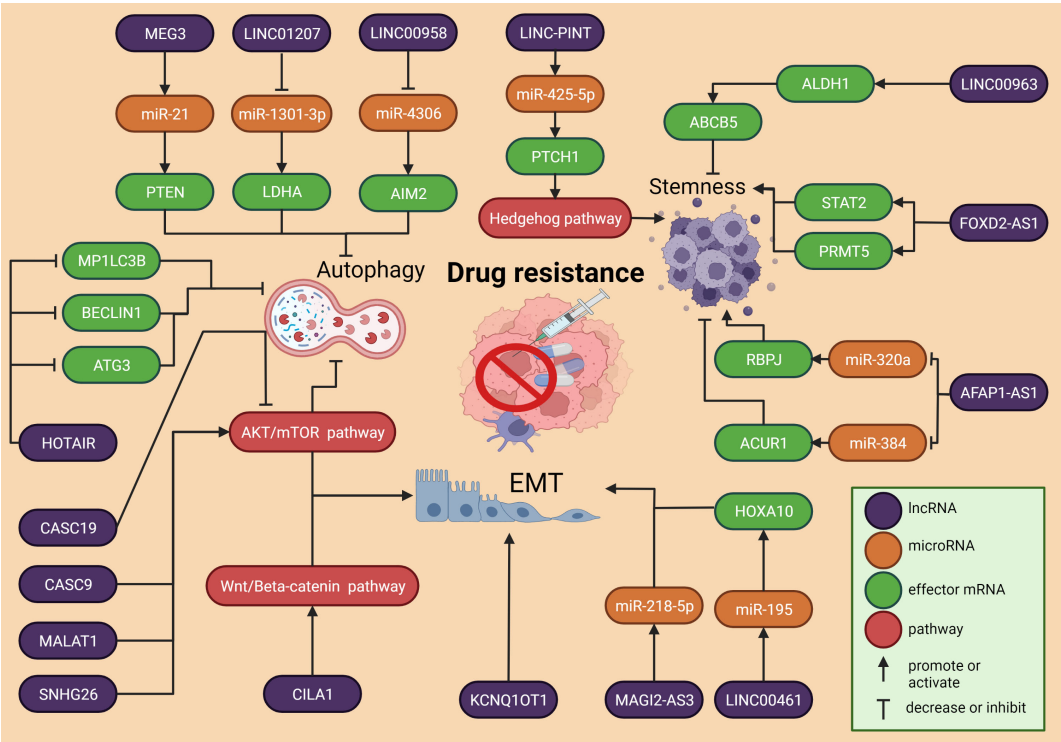

FIGURE 3
Overview of the molecular mechanisms of lncRNA in HSCC drug resistance. Three of the most important cellular processes involved with drug resistance are autophagy, EMT and stemness. All of them are ruled by regulatory axes that comprise the interaction between lncRNAs, microRNAs and expression of genes.

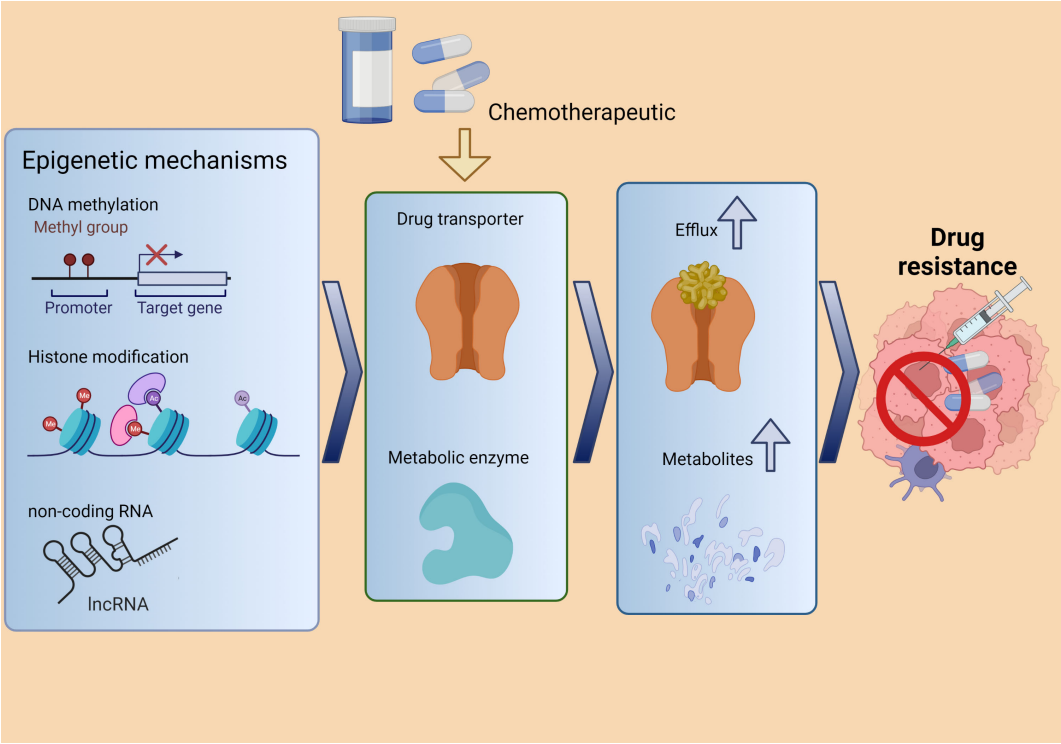

FIGURE 4
The epigenetic processes involved in cancer chemoresistance includes DNA methylation, histone acetylation, and lncRNA interaction. These processes regulate drug transporters and metabolic enzymes, promoting drug resistance. Adapted from Zhou et al. (148).

through a phase of active intracellular metabolism along with the degradation by the liver and other metabolic organs and tissues (142). Furthermore, the dysregulation of enzymes and other proteins responsible for cellular metabolism offers additional challenges that reduce the effectiveness of anti-tumor drugs (143).

Three main phases of drug metabolism and disposition have been observed: phases I and II concerning drug metabolism and phase III concerning drug disposition (144). In phase I, enzymes are mostly cytochrome P450 (CYPs) and are involved in the oxidation, reduction, and/or hydrolysis processes that activate or inactivate the agent (145). During phase II, metabolic reactions are carried out by transferases whose primary mission is to deactivate pharmacologically active drugs, facilitating their elimination by making them more soluble in water (146). Finally, phase III consists of drug transporters active in the absorption, distribution, and elimination of drugs (147). Increased expression, transcription activation of involved genes, and activity of efflux drug transporters represent a major mechanism for developing chemoresistance, mostly under the control of epigenetic processes like DNA methylation, histone acetylation, and ncRNA interaction (Figure 4) (142, 144, 148, 149). The involvement of lncRNAs in drug metabolism and efflux phases has been investigated to elucidate the DR mechanisms of cancer (141).

Some studies have demonstrated the involvement of lncRNAs in the metabolism and disposition of anti-cancer drugs, influencing directly the development of DR (142, 148). In HNSCC, DR has become an increasingly concerning challenge for the scientific community and clinicians (16, 31, 135). Along with many others, the long intergenic non-coding RNA 00958 (LINC00958) has been studied as apoptosis- and autophagy-related lncRNA in HNSCC as part of prognostic signature, establishing a worse overall survival rate of patients when the signature is present (140, 150). Moreover, Jian et al. concluded that LINC00958 downregulates miR-4306 levels to activate the pyroptosis pathway mediated by AIM2 and promotes cancer cell survival in OSCC (96). Another study suggests that LINC00958 interplay with c-Myc as a feedback loop facilitating HNSCC development and resistance to chemo- and radiotherapy, and its upregulation is associated with poor tumor differentiation, advanced tumor stage, and shorter overall survival of patients (31) Another lncRNA related to DR is the plasmacytoma variant translocation 1 (PVT1), identified as upregulated in cisplatin-resistant cancer cell lines and tissue samples (108) and as a promoter of decreased sensitivity to

TABLE 2 LncRNAs and their influence on HNSCC drug resistance.

LNCRNA	INFLUENCE	REFERENCE
OSCC		
UCA1	Accelerates proliferation, increases CDDP chemoresistance, and restrains apoptosis.	(97)
HOXA11-AS	Promotes proliferation in CDDP-sensitive cells and inhibits CDDP-induced cytotoxicity through the HOXA11-AS/miR214-3p/PIM1 axis.	(98)
XIST	Upregulation of XIST promotes cell proliferation, enhances CDDP resistance, and inhibits apoptosis.	(99)
MALAT1	Induces EMT and CDDP resistance *via* activation of PI3K/AKT/m-TOR signaling pathway and the upregulation of P-gp.	(100)
ANRIL	CAF-secreted midkine enhances tumor cell resistance to cisplatin by inducing ANRIL expression and increasing anti-apoptotic protein Bcl-2 expression.	(101)
OIP5-AS1	Its knockdown enhances DDP sensitivity *in vivo*. Improves DDP resistance through the upregulation of TRIM14 mediated by miR-27b-3p.	(102)
KCNQ1OT1	Promotes DDP resistance of tongue cancer by sponging miR-124-30 to regulate TRIM14 expression. Facilitates tumor growth and chemo-resistance by acting as a competing endogenous RNA (ceRNA) to modulate the expression of miR-211-5p.	(28) (103)
SNHG26	Its expression is positively correlated with proliferation, EMT, migration, invasion, and cisplatin resistance by binding directly to PGK1 protein, inhibiting its ubiquitination and activating the Akt/mTOR signaling pathway.	(104)
CYTOR	Acts as a ceRNA to inhibit miR-1252-5p and miR-3148 upregulating LPP expression. CYTOR/LPP axis is essential for FOXD1-induced EMT and chemoresistance.	(15)
LHFLP3-AS1	It is upregulated in cisplatin-resistant tumors promoting proliferation, migration, and invasion.	(105)
CEBPA-DT	Regulates cisplatin chemosensitivity through CEBPA-BCL12-mediated cell apoptosis.	(106)
MPRL	High expression of MPRL and pre-miR-483 and low expression of miR-143-5p are associated with neoadjuvant chemosensitivity and better prognosis.	(107)
PVT1	Its upregulation in cisplatin-resistant tissues and cell lines is strongly correlated with worse overall survival acting as a ceRNA on miR-194-5p.	(108)
HEIH	Exosomal HEIH acts as a ceRNA for miR-3619-5p to upregulate HDGF, promoting DDP resistance.	(109)
CILA1	High CILA1 expression levels and low levels of phosphorylated beta-catenin are associated with cisplatin resistance and advanced disease stage.	(110)
APCDD1L-AS1	Its expression is related to worse prognosis and confers resistance to 5-FU *via* miR-1224-5p/NSD2 axis.	(111)
TUG1	Its upregulation promotes cisplatin resistance by mediating miR-133b and CXCR4.	(112)
LINC00963	Its suppression reduces the activity of ALDH1, percentage of self-renewal, chemoresistance and expression of multidrug-resistance transporter ABCB5.	(17)
NPC		
HOXA11-AS1	Enhances DDP resistance *via* the miR-98/PBX3 axis. Its silencing inhibits the c-Met/AKT/mTOR pathway by specifically upregulating miR-454.3p, promoting cell apoptosis and enhancing the sensitivity of cisplatin-resistant cells.	(116) (117)
KCNQ1OT1	Enhances DDP resistance, proliferation, migration, and invasion *via* the miR-454/USP47 axis.	(118)
MIAT	Upregulates HMB1 expression, contributing to cisplatin resistance and poor clinical outcome *via* the MIAT/HMGB1/IL6 axis.	(121)
NEAT1	NEAT1/let-7a-5p axis regulates the cisplatin resistance by targeting Rsf-1 and modulating the Ras-MAPK signaling pathway. NEAT1 increases in tissues and manages to facilitate SAHA tolerance by modulating the miR-129/Bcl-2 axis.	(122) (126)
n375709	Its inhibition increases the paclitaxel sensitivity.	(125)
MAGI2-AS3	MAG2-AS1/mR-218-5p/GDPD5/SEC61A1 axis drives cell proliferation, migration, and EMT, and conferred cisplatin resistance.	(124)
LINC00346	LINC00346 regulates the cisplatin resistance by inhibiting miR-342-5p.	(123)
TINCR	Acts as a crucial driver of progression and chemoresistance, and highlights the INCR-ACLY-PADI1-MAPK-MMP2/9 axis.	(119)
CCAT1	Its upregulation enhances paclitaxel resistance *via* miR-181a/CPEB2 axis.	(127)
MRVI1-AS1	MRVI1-AS1/ATF3 signaling pathway increases paclitaxel chemosensitivity by modulating the Hippo-TAZ.	(128)
DLEU1	Acts as an oncogene to promote DDP resistance and BIRC6 expression through interacting with miR-381-3p.	(129)
LSCC		
H19	Exerts inhibiting effect on autophagy and drug resistance by downregulating HMGB1 through targeting miR-107.	(131)
MALAT1	Its over-expression enhances chemoresistance and demonstrates poorer 5-year overall survival.	(132)
FOXD2-AS1	FOXD2-AS1 acts as a scaffold for STAT3 and PRMT5, promoting STAT3 transcriptional activity, essential to maintain cancer stemness and promote chemotherapeutic resistance.	(18)

(Continued)

TABLE 2 Continued

LNCRNA	INFLUENCE	REFERENCE
FGD5-AS1	Its overexpression increases cisplatin-resistance by modulating miR-497-5p/SEPT2 axis.	(133)
HOXA11-AS1	Enhances CDDP resistance of LSCC *via* miR-518a/SPATS2L axis. Its knockdown inhibits viability, migration, and invasion, but promoted apoptosis.	(134)
LINC-PINT	Targets miR-425-5p which also targeted PTCH1, affecting the Hedgehog pathway, thus increasing cancer stemness and chemoresistance to cisplatin.	(19)
AFAP1-AS1	Increases RBPJ expression by negatively regulating miR-320a and RBPJ overexpression rescues stemness and chemoresistance inhibited by AFAP1-AS1 silencing.	(120)
BANCR	Its downregulation reverses cisplatin resistance.	(30)
HNSCC		
PVT1	Decreases the sensitivity of HNSCC cells to cetuximab by enhancing methylation-mediated inhibition of miR-124-3p.	(135)
LINC00461	Downregulates expression of miR-195 to subsequently upregulate expression of HOXA10, promoting EMT and enhancing chemoresistance.	(16)
Lnc-POP1-1	lnc-POP1-1 promotes DNA repair through interaction with MCM5 and deceleration of its degradation. VN1R5 promotes cisplatin resistance in a lnc-POP1-1-dependent manner.	(32)
LINC00958	LINC00958 interplays with c-Myc as a feedback loop facilitating development and resistance to chemo- and radiotherapy.	(31)

cetuximab (135). Table 2 summarizes the most important dysregulated lncRNAs that influence DR on HNSCC.

The homeobox A11 antisense lncRNA (HOXA11-AS) has also been related to chemoresistance. In this regard, OSCC tumor tissues and cell lines were analyzed, concluding that the upregulation of HOXA11-AS promoted proliferation in CDDP-sensitive cells and inhibited CDDP-induced cytotoxicity by intervention in the miR214-3p/PIM1 axis (98). Other studies demonstrated that the knockdown of HOXA11-AS enhances CDDP resistance *via* the miR-98/PBX3 axis (116) and can inhibit the c-Met/AKT/mTOR pathway by specifically upregulating miR-454-3p, thus promoting cell apoptosis and enhancing the sensitivity of cisplatin-resistant NPC cells to cisplatin (117). Conversely, Shen et al. analyzed LSCC tissues and cell lines, showing that HOXA11-AS1 knockdown inhibits the viability, migration, and invasion but promotes the apoptosis of cells (134).

Another lncRNA actively involved in DR in HNSCC is the KCNQ1 overlapping transcript 1 (KCNQ1OT1). It has been established that its upregulation facilitates tumor growth and chemoresistance in tongue SCC by sponging miR-124-3p (28) and by acting as a ceRNA to modulate the expression of miR-211-5p (103). Moreover, Yuan et al. showed that KCNQ1OT1 knockdown promotes chemosensitivity in DDP-resistant NPC cells by significantly decreasing cell proliferation, migration, and invasion *via* the miR-454/USP47 axis (118). NEAT1 (nuclear paraspeckle assembly transcript 1) has also been associated with HNSCC chemoresistance, especially in NPC where its depletion repressed the cisplatin resistance of NPC cells and phenocopied the effect of miR-129 overexpression, which also enhanced apoptosis by the histone deacetylase inhibitor SAHA (122, 126).

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been identified as a prognostic factor in patients with lung cancer (151), and its overexpression is related to poor clinical outcome, chemoresistance, and progression in many cancer types, including kidney (152, 153), pancreatic (154), prostate (155, 156), esophageal (157, 158), breast (159, 160), gastric (161), ovarian (162), and colorectal (163, 164). In both oral and laryngeal SCC, the over-expression of MALAT1 contributes to the enhanced chemoresistance and metastatic power of several cell lines (100, 132).

In the case of H19, it has been demonstrated that it is upregulated in LSCC, exerting an inhibiting effect at the autophagy level and DR by downregulating HMGB1 by targeting miR-107 (131). A similar effect has been observed in many other cancer types, with anti-apoptotic, pro-proliferative, and pro-migratory functions, along with the induction of EMT, activation of oncogenic signaling pathways, and changes in the tumor microenvironment, contributing to anti-cancer DR (165). Another well-studied lncRNA that promotes the proliferation, migration, and chemoresistance of several cancer types is the myocardial infarction-associated transcript (MIAT) (166–169). According to Zhu et al., an elevated MIAT level upregulates HMB1 expression, contributing to cisplatin resistance and poor clinical outcomes (121).

An increasing number of other lncRNAs have been proposed as promoters of DR in HNSCC. For instance, the urothelial cancer-associated lncRNA 1 (UCA1) plays an important role in the tumorigenesis, progression, and diagnosis of many cancers, mainly bladder cancer (170, 171). In oral cancer tissues and cell lines, UCA1 accelerates proliferation, increases CDDP chemoresistance and restrains apoptosis partly by modulating SF1 by sponging miR-184 (97). In another study, the results showed that CAF-secreted midkine enhanced OSCC resistance to cisplatin by inducing ANRIL expression and increasing the anti-apoptotic protein Bcl-2 expression (101). Chen et al. studied 146 paraffin-embedded OSCC specimens along with OSCC cell lines CAL-27 and SCC4 and found that the cytoskeleton regulator RNA

(CYTOR) acts as a ceRNA to inhibit miR-1252-5p and miR-3148, upregulating the lipoma-preferred partner (LPP) protein and therefore proving essential for FOXD1-induced EMT and chemoresistance (15). Another forkhead box (FOX) reported as important for laryngeal SCC DR is FOXD2-AS1, which acts as a scaffold for STAT3 and PRMT5, promoting STAT3 transcriptional activity, maintaining cancer stemness, and promoting chemotherapeutic resistance (18).

LncRNAs in autophagy

The intracellular degradation systems encompass two major protein pathways that are directly involved in the maintenance of metabolic homeostasis; one of them is the ubiquitin–proteasome pathway, responsible for degrading short-lived and damaged proteins; and the other is the lysosome-autophagy system, whose target is long-lived macromolecular complexes and organelles (172). Autophagy is a highly conserved and successive cellular process in which damaged organelles, intracellular microbes, and pathogenic long-lived proteins are degraded, recycling amino acids, nucleotides, and fatty acids to maintain cellular homeostasis (173–175). Thus, autophagy is closely related to the occurrence of a wide variety of human diseases (176).

Autophagy-related genes (ATGs) are responsible for autophagy occurring under micro-environmental stress such as hypoxia, heat, nutrient deficiency, and accumulation of reactive oxygen species (175, 176). The main successive autophagy stages are the initiation of phagophore assembly, autophagosomal formation, and lysosomal fusion (177, 178). Two highly conserved serine–threonine kinases, the mammalian target of rapamycin (mTOR) and the mammalian homologs of yeast ATG1-Unc-51-like kinases 1 (ULK1), regulate cell growth and survival and are the central modulators of autophagy, responding to intra- and extra-cellular changes by forming autophagosomes (179, 180). mTOR is activated under favorable conditions, inhibiting autophagy and protein degradation, whereas it is inactivated in hostile environments related to poor nutritional conditions (172).

Recent studies have demonstrated an important role for autophagy in tumorigenesis and the progression of cancer (181). A dual function has been proposed since autophagy can stabilize the genome and prevent the formation of tumor cells, while once the tumor cells have been formed, autophagy plays a pivotal role in tumor initiation, progression, and resistance to chemotherapy (172, 177). LncRNAs are involved in autophagy, modulating the expression of ATG genes by acting as ceRNAs for miRNAs (36, 180). Recently, several autophagy-related lncRNAs have been proposed as biomarkers and signatures for diagnostic and prognostic purposes for many cancer types, including breast (182, 183), bladder (184, 185), pancreatic (186), colorectal (187, 188), and lung (189, 190). In most cases, the overall survival of patients in high-risk groups is significantly lower based on the presence of each proposed signature.

Many studies have established prognostic signatures in HNSCC. For instance, in a recent study, 910 autophagy-related (AR) lncRNAs from mRNA sequences and clinical data of HNSCC patients and controls from The Cancer Genome Atlas (TCGA) were analyzed. The principal component analysis distinguished two categories based on the nine lncRNA prognostic signatures, resulting in a significantly worse overall survival rate in the high-risk group (150). Another study consulted the TCGA database to obtain 155 HNSCC samples (mainly laryngeal, nasopharyngeal, tonsil, and lip cancer) and the RNA profile indicated that ATG12, BECN1, and MAP1LC3B have prognostic value, and their related pathways may be involved in regulating HNSCC prognosis (191). Guo et al. included 17 prognostic-related autophagy- and ferroptosis-related lncRNAs as the main components of a ceRNA network that regulates differentially expressed genes mainly enriched in immune-related pathways (12). Similarly, gene set enrichment analysis (GSEA) and gene ontology (GO) functional annotation proved that autophagy-related pathways are mainly enriched when 13 autophagy-related lncRNAs are present in HNSCC patients (140). Regarding oral and oropharyngeal SCC, the signature-based on nine autophagy-related lncRNAs acted as an independent prognostic indicator, showing a significantly lower overall survival in high-risk groups (13). The autophagy-related (AR) signatures of lncRNAs proposed as biomarkers in HNSCC are summarized in Table 3.

Quantitative reverse transcription PCR (RT-qPCR) was performed to analyze the cancer susceptibility candidate 9 (CASC9) expression in OSCC tissues and cell lines, demonstrating that CASC9 promotes progression by enhancing cell proliferation and suppressing autophagy-mediated cell apoptosis *via* the AKT/mTOR pathway (92). Another study by Chen et al. showed that when the gastric cancer-associated transcript 1 (GACAT1) was inhibited in OSCC samples, it promoted apoptosis and autophagy, mainly related to the targeting of miRNA-149 (93). Long intergenic non-coding RNA 01207 (LINC01207) and LINC00958 were also upregulated in OSCC tissues and cells. LINC01207 upregulates LDHA expression to promote cell proliferation and migration and inhibits apoptosis and autophagy by acting as a ceRNA that sponges miR-1301-3p (94), whereas LINC00958 downregulates miR-4306 levels to activate a pyroptosis pathway mediated by AIM2 and promotes cancer cell survival (96).

A broadly studied oncogenic trans-acting lncRNA is the HOX transcript antisense RNA (HOTAIR), which is found overexpressed in a wide variety of cancers and is mainly associated with metastasis and poor prognosis (192). In OSCC cells, HOTAIR silencing inhibited autophagy with the downregulated expression of MAP1LC3B, Beclin1, ATG3, and ATG7; proliferation, migration, and invasion of OSCC cells were

TABLE 3 Autophagy-related (AR) signatures of lncRNAs proposed as biomarkers in HNSCC.

AR LNCRNAS	INFLUENCE	REFERENCE
PTCSC2, **AC099850.3**, **LINC01963**, **RTCA-AS1**, **AP002884.1**, **UBAC2-AS1**, **AL512274.1**, **MIR600HG**, **AL354733.3**	The overall survival of the high-risk group was significantly lower than that of the low-risk group. The signature-based on autophagy/related lncRNAs potentially acts as an independent prognostic indicator for patients with OSCC/OPSCC.	(13)
ATG12, **BACN1**, **MAP1LC3B**	All three autophagy-related lncRNAs have prognostic value with respect to HNSCC, and their related pathways may be involved in regulating HNSCC prognosis.	(191)
MIR4435-2HG, **PCED1B-AS1**, **AL512274-1**, **MYOSLID**, **LINC01871**, **LINC02541**, **AC012236-1**, **C5orf66-AS1**, **AC004687-1**, **AL354836.1**, **LINC02454**, **AC024075.2**, **LINC00460**, **AATBC**, **ITGB2-AS1**, **MIR9-3HG**, **AF131215.5**	Differentially expressed genes (DEGs) between high- and low-risk groups were mainly enriched in immune-related pathways and regulated by a PAF-lncRNA-directed ceRNA network.	(12)
AC008115.3, **AL139158.2**, **AC136475.2**, **AL160006.1**, **AL357033.4**, **AC007991.2**, **AC104083.1**, **AL139287.1**, **AL450992.2**, **LINC00958**, **AC103702.2**, **PSMA3-AS1**, **UBAC2-AS1**	Overall survival in the high-risk group was shorter than the low-risk group. Gene set enrichment analysis (GSEA) and gene ontology (GO) functional annotation proved that autophagy-related pathways are mainly enriched in the high-risk group.	(140)

suppressed, along with an enhanced apoptosis rate and an improvement in sensitivity to cisplatin (95).

As previously addressed, MEG3 is considered a tumor-suppressor lncRNA. Lin et al. concluded that MEG3 promotes autophagy and apoptosis of NPC cells by enhancing PTEN expression by binding to miR-21 (113). Another important tumor-suppressor lncRNA involved in autophagy activation is the growth arrest-specific 5 RNA (GAS5). In LSCC, GAS5 inhibited the viability of AMC-HN-8 cells and induced apoptosis, acting as a tumor suppressor by regulating the miR-26a-5o/ULK2 axis (130). Conversely, CASC19 suppressed cellular autophagy by inhibiting the AMPK/mTOR pathway, contributing to the radioresistance of NPC by regulating autophagy (114).

LncRNAs in EMT

Epithelial-to-mesenchymal transition (EMT) is described as a process where epithelial cells are transformed into mesenchymal stem cells and plays an important role in both development and tumorigenesis (177, 193). Moreover, EMT has been broadly related to tumor proliferation, metastasis, and DR (194, 195). However, this transition is reversible since tumor cells will go through the opposite process of mesenchymal-to-epithelial transition (MET) once they have reached a suitable place where they can metastasize, re-expressing epithelial characteristics (196). A wide variety of signaling pathways can be involved in EMT, including the transforming growth factor-beta (TGF-beta) pathway (196, 197), the Wnt/beta-catenin pathway (198, 199), the Notch signaling pathway (200), the Hedgehog pathway (201), and the signal transducer and activator of transcription 3 (STAT3) pathway (202), among others. These signaling molecules can subsequently activate different EMT transcription factors like Snail, basic helix-loop-helix (TWIST), and zing-finger E-box-binding homeobox (ZEB) to repress epithelial markers and activate the EMT program (6, 177, 196).

Recently, several lncRNAs have been linked to EMT since they play fundamental roles in the above-mentioned signaling cascades, epigenetics, and transcription factors (203–205). The lncRNA MALAT1 induces EMT and CDDP resistance in OSCC cells *via* the activation of the PI3K/AKT/mTOR signaling pathway in cell lines CAL-27 and SCC-9 (100). LncRNA KCNQ1OT1 has also been related to EMT in tongue cancer tissues and cells, promoting survival rate proliferation, migration, and invasion (28). Similarly, quantitative PCR performed in pituitary adenoma samples found the same lncRNA to be upregulated, and its knockdown inhibited cell stemness, angiogenesis, and EMT (206).

A novel lncRNA named chemotherapy-induced lncRNA 1 (CILA1) was upregulated in cisplatin-resistant tongue SCC cells, displaying EMT features, promoting invasiveness and chemo-resistance, mainly activating the Wnt/beta-catenin pathway (110). Another lncRNA related to EMT is the recently discovered small nucleolar RNA host gene 26 (SNHG26), first described by Tong et al. as part of a prognosis signature along with the other 13 lncRNAs in bladder cancer (207). Similarly, a four-lncRNA signature that included SNHG26 was associated with immune infiltration and prognosis in colon cancer; the signature-divided colon cancer patients of TCGA into high- and low-risk groups with significantly different outcomes (208). In TSCC, SNHG26 expression was positively correlated with proliferation, EMT, migration, invasion, and cisplatin resistance by activating the AKT/mTOR signaling pathway (104).

Another lncRNA recently linked to EMT in cancer is the long intergenic non-protein coding RNA 461 (LINC00461), highly expressed in non-small cell lung cancer and directly

involved in cell proliferation, migration, and EMT by targeting the miR-4478/E2F1 axis (209). Similarly, Wu et al. showed that the upregulation of LINC00461, LINC00402, and SFTA1P had suppressive effects on the homologous pleckstrin-homology (PH)-domain leucine-rich-repeat protein phosphatases (PHLPP2), reported previously as a tumor suppressor in colon cancer (210). In HNSCC, LINC00461 was highly expressed in 52 tissues analyzed, and it was found that LINC00461 downregulates the expression of miR-195 to subsequently upregulates the expression of HOXA10, promoting EMT and enhancing chemoresistance in HSNCC (16).

The membrane-associated guanylate kinase 2 antisense RNA 3 (MAGI2-AS3) was recently identified in NPC, driving cell proliferation, migration, and EMT through the miR-218-5p/GDPD5/SEC61A1 axis, conferring cisplatin resistance in cell lines (124). The same lncRNA expression was detected by quantitative real-time PCR in pancreatic cancer cells, and its upregulation promoted EMT through the regulation of miR-490-5p (211). Moreover, MAGI2-AS1 was identified to be EMT-related and highly co-expressed with ZEB1/2 in both gastric tissues and normal stomach tissue (212). Conversely, MAGI2.AS3 overexpression inhibited bladder cancer progression by regulating MAGI2/PTEN/EMT in 80 bladder cancer tissues (213).

Although several studies have encompassed the involvement of lncRNAs as regulators of EMT and, consequently, DR in HNSCC, more studies must complement the information available at present time.

LncRNAs in stemness

The stemness of cancer cells is an important cellular feature that grants tumor heterogeneity, enhanced growth capacity, DR, and augmented metastatic ability through the CSC properties of self-renewal, quiescent state, high cell turnover, increased expression of drug transporters, and other resistance genes (177, 214, 215). Evidence suggests that CSCs retain properties that make them highly resistant to currently available chemotherapy drugs since CSCs remain in an inactive state of the cell cycle and most of these treatments attack cells with a high proliferative rate (216, 217). Additional properties like rapid DNA repair (218), tumor microenvironment (219, 220), and extracellular matrix contribute to maintaining cancer stemness and chemoresistance (221, 222).

As previously addressed, even though lncRNAs have no protein-coding capacity, they are emerging as master regulators of gene transcription and act as proto-oncogenes or tumor suppressors (223, 224). LncRNAs like LINC00617 (225), lncSOX2OT (226), and HOTAIR (227, 228) play an important role in CSC regulation of various types of cancer through several mechanisms and signaling pathways. Furthermore, in colorectal cancer, the lncTCF7 interacts with subunits of the SWI/SNF chromatin remodeling complex, regulating the transcription of the TCF7 gene and activating the Wnt cascade, involved in stem cell self-renewal (229). H19 is another lncRNA overexpressed in many cancers and confer stem-like properties in correlation with stem cell markers like SOX2, OCT4, NOTCH1, c-Myc, and ABCG2 (230–232).

Little research has been done concerning the role of lncRNAs in DR associated with cancer cell stemness. Lee et al. studied OSCC tumor tissues and cell lines and concluded that the downregulation of the long intergenic non-coding RNA 963 (LINC00963) inhibited CSC hallmarks, such as migration, invasion, and colony formation capacity. Moreover, the suppression of LINC00963 reduced the activity of ALDH1, the percentage of self-renewal, chemoresistance, and the expression of multidrug-resistance transporter ABCB5 (17). Another long intergenic non-coding RNA recently involved in chemoresistance driven by cancer cell stemness is the p53-induced non-coding transcript (LINC-PINT). Interestingly, the tumor suppressor PTCSC3 was studied along with LINC-PINT in gastric cancer tissues, inhibiting tumor growth and stemness when both were over-expressed (233). The same lncRNA was observed in 24 LSCC samples and cells, targeting miR-425-5p and subsequently targeting PTCH1, affecting the Hedgehog pathway and its downregulation was associated with increased cancer stemness and chemoresistance to cisplatin (19).

LncRNA FOXD2-AS1 has been related to different forms of malignancy and CSCs, mainly involving gliomas (234). In laryngeal SCC chemotherapy-resistant patients, FOXD2-AS1 showed increased expression and acted as a scaffold for STAT2 and PRMT5, both essential to maintain cancer stemness and promote chemotherapy resistance (18). Oncogenic actin filament-associated protein 1-antisense RNA 1 (AFAP1-AS1) is a recently discovered lncRNA related to cancer stemness (235). The first documented association between AFAP1-AS1 high expression, stemness, and DR was found in LSCC specimens, increasing RBPJ expression by negatively regulating miR-320a; subsequently, RBPJ overexpression rescued stemness and chemoresistance inhibited by AFAP1-AS1 silencing (120). Another study suggested that AFAP1-AS1 functions as an endogenous RNA by competitively binding to miR-384 to regulate ACVR1, thus conferring inhibitory effects on pancreatic cell stemness and tumorigenicity (236).

Clinical relevance of lncRNAs in drug resistance mechanisms in HNSCC

The clinical importance of establishing a correlation between the increasing number of newly discovered lncRNAs and the various mechanisms of DR lies in the implications that they have on the prognosis, molecular staging, and treatment possibilities of many tumors. In this regard, CASC9 was strongly associated

with tumor size, clinical stage, regional lymph node metastasis, and overall survival time in OSCC patients (92). Similarly, the upregulation of PVT1 was strongly correlated with the worse overall survival of 83 OSCC patients (108). Lin et al. proposed CILA1 as a biomarker in TSCC, correlating its expression levels with cisplatin resistance and advanced disease stage (110). Moreover, SNHG26 expression was also associated with the occurrence, progression, and poor prognosis of TSCC (104).

It is important to remember that DR can be categorized as intrinsic and acquired resistance, the first being defined as the lack of tumor regression following treatment (which is the result of mechanisms that existed before therapy). Meanwhile, acquired resistance denotes the elimination of an observed response after an initial clinical benefit following treatment (237). Because stemness of cancer cells is an intrinsic mechanism of DR, its clinical relevance stands out given that lncRNAs such as LINC00963, LINC-PINT, FOXD2-AS1, and AFAP1-AS1 have been found overexpressed, conferring the stemness state to cells. Nevertheless, to date, there is no evidence of a relationship between these specific lncRNAs and a particular drug.

As prognostic signatures, several studies have linked lncRNAs with the prognosis of HNSCC, particularly autophagy- and ferroptosis-related lncRNAs (12, 13, 140, 150, 191). In all cases, the signatures proposed exhibited prognostic value concerning HNSCC (**Table 3**), and their related pathways may be involved in regulating HNSCC prognosis.

Future directions for lncRNA research

The role of lncRNAs in many diseases has become a widely investigated field, especially in cancer research. Given the increasing evidence of the involvement of lncRNAs in several drug resistance mechanisms, research should be directed toward new horizons to elucidate the molecular pathways by which lncRNAs interact to drive the resistance of certain cell lines (51). This understanding would help in the improvement of the diagnosis and treatment strategies of HNSCC (3, 238). A potential line of research could involve the upstream regulatory mechanisms of lncRNAs since previous evidence suggests regulation by histone status, DNA methylation patterns (107), transcription factors (239), and post-transcriptional regulation (240, 241).

With the development of high-throughput sequencing technology, the library of lncRNAs has notably increased. However, most of the mechanisms of influence on DR through phenomena such as EMT (15), autophagy (131), and stemness (18) have not been fully understood, and some even remain unexplored. A better comprehension of the regulatory networks between lncRNAs, genetic, and epigenetic alterations could give rise to therapeutic strategies that promote improvements in dealing with DR mechanisms (107). Also, the elucidation of all regulatory networks could lead to the formulation of clinical trials targeting specific lncRNAs.

To achieve the clinical application of lncRNAs, molecular techniques such as microarrays, RNA-seq, and qRT–PCR (242) have been used to quantify their expression, but still numerous limitations that need to be overcome. For instance, technical procedures such as ensuring stability, sample preparation, lncRNA extraction, and detection must be standardized. Besides, the sensitivity and specificity of lncRNAs must be ensured. Thus, until all the technical difficulties have been overcome, the detection of circulating lncRNAs would be applied in regular clinical practice (243).

Conclusion

In recent years, the pivotal role of lncRNAs in DR has begun to gain importance in the mechanisms that harbor and promote chemoresistance in HNSCC. As the involvement of more lncRNAs is elucidated, an improvement in diagnostic and prognostic tools could promote an advance in targeted and specific therapies in precision oncology.

Author contributions

JP-F, MB, and RR-P conceived and designed the content of this review. JP-F, MB, CV-M, US-B, DM-C, AA-R, BP-A, AL-P, CL-C, JL-G, JG-P, ME-M, JC-Q, and AB-G wrote the paper. All authors contributed to the final version of the paper and approved the submitted version.

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.

Publisher's note

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LncRNA SCAMP1 disrupts the balance between miR-26a-5p and ZEB2 to promote osteosarcoma cell viability and invasion

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Osteosarcoma often occurs in children and adolescents and affects their health. The survival rate of osteosarcoma patients is unsatisfactory due to the lack of early detection and metastasis development and drug resistance. Hence, dissection of molecular insight into osteosarcoma initiation and progression is pivotal to provide the new therapeutic strategy. In recent years, long noncoding RNAs (lncRNAs) have burst into stage in osteosarcoma development and malignant behaviors. LncRNA SCAMP1 has been discovered to play an essential role in carcinogenesis and progression. However, the mechanisms of lncRNA SCAMP1-involved tumorigenesis have not been reported in human osteosarcoma. In this study, we utilized multiple cellular biological approaches to determine the function of lncRNA SCAMP1 in osteosarcoma cells. Moreover, we performed several molecular biological approaches to define the mechanism by which lncRNA SCAMP1 regulated cell viability and invasion in osteosarcoma. We dissected that lncRNA SCAMP1 promoted progression of osteosarcoma *via* modulation of miR-26a-5p/ZEB2 axis. In conclusion, targeting lncRNA SCAMP1 and its downstream targets, miR-26a-5p and ZEB2, might be a useful approach for osteosarcoma therapy.

KEYWORDS

miR-26a-5p, SCAMP1, ZEB2, viability, invasion

Introduction

Osteosarcoma often occurs in children and adolescents, which affects their health due to that osteosarcoma is one of bone malignant tumors (1). Osteosarcoma patients often have a worse prognosis due to the distant metastasis, such as pulmonary metastasis (2). The conventional therapy for osteosarcoma includes surgery, neoadjuvant, radiotherapy and chemotherapy (3, 4). The five-year survival rate is unsatisfactory in osteosarcoma patients because of the lack of early detection and development of metastasis as well as radio-resistance and drug resistance (5–7). Moreover, the molecular mechanisms of osteosarcoma development and progression are not fully determined. Hence, exploration of molecular insight into osteosarcoma initiation and progression is pivotal to discover the new therapeutic strategy.

In recent years, noncoding RNAs have burst into stage in cancer development and malignant behaviors (8–12). Evidence has suggested that noncoding RNAs are involved in regulation of some cellular biological functions, such as proliferation, cell cycle, autophagy, motility, metastasis, epithelial-to-mesenchymal transition, cancer stem cell, drug resistance and immunotherapy in a variety of cancers (13–17). Noncoding RNAs conduct protein modification, epigenetic modulation, RNA degradation, chromatin remodeling, etc. (18). Based on their size, noncoding RNAs are classified into small noncoding RNAs (< 200 nucleotides), long noncoding RNAs (lncRNA, >200 nucleotides). Accumulated studies have suggested that lncRNAs are important factors to drive osteosarcoma development (19).

SCAMP1 (secretory carrier membrane protein 1) has been implied to associate with tumorigenesis. In pancreatic cancer tissues, SCAMP1 was remarkable upregulated in tissues with lymph node metastasis compared with tissues without metastasis (20). Silencing of SCAMP1 by siRNA transfection led to a marked suppression in invasion and migration in pancreatic cancer cells and gallbladder cancer cells (20). Knockdown of SCAMP1 reduced the activity of vascular endothelial growth factor (VEGF) in gallbladder cancer and pancreatic cancer (20). SCAMP1 expression was correlated with the patient clinicopathological features in pancreatic cancer, including TNM stage, neural invasion, poor prognosis (21). In breast cancer cells, SCAMP1 prevented cell invasion *via* cooperation of MTSS1 (metastasis suppressor protein 1) in breast cancer (22). Loss of SCAMP1 and MTSS1 in breast cancer tissues associated with poor disease-specific survival in HER2+ breast cancer patients (22). SCAMP1-transcript variants (SCAMP1-TV2) knockdown repressed invasion, migration and viability, and induced apoptosis in breast cancer cells (23). Depletion of SCAMP1-TV2 reduced its interaction with PUM2 and enhanced the PUM2 and INSM1 interactions, leading to INSM1 mRNA degradation in breast cancer (23).

LncRNA SCAMP1 has been discovered to play a necessary role in carcinogenesis and progression. For example, lncRNA SCAMP1 was remarkable elevated in ovarian cancer cells and tissues, and overexpression of lncRNA SCAMP1 induced angiogenesis and invasion (24). Moreover, lncRNA SCAMP1 can bind with miR-137 and upregulate the expression of CXCL12 (C-X-C motif chemokine ligand 12) in ovarian cancer cells (24). The mechanisms of lncRNA SCAMP1-mediated tumorigenesis have not been determined in human osteosarcoma. In this study, we utilized multiple cellular biological approaches to determine the function of lncRNA SCAMP1 in osteosarcoma cells. Moreover, we performed several molecular biological approaches to define the underlying mechanism by which lncRNA SCAMP1 regulated cell viability and invasion in osteosarcoma. We dissected that lncRNA SCAMP1 promoted progression of osteosarcoma *via* modulation of miR-26a-5p/ZEB2 axis.

Materials and methods

Cell culture

The human osteosarcoma cell lines MG63 and U2OS cells were bought from American Type Culture Collection (ATCC) Company. The MG63 and U2OS cells were maintained in DMEM medium with 1% penicillin/streptomycin and 5% fetal bovine serum at 37°C incubator under 5% CO_2 atmosphere.

Transfection

The human osteosarcoma MG63 and U2OS cells were seeded in 6-well plates for overnight. Then, osteosarcoma cells were transfected with negative control or miR-26a mimics, miR-26a inhibitors, ZEB2 cDNA, shR-ZEB2, shR-SCAMP1, or SCAMP1 cDNA by Lipofectamine 2000 according to the instruction's approach as described previously (25). After different time transfection, further analysis was performed in transfected osteosarcoma cells for their viability, migration and invasion.

Quantitative real-time reverse transcription-PCR

Total RNA was extracted from the transfected osteosarcoma cells using 1ml TRLzol Reagent. Then, 1microgram RNA was used for reverse transcription to generate first-strand cDNA. PCR was conducted using SYBR Green Kit following the manufacturer's instructions as described before (26).

Cell viability assay

The transfected osteosarcoma cells were seeded in 96-well plates for overnight. After 24, 48 and 72 hours, CCK8 cell viability assay was used to measure the viability of osteosarcoma cells. Briefly, after different time treatments, 10 μL CCK8 reagent was put into each well and maintained for 3 hours at 37 °C in a humidified incubator. The OD values were measured by the microplate reader at 450 nm wavelength.

Colony formation assay

The human osteosarcoma MG63 and U2OS cells were seeded in 6-well plates and cultured with full DMEM medium for 10-14 days at 37°C incubator under 5% CO_2 atmosphere. Then, the cells were washed by PBS three times after discarding the medium. 4% paraformaldehyde was used to fix the osteosarcoma cells for 30-40 minutes. The colonies were stained by 0.1% crystal violet for 15 minutes. The images were taken by microscope and the colony numbers were calculated.

Transwell invasion assay

The transfected osteosarcoma cells were seeded in top Transwell plates with serum-free DMEM on 24-well plates. The bottom Transwell plates were filled with full DMEM medium, which make the cells transfer from the top plates to the bottom plates *via* the transwell membranes with Matrigel. After 20 hours, the culture medium in the top plates were removed and the plates were washed three times by PBS. Then, the plates were fixed by 4% paraformaldehyde for half hour and stained by 0.1% crystal violet. The invasive cells were imaged by an inverted microscope.

Wound healing assay

The transfected osteosarcoma cells were seeded on 6-well plates. Until the cell fluence reached high than 90%, the pipette tip was used to scratch a wound. The cells were washed three times to remove the floatage cells. After 20 hours, the wound location was taken photographs as described before (27).

Luciferase report assay

To confirm the interaction between lncRNA SCAMP1 and miR-26a-5p, we performed luciferase report assay. The mutant binding sequences of miR-26a-5p in lncRNA SCAMP1 were cloned into pmirGLO dual-luciferase vector. The osteosarcoma cells were transfected with various plasmids. The lncRNA SCAMP1 mutant had the mutant binding sequences of miR-26a-5p. The luciferase activity was measured following manufacturer's instructions.

Statistical analysis

In this study, all data were analyzed by GraphPad Prism 5.0. Student t test was performed to measure significance between two groups. ANOVE was used to compare three or more groups. The results were descripted as means ± SD. $P < 0.05$ was considered as statistically significant.

Results

LncRNA SCAMP1 overexpression stimulates viability and colony formation of osteosarcoma cells

To check the function of lncRNA SCAMP1 in osteosarcoma cells, we transfected the SCAMP1 cDNA into MG63 and U2OS cells using Lipofectamine 2000. Our RT-PCR data indicated that lncRNA SCAMP1 was highly elevated in MG63 and U2OS cells after SCAMP1 cDNA transfection (Figure 1A). Moreover, shR-SCAMP1 was transfected into MG63 and U2OS cells by Lipofectamine 2000. We found that lncRNA SCAMP1 expression was remarkable reduced in MG63 and U2OS cells after shR-SCAMP1 transfection (Figure 1A). To examine whether the cell viability was governed by lncRNA SCAMP1, CCK8 assay was utilized in MG63 and U2OS cells after lncRNA SCAMP1 modulation. As expected, shR-SCAMP1 transfected MG63 cells exhibited the less viability at 48 hours and 72 hours (Figure 1B). Consistently, SCAMP1 cDNA transfection led to high viability at 48 hours and 72 hours in U2OS cells (Figure 1C). To validate this role of lncRNA SCAMP1 in osteosarcoma cells, colony formation assay was conducted in MG63 and U2OS cells after lncRNA SCAMP1 knockdown and overexpression, respectively. The data showed that knockdown of lncRNA SCAMP1 reduced the number of colony formation in MG63 cells (Figure 2A). In line with this result, lncRNA SCAMP1 overexpression increased the number of colony formation in U2OS cells (Figure 2B). Therefore, lncRNA SCAMP1 could govern the viability of osteosarcoma cells.

LncRNA SCAMP1 overexpression increased invasion and migration of osteosarcoma cells

It is known that Transwell invasion assay is a good approach for detection of cell invasive ability in cancer. We used Transwell

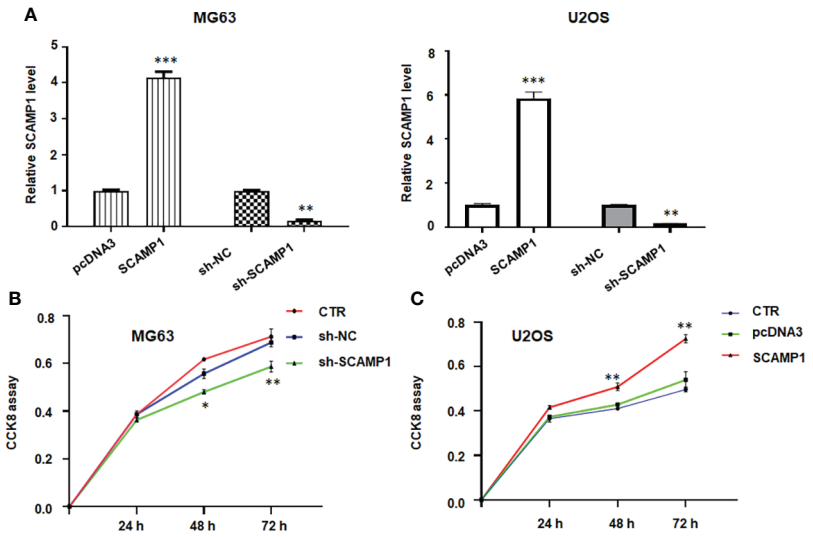

FIGURE 1
LncRNA SCAMP1 overexpression stimulates viability of osteosarcoma cells. **(A)**: RT-PCR data showed that SCAMP1 cDNA transfection increased the SCAMP1 expression, while sh-SCAMP1 transfection decreased the expression of SCAMP1 in MG63 and U2OS cells. **(B)**: CCK8 assays showed that sh-SCAMP1 transfection reduced viability of MG63 cells. **(C)**: CCK8 assays showed that SCAMP1 cDNA transfection induced viability of U2OS cells. *p<0.05; **p<0.01; ***p<0.001 *vs.* control group. CTR: control; sh-NC: shRNA negative control; sh-SCAMP1: shRNA SCAMP1; SCAMP1: SCAMP1 cDNA.

invasion assay to test the function of lncRNA SCAMP1 in osteosarcoma cells. As shown in Figures 3A, B, SCAMP1 cDNA transfection resulted in promotion of invaded cell numbers in both MG63 and U2OS cells. The invasiveness ability in shR-SCAMP1 transfected osteosarcoma cells was also examined using Transwell invasion assay. Expectedly, shR-SCAMP1 transfected cells had a reduction in invaded cell numbers in U2OS and MG63 cells (Figures 3A, B). Wound

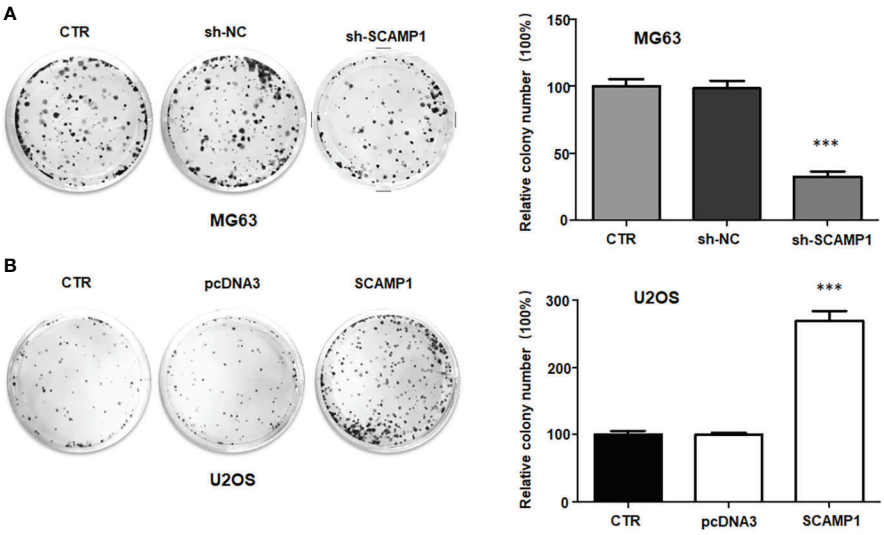

FIGURE 2
LncRNA SCAMP1 overexpression increases colony formation of osteosarcoma cells. **(A)**: Left panel: Colony formation assays showed that sh-SCAMP1 transfection reduced colony formation of MG63 cells. Right panel: quantitative data is shown for colony formation of MG63 cells. **(B)**: Left panel: Colony formation assays showed that SCAMP1 cDNA transfection increased colony formation of U2OS cells. Right panel: quantitative data is shown for colony formation of U2OS cells. ***p<0.001 *vs.* control group.

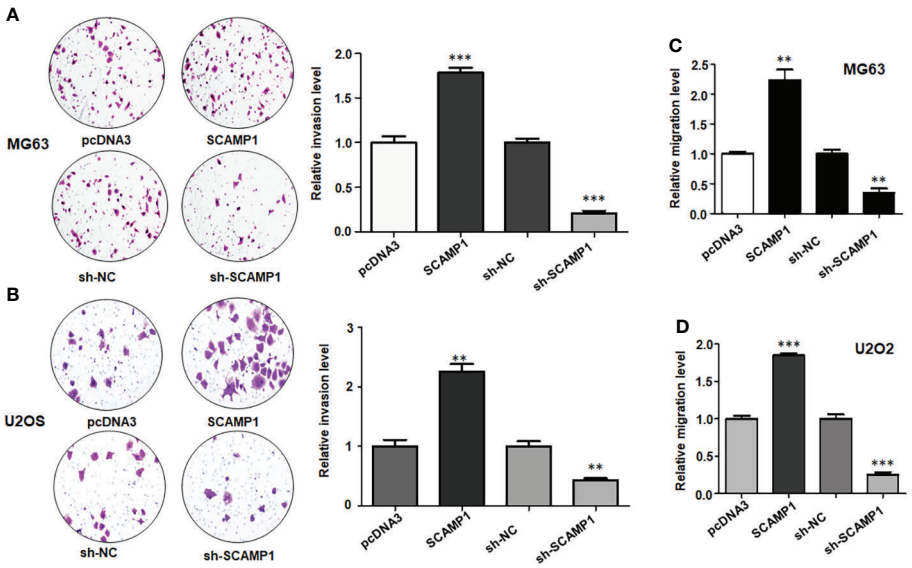

FIGURE 3
LncRNA SCAMP1 overexpression increases invasive and migratory abilities of osteosarcoma cells. **(A)**: Left panel: Transwell invasion assays showed that sh-SCAMP1 transfection reduced invasion of MG63 cells, while SCAMP1 cDNA transfection increased invasion of MG63 cells. Right panel: quantitative data is shown for invasive ability of MG63 cells. **(B)**: Left panel: Transwell invasion assays showed that sh-SCAMP1 transfection and SCAMP1 cDNA transfection modulated invasion of U2OS cells. Right panel: quantitative data is shown for invasion ability of U2OS cells. **(C)**: Wound healing assays showed that sh-SCAMP1 transfection reduced migration of MG63 cells, while SCAMP1 cDNA transfection increased migration of MG63 cells. **(D)**: Wound healing assays showed that sh-SCAMP1 transfection and SCAMP1 cDNA transfection modulated migratory ability of U2OS cells. **p<0.01; ***p<0.001 *vs.* control group.

healing assay is often used for measuring the migrative ability in cancer cells. Therefore, we used the wound healing assay in our study to check the role of lncRNA SCAMP1 in regulation of migration in osteosarcoma cells. The wound healing assay data showed that shR-SCAMP1 transfected cells had a slower rate to close the wound area in U2OS and MG63 cells (Figures 3C, D). On the contrary, both osteosarcoma cell lines with SCAMP1 cDNA transfection had a faster rate to close the wound area (Figures 3C, D). Hence, lncRNA SCAMP1 could govern the invasive and migrative abilities in osteosarcoma cells.

LncRNA SCAMP1 interacts with and regulates miR-26a-5p in osteosarcoma cells

It has been known that lncRNAs often sponge with miRNAs to suppress their expressions. Therefore, we aimed to explore the lncRNAs that could bind to lncRNA SCAMP1 in osteosarcoma cells. Based on the TargetScan database from website, we saw the binding sites between lncRNA SCAMP1 and miR-26a-5p (Figure 4A). The luciferase reporter gene assay is a regular approach to confirm the interaction between lncRNAs and miRNAs. The data from the dual luciferase assay showed that miR-26a-5p mimic transfection led to a reduction in the luciferase activity in the lncRNA SCAMP1 wild-type group (Figure 4B). On the contrary, this phenotype was not observed in the lncRNA SCAMP1 mutant group (Figure 4B). Moreover, miR-26a-5p inhibitor increased the luciferase activity in lncRNA SCAMP1 wild-type group, but not in lncRNA SCAMP1 mutant group (Figure 4B). We also measured the expression of miR-26a-5p in U2OS and MG63 cells after miR-26a-5p mimics transfection and miR-26a-5p inhibitor treatment, respectively. Our data showed that miR-26a inhibitors decreased the expression of miR-26a-5p in MG63 cells, while miR-26a mimic transfection increased the miR-26a-5p expression levels in U2OS cells (Figure 4C). Notably, upregulation of wild-type lncRNA SCAMP1 suppressed the expression of miR-26a-5p in MG63 cells, but this phenotype did not exhibit in mutant lncRNA SCAMP1 group (Figure 4D). Strikingly, shR-SCAMP1 group showed the high expression of miR-26a-5p compared with shR-NC group in U2OS cells (Figure 4D). Hence, lncRNA SCAMP1 can interact with and regulate miR-26a-5p inU2OS and MG63 cells.

LncRNA SCAMP1 overexpression promotes cell viability *via* miR-26a-5p

To check whether miR-26a-5p participates in lncRNA SCAMP1-mediated promotion of cell viability in osteosarcoma

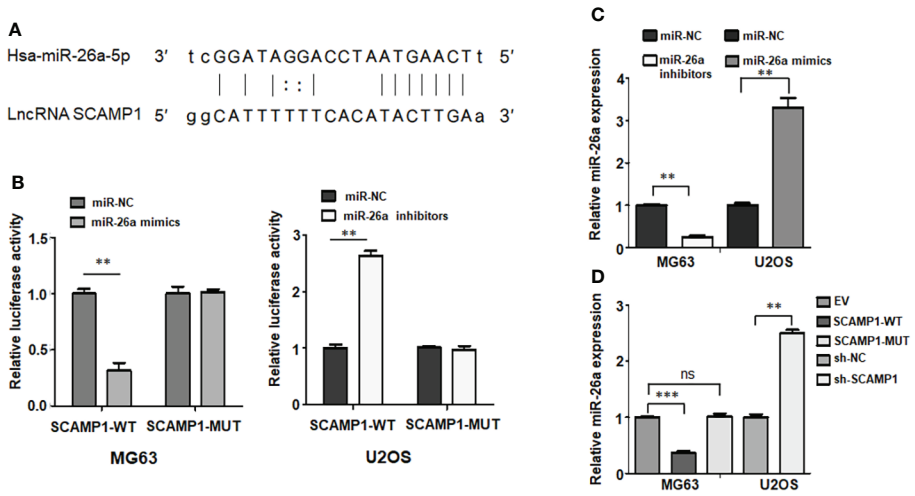

FIGURE 4
LncRNA SCAMP1 interacts with and regulates miR-26a-5p in osteosarcoma cells. **(A)**: Potential binding sites between miR-26a-5p and lncRNA SCAMP1. **(B)**: Dual luciferase reporter assays showed that miR-26a interacted with lncRNA SAMP1. miR-26a-5p modulation regulated luciferase activity in SCAMP1 wild-type group. **(C)**: RT-PCR data showed that miR-26a inhibitors decreased the expression of miR-26a-5p in MG63 cells, while miR-26a mimic transfection increased the miR-26a-5p expression levels in U2OS cells. **(D)**: RT-PCR data showed that upregulation of SCAMP1 suppressed the expression of miR-26a-5p in MG63 cells, while sh-SCAMP1 increased miR-26a-5p expression in U2OS cells. SCAMP1-WT: SCAMP1 wild type; SCAMP1-MUT: SCAMP1 mutant. **P < 0.01; ***P < 0.001 vs control group; ns, no significance.

cells, the MG63 cells were treated with miR-26a-5p mimics in combination with lncRNA SCAMP1 cDNA transfection. Our CCK8 results indicated that miR-26a-5p mimic transfection suppressed viability of MG63 cells at 48 hours and 72 hours (Figure 5A). Overexpression of lncRNA SCAMP1 abolished miR-26a-5p mimics-mediated inhibition of cell viability in MG63 cells (Figure 5A). Moreover, inhibition of miR-26a-5p enhanced viability of U2OS cells, which was abrogated by shR-SCAMP1 transfection (Figure 5B). In line with these results, colony formation data revealed that miR-26a-5p mimics reduced the colony numbers in MG63 cells, while miR-26a-5p inhibitors increased the colony numbers in U2OS cells (Figure 5C *and* 65D). In SCAMP1-overexpressing MG63 cells, miR-26a-5p mimics-mediated suppression of colony formation was abolished in MG63 cells (Figure 5C). In U2OS cells with shR-SCAMP1 transfection, miR-26a-5p-inhibitor-induced colony formation was blocked in U2OS cells (Figure 5D). Taken together, lncRNA SCAMP1 overexpression promotes cell viability *via* miR-26a-5p in osteosarcoma cells.

ZEB2 is a downstream target of miR-26a-5p in osteosarcoma cells

It is known that miRNAs performed their functions in tumorigenesis *via* inhibiting the expression of their downstream genes. According to the TargetScan database from website, we observed the binding sites between miR-26a-5p and ZEB2, indicating that miR-26a-5p could interact with ZEB2 and suppress its expression (Figure 6A). In MG63 cells, we found that miR-26a-5p mimics attenuated the mRNA levels of ZEB2 (Figure 7B). Similarly, in U2OS cells, we found that miR-26a-5p inhibitors elevated the mRNA levels of ZEB2 (Figure 6B). Moreover, we tested whether lncRNA SCAMP1 regulated the expression of ZEB2 in osteosarcoma cells. We found that SCAMP1-overexpressing cells had the high expression of ZEB2 in MG63 cells (Figure 6B). In shR-SCAMP1 transfected U2OS cells, ZEB2 mRNA levels were remarkable downregulated (Figure 6B). Altogether, miR-26a-5p could target ZEB2 in osteosarcoma cells.

LncRNA SCAMP1 increases cell viability and colony formation *via* miR-26a/ZEB2 axis

To determine whether lncRNA SCAMP1 increased viability of osteosarcoma cells *via* targeting miR-26a/ZEB2 axis, MG63 cells were co-transfected with miR-26a-5p mimics and ZEB2 cDNA, or SCAMP1 and sh-ZEB2. Our CCK8 assay data demonstrated that pZEB2 cDNA transfection increased cell viability in MG63 cells (Figure 7A). Upregulation of ZEB2 abrogated miR-26a-5p-induced inhibition of cell viability in MG63 cells (Figure 7A). Moreover, SCAMP1 overexpression elevated cell viability, which was blocked by downregulation of ZEB2 in MG63 cells (Figure 7A). Similarly, ZEB2 cDNA transfection increased colony formation of MG63

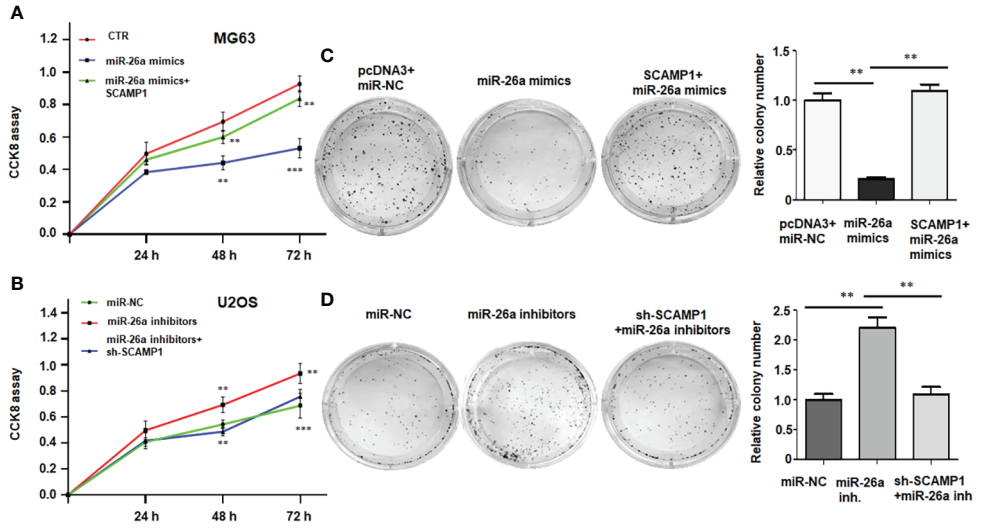

FIGURE 5
LncRNA SCAMP1 overexpression promotes cell viability and colony formation *via* miR-26a-5p. **(A)**: CCK8 assays showed that overexpression of lncRNA SCAMP1 abolished miR-26a-5p mimics-mediated inhibition of cell viability in MG63 cells. **(B)**: CCK8 assays showed that inhibition of miR-26a-5p enhanced viability of U2OS cells, which was abrogated by shR-SCAMP1 transfection. **(C)**: Left panel: Colony formation assays showed that in SCAMP1-overexpressing MG63 cells, miR-26a-5p mimics-mediated suppression of colony formation was abolished in MG63 cells. Right panel: quantitative data is shown for colony formation of MG63 cells. **(D)**: Left panel: Colony formation assays showed that in U2OS cells with shR-SCAMP1 transfection, miR-26a-5p-inhibitor-induced colony formation was blocked. Right panel: quantitative data is shown for colony formation of U2OS cells. miR-26a inh: miR-26a inhibitors. **p<0.01; ***p<0.001 *vs.* control group.

cells, which was abrogated by miR-26a mimics transfection (Figure 7B). LncRNA SCAMP1 upregulation elevated colony formation of MG63 cells, which was blocked by sh-ZEB2 transfection (Figure 7B).

LncRNA SCAMP1 increases cell invasion and migration *via* miR-26a/ZEB2 axis

To examine whether lncRNA SCAMP1 promoted invasion and migration of osteosarcoma cells *via* targeting miR-26a/ZEB2 axis, MG63 cells were co-transfected with miR-26a-5p mimics plus ZEB2 cDNA, or SCAMP1 plus shZEB2. Our Transwell invasion assay data dissected that pZEB2 cDNA transfection increased cell invasion ability in MG63 cells (Figure 7C). Upregulation of ZEB2 abrogated miR-26a-5p-induced suppression of cell invasion in MG63 cells (Figure 7C). Moreover, SCAMP1 overexpression elevated cell invasion, which was reduced by downregulation of ZEB2 in MG63 cells (Figure 7C). Similarly, ZEB2 cDNA transfection increased migrative ability of MG63 cells, which was abrogated by miR-26a mimics transfection (Figure 7D). LncRNA SCAMP1 upregulation elevated migrative ability of MG63 cells, which was blocked by sh-ZEB2 transfection (Figure 7D). In a word, lncRNA SCAMP1 regulated cell invasiveness and migrative ability through targeting miR-26a and ZEB2 pathways in osteosarcoma.

Discussion

LncRNAs are involved in osteosarcoma development, progression, metastasis, prognosis and drug resistance (28, 29). LncRNAs utilized their functions in oncogenesis *via* targeting various cellular signaling pathways (30). For instance, lncRNA CBR3-AS1 targeted the network of miR-140-5p-DDX54-NUCKS1-mTOR signaling pathway and contributed to osteosarcoma progression (31). One study found that lncRNA DARS-AS1 regulated miR-532-3p/CCR7 axis and facilitated progression of osteosarcoma (32). Another study revealed that lncRNA SNHG1 stimulated osteosarcoma development *via* sponging miR-493-5p and elevating the expression of S100A6 (33). Moreover, lncRNA ODRUL acted as a sponge of miR-6874-3p to elevate the expression of IL-6, leading to osteosarcoma progression (34). Furthermore, lncRNA MELTF-AS1 regulated the expression of MMP14 and enhanced osteosarcoma metastasis (35). In addition, lncRNA MALAT1 sponged miR-150-5p and increased the expression of VEGFA and enhanced tumor angiogenesis in osteosarcoma (36). LncRNA PURPL affected tumor-associated macrophages through modulating miR-363 and PDZD2 in osteosarcoma cells (37). These studies indicated that lncRNAs critically participate in osteosarcoma progression.

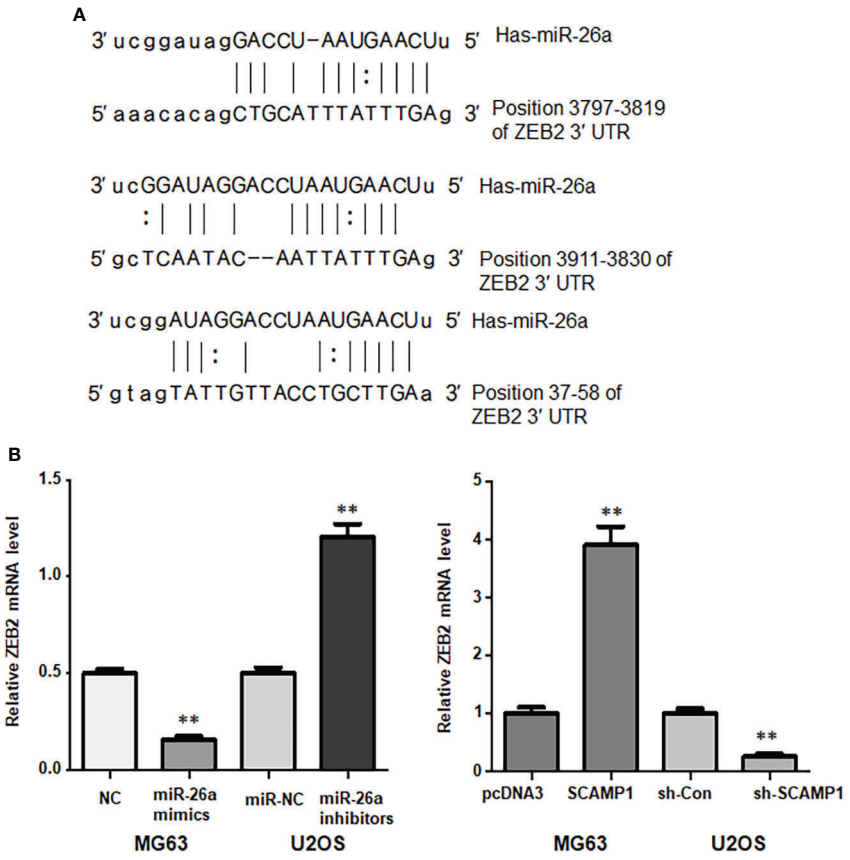

FIGURE 6
ZEB2 is a downstream target of miR-26a-5p in osteosarcoma cells. **(A)**: The potential binding sites between miR-26a-5p and ZEB2. **(B)**: Left panel: RT-PCR data showed that miR-26a-5p mimics attenuated the mRNA levels of ZEB2 in MG63 cells. miR-26a-5p inhibitors elevated ZEB2 mRNA levels in U2OS cells. Right panel: RT-PCR data showed that SCAMP1-overexpressing cells had the high expression of ZEB2 in MG63 cells. In shR-SCAMP1 transfected U2OS cells, ZEB2 mRNA levels were remarkable downregulated. **p<0.01 *vs.* control group.

LncRNA SCAMP1 was reported to be significantly upregulated in pancreatic cancer tissues compared with normal tissues (38). Pancreatic cancer patients with unfavorable survival often had higher expression of lncRNA SCAMP1. The mRNA-miRNA-lncRNA regulatory network predicted that lncRNA SCAMP1 could bind to miR-132-3p and MMP9 in pancreatic cancer cells (38). LncRNA SCAMP1 has been reported to regulate ZEB1/JUN axis in renal cell carcinoma (39). In renal cell carcinoma specimens, the expression of lncRNA SCAMP1 was highly elevated. Knockdown of lncRNA SCAMP1 induced apoptosis and reduced cell viability in renal cell carcinoma cells after H_2O_2 treatment (39). Moreover, miR-429 was found to interact with lncRNA SCAMP1 in renal cell carcinoma cells. Consistently, miR-429 expression was remarkable decreased in human renal cancer samples. Moreover, miR-429 targets both ZEB1 and JUN in renal cell carcinoma cells. Furthermore, lncRNA SCAMP1 also affected autophagy and miR-429-mediated tumorigenesis in renal cell carcinoma (39). Therefore, lncRNA SCAMP1 enhanced progression of renal cell carcinoma *via* regulation of autophagy and miR-429/ZEB1/JUN axis under oxidative stress. In our study, we reported that lncRNA SCAMP1 modulated the expression of ZEB2 *via* sponging miR-26a-5p in osteosarcoma, which led to promotion of cell viability and colony formation. It is required to investigate whether lncRNA SCAMP1 targets ZEB1 in osteosarcoma cells. Since ZEB2 is the critical driver in EMT progression, it is needed to address whether lncRNA SCAMP1 can regulate the EMT in osteosarcoma *via* targeting ZEB2.

Zong et al. reported that silencing of lncRNA SCAMP1 restrained viability, invasive and migratory abilities and induced apoptosis *via* sponging miR-499a-5p in glioma (40). LMX1A was found to be a downstream target of miR-499a-5p and participated in lncRNA SCAMP1-induced oncogenesis in glioma. LMX1A can regulate the expression of NLRC5 (NLR family, CARD domain containing 5) and activate Wnt/β-catenin signaling pathway in glioma (40). In the current study, we observed that lncRNA

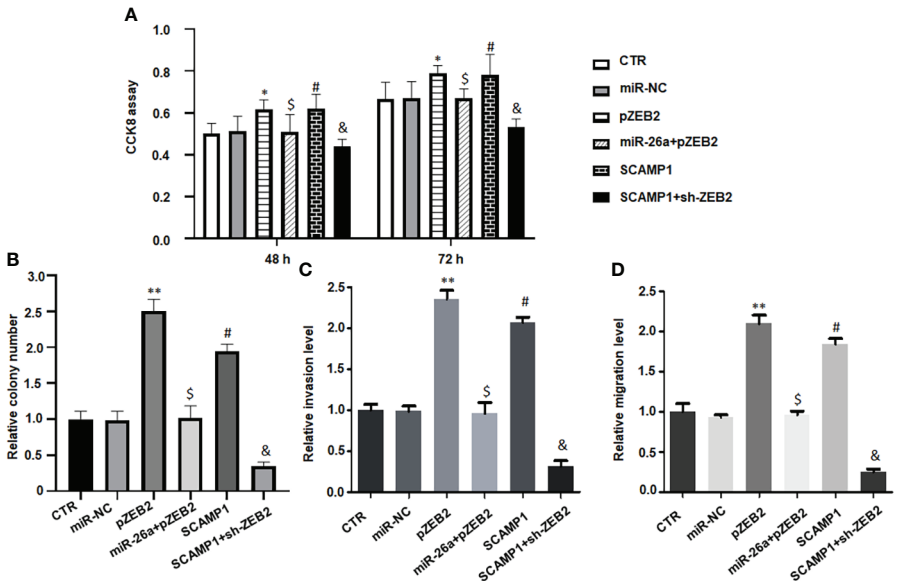

FIGURE 7
LncRNA SCAMP1 increases cell malignant progression *via* miR-26a/ZEB2 axis. **(A)**: CCK8 assays data showed that upregulation of ZEB2 abrogated miR-26a-5p-induced inhibition of cell viability in MG63 cells. SCAMP1 overexpression elevated cell viability, which was blocked by downregulation of ZEB2 in MG63 cells. **(B)**: Colony formation data showed that ZEB2 cDNA transfection increased colony formation of MG63 cells, which was abrogated by miR-26a mimics transfection. SCAMP1 upregulation elevated colony formation of MG63 cells, which was blocked by sh-ZEB2 transfection. **(C)**: Transwell invasion assay data showed that upregulation of ZEB2 abrogated miR-26a-5p-induced suppression of cell invasion in MG63 cells. SCAMP1 overexpression elevated cell invasion, which was reduced by downregulation of ZEB2 in MG63 cells. Right panel: quantitative data is shown for invasion assay of left panel. **(D)**: Wound healing assays showed that ZEB2 cDNA transfection increased migrative ability of MG63 cells, which was abrogated by miR-26a mimics transfection. LncRNA SCAMP1 upregulation elevated migrative ability of MG63 cells, which was blocked by sh-ZEB2 transfection. **p<0.01 *vs.* control group; $p<0.01 *vs.* pZEB2 group; #p<0.01 *vs.* control group; &p<0.01 *vs.* SCAMP1 group. ; *p < 0.05 vs control group.

SCAMP1 promoted cell invasion and migration in osteosarcoma cells. Moreover, lncRNA SCAMP1 performed their functions on cell motility *via* targeting miR-26a-5p/ZEB2 axis in osteosarcoma.

Conclusions

In summary, lncRNA SCAMP1 modulated the expression of ZEB2 *via* sponging miR-26a-5p in osteosarcoma, which resulted in promotion of viability and colony formation of osteosarcoma cells. We also reported that lncRNA SCAMP1 promoted cell invasiveness and migrative capacity in osteosarcoma cells *via* regulating miR-26a-5p/ZEB2 axis. This study has several limitations. For instance, the current study used cell culture system to define the role of lncRNA SCAMP1 in osteosarcoma. This study did not use the mouse model and clinical tissues from osteosarcoma patients to dissect the function of SCAMP1. Therefore, it is necessary to describe that *in vivo* experiments and clinical sample study are required to determine the role of lncRNA SCAMP1 in osteosarcoma.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

Author contributions

RL and LD designed this study. RL, ZC, YZ, GM, and QY performed the experiments. YL, AdM, CZ, JR, CL, AbM, and PZ analyzed the data. RL and LD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.967000/full#supplementary-material

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Long non-coding RNA LINC00152 in cancer: Roles, mechanisms, and chemotherapy and radiotherapy resistance

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Long non-coding RNA LINC00152 (cytoskeleton regulator, or LINC00152) is an 828-bp lncRNA located on chromosome 2p11.2. LINC00152 was originally discovered during research on hepatocarcinogenesis and has since been regarded as a crucial oncogene that regulates gene expression in many cancer types. LINC00152 is aberrantly expressed in various cancers, including gastric, breast, ovarian, colorectal, hepatocellular, and lung cancer, and glioma. Several studies have indicated that LINC00152 is correlated with cell proliferation, apoptosis, migration, invasion, cell cycle, epithelial-mesenchymal transition (EMT), chemotherapy and radiotherapy resistance, and tumor growth and metastasis. High LINC00152 expression in most tumors is significantly associated with poor patient prognosis. Mechanistic analysis has demonstrated that LINC00152 can serve as a competing endogenous RNA (ceRNA) by sponging miRNA, regulating the abundance of the protein encoded by a particular gene, or modulating gene expression at the epigenetic level. LINC00152 can serve as a diagnostic or prognostic biomarker, as well as a therapeutic target for most cancer types. In the present review, we discuss the roles and mechanisms of LINC00152 in human cancer, focusing on its functions in chemotherapy and radiotherapy resistance.

KEYWORDS

long non-coding RNA, LINC00152, cancer, chemotherapy resistance, radiotherapy resistance

Introduction

Long non-coding RNAs (lncRNAs) are transcripts of more than 200 nucleotides that generally do not encode proteins and include cyclic RNAs (circRNAs) and pseudogenes (1). These lncRNAs play a vital role in regulating cell homeostasis and disease progression by serving as competitive endogenous RNAs (ceRNAs) or binding directly to regulate tumor occurrence and growth. The lncRNA cytoskeletal regulatory RNA (CYTOR), also known as LINC00152, is located in the chromosomal region 2p11.2, and is overexpressed in many cancers (Figure 1). LINC00152 was initially detected with variable hypomethylation levels during the development of hepatocellular cancer (2).

The function of lncRNAs is highly correlated with their subcellular distribution. LncRNAs act as endogenous miRNA sponges to modulate miRNA targets in the cytoplasm. Cytoskeletal regulators, such as long intergenic non-coding RNA 00152 (LINC00152), can regulate gene expression through various mechanisms. LINC00152 acts as a ceRNA in the cytoplasm and binds to multi-comb inhibition complex 2 (PRC2) in the nucleus to regulate epigenetic gene regulation. LINC00152 is primarily found in the cytoplasm, where cytoplasmic lncRNAs operate as microRNA sponges, thus inhibiting the action of target microRNA (3). Mechanistic investigations have revealed that LINC00152 can act as a ceRNA by sponging miRNA, thus influencing the amount of protein encoded by a gene and altering gene expression at the epigenetic level.

LINC00152, which was later called STAiR18, was identified in 2013 by analyzing the expression profile of signal transducer and activator of transcription 3 (STAT3)-dependent genes in gastric cancer (4). The capacity of LINC00152 to sponge various miRNAs influences cell cycle arrest, apoptosis, EMT, migration, and invasion. Sponging miRNAs eliminates their inhibitory effect on target genes, thereby altering their expression level (4). Subsequent studies have demonstrated that LINC00152 is overexpressed in many human malignancies, including lung, liver, pancreatic, and breast cancers. In addition, LINC00152 has been implicated in regulating cancer cell proliferation, the cell cycle, epithelial-mesenchymal transition (EMT), and chemotherapy and radiotherapy resistance. Ongoing investigations into the role of LINC00152 are therefore required. LINC00152 is a pivotal oncogenic long non-coding RNA in human cancers (5). The expression of LINC00152 could contribute to tumor diagnosis, targeted therapy and curative effect evaluation (6).

The present review highlights the current research on the function, regulatory mechanisms, and chemotherapy and radiotherapy resistance of LINC00152 in human cancers.

The role of LINC00152 in various cancers

The role of LINC00152 in human cancer has been explored in numerous clinical, translational, and basic studies (5). Accumulating evidence has demonstrated that the expression of LINC00152 is abnormally dysregulated in most tumor types. High expression of LINC00152 has been observed in multiple types of tumors, including breast cancer, ovarian cancer, hepatocellular cancer, lung cancer, leukemia, bladder cancer, nasopharyngeal cancer, gallbladder cancer, osteosarcoma, laryngeal cancer, thyroid cancer, retinoblastoma, head and neck squamous cell cancer, and pancreatic cancer (Figure 2). In contrast, LINC00152 is expressed at low levels in colon cancer

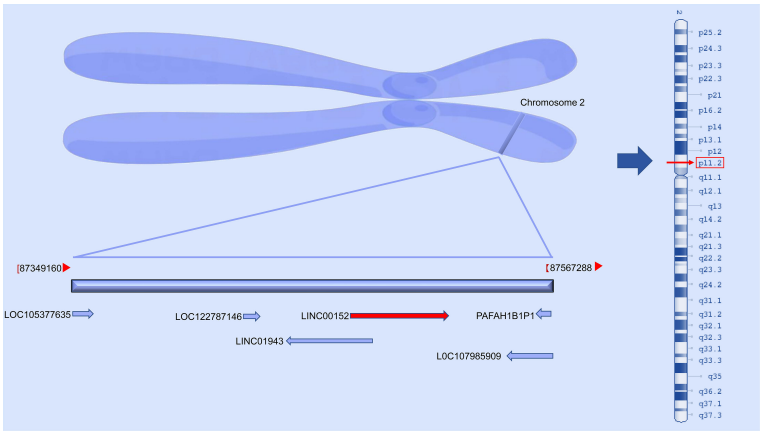

FIGURE 1
Location of LINC00152 in chromosomal region 2p11.2.

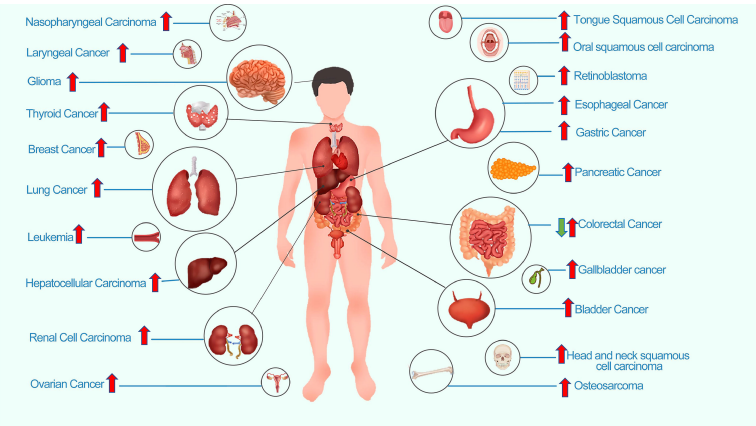

FIGURE 2
Dysregulation of LINC00152 expression in human cancers.

tissue and cells (7). A specific explanation for the downregulation of LINC00152 expression in colon cancer remains unknown.

LINC00152 has essential roles in almost all aspects of tumor occurrence and progression, including tumorigenesis, cancer cell proliferation, apoptosis, invasion, metastasis, autophagy, and the response to anti-tumor treatment. The functions and underlying molecular mechanisms of LINC00152 in various cancers are summarized in Table 1. Potential biomarkers for the diagnosis and prognosis of LINC00152 in cancer are presented in Table 2. Table 3 summarizes the role of LINC00152 in chemotherapy and radiation resistance and will be explained in detail in later sections.

Function and mechanisms of LINC00152 in human cancer

Oral squamous cell cancer

Oral squamous cell cancer (OSCC) is an aggressive form of head and neck squamous cell cancer (HNSCC) (78). OSCC accounts for 4% of all newly diagnosed cancers and ranks eighth among all estimated new cases among men worldwide (79).The five-year survival rate of patients with OSCC can reach 68%. Chen et al. found that the LINC00152/lipoma preferred partner (LPP) axis is the key to Forkhead boxD1 (FOXD1)-induced EMT and chemotherapy resistance in OSCC. FOXD1 may bind directly to the LINC00152 promoter and activate LINC00152 transcription. LINC00152 then specifically inhibits miR-1252-5p and miR-3148, thus upregulating the expression of LPP and promoting EMT and chemoresistance in OSCC (48).

Tongue squamous cell cancer

Squamous cell carcinoma of the tongue (TSCC) is the most common oral malignancy and has a poor prognosis. The five-year survival rate of patients with TSCC can reach 68.8%. Li et al. demonstrated that LINC00152 expression is significantly upregulated in TSCC tissue compared to that in normal tissue. Li et al. also revealed that increased LINC00152 expression could promote TSCC cell growth and cell cycle progression, migration and invasion, as well as inhibit apoptosis. Mechanistic analyses have indicated that LINC00152 acts as a sponge for miR-193b-3p to promote the phosphorylation and activation of the phosphoinositide 3-kinase (PI3K) signaling pathway and downstream protein kinase B (AKT), which contributes to the development of TSCC (58). LINC00152, therefore, promotes the oncogenic potential of TSCC and may be a potential therapeutic target.

Esophageal cancer

Esophageal cancer (EC) is one of the most common cancers of the digestive system (Figure 3), ranking seventh among the causes of cancer-related death (79). EC has a unique geographical distribution and is widespread in Eastern Asia and Southern Africa but rare in Central America (80). The five-year survival rate of patients with EC only reaches 20.6% (https://seer.cancer.gov/). EC is frequently identified at advanced cancer stages owing to the lack of early clinical signs and symptoms (81).

Yang et al. (64). studied LINC00152 overexpression in esophageal squamous cell carcinoma (ESCC) tissue. LINC00152 is closely related to TNM staging and lymphatic metastasis in ESCC. High expression of LINC00152 is related

Table 1 The molecular mechanisms of LINC00152 in various cancers.

Cancer	Role	Expression	Regulated molecules	Related pathway	reference
Leukemia Stem Cells	oncogene	overexpression	PARP1	LINC00152/PARP1	(8)
Acute lymphoblastic leukemia	oncogene	overexpression	Not reported	Not reported	(9)
Bladder Cancer	oncogene	overexpression	Wnt/β-Catenin	LINC00152/Wnt/β-Catenin	(10)
Nasopharyngeal carcinoma	oncogene	overexpression	miR-613/ANXA2	LINC00152/miR-613/ANXA2	(11)
Gallbladder cancer	oncogene	overexpression	SP1/PI3K/AKT	SP1/LINC00152/PI3K/AKT	(12)
Gallbladder cancer	oncogene	overexpression	miR-138/HIF-1a	LINC00152/miR-138/HIF-1a/Slug	(13)
Lung cancer	oncogene	overexpression	EGFR/PI3K/AKT	LINC00152/EGFR/PI3K/AKT/Fibronectin/Vimentin	(14)
Lung cancer	oncogene	overexpression	LINC00152	LINC00152/miR-16-5p/BCL2L2	(15)
Lung cancer	oncogene	overexpression	LINC00152	Not reported	(16)
Lung cancer	oncogene	overexpression	miR-206/PTMA	LINC00152/miR-206/PTMA	(17)
Lung cancer	oncogene	overexpression	Not reported	Not reported	(14)
Lung cancer	oncogene	overexpression	miR-195	Linc00152/miR-195	(18)
Lung cancer	oncogene	overexpression	EZH2/IL24	LINC00152/EZH2/LSD1/IL24	(19)
Hepatocellular cancer	oncogene	overexpression	Not reported	Not reported	(20)
Hepatocellular cancer	oncogene	overexpression	EpCAM/mTOR	LINC00152/EpCAM/mTOR	(21)
Hepatocellular cancer	oncogene	overexpression	miR-125b/SEMA4C	LINC00152/miR-125b/SEMA4C	(2)
Hepatocellular cancer	oncogene	overexpression	miR-193a/b-3p/CCND1	LINC00152/miR-193a/b-3p/CCND1	(22)
Hepatocellular cancer	oncogene	overexpression	Not reported	Not reported	(23)
Hepatocellular cancer	oncogene	overexpression	HBx	LINC00152/HBx	(24)
Hepatocellular cancer	oncogene	overexpression	miR-125b-5p/KIAA1522	LINC00152/miR-125b-5p/KIAA1522	(25)
Hepatocellular cancer	oncogene	overexpression	LINC00152/miR-215/CDK13	LINC00152/LINC00152/miR-215/CDK13	(26)
Hepatocellular cancer	oncogene	overexpression	Not reported	Not reported	(27)
Osteosarcoma	oncogene	overexpression	miR-1182/CDK14/TCF3-	TCF3/LINC00152/miR-1182/CDK14	(28)
Osteosarcoma	oncogene	overexpression	miR-193b-3p	LINC00152/miR-193b-3p	(29)
Human multiple myeloma	oncogene	overexpression	STAT3/miR-21Mcl-1	IL-6/STAT3/LINC00152/miR-21/Mcl-1	(4)
Laryngeal cancer	oncogene	overexpression	miR-613	LINC00152/miR-613	(30)
Papillary thyroid cancer	oncogene	overexpression	miR-497/BDNF	LINC00152/miR-497/BDNF	(31)
Papillary thyroid cancer	oncogene	overexpression	TRIM29/miR-873-5p/FN-1	TRIM29/LINC00152/miR-873-5p/FN-1	(32)
Glioma	oncogene	overexpression	3′ end of LINC00152	Not reported	(33)
Glioma	oncogene	overexpression	PI3K/AKT	LINC00152/miR-613/CD164/PI3K/AKT	(34)
Glioma	oncogene	overexpression	Not reported	Not reported	(35)
Glioma	oncogene	overexpression	Epigenetic	Not reported	(36)
Glioma	oncogene	overexpression	miR-103a-3p/FEZF1/ CDC25A	LINC00152/miR-103a-3p/FEZF1/CDC25A/PI3K/ AKT	(37)
Glioma	oncogene	overexpression	UPF1	UPF1/LINC00152	(38)
Colorectal cancer	oncogene	overexpression	miR-193a-3p/ERBB4/AKT	LINC00152/miR-193a-3p/ERBB4/AKT	(39)
Colorectal cancer	oncogene	overexpression	Wnt/b-Catenin	Wnt/b-Catenin Signaling	(40)
Colorectal cancer	oncogene	overexpression	GACAT3/miR-103	GACAT3/LINC00152/miR-103	(41)
Colorectal cancer	suppressor oncogene	downregulation	miRNA-105/PTEN/akt	LINC00152/miRNA-105/PTEN	(42)
Colorectal cancer	oncogene	overregulation	miR-376c-3p	LINC00152/miR-376c-3p/Ki-67, Bcl-2, Fas	(7)
Colorectal cancer	oncogene	overexpression	LINC00152	Not reported	(43)
Colorectal cancer	oncogene	overexpression	NCL, Sam68	LINC00152, NCL and Sam68/NF-κB/EMT	(44)
Colorectal cancer	oncogene	overexpression	miR-3679-5p/MACC1	LINC00152/miR-3679-5p/MACC1	(45)
Colorectal cancer	oncogene	overexpression	hypomethylation	PI3K/Akt, Ras, WNT, TP53, Notch and ErbB.	(46)
Colorectal cancer	oncogene	overexpression	YAP1/miR-632-miR-185-3p/ FSCN	YAP1/LINC00152/miR-632-miR-185-3p/FSCN	(47)
Oral squamous cell cancer	oncogene	overexpression	FOXD1/LPP	FOXD1/LINC00152 transcription/miR-3148/miR-1252-5p/LPP	(48)

(Continued)

Continued

Cancer	Role	Expression	Regulated molecules	Related pathway	reference
Pan-Cancer	oncogene	overexpression	EZH2	LINC00152/EZH2	(49)
Breast Cancer	oncogene	overexpression	YY1/PTEN	YY1/LINC00152/PTEN	(50)
Breast Cancer	oncogene	overexpression	DNMTs/BRCA1/PTEN	LINC00152/DNMTs/BRCA1/PTEN	(51)
Breast Cancer	oncogene	overexpression	miR-125a-5p	LINC00152/miR-125a-5p/SRF/MAPK/ERK pathway/ TAZ	(52)
Breast Cancer	oncogene	overexpression	mTOR	LINC00152/mTOR	(53)
Breast Cancer	oncogene	overexpression	KLF5	LINC00152/KLF5/PTEN and b-Catenin	(54)
Ovarian cancer	oncogene	overexpression	BCL6	LINC00152/BCL6	(55)
Ovarian cancer	oncogene	overexpression	miR-125b/MCL-1	LINC00152/miR-125b/MCL-1	(56)
Ovarian cancer	oncogene	overexpression	TNF/CDKN1C	LINCOO152/TNF/CDKN1C	(57)
Tongue squamous cell cancer	oncogene	overexpression	miRNA-193b-3p	LINC00152/miRNA-193b-3p/PI3K/AKT	(58)
Renal cell cancer	oncogene	overexpression	P16/miR-205	LINC00152/P16/miR-205	(59)
Renal cell cancer	oncogene	overexpression	Not reported	Not reported	(60)
Retinoblastoma cells	oncogene	overexpression	Sp1/miR-30d/SOX9/ZEB2/ EMT	Sp1/miR-30d/SOX9/ZEB2/EMT	(61)
Retinoblastoma	oncogene	overexpression	miR-613/YAP1	LINC00152/miR-613/YAP1	(62)
Esophageal Squamous Cell Cancer	oncogene	overexpression	EGFR	LINC00152/EGFR/PI3K/AKT/P21	(63)
Esophageal Squamous Cell Cancer	oncogene	overexpression	Not reported	Not reported	(64)
Esophageal Squamous Cell Cancer	oncogene	overexpression	LINC00152/miR-107/Rab10	LINC00152/miR-107/Rab10	(65)
Esophageal Squamous Cell Cancer	oncogene	overexpression	miR-153-3p/FYN	LINC00152/miR-153-3p/FYN	(66)
Head and neck squamous cell cancer	oncogene	overexpression	miR-608/EGFR	LINC00052/miR-608/EGFR	(67)
Head and neck squamous cell cancer	oncogene	overexpression	Not reported	Not reported	(68)
Gastric cancer	oncogene	overexpression	EZH2/CXCL9, 10/CXCR3	LINC00152/EZH2/CXCL9,10/CXCR3	(69)
Gastric cancer	oncogene	overexpression	ERK/MAPK	LINC00152/ERK/MAPK	(70)
Gastric cancer	oncogene	overexpression	Bcl-2	LINC00052/Bcl-2	(71)
Gastric cancer	oncogene	overexpression	Not reported	Not reported	(72)
Gastric cancer	oncogene	overexpression	microRNA-193a-3p/MCL1	LINC00152/microRNA-193a-3p/MCL1	(73)
Gastric cancer	oncogene	overexpression	EGFR/PI3K/AKT	EGFR/PI3K/AKT	(74)
Pancreatic cancer	oncogene	overexpression	miR-205-5p/CDK6	LINC00152/miR-205-5p/CDK6	(75)
Pancreatic cancer	oncogene	overexpression	miR-150	LINC00152/miR-150	(76)

to poor prognosis in ESCC patients. Functionally, the overexpression of LINC00152 promotes the proliferation, invasion, and migration of ESCC cells *in vitro* and also regulates the interaction between mitotic arrest-deficient 2-like 1 (MAD2L1) and cyclin-dependent kinase 6 (CDK6) in vesicle transport pathway proteins, and syntaxin 3 (STX3) and STX12 soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) family members (64). Ding et al. (63) found that LINC00152 knockdown might inhibit proliferation and induce apoptosis of Eca-109 and KYSE-150 cells by inhibiting the anti-tumor epidermal growth factor receptor EGFR/PI3K/AKT pathway and enhancing P21 expression in EC (63). In addition, Zhou et al. (38) found that LINC00152 regulates Rab10 by sponging miR-107 to promote cell proliferation, migration, and invasion in ESCC (65). Liu et al. (66) found that LINC00152 promotes ESCC proliferation and inhibits apoptosis by downregulating miR-153-3p and promoting FYN expression (66). Therefore, LINC00152 is an optimal candidate as a therapeutic target for the treatment of EC.

Gastric cancer

Gastric cancer (GC) is the fifth most common cancer and the third most common cause of cancer-related deaths

TABLE 2 Biomarkers of LINC00152 in various cancers.

Cancer	biomarker type	functional role	Reference
Leukemia	prognostic marker	chemoresistance	(8)
Leukemia	early relapse and mortality	metastasis, relapse and chemoresistance	(9)
Bladder cancer	diagnosis and prevention	proliferation, metastasis, invasion, clonogenicity, apoptosis	(10)
Nasopharyngeal cancer	therapeutic targets	invasion and metastasis	(11)
Gallbladder cancer	prognostic markers	proliferation, metastasis, apoptosis	(12)
Gallbladder cancer	prognostic	metastasis and progression	(13)
Lung cancer	not reported	proliferation, invasion and migration	(16)
Lung cancer	prognosis	proliferation, invasion, migration, growth	(15)
Lung cancer	progression, prognosis	proliferation, migration, growth invasion	(16)
Lung cancer	diagnosis, prognosis and attenuation	proliferation, migration and invasion	(17)
Lung cancer	diagnosing and monitoring	not reported	(14)
Lung cancer	prognosis	proliferation, migration, invasion and radiosensitivity	(18)
Lung cancer	diagnostic markers	proliferation, cell apoptosis	(19)
Hepatocellular Cancer	not reported	not reported	(20)
Hepatocellular Cancer	diagnosis	proliferation, growth	(21)
Hepatocellular Cancer	not reported	proliferation, tumor growth, apoptosis	(2)
Hepatocellular carcinoma	not reported	proliferation	(22)
Hepatocellular Carcinoma	prognostic marker	tumor autophagy	(23)
Hepatocellular Carcinoma	not reported	proliferation and invasion	(24)
Hepatocellular carcinoma	prognosis	autophagy	(27)
Hepatocellular Carcinoma	not reported	proliferation, cell cycle and apoptosis	(25)
Hepatocellular Carcinoma	not reported	colony formation, apoptosis, migration and invasion	(26)
Osteosarcoma	not reported	proliferation, migration, invasion	(28)
Osteosarcoma	not reported	G0/G1 cell cycle, proliferation, apoptosis	(29)
Human multiple myeloma	not reported	cell cycle, apoptosis, migration and invasion,	(4)
Laryngeal cancer	diagnostic biomarker	apoptosis, cell proliferation, cell migration and invasion	(30)
Papillary thyroid carcinoma	not reported	growth and proliferation, colony formation, migration, invasion	(31)
Papillary thyroid carcinoma	not reported	migratory and invasive	(32)
Glioma	not reported	proliferation, apoptosis, migration and invasion	(34)
Glioma	prognostic biomarker	proliferation, growth, chemotherapy migration, and invasion	(35)
Glioma	not reported	migration, invasion, proliferation, EMT, epigenetic	(36)
Glioma	not reported	proliferation, migration, invasion, apoptosis	(37)
Glioma	prognosis	invasion, EMT	(33)
Glioma	not reported	proliferation, invasion, growth	(38)
Colorectal cancer	prognostic	apoptosis, chemoresistance, cell viability	(39)
Colorectal cancer	not reported	EMT and metastasis	(47)
Colorectal cancer	diagnostic biomarker	growth, proliferation	(41)
Colorectal cancer	diagnosis and therapeutic	proliferation and metastasis	(42)
Colorectal cancer	therapeutic target	invasion and metastasis	(43)
Colorectal cancer	diagnosis and treatment.	apoptosis, viability	(7)
Colorectal cancer	not reported	proliferation, invasion, and metastasis	(47)
Colorectal cancer	prognostic biomarker, therapies	progression, metastasis, invasion, EMT	(44)
Colorectal cancer	prognosis, diagnostic marker	tumorigenesis	(45)
Colorectal cancer	not reported	proliferation, invasion, cell cycle, EMT, apoptosis, migration	(46)
Oral squamous cell carcinoma	prognostic marker	chemotherapy resistance, EMT	(48)

(Continued)

TABLE 2 Continued

Cancer	biomarker type	functional role	Reference
Pan-cancer	diagnosed	proliferation, migration and invasion	(49)
Ovarian cancer	prognosis	tumor growth and metastasis	(55)
Ovarian cancer	not reported	apoptosis	(77)
Ovarian cancer	prognostic biomarker	cell proliferation, apoptosis	(56)
Ovarian cancer	not reported	proliferation and cell cycle	(57)
Breast cancer	not reported	progression	(50)
Breast cancer	treatment marker	invasion, migration, colony, growth, apoptosis	(51)
Breast cancer	not reported	tamoxifen resistance	(52)
Breast cancer	prognosis	growth, proliferation and tumorigenicity	(54)
Breast cancer	prognosis and treatment	proliferation, migration	(53)
Tongue squamous cell carcinoma	therapeutic	proliferation, migration, invasion and apoptosis	(58)
Renal cell carcinoma	prognostic marker	proliferation	(59)
Renal cell carcinoma	prognostic marker	proliferation, invasion, cell cycle, apoptosis	(60)
Esophageal squamous cell carcinoma	therapeutic	proliferation, migration, invasion, cell viability	(65)
Esophageal squamous cell carcinoma	not reported	apoptosis, cell cycle, proliferation	(63)
Esophageal squamous cell carcinoma	not reported	proliferation, apoptosis, cell cycle, migration and invasion	(64)
Esophageal squamous cell carcinoma	prognostic biomarkers	proliferation, apoptosis	(66)
Head and neck squamous cell carcinoma	not reported	proliferation, migration, differentiation	(67)
Head and neck squamous cell carcinoma	prognostic biomarkers	metastasis, apoptosis, proliferation	(68)
Gastric cancer	not reported	tumor growth	(69)
Gastric cancer	therapy	migration, apoptosis, invasion	(70)
Gastric cancer	therapy	migration, invasive, apoptosis	(71)
Gastric cancer	not reported	cell cycle, apoptosis, EMT, cell migration and invasion	(72)
Gastric cancer	prognosis	proliferation, migration, growth, invasion	(73)
Gastric cancer	therapy	proliferation and tumor growth	(74)
Pancreatic cancer cell	therapy	proliferation and migration	(75)
Pancreatic cancer cell	therapy	proliferation, migration and invasion	(76)
Retinoblastoma	prognosis	invasion and metastasis	(61)
Retinoblastoma	therapy	proliferation, apoptosis, invasion, autophagy and chemoresistance	(62)

worldwide (82). Stomach cancer has a unique geographic distribution and is common in Eastern Asian countries such as Japan and Mongolia but uncommon in Southern Africa (80). Men are twice more likely than women to have GC. As a result, novel molecular targets for GC treatment are urgently required.

LINC00152 is highly expressed in GC tissue and cells. Huang et al. (73) showed that LINC 00152 overexpression promotes GC cell proliferation through the LINC00152/miR-193a-3p/myeloid leukemia 1 (MCL1) pathway (73). *In vivo* experiments have confirmed that knockdown of LINC00152 inhibits the growth of GC xenografts by upregulating mir-193b-3p and downregulating ETS1 (72). Further research revealed that LINC00152 might directly bind to Bcl-2 to activate cell cycle signaling, promote migration and invasion, and suppress apoptosis (71). LINC00152 activates PI3K/AKT signaling by directly binding to EGFR to increase GC cell proliferation (74). An enhanced extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway significantly reverses the biological effects of GC caused by LINC00152 (70). LINC00152 can also promote the growth of tumor cells, both *in vivo* and *in vitro*, by binding enhancer of zeste homolog 2 (EZH2) and regulating the CXC motif chemokine ligand 9 (CXCL9) and CXCL10/CXCR3 axes in CD8T cells (69). LINC00152 may, therefore, be a potential prognostic biomarker and therapeutic target for GC in the future.

Colorectal cancer

Colorectal cancer (CRC) is the fourth most common cancer globally. Li et al. (45) monitored the overexpression of LINC00152 in colon cancer and found that it was significantly associated with poor prognosis. LINC00152 is positively linked to invasion depth, TNM stage, lymph node metastasis, and

TABLE 3 The role of LINC00152 in chemotherapy and radiation resistance.

Cancer	Drug	Chemotherapy resistance	Radiation therapy resistance	References
Lung Cancer	Not reported	Not reported	silencing LINC00152 promoted miR−206 to enhance the radiosensitivity of NSCLC cells	(17)
Lung Cancer	Not reported	Not reported	overexpression of LINC00152 decreased miR-195 expression in H1299 and H1581 and suppressed the radiosensitivity of NSCLC cells	(18)
Colorectal Cancer	Not reported	the invasion and metastasis of residual CRC cells increased following radiotherapy and chemotherapy	invasion and metastasis of residual CRC cells increased following radiotherapy and chemotherapy	(43)
Colorectal Cancer	Oxaliplatin	AKT activation mediated by ERBB4 contributes to Linc00152-conferred L-OHP resistance	not reported	(39)
Leukemia	Adriamycin	LINC00152 Regulates LSC Chemoresistance *Via* PARP1	not reported	(8)
Glioma	Temozolomide	Knockdown of LINC00152 increases sensitivity to chemotherapy	not reported	(35)
Oral Squamous Cell Carcinomas	Cisplatin	overexpression of FOXD1 promotes chemoresistance *in vivo*	not reported	(48)
Pan-Cancer	Anthracycline	Linc00152 induces chemoresistance in pan-cancer	not reported	(49)
Ovarian Cancer	Cisplatin	LINC00152 knockdown enhances the sensitivity of ovarian cancer cells to cisplatin.	not reported	(77)
Breast Cancer	Tamoxifen	LINC00152 regulates tamoxifen sensitivity *via* SRF in breast cancer cells	not reported	(52)
Retinoblastoma	Adriamycin, Carboplatin	LINC00152 enhanced the aggressiveness of retinoblastoma and boosted carboplatin and Adriamycin resistance by regulating YAP1 by sponging miR-613 in human retinoblastoma.	not reported	(62)

carbohydrate antigen 19-9 (CA19-9) levels according to clinicopathological examinations (41). LINC00152 was reported to regulate the biological characteristics of residual CRC cells after radiotherapy and chemotherapy, and promote the migration and increased invasion of residual cells (43). The heterotrimeric complex of LINC00152, NCL, and SAM68 activates the nuclear factor-kappa B (NF-κB) pathway and EMT and thus promotes CRC progression (44). High SAM68 expression was inversely related to the overall survival rate. Our current research suggests that SAM68 can specifically recognize the binding site in exon1 of LINC00152, and the formation of the NCL-LINC00152-SAM68 complex can activate the NF-κB signaling pathway, thus promoting the EMT and metastasis of CRC (44). In addition, LINC00152 can promote tumor

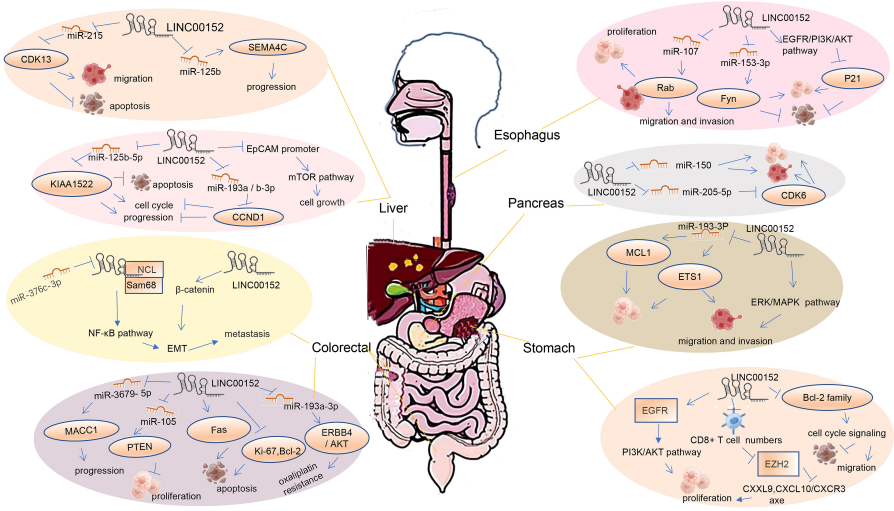

FIGURE 3
Mechanism of LINC00152 in regulating digestive system cancer.

progression and proliferation through the LINC00152/miR-3679-5p/MACC1 axi (45). LINC00152 was used as a competitive endogenous RNA to make oxaliplatin-resistant colon cancer-sponging miR-193a-3p *via* the LINC00152/miR-193a-3p/erbb4/Akt signaling axis (39). LINC00152 is negatively regulated by miR-376c-3p and may suppress the viability of colon cancer cells and contribute to apoptosis by regulating the expression of Ki-67, Bcl-2, and Fas (7). Hypomethylation of the LINC00152 promoter is closely related to its increased expression (46). In addition, Yue et al. (40) found a positive feedforward loop between LINC00152 and Wnt/β-catenin signaling that promotes colon cancer metastasis and EMT (40). Ou et al. (2019) identified that YAP1 target LINC00152, which promoted the biological characteristics of CRC cells by sponging miR-185-3p and miR-632 for upregulating its target FSCN1, as an "YAP1/LINC00152/FSCN1" axis to promote the malignant proliferation, migration and metastasis in CRC (47).

However, Zhang et al. (42) reported that LINC00152 inhibits proliferation and metastasis of colon cancer cells through regulating miRNA-105/PTEN axis (42). This is oppositive with other studies in colon cancer. More experiments need to identify the function of LINC00152 in colon cancers.

Hepatocellular cancer

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer and is the sixth most common neoplasm (80). LINC00152 expression was elevated in HCC tissue compared to that in normal and precancerous tissue. Hu et al. (25) reported that interfering with LINC00152 can inhibit proliferation, arrest the cell cycle, and promote apoptosis of hepatocellular cancer cells by regulating the miR-125b-5p/KIAA1522 axis (25). Wang et al. (26) demonstrated that silencing LINC00152 inhibited HCC development by modulating miR-215 to upregulate CDK13 (26). Deng et al. (24) found that HBx enhances the expression of LINC00152 and promotes the proliferation and invasion of HCC cells (24). LINC00152 acts as a ceRNA by sponging miR-193a/b-3p to regulate CCND1 expression to inhibit cell cycle progression (22). Deng et al. (23) found that autophagy-associated genes (ARG) are associated with the prognosis of HCC patients (23). LINC00152 promotes the proliferation and tumor growth of HCC cells by sponging miR-125b and upregulating the expression of semaphorin-4C (SEMA4C) (2). Similarly, Ji et al. (21) showed that LINC00152 could activate the mammalian target of rapamycin (mTOR) signaling pathway through a combination of EpCAM promoters in a cis-regulated manner, which promotes HCC cell proliferation *in vitro* and tumor growth *in vivo* (21). In hepatocellular cancer, a signature of immunoautophagy-related lncRNA (IAR-lncRNA) predicts survival (27). LINC00152 can be used as a biomarker for the differential diagnosis of liver cancer (20). Our growing understanding of LINC00152 suggests that targeting it may be a unique therapeutic strategy for hepatocellular carcinoma.

Gallbladder cancer

Gallbladder cancer (GBC) is the most common and aggressive malignancy of the biliary system (83). Some gallbladder cancers can be cured by radical cholecystectomy, whereas metastases to other organs require chemotherapy, and some patients also require postoperative adjuvant chemotherapy (84). LINC00152 is significantly upregulated in gallbladder cancer, and the upregulation of LINC00152 by SP1 promotes gallbladder cancer cell growth and tumor metastasis by targeting the PI3K/AKT signaling pathway (12). LINC00152 can inhibit the expression of HIF-1a by functioning as a miRNA sponge to abrogate the endogenous effect of miR-138, which promotes GBC metastasis and EMT (13). This suggests that LINC00152 could be used as a therapeutic target for GBC treatment.

Pancreatic cancer

Pancreatic cancer (PC) remains a life-threatening disease, with a five-year survival rate of only 10% and an overall poor prognosis. PC lacks tools for early diagnosis, and treatment choices are limited. LINC00152 is remarkably upregulated in PC tissue and cell lines. Yuan et al. (76) found that LINC00152 promotes the proliferation, migration, and invasion of pancreatic cancer cells by inhibiting the expression of miR-150 (76). Inhibition of CDK6 expression by LINC00152 sponges miR-205-5p and promotes the proliferation and migration of tumor cells (75). These results indicate that LINC00152 may be an effective diagnostic biomarker and therapeutic target for PC.

Nasopharyngeal cancer

The expression of LINC00152 in nasopharyngeal cancer tissue and cells is increased compared to in normal tissue and cells. LINC00152 competitively binds to miR-613 to induce ANXA2 upregulation, thus promoting the invasion and metastasis of nasopharyngeal cancer cells (11)

Laryngeal cancer

LINC00152 is significantly upregulated in laryngeal squamous cell carcinoma (LSCC) tissue and is correlated with poor prognosis (85). LINC00152 sponges miR-613, thus promoting the proliferation, migration, and invasion of

laryngeal cancer cells, and inducing apoptosis (30). Our results highlight the role of LINC00152 as a therapeutic target for laryngeal cancer.

Lung cancer

Lung cancer is the second most common type of cancer worldwide. LINC00152 promotes the growth, invasion, and migration of lung adenocarcinoma cells and is associated with a poor prognosis (16). Chen et al. (19) found that the interaction of LINCOO152 with EZH2 inhibits interleukin-24 (IL24) transcription to promote lung adenocarcinoma proliferation, and ectopic expression of IL24 partially reversed the LAD cell growth promotion induced by LINC00152 overexpression (19) (Figure 4). LINC00152 enhances non-small cell lung cancer (NSCLC) cell proliferation, migration, and invasion, and decreases radiosensitivity in NSCLC cells *in vitro* by sponging miR-195 (18). LINC00152 knockdown inhibits the proliferation, invasion, and migration of lung cancer cells through the EGFR/PI3K/AKT pathway, and improves apoptosis and the G1 phase ratio (14).Silencing LINC00152 enhanced the radiosensitivity of NSCLC cells by upregulating miR-206 and inhibiting prothymosin alpha (PTMA). Gaining an understanding of the role of LINC00152 in the radiosensitivity of NSCLC identified new potential targets for the clinical treatment of NSCLC (17). Hu et al. (15) found that LINC00152 silencing restrained tumorigenesis in NSCLC by regulating the miR-16-5p/BCL2L2 axis (15). LINC00152 may be a valuable biomarker for diagnosing and monitoring NSCLC (15).

Ovarian cancer

The expression of LINC00152 in epithelial ovarian cancer tissue was significantly upregulated compared to in normal tissue (57). An *in vitro* study found that LINC00152 regulates cell proliferation and cell cycle in SKOV3 cells (57). LINC00152 may competitively inhibit miR125b upregulation of MCL1 expression, which modulates the mitochondrial apoptosis pathway during ovarian cancer progression (56). LINC00152 knockdown boosted epithelial ovarian cancer cell chemosensitivity to cisplatin by enhancing apoptosis and reducing the expression levels of MDR1, MRP1, and GST (77). Wang et al. (55) found that LINC00152 binds to Ser333/Ser343 of B-cell lymphoma 6 (BCL6) and stabilizes it against ubiquitination to promote ovarian tumor proliferation and invasion (55).

Breast cancer

Breast cancer (BC) is the most common malignancy worldwide, accounting for 15% of deaths among women (79). The expression of LINC00152 was significantly increased in triple-negative breast cancer tissue and cells. YY1 binds to the LINC00152 promoter to inhibit the transcription of LINC00152, which weakens the stability of PTEN and promotes the progression of triple-negative breast cancer (50). LINC00152 regulates genes involved in the rapamycin pathway of EGFR/mTOR and is required for cell proliferation, migration, and cytoskeleton organization (53).

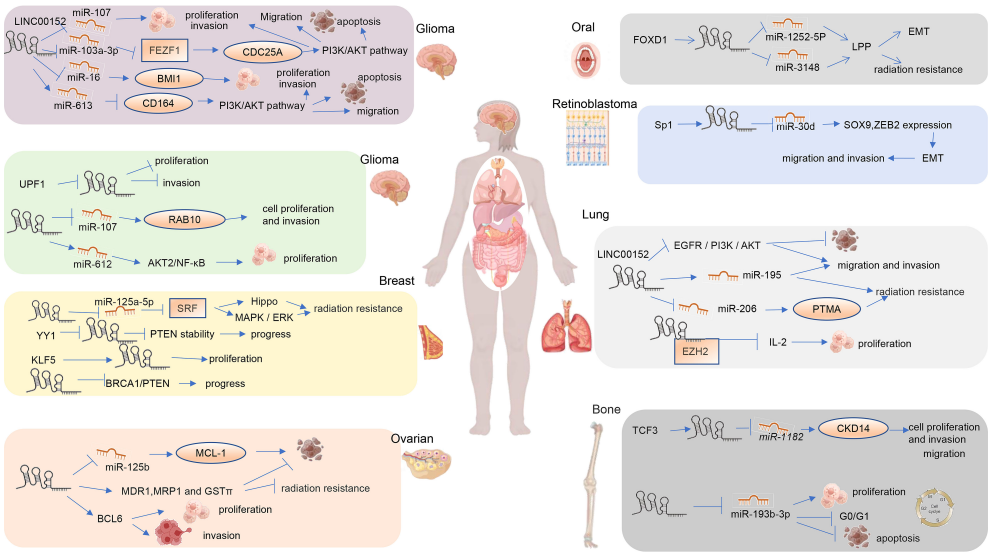

FIGURE 4
Mechanism of LINC00152 in regulating other human cancers.

Positive feedback loops of LINC00152 and KLF5 promote breast cancer growth and proliferation (54). LINC00152 binding to miR-125a-5p promotes tamoxifen resistance by inhibiting serum response factor (SRF), thereby activating the MAPK/ERK and Hippo pathways. LINC00152 also promotes tamoxifen resistance in breast cancer cells by sponging miR-125a-5p (52). Wu et al. (51) found that LINC00152 knockdown inhibits breast cancer cell invasion, migration, tumor growth, and colony growth, and triggers apoptosis through a mechanism that activates breast cancer type 1 (BRCA1)/phosphatase and tensin homolog (PTEN) *via* DNA methyltransferase (DNMT) inactivation (51).

Bladder cancer

Tang et al. (2019) found that LINC00152 promotes bladder cancer cell viability, migration, invasion, and EMT by activating the Wnt/ß-Catenin signaling pathway (10). This is rare research about the role of LINC00152 in bladder cancer. This is an area for urgent attention, and more intensive research is warranted going forward.

Renal cell cancer

LINC00152 is involved in the progression of clear cell renal cell cancer (ccRCC) and is a potential prognostic biomarker and therapeutic target for ccRCC (60). By epigenetically suppressing P16 expression and interacting with miR-205, LINC00152 may contribute to renal cell cancer progression (59). Despite the potential role of LINC00152 in ccRCC, there has not been sufficient focus on this field in recent years. Additional research is required to determine if LINC00152 is a suitable diagnostic or prognostic biomarker for renal cell carcinoma.

Leukemia

Leukemia is the most common childhood cancer, accounting for 28% of cases. High LINC00152 expression is associated with poor survival in acute myeloid leukemia (AML) patients. LINC00152 promotes poly [ADP-ribose] polymerase 1 (PARP1) expression to induce chemoresistance and regulate the self-renewal of leukemic stem cell (LSC) self-renewal. The inhibition of LINC00152 increased the sensitivity of leukemic cells to doxorubicin. These results suggest that LINC00152 may serve as a potential prognostic marker in AML patients (8). Transcriptome analysis has identified LINC00152 as a biomarker for early relapse and mortality in acute lymphoblastic leukemia (9)

Thyroid cancer

Thyroid cancer (PTC) is the most common endocrine cancer. TRIM29 reduces miR-873-5p expression by upregulating LINC00152 to upregulate FN1, thereby promoting PTC migration and invasion (32). LINC00152 acts as a ceRNA miR-497 sponge, downregulating its downstream target brain-derived neurotrophic factor (BDNF) to promote cell proliferation, colony formation, migration, and invasion (31).

Glioma

LINC00152 is upregulated in glioma tissue and cells and negatively correlates with UPF1 levels. LINC00152 promotes the proliferation and invasion of glioma cells by inducing BMI1 expression by sponging miR-16 (86). Peng et al. (87) found that LINC00152 promotes tumor proliferation and invasion through the LINC00152/miR-107/RAB10 axis (87). LINC00152 functions as an oncogene in glioblastoma cells, promoting cell proliferation and invasion, in part by targeting miR-107 expression (88). Zou et al. (38) found that UPF1 downregulates LINC00152 to suppress the growth and invasion of glioma cells (38). Functionally, LINC00152 promotes the proliferation, migration, invasion, and induction of apoptosis of glioma cells, and reduces their sensitivity to *in vitro* chemotherapy (35). Mechanistically, LINC00152 binds to miR-103a-3p to suppress FEZ family zinc finger 1 (FEZF1), thereby promoting cell division cycle 25 A (CDC25A) expression to promote the PI3K/AKT pathway to exert these functions in malignant glioma (37). Through the PI3K/AKT pathway, the LINC00152/miR-613/CD164 axis affects cell proliferation, apoptosis, migration, and invasion in glioma (34). LINC00152 promotes invasion through a 3′-hairpin structure and is related to glioblastoma prognosis (33). Blocking LINC00152 reduces glioblastoma malignancy by affecting the mesenchymal phenotype *via* the miR-612/AKT2/NF-B pathway (89). Consequently, blocking LINC00152 decreases glioblastoma malignancy (33). However, LINC00152 has opposing effects in different types of glioblastoma cells (36). LINC00152 knockdown stimulates migration and invasion of A172 GBM cells, whereas knockdown of LINC00152 in other glioblastoma cell lines (U87-MG and LN299) leads to tumor suppression, as it serves as an oncogene (36). In summary, LINC00152 may serve as a prognostic marker and new therapeutic target for glioma.

Head and neck squamous cell cancer

LINC00152 is involved in multi-step pathological processes in head and neck squamous cell cancer (HNSCC), such as

ribosomal biogenesis and maintenance of genomic stability (68). LINC000152 is positively correlated with lymph node metastasis and negatively correlated with overall survival (OS) and disease-free survival (DFS) in HNSCC patients (68). Upregulated LINC00052 expression in head and neck cancers is associated with poor prognosis. LINC00052 acts as a ceRNA for miR-608 to regulate the expression of epidermal growth factor receptor (EGFR), thus promoting the proliferation, migration, and invasion of HNSCC (67).

Osteosarcoma

LINC00152 acts as a ceRNA binding miR-193b-3p, leading to increased cell proliferation, G0/G1 cell cycle arrest, and reduced apoptosis, thus promoting osteosarcoma development (29). Zheng et al. (28) found that transcription factor 3 (TCF3) activates LINC00152 to act as a ceRNA to sponge miR-1182 and upregulate the expression of CDK14, thus promoting the proliferation, migration, and invasion of osteosarcoma cells (28).

Multiple myeloma

IL-6 mediates STAT3 activation, and positive feedback induces LINC00152 expression, which is a critical factor for the survival of INA-6 multiple myeloma cells (4). LINC00152 is overexpressed in osteosarcoma cells (4). At present, there are rare studies on the role and mechanism of LINC00152 in multiple myeloma, and further studies are needed.

Retinoblastoma

LINC00152 is upregulated in retinoblastoma tumor tissue. 61 found that LINC00152, which is activated by Sp1, can sponge miR-30d, thus significantly increasing the expression of SOX9 and zinc finger E-box-binding homeobox 2 (ZEB2), inducing EMT, and promoting the invasion and metastasis of retinoblastoma cells (61). LINC00152 regulates the expression of YAP1 in retinoblastoma cells by sponging miR-613, thus promoting proliferation, invasion, apoptosis, autophagy, and chemical resistance of retinoblastoma cells (62).

We summarized the role and mechanisms of LINC00152 in various cancer types. It indicated the potential cancer diagnosis and prognosis value of LINC00152. More importantly, LINC00152 also play an important role in radiotherapy and chemotherapy resistance.

The role and mechanism of LINC00152 in radiotherapy and chemotherapy resistance

LINC00152 plays a vital role in the resistance to radiotherapy and chemotherapy. We summarized the mechanisms by which LINC00152 confers resistance to chemotherapy in Figure 5. LINC00152 is highly expressed in NSCLC, and silencing of LINC00152 enhances the radiosensitivity of NSCLC cells by upregulating miR-206 and inhibiting prothymosin α(PTMA). LINC00152 knockdown and

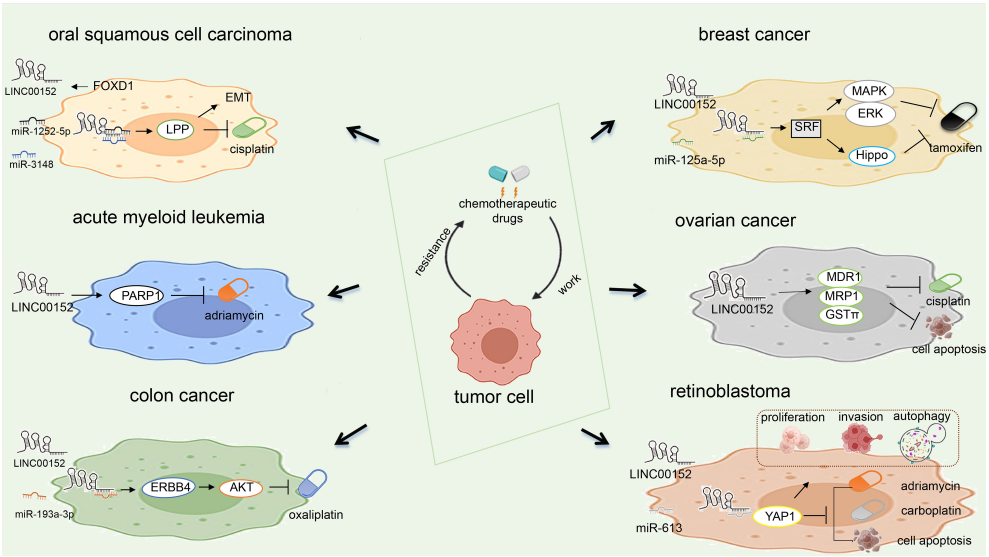

FIGURE 5
Mechanism of LINC00152 in chemotherapy resistance.

control cells were administered subcutaneously into mice as part (17). The tumor weight and size in the knockdown group were significantly reduced after radiation, demonstrating that LINC00152 knockdown improved the radiosensitivity of xenograft tumors in mice in an animal study (17). Silencing LINC00152 may therefore represent a strategy for the treatment of NSCLC. However, this study was conducted in the context of radiation therapy in NSCLC and did not explore the role of LINC00152 in other cancer cells, which should be explored further in the future.

LINC00152 enhances NSCLC proliferation, migration, and invasion, and alleviates radiosensitivity *in vitro* by sponging miR-195 (18). Further research showed LINC00152 inhibited radiosensitivity of NSCLC cells *in vitro* and *in vivo*. The increased radiosensitivity achieved by knocking down LINC00152 sponging of miR-195 can improve the prognosis of patients with NSCLC. LINC00152 may serve as a prognostic marker and promising therapeutic target for patients with NSCLC. However, the role of LINC00152 in chemotherapy or molecular-targeted therapy has not been reported for lung cancer. Therefore, the regulatory role of LINC00152 in drug resistance in lung cancer remains unknown.

Chen et al. (43) found that LINC00152 is involved in regulating the invasion and metastasis of residual CRC cells after chemoradiotherapy. Author established residual CRC cells models, which was intended to mimic the clinical treatment model as far as possible. Transwell experiments prove that the migration and invasion of the residual CRC cells were significant increased compared with the original cells. LINC00152 is a potential biomarker of altered biological characteristics caused by chemoradiotherapy in CRC cells (43). There is a solid theoretical basis for further research to improve the CRC therapy and improve the prognosis of patients with CRC.

Cui et al. (8) demonstrated that LINC00152 promotes PARP1 expression, which induces chemoresistance in acute myeloid leukemia and regulates the self-renewal of LSCs (8). In addition, knockdown of LINC00152 can inhibit PARP1 expression to improve the sensitivity of leukemia cells to chemotherapy, thus improving the prognosis of leukemia patients. These findings indicate that the LINC00152/PARP1 pathway could be used as a new therapeutic target for AML.

LINC00152 may serve as a potential prognostic biomarker for high-grade glioma (HGG) patients and is, therefore, a potential therapeutic target for gliomas. Further studies are needed to identify the mechanisms by which LINC00152 regulates glioma and verify its clinical application in patients with glioma. In addition, further research suggests that patients with low expression of LINC00152 had longer OS than that of the other groups. Moreover, assay showed knockdown of LINC00152 increased the sensitivity of chemotherapy in TMZ-resistant LN229 and SNB19 cells. Wang et al. (44) reported that knockdown of LINC00152 suppresses the proliferation, invasion, and migration of glioma cells *in vitro* and increases their sensitivity to chemotherapy (35).

Yue et al. found that colon cancer cells display different response to oxaliplatin treatment and LINC00152 antagonize oxaliplatin-induced apoptosis LINC00152 regulates oxaliplatin resistance by sponging miR-193a-3p and then regulates ERBB4 *in vitro*. Besides, LINC00152 mediates oxaliplatin resistance through sponging miR-193a-3p in xenograft model. Further research found that AKT activation mediated by ERBB4 contributes to LINC00152-conferred oxaliplatin resistance. Collectively, LINC00152 promotes oxaliplatin resistance by sponging miR-193A-3P to participate in the LINC00152/miR-193A-3P/ERBB4/AKT signal axis as a competitive endogenous RNA (39).

Chen et al. (11) found that FOXD1 upregulates LINC00152 as a ceRNA to inhibit miR-1252-5p and miR-3148, thereby upregulating LPP expression to promote EMT and chemotherapy resistance in OSCC (90). In this previous study, Further studies reduced the role of EMT in OSCC by silencing FOXD1, thus increasing chemosensitivity and promoting apoptosis. This finding indicates that overexpression of FOXD1 promotes cisplatin resistance *in vitro* and *in vivo* by regulating the EMT of OSCC cells. Whereas silencing FOXD1 inhibits cisplatin resistance, suggesting that FOXD1 may be a potential prognostic marker and anti-drug resistance therapeutic target. New evidence is expected for the role of FOXD1 and the chemical resistance of OSCC involved by FOXD1. However, the detailed mechanisms of FOXD1 upregulation in OSCC remain unexplored and will be the focus of our future research.

Xu et al. found that LINC00152 induces chemoresistance in pan-cancer, resulting in a poor prognosis for pan-cancer patients (49). The mechanisms underlying LINC00152's upregulation in pancreatic cancer is unknown. This research broadened the carcinogenic role of lncRNA in pancreatic cancer and revealed that LINC00152 might be a potential therapeutic target and contribute to the comprehensive management of pancreatic cancer.

The expression level of LINC00152 in epithelial ovarian cancer cells is upregulated. The knock-down of LINC00152 increases the chemosensitivity of epithelial ovarian cancer cells to cisplatin by increasing apoptosis and decreasing the expression levels of MDR1, MRP1, and GSTπ (77). This study only investigated the effect of LINC00152 silencing on cisplatin resistance in COC1 and COC1/DDP cells but did not explore the effect of LINC00152 overexpression on drug resistance. This needs to be further verified on other ovarian cancer cell lines and animal models.

Liu et al. (66) found that LINC00152 improves serum response factor (SRF) expression by sponging miR-125a-5p to activate the MAPK/ERK and Hippo pathways to promote tamoxifen resistance in breast cancer cells. In addition, the prognosis of patients with breast cancer can be improved by

promoting tamoxifen sensitivity in breast cancer cells by knocking down LINC00152 to inhibit SRF (52).

LINC00152 regulates the expression of YAP1 in retinoblastoma cells by sponging miR-613, and knockdown of YAP1 eliminates the miR-613-mediated effects on retinoblastoma cell proliferation, invasion, apoptosis, autophagy, and chemical resistance (62). In addition, Wang et al. (55) also found that knockdown of LINC00152 increased the chemosensitivity of retinoblastoma to carboplatin and doxorubicin by regulating miR-613.

In total, LINC00152 plays an important role in chemotherapy and radiotherapy resistance through regulating microRNA, protein, or classical signaling pathway. LINC00152 may be a potential sensitizer for radiotherapy and chemotherapy in the future.

The role and mechanism of LINC00152 in cancer recurrence

LINC00152 as a tumor marker to predict tumor recurrence has been reported in various cancers. A meta-analysis showed that LINC00152 overexpression is significantly related to poor overall survival and poor disease-free survival (91). Meanwhile, LINC00152 is a biomarker of early relapse and mortality in acute lymphoblastic leukemia according to transcriptome analysis (9). In retinoblastoma, LINC00152 is activated by SP1 to inhibit miR-30d and thus regulate the expression of SOX9 and ZEB2 to promote tumor recurrence (61). The Kaplan-Meier analysis suggested that high LINC00152 expression leads to significantly lower DFS rates in lung adenocarcinoma. CCK8 assay and the colony-forming assay showed LINC00152 stimulated tumor cell proliferation in lung adenocarcinoma (92). Immunochemistry staining found that LINC00152 was related to nuclear accumulation of β-catenin in colon cancer tissues and have a prognostic value (40). Li et al. (14) found that LINC00152 binds to KLF5 to induce breast cancer cell proliferation and predicts poor prognosis. Yu et al. (5) found that LINC00152 expression was significantly upregulated in tongue squamous cell carcinoma and high LINC00152 expression was closely associated with progression and poor prognosis (93).More studies about the role and mechanism of linc00152 in cancer recurrence are needed.

The role and mechanism of LINC00152 in immunotherapy response

LINC00152 is also reported to involved in immunotherapy response. Ou et al. (69) found that LINC00152 mediates $CD8^+$ T cell infiltration in gastric cancer by binding to EZH2 and regulating CXCL9,10/CXCL9 axis. The inhibition of LINC00152 may inhibit the progression of gastric cancer *in vivo* by promoting $CD8^+$ T cell infiltration immune response (69). TCGA database indicated that LINC00152 and HMGA1 regulate each other. Chen et al. (92) found that LINC00152 acts as a competitive endogenous RNA to regulate the expression of HMGA1. LINC00152 and HMGA1 play an important role in the cell cycle and proliferation of GC cells, through reducing the infiltration of immune cells and the 28 types of tumor-infiltrating lymphocytes (TILs) found in human cancers (94). More studies about the role and mechanism of LINC00152 in immunotherapy response are needed.

Discussion

Dysregulation of lncRNAs is associated with various malignant behaviors of cancer cells, such as cancer progression and metastasis. LINC00152 is significantly upregulated in most cancer tissue and cell lines, and is associated with poor prognosis. Clinicopathological analysis has shown that LINC00152 is positively associated with tumor infiltration depth, TNM stage, lymph node metastasis, and CA19-9 levels (41). LINC00152 research has recently flourished, confirming their role in regulating diverse functions such as proliferation, apoptosis, EMT, migration, invasion, cell cycle, and chemotherapy and radiotherapy resistance in various human cancers.

LINC00152 is overexpressed and plays an oncogenic role in many types of tumors, including lung, hepatocellular, ovarian, and esophageal cancer. LINC00152 can contribute to tumor progression in certain cancer types. Chen et al. (19) found that the interaction between LINCOO152 and EZH2 inhibits IL24 transcription to promote lung adenocarcinoma proliferation. However, downregulation of LINC00152 in serum-derived exosomes has been observed in CRC patients (95).

The mechanisms by which LINC00152 promotes tumor development are highly complex, including serving as a ceRNA sponge for miRNA, interacting with proteins, activating signaling pathways, and regulating epigenetic regulation. LINC00152 is involved in various signaling pathways leading to cancer progression, including the ERK/MAPK, β-catenin, mTOR, and PI3K signaling pathways. Several experiments have confirmed the role of lncRNAs in epigenetics, transcription, and gene expression, and lncRNAs, circRNAs, and miRNAs can act as ceRNAs to interact with mRNA and regulate cell function (96) (Figure 6). LINC00152 can act as a ceRNA to regulate HMGA1 expression in GC cells (94). The molecular mechanism by which LINC00152 participates in multiple cancers has been preliminarily explored. However, further in-depth analysis is warranted, particularly in cancers that are poorly understood or have limited treatment options.

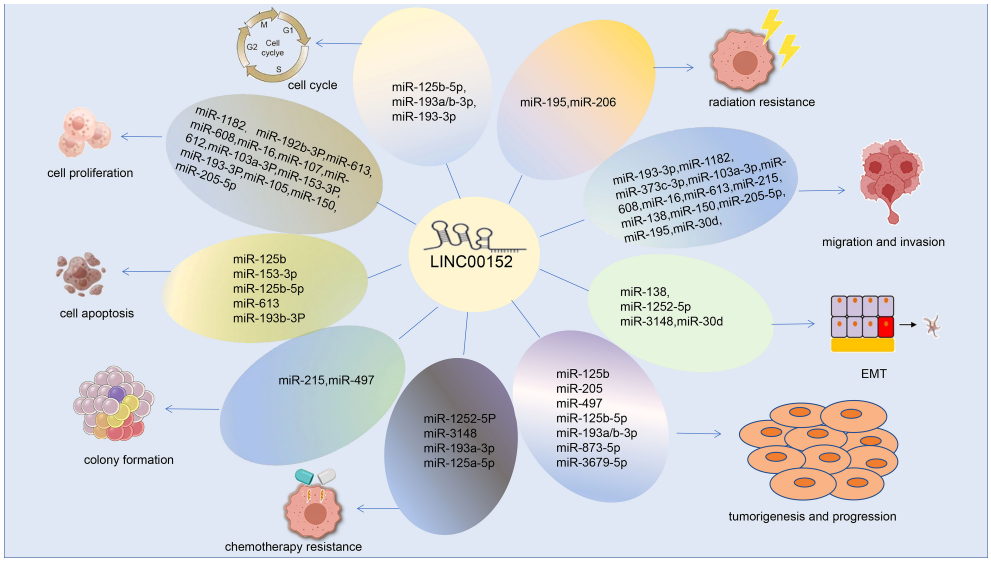

FIGURE 6
LINC00152 and miRNA-related regulatory mechanisms.

LINC00152 play an important role in radiotherapy and chemotherapy. LINC00152 was reported to induce chemoresistance in pan-cancer, resulting in poor patient prognosis (49). Wang et al. (44) reported that knockdown of LINC00152 increases the sensitivity to chemotherapy in glioma (35). In addition, knockdown of LINC00152 increased the chemosensitivity of carboplatin and doxorubicin in retinoblastoma (62). We summarized the role and mechanism of LINC00152 in chemotherapy in Figure 5. There are rare studies about the role and mechanism of LINC00152 in radiotherapy. Only 2 papers reported that LINC00152 reduced the radiosensitivity by sponging miR-195 or miR-206 in NSCLC (17, 18). It remains unknow about the role of LINC00152 in radiotherapy in other cancers. LINC00152 could be used as a potential chemotherapy and radiotherapy sensitization targets and may contribute to the prognosis of cancer patients.

This review provides a comprehensive description of the role and mechanisms of LINC00152 in various cancer types, with an emphasis on chemotherapy and radiotherapy resistance. More studies are needed on LINC00152 to elucidate the mechanisms of chemoradiotherapy resistance and improve the prognosis of patients with cancer.

LINC00152 could be a potential biomarker for cancer diagnosis and prognosis, and may be a promising therapeutic target due to its important role in cancer. The source of LINC00152, the mechanism of LINC00152, and its clinical application require further investigation. Only once these mechanisms are fully understood can LINC00152 be used in the clinical setting for treating cancer.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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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.

Publisher's Note

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Epigenetic regulation of cutaneous T-cell lymphoma is mediated by dysregulated lncRNA MALAT1 through modulation of tumor microenvironment

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Cutaneous T-Cell Lymphoma (CTCL) is a rare non-Hodgkin lymphoma marked by migration of T-lymphocytes to the skin. It has many subtypes some of which are aggressive with documented metastasis. We investigated a possible role of lncRNA MALAT1 in CTCL cells because of its documented involvement in cancer metastasis. A screening of MALAT1 in CTCL patients revealed its elevated levels in the patients, compared to healthy individuals. For our investigation, we employed HH and H9 CTCL cells and silenced MALAT1 to understand the MALAT1 mediated functions. Such silencing of MALAT1 resulted in reversal of EMT and inhibition of cancer stem cell phenotype, along with reduced cell growth and proliferation. EMT reversal was established through increased E-cadherin and reduced N-cadherin while inhibition of cancer stem cell phenotype was evident through reduced Sox2 and Nanog. CTCL patients had higher circulating levels of IL-6, IL-8, IL-10, TGFβ, PGE2 and MMP7 which are factors released by tumor-associated macrophages in tumor microenvironment. MALAT1 sponged miR-124 as this tumor suppressive miRNA was de-repressed upon MALAT1 silencing. Moreover, downregulation of miR-124 attenuated MALAT1 silencing effects. Our study provides a rationale for further studies focused on an evaluation of MALAT1-miR-124 in CTCL progression.

KEYWORDS

cutaneous T-Cell lymphoma, MALAT1, miR-124, EMT, cancer stem cells

Introduction

Cutaneous T-Cell Lymphoma (CTCL) is rather a rare cancer that starts in T-lymphocytes and affects skin. It represents the most common lymphoma of skin (1) with indications of some connections with the industrialization of communities (1). Some of the most common subtypes of CTCL are Mycosis Fungoides (MF), Sézary Syndrome (SS) and the primary cutaneous anaplastic large cell lymphoma (cALCL) which together contribute to almost four-fifths of all diagnosed CTCL cases (2). The incidence of CTCL is a little over 6 cases per a population size of one million persons in the United States (3). There is indication for geographical variation in incidence rate with some Asian populations reporting relatively higher incidence of particularly rare CTCL subtypes (4).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known by its alternate name, nuclear enriched abundant transcript 2 (NEAT2), is an oncogenic long non-coding RNA (lncRNA) that has gained lot of interest in recent years (5–7). As suggested by its name, it induces metastasis of cancer cells and is therefore connected with overall increased metastatic potential of different cancers (8) although some research has even suggested its metastasis-suppressing function (9). Thus, according to many researchers, the role of MALAT1 is controversial and may need to be better elucidated through more comprehensive studies (10). Incidentally, the role of MALAT1 in CTCL is almost unknown with little evidence in the literature. This prompted us to undertake this study wherein we planned to study first the relative abundance of this lncRNA in patient samples followed by the study of its mechanism in CTCL cell lines. In its evaluation in CTCL patients, MALAT1 has previously been shown to be elevated (11). To the best of our knowledge this is only report on MALAT1 in CTCL. In view of the controversy surrounding the oncogenic vs. tumor suppressor role of MALAT1 in human cancers, we started with the quantitation of CTCL in serum of CTCL patients in our cohort and we further employed CTCL cell lines HH and H9 to further characterize the role of MALAT1 in CTCL.

Materials and methods

Cell culture

HH cells (CRL-2105) and H9 cells (HTB-176) were obtained from ATCC (USA). According to ATCC, HH is a mature T cell line derived in 1986 from peripheral blood of a patient with aggressive cutaneous T cell leukemia/lymphoma while H9 cell line is a clonal derivative of the Hut 78 cell line and the H9 clone was selected for permissiveness for HIV-1 replication. Both the cell lines were cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate (ATCC, USA). Cells were cultured in incubators with 5% CO_2.

Patients and healthy controls

A total of 10 CTCL patients and 8 healthy controls were recruited to the study. Both, CTCL patients and healthy volunteers were enrolled at the the first Hospital of Jilin University. The study involving volunteer and patient recruitment and procedures was approved by the Ethics Committee at the First Hospital of Jilin University (Approval Number 2021-680). Further, the study complied with the standards set by the Declaration of Helsinki, as enforced by the Ethics Committee at the Jilin University. All the patients and volunteers provided a written consent to be part of the study.

Serum RNA extraction

RNA was extracted from serum of CTCL patients as well as healthy controls, as described in published report (12). First, we extracted the total RNA by mixing 500 µL of serum with 1.5 mL of Trizol reagent (Thermo Fisher Scientific, China). After incubation at room temperature for 5 min, we added 40 µL of chloroform and mixed it well before further incubation for 15 minutes, followed by centrifugation at 4°C for 25 min at 12,000g. At the end of centrifugation, aqueous phase was collected and transferred to a clean and fresh new tube. Then, 1 mL of isopropanol was added and the tubes kept at room temperature for 10 min. Finally, the tubes were further centrifuged at 4°C for 15 min at 21,100 g to pellet the RNA, and the obtained pellet once washed with ethanol.

MALAT1 expression

We evaluated the expression of MALAT1 by using TaqMan Gene Expression Assay (Thermo Fisher Scientific, China; cat# Hs00273907_s1), following the protocol provided by the company and as earlier described (12). RT-qPCR was performed using the BioRad RT-qPCR machine. GAPDH (cat# Hs02786624_g1) was used as the experimentally verifiable control gene. The calculations were done using the $2^{-\Delta\Delta CT}$ method.

Cell proliferation

For cell proliferation/cell growth, we used cell counting Kit-8 (CCK-8) (Sigma, China), which uses WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-

tetrazolium, monosodium salt), to produce a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. The CCK-8 solution was directly added on top of the media in the plates with CTCL cells. The number of CTCL cells in individual wells of the 96-well plates ranged from 3000 to 5000, depending upon the cell line, length of incubation and the individual experiment. The principle of this assay is that WST-8 is bioreduced by cellular dehydrogenases to form an orange formazan product which is soluble in normal cell culture medium. The amount of formazan produced is directly proportional to the number of living cells. The WST-8 was added to cells for 4 hours and the color development was stopped by adding 10 µl of 0.1 mol/l hydrochloric acid. O.D. was read to 450 nm using a Shimadzu spectrophotometer.

qRT-PCR for miRNA and mRNA detection

RNA extractions were carried out using Trizol (Thermo Fisher Scientific, China) and re-suspended in nuclease-treated H_2O. cDNA synthesis was prepared by the miR-specific, U6 snRNA-specific or oligo-dT primers method using the Superscript II Reverse Transcription kit (Thermo Fisher Scientific, China). Quantitative PCR was performed using BioRad machine. miRNA or mRNA levels were determined, relative to U6 or GAPDH expression using the SYBR Green PCR kit (Thermo Fisher Scientific, China), respectively. Fold change in expression was determined using the formula of $2^{-\Delta\Delta CT}$.

MALAT1 and miR-124 silencing

MiR-124 inhibitor, its scramble control and small interference RNAs targeting MALAT1 along with scramble control were obtained from Gene Pharma Co., Ltd. (Shanghai, China). siRNA against MALAT1 as well as miR-124 inhibitors were transfected into CTCL cells using Lipofectamine 2000 (Thermo Fisher Scientific, China) according to the manufacturer's instructions.

ELISA

All kits for the ELISA assays were purchased from Enzo Life Sciences (USA). The kits provide all the reagents needed for quantitative assessment of various factors in human serum. The individual kits contain specific antibodies immobilized on microtiter plates. For the assay, serum samples (100 µl) were applied to the individual wells in the provided 96-well plates, and the plates were incubated at room temperature for 1 hour with gentle shaking. At the end of incubation, the contents were emptied out and replaced with 400 µl of wash buffer for washing. The washings were done a total of 3 times and the wash buffer were aspirated out. Then, biotinylated antibody specific to the factor being assayed by specific kits was added to wells and plates further incubated at room temperature for 1 hour with gentle shaking. This was followed by 3x washings and the aspiration of wash buffer every time. Finally, streptavidin conjugated to horseradish peroxidase was added to each wells and the color allowed to develop by shaking the plates gently for 30 minutes at room temperature. 100 µl of stop solution was then added to each well and the O.D. was read at 450 nm using a Shimadzu spectrophotometer.

Statistical analysis

All results are expressed as mean ± standard deviation. We used Student's *t*-test or ANONA (One-way) to compare groups. $P < 0.05$ was considered significantly different.

Results

MALAT1 in elevated in CTCL patients

The status of lncRNA MALAT1 in CTCL patients is not clear and moreover there is quite some controversy regarding the oncogenic vs tumor suppressive role of MALAT1 in different cancers. Therefore, we started with an evaluation of MALAT1 levels in the serum of CTCL patients (n=10), as compared to the levels of MALAT1 in the serum of healthy control volunteers (n=8). In our cohort, we found (Figure 1) significantly increased

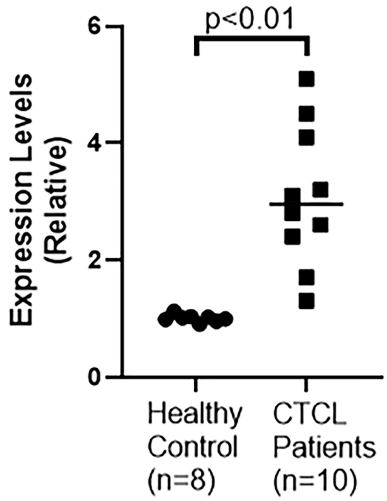

FIGURE 1
MALAT1 levels in CTCL patients (n=10) vs. healthy controls (n=8).

MALAT1 in patients' serum with a p value of p<0.01. Thus, MALAT1 is clearly overexpressed in CTCL patients, atleast in our cohort.

MALAT1 silencing affects EMT and cell growth parameters

MALAT1 influences cancer metastasis through induction of epithelial-mesenchymal transition (EMT) (13, 14). This made us next evaluate the effect of MALAT1 on EMT in CTCL cells. For this, we started with CTCL cell line, HH, and silenced MALAT1 in this cell line by using specific MALAT1-tergeting siRNA. We first tested three different siRNAs, si-1, si-2 and si-3 (Figure 2A) to find the most effective siRNA. Our observations proved that siRNA # 2 was the best in terms of effective silencing of MALAT1 because this particular siRNA reduced the expression of MALAT1 by more than half in HH cells with a p value of p<0.01. In contrast, while one siRNA (siRNA#1) did not significantly reduce MALAT1, another (siRNA#3) was comparatively less effective (p<0.05). Subsequently, we used siRNA#2 for all of the remaining experiments. A screening of siRNA#2 in the other tested CTCL cell line H9 revealed a potent silencing effect of siRNA#2 in these cells as well with significantly reduced MALAT1 levels (p<0.01) (Figure 2B).

We further used the siRNA to reduce MALAT1 levels in two different CTCL cells, HH and H9 and first studied the cell growth pattern before studying the levels of EMT markers. Cell growth was measured by CCK-8 method which allows quantification of viable cells and is therefore a reliable test for proliferation and cell growth. Post-silencing of MALAT1, CTCL cells were subjected to CCK-8 assay for 24 hours with reading taken at 1, 12 and 24 hours. We observed that whereas the cells grew normally under control conditions (cells transfected with control siRNA), the ones with silenced MALAT1 were significantly slower in their growth. Same results were seen for both HH and H9 cells (Figures 2C, D) with a p- value of p<0.01 for both CTCL cell lines and at 12 and 24 hours post silencing of MALAT1.

After checking the effect of MALAT1 silencing on cell growth, we next checked for the effect of MALAT1 silencing on EMT. We chose one marker of epithelial phenotype and another marker for mesenchymal phenotype. E-cadherin was our chosen epithelial marker and N-cadherin was our chosen mesenchymal marker. We observed that silencing of MALAT1 increased E-cadherin while reducing N-cadherin in HH cells (Figure 3A). These results had a p-value of p<0.01. Similar observations were made in H9 cells as well (Figure 3B). We also checked the cancer stem cell markers, Sox2 and Nanog in both of these CTCL cells and observed that both the markers, that we evaluated, were significantly reduced upon silencing of MALAT1 in HH (Figure 3C) as well as in H9 (Figure 3D) cells.

Levels of macrophages M2-related factors in CTCL patients

Tumor microenvironment plays a very important role in cancer metastasis and tumor-associated macrophages (TAMs), particularly the M2 type play an important role in cancer metastasis. M2 macrophages secrete many factors, such as, IL-6, IL-8, IL-10, TGF-β, PGE2 and MMP7, and these macrophages promote immunosuppression (15) and tumor growth. Therefore, we checked for the levels of these factors in serum of CTCL patients. We observed that all of these factors were significantly elevated in CTCL patients, compared to the healthy control volunteers (Figure 4). While IL-6 and IL-8 had p-values of 0.05, IL-10, TGFβ, PGE2 and MMP7 were even more significantly elevated in patients with p-values of p<0.01. Based on these results, it is apparent that

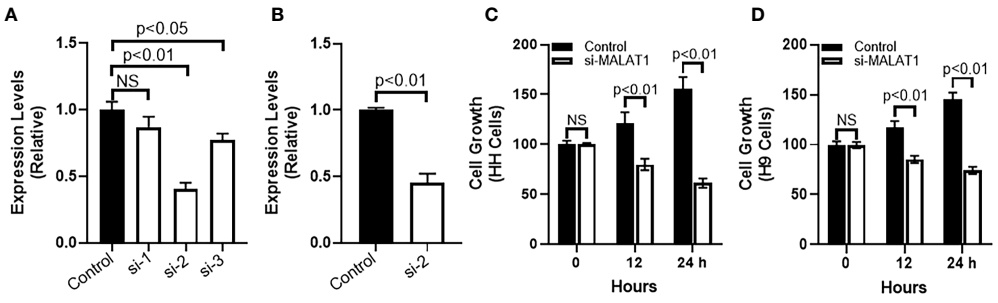

FIGURE 2
MALAT1 silencing in CTCL cells and its effects on cell growth. **(A)** Different siRNAs were checked for their efficacy to inhibit MALAT1 in HH cells. **(B)** siRNA#2 was further evaluated in H9 cells. Cell growth was assessed by CCK-8 method in HH **(C)** and H9 **(D)** cells. NS is 'not significant'.

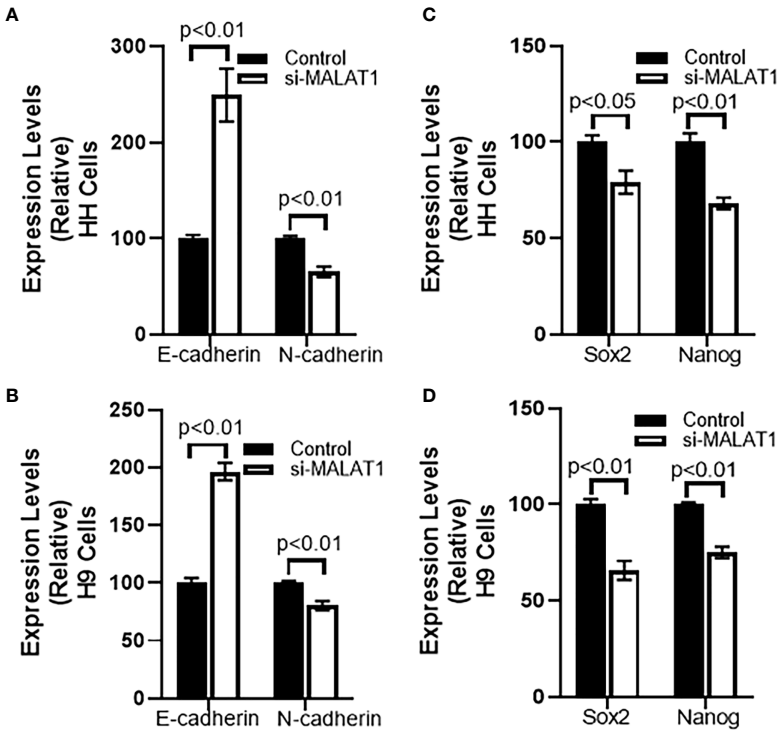

FIGURE 3
Effect of MALAT1 silencing on EMT and cancer stem cell characteristics of CTCL cells. Epithelial marker E-cadherin and mesenchymal marker N-cadherin was evaluated in HH **(A)** and H9 **(B)** cells upon MALAT1 silencing. Similarly, cancer stem cells markers Sox2 and Nanog were evaluated in HH **(C)** and H9 **(D)** cells upon MALAT1 silencing.

the factors related to M2 TAMs are upregulated in the CTCL patients.

MALAT1 sponges multiple miRNAs in CTCL cells

It is well established in the literature that lncRNAs function *via* sponging of miRNAs (16). miRNAs that have previously been shown to be sponged by MALAT1 and also connected to EMT, namely, miR-101, miR-124 and miR-200c were screened for their possible sponging by MALAT1 in CTCL cells HH. We checked the levels of these three miRNAs in the HH cells with MALAT1 silenced, and observed that all of these miRNAs were upregulated in the cells when MALAT1 was silenced (Figure 5A). All the miRNAs had a p-value of $p<0.01$. However, it was also observed that among the three tested miRNAs, miR-124 was the most upregulated miRNA in HH cells (Figure 5A) leading us to further evaluate this miRNA in H9 cells. Similar to our findings in HH cells, we found miR-124 to be significantly elevated in H9 cells (Figure 5B). Therefore, we further chose this miRNA for more studies, as discussed below.

Anti-miR-124 reverses MALAT1 silencing effects

Further mechanism was studied because of the observation that miRNA-124 was upregulated in MALAT1 silenced cells. To find a role of miR-124 in MALAT1 functions, we countered the increased miR-124 in MALAT1 silenced cells by silencing miR-124 in these cells. First, we checked the cell growth using CCK-8 method as described above. We observed that when miR-124 was silenced in MALAT1 silenced HH CTCL cells, the reduced proliferation associated with MALAT1 silencing was reversed (Figure 6A). Since our results above also found an inhibitory effect of MALAT1 silencing on markers of EMT and cancer stem cells, we also checked for the effect of miR-124 silencing on these markers. We observed that the epithelial marker E-cadherin was downregulated by miR-124 silencing whereas mesenchymal marker N-cadherin was upregulated by miR-124 (Figure 6B). When we studied cancer stem cell markers Sox2 and Nanog, we

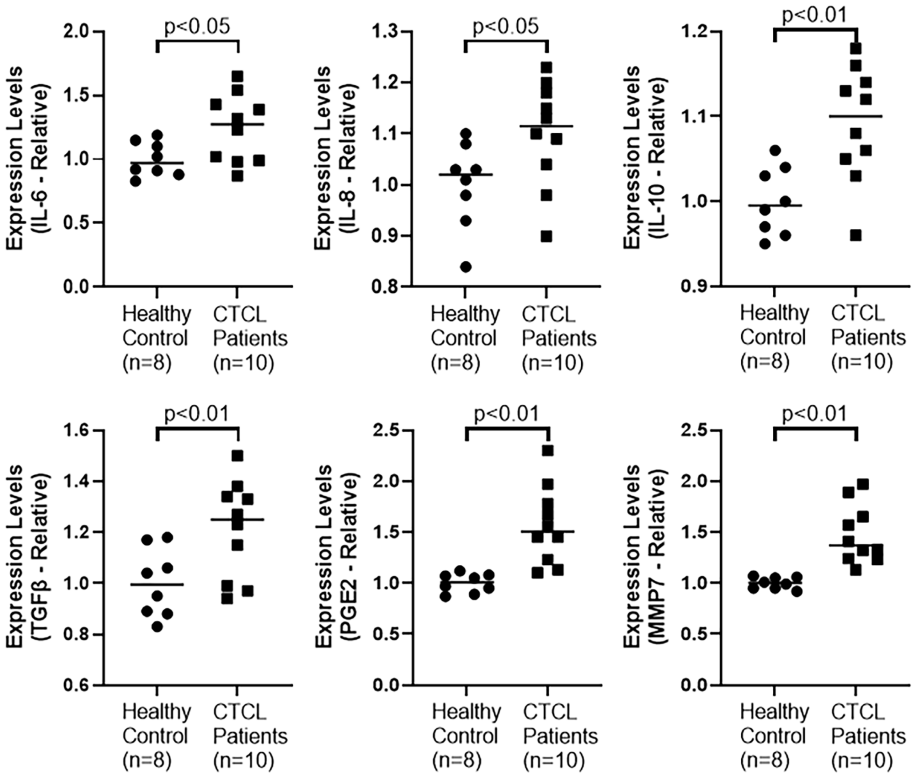

FIGURE 4
Levels of macrophages M2-related factors in CTCL patients were measured by ELISA, as described in Methods.

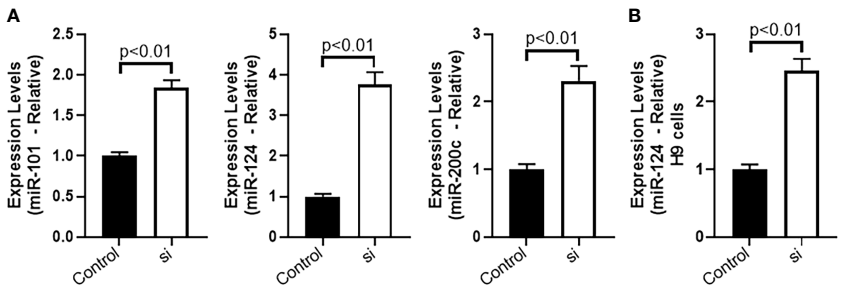

FIGURE 5
Sponging of miRNAs by MALAT1 in CTCL cells. **(A)** miR-101, miR-124 and miR-200c were quantitated in HH cells followed by evaluation of miR-124 in H9 cells **(B)**.

observed that silencing of miR-124 upregulated both the stem cell markers significantly (Figure 6C) with a p-value of p<0.01. We also wanted to check the effect of anti-miR-124 on M2 macrophages-released factors. As a proof-of-principle, we checked the levels of IL-6 and IL-10 in control and MALAT1 silenced cells. We observed that MALAT1 silencing significantly reduced both of these factors in HH cells (Figure 7). Furthermore, when we silenced miR-124 in MALAT1 silenced cells, the levels of these factors were almost restored (Figure 7).

Discussion

Both lncRNAs and miRNAs belong to the class of non-coding RNAs which were, not very long ago, considered to be 'junk' (17). However, now these non-coding RNAs are considered to be very important regulators that determine disease progression, particularly cancer. MALAT1 is one such lncRNA that has now been extensively studied in various different cancers (6, 7, 13, 18) with hundreds of available

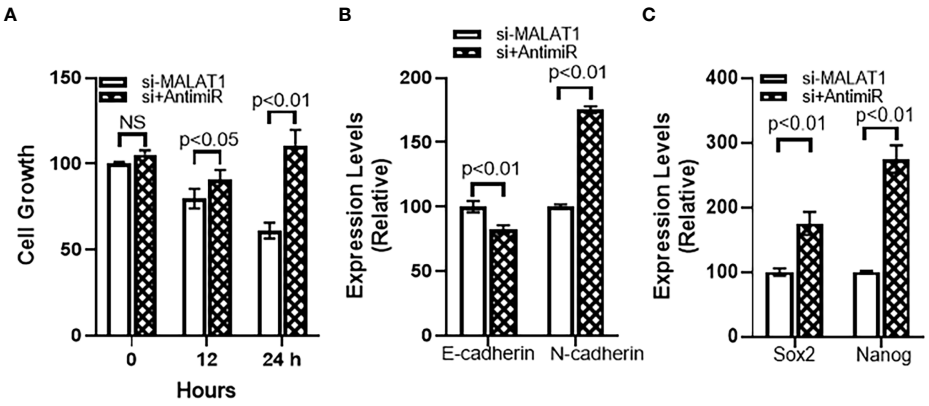

FIGURE 6
miR-124 reverses MALAT1-silencing effects on **(A)** cell growth, **(B)** EMT and **(C)** cancer stem cells characteristics. si+AntimiR: siMALAT1+Anti-miR-124. NS, not significant.

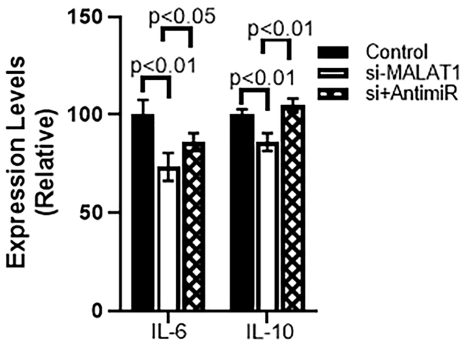

FIGURE 7
miR-124 reverses MALAT1-silencing effects on IL-6 and IL-10 in HH cells. si+AntimiR: siMALAT1+Anti-miR-124.

reports on this lncRNA in the scientific literature. However, there is not much information on this lncRNA in CTCL and that was the primary reason behind our planned study. In the one published report, MALAT1's role in CTCL was suggested a few years back (11). It was shown that in CTCL MyLA cells, C-C motif chemokine ligand 21 (CCL21) activates mTOR leading to MALAT1 upregulation with observed surge in cell migration (11). Therefore, this early publication on MALAT1 in CTCL supported our current findings by validating an oncogenic role of MALAT1 in CTCL cells. The published report had MyLA cells as the experimental model while our study examined HH and H9 CTCL cells. Combined, it seems like MALAT1 is oncogenic in CTCL cells across multiple cell lines and thus could be an important therapeutic target.

As one of the mechanisms by which MALAT1 can influence CTCL progression, we observed induction of EMT by MALAT1. For establishing this action of MALAT1, we studied two different biomarkers for EMT – E-cadherin and N-cadherin. These markers are representative of two entirely different facets of EMT. While E-cadherin is marker of epithelial phenotype, N-cadherin is a marker for mesenchymal phenotype. During the induction of EMT, cancer cells loose epithelial characteristics and move towards a mesenchymal phenotype. This is marked by loss/downregulation of E-cadherin and gain/upregulation of N-cadherin. In our experiments, we observed gain of E-cadherin and loss of N-cadherin upon MALAT1 silencing. This means that when MALAT1 is downregulated, a reversal of EMT happens. This provides an evidence for a positive correlation between MALAT1 and EMT induction in CTCL cells because upregulation of epithelial marker with simultaneous downregulation of mesenchymal marker is reliable indication of reversal of EMT. The EMT induced by lncRNAs plays an important role in cancer metastasis (19) and accordingly MALAT1-induced EMT has been reported to regulate cancer metastasis (13, 20, 21). Thus, MALAT1 upregulation in CTCL can be related to a more aggressive disease.

In our study, we also checked for the cancer stem cell markers because in our results we observed induction of EMT by MALAT1 and it is known in literature that EMT promotes cancer stem cell phenotype (22). Moreover, MALAT1 has itself been shown to promote cancer stem cell characteristics (23, 24) with silencing of MALAT1 linked to reduced EMT and cancer stem cells phenotype (25), which is in complete agreement with the results that we have presented in our study. In our experiments, we observed reduced levels of stem cells markers, both Sox2 and Nanog in CTCL cells, upon silencing of MALAT1, which is a clear indication that MALAT1 promotes cancer stem cell phenotype as silencing of MALAT1 reduces stem cell markers.

Tumor microenvironment provides a sanctuary for the growth of cancer cells and this is facilitated by mutual interactions between several cellular components of the

microenvironment. There is a functional crosstalk between the many different cell types within this microenvironment (26). TAMs represent a major component of tumor microenvironment and they play a very important role in tumor progression through their contribution to evasion of host immune responses (15). TAMs also release many factors such as IL-6, IL-8, IL-10 and TGFβ (27, 28). It was therefore important to find out if these factors are detectable in CTCL patients. Our observations that these factors can be detected in circulation in CTCL patients is a clear indication for the activity of M2-type macrophages in CTCL patients. Further, immunosuppressive activity of TAMs is supported through dysregulated miRNAs (15) and therefore, it was important for us to find miRNA that was central to the MALAT1 activity.

For the identification of miRNAs sponged by MALAT1 in CTCL cells, we focused on three specific miRNAs that have been shown to regulate EMT and also sponged by MALAT1. This was based on the observation that MALAT1 regulates EMT and CSC. All the three tested miRNAs, miR-101, miR-124 and miR-200c regulate EMT (29–31) and there are also reports on their sponging by MALAT1 (32–34). Our observations further add to this knowledge by establishing sponging of these miRNAs by MALAT1 in CTCL cells as well. In our results, we observed miR-124 to be the most dysregulated miRNA when MALAT1 was silenced. This guided us to perform experiments wherein we rescued cancer cell characteristics in MALAT1-silenced cells through targeted dysregulation of miR-124. Since, MALAT1 is oncogenic in our model, we expected the miRNAs sponged by it to be tumor suppressive and that was the case as all sponged miRNAs, including the most affected miRNA, miR-124, are all tumor suppressive miRNAs. miR-124 was upregulated when MALAT1 was silenced and downregulation of miR-124 restored the cancer cell characteristics that would otherwise be seen in MALAT1 expressing cells. Thus, our study established a MALAT1-miR-124 axis in CTCL cells.

Finally, we report in this study that we checked the levels of IL6 and IL10 in CTCL HH cells. For this particular experiment, we had several experimental conditions which included MALAT1 silencing followed by miR-124 downregulation. The rationale for this experiment came from an earlier observation in this study where we saw elevated IL-6 and IL-10 in serum from CTCL patient's serum. Since manipulation of MALAT1 and miR-124 was not possible in humans, we performed the experiments using CTCL cells as a proof of principle. We were able to show that MALAT1 silencing reduces the levels of these factors and furthermore, downregulation of miR-124 can reverse MALAT1-silencing effects.

Our study thus provides a novel involvement of MALAT1 in CTCL cells. MALAT1 induces EMT and cancer stem cell phenotype and this is facilitated by sponging of miR-124 by MALAT1. Further pre-clinical studies should provide additional verification of this phenomenon with identification of key steps that can targeted for therapeutic benefit.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was reviewed and approved by the Ethics Committee of the First Hospital of Jilin University (Approval Number 2021-680). The patients/participants provided their written informed consent to participate in this study.

Author contributions

WG, G-ML, J-YG, Y-JC performed experiments. WG, J-YG, Y-ZZ and KW analyzed data. WG and G-ML prepared first draft. OB supervised study and edited draft of manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Expression pattern of non-coding RNAs in non-functioning pituitary adenoma

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Non-functioning pituitary adenoma (NFPA) is a benign tumor arising from the adenohypophyseal cells. They can be associated with symptoms arising from mass effect. Although these tumors are regarded to be benign tumors, they are associated with increased comorbidity and mortality. Several studies have indicated abnormal expression of genes in these tumors. In the current study, we have used existing methods to identify differentially expressed genes (DEGs) including DE long non-coding RNAs (DElncRNAs) and DE microRNAs (DEmiRNAs) in NFPAs compared with normal samples. Then, we have assessed the relation between these genes and important signaling pathways. Our analyses led to identification of 3131 DEGs, including 189 downregulated DEGs (such as RPS4Y1 and DDX3Y) and 2898 upregulated DEGs (such as ASB3 and DRD4), and 44 DElncRNAs, including 8 downregulated DElncRNAs (such as NUTM2B-AS1 and MALAT1) and 36 upregulated DElncRNAs (such as BCAR4 and SRD5A3-AS1). GnRH signaling pathway, Tight junction, Gap junction, Melanogenesis, DNA replication, Nucleotide excision repair, Mismatch repair and N-Glycan biosynthesis have been among dysregulated pathways in NFPAs. Taken together, our study has revealed differential expression of several genes and signaling pathways in this type of tumors.

KEYWORDS

non-functioning pituitary adenoma, lncRNA, miRNA, expression, biomarker

Introduction

Non-functioning pituitary adenoma (NFPA) is a benign tumor arising from the adenohypophyseal cells. This type of tumor is described by the lack of clinical signs of hypersecretion of hormones. Statistics show a prevalence of 7–41.3/100,000 for NFPA (1–3). The incidence of this type of tumors seems to be increased during recent years, possibly due to enhanced numbers of incidentally identified adenomas in brain imaging conducted for other purposes (4).

Eight subtypes identified for NFPA are as follow: silent gonadotroph, corticotroph, somatotroph, thyrotroph, lactotroph, plurihormonal Pit-1, null-cell, and double/triple NFPAs (5). NFPA has variable clinical manifestations ranging from asymptomatic to symptoms resulting from effects of mass on neighboring regions leading to headache, visual defect, and/or hypopituitarism (2, 6).

Although these tumors are regarded to be benign tumors from a histological point of view, they are associated with increased comorbidity and mortality (3, 7).

Recent studies have indicated abnormal expression pattern of several coding and non-coding genes in NFPA (8, 9). For instance, transcriptome analysis has shown distinct profiles in pituitary adenomas compared to the non-tumoral tissues, irrespective of the identified immunophenotype. Notably, calcium metabolism and immune-related genes are among the mostly altered genes in adenomas (9).

In the current study, we have used existing methods to identify differentially expressed genes (DEGs) including DE long non-coding RNAs (DElncRNAs) and DE microRNAs (DEmiRNAs) in NFPAs compared with normal samples. Then, we have assessed the relation between these genes and important signaling pathways.

Methods

Microarray data collection

We used the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) to obtain the human expression profiles of GSE62960 (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F) and GSE63357 (Affymetrix Human Genome U133 Plus 2.0 Array), which contained 28 and 25 samples, respectively. We selected 10 non-functioning pituitary adenoma samples from GSE62960 and 5 normal pituitary samples from GSE63357 for further analysis. The expression data contained both lncRNAs and mRNAs expression signatures.

Microarray data processing, integrative meta-analysis and assessment of data quality

Processing and integration of all microarray data were performed using the R statistical programming language, the mentioned datasets have different and trendy platforms (Agilent and Affymetrix), a sensitive step in the integration of heterogeneous data is normalization (10). Batch effects (non-biological differences) were removed by applying the ComBat function from the R Package Surrogate Variable Analysis (SVA) (11). Batch effect removal was checked by PCA and boxplot. The meta-analysis outcome is a unit expression matrix (the combination of four datasets of this study). Then, we used quantile normalization method to normalize data expression matrix.

To accomplish quantile normalization, we used the preprocessCore R package. Also, we utilized ComBat function based on its description in sva package (ComBat permits adjustment of batch effects in datasets where the batch covariate is known (12)) and bioconductor (The sva package can be utilized to eliminate artifacts by three methods: (1) recognizing and appraising surrogate variables for unknown sources of variation in high-throughput experiments (13), (2) directly eliminating identified batch effects using ComBat (12) and (3) removing batch effects with known control probes. We used this function after merging two datasets. This method has been used in recent publications as well (14).

Analysis of differentially expressed lncRNAs and mRNAs

The Limma package in R language (15) was used to obtain DEGs and DElncRNAs between NFPA and normal samples. Furthermore, we used Bonferroni in the multtest package to adjust the P value into the FDR. We used the FDR < 0.05 and $|\log_2 FC| > 1$ as the cutoff criteria for DEGs and DElncRNAs. Then we identified DElncRNAs using HUGO gene nomenclature committee.

Two-way clustering of DEGs and DElncRNAs

The gene expression parameters of substantial differentially expressed genes and lncRNAs were obtained. Then, the pheatmap package in R language (version 1.0.12) (16) was used to conduct the two-way clustering based on the Euclidean distance.

Gene ontology (GO) enrichment analyses

In order to find the function of the obtained considerably downregulated and upregulated DEGs, we performed Gene Ontology (GO) enrichment analysis using the clusterProfiler R package (17). We set p-value < 0.05 as the thresholds of the functional categories.

Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis

KEGG pathway analysis of considerably downregulated and upregulated DEGs was performed to find the potential function of these genes contributing to the pathways based on the KEGG database (18).

Constructing the ceRNA network

We built a ceRNA network through the following steps: 1) Searching the miR2Disease database (http://watson.compbio.iupui.edu:8080/miR2Disease/index.jsp) (19) for the pituitary adenoma (PA)-related miRNAs using the keyword "Pituitary Adenoma". 2) Using miRcode (http://www.mircode.org/) for assessment of interaction between lncRNAs and miRNAs based on the PA-related miRNAs; 2) Application of miRDB (http://www.mirdb.org/) (20), TargetScan (http://www.targetscan.org/) (21) and miRWalk (http://129.206.7.150/) (22) for prediction of miRNAs-targeted mRNAs; 3) Finding the intersections of the differentially expressed lncRNAs and mRNAs, and establishment of lncRNA/mRNA/miRNA ceRNA network using Cytoscape v3.0 (23); and 4) we used cytohubba (24) to detect 15 hub genes with best degree in ceRNA network.

Survival analysis

GEPIA (25) was used to depict survival curves according to prognostic value of top 10 genes with best degree in ceRNA network. The clinical data for patients with low grade glioma was obtained from TCGA. The TCGA-LGG data (https://portal.gdc.cancer.gov/) included 515 primary solid tumor samples. This analysis was done on June 9, 2022.

Kaplan–Meier curves were depicted for evaluation of univariate survival. P-values less than 0.05 were considered statistically significant.

Results

Dataset quality assessment

Figure 1 demonstrates the boxplot of raw data before and after batch effect removal. These boxplots indicates that the quality of the expression data was reliable.

Figure 2 displays the Euclidean distances between the samples after batch effect removal. Tumor and healthy samples were divided into two groups and put into two clusters.

The 15 samples are displayed in the 2D plane covered by their first two principal components in the PCA plot (PC1 and PC2) (Figure 3). This plot shows the good relative variance between the NFPA and normal samples.

DEGs analysis

According to analyses of the microarray data between NFPA and normal samples by limma, we obtained 3131 DEGs, including 189 downregulated DEGs (such as RPS4Y1 and DDX3Y) and 2898 upregulated DEGs (such as ASB3 and DRD4), and 44 DElncRNAs, including 8 downregulated DElncRNAs (such as NUTM2B-AS1 and MALAT1) and 36 upregulated DElncRNAs

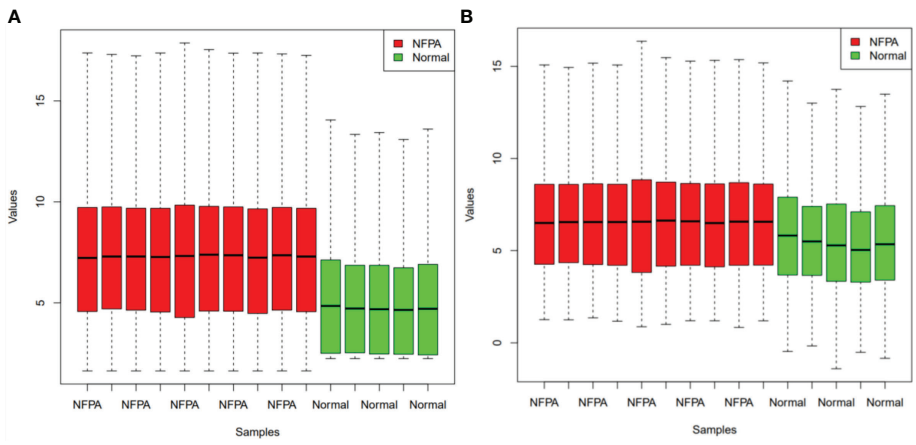

FIGURE 1
Boxplots for the data before **(A)** and after **(B)** batch effect removal. Red boxes indicate NFPA samples and green boxes show healthy samples.

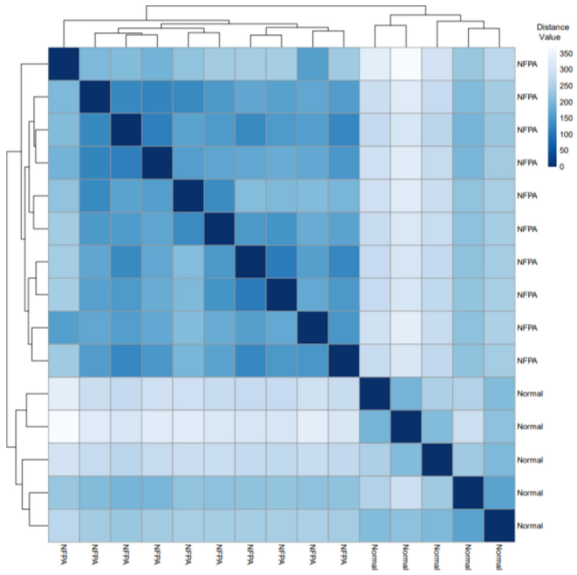

FIGURE 2
The Euclidean distances between the samples. Based on the Euclidean distance, hierarchical clustering between the samples has been established; the distance values between samples are shown.

(such as BCAR4 and SRD5A3-AS1). Table 1 lists the top 10 markedly downregulated and upregulated DEGs.

Table 2 lists the markedly downregulated and upregulated lncRNAs.

Volcano plots were depicted to visualize and assess variation (or reproducibility) of lncRNA and mRNA expressions between NFPA and normal samples (Figure 4). Some of the differentially expressed genes included in the tables were displayed in this plot.

Besides, the two-way clustering showed that lncRNAs and mRNAs expression pattern between PA and healthy controls was distinctive (Figure 5). Also, a heatmap depicts the expression of these DElncRNAs (Figure 6).

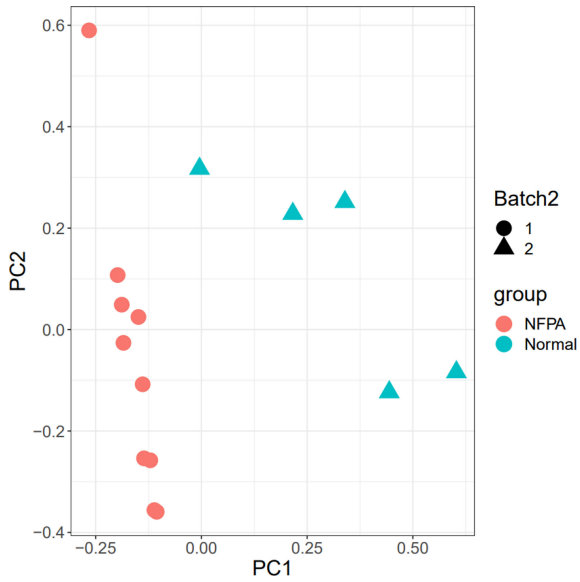

FIGURE 3
PCA plot. The Batch implies that the data includes two platforms. Also, healthy and tumor samples were divided into two groups.

TABLE 1 The top 10 up- and downregulated DEGs between NFPA and normal samples.

Down-regulated DEG	Log FC	Adjusted *P* value	Up-regulated DEG	Log FC	Adjusted *P* value
RPS4Y1	-6.247364	0.033317521	ASB3	9.711828	0.002707552
DDX3Y	-5.227572	0.026558724	DRD4	9.339609	0.003678029
POMC	-4.809255	0.020522011	LOC646626	8.928223	0.002707552
SFPQ	-4.447223	0.016269745	LOC100130331	8.411643	0.002707552
KDM5D	-4.205645	0.044775414	HIST1H2BO	8.230407	0.002765520
CRYAB	-4.043984	0.006188775	RRN3P3	8.210715	0.002707552
TSHB	-3.925312	0.003632825	SNORA78	8.092251	0.002859834
PAX6	-3.889940	0.002707552	EGLN2	7.175348	0.002707552
SCUBE3	-3.822918	0.003706830	HIST1H3C	7.109154	0.002795396
CGA	-3.685164	0.013048913	TACR2	7.056791	0.003065875

GO enrichment analysis of DEGs

The noticeably DEGs were enriched in 3171 GO terms. We used Clusterprofiler package to perform analysis. in GO functional enrichment analysis, 25 GO entries satisfy Adjusted P value less than 0.05, most of which are biological processes, followed by cellular component and molecular function. The first 25 entries are integral component of endoplasmic reticulum membrane, intrinsic component of endoplasmic reticulum membrane, extracellular exosome, extracellular organelle,

TABLE 2 The significantly up- and downregulated DElncRNAs between NFPA and normal samples.

Down-regulated DEG	Log FC	Adjusted *P* value	Up-regulated DEG	Log FC	Adjusted *P* value
NUTM2B-AS1	-2.536098	0.01392066	LINC00174	7.536962	0.003117761
MALAT1	-2.070053	0.04329433	ARIH2OS	4.488056	0.004377148
LINC00641	-1.981903	0.04098596	SRD5A3-AS1	4.34513	0.002795396
RNF157-AS1	-1.707092	0.04113265	PXN-AS1	4.322818	0.008798058
LINC00899	-1.649536	0.0258828	LIFR-AS1	4.262626	0.007013894
MIR31HG	-1.624021	0.006210766	URB1-AS1	3.614134	0.01095202
LINC00844	-1.348868	0.04505837	BCAR4	3.22296	0.002795396
SPANXA2-OT1	-1.287386	0.04430635	LINC00886	3.089068	0.02782602
			NEBL-AS1	2.937485	0.04471735
			ATP6V0E2-AS1	2.856039	0.01387096
			LINC01003	2.855841	0.004992219
			SCAMP1-AS1	2.807056	0.01915909
			LOH12CR2	2.751739	0.02594852
			RAP2C-AS1	2.732042	0.02622078
			APTR	2.724516	0.01066791
			LINC00667	2.673574	0.0145873
			EPB41L4A-AS1	2.672387	0.00749758
			TP53TG1	2.642199	0.009631829
			ST7-AS1	2.60673	0.04587292
			FGD5-AS1	2.541793	0.02155033
			LINC00842	2.468281	0.01051766
			TRAM2-AS1	2.380206	0.04869672
			RBM26-AS1	2.318657	0.03268624
			WWC2-AS2	2.278828	0.02880773
			UMODL1-AS1	2.172951	0.04606644
			HEIH	2.148043	0.04100989
			IDH1-AS1	1.98541	0.01740445
			GSN-AS1	1.926714	0.02052201
			ADORA2A-AS1	1.917936	0.03131871
			GAS5	1.72566	0.01252509
			DUBR	1.513353	0.01594373
			C2orf27A	1.48974	0.04095487
			MAPKAPK5-AS1	1.360005	0.01369419
			HOXA-AS3	1.262868	0.02831569
			ARHGAP5-AS1	1.240983	0.04245685
			TRAF3IP2-AS1	1.204049	0.03087259

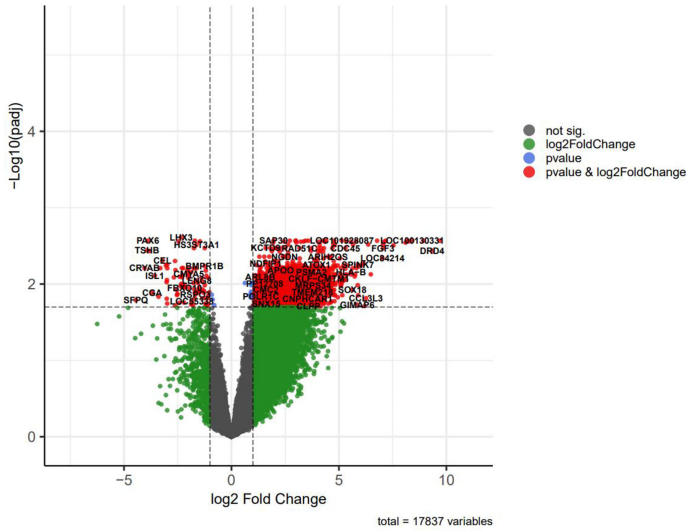

FIGURE 4
The volcano plot of differentially expressed genes (DEGs); horizontal axis, log_2(FC); vertical axis, -log10 (adjusted P value).

extracellular vesicle, mitochondrial inner membrane, mitochondrial envelope, mitochondrial membrane, carbohydrate binding, antigen binding, hormone activity, G protein-coupled receptor activity, diencephalon development, endocrine system development, cell fate specification, small molecule metabolic process, immune response, sensory organ development, immune effector process, cell fate commitment, adaptive immune response, forebrain development, pancreas development, cell differentiation in spinal cord and response to interferon-gamma. Figures S1 and S2 show the barplots of function enrichment analyses and GO enrichment analysis of DEGs, respectively.

Pathway analysis

Using Pathview (26) and gage (27) packages in R, KEGG pathways analysis of 189 downregulated and 2898 upregulated DEGs were conducted to detect the potential functional genes (Table 3 and Figure 7).

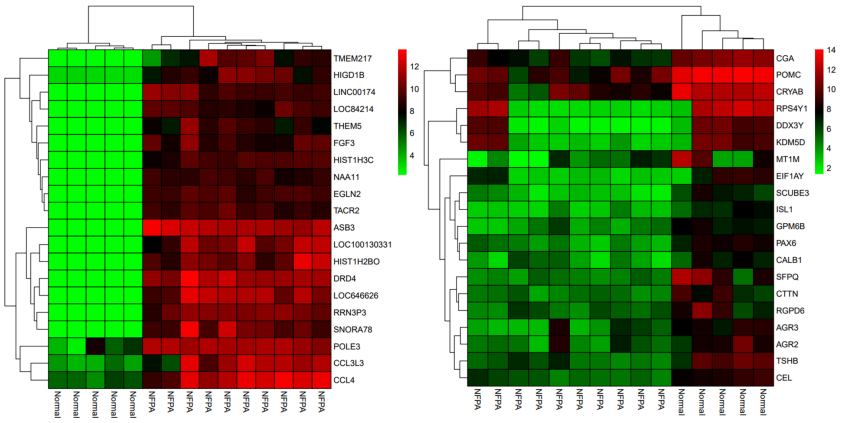

FIGURE 5
The two-way clustering of top 20 DEGs between NFPA samples and normal tissue samples; horizontal axis, the samples; vertical axis, DEGs between Normal tissues samples and NFPA samples.

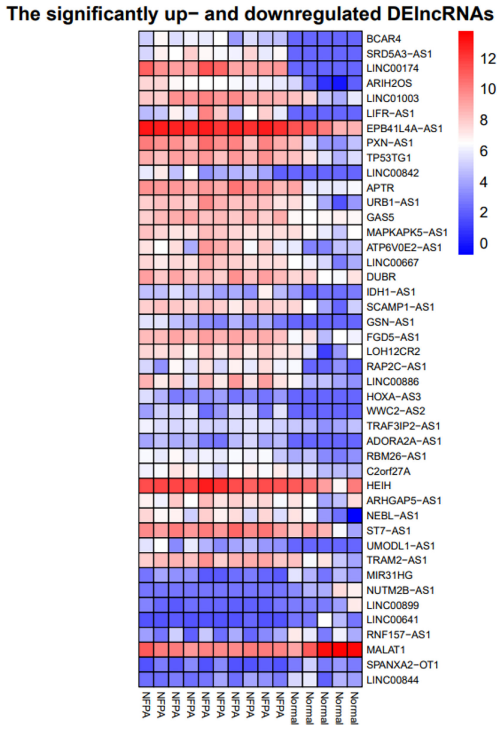

FIGURE 6
Heatmap of differentially expressed lncRNAs. The horizontal axis shows the names of 15 samples. The vertical axis presents the lncRNAs names.

ceRNA network construction in NFPA

According to the miR2Disease database, we identified 26 PA-related miRNAs. Then, miRcode was used to assess interaction between lncRNAs and miRNAs. This step showed that 14 of 26 PA-specific miRNAs may target to the 11 of 44 lncRNAs (Table 4). Subsequently, miRDB, TargetScan and miRWalk were used for prediction of these 12 miRNA-targeted mRNAs to find the relationship between miRNAs and mRNAs. Only 5 PA-specific miRNAs were found that might target 51 of the 3131 NFPA-specific mRNAs (Table 5). miRNA-targeted mRNAs were excluded in the case that they were not detected in DERNAs. Accordingly, Cytoscape 3.9 was used for construction of lncRNA-miRNA-mRNA ceRNA network. A total of 11 lncRNAs, 51 mRNAs, and 14 miRNAs were included in the ceRNA network (Figure 8). Finally, we computed nodes degrees and displayed 10 hub genes in the network using cytohubba app (24) (Figure 9). We found has-miR-15a, has-miR-132, has-miR-26a, has-miR-26b, has-miR-223, has-miR-16-1, MALAT1, GAS5, EPB41L4A-AS1 and FGD5-AS1 as 10 hub genes in ceRNA network.

Survival analysis

In this section, we retrieved RNA-seq data of brain low grade glioma. Survival analysis was performed based on Kaplan-Meier curve analyses using Survival package in R. We performed the survival analysis based on the hub genes in ceRNA network. The difference was regarded significant with log-rank $P < 0.05$. This

TABLE 3 Up-regulated and down-regulated pathways.

Down-regulated Pathway	P value	Up-regulated DEG	P value
GnRH signaling pathway	0.01958461	DNA replication	0.01894638
Tight junction	0.02421712	Nucleotide excision repair	0.03886668
Gap junction	0.04006672	Mismatch repair	0.04046353
Melanogenesis	0.04636715	N-Glycan biosynthesis	0.04360584

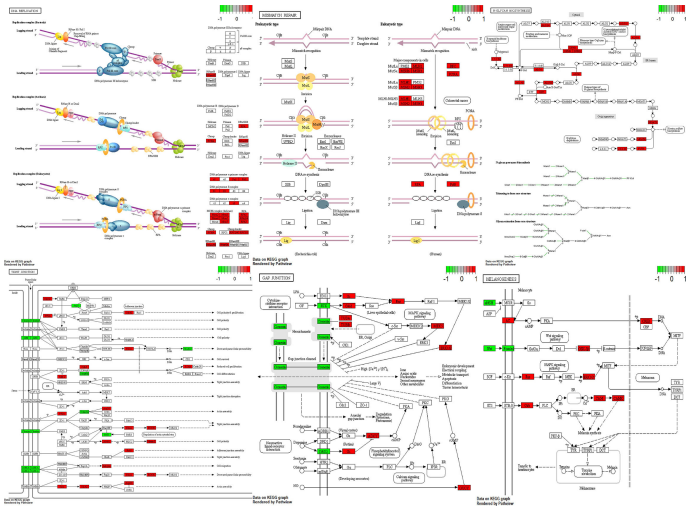

FIGURE 7
Visualization of pathways. Green boxes are downregulated genes and red boxes are upregulated genes.

analysis showed that EPB41L4A-AS1 and GAS5 were correlated with low survival time in patients with brain low grade glioma (Figure 10).

Discussion

The current study aimed to identify DEGs between NFPAs and normal samples and find the importance of these genes in the pathoetiology of this disorder. Our analyses led to identification of 3131 DEGs, including 189 downregulated DEGs (such as RPS4Y1 and DDX3Y) and 2898 upregulated DEGs (such as ASB3 and DRD4). RPS4Y1 and DDX3Y have been among downregulated genes in 12 cancers in a recent whole transcriptome analysis (28). The dopamine receptor DRD4 is also among important gens in the carcinogenic processes (29).

Moreover, we found 44 DElncRNAs, including 8 downregulated DElncRNAs (such as NUTM2B-AS1 and MALAT1) and 36 upregulated DElncRNAs (such as BCAR4 and SRD5A3-AS1). Notably, MALAT1 is commonly regarded as an oncogene in the carcinogenic processes. However, some reports have suggested a tumor-suppressing effect for MALAT1 (30, 31). It seems that MALAT1 exerts an anti-

TABLE 4 The MiRcode database revealed interactions between 12 DElncRNAs and 14 DEmiRNAs.

lncRNA	miRNA
MIR31HG, LINC00174, EPB41L4A-AS1	hsa-miR-7-1
MALAT1, EPB41L4A-AS1, C2orf27A, TRAF3IP2-AS1, FGD5-AS1	hsa-miR-16-1
MALAT1, EPB41L4A-AS1, C2orf27A, TRAF3IP2-AS1, FGD5-AS1	hsa-miR-15a
MALAT1, LIFR-AS1, ST7-AS1	hsa-miR-192-3
MALAT1, LINC00174, GAS5	hsa-miR-26a
MALAT1, LINC00174, GAS5	hsa-miR-26b
MALAT1, LINC00174, LIFR-AS1, GAS5, C2orf27A, TRAF3IP2-AS, FGD5-AS1	hsa-miR-24-1
MALAT1, LINC00174, LIFR-AS1, GAS5, ST7-AS1	hsa-miR-138-2
SPANXA2-OT1, EPB41L4A-AS1, TRAF3IP2-AS1	hsa-miR-9-3
LINC00174, C2orf27A	hsa-let-7a-1
LINC00174, TRAF3IP2-AS1	hsa-miR-103
LINC00174, TRAF3IP2-AS1	hsa-miR-103-2
EPB41L4A-AS1, GAS5, FGD5-AS1	hsa-miR-223
GAS5	hsa-miR-132

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TABLE 5 miRWalk, miRDB and TargetScan databases revealed interactions between 5 DEmiRNAs and 51 DEmRNAs.

miRNA	mRNA
hsa-miR-15a	SPTLC1, CFAP45, POLR3F, GDI2, CDC27, PSMD7, CCDC28A, SLC39A10, DCAF10, IP6K1, EGLN1, RRAGA, TBP, VTI1B, BCL2L2, PDIA6, SESN1, C16orf72, DYNC1I1, PCDHA1, CCNYL1, CDK6, CCNE1, KLF7, EYA4
hsa-miR-26a	ADAM17, LARP4B, ZDHHC20, NXPE3, CIPC
hsa-miR-26b	ZDHHC20, NXPE3, CIPC
hsa-miR-223	GTPBP8, SLC23A2, DENND5B,
hsa-miR-132	CFL2, PIK3IP1, DYRK2, PAIP2, SMAD2, PRICKLE2, CBLL1, CDC40, GRM3, MAPK1, SLC31A1, MED9, SLC23A2, KDM5A, KLF7

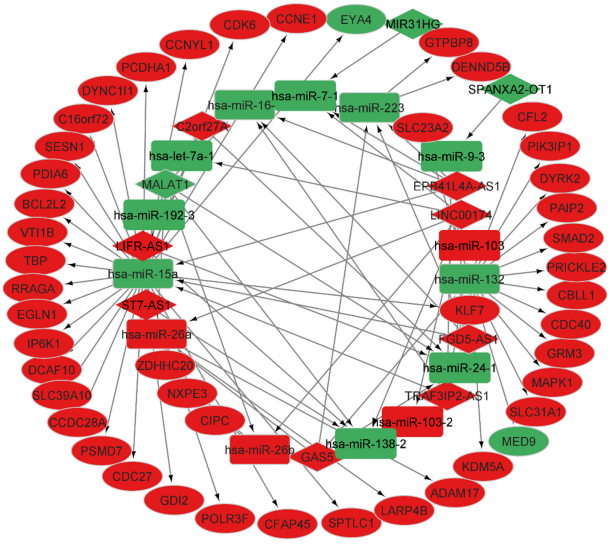

FIGURE 8
CeRNA network in NFPA. Red nodes show a high level of expression, while green nodes show a low level of expression. Ellipses represent protein-coding genes; round rectangles represent miRNAs; diamonds show lncRNAs; gray edges indicate lncRNA-miRNA-mRNA interactions.

cancer effect in NFPAs. In addition, NUTM2B-AS1 has been among up-regulated lncRNAs in hepatocellular carcinoma (HCC) whose expressions have been associated with poor prognosis of affected persons. This lncRNA has also been found to participate in the construction of ceRNA network in this type of cancer (32). Thus, the aforementioned results indicate distinct role of some lncRNAs in the pathogenesis of different types of cancers. BCAR4 has been shown to exert an oncogenic role in breast cancer inducing endocrine resistance in these cells (33). SRD5A3-AS1 is transcribed from the antisense region of SRD5A3, a gene that induces tumor growth and is associated with poor survival of HCC (34).

Hsa-miR-15a, hsa-miR-26a, hsa-miR-26b, hsa-miR-223 and hsa-miR-132 are related miRNAs with these lncRNAs. miR-26a and miR-26b are two tumor suppressor miRNAs in colorectal cancer that can suppress aggressive behavior of cancer cells through regulating FUT4 (35). In addition, miR-15a has been shown to target several oncogenes, such as BCL2, MCL1, CCND1, and WNT3A. This miRNA has been reported to be down-regulated in chronic lymphocytic lymphoma, pituitary adenomas, and prostate carcinoma (36). miR-132 and miR-223 have been shown to regulate positive feedback circuit through regulation of FOXO3a (37).

GnRH signaling pathway, Tight junction, Gap junction, Melanogenesis, DNA replication, Nucleotide excision repair, Mismatch repair and N-Glycan biosynthesis have been among dysregulated pathways in NFPAs. Thus, DNA repair systems are implicated in the pathogenesis of NFPAs.

Then, we constructed a ceRNA network which included 11 lncRNAs, 51 mRNAs, and 14 miRNAs. This ceRNA network

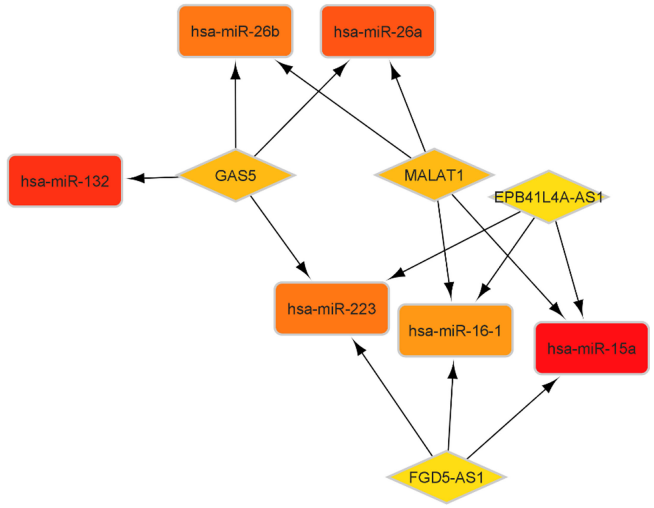

FIGURE 9
Top 10 genes with best degree in ceRNA network.

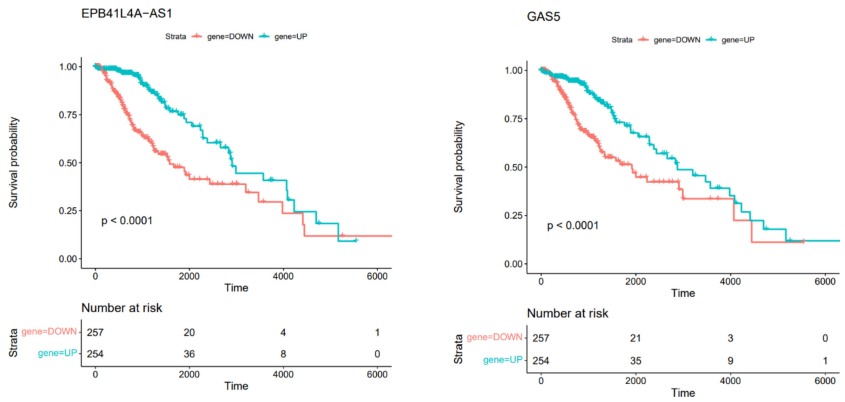

FIGURE 10
Kaplan–Meier survival curves of DElncRNAs associated with overall survival of patients with low grade glioma.

not only represents complicated pathoetiology of NFPAs, but also provides candidates for targeted therapy of this kind of tumor.

Two DElncRNAs, including EPB41L4A-AS1 and GAS5 have been associated with survival time of patients with brain tumors. Although the association between expression pattern of these lncRNAs and mortality or morbidity of patients with NFPAs has not been investigated yet, this finding suggests the importance of these lncRNAs in this regard. GAS5 lncRNA is mainly regarded as a tumor suppressor in human cancers. This lncRNA is down-regulated in several kinds of cancer, regulating cellular processes such as cell proliferation, apoptosis and invasion. Down-regulation of GAS5 expression is associated with higher ability of proliferation and poor prognosis in some malignancies (38). Association between expression of EPB41L4A-AS1 and survival of patients has been less studied. A single study in colorectal cancer has revealed an oncogenic role for this lncRNA and indicated it as a regulator of Rho/ROCK pathway (39).

Taken together, our study has revealed differential expression of several genes and signaling pathways in this type of tumors. Some of the identified DE genes in NFPAs are predicted to exert a specific role in this type of tumor. Others have common effects in the regulation of cell proliferation in several types of cancers.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

SG-F wrote the draft and revised it. MT and MA-B designed and supervised the study. BH, AS, and ND collected the data and designed the figures and tables. AS performed the bioinformatic analysis. All the authors read the submitted version and approved it.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.978016/full#supplementary-material

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LncRNA MALAT1 regulates METTL3-mediated PD-L1 expression and immune infiltrates in pancreatic cancer

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Pancreatic cancer is the fourth leading cause of cancer death in the United States. The main methods of treating pancreatic cancer are surgery and chemotherapy, but the treatment efficacy is low with a poor prognosis. Immunotherapy represented by PD-1/PD-L1 has brought a milestone progress in the treatment of pancreatic cancer. However, the unique tumor microenvironment of pancreatic cancer presents challenges for immunotherapy. In addition, m6A is a common RNA modification and a potential molecular target in tumor therapy. The expression pattern of m6A in pancreatic cancer is still unclear. LncRNAs also play an essential role in pancreatic cancer development and treatment. In this study, we found that some m6A regulators were significantly elevated in pancreatic cancer and associated with the expression of PD-1/PD-L1. Moreover, we observed that METTL3 can increase the expression of PD-L1. Notably, METTL3 positively regulates the expression of lncRNA MALAT1 in pancreatic cancer cells. Strikingly, lncRNA MALAT1 increased the expression of PD-L1 in pancreatic cancer cells. This finding indicated that METTL3 regulated the expression of PD-L1 possibly *via* targeting lncRNA MALAT1 in pancreatic cancer cells. Lastly, MALAT1 governed the viability of pancreatic cancer cells. Taken together, lncRNA MALAT1 is involved in METTL3-mediated promotion of PD-L1 expression in pancreatic cancer.

KEYWORDS

lncRNA, MALAT1, PD-L1, METTL3, TME, pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the common malignant tumors of the pancreas and more than 90% of pancreatic cancer are exocrine PDAC (1). According to an epidemiology report, the five-year survival rate of pancreatic cancer after diagnosis is about 10%, and it is estimated that the pancreatic cancer will surpass breast

cancer as the third leading cause of death (2). Cancer statistics suggest that there were 32,970 new cases of pancreatic cancer in men and 29,240 new cases of pancreatic cancer in women in the United States (3). The number of people who died from pancreatic cancer was 49,830 for men and 25,970 for women, respectively (3). Surgery and chemotherapy remain the mainstays of treatment for pancreatic adenocarcinoma (4). However, pancreatic cancer is aggressive with no obvious symptoms in the early stage. Most PDAC patients are late to be treated when diagnosed, and less than 20% of the patients are eligible for surgery (5). For unresectable patients, chemotherapy is the main treatment, but its efficacy is not ideal, and the median OS (Overall survival) is basically less than 1 year (6). Recently, immunotherapy, represented by immune checkpoint inhibitors (ICIs), has brought milestone progress to tumor treatment. However, ICIs are almost completely wiped out in pancreatic cancer, and most of them have failed in phase I and II clinical trials (7).

However, studies have shown that pancreatic cancer has a special tumor microenvironment (TME), which brings challenges to immunotherapy and deserves further study (8, 9). Tumor immune microenvironment (TIME) refers to the internal environment in which tumor cells are generated and live, which includes not only tumor cells themselves but also fibroblasts, immune and inflammatory cells, and glue cells that are closely related to tumor cells. However, compared with other cancers, pancreatic cancer has a unique TIME, which presents challenges for immunotherapy, and this may be one of the reasons why programmed death (PD-1)/PD ligand 1 (PD-L1) therapy is not highly sensitive to pancreatic cancer. PD-1, a 288 amino acid type 1 transmembrane protein, is often expressed on the surfaces of several immune cell types, while PD-L1, a 290 amino acid type 1 transmembrane protein, is expressed on hematopoietic cells (10, 11). Pancreatic tumor cells promote the activation of peripheral stromal cells and immunosuppressive cells, including regulatory T cells (Tregs), bone-marrow derived inhibitory cells (MDSCs), and tumor-associated macrophages (TAMs). At the same time, they secrete a series of cytokines and chemokines that cause these cells to flock to the tumor site. On the other hand, activated stromal cells generate a large amount of extracellular matrix that forms a fibrous "barrier" around pancreatic tumor cells, preventing effector cells (T and NK cells) from infiltrating into the tumor which allows tumor cells to evade immune surveillance. Activated immunosuppressive cells secrete immunosuppressive factors and express ligands (e.g., PD-L1 and B7-1/2), forming an immunosuppressive microenvironment. This plays an important role in the occurrence, development, invasion, metastasis and drug resistance of pancreatic cancer (12). The antitumor immune response is a complex, multistep process (13). Therefore, the mechanism of TIME should be further studied to explore new and potentially beneficial targets to improve the efficacy of pancreatic cancer immunotherapy.

Meanwhile, m6A is the most common RNA modification in eukaryotic RNA and plays an important role in cancer progression (14). It has been demonstrated that m6A modification is a dynamic and reversible process, which is composed of methyltransferase complex (Writers), demethylase (Erasers) and function managers (Readers) (15). It is believed that the function of m6A writers is stability of mRNA (2). A recent study show that there are 28 m6A regulators, including METTL3, METTL14, METTL16, WTAP, RBM15, RBM15B, ZC3H13, VIRMA, CBLL1, ZCCHC4, LRPPRC, ELAVL1, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, HNRNPC, HNRNPA2B1, EIF3A, EIF3H, IGF2BP1, IGF2BP2, IGF2BP3, CBLL1, PRRC2A, FTO, ALKBH5 (16). m6A modification plays a role in pre-mRNA splicing, 3'-end processing, nuclear output, translation regulation, mRNA decay and miRNA processing, and its dynamic reversible changes control and determine cell growth and differentiation, suggesting that abnormalities of m6A and modified proteins may also produce pathological effects in the occurrence and progression of tumors (17). Besides, as the most common modification in mRNA, m6A links epigenomics with tumorigenesis and development, and affects the processes of tumor stem cell self-renewal and differentiation, proliferation and apoptosis, invasion and metastasis, drug resistance, and immunosuppression. Therefore, m6A is involved in m6A-modified key proteins that are expected to be potential molecular targets for cancer diagnosis and treatment and drug development. For instance, a study examining DNA and RNA methylation status in circulating tumor cells (CTCs) from lung cancer patients demonstrated for the first time elevated m6A modification levels in CTCs from lung cancer patients (18).

LncRNA, one type of noncoding RNA, participates in tumorigenesis and progression (19–22). LncRNA MALAT1 has been reported to regulate pancreatic oncogenesis. The expression of MALAT1 was highly elevated in PDAC compared with the adjacent normal specimens (23). MALAT1 expression was linked to invasion, tumor stage, poor survival, tumor size and metastasis in PDAC patients (23, 24). Moreover, MALAT1 enhanced invasion, migration and viability of pancreatic cancer cells *via* reduction of EMT and cancer stem cells as well as induction of apoptosis and cell cycle arrest (25). The role of MALAT1 in PDAC development is not fully elucidated. The expression pattern and pathophysiological role of m6A in pancreatic cancer remain unknown. In addition, the association between m6A methylation modulator and PD-L1 remains unexplored. Therefore, the main purpose of this paper aims to analyze the relationship between m6A RNA methylation regulators, PD-L1 and MALAT1 in pancreatic cancer.

Materials and methods

Data collection and m6A-related regulators

The data was collected from The Cancer Genome Atlas (TCGA) database and the basic information including age, gender, grade, stage, T, M and N of 178 samples was obtained. In this study, we collected 24 m6A-related regulators, including writers, erasers and readers. The writers include RBM27, METTL3, WTAP, RBM15, ZC3H7B, CAPRIN1 and METTL14. The erasers include FTO and ALKBH5. The readers include YTHDF1, YTHDF2, YTHDF3, IGF2BP1/2/3, YTHDC1/2, IGF2BPs, KIAA1429 (VIRMA), EIF3A, EIF3H, HNRNPC, HNRNPA2B1, LRPPRC, ELAVL1 and PRRC2A (26). To determine the interaction of these 20 regulators, they were searched in the Genes/Protein database to gain a preliminary understanding of their biological functions (27).

Bioinformatic analysis

Consensus clustering is a method of providing quantitative evidence for determining the number and members of possible clusters in a data set, such as microarray gene expression (28). This approach is widely used in cancer genomics. In this study, we adopted this method to explore two PDAC clusters and their association with clinicopathological parameters. Gene set enrichment analysis (GSEA) 3.0 was used to predict the underlying downstream pathways of the two clusters (29). Immune score, stromal score, and tumor purity of each sample were calculated by using the ESTIMATE algorithm (30).

Genes with significant ($p<0.01$) prognosis were screened from DEGs using univariate COX regression analysis, and gene prognosis models were established by lasso-cox regression. The K-M curve was drawn using the R package survival, and the survival difference between different groups was calculated by the log-rank test to draw the K-M curve. The ROC of the model was calculated using the R package time ROC. Finally, the independent prognostic ability of risk scores was tested by univariate and multivariate Cox regression models. Groups will be divided into two groups including high-risk group and low-risk group by evaluating the distribution of clinical case characteristics using the R package "heatmap". This study adopted Cox regression models to assess whether the risk score combining with other clinical characteristics, could be an independent prognostic factor.

Cell culture and transfection

The human pancreatic cancer cell lines, BxPC-3 (with epithelial properties) and PANC-1 (with more mesenchymal properties), were cultured in DMEM medium supplemented with and 10% FBS and 1% penicillin/streptomycin solution. The cells were cultured in an incubator with 5% CO_2 at 37°C. Human MALAT1 cDNAs were subcloned into pcDNA3 vector. Human METTL3 cDNAs were subcloned into pLenti-C-mGFP vector. Specific small hairpin RNAs (shRNAs) targeting METTL3 (shMETTL3) or MALAT1 (shMALAT1) and the control shRNA (shNC) were obtained (GenePharma, Shanghai, China).

Quantitative real-time reverse transcription-PCR

Total RNA was extracted from pancreatic cancer cells using 1ml TRLzol Reagent. Then, RNA was used for reverse transcription and PCR was performed using SYBR Green Kit Data were analyzed by the ΔΔCt approach. GAPDH was used as the control. MALAT1: FW: GGA TCC TAG ACC AGC ATG CC; RV: AAA GGT TAC CAT AAG TAA GTT CCA GAA AA (31). The detailed method for PCR was described previously (32).

Western blotting analysis

The pancreatic cells were lysed by RIPA buffer and a bicinchoninic acid (BCA) assay was used for detection of protein qualification. After proteins were separated onto SDS-PAGE, the proteins were transferred onto a PVDF membrane. The membrane was incubated with 5% non-fat milk and then incubated with METTL3 (1:1000), PD-L1 (1:1,000) or Tubulin (1:1,000) antibody for overnight at 4°C. The membranes were washed by TBST and then incubated with the secondary antibody for 1 h. Then, ECL method was used to examine the protein expression (33).

Cell viability assay

The treated pancreatic cancer cells were cultured in 96-well plates for 48 and 72 hours. The viability of pancreatic cancer cells was determined by CCK8 assay as described previously (34). Briefly, 10 μL CCK8 reagent was added to each well and incubated for 2.5-3 hours in a cell culture incubator. The OD450 values were obtained by the microplate reader.

Statistical analysis

In this study, the Pearson correlation coefficient was widely used to measure the degree of correlation between two variables, and its value was between -1 and 1. To assess the impact of M6A-related risk characteristics on the prognosis of PDAC, we compared the prognostic differences between the high-risk and low-risk groups. Kaplan-Meier plotter was used to analyze the relationship between gene expression profile and survival information in PATIENTS with PDAC. In addition, multivariate Cox regression analysis was performed to determine prognostic factors for PDAC patients. Student t test was used to validate significance between two groups. ANOVE was used to validate significance among three or more groups. $P < 0.05$ is considered statistically significant.

Results

Expression of m6A RNA Methylation Regulators in PDAC

Initially, we analyzed the frequency of mutations in 24 expressed m6A regulators and found that 18 regulators all had a mutation frequency of 1%~2%. Mutations occurred in 7.3% of the 178 samples, the most common mutation being missense mutation (Figure 1A). To further understand the expression of m6A RNA methylation regulator in tumor and normal samples, the heatmap was conducted and the results have revealed that compared with normal samples, there were 24 regulators of m6A, whose expression was relatively higher in cancer tissues (Figure 1B). Besides, the expression difference of m6A RNA methylation regulators between tumor and normal

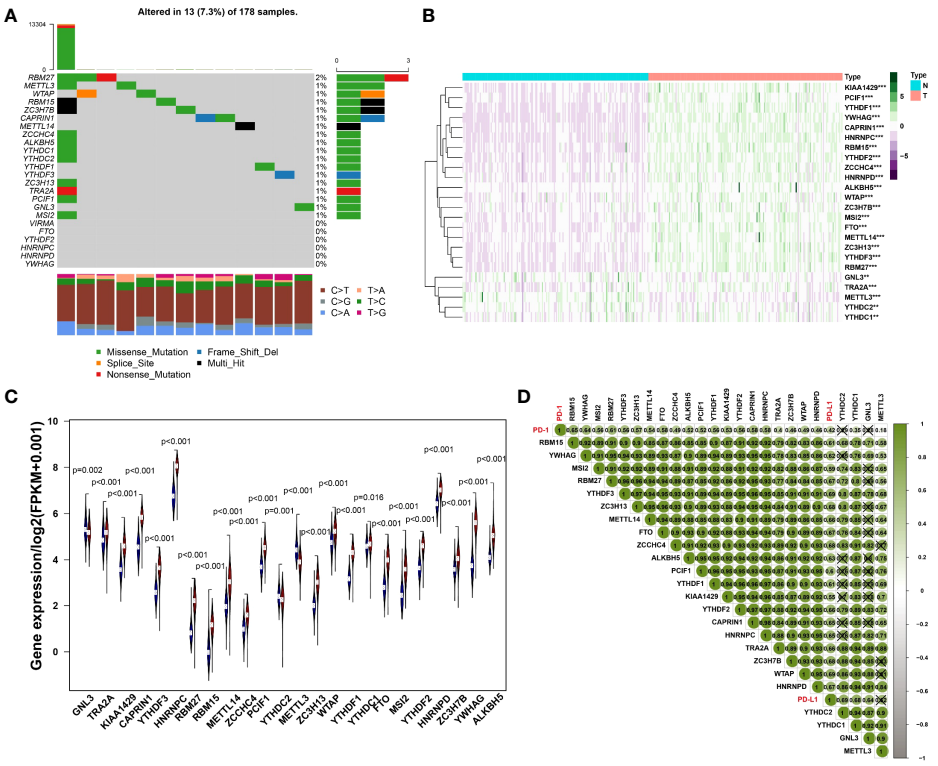

FIGURE 1
(A) The mutation frequency in each regulator. **(B)** Heatmap of m6A RNA methylation regulator expression level in each sample. **p<0.01; ***p<0.001. **(C)** The expression difference of m6A RNA methylation regulator between tumor and normal samples. **(D)** Correlation among PD-1, PD-L1 and m6A RNA methylation regulators.

samples was significant since the p-value is less than 0.05 (Figure 1C).

Correlations among PD-L1, PD1, and m6A RNA methylation regulators

The results of Pearson correlation suggested that PD-1 is associated with 20 regulators, including RBM15, YWHAG, MSI2, RBM27, YTHDF3, ZC3H13, METTL14, FTO, ZCCHC4, ALKBH5, PCIF1, YTHDF1, KIAA1429, YTHDF2, CAPRIN1, HNRNPC, TRA2A, ZC3H7B, WTAP, HNRNPD, YTHDC1 and METTL3. The correlation coefficient of PD-1 and RBM15 was the strongest. Similarly, the correlation between PD-L1 and WTAP was the strongest with a correlation coefficient of 0.69, while the relationship between PD-L1 and KIAA1429 was the weakest with a correlation coefficient of 0.55. All regulators of PD-1, PD-L1 and M6A were positively correlated (Figure 1D).

Analysis of consensus clustering

Consensus clustering method was adopted to aggregates data such as transcriptome and proteome profiles. From Figure 2A, it is believed that k = 2 has the optimal clustering stability from k = 2 to 9. Then, the consensus clustering has identified the PDAC cohort of TCGA into two clusters and demonstrated their relationship with clinicopathological parameters (Figure 2A). Subsequently, the heatmap of correlation of m6A RNA methylation regulators with characteristics of PDAC patients and the tracking plot have been concisely displayed (Figures 2B, C). Furthermore, the overall survival (OS) for PDAC patients was analyzed by using Kaplan-Meier curves. It is noteworthy that cluster2 had a significantly higher survival rate than cluster1 (Figure 2D).

Infiltrating levels of immune cell types in cluster1/2 with PDAC

We analyzed the infiltrating levels of various immune cells in cluster1/2 in PDAC and the results were displayed (Figure 3A). Meanwhile, the graph of estimated proportion of 22 immune cell types in cluster1/2 suggested that the estimated proportion of Macrophages MO of cluster1 was higher than that of Macrophages MO of cluster2, (Figure 3B). In order to further understand the infiltrating levels in cluster1/2 with PDAC, we compared the StromalScore, ImmuneScore and EstimateScore. We found the StromalScore and EstimateScore of cluster2 were higher than that of cluster1, which indicated that cluster2 had a higher degree of immune infiltration than cluster1 (Figures 4A–C). Moreover, cluster1 and cluster2 were involved

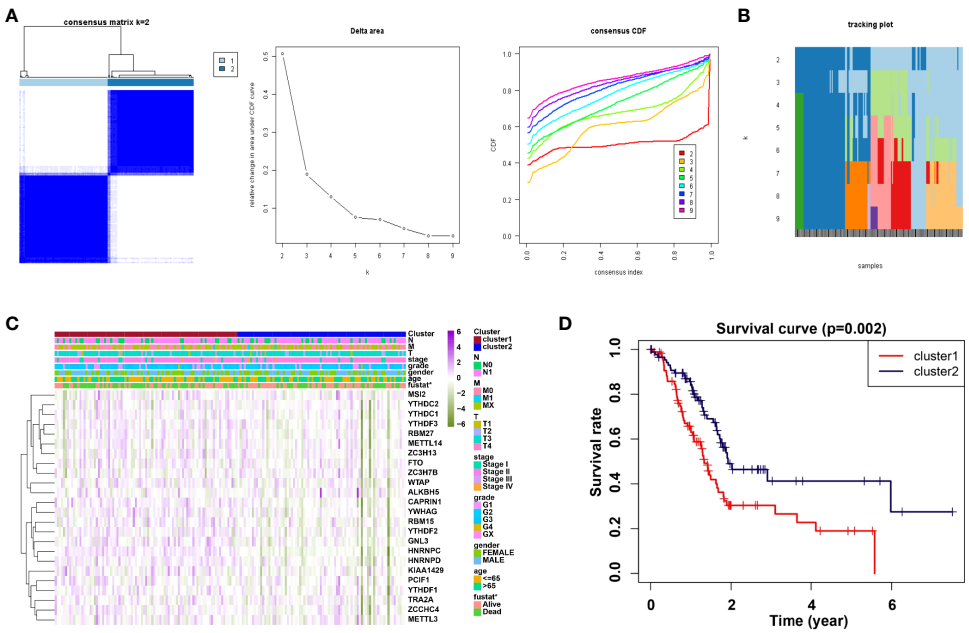

FIGURE 2
Correlation of consensus clustering for m6A RNA methylation regulators with the characteristics and survival of PDAC patients. **(A)** Consensus clustering matrix for k=2 (left panel); Consensus clustering cumulative distribution function (CDF) for k=2 to 9 (middle panel); relative change in area under CDF curve for k=2 to 9. **(B, C)** Heatmap of correlation of m6A RNA methylation regulators with characteristics of PDAC patients. **(D)** Kaplan-Meier curves of overall survival (OS) for patients.

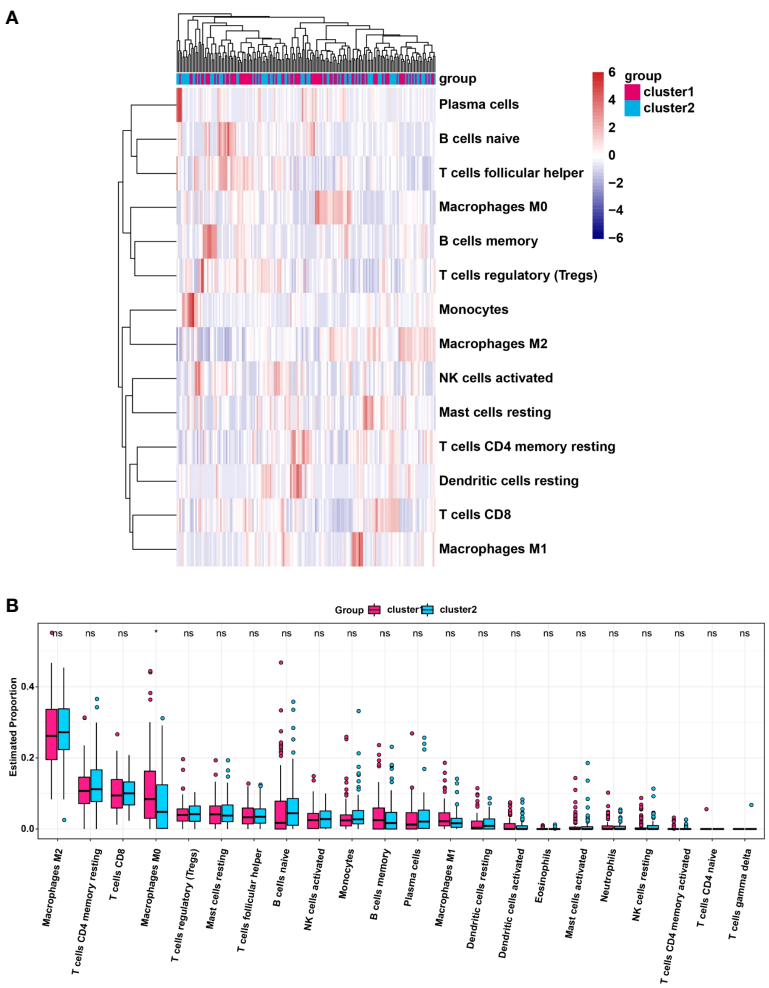

FIGURE 3
(A) Heatmap of infiltrating levels of various immune cells in cluster1/2 in pancreatic cancer. **(B)** Estimated proportion of 22 immune cell types in cluster1/2 in pancreatic cancer. *p<0.05; ns, no significance.

in the following five signaling pathways: cell cycle, mismatch repair, p53 signaling pathway, RNA degradation and Spliceosome (Figure 4D).

Construction and validation of prognostic characteristics of m6A regulators

Univariate analysis of 24 m6A RNA methylation regulators was performed to identify genes, which may significantly associate with prognosis. Indeed, the results revealed that GNL3, CAPRIN1, PCIF1, METTL3, YWHAG and ALKBH5 were significantly associated with OS with hazard ratios of 1.807, 2.289, 0.534, 0.647, 1.759 and 0.474, respectively. The 95 percent confidence intervals were (1.128-2.893), (1.315-3.984), (0.325-0.897), (0.443-0.945), (1.068-2.895) and (0.306-0.735), respectively (Figure 5A). Overall, the hazard ratio of GNL3, CAPRIN1 and YWHAG was greater than 1, while the hazard ratio of PCIF1, METTL3 and ALKBH5 was less than 1. Then through the lasso regression algorithm, the coefficient of prognostic genes was identified (Figures 5B, C).

Moreover, survival analysis showed higher survival rates in the low-risk group than in the high-risk group (Figure 5D). The AUC at 1 years, 3 years and 5 years is 0.615, 0.756 and 0.801 (Figure 5E). Furthermore, to determine whether prognostic marker-based risk scores are independent prognostic indicators for pancreatic cancer patients, univariate and multivariate Cox regression analyses of risk scores were performed. The results proposed that N and risk score were independent prognostic indexes (p-value=0.014, HR=0.017; p-value<0.001, HR=2.247) (Figures 5F, G). The clinical features of PDAC cohort has been displayed in Figure 6A. The riskscore of cluster2 was higher than that of cluster1, and similarly, the

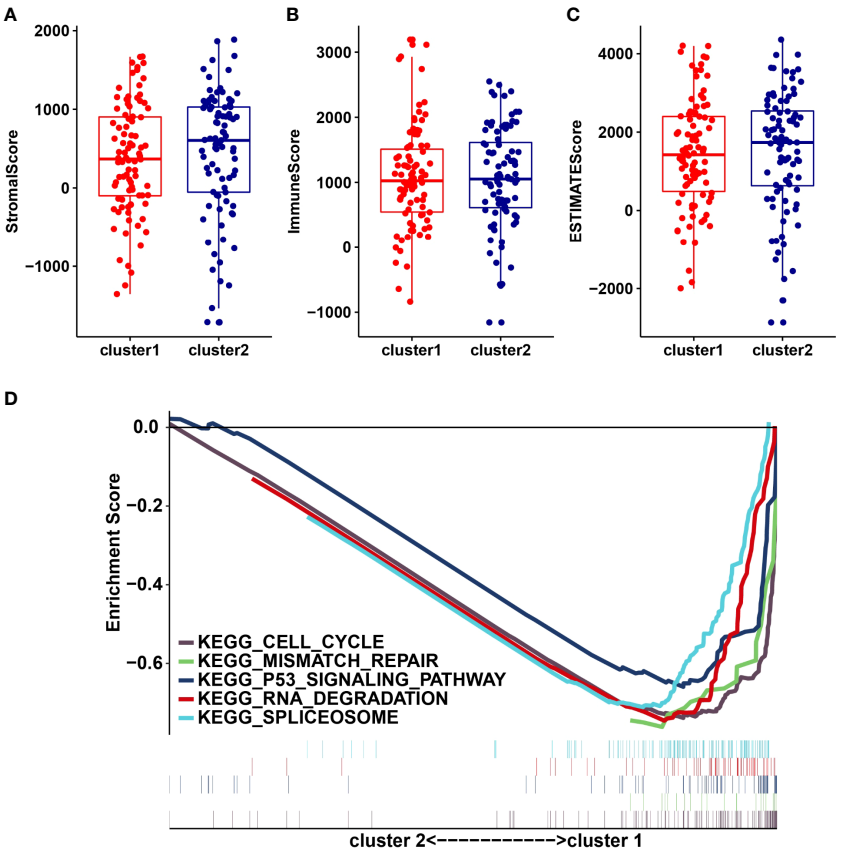

FIGURE 4
StromalScore **(A)**, ImmunoScore **(B)**, EstinateScore **(C)** in the cluster1/2 subtypes are illustrated. **(D)**: The signaling pathways are involved in cluster1 and cluster2.

riskscore of the low-risk group was higher than that of the high-risk group. Among G1, G2, G3 and G4 groups, G3 group had the highest riskscore, while G4 group had the lowest riskscore (Figure 6B). It is noteworthy that PD-1 and PD-L1 were highly expressed in pancreatic cancer cells compared with normal cells. Compared with cluster2, PD-L1 was highly expressed in cluster1(Figure 6C).

METTL3 regulates the expression of PD-L1 and lncRNA MALAT1.

To confirm the association between METTL3 and PD-L1 in pancreatic cancer cells, we transfected METTL3 cDNA and shMETTL3 plasmids to BxPC-3 and PANC-1 cells. We observed that overexpression of METTL3 increased the expression of PD-L1, whereas shMETTL3 infection led to downregulation of PD-L1 in pancreatic cancer cells (Figure 7A). It has been known that METTL3 can regulate the expression of lncRNA MALAT1. Next, we tested whether METTL3 modulation can govern the expression level of lncRNA MALAT1 in pancreatic cancer cells. Indeed, upregulation of METTL3 increased the MALAT1 level, and downregulation of METTL3 reduced the expression of MALAT1 in pancreatic cancer cells (Figure 7B). Moreover, MALAT1 overexpression increased PD-L1 expression level, while reduction of MALAT1 reduced PD-L1 level in pancreatic cancer cells (Figure 7C). Taken together, METTL3 regulates the expression of PD-L1 partly due to regulation of lncRNA MALAT1 in pancreatic cancer.

LncRNA MALAT1 regulates viability of pancreatic cancer cells.

To define the role of lncRNA MALAT1 in regulation of viability of pancreatic cancer cells, we used shMALAT1 or MALAT1 cDNA to modulate the expression of MALAT1 in pancreatic cancer cells. We found that shMALAT1 transfection suppressed the expression of lncRNA MALAT1, while MALAT1 cDNA transfection elevated the expression of MALAT1 in pancreatic cancer cells (Figures 7D, E). Moreover, increased

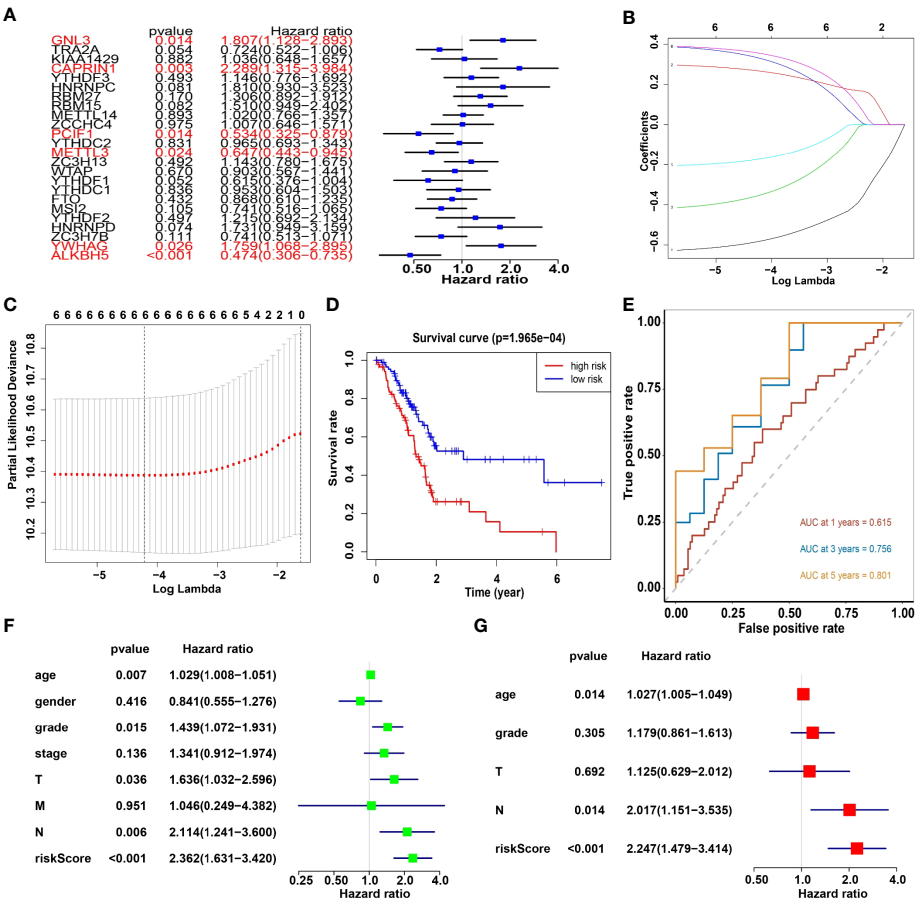

FIGURE 5
(A) Univariate analysis of 24 regulators. **(B, C)** LASSO Cox regression algorithm. **(D)** The Kaplan-Meier curve of high risk and low risk group. **(E)** Time-dependent ROC curves. **(F, G)** Univariate and multivariate Cox regression analysis of the risk scores in TCGA.

expression of MALAT1 promoted the viability of BxPC-3 and PANC-1 (Figure 8A). Furthermore, depletion of MALAT1 attenuated the cell viability at 48 h and 72 h in pancreatic cancer cells (Figure 8B). Altogether, lncRNA MALAT1 regulates viability of pancreatic cancer cells.

Discussion

Pancreatic cancer is one of the common malignant tumors of the digestive tract and is known as the “king of cancer” in the field of tumor (35). The current treatment methods are mainly chemotherapy and surgery (36). However, the five-year survival rate after diagnosis of pancreatic cancer is about 10%, and it is one of the malignant tumors with poor prognosis (37). Immunotherapy of PD-1/PD-L1 has brought the hope for the treatment of pancreatic cancer (38). However, studies have shown that pancreatic cancer has a special tumor immune microenvironment, which brings challenges to immunotherapy and deserves further study (8). m6A methylation is the most common form of mRNA modification and involves in tumorigenesis (39). However, the role of m6A methylation in pancreatic cancer and the relationship between m6A, PD-1 and the infiltration of TIME in pancreatic cancer were unelucidated.

This study analyzed the relationship between m6A RNA methylation regulators, PD-L1, prognosis and TIME in pancreatic cancer. We found a total of 24 m6A genes that were highly expressed in the tumor samples. PD-1/PD-L1 was significantly associated with 20 m6A regulators. Subsequently, we used consensus clustering to identify two subgroups (cluster 1 and cluster 2) and found that patients in cluster2 displayed better prognosis than cluster1. Furthermore, cluster1 and cluster2 may be associated with cell cycle, p53 pathway, mismatch repair, RN degradation and Spliceosome. Notably, we identified risk signatures, including GNL3, CAPRIN1, METTL3, YWHAG, ALKBH5 and PCIF1.

A recent study found that there is a significantly increasing of METTL3 expression in PDAC cells (40), and this is consistent

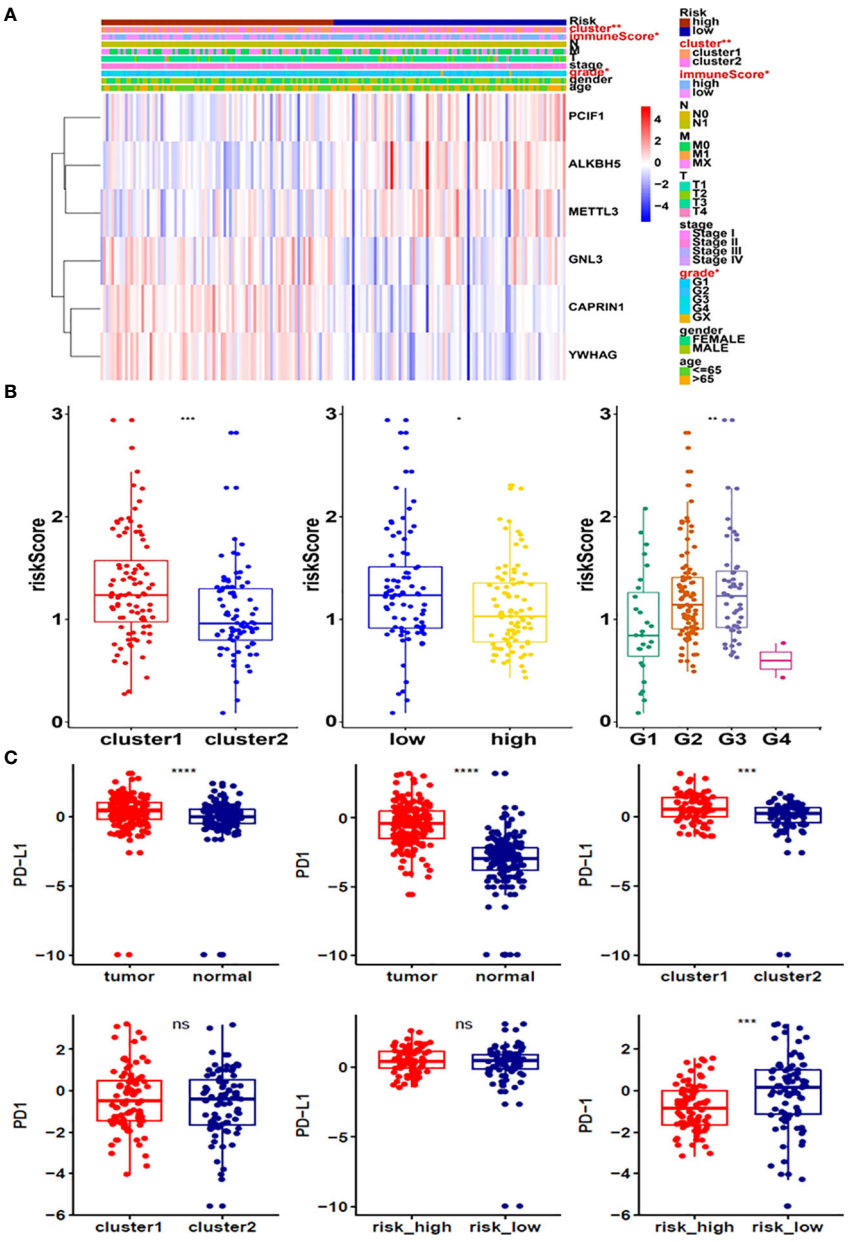

FIGURE 6
(A) Heatmap of clinicopathological features of pancreatic cancer cohort. **(B)** Distribution of risk scores stratified by cluster1/2. **(C)** The expression of PD-1 and PD-L1 in tumors, cluster1/2 and high/low-risk groups. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ns, no significance.

with our study. Our findings suggested that the expression difference of m6A RNA methylation regulator between tumor and normal samples was significant. Moreover, other evidence suggested that the upregulation of METTL3 can promote proliferation and invasion of pancreatic cancer (41). The chemical resistance in tumor cells will increase because of the rising expression of METTL3 (42). More specifically, a study pointed out that hypomethylation of the METTL3 promoter leads to overexpression of METTL3, which cooperates with NF-κB activating protein (NKAP) to coordinate m6A modification of the primary transcript of miR-25, making it mature miR-25-3p. Moreover, miR-25-3p inhibits PHLPP2 and activates oncogenic AKT-p70S6K signaling, and promotes the occurrence and progression of pancreatic cancer (43). METTL3 expression plays an important role in the TIME of pancreatic cancer (44, 45).

METTL3 has been reported to regulate the expression of PD-L1 in various cancer types. One study showed that METTL3

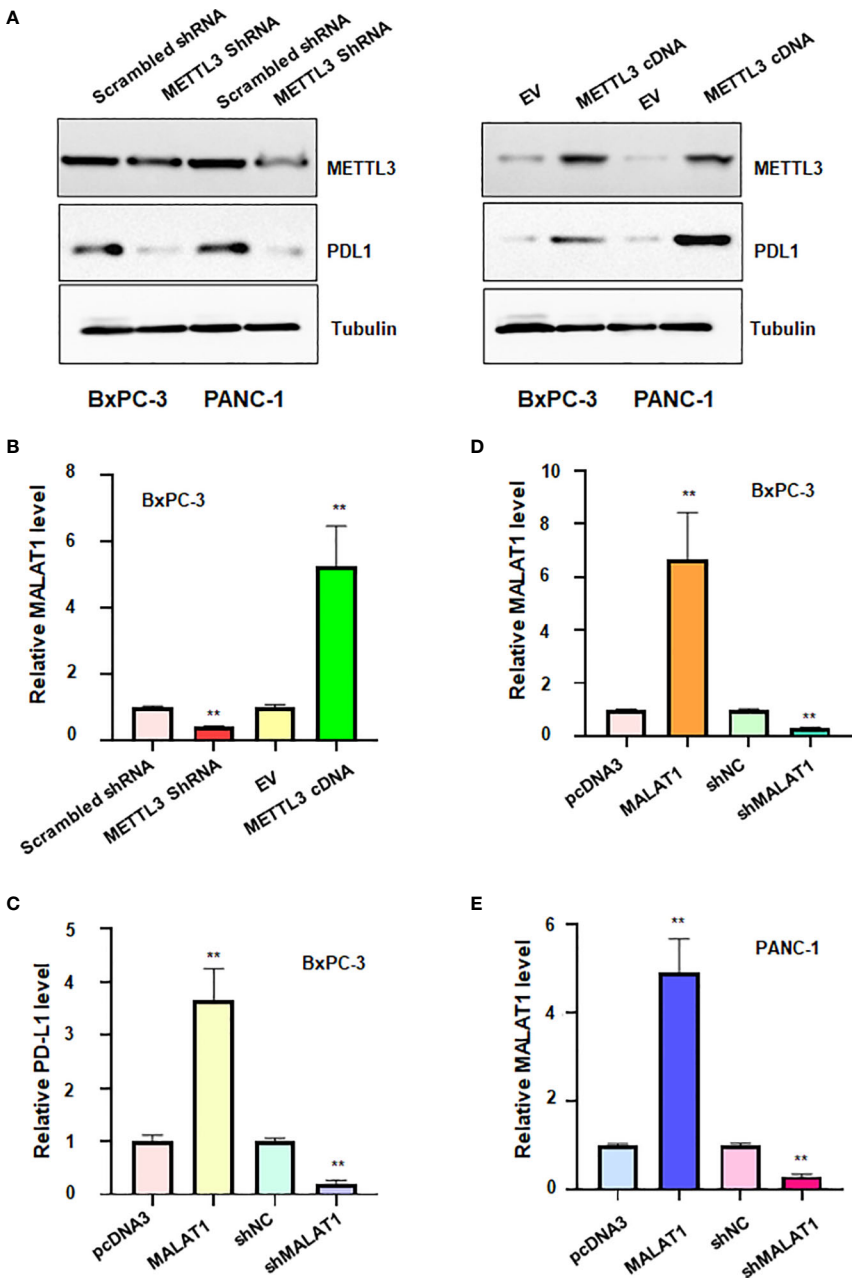

FIGURE 7
The relationship between METTL3, lncRNA MALAT1 and PD-L1 in PADC cells. **(A)** Western blotting was used to measure the expression of PD-L1 in BxPC-3 and PANC-1 cells after METTL3 modulation. **(B)** RT-PCR was used to measure the expression of lncRNA MALAT1 in BxPC-3 cells after METTL3 modulation. **(C)** RT-PCR was used to test the expression of PD-L1 in BxPC-3 cells after lncRNA MALAT1 changes. D-E: RT-PCR was used to measure the expression of MALAT1 in BxPC-3 **(D)** and PANC-1 cells **(E)** after MALAT1 modulation. **p<0.01.

increased the expression of PD-L1 and intensified the malignant phenotype in oral squamous cell carcinoma (46). Another group identified that METTL3 can upregulate the expression of PD-L1 mRNA in breast cancer cells (47). In line with this report, METTL3 also elevated the PD-L1 mRNA in bladder cancer cells (48). In the present study, we found that METTL3 had a weak association with PD-L1 expression in pancreatic cancer patients. Moreover, METTL3 positively regulates the expression of PD-L1 in pancreatic cancer cells. METTL3 has been known to upregulate the expression of MALAT1 in several cancer types. METTL3 promoted the stability of MALAT1 and enhanced the glioma progression (49). METTL3 targeted MALAT1/miR-26b/

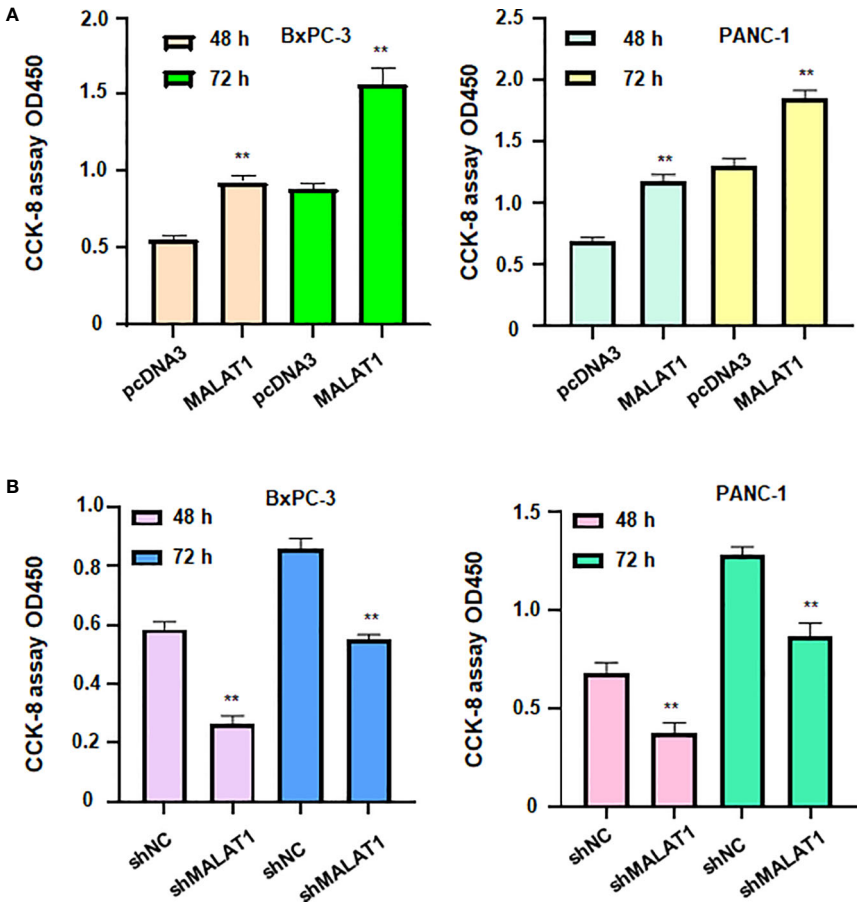

FIGURE 8
LncRNA MALAT1 regulates viability of pancreatic cancer cells. **(A)** CCK-8 assay was used to measure the viability of BxPC-3 and PANC-1 cells after MALAT1 overexpression. **(B)** CCK-8 assay was conducted to measure the viability of BxPC-3 and PANC-1 cells after MALAT1 downregulation. **p<0.01.

HMGA2 axis and caused EMT and promotion of migration and invasion in breast cancer (50). METTL3 regulated MALAT1/E2F1/AGR2 pathway and subsequently controlled Adriamycin resistance in breast cancer (51). We also found that METTL3 controlled the expression of MALAT1 in pancreatic cancer cells.

LncRNA MALAT1 upregulated the expression of PD-L1 *via* sponging miR-195, leading to promotion of tumorigenesis in diffuse large B cell lymphoma (52). MALAT1 elevated the PD-L1 expression level *via* inhibition of miR-200a-3p, resulting in non-small lung cancer progression (53). Our data showed that MALAT1 can regulate the expression of PD-L1 in pancreatic cancer cells. LncRNA MALAT1 has been found to maintain the cancer stem cell-like properties in pancreatic cancer cells, including self-renewing ability, chemoresistance and angiogenesis (54). Han et al. reported that MALAT1 interacted with EZH2 and suppressed E-cadherin expression, leading to EZH2-induced invasion and migration in pancreatic cancer (55). Li et al. found that MALAT1 facilitated tumor cell metastasis and proliferation *via* the promotion of autophagy in pancreatic cancer (56). Zhang et al. revealed that miR-216a triggered apoptosis and G2/M arrest in pancreatic cancer cells *via* inhibition of MALAT1 expression (57). MALAT1 downregulation retarded pancreatic cancer progression *via* targeting Hippo-YAP signaling pathway (58). Moreover, MALAT1 was involved in efficacy of gemcitabine treatment in pancreatic cancer patients (59). Recently, MALAT1 was identified to govern pancreatic cancer progression *via* modulation of miR-129-5p (60). In the current study, we found that depletion of MALAT1 reduced cell viability, whereas overexpression of MALAT1 enhanced viability of pancreatic cancer cells.

Conclusion

In summary, this study is the first time to apply a bioinformatic approach to describe the relationship between m6A and PD-L1 and the TIME in pancreatic cancer. There is a limitation that this work mainly used a bioinformatic strategy to explore the association among m6A, PD-L1 and TME in pancreatic cancer. The role of METTL3 in pancreatic cancer development should be validated in animal study and clinical tissue samples. The mechanism of lncRNA MALAT1-mediated pancreatic oncogenesis should be dissected. How METTL3 regulates PD-L1 expression *via* regulation of lncRNA MALAT1 is required to be determined in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

ZS, XW, FC, WL, QC, XY and XZ performed the experiments, analyzed the data. ZS, QC and XL wrote the manuscript. PW edited the manuscript and supervised this study. All authors read and approved the final manuscript.

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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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LncRNA SNHG6 sponges miR-101 and induces tamoxifen resistance in breast cancer cells through induction of EMT

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Acquired resistance is a major clinical challenge for tamoxifen-based therapy. In this study, we focused on lncRNA SNHG6 which plays a role in chemoresistance of cancer cells, but has never been investigated in the context of tamoxifen resistance. We found elevated levels of SNHG6 in tamoxifen-resistant estrogen receptor (ER)-positive MCF-7 cells (MCF7TR), relative to naïve MCF-7 cells, as well as in tamoxifen-resistant T47D cells (T47DTR), relative to naïve T47D cells, which correlated with induced vimentin, ZEB1/2 and decreased e-cadherin, thus implicating a role of EMT in SNHG6-mediated tamoxifen resistance. Downregulation of SNHG6, using specific siRNA, sensitized MCF7TR as well as T47DTR cells to tamoxifen along with markedly reduced proliferation, invasion and anchorage-independent clonogenicity. Further, SNHG6 was found to sponge and inhibit miR-101 as the endogenous expression levels of SNHG6 and miR-101 inversely correlated in paired parental and tamoxifen-resistant cells and, moreover, silencing of SNHG6 in tamoxifen-resistant cells resulted in de-repression of miR-101, along with reversal of EMT. SNHG6 expression also directly correlated with increased stem cells markers Sox2, Oct4 and EZH2. miR-101 levels, manipulated by transfections with pre/anti-miR-101 oligos, directly affected tamoxifen sensitivity of ER-positive cells with pre-miR-101 sensitizing MCF7TR and T47DTR cells to tamoxifen whereas anti-miR-101 inducing resistance of parental MCF-7 and T47D cells to tamoxifen. Further, miR-101 was found to attenuate SNHG6-mediated effects on tamoxifen resistance, EMT as well as stem cell markers, thereby making a case for SNHG6-miR-101 axis in tamoxifen resistance of ER-positive breast cancer cells. Thus, lncRNA SNHG6 is a novel modulator of tamoxifen resistance through its sponging of miR-101 and the resulting effects on EMT.

KEYWORDS

tamoxifen resistance, SNHG6, miR-101, epithelial-to-mesenchymal transition (EMT), epigenetic

Introduction

Breast cancer is a major cancer that mostly affects women with 287,850 new cases of invasive breast cancer estimated for the current year 2022 in the United States (1). Worldwide, approximately 2.3 million breast cancer cases were diagnosed in the year 2020 (2). There has been an increase in the newly diagnosed cases over past several years, primarily due to aggressive screening and vigilance (3), with the rate of increase around 0.5% since the mid-2000s (1). With these numbers, breast cancer ranks number one among all cancers that are diagnosed in women in the US, accounting for almost one-third of all cancer diagnoses in the women. Treatment of breast cancer patients is particularly challenging because of the many individual breast cancer subtypes. The estrogen receptor (ER)-positive breast cancers make up to 60% of all breast cancers (4) and are therefore a major subtype. The ER-positive breast cancers are managed through administration of ER antagonist tamoxifen, however, acquired resistance against tamoxifen remains a major clinical challenge (5).

Recent years have witnessed a growing interest in the involvement of non-coding RNAs in tamoxifen resistance of ER-positive breast cancers (6, 7). Various non-coding RNAs, ranging from microRNAs (miRNAs) to long non-coding RNAs (lncRNAs) have been reported to mechanistically and functionally affect the tamoxifen resistance. Our own earlier work demonstrated a role of miR-10b in the tamoxifen resistance of ER-positive breast cancers (8) and such role of miRNAs in tamoxifen resistance has been investigated by several other researchers as well (6, 7, 9). Further, lncRNAs are novel molecules of interest in terms of their possible role in the modulation of response to tamoxifen in ER-positive breast cancers (10). Among the many lncRNAs currently being investigated for their possible role in cancer drug resistance, lncRNA SNHG6 is a promising lncRNA that has been associated with increased risk of poor overall survival of cancer patients (11). High expression of SNHG6 has been demonstrated to correlate with tumor progression and poor prognosis in multiple human cancers (12). Even though a role of SNHG6 in cancer radio-resistance (13) as well as chemoresistance (14), particularly resistance against cisplatin (15) and paclitaxel (16) has been described, there is no report on it's possible role in acquired tamoxifen resistance of ER-positive breast cancers, prompting us to plan this study. Using a paired ER-positive breast cancer cell line comprising of MCF-7 cells that are sensitive to tamoxifen along with their derivate cells that are resistant to tamoxifen (MCF7TR), we investigated the SNHG6-regulated mechanism of tamoxifen resistance. We further confirmed the findings using a parallel cell line pair comprising of tamoxifen-sensitive ER-positive T47D cells and their tamoxifen-resistant derivatives, the T47DTR cells.

Materials and methods

Cell lines and reagents

MCF-7 and T47D breast cancer cells were purchased from ATCC (USA) and cultured in DMEM and RPMI cell culture medium (ThermoFisher Scientific, USA), respectively, with 10% fetal bovine serum, 100 units/ml penicillin, and 100µg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. The tamoxifen resistant MCF-7 and T47D cell lines, MCFTR and T47DTR, respectively, were generated by culturing cells in their respective culture mediums supplemented with 5% FBS, antibiotics and 10^{-6} M 4-hydroxy tamoxifen (TAM), as described previously (8). TAM concentration was gradually increased over the course of six months until the final concentration was 10^{-6}M.

lncRNA downregulation

siRNAs (ThermoFisher Scientific, USA) designed against SNHG6 were transfected into MCF7TR cells using Lipofectamine RNAiMAX Transfection Reagent. Knockdown was evaluated by qRT-PCR. Our detailed preliminary validation revealed that the siRNAs were effective at 30nM final concentration and at 24 hours post-transfection, therefore, we used 30nM concentration of si-SNHG6 for our experiments. The siRNA used in our study was locked nucleic acid (LNA) modified siRNA (ThermoFisher Scientific, USA) and was used for increased potency and lower off-target effects.

siRNA transfections

We used Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific, USA) for the transfections of si-SNHG6 in MCF7TR cells for effective downregulation of the lncRNA. Cells were first seeded in 24-well plates in a total volume of 500µl in culture medium without antibiotics. Cells were seeded at a density so that they were 50% confluent the next day. On the day of transfections, siRNA-Lipofectamine complexes were prepared by diluting siRNA in 50µl of Opti-MEM ® I Reduced Serum Medium without serum. In a separate tube 1µl of Lipofectamine RNAiMAX Transfection Reagent was diluted in 50µl of Opti-MEM ® I Reduced Serum Medium. The contents of two individual tubes were then mixed and incubated for 20 minutes at room temperature. The contents were then added on to the individual wells without removing the overnight culture medium. The calculations were done to ensure 30nM concentration of siRNA in the total final volume of 600µl. Contents of the wells were mixed by gentle rocking and plates

were transferred back to cell culture incubators. Effect of siRNA transfections were tested after an incubation of 24 hours.

Cell growth inhibition studies by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Parental as well as tamoxifen-resistant cells were seeded overnight at a density of 5 x 10^3 cells per well in 96-well culture plates. Thereafter, culture medium was aspirated and replaced with fresh complete culture medium containing DMSO (vehicle control) or different concentrations of tamoxifen, as indicated. After 48 hours, 25µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml in phosphate-buffered saline, PBS) was added to individual assay wells and incubated further for 2 h at 37°C. Upon termination, the supernatant was removed and the MTT formazan, formed by metabolically viable cells, was dissolved in DMSO (100µl) by mixing for 30 min on a gyratory shaker. The absorbance was measured at 595 nm on Ultra Multifunctional Microplate Reader (TECAN, USA).

Apoptosis assay (Histone/DNA ELISA)

We used Cell Death Detection ELISA Kit (Roche) to detect apoptosis, as described earlier (8). Briefly, after the cells were appropriately treated, as indicated for individual experiments, the cytoplasmic histone/DNA fragments were extracted and incubated in microtiter plate modules coated with anti-histone antibody. Thereafter, peroxidase-conjugated anti-DNA antibody was used to detect immobilized histone/DNA fragments, followed by color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined using Ultra Multifunctional Microplate Reader (TECAN, USA) at 405 nm.

Cell invasion assay

Cell invasion was assessed using 24 well transwell permeable supports with 8 µM pores (Corning, USA). After appropriate experimental set-up, as indicated for individual experiments, cells were trypsinized and re-suspended in serum free medium before seeding into the transwell inserts coated with growth factor reduced Matrigel (BD Biosciences, USA). Bottom wells were filled with complete media. After 24 hours, cells were stained with 4 µg/ml calcein AM (ThermoFisher Scientific, USA) in PBS at 37°C for 1 h. Cells were detached from inserts by trypsinization and fluorescence of the invaded cells was read using ULTRA Multifunctional Microplate Reader (TECAN, USA).

Anchorage-independent clonogenicity assay

Tamoxifen-resistant cells were first transfected with appropriate siRNAs (non-specific control siNS or siSNHG6), allowed to grow for 24 h and then collected by trypsinization. 3 × 10^4 cells were then seeded in 0.5 ml of complete culture medium containing 0.3% (w/v) top agar layered over a basal layer of 0.7% (w/v) agar (with culture medium and the supplements) in 24-well plates. After 21 days of culture, colonies were counted using a phase contrast microscope (Nikon, USA).

Prediction of miRNA targets of lncRNA SNHG6

miRNA targets of lncRNA SNHG6 were predicted using DIANA-LncBase v3 (https://diana.e-ce.uth.gr/lncbasev3), as described recently by others (17). DIANA-LncBase v3 is a reference repository that lists experimentally supported miRNA targets on non-coding transcripts. As of the date of access of DIANA-LncBase v3, the database consisted of approximately ~500,000 entries, corresponding to ~240,000 unique tissue and cell-type specific miRNA-lncRNA interactions. As per the information on database webpage, the incorporated interactions between lncRNAs and miRNAs are defined by 15 distinct low-/high-throughput methodologies, corresponding to 243 distinct cell types/tissues and 162 experimental conditions. We listed the miRNA targets of lncRNA SNHG6 without any bias for cell type or other parameters that could have affected the listing of miRNA targets in any way.

miRNA transfections

Transfections of pre/anti-miR-101 were done using methodology previously described (8). Briefly, cells were seeded (2.5 x 10^5 cells per well) in six well plates and transfected with pre/anti-miR-101 or non-specific pre/anti-miRNA controls (ThermoFisher Scientific, USA) at a final concentration of 200 nM, using DharmaFECT transfection reagent (Dharmacon, USA). After 48 hours of transfection, cells were passaged and transfected twice again, using the same methodology, before being used in the individual experiments.

qRT-PCR and miRNA detection

Real-Time quantitative (q)RT-PCR analyses were also done as described previously (8). Total RNA was isolated from the cells, after the completion of individual experiments, using the

mirVana miRNA isolation kit (ThermoFisher Scientific, USA). RNAse-free water was used throughout the analysis. RT^2 First Strand Kit (Qiagen, USA) was used to synthesize cDNA first strand using 1µg RNA, to which 2µl of genomic DNA elimination mix was added, mixed and incubated 10 minutes at 42^{0}C, followed by immediate transfer to ice for 1 minute. Reverse transcription mix, consisting of 5x buffer and Reverse Transcriptase, was then prepared and added to RNA which was then subjected to incubation for 15 minutes at 42^{0}C. Finally, the reaction was stopped by incubation at 95^{0}C for 5 minutes. The levels of miR-101 were determined using miRNA-specific Taqman probes from the Taqman MicroRNA Assay (ThermoFisher Scientific, USA). The relative amounts of miRNA were normalized to RNU6B.

Statistical analysis

All experiments were performed a minimum of three times with triplicate repeats in individual experimental setup. To evaluate if 2 datasets were significantly different, a p value was calculated using Student *t* test or one way ANOVA assuming equal variables and 2-tailed distribution. Prior to the statistical tests, datasets were log-transformed to ensure normal distribution. In all of our experiments, the p values <0.05 were considered to be statistically significant.

Results

SNHG6 is elevated in tamoxifen resistant cells and positively regulates acquired resistance against tamoxifen

We started our investigation by assessing the relative levels of lncRNA SNHG6 in parental MCF-7 cells and their tamoxifen resistant counterparts, the derived MCF7TR cells. SNHG6 levels were more than 12-folds elevated in the MCF7TR cells, compared to MCF-7 cells (Figure 1A) indicating a correlation of lncRNA SNHG6 with tamoxifen resistance. To further increase confidence in our findings and rule out a cell line-specific effect, we measured the levels of SNHG6 in another ER-positive breast cancer cell line T47D. A comparison of levels in parental T47D vs. the tamoxifen resistant derivatives T47DTR revealed a >5-folds increase in SNHG6 levels in the resistant cells (Figure 1B). The increase in SNHG6 levels in both of the cell line pairs was found to be highly significant ($p<0.01$). As described in the Methods, resistant MCF7TR and T47DTR were generated by prolonged exposure of respective native cells to tamoxifen in cell culture set-up. To check whether such prolonged exposure of native cells to tamoxifen had indeed resulted in generation of tamoxifen-resistant derivatives, we checked the tamoxifen sensitivity of the paired cells. When exposed to increasing concentrations of tamoxifen for 48 hours, followed by MTT assay, we observed a remarkable increase ($p<0.01$) in the resistance against tamoxifen in 'resistant' cells, both MCF7TR (Figure 1C) and T47DTR (Figure 1D), against tamoxifen. The IC-50 for MCF7TR cells was >10-folds while the IC-50 for T47DTR cells was >8-folds, relative to the respective native cells (Table 1). In continuation of our observation that SNHG6 levels were elevated in resistant cells, we next silenced SNHG6 using LNA-modified siRNAs against lncRNA SNHG6. We first tested four different siRNA preparations (Results not shown) and chose the siRNA that demonstrated more than 80% downregulation of SNHG6. The testing was initially done in MCF7TR cells and the chosen siRNA was tested for its efficacy in T47DTR cells as well wherein, the siRNA again demonstrated more than 80% downregulation of SNHG6. siRNA against SNHG6 significantly sensitized MCF7TR cells against tamoxifen, as observed by significantly reduced ($p<0.01$) cell proliferation in the presence of tamoxifen (Figure 1C). Very similar results were observed in T47D cells, as well, with silencing of SNHG6 resulting in significantly reduced ($p<0.01$) proliferation when cells were treated with increasing concentrations of tamoxifen (Figure 1D).

SNHG6 affects EMT, apoptosis, invasion and clonogenicity

As a mechanism of tamoxifen resistance possibly induced by SNHG6, we first evaluated the process of epithelial-to-mesenchymal transition (EMT) because of the published reports demonstrating a profound modulation of EMT by SNHG6 (18, 19) and the role of EMT in tamoxifen resistance (20, 21). We evaluated the expression of EMT markers, E-cadherin, vimentin, ZEB1 and ZEB2 in paired cell lines. While E-cadherin is a marker of epithelial phenotype, the rest three are markers of mesenchymal phenotype. We observed that E-cadherin was markedly downregulated ($p<0.01$) whereas vimentin, ZEB1 and ZEB2 were markedly upregulated ($p<0.01$) in MCF7TR cells, relative to the parental MCF-7 cells, indicating the induction of EMT (Figure 2A). Similar induction of EMT was apparent in T47DTR cells as well, relative to parental T47D cells (Figure 2B), as evidenced by significantly downregulated ($p<0.01$) E-cadherin and significantly upregulated ($p<0.01$) vimentin, ZEB1 and ZEB2. We further studied the effect of silencing SNHG6 in these resistant cells to check if the silencing of oncogenic SNHG6 can reverse EMT. As shown in Figures 2A, B, we observed that silencing of SNHG6 significantly ($p<0.01$) attenuated the tamoxifen resistance-associated changes in EMT markers in both of the cell lines. The reduced levels of E-cadherin were attenuated by silencing of SNHG6 while the elevated levels of vimentin, ZEB1 and ZEB2 in tamoxifen-resistant cells were

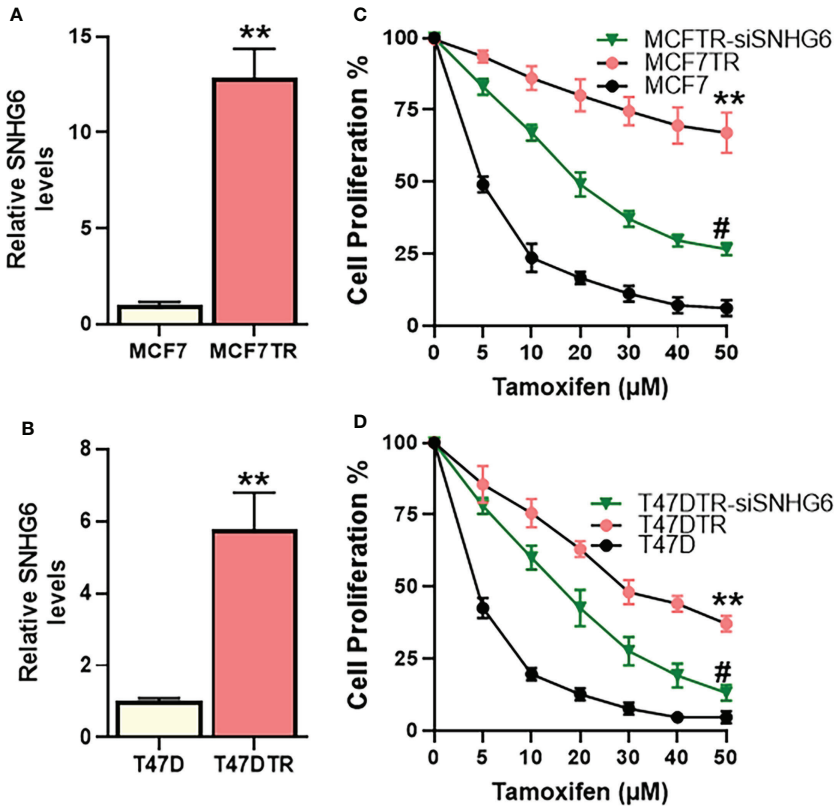

FIGURE 1
SNHG6 expression and function in tamoxifen resistant ER-positive Breast cancer cells. Expression of SNHG6 was quantitated in tamoxifen-resistant **(A)** MCF-7 cells (MCF7TR) and **(B)** T47D cells (T47DTR) by qRT-PCR. The levels of SNHG6 in parental cells (MCF-7/T47D) were assigned a value of 1 and the relative levels in tamoxifen-resistant derivatives are plotted. Cell proliferation was measured by MTT assay in paired cell lines **(C)** MCF-7/MCF7TR and **(D)** T47D/T47DTR after the cells were exposed to indicated concentrations of tamoxifen for 48 hours. Further, tamoxifen resistant MCF7TR and T47DTR were subjected to SNHG6 silencing and then treated with increasing concentrations of tamoxifen for 48 hours before the proliferation was assessed using MTT assay. **p<0.01, compared to native controls and #p<0.01, compared to respective resistant cells without SNHG6 silencing.

significantly reduced by the silencing of SNHG6. Further, silencing of SNHG6 induced apoptosis in both of the resistant cell lines (Figure 2C) in addition to reducing the invasion potential (Figure 2D) and the colony forming ability (clonogenicity) (Figure 2E) of both cell lines. These results suggest that SNHG6 silencing sensitizes tamoxifen resistant cells to tamoxifen through reversal of EMT, induction of apoptosis and associates with reduced aggressiveness and significantly reduced invasion potential and colony forming ability.

Identification of miR-101 as the miRNA sponged by SNHG6

lncRNAs function through sponging of target miRNAs and, therefore, we next evaluated the miRNAs that are sponged by SNHG6 as evidenced through their negative regulation by SNHG6. For the screening of putative miRNAs, we searched the literature for published miRNAs that are sponged by SNHG6, and, additionally, employed bioinformatics-based

TABLE 1 IC-50 values.

Cell line	IC-50	Cell line	IC-50
MCF-7	4.6 ± 0.1 μM	T47D	3.5 ± 0.2 μM
MCF7TR	> 50 μM	T47DTR	28.7 ± 0.8 μM
MCF7TR-siSNHG6	19.1 ± 0.6 μM	T47DTR-siSNHG6	10.0 ± 0.3 μM

The values were calculated based on the experiments reported in Figures 1 C, D.

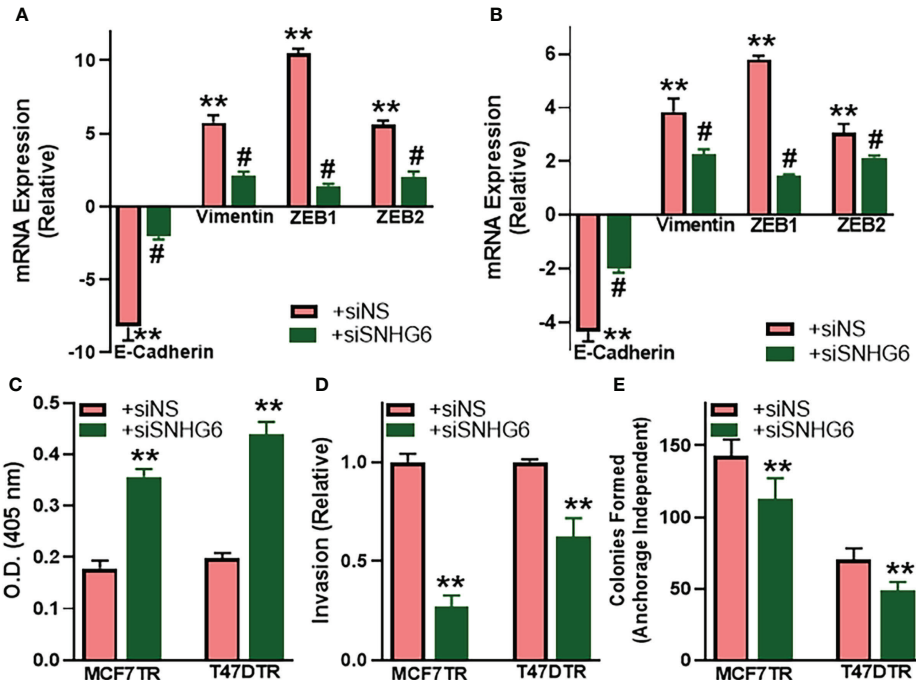

FIGURE 2
SNHG6 affects EMT, apoptosis, invasion and clonogenicity. **(A)** MCF7TR and **(B)** T47DTR cells were transfected with control siRNA (siNS) or siRNA against SNHG6 and then the levels of EMT markers, E-cadherin, vimentin, ZEB1 and ZEB2 were evaluated by qRT-PCR. The levels of these markers in respective parental cells were assigned a value of 1 and the relative levels in siNS/siSNHG6 transfected tamoxifen-resistant cells are plotted. **(C)** Apoptosis was assessed in tamoxifen-resistant cells by Histone/DNA ELISA method, as described in the Methods, by measuring the O.D. at 405 nm. **(D)** Invasive potential of tamoxifen-resistant cells was evaluated by quantitating the fluorescence of cells that invaded through the matrigel-coated membranes. Fluorescence of siNS cells assigned a value of 1 and the relative fluorescence of siSNHG6 transfected tamoxifen-resistant cells is plotted. **(E)** Clonogenicity was measured by counting the colonies formed by cells in an anchorage-independent manner, as described in the Methods. **p<0.01, compared to respective controls, #p<0.01, compared to siNS.

approach to list the putative miRNAs that can be sponged. DIANA-LncBase v3 was used to list such miRNAs. A number of miRNAs were shortlisted and tested but only those that were significantly modulated in MCF7TR cells, relative to native MCF-7 cells, are reported in Figure 3. miR-101 (also referred to as miR-101-3p) was the most significantly ($p<0.01$) downregulated miRNA among all the tested miRNAs. Several other miRNAs were also found to be downregulated significantly (let-7d, let-7e and miR-325 with $p<0.05$ and miR-26a and miR-485 with $p<0.01$) whereas two miRNAs (miR-186 and miR-1297) were found to be upregulated in MCF7TR cells, relative to the MCF-7 cells. Based on these results, we chose miR-101 as the miRNA of interest for further mechanistic studies.

miR-101 effects on tamoxifen resistance in MCF-7 cells

The effect of miR-101 on tamoxifen sensitivity was checked by manipulating the levels of miR-101 in MCF-7 as well as MCF7TR cells. First, we checked the levels of miR-101 in these two paired cells and found significantly reduced ($p<0.01$) miR-101 levels in MCF7TR cells, compared to the parental MCF-7 cells (Figure 4A). We also checked for a regulation of miR-101 by SNHG6 in our experimental model and observed that silencing of SNHG6 significantly ($p<0.01$) attenuated the tamoxifen resistance-associated down-regulation of miR-101 in MCF7TR cells (Figure 4A). Since the levels of miR-101 were relatively higher in MCF-7 cells, we downregulated miR-101 levels in these cells, through the use of specific anti-miR-101 oligomers and subjected the cells to tamoxifen treatment for 48 hours. As shown in Figure 4B, such downregulation of miR-101 significantly increased the resistance of MCF-7 cells, relative to the MCF-7 cells with non-specific control oligomers. As an experiment to further confirm the role of miR-101 in tamoxifen resistance, we upregulated miR-101 in MCF7TR cells through transfections with pre-miR-101 and observed significantly reduced tamoxifen resistance (Figure 4B). Next, we checked for the functional relevance of miR-101 upregulation in SNHG6-silenced MCF7TR cells through the evaluation of tamoxifen sensitivity and found that antagonizing such elevated levels of miR-101 in SNHG6-silenced MCF7TR cells, through anti-miR-

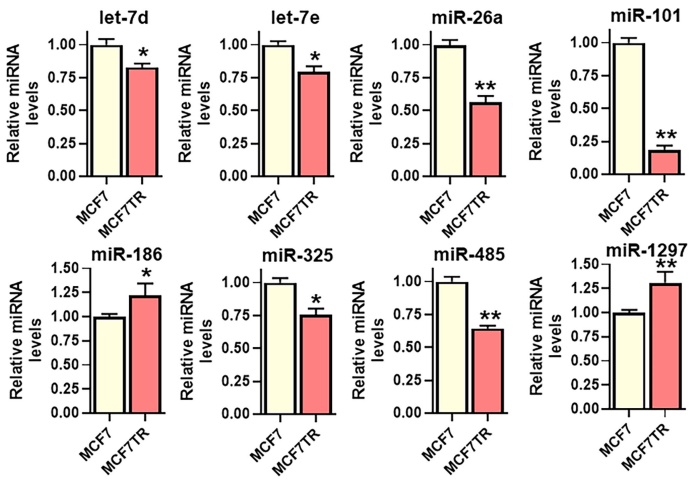

FIGURE 3
Identification of SNHG6 target miRNAs. Several miRNAs, as identified, were quantitated in MCF-7 and MCF7TR cells, using qRT-PCR. The levels of miRNAs in parental MCF-7 cells were assigned a value of 1 and the relative levels in tamoxifen-resistant MCF7TR are plotted. *p<0.05 and **p<0.01, compared to respective controls.

101 oligomers, significantly increased the resistance of MCF7TR cells against tamoxifen (Figure 4C).

miR-101 effects on SNHG6-mediated EMT and cancer stem cells

As reported above, SNHG6 had modulating effects on EMT induction, therefore, we next checked the mechanistic involvement, if any, of miR-101, the miRNA sponged by SNHG6, in the process. As shown in Figure 5A, relative to MCF7TR cells silenced for SNHG6, the cells with added anti-miR-101 oligomers had significantly reduced (p<0.01) epithelial marker E-cadherin levels along with significantly elevated (p<0.01) mesenchymal markers vimentin, ZEB1 and ZEB2. Also, in view of the intricate connection between drug resistance, EMT and the cancer stem cell phenotype (22, 23), we evaluated various markers of cancer stem cell phenotype in our experimental model system and found that all the tested markers, Sox2, Oct4 as well as EZH2 were significantly downregulated in SNHG6-silenced MCF7TR cells, relative to the control MCF7TR cells (Figure 5B). Transfection of anti-miR-

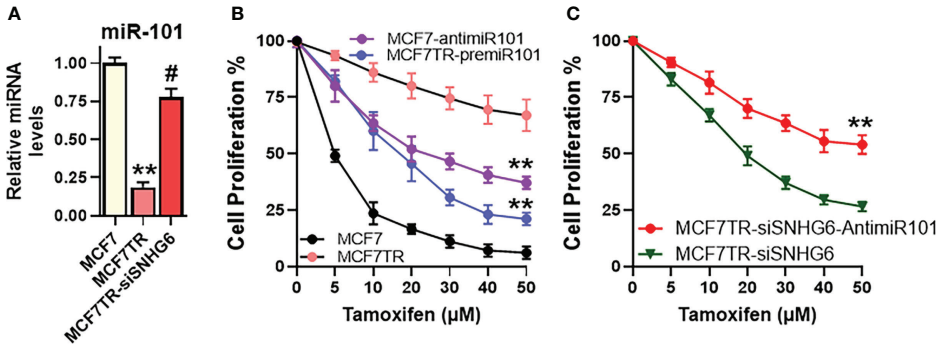

FIGURE 4
miR-101 affects tamoxifen resistance. **(A)** Levels of miR-101 were assessed in MCF-7, MCF7TR and MCF7TR cells silenced for SNHG6. Levels of miR-101 in parental MCF-7 cells were assigned a value of 1 and the relative levels in other cells are plotted. **(B)** Cell proliferation was measured by MTT assay in MCF-7/MCF7TR cells after transfections with pre/anti-miR-101 oligomers, as appropriate, followed by exposure to indicated concentrations of tamoxifen for 48 hours. **(C)** Tamoxifen resistant MCF7TR either just silenced for SNHG6 or additionally transfected with anti-miR-101 oligomers were treated with increasing concentrations of tamoxifen for 48 hours before the proliferation was assessed using MTT assay. **p<0.01, compared to respective controls, #p<0.01, compared to MCF7TR.

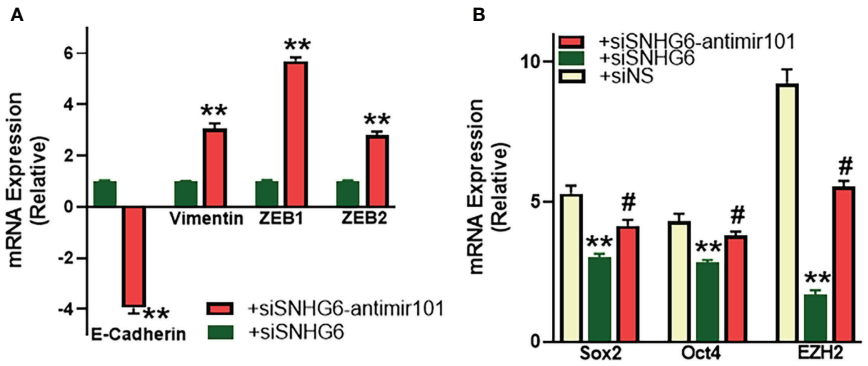

FIGURE 5
miR-101 affects EMT and cancer stem cell markers. **(A)** MCF7TR cells were silenced for SNHG6 and then the levels of EMT markers, E-cadherin, vimentin, ZEB1 and ZEB2 were evaluated by qRT-PCR in these cells as well as cells that were additionally transfected with anti-miR-101 oligomers. The levels of markers in SNHG6-silenced cells were assigned a value of 1 and the relative levels in siSNHG6+anti-miR-101-transfected cells are plotted. **(B)** Molecular markers of cancer stem cell phenotype, Sox2, Oct4 and EZH2 were quantitated, using qRT-PCR. The levels of miRNAs in parental MCF-7 cells were assigned a value of 1 and the relative levels in tamoxifen-resistant control MCF7TR (siNS) and those silenced for SNHG6 with and without additional transfections with anti-miR-101, are plotted. **p<0.01, compared to siNS, #p<0.01, compared to siSNHG6.

101 oligomers in SNHG6-silenced MCF7TR cells resulted in significant ($p<0.01$) attenuation of SNHG6 silencing effects (Figure 5B) which further established the mechanistic role of miR-101 in SNHG6 mediated effects.

miR-101 mediates SNHG6 effects in T47D cells as well

We identified miR-101 as the miRNA sponged by SNHG6 in experiments that were carried out using MCF7 and MCF7TR cells, and further established the mechanistic role of miR-101 in SNHG6 effects in those cells. To further validate these findings, we performed similar experiments in T47D cells as well. We started with an evaluation of the relative levels of miR-101 in parental and tamoxifen resistant T47D cells, and observed significantly reduced ($p<0.01$) miR-101 levels in T47DTR cells (Figure 6A) thus confirming the earlier results from paired MCF7 cells. miR-101 manipulations had a profound effect on tamoxifen sensitivity as antagonizing miR-101 in parental T47D cells resulted in significantly ($p<0.01$) induced tamoxifen resistance while overexpression of miR-101, through pre-miR-101 oligomers, resulted in significant ($p<0.01$) sensitization of T47DTR cells to tamoxifen (Figure 6B). In context of the mechanistic involvement of miR-101 in SNHG6 induced tamoxifen resistance, anti-miR-101 oligomers significantly ($p<0.01$) increased the sensitivity to tamoxifen of T47DTR cells silenced for SNHG6 (Figure 6C) which involved modulation of SNHG6-mediated EMT because, relative to T47DTR cells with silenced SNHG6, the ones that additionally were transfected with anti-miR-101, showed induction of EMT, as evidenced by downregulated E-cadherin and upregulated vimentin, ZEB1 and ZEB2 (Figure 6D). Further, SNHG6 silencing reduced cancer stem cell markers Sox2, Oct4 and EZH2 in T47DTR cells, relative to parental T47D cells, and anti-miR-101 oligomers significantly ($p<0.01$) attenuated this effect (Figure 6E).

Discussion

Acquired resistance against tamoxifen is a major clinical challenge that severely impacts the clinical management of ER-positive breast cancer patients. A number of lncRNAs are now known to play a role in resistance against cancer therapies (24, 25), including resistance against tamoxifen (7, 10). In the present study, we focused on lncRNA SNHG6 for its potential role in acquired tamoxifen resistance in ER-positive breast cancer cells. Our hypothesis to focus on SNHG6 for this study was based on the published literature supporting a role of SNHG6 in cancer drug resistance, coupled with the fact that its role specifically in tamoxifen resistance has never been elucidated. As per the published literature, SNHG6 can impact cisplatin resistance in gastric cancer (15, 26), 5-FU resistance in colorectal cancer (14), paclitaxel resistance in prostate cancer (16) as well as radio-resistance in cervical cancer (13). We now provide first evidence for a role of lncRNA SNHG6 in drug resistance of breast cancer, in general, and tamoxifen resistance, in particular.

We report elevated levels of SNHG6 in tamoxifen-resistant cells, which is in agreement with the published literature about the oncogenic role of SNHG6 in human cancers (12, 27, 28). Since SNHG6 is itself elevated in tamoxifen resistant cells, the miRNAs that it sponges are expected to be tumor-suppressive. In agreement with this, not only the miR-101 that we shortlisted for

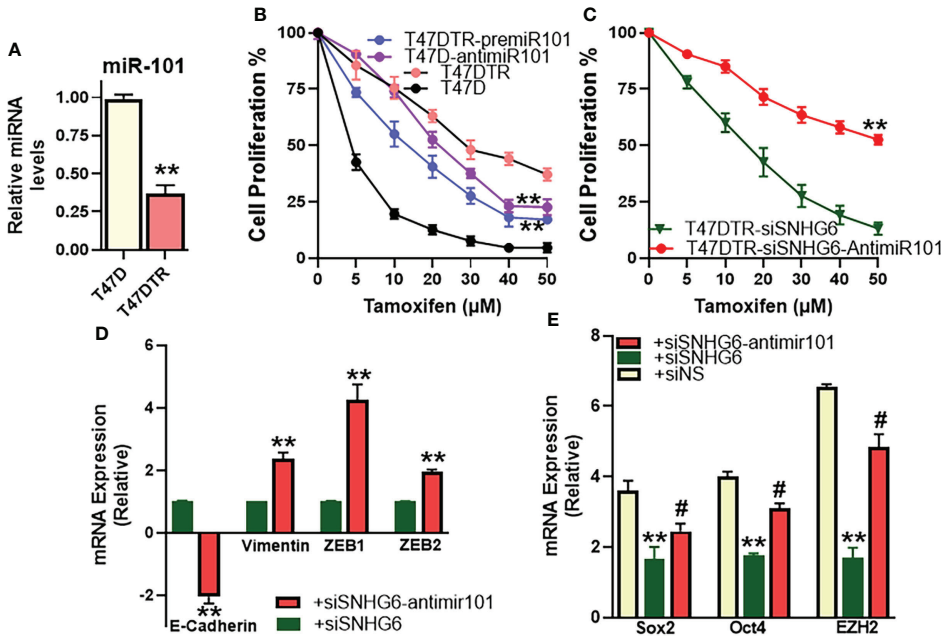

FIGURE 6
miR-101 affects validated in T47D cells. **(A)** Levels of miR-101 were assessed in T47DTR cells, relative to levels in parental T47D cells. Levels of miR-101 in parental cells were assigned a value of 1 and the relative levels in tamoxifen-resistant cells are plotted. **(B)** Cell proliferation was measured by MTT assay in T47D/T47DTR cells after transfections with pre/anti-miR-101 oligomers, as appropriate, followed by exposure to indicated concentrations of tamoxifen for 48 hours. **(C)** Tamoxifen resistant T47DTR either just silenced for SNHG6 or additionally transfected with anti-miR-101 oligomers were treated with increasing concentrations of tamoxifen for 48 hours before the proliferation was assessed using MTT assay. **(D)** T47DTR cells were silenced for SNHG6 and then the levels of EMT markers, E-cadherin, vimentin, ZEB1 and ZEB2 were evaluated by qRT-PCR in these cells as well as cells that were additionally transfected with anti-miR-101 oligomers. The levels of markers in SNHG6-silenced cells were assigned a value of 1 and the relative levels in siSNHG6+anti-miR-101-transfected cells are plotted. **(E)** Molecular markers of cancer stem cell phenotype, Sox2, Oct4 and EZH2 were quantitated, using qRT-PCR. The levels of miRNAs in parental T47D cells were assigned a value of 1 and the relative levels in tamoxifen-resistant control T47DTR (siNS) and those silenced for SNHG6 with and without additional transfections with anti-miR-101, are plotted. **p<0.01, compared to respective controls, #p<0.01, compared to siSNHG6.

detailed mechanistic evaluation based on the observation, was the most down-regulated miRNA in tamoxifen resistant cells, but several other putative miRNAs, such as let-7d, let-7e, miR-26a, miR-325 and miR-485 were also found to be significantly downregulated. Incidentally, all of these miRNAs that were down-regulated in our experimental model, are known to be tumor suppressor miRNAs (29–33). Of note, we observed two individual miRNAs, miR-186 and miR-1297 to be upregulated which was a little confusing, given the earlier reports suggesting sponging of these miRNAs by SNHG6. However, both of these miRNAs seem to have a dual effect (34, 35), both oncogenic as well as tumor suppressive, and our results support their reported oncogenic function. It would also be of interest to readers to know that we combined the information from published literature as well as bioinformatics analysis to shortlist the potential miRNAs that can be sponged by SNHG6. miR-26a (14), miR-186 (16), miR-325 (15), miR-485 (13) and miR-1297 (26) represent miRNAs that were shortlisted based on published reports while let-7d/e and miR-101 represent miRNAs that were identified through DIANA-LncBase v3 platform.

One of the primary mechanism through which SNHG6 mediates its oncogenic effects is the induction of EMT. Accordingly, SNHG6 has been shown to induce EMT leading to increased proliferation/migration/invasion of gastric cancer cells (36), colorectal cancer cells (37, 38), pituitary adenoma (18), glioma (19) and even breast cancer cells (39). However, our work is the first to demonstrate induction of EMT by SNHG6 in breast cancer cells with functional implications in acquired resistance against tamoxifen. We show here that SNHG6 silencing reversed EMT leading to acquisition of a tamoxifen sensitive phenotype. This is a clear proof supporting our hypothesis that SNHG6 plays a role in tamoxifen resistance. Further, we demonstrate a negative correlation between SNHG6 and miR-101 levels. In the SNHG6-silenced cells, miR-101 levels are higher, which makes sense given the tumor suppressive nature of miR-101. Furthermore, antagonizing miR-101 in SNHG6 silenced cells once again induced EMT, as evidenced by downregulated epithelial marker E-cadherin and the upregulated mesenchymal markers, vimentin, ZEB1 and

ZEB2, thus supporting EMT induction as well as significantly increased tamoxifen resistance.

While our present report is the first one to demonstrate sponging of miR-101 by SNHG6 in breast cancer, particularly in tamoxifen-resistant breast cancer, there is ample evidence from other cancer as well as non-cancer models to confirm such miR-101 sponging by SNHG6. In one of the early report on the subject, SNHG6 was reported to sponge miR-101 in hepatocellular carcinoma cells (40). This work also reported an effect of SNHG6 on mesenchymal marker ZEB1, similar to one shown in our present study. Thereafter, SNHG6 was shown to sponge miR-101 in gastric (36), glioma (41), colorectal (42), non-small cell lung (43), cholangiocarcinoma (44), melanoma (45) and esophageal (46) cancer cells. In addition, SNHG6 has been reported to sponge miR-101 in rat degenerate nucleus pulposus cells (47) and such sponging of miR-101 by SNHG6 has been suggested to contribute to ventricular septal defect formation (48). Thus, specific targeting of miR-101 by SNHG6 is functionally relevant not only in various cancers but in several other physiological phenomena as well. Our novel information on the modulation of tamoxifen resistance by this SNHG6-miR-101 axis adds new knowledge, and should further generate interest in the evaluation of miR-101 targeting by SNHG6 in the context of cancer drug resistance.

In our present work, we also report that miR-101 itself affected tamoxifen resistance. Just the manipulation of miR-101 levels in parental as well as tamoxifen-resistant cells, through the use of pre- or anti-miR-101 oligomers, as appropriate, resulted in differential sensitization of cells to tamoxifen exposure. More importantly, we determined miR-101 to be mechanistically involved in SNHG6 effects because miR-101 was found to attenuate the SNHG6 effects on tamoxifen sensitization/resistance in both MCF-7/MCF7TR and T47D/T47DTR paired cell lines. We thus provided novel evidence to support a role of miR-101 in tamoxifen resistance of ER-positive breast cancer. An earlier report has documented an estrogen-independent growth stimulation by miR-101 in MCF-7 cells (49). There, however, has been no attempt yet to connect miR-101 with tamoxifen resistance of breast cancers, which underlines another important revelation from our work.

In our experiments, we found ZEB1 to be the most affected EMT biomarker. It was found to be the most differentially regulated molecular marker, among all the markers of EMT tested, in the tamoxifen resistant cells, when compared to the parental cells. ZEB1, also happened to be to most affected EMT marker when SNHG6 was silenced in tamoxifen-resistant cells and finally, ZEB1 was again the most affected EMT marker when anti-miR-101 oligos were added to SNHG6-silenced cells. In a similar observation, EZH2 was found to be the most affected cancer stem cell marker as it was found to be the most up-regulated stem cell marker in tamoxifen-resistant cells, most affected marker in SNHG6-silenced cells and then the best marker rescued by miR-101 manipulation in SNHG6-silenced cells. Interestingly, miRDB prediction lists ZEB1 and EZH2 as the predicted targets of miR-101 which is also supported by published evidence of ZEB1 (40) and EZH2 (46) targeting by miR-101. Taken together, our study provides evidence to support a role of lncRNA SNHG6-miR101 axis in tamoxifen resistance of ER-positive breast cancers which involves modulation of EMT and cancer stem cells phenotype through targeting of ZEB1/EZH2. Further clinical studies will be needed to exploit this novel information for future benefit of breast cancer patients, particularly those with acquired tamoxifen resistance.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization, validation, resources, writing—review and editing, supervision, and project administration, AA. Methodology, validation, investigation, data curation, and writing—original draft preparation, MK and AA. Formal analysis and funding acquisition, MK. Both authors have read and agreed to the published version of the manuscript.

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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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In silico characterization of competing endogenous RNA network in glioblastoma multiforme with a systems biology approach

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Glioblastoma multiforme (GBM) is the most frequent malignant type of primary brain cancers and is a malignancy with poor prognosis. Thus, it is necessary to find novel therapeutic modalities based on molecular events occur at different stages of tumor progression. We used expression profiles of GBM tissues that contained long non-coding RNA (lncRNA), microRNA (miRNA) and mRNA signatures to make putative ceRNA networks. Our strategy led to identification of 1080 DEmRNAs, including 777 downregulated DEmRNAs (such as GJB6 and SLC12A5) and 303 upregulated DEmRNAs (such as TOP2A and RRM2), 19 DElncRNAs, including 16 downregulated DElncRNAs (such as MIR7-3HG and MIR124-2HG) and 3 upregulated DElncRNAs (such as CRNDE and XIST) and 49 DEmiRNAs, including 10 downregulated DEmiRNAs (such as hsa-miR-10b-5p and hsa-miR-1290) and 39 upregulated DEmiRNAs (such as hsa-miR-219a-2-3p and hsa-miR-338-5p). We also identified DGCR5, MIAT, hsa-miR-129-5p, XIST, hsa-miR-128-3p, PART1, hsa-miR-10b-5p, LY86-AS1, CRNDE, and DLX6-AS1 as 10 hub genes in the ceRNA network. The current study provides novel insight into molecular events during GBM pathogenesis. The identified molecules can be used as therapeutic targets for GBM.

KEYWORDS

glioblastoma multiforme (GBM), ceRNA, lncRNA, miRNA, biomarker

Introduction

Glioblastoma multiforme (GBM) is the most frequent malignant type of primary brain cancers (1). This type of cancer is classified into primary and secondary subtypes based on the presence of mutations in isocitrate dehydrogenase (IDH) genes (2). Moreover, the mutation status of *IDH1* and *IDH2* genes is considered as an important factor in defining prognosis of GBM (2). GBM is a highly invasive tumor with high tendency to diffuse all over the brain parenchyma. In addition, high level of vascularity of GBM makes it exceedingly recidivist, leading to a short survival time even after surgical resection and chemoradiotherapy (2). From an immunological point of view, GBM is regarded as a cold tumor with an extremely immunosuppressive tumor microenvironment that acts in favor of tumor progression, recurrence and poor prognosis (2). Therefore, it is necessary to find novel therapeutic modalities based on molecular events occur at different stages of tumor progression.

Recent studies have used expression data of differentially expressed RNAs in GBM to construct competitive endogenous RNA (ceRNA) networks with the potential to be used as prognostic factors (3–6). In one of the recent studies, one lncRNA with differential expression related to survival, IL10RB-AS1, was discovered using a combination of bioinformatic techniques. This may have predictive utility and present novel therapy options for GBM, along with a number of associated signaling pathways and ceRNA systems that were discovered in GBM (7). This strategy can also been used to identify subtype-specific modules with distinctive biological functions that influence patients' prognosis in different GBM subtypes (8). Therefore, it is very important to look into probable genetic causes of GBM. One of the most urgent difficulties in cancer therapy is the creation of alternate and acceptable biomarkers to accurately identify and treat GBM (9).

In the current study, we used expression profiles of GBM tissues that contained long non-coding RNA (lncRNA), microRNA (miRNA) and mRNA signatures to make putative ceRNA networks. Then, we find the molecular pathways which are associated with these ceRNA networks.

Abbreviations: GBM, Glioblastoma Multiforme; lncRNA, Long non-coding RNA; miRNA, MicroRNA; mRNA, Messenger RNA; ceRNA, Competitive Endogenous RNA; DEG, Differentially Expressed Genes; GEO, Gene Expression Omnibus; SVA, Surrogate Variable Analysis; PCA, Principal Component Analysis; Limma, Linear Models for Microarray Data; FDR, False Discovery Rate; Log2FC, Log2 Fold Change; HGNC, HUGO gene nomenclature; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, Protein-Protein Interaction; TCGA, The Cancer Genome Atlas; BP, Biological Process; MF, Molecular Function; CC, Cellular Component; CCLE, Cancer Cell Line Encyclopedia.

Methods

Microarray data collection

Expression profiles of GSE50161 ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array), GSE36245 ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array), GSE83300 (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Probe Name version)) and GSE65626 ([miRNA-4] Affymetrix Multispecies miRNA-4 Array), which included 130, 46, 50 and 6 samples, respectively, were acquired using the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/). We selected 34 GBM and 13 normal tissue samples from GSE50161, 46 GBM samples from GSE36245, 50 GBM samples from GSE83300 and 3 GBM and 3 normal samples from GSE65626 for further analysis (Table 1). The expression data contained lncRNAs, miRNAs and mRNAs expression signatures.

Microarray data processing, integrative meta-analysis and assessment of data quality

The described datasets contain different and trendy platforms (Agilent and Affymetrix), and normalization is a critical step in the integration of heterogeneous data. All microarray data was processed and integrated using the statistical programming language R. For pre-processing, data from Affymetrix and Agilent was first normalized separately using the normalizeQuantiles function in the preprocessCore package (https://bioconductor.org/packages/release/bioc/html/preprocessCore.html). The program R was used to combine data from both platforms. In order to exclude batch effects (non-biological differences), the ComBat function from the R Package Surrogate Variable Analysis (SVA) was used (10). By using PCA and a boxplot, batch effect removal was evaluated. The result of the meta-analysis is a unit expression matrix (the combination of three datasets of this study).

Analysis of differentially expressed lncRNAs, miRNAs and mRNAs

We used the Limma package in R language (11) to screen differentially expressed mRNAs (DEmRNAs), lncRNAs (DElncRNAs), and miRNAs (DEmiRNAs) between GBM and normal samples. GSE50161, GSE36245 and GSE83300 were used to obtain DEmRNAs and DElncRNAs. GSE65626 was utilized to acquire DEmiRNAs. DEmRNAs and DElncRNAs were evaluated with the cut-off criteria of false discovery rate (FDR; adjusted p value) < 0.05 and $|\log2 \text{ fold Change (FC)}| > 2$ while the cut-off

TABLE 1 Information of datasets.

Datasets	Platform	Use	Patient	Control	Tissue
GSE50161	GPL570	DEmRNA – DElncRNA	34	13	Brain
GSE36245	GPL570	DEmRNA – DElncRNA	46	-	Brain
GSE83300	GPL6480	DEmRNA – DElncRNA	50	-	Brain
GSE65626	GPL19117	DEmiRNA	3	3	Brain

criteria of false discovery rate (FDR; adjusted p value) < 0.05 and | log2 fold Change (FC)| > 3.5 was considered for DEmiRNAs. Then, we identified DElncRNAs using HGNC (HUGO gene nomenclature) database.

Two-way clustering of DEGs

We determined gene expression levels of significant DEmRNAs, DElncRNAs, and DEmiRNAs. We used this data in the pheatmap package in R language (12) to perform two-way clustering based on the Euclidean distance.

Gene ontology enrichment analysis

We used the clusterProfiler R package (13) to perform gene ontology (GO) enrichment analysis to investigate the functions of the remarkably upregulated and downregulated DEGs that we discovered. The functional category criteria were established at an adjusted p-value of 0.05 or below.

Kyoto encyclopedia of genes and genomes pathway analysis

KEGG pathway analysis of considerably DEGs was carried out to discover the possible functions of these genes that participated in the pathways based on the KEGG database (14).

PPI network construction and hub genes identification

The STRING database (15) was utilized to create the PPI network for DEGs. Highest level of confidence was used to establish the interactions parameter (confidence score >0.9). The Cytoscape software v3.9 (16) was used to visualize the interactions between the proteins. The top 20 DEGs related to hub genes were ultimately found using the Cytohubba plugin (17) of Cytoscape using the betweenness approach.

Constructing the ceRNA network and hub genes identification

We built a ceRNA network through the following steps: 1) assessing the interactions between lncRNAs and miRNAs based on the GBM-related miRNAs using miRcode (http://www.mircode.org/); 2) Application of miRDB (http://www.mirdb.org/) (18), miRTarBase (https://mirtarbase.cuhk.edu.cn/) (19), TargetScan (http://www.targetscan.org/) (20) and miRWalk (http://129.206.7.150/) (21) for prediction of miRNAs-targeted mRNAs; 3) Finding the intersections of the differentially expressed lncRNAs and mRNAs, and establishment of lncRNA/mRNA/miRNA ceRNA network using Cytoscape v3.9 and 4) predicting hub genes using cytohubba plugin based on degree method.

Validation of hub genes *via* expression values

The expression value of hub genes was assessed using the ualcan database (22). The hub genes in the TCGA-GBM RNA-seq data were examined, and those were present in the PPI and ceRNA networks as well as in the TCGA-GBM were chosen for gene expression validation.

Expression of the hub genes in various GBM cell lines

We selected GBM and normal brain cell lines using cancer cell line encyclopedia (CCLE) (https://sites.broadinstitute.org/ccle/) and DepMap portal gene expression datasets (https://depmap.org/portal/). In order to determine how the hub genes are expressed, we finally employed the limma package of the R programming language to analyze this data.

Survival analysis

We used survival package (https://CRAN.R-project.org/package=survival) in R to define survival curves, which were grouped by the prognostic value of hub genes with highest degree in ceRNA network. The clinical data for patients with GBM derived from TCGA (PRAD-TCGA). Univariate survival analysis was evaluated using Kaplan-Meier curves. Statistics were considered significant for P-values under 0.05.

Results

Microarray data processing, integrative meta-analysis and assessment of data quality

Figure 1 shows the boxplot of raw data and normalized data after batch effect removal. These boxplots indicate that the quality of the expression data was reliable, and the boxplot of the preprocessed data presented the good normalization. In the PCA plot (Figure 2), 143 samples are shown in the 2D plane spanned by their first two principal components (PC1 and PC2). According to this plot, the samples had a good variation after batch effect removal.

DEGs analysis

Based on the microarray data analysis between GBM and normal samples using Limma package, we obtained 1080 DEmRNAs, including 777 downregulated DEmRNAs (such as GJB6 and SLC12A5) and 303 upregulated DEmRNAs (such as TOP2A and RRM2), 19 DElncRNAs, including 16 downregulated DElncRNAs (such as MIR7-3HG and MIR124-2HG) and 3 upregulated DElncRNAs (such as CRNDE and XIST) and 49 DEmiRNAs, including 10 downregulated DEmiRNAs (such as hsa-miR-10b-5p and hsa-miR-1290) and 39 upregulated DEmiRNAs (such as hsa-miR-219a-2-3p and hsa-miR-338-5p). The most significantly upregulated and downregulated DEmRNAs, DElncRNAs, and DEmiRNAs are shown in Tables 2–4, respectively.

We used the volcano plot with the Enhanced Volcano package (23) in R to compare the variation in miRNA, lncRNA, and mRNA expression between GBM and normal samples (Figure 3). In addition, the two-way clustering demonstrated that 20 clearly distinct DEmRNA expression patterns between GBM and normal samples were identifiable (Figure 4A). The expression of DElncRNAs and DEmiRNAs is also shown in two heatmaps (Figure 4B).

GO enrichment analysis of DEGs

DEGs were enriched in 816 GO terms. We used Clusterprofiler package to perform analysis. In GO functional enrichment analysis, 816 GO entries satisfy adjusted P value less than 0.05, most of which are biological processes (BP), followed by cellular components (CC) and molecular functions (MF). The first 30 entries are synaptic membrane (CC), modulation of chemical synaptic transmission (BP), regulation of trans-synaptic signaling (BP), glutamatergic synapse (CC), synapse organization (BP), synaptic vesicle cycle (BP), vesicle-mediated transport in synapse (BP), neurotransmitter transport (BP), regulation of membrane potential (BP), neuron to neuron synapse (CC), postsynaptic specialization (CC), postsynaptic membrane (CC), neurotransmitter secretion (BP), signal release from synapse (BP), ion channel complex (CC), regulation of neurotransmitter levels (BP), postsynaptic density

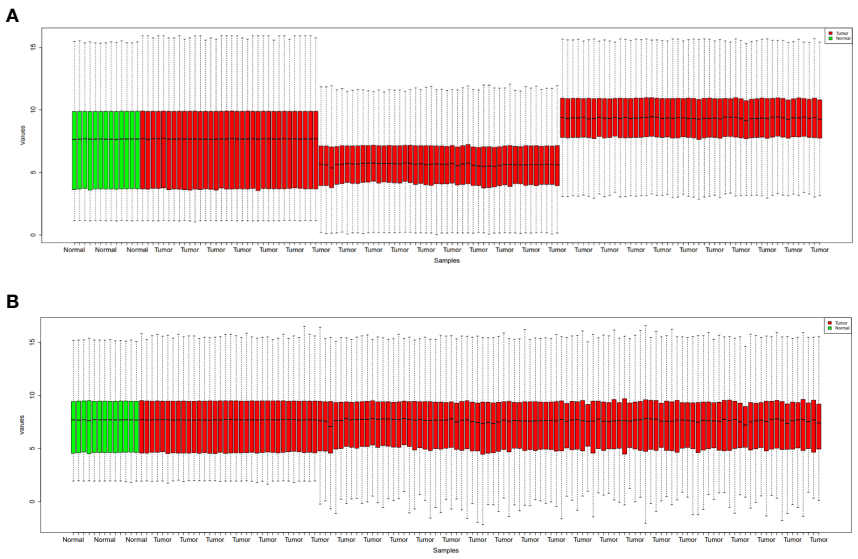

FIGURE 1
Boxplots for the raw data **(A)** and normalized data after batch effect removal **(B)**. GBM samples are indicated by red boxes, whereas healthy samples are shown by green boxes.

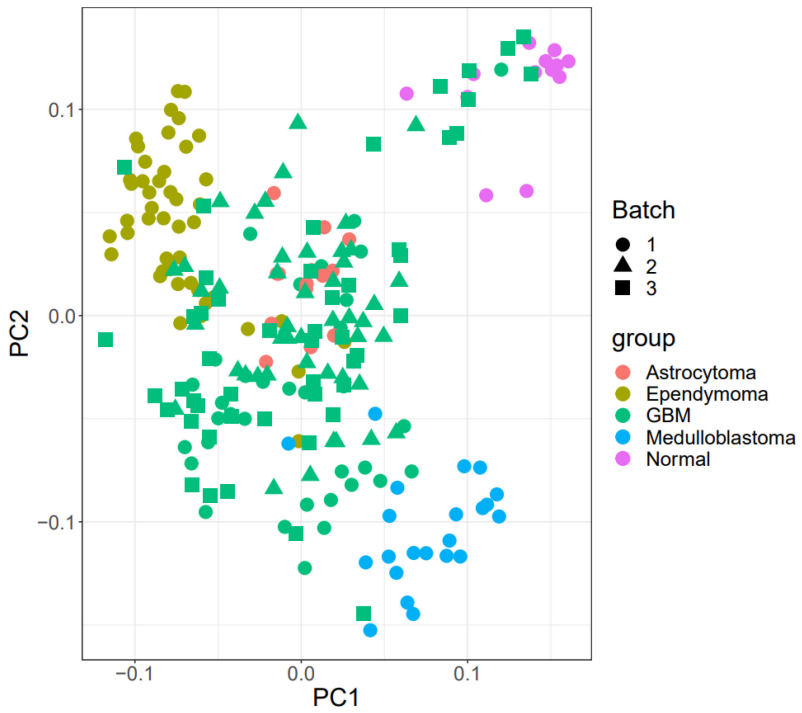

FIGURE 2
PCA plot. The Batch implies that the data includes three platforms. Also, healthy benign and tumor samples were divided into three groups.

(CC), asymmetric synapse (CC), transmembrane transporter complex (CC), transporter complex (CC), cation channel complex (CC), regulation of synaptic plasticity (BP), presynaptic membrane (CC), synaptic vesicle membrane (CC), exocytic vesicle membrane (CC), synaptic vesicle (CC), regulation of ion transmembrane transport (BP), exocytic vesicle (CC), synaptic vesicle exocytosis (BP) and gated channel activity (MF). Figure 5 shows the barplots of top 10 enriched functions.

Figures 6 and 7 depict visualization of the dotplots of top 10 enriched functions and enriched GO induced graph, respectively.

Figure 8 indicates the gene-concept network of top 5 GO terms (Modulation of chemical synaptic transmission, regulation of trans-synaptic signaling, synapse organization, synaptic vesicle cycle and vesicle-mediated transport in synapse).

In Figure 9, the UpSet plot visualizes the intersection between top 10 GO terms. It highlights the gene overlap between several gene sets.

TABLE 2 The top 10 up- and down-regulated DEmRNAs between GBM and normal samples.

Down-regulated			Up-regulated		
DEmRNA	**Log FC**	**Adjusted *P* value**	**DEmRNA**	**Log FC**	**Adjusted P value**
GJB6	-7.096688	3.082137e-24	**TOP2A**	5.106081	5.083076e-14
SLC12A5	-6.726824	3.298847e-17	**RRM2**	4.991494	9.047466e-15
PACSIN1	-6.712373	1.336264e-18	**KIAA0101**	4.748463	1.192890e-17
SYNPR	-6.505139	2.538197e-14	**UHRF1**	4.484171	8.187032e-22
VSNL1	-6.469234	2.949472e-12	**ASPM**	4.420167	1.604840e-10
CAMK2A	-5.812767	8.827178e-20	**NUSAP1**	4.218903	1.390467e-14
SV2B	-5.752584	1.830778e-15	**PBK**	4.210402	6.369782e-10
NEFM	-5.737146	5.630558e-12	**WEE1**	4.195263	1.993886e-22
ETNPPL	-5.735033	5.781047e-10	**CDK1**	4.164108	1.641432e-16
SVOP	-5.733442	6.691090e-20	**CFI**	4.054792	2.503017e-08

TABLE 3 The up- and down-regulated DElncRNAs between GBM and normal samples.

Down-regulated			Up-regulated		
DElncRNA	**Log FC**	**Adjusted *P* value**	**DElncRNA**	**Log FC**	**Adjusted P value**
MIR7-3HG	-3.826937	2.943952e-14	**CRNDE**	3.762562	1.547281e-08
MIR124-2HG	-3.483949	3.114815e-10	**XIST**	3.637404	0.01229272
DGCR5	-3.466361	2.495893e-19	**KIFC1**	2.133776	7.987507e-06
DCTN1-AS1	-3.326676	1.312921e-23			
RFPL1S	-3.284252	1.929947e-12			
DPP10-AS1	-3.174864	3.760156e-08			
SLC26A4-AS1	-2.994545	1.566864e-14			
HAR1A	-2.760872	5.007026e-21			
LY86-AS1	-2.705636	9.604924e-16			
LINC00622	-2.67454	1.846807e-10			
MEG3	-2.551883	1.11688e-11			
MIAT	-2.33424	8.580568e-05			
PART1	-2.318588	1.6899e-11			
LINC01102	-2.121837	4.563676e-05			
DLX6-AS1	-2.06398	0.0008082298			
TTTY15	-2.01289	0.006006504			

Pathway analysis

Using Pathview (24) and gage (25) packages in R, KEGG pathways analysis of 177 downregulated and 177 upregulated DEGs were performed to identify the potential functional genes (Table 5; Figure 10).

PPI network construction and selection of hub genes

A PPI network of DEGs (Figure 11) with 411 nodes and 727 edges that was generated from STRING was put into the Cytohubba plugin of Cytoscape 3.9 in order to identify the hub genes. The 20 hub genes with the highest dgree of connectivity were DLG4, CAMK2B, BUB1B, LIN7B, CDK2, SYT1, DNM1, STX1A, GRIA4, CCNB1, AURKA, AURKB, BUB1, STXBP1, TP53, CCNB2, SNAP25, CDK1, GRIA2 and CDK4. Table 6 is a list of this hub's information. The greatest degree to lowest degree is used to order these hubs.

CeRNA network construction in GBM

Using miRcode, the relationship between lncRNAs and miRNAs was assessed. This step demonstrated that 20 of the 39

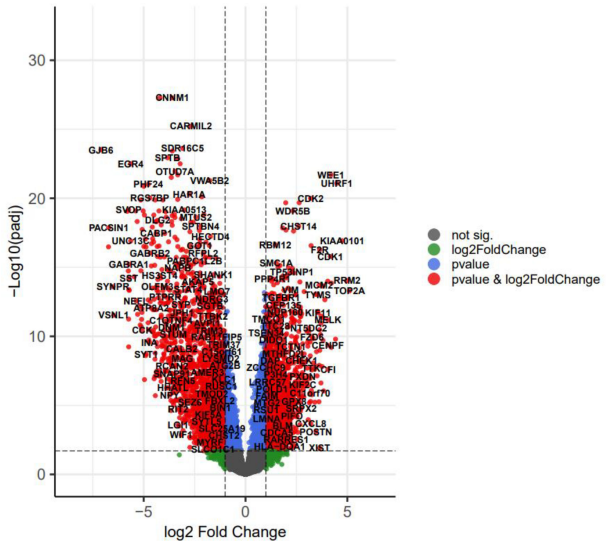

FIGURE 3
The volcano plot of differentially expressed genes (DEGs); horizontal axis, log_2(FC); vertical axis, -log_{10}(adjusted P value).

TABLE 4 The top 10 up- and down-regulated DEmiRNAs between GBM and normal samples.

Down-regulated			Up-regulated		
DEmiRNA	**Log FC**	**Adjusted *P* value**	**DEmiRNA**	**Log FC**	**Adjusted P value**
hsa-miR-10b-5p	-4.7771098	0.00071	**hsa-miR-219a-2-3p**	8.6479768	0.011486
hsa-miR-1290	-4.4993318	0.03931	**hsa-miR-338-5p**	7.9211592	0.000161
hsa-miR-371b-5p	-4.4205838	0.005439	**hsa-miR-139-3p**	6.5955712	0.000161
hsa-miR-199a-5p	-4.4046581	0.006768	**hsa-miR-383-5p**	6.1274595	0.001604
hsa-miR-21-3p	-4.274357	0.000912	**hsa-miR-330-3p**	6.0482004	0.004003
hsa-miR-199a-3p	-4.1722017	0.004278	**hsa-miR-584-5p**	5.8478087	0.007511
hsa-miR-199b-3p	-4.1722017	0.004278	**hsa-miR-129-5p**	5.4260841	0.004825
hsa-miR-21-5p	-3.9891862	0.001823	**hsa-miR-330-5p**	5.0897962	0.000547
hsa-miR-431-5p	-3.7024153	0.001823	**hsa-miR-138-2-3p**	4.8481859	0.000161
hsa-miR-424-3p	-3.5577576	0.004493	**hsa-miR-1250-5p**	4.6317277	0.003127

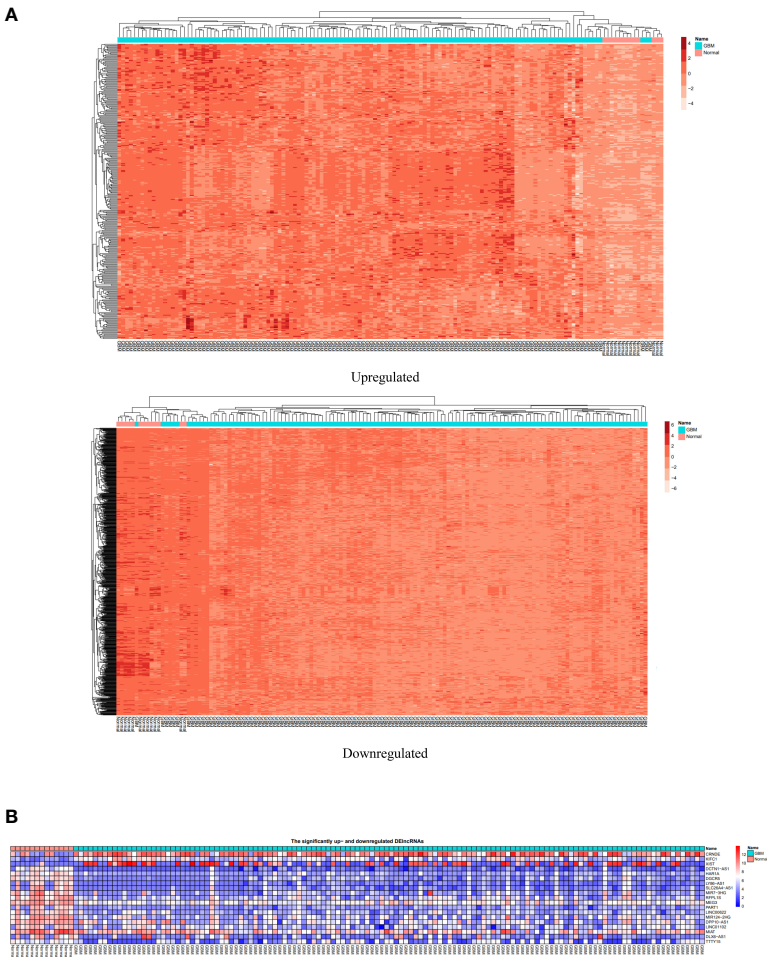

FIGURE 4
(A) The two-way clustering of DEmRNAs between GBM samples and normal samples; horizontal axis, the samples; vertical axis, DEmRNAs.
(B) Two heatmaps depicting expression of DElncRNAs and DEmiRNAs.

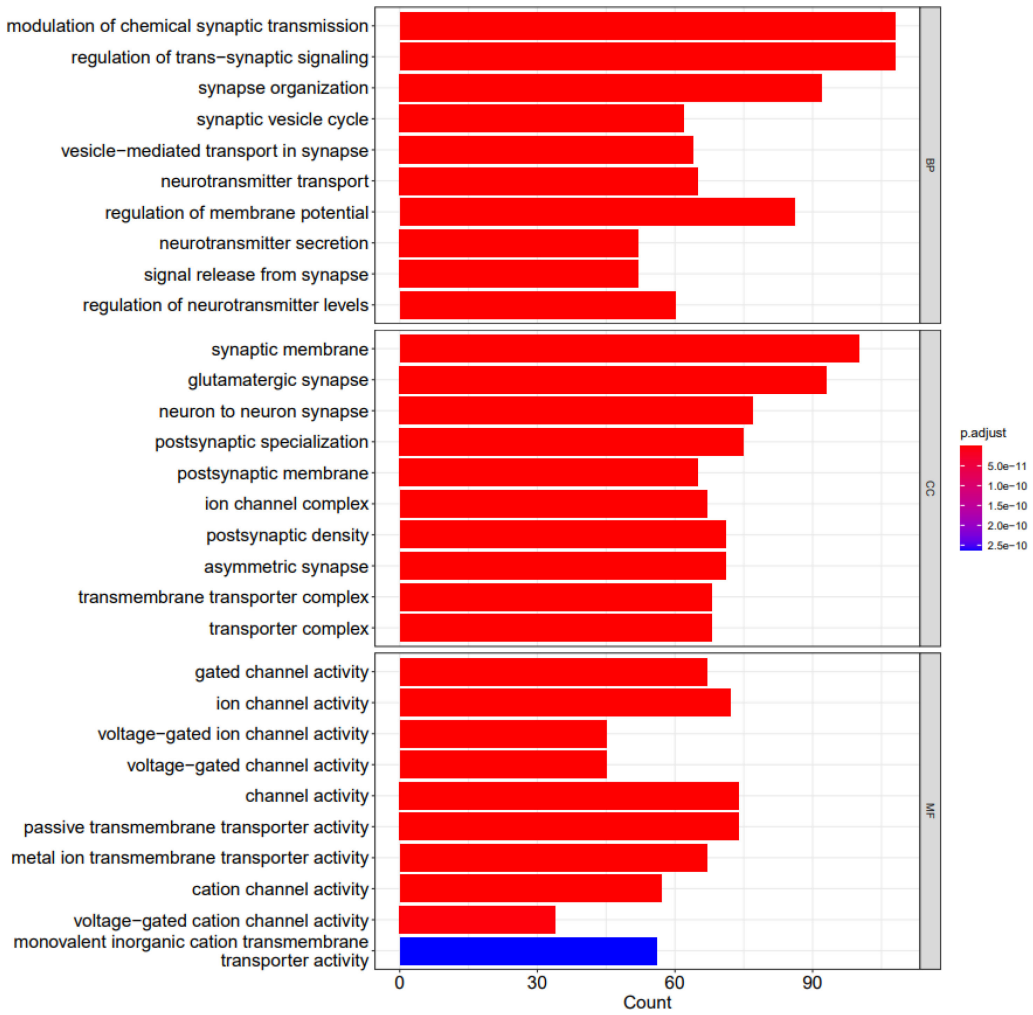

FIGURE 5
The barplots of top 10 enriched functions; BP (biological process), CC (cellular component) and MF (molecular function). X axis shows the count of geneset; Y axis shows the geneset function; Bar color represents the adjusted P.value, ranging from red (most significant) to blue (least significant).

GBM-specific DEmiRNAs may be targeted by 10 of the 19 lncRNAs (Table 7). Then, in order to investigate the connection between miRNAs and mRNAs, we used miRWalk with miRTarBase, TargetScan and miRDB filters to predict targeted mRNAs by these 20 miRNA. The findings suggested that 3 miRNAs may target 6 of the 1080 mRNAs (Table 8). If miRNA-targeted mRNAs were not found in DEmRNAs, they were eliminated. Cytoscape 3.9 was used to build the lncRNA-miRNA-mRNA ceRNA network using the data from Tables 7 and 8. The ceRNA network contained a total of 3 miRNAs, 10 lncRNAs, and 6 mRNAs (Figure 12). We displayed this ceRNA network using a Sankey diagram generated by the ggalluvial R package (Version: 0.12.3) (26) in order to better understand the impact of lncRNAs on mRNAs in GBM that is mediated by their interaction with miRNAs (Figure 13). Finally, using the cytohubba app, we calculated nodes closeness and exhibited the top 10 nodes in the network with the highest closeness centrality (Figure 14). We identified DGCR5, MIAT, hsa-miR-129-5p, XIST, hsa-miR-128-3p, PART1, hsa-miR-10b-5p, LY86-AS1, CRNDE, and DLX6-AS1 as 10 hub genes in the ceRNA network.

Validation of hub genes *via* expression value

We first obtained the TCGA-GBM RNA-seq data using the TCGAbiolinks package (27), and then we used the R packages

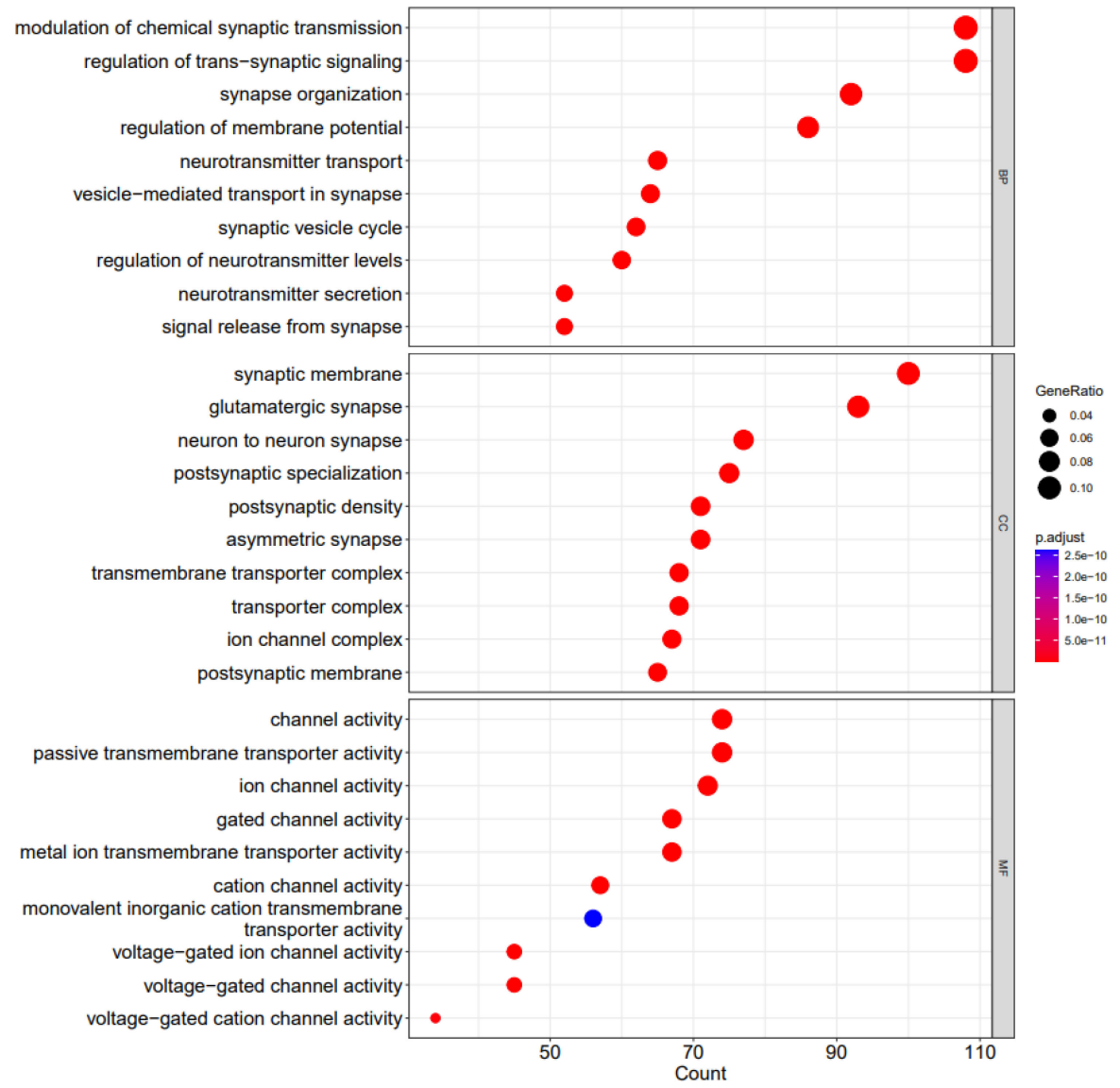

FIGURE 6
The dotplots of top 10 enriched functions. X axis shows the count of geneset; Y axis shows the geneset function; Dot color represents the adjusted P value ranging from dark blue (most significant) to red (least significant). Dot size represents the GeneRatio and the larger the size of the dot, the higher the value of the gene ratio.

limma and edgeR to analyze it. Then, by using Venny 2.0.2 (28), we were able to obtain the genes that were present in both the PPI and ceRNA networks' Hub genes and TCGA-GBM DEGs (adjusted p value < 0.05 and |log2 fold Change (FC)| > 2) (Figure 15). As a result, 18 of the 20 PPI network genes and 6 of the 10 ceRNA network genes were also included in the TCGA-GBM DEGs. Utilizing the ualcan, the expression value of these hub genes was evaluated. Therefore, all hub genes in PPI network and CRNDE, DGCR5, LY86-AS1, MEG3, MIAT, PART1 in ceRNA network revealed excellent statistical significance (Figure 16; Table 9).

Expression of the hub genes in various GBM cell lines

Using the cancer cell line encyclopedia (CCLE), we gathered cell line expression data (DepMap Public 22Q2) (29) and chose four GBM cell lines and hub genes. We selected four primary cell

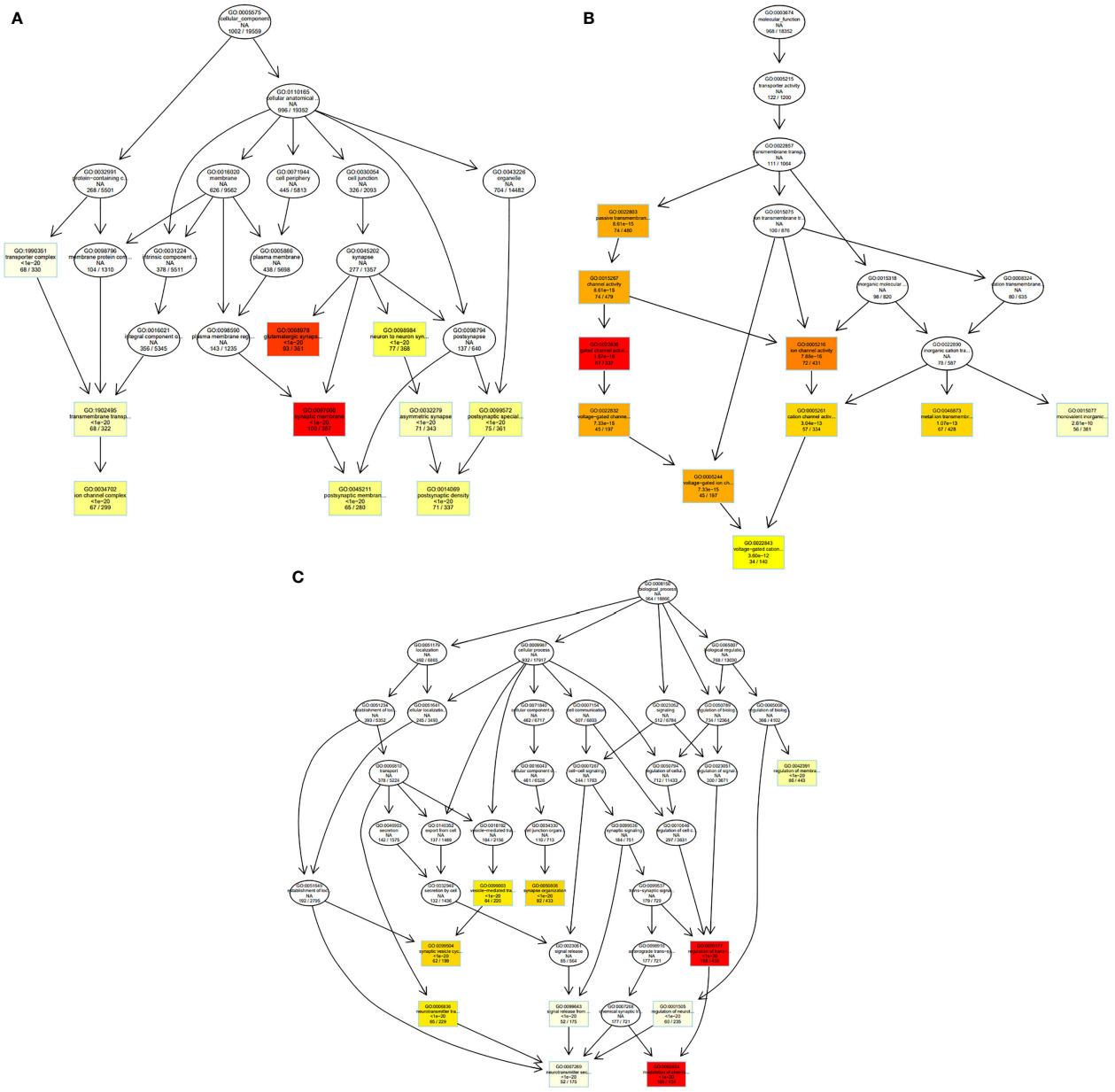

FIGURE 7
GO graph visualization of top GO terms enriched. **(A)** The top 10 GO terms in the category "Cellular Component" have generated a GO sub-graph. **(B)** The top 10 GO terms in the category "Molecular Function" have generated a GO sub-graph. **(C)** The top 10 GO terms in the category "Biological process" have generated a GO sub-graph. Boxes indicate the most significant terms. From dark red (most significant) to light yellow (least significant), the color of the box indicates the relative significance.

lines for GBM, namely A172, U251, U87, and T98G. NHA cell line was utilized as a normal brain cell line. Finally, we analyzed this data using the limma package in the R programming language, and we discovered how the hub genes are expressed in diverse GBM cell lines (Table 10). As a threshold, we utilized Log2FC > |0.5| and an adjusted P.value of 0.05.

Survival analysis

A Kaplan-Meier curve analysis was used to perform a survival analysis using the R survival package. We carried out a survival analysis using hub genes in PPI and ceRNA networks. The difference was statistically significant with a log-rank P value

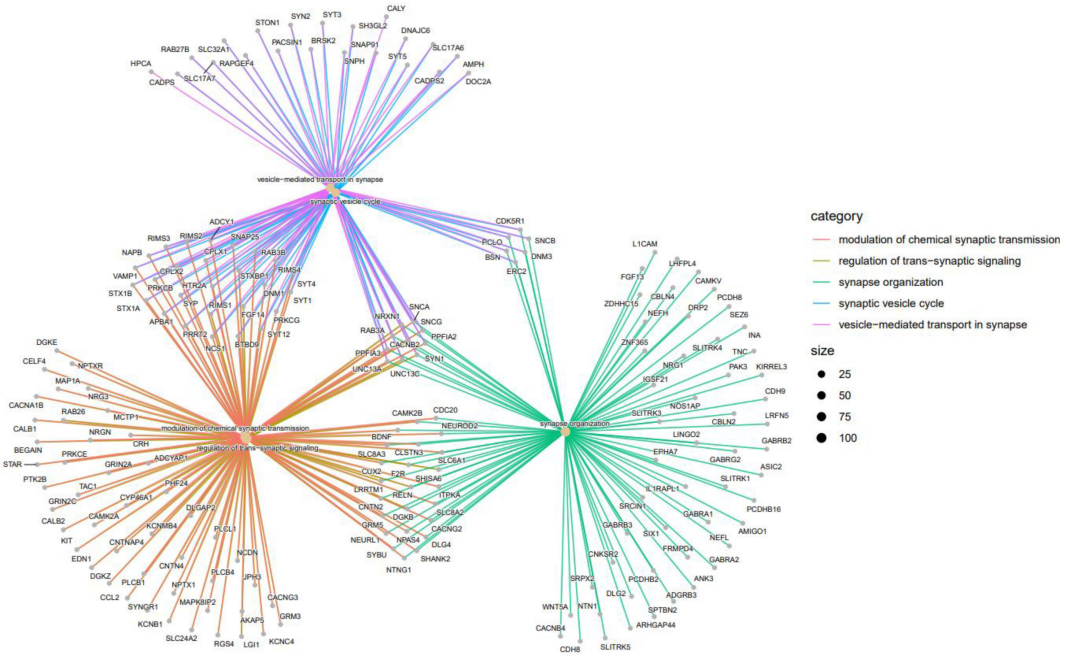

FIGURE 8
Top 5 GO terms as a network plot. These GO terms are connected to genes in this graph. The connection of genes to the corresponding GO is marked with a special color; There are more genes for a specific GO term if the dot relating to it is bigger.

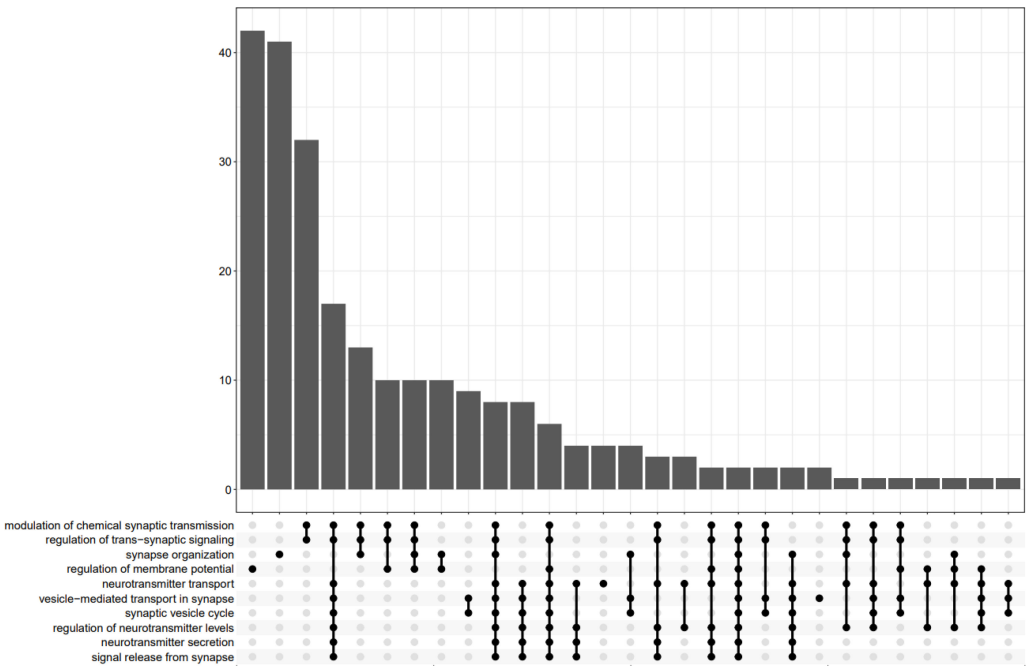

FIGURE 9
UpsetPlot of 10 GO terms.

TABLE 5 Down-regulated and Up-regulated Pathways.

Down-regulated		Up-regulated	
Pathway	**P value**	**Pathway**	**P value**
Calcium signaling pathway	0.002285132	**Cell cycle**	2.009266e-06
Long-term potentiation	0.018132471	**p53 signaling pathway**	2.197253e-05
Gastric acid secretion	0.025751413	**ECM-receptor interaction**	4.957866e-03
Pancreatic secretion	0.034688834	**Focal adhesion**	3.223350e-02

TABLE 6 The information of hub genes in PPI network.

Hub Gene	Adjusted P.value	Log2FC	Clustering Coefficient	Degree	Closseness Centrality	Betweenness Centrality
DLG4	2.36E-15	-2.598617544	0.071428571	28	0.224764468	0.198126639
TP53	1.87E-12	2.464542358	0.043333333	25	0.242556282	0.292493307
CDK1	1.64E-16	4.164108388	0.204761905	21	0.236376504	0.17080442
SNAP25	1.34E-07	-3.669305973	0.205263158	20	0.207840697	0.082044934
CCNB1	2.23E-06	2.248216201	0.267973856	18	0.210327456	0.031022432
STX1A	1.45E-15	-4.103297234	0.196078431	18	0.195321637	0.050648257
CAMK2B	8.26E-12	-3.073502871	0.352380952	15	0.212603437	0.034827756
GRIA4	0.001527989	-2.209019827	0.384615385	14	0.207970112	0.02887363
SYT1	1.99E-09	-4.875013677	0.318681319	14	0.19716647	0.049932638
AURKB	1.88E-05	2.02463802	0.179487179	13	0.214515093	0.060609355
BUB1	6.85E-07	2.835182173	0.358974359	13	0.203039514	0.020336039
CDK2	1.01E-20	3.199041769	0.358974359	13	0.21618123	0.028807346
DNM1	2.27E-11	-3.62934314	0.128205128	13	0.166003976	0.082152881
GRIA2	0.002650131	-2.194510124	0.294871795	13	0.206427689	0.021606876
AURKA	2.22E-07	2.456079967	0.136363636	12	0.234057463	0.122769569
BUB1B	3.32E-11	3.639344188	0.363636364	12	0.20291616	0.017593973
CCNB2	3.40E-12	3.703784989	0.424242424	12	0.203410475	0.00663464
LIN7B	2.52E-11	-2.039148743	0.484848485	12	0.187640449	0.010884111
STXBP1	6.03E-14	-2.263599416	0.484848485	12	0.192618224	0.015683204
CDK4	1.08E-11	2.368774869	0.436363636	11	0.214652956	0.010245679

less than 0.05. Therefore, in patients with GBM, DLG4, DNM1, STX1, and CRNDE exhibited a significant correlation with a shorter overall survival time (Figure 17).

Discussion

Using an *in-silico* approach, we aimed to identify ceRNA networks in GBM. The ceRNA network between three mentioned classes of RNAs is a recently revealed regulatory relationship. This network has an essential role in the modulation of biological features of cancer. Our strategy led to identification of 1080 DEmRNAs, including 777 downregulated DEmRNAs (such as GJB6 and SLC12A5) and 303 upregulated DEmRNAs (such as TOP2A and RRM2), 19 DElncRNAs, including 16 downregulated DElncRNAs (such as MIR7-3HG and MIR124-2HG) and 3 upregulated DElncRNAs (such as CRNDE and XIST) and 49 DEmiRNAs, including 10 downregulated DEmiRNAs (such as hsa-miR-10b-5p and hsa-miR-1290) and 39 upregulated DEmiRNAs (such as hsa-miR-219a-2-3p and hsa-miR-338-5p).

In line with our results, a previous study has shown that lncRNA XIST has as oncogenic function in human glioma through influencing expression of miR-137 (30). Moreover, ceRNA network analyses have shown that CRNDE enhances glioblastoma progression *via* sponging miR-9-5p (31).

Modulation of chemical synaptic transmission, regulation of trans-synaptic signaling, synapse organization, synaptic vesicle cycle and vesicle-mediated transport in synapse were identified as the top five GO terms. Therefore, the most important pathways are related with synaptic function.

DGCR5, MIAT, hsa-miR-129-5p, XIST, hsa-miR-128-3p, PART1, hsa-miR-10b-5p, LY86-AS1, CRNDE, and DLX6-AS1 were identified as 10 hub genes in the ceRNA network. These different types of RNAs are possible therapeutic targets and markers for GBM. Further experiments revealed association

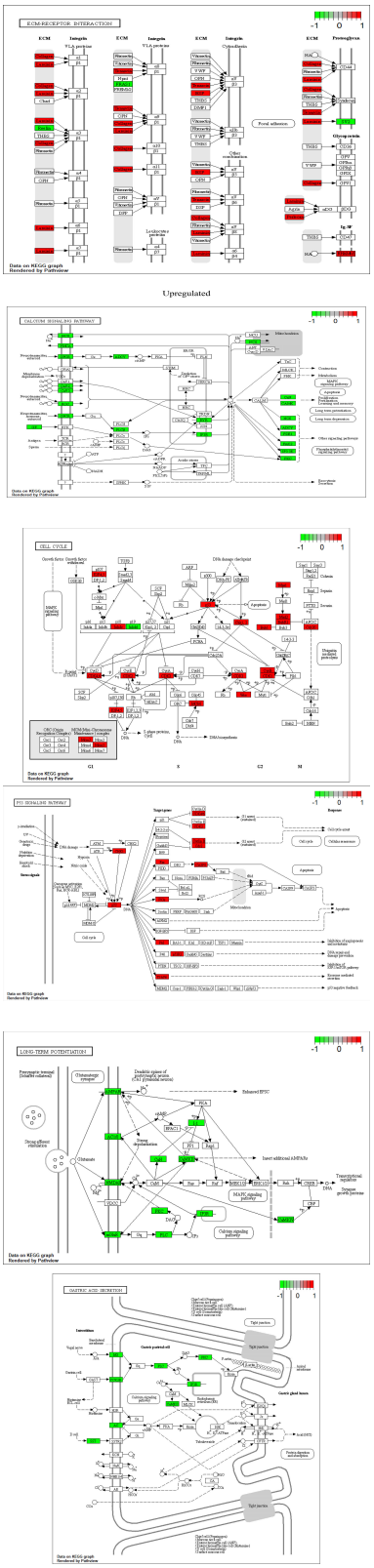

FIGURE 10
Visualization of the first two upregulated and downregulated pathways. Green boxes are downregulated genes and red boxes are upregulated genes.

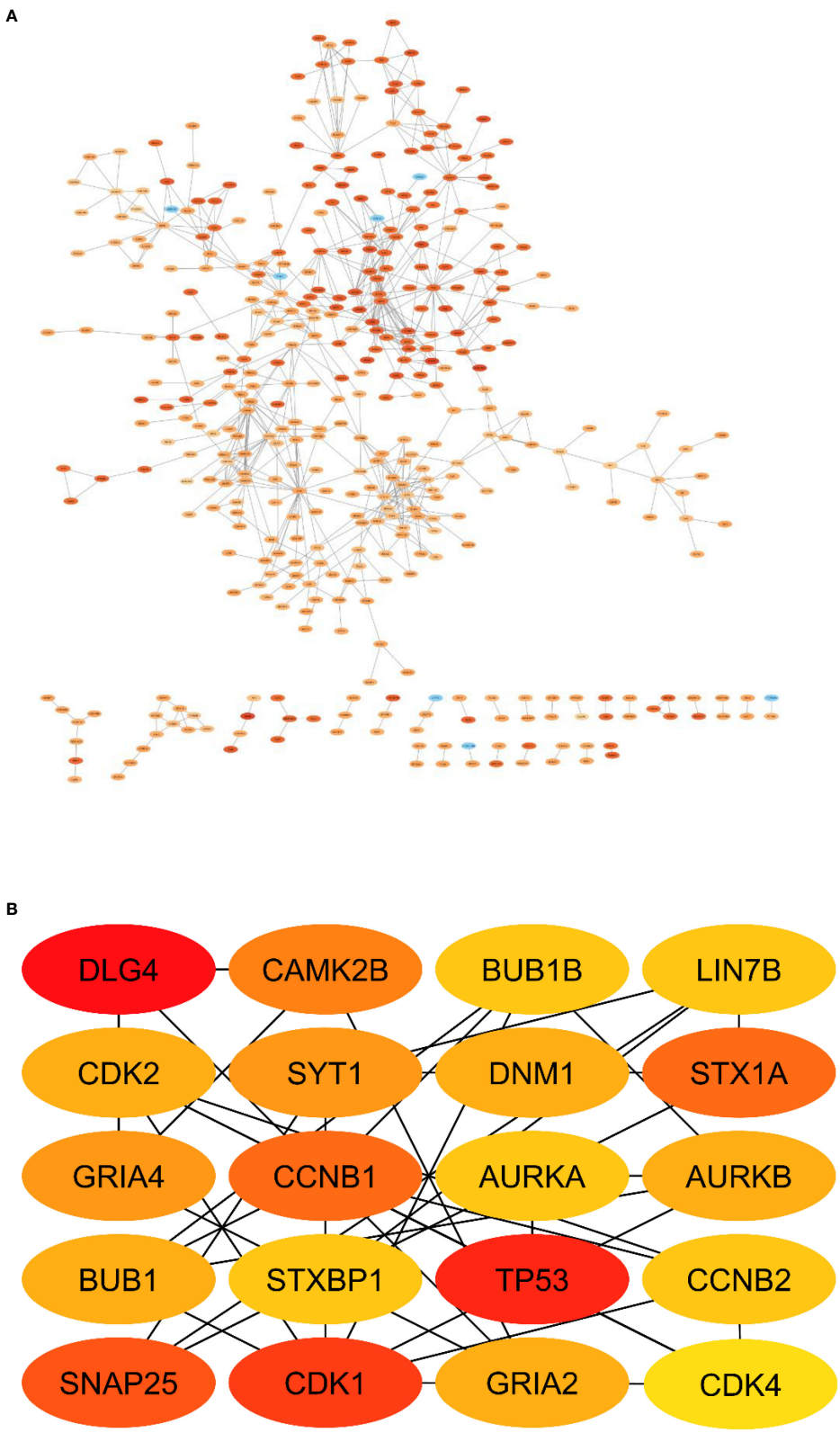

FIGURE 11
PPI network of DEmRNAs. **(A)** Total PPI network, **(B)** Subnetwork of hub genes.

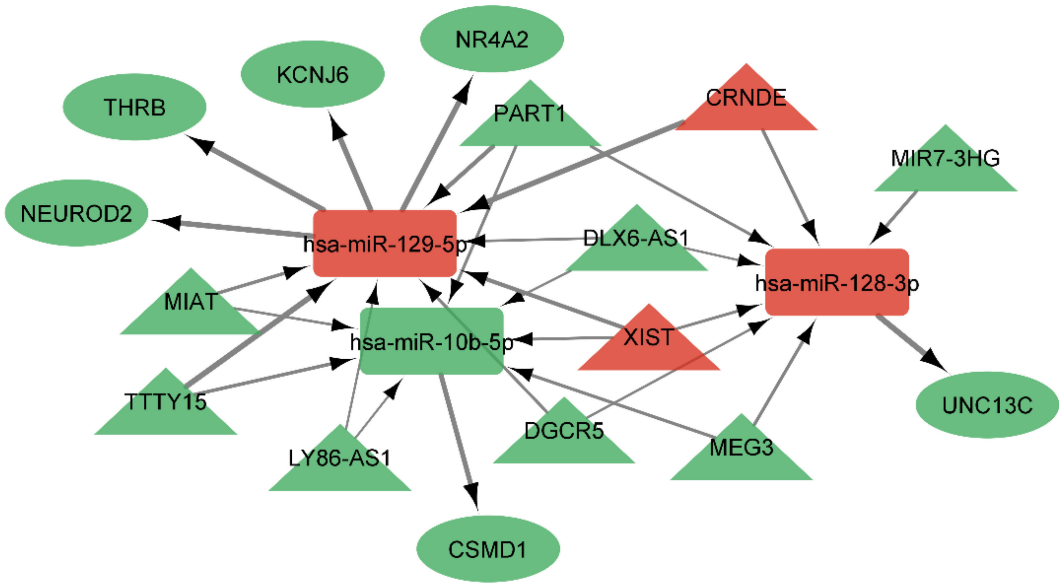

FIGURE 12
ceRNA network in GBM. Red nodes signify a strong expression level, while green nodes signify a low level of expression. Ellipses represent protein-coding genes; rectangles represent miRNAs; Triangles represent lncRNAs; gray edges indicate lncRNA-miRNA-mRNA interactions. Greater edge thickness indicates greater betweenness.

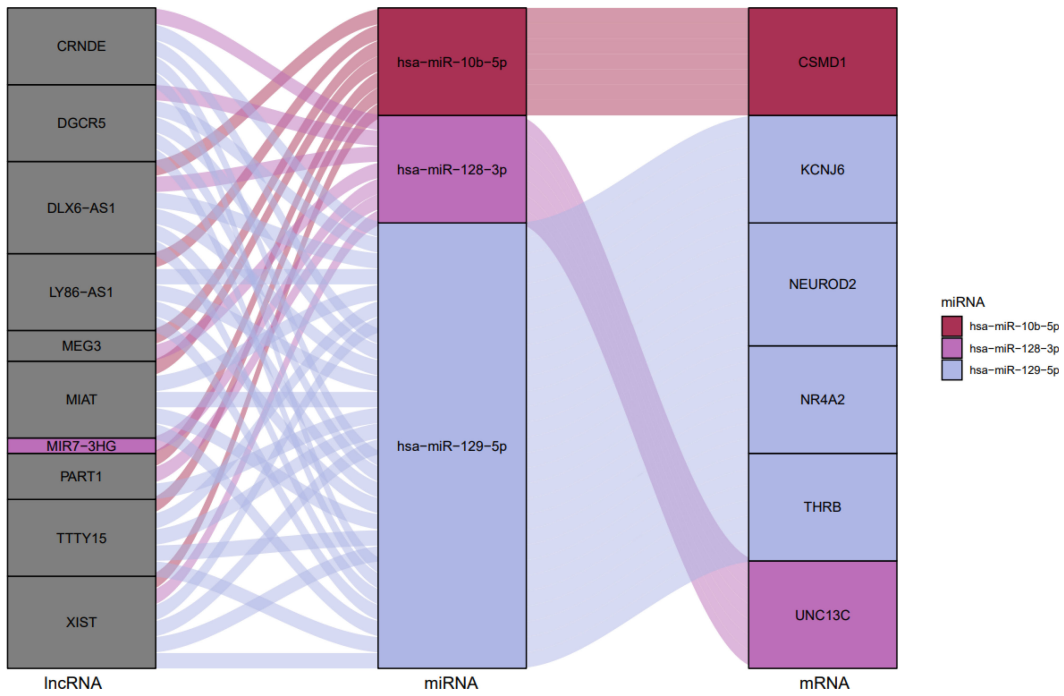

FIGURE 13
The ceRNA network in GBM is shown by a Sankey diagram. Each rectangle represents a gene, and depending on the size of the rectangle, the degree of relationship between each gene is shown.

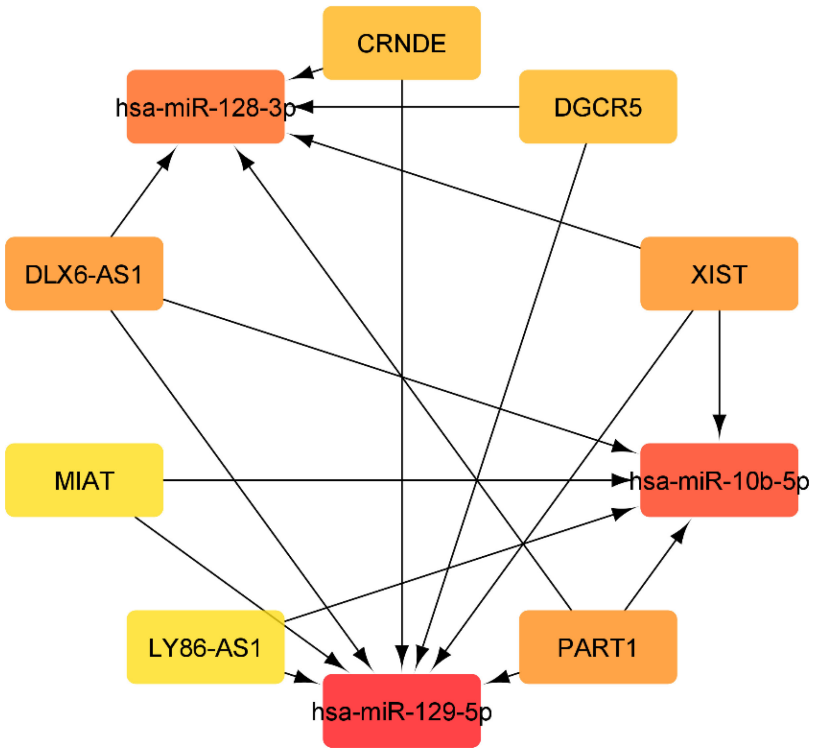

FIGURE 14
Top 10 genes with highest closeness centrality in ceRNA network.

TABLE 7 The MiRcode database revealed interactions between 10 DElncRNAs and 20 DEmiRNAs.

lncRNA	miRNA
CRNDE, XIST, DGCR5, MEG3, MIAT, DLX6-AS1	hsa-miR-338-5p
CRNDE, XIST, MEG3, MIAT	hsa-miR-1244
CRNDE, XIST, DGCR5, MIR7-3HG, MEG3, PART1, DLX6-AS1	hsa-miR-128-3p
CRNDE, XIST, DGCR5, LY86-AS1, PART1, MIAT, DLX6-AS1, TTTY15	hsa-miR-129-5p
CRNDE, XIST, DGCR5, MEG3, MIAT, DLX6-AS1	hsa-miR-338-3p
CRNDE, XIST, DGCR5, LY86-AS1, MEG3, MIAT, DLX6-AS1	hsa-miR-23b-5p
CRNDE, DGCR5, MEG3, PART1, MIAT, DLX6-AS1	hsa-miR-199a-5p
XIST, DGCR5, LY86-AS1, MIR7-3HG, MEG3, PART1	hsa-miR-138-2-3p
XIST, DGCR5, LY86-AS1, MIAT, DLX6-AS1, TTTY15	hsa-miR-29c-5p
XIST, DGCR5, LY86-AS1, MEG3, DLX6-AS1,	hsa-miR-383-5p
XIST, LY86-AS1, MIAT, DLX6-AS1, TTTY15	hsa-miR-139-5p
XIST, DGCR5, LY86-AS1, MEG3, PART1, MIAT	hsa-miR-34c-3p
XIST, DGCR5, LY86-AS1, MIAT, DLX6-AS1, TTTY15	hsa-miR-29b-2-5p
XIST, LY86-AS1, MEG3, PART1, MIAT, DLX6-AS1, TTTY15	hsa-miR-10b-5p
XIST, DGCR5, MEG3, PART1, TTTY15	hsa-miR-21-3p
XIST, DGCR5, MEG3, PART1, TTTY15	hsa-miR-21-5p
XIST, DGCR5, LY86-AS1, MEG3, PART1, MIAT, DLX6-AS1	hsa-miR-424-3p
LY86-AS1, MIAT	hsa-miR-139-3p
LY86-AS1, MEG3, MIAT	hsa-miR-184
LY86-AS1	hsa-miR-129-2-3p

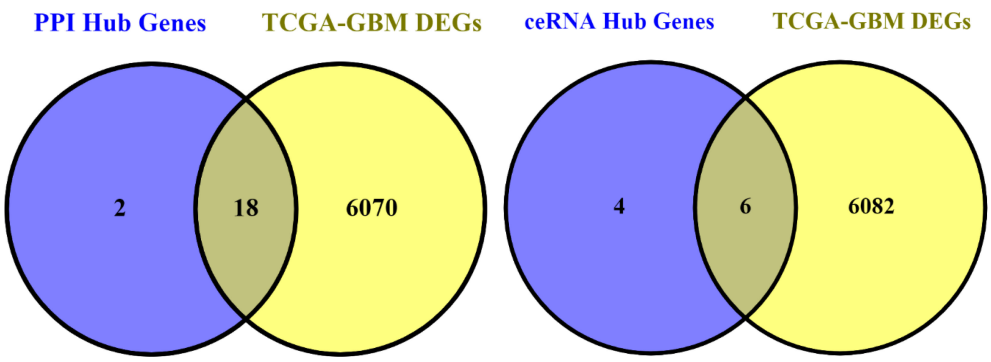

FIGURE 15
Venn diagram. The number of hub genes in PPI and ceRNA networks and TCGA-GBM DEGs.

between expression of DLG4, DNM1, STX1, and CRNDE and overall survival time of GBM patients, indicating their importance as prognostic factors. DLG4 has been identified as a core biomarker Biomarkers related with clinical outcome in glioma patients through a bioinformatics approach (32). Bioinformatics analyses have also identified DNM1 as a marker of invasion in this type of cancer (33). Besides, functional studies have shown that interference with the Stx1 function can impair progression of GBM *in vivo*.

Additionally, a prior study has shown that blocking the SNARE protein Stx1 *via* three distinct methods, including STX1A knockdown, consistently results in a marked slowing of the growth of glioblastoma tumors in an orthotopic mouse model (34).

According to reports, DNM1 promotes the growth of tumors in a number of malignancies, including gastric adenocarcinoma (35). We also found DNM1 to be a significant biomarker in GBM.

DLG4, which was also recognized as a key gene hub in this illness was another gene that we discovered to be a biomarker in GBM (36).

Taken together, ceRNA network analyses in GBM have provided new insights into molecular mechanisms in this type of cancer, representing novel markers and therapeutic targets in GBM. Future assessment of their expression in clinical samples and functional studies in animal models would lead to identification of detailed data in this regard.

TABLE 8 miRWalk (miRTarBase, TargetScan and miRDB filters) database revealed interactions between 3 DEmiRNAs and 6 DEmRNAs.

miRNA	mRNA
hsa-miR-128-3p	UNC13C
hsa-miR-129-5p	NEUROD2, NR4A2, THRB, KCNJ6
hsa-miR-10b-5p	CSMD1

TABLE 9 Statistical significance of hub genes based on sample types in GBM.

Hub genes	Statistical significance of expression value*
DLG4	4.066400E-02
TP53	1.62625468647093E-12
CDK1	1.62436730732907E-12
SNAP25	3.176000E-02
STX1A	4.005600E-02
CCNB1	<1E-12
CAMK2B	3.325700E-02
SYT1	3.254000E-02
DNM1	3.230500E-02
AURKB	1.62447832963153E-12
BUB1	<1E-12
CDK2	1.62447832963153E-12
AURKA	<1E-12
STXBP1	3.203700E-02
BUB1B	1.62447832963153E-12
CCNB2	1.62447832963153E-12
APBA1	2.727800E-02
DGCR5	2.698251E-02
MEG3	4.831681E-02
MIAT	3.234847E-02
LY86-AS1	1.431129E-02
PART1	1.269176E-02
CRNDE	3.024283719995E-31

*Low number (<10) of normal samples considered.

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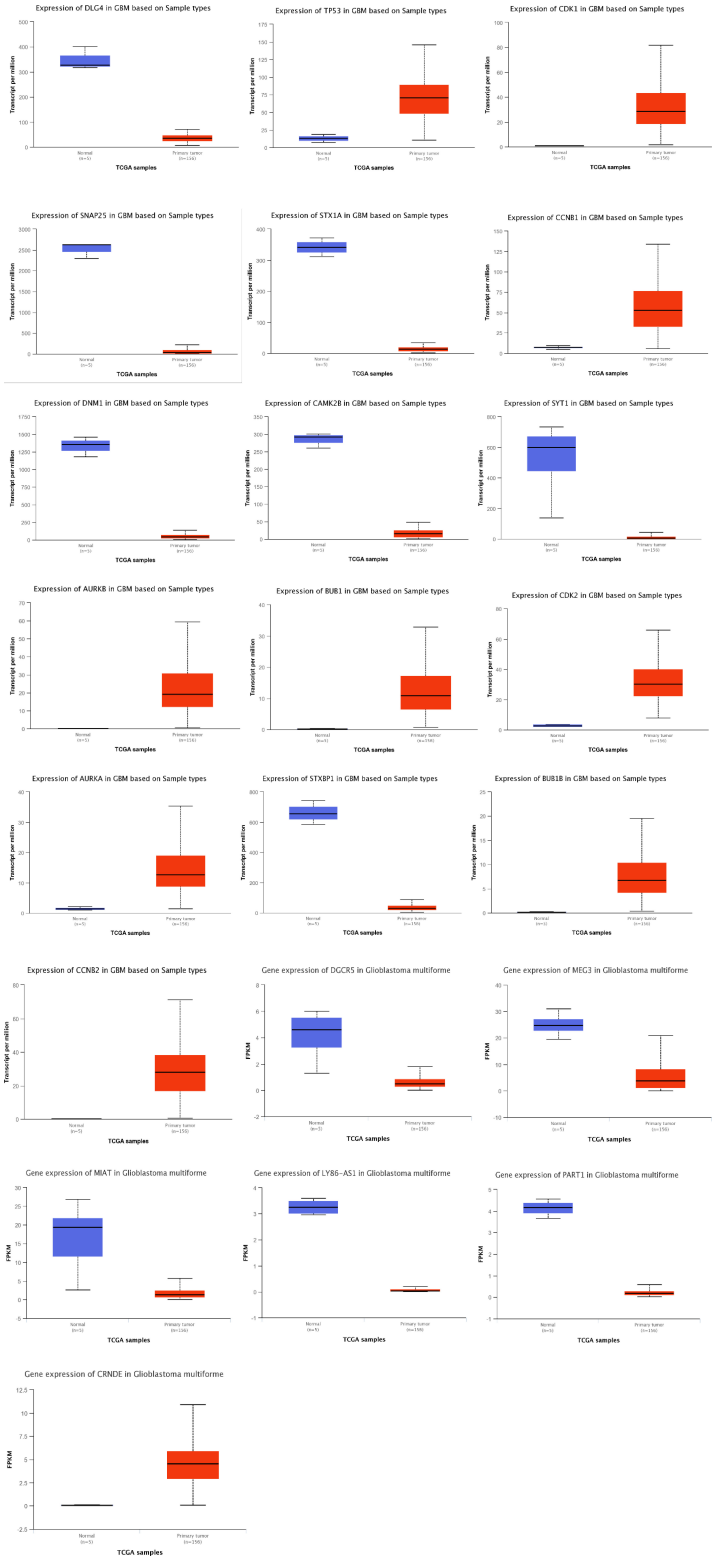

FIGURE 16
Box plots of gene expression of hub genes in GBM and normal samples based on TCGA. Red and blue boxes show gene expression of hub genes in GBM and normal samples, respectively.

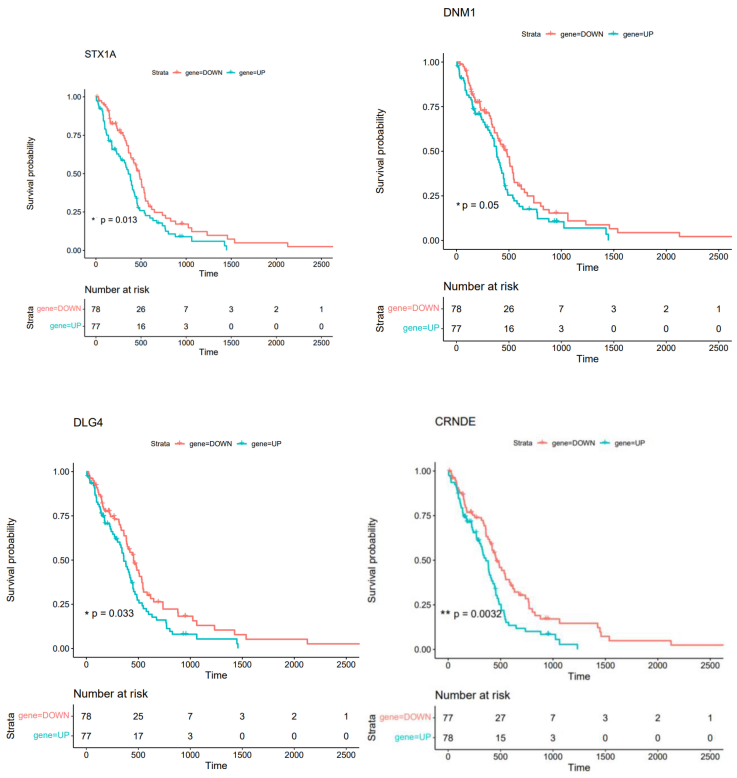

FIGURE 17
The overall survival of GBM patients is related to the Kaplan-Meier survival curves of STX1A, DNM1, DLG4, and CRNDE (* shows P<=0.05, ** shows P<0.01).

TABLE 10 Expression pattern of the hub genes in A172, U251, U87, and T98G cell lines.

Hub gene	A172	U87	U251	T98G
DLG4	Not significant	Downregulated	Downregulated	Downregulated
TP53	Not significant	Downregulated	Upregulated	Upregulated
CDK1	Upregulated	Upregulated	Upregulated	Upregulated
SNAP25	Not significant	Upregulated	Downregulated	Downregulated
STX1A	Not significant	Upregulated	Downregulated	Downregulated
CCNB1	Upregulated	Upregulated	Downregulated	Upregulated
CAMK2B	Upregulated	Downregulated	Downregulated	Downregulated
SYT1	Not significant	Downregulated	Downregulated	Downregulated
DNM1	Not significant	Upregulated	Upregulated	Downregulated
AURKB	Upregulated	Upregulated	Upregulated	Upregulated
BUB1	Upregulated	Upregulated	Upregulated	Upregulated
CDK2	Upregulated	Upregulated	Upregulated	Upregulated
AURKA	Not significant	Upregulated	Upregulated	Upregulated
STXBP1	Not significant	Upregulated	Downregulated	Downregulated
BUB1B	Upregulated	Upregulated	Downregulated	Upregulated
CCNB2	Upregulated	Upregulated	Upregulated	Upregulated
APBA1	Not significant	Downregulated	Upregulated	Downregulated

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/ (GSE50161, GSE36245, GSE83300 and GSE65626).

Author contributions

SG-F wrote the draft and revised it. GS and MT designed and supervised the study. BH, AS, and MA-B performed the bioinformatic analysis and data collection. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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The role of lncRNAs in regulation of DKD and diabetes-related cancer

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Diabetes mellitus often results in several complications, such as diabetic kidney disease (DKD) and end-stage renal diseases (ESRDs). Cancer patients often have the dysregulated glucose metabolism. Abnormal glucose metabolism can enhance the tumor malignant progression. Recently, lncRNAs have been reported to regulate the key proteins and signaling pathways in DKD development and progression and in cancer patients with diabetes. In this review article, we elaborate the evidence to support the function of lncRNAs in development of DKD and diabetes-associated cancer. Moreover, we envisage that lncRNAs could be diagnosis and prognosis biomarkers for DKD and cancer patients with diabetes. Furthermore, we delineated that targeting lncRNAs might be an alternative approach for treating DKD and cancer with dysregulated glucose metabolism.

KEYWORDS

lncRNAs, cancer, diabetes, DKD, miRNAs, treatment

Abbreviations: α-SMA, alpha smooth muscle actin; α1-MG, α1-microglobulin; APEX2, ascorbate peroxidase; β2-MG, β2-microglobulin; BSA, bovine serum albumin; CASC2, cancer susceptibility candidate 2; ChREBP, carbohydrate response element binding protein; CTGF, connective tissue growth factor; DRAIR, diabetes regulated anti-inflammatory lncRNA; DKD, diabetic kidney disease; HbA1c, glycosylated hemoglobin; HDAC1, histone deacetylase 1; HKDC1, hexokinase domain-containing 1; HOTAIR, HOX antisense intergenic RNA; LncRNAs, long noncoding RNAs; MEG3, maternally expressed gene 3; MLX, MAX dimerization protein; MXD1, MAX dimerization protein 1; NEAT, nuclear-enriched abundant transcript; PBMC, peripheral blood mononuclear cells; PGC-1α, peroxisome proliferator-activated receptor γ coactivator α; SOD, superoxide dismutase; TGF-β1, transforming growth factor-β1; TME, tumor microenvironment; TUG1, taurine upregulated gene 1.

Introduction

Noncoding RNAs have been known to play an essential role in development of many diseases (1, 2). Noncoding RNAs include short non-coding RNAs, such as microRNAs (miRNAs), small interfering RNA (siRNAs), piwi-interacting RNA (piRNAs), transfer RNA (tRNAs), small nuclear RNA (snRNAs) and small nucleolar RNA (snoRNAs), and long non-coding RNAs (lncRNAs), which is based on their length (3). LncRNAs often have more than 200 nucleotides and cancer serve as signal molecules, decoy molecules, guide molecules, and scaffold molecules to perform their functions *via* regulation of gene expression at epigenetic, transcriptional and post-transcriptional levels (4, 5). Accumulated evidence has dissected that lncRNAs participate in cellular biological processes *via* regulation of protein degradation and governing gene transcription as well as controlling protein coding sections (6–8). Dysregulated lncRNAs have been reported to participate in numerous diseases, including cancer, inflammatory bowel disease, cardiovascular disease, neurological disorders and diabetes (9–14).

Diabetes mellitus (DM) has become a major health problem in the world, which often results in several complications, such as diabetic kidney disease (DKD) (15). DKD is often known as diabetic nephropathy. DM has three types: type 1 diabetes, type 2 diabetes and gestational diabetes (GDM). Type 1 diabetes is insulin-dependent and often appears during childhood and adolescence. Type 2 diabetes often appears in older adults due to that pancreas does not make enough insulin or cells respond poorly to insulin. GDM often happens during the pregnancy after insulin secretion is not enough. DKD is one of causes to develop end stage kidney disease (ESKD) and kidney failure (16). It has been known that chronic stimuli such as high glucose in the bloodstream can lead to pathological gene modulation and DKD in diabetic patients (17). EMT and endothelial-mesenchymal transition (EndMT) have been characterized to integrate into the fibrosis and DKD (18, 19). EMT is a process in which epithelial cells acquire mesenchymal characteristics after various stimulations. Similarly, EndMT is a process in which endothelial cells have the phenotype toward mesenchymal cells, which often appears in cardiovascular diseases. Cancer patients often have the dysregulated glucose metabolism. Abnormal glucose metabolism can enhance the tumor malignant progression (20).

Recently, noncoding RNAs, including lncRNAs, have been reported to regulate the key proteins and signaling pathways in DM and DKD development and progression as well as in cancer with diabetes (21–24). In this review article, we elaborate the evidence to support the function of lncRNAs in development of DKD and cancer patients with diabetes. Moreover, we envisage that lncRNAs could be diagnostic and prognosis biomarkers for DKD and diabetes-related cancers. Furthermore, we delineated that targeting lncRNAs might be an alternative approach for treating DKD and diabetes-associated cancer.

Role of lncRNAs in DKD

Emerging evidence has suggested that lncRNAs are useful for precision medicine in DKD (25–28). Zhang and colleagues used the integrate biological, computational, and statistical strategies to analyze the pathogenesis and progression of DKD through analysis of regulatory networks including miRNAs, lncRNAs and mRNAs (29). This study reported that 127 lncRNAs were changes in DKD, among which 26 were decreased and 101 were increased. In particular, this work identified that miR-223-3p might be a biomarker for prediction of DKD disease process (29).

LncRNA HOTAIR

Evidence showed that lncRNA HOTAIR is critically involved in DKD development (30). One group used several mouse models, such as podocyte-specific Hotair knockout mice, streptozotocin-induced diabetes in mice, and the db/db mouse model of type 2 diabetes. In these mouse models, glomerular HOTAIR was upregulated. Depletion of Hotair in podocytes did not affect structure, ultrastructure, function of kidneys (30). In mouse podocytes, high glucose treatment increased the expression of HOTAIR. Interestingly, silencing of HOTAIR did not affect the kidney damage in diabetic mice. Moreover, HOTAIR expression was linked to HOXC11 expression in human kidney tissues according to a bioinformatic assay (30). Notably, the serum level of HOTAIR was increased in type 2 DM patients (31). HOTAIR can be a useful biomarker in prediction of diabetic retinopathy and DKD in patients with type 2 DM. In addition, HOTAIR facilitated high glucose-mediated fibrosis and proliferation of mesangial cells *via* affecting miR-147a/WNT2B axis in diabetic nephropathy (32). The role of HOTAIR in DKD needs to be ascribed to validate its function in the pathogenesis of DKD.

LncRNA GAS5

Wang et al. reported that lncRNA GAS5 promoted renal tubular epithelial fibrosis *via* sponging miR-96-5p (33). Renal fibrosis is often observed in DKD. Higher expression of lncRNA GAS5 was reported in renal proximal tubular cells after TGF-β1 treatment. The kidneys of high-fat diet (HFD)/streptozotocin (STZ) mice had the upregulation of lncRNA GAS5 (33). Silencing of lncRNA GAS5 reduced renal fibrosis *via* inhibition of miR-96-5p. Consistently, DKD mice had the lower expression of miR-96-5p, leading to upregulation of

fibronectin. Hence, depletion of lncRNA GAS5 could have antifibrosis *via* sponging miR-96-5p and regulating fibronectin. Zhang et al. found that lncRNA GAS5 attenuated TGF-β-mediated renal fibrosis by inhibition of collagen type 1 an fibronectin *via* targeting the Smad3/miR-142-5p axis (34). LncRNA GAS5 suppressed fibrosis and cell proliferation through attenuating miR-221 and upregulating SIRT1 expression in diabetic nephropathy (35). LncRNA GAS5 inhibited pyroptosis and oxidative stress in renal tubular cells after high glucose stimulation (36). LncRNA GAS5 alleviated fibrosis *via* inhibition of MMP9 by recruitment of EZH2 in diabetic nephropathy (37). Altogether, modulation of lncRNA GAS5 might be useful for preventing DKD.

LncRNA MALAT1

LncRNA MALAT1 has been identified to play key roles in DKD pathophysiology (38). One work assessed urinary albumin in 136 patients with type 2 DM and 25 normal people. This work found that urinary lncRNA MALAT1 was positively associated with urinary podocalyxin, synaptopodin, UACR (urinary albumin), NAG (N-acetyl-D-glucosaminidase), KIM-1 (kidney injury molecule 1), miR-21, miR-93, miR-29a (38). LncRNA MALAT1 was negatively correlated with eGFR, miR-29a and miR-93. In addition, urinary lncRNA MIAT was positively linked to miR-29a, miR-93 and eGFR, while lncRNA MIAT was negatively associated with miR-21, miR-124, UACR, NAG and KIM-1 (38). In line with this report, the expression of lncRNA MALAT1 in PBMC was increased in type 2 DM and DKD (39). MALAT1 was associated with ACR, HbA1c, SOD, creatinine, α1-MG and β2-MG in type 2 DM and DKD patients. MALAT1 in combination with ACR, α1-MG and creatinine could be helpful for prediction of DKD in DM patients (39). MALAT1 enhanced diabetic nephropathy *via* suppression of miR-15b-5p and upregulation of TLR4 signaling (40).

MALAT1 activated LIN28 and Nox4/AMPK/mTOR pathway, resulting in promotion of renal tubular injury in diabetic nephropathy (41). Huang et al. reported that MALAT1 aggravated renal fibrosis *via* modulation of miR-2355-3p/IL6ST axis in diabetic nephropathy (42). One study showed that podocyte injury could be due to abnormal MALAT1 expression and subsequent dysregulated let-7f and KLF5 in diabetic nephropathy (43). MALAT1 was also reported to participate in high glucose-mediated HK-2 cell EMT *via* activation of Wnt/β-catenin pathway and injury (44). Consistently, MALAT1 was involved in high glucose-mediated podocyte injury in diabetic nephropathy *via* its interaction with β-catenin (45). MALAT1 aggravated high glucose-triggered EndMT and fibrosis through regulation of miR-145/ZEB2 axis (46). Additionally, MALAT1 participated in high glucose-mediated HK-2 cell injury *via* interplay with Foxo1 to affect SIRT expression (47).

LncRNA MIAT

Urinary lncRNA MIAT was positively linked to miR-29a, miR-93 and eGFR, while lncRNA MIAT was negatively associated with miR-21, miR-124, UACR, NAG and KIM-1 in type 2 DM (38). Depletion of lncRNA MIAT mitigated apoptosis and inflammation in podocyte after high glycose stimulation through modulating miR-130a-3p and TLR4 pathway (48). Ablation of lncRNA MIAT ameliorated fibrosis and cell proliferation *via* suppression of E2F3 expression in diabetic nephropathy (49). Loss of lncRNA MIAT blocked podocyte injury and mitotic damage in diabetic nephropathy (50). However, one study showed that lncRNA MIAT blocked the high glucose-mediated cell damage and activation of NF-κB *via* sponging miR-182-5p and elevating the GPRC5A expression in diabetic nephropathy, leading to suppression of diabetic nephropathy progression (51).

LncRNA NEAT1

Evidence has suggested that lncRNA NEAT1 governed renal tubular EMT *via* regulation of the ERK1/2 signaling pathway in DKD (52). LncRNA NEAT1 was increased in BSA-treated HK2 cells and HFD/STZ-induced DKD mice. Depletion of NEAT1 suppressed the expression of the EMT-related markers, such as vimentin and a-SMA, and the renal fibrosis-associated markers, including TGF-β1 and CTGF (52). LncRNA NEAT1 regulated DKD progression *via* modulation of the ERK1/2 signaling pathway. Li et al. discovered that NEAT1 interacted with miR-129 to promote renal fibrosis *via* upregulation of collagen type 1 and promotion of EMT process (53). Additionally, urinary lncRNA NEAT1 was positively correlated with miR-21, miR-124, KIM-1, synaptopodin, and NAG in type 2 DM. Urinary lncRNA NEAT1 had a negative association with miR-29a, miR-93 and eGFR (38).

LncRNA NEAT1 activated Akt/mTOR pathway and accelerated cell fibrosis and proliferation in diabetic nephropathy (54). LncRNA NEAT1 enhanced EMT and accumulation of extracellular matrix in diabetic nephropathy *via* sponging miR-27b-3p and ZEB1 (55). Ablation of lncRNA NEAT1 attenuated proliferation, fibrosis and inflammation of mouse mesangial cells in diabetic nephropathy (56). In addition, lncRNA NEAT1 accelerated diabetic nephropathy occurrence and progression *via* suppression of miR-23c (57). LncRNA NEAT1 affected pyroptosis *via* targeting the miR-34c and NLRP3 in diabetic nephropathy (58). One group showed that lncRNA NEAT1 accelerated high glucose-triggered hypertrophy in mesangial cells through modulating miR-222-3p and CDKN1B (59). Yang et al. found that lncRNA NEAT1 enhanced tubular epithelial cell damage in kidney through regulation of mitophagy by targeting miR-150-5p and

DRP1 in diabetic nephropathy (60). Moreover, lncRNA NEAT1 promoted fibrosis, inflammation, proliferation and oxidative stress by modification of the miR-423/5p and GLIPR2 pathway in diabetic nephropathy (61). Hence, lncRNA NEAT1 might be a promising therapeutic target for the treatment of DKD.

LncRNA TUG1

LncRNA TUG1 has been identified to play a crucial role in DKD progression (62). One study revealed that ChREBP controlled lncRNA TUG1 transcription when glucose levels were increased in podocytes (62). Besides ChREBP, other coregulates, such as MXD1, MLX and HDAC1, were increased at the TUG1 promoter in response to high glucose exposures. This work suggested that ChREBP coordinated glucose homeostasis *via* regulation of lncRNA TUG1 (62). In addition, lncRNA TUG1 was discovered to regulate mitochondrial bioenergetics *via* regulation of PGC-1α in podocytes in diabetic nephropathy (63, 64). Overexpression of TUG1 in podocytes ameliorated diabetes-mediated chronic kidney disease in mice (63). Zhang et al. reported that knockdown of lncRNA TUG1 retarded the EMT of renal tubular epithelial cells *via* targeting miR-141-3p/β-catenin (65). Another work also demonstrated that lncRNA TUG1 reduced accumulation of extracellular matrix by sponging miR-377 and targeting PPARγ in diabetic nephropathy (66). Moreover, lncRNA TUG1 interacted with miR-9 and upregulated SIRT1, resulting in protection of podocytes from high glucose-triggered apoptosis and mitochondrial dysfunction (67). Urinary lncRNA TUG1 was positively associated with miR-29a, miR-93 and eGFR in type 2 DM, while lncRNA TUG1 had a negative association with miR-21, miR-124, podocalyxin, NAG and synaptopodin (38).

LncRNA TUG1 participated in regulation of podocyte apoptosis *via* modulation of TRAF5 pathway in diabetic nephropathy rats (68). LncRNA TUG1 influenced podocyte apoptosis *via* promotion of endoplasmic reticulum stress in diabetic nephropathy progression (69). Additionally, lncRNA TUG1 repressed the PI3K/AKT pathway and suppressed the fibrosis and proliferation in mesangial cells in diabetic nephropathy (70). LncRNA TUG1 inhibited the expression of miR-21 and enhanced the TIMP3 expression, leading to ameliorating diabetic nephropathy (71). LncRNA TUG1 repressed the PU.1/RTN1 pathway and improved diabetic nephropathy (72). Notably, lncRNA TUG1 affected high glucose-stimulated renal epithelial cell injury *via* regulation of endoplasmic reticulum stress by targeting miR-29c-3p and SIRT1 in diabetic nephropathy (73).

LncRNA MEG3

LncRNA MEG3 has been revealed to regulate glucose metabolisms in diabetic mice (74). STZ-mediated diabetic mice had an increased expression of lncRNA MEG3, which was associated with the podocyte numbers. Mice with knockdown of MEG3 in podocyte had improved renal physiological and histopathological features (74). These mice also had a reduced mitochondrial translocation of Drp1 and a decreased podocyte damage (74). Overexpression of lncRNA MEG3 in podocyte led to podocyte injury and enhanced mitochondria damage and upregulated expression and phosphorylation of Drp1 (74). LncRNA MEG3 increased fibrosis and inflammation through regulating miR-181a, Egr-1 and TLR4 in diabetic nephropathy (75). Moreover, lncRNA MEG3 sponged miR-145 and impacted the development of diabetic nephropathy (76). Strikingly, lncRNA MEG3 inactivated the Wnt/β-catenin pathway and reduced podocyte injury in diabetic nephropathy (77). Therefore, MEG3 plays an essential role in diabetic mice and DKD.

LncRNA KCNQ1OT1

Downregulation of KCNQ1OT1 attenuated oxidative stress and inflammation and reduced pyroptosis in renal tubular epithelial cells after high glucose stimulations through regulation of miR-506-3p (78). One study showed that KCNQ1OT1 participated in governing fibrosis, apoptosis and proliferation *via* regulation of miR-18b-5p and SORBS2 and NF-κB in diabetic nephropathy (79). Another study revealed that KCNQ1OT1 sponged miR-18b and increased the expression of HMGA2 and led to controlling high glucose-triggered oxidative stress, proliferation and extracellular matrix promotion in mesangial cells (80). In addition, KCNQ1OT1 was reported to accelerate diabetic nephropathy development *via* modulating miR-93-5p/ROCK2 axis (81). Xu et al. dissected that KCNQ1OT1 governed cell oxidative stress, proliferation, inflammation and extracellular matrix enhancement through miR-147a/SOX6 pathway in diabetic nephropathy (82). Recently, KCNQ1OT1 expression in diabetic nephropathy was increased and associated with activation of MEK/ERK pathway in diabetic nephropathy (83). LncRNA KCNQ1OT1 participates in DKD development and progression.

LINC00472

Wang et al. used the data from Gene Expression Omnibus (GEO) database to explore the differentially expressed profiles

between DKD patients and the normal patients. This study found that among 252 lncRNAs, 14 lncRNAs were differentially expressed. LINC00472 was identified to be differentially expressed in DKD patients, suggesting that LINC00472 could act as the diagnostic biomarkers for DKD patients (84). It is required to explore the detailed role of LINC00472 in DKD.

LncRNA NONMMUG023520.2 and NONMMUG032975.2

Smad3 has been reported to enhance the development of type 2 DM and involve in DKD pathogenesis (85–87). One group discovered the Smad3-associated genes *via* analysis of whole transcriptome profile in three types of transgenic mouse models, including Smad3 WT-db/db, Smad3 KO-db/db, $Smad3^{+/-}$ db/db mice (88). Smad3 KO-db/db mice displayed dysregulated genes involved in metabolism and RNA splicing, $Smad3^{+/-}$ db/db mice exhibited dysregulated genes that were associated with cell cycle and cell division (88). Two lincRNAs, NONMMUG023520.2 and NONMMUG032975.2, were further validated to be linked to the pathogenesis of diabetic nephropathy. Moreover, Upk1b, Psca and Gdf15 were identified to be correlated with diabetic nephropathy development [26. Without a doubt, further investigation is pivotal to determine the function of lncRNA NONMMUG023520.2 and NONMMUG032975.2 in DKD development and pathogenesis.

LncRNA 254693

Increased evidence has revealed that lncRNA ENSG00000254693 participated in DKD development (89). One research used RNA sequencing data and observed numerous differentially expressed lncRNAs in renal specimens of DKD. Among these dysregulated lncRNAs, lncRNA ENSG00000254693 was drastically changed. Moreover, DKD patients had higher expression of lncRNA ENSG00000254693 (89). Consistently, lncRNA ENSG00000254693 was upregulated in human podocytes after high glucose exposures. Depletion of lncRNA 254693 attenuated apoptosis, inflammation, and podocyte injury that were induced by high glucose (89). Furthermore, lncRNA 254693 was found to combine with HuR, and depletion of lncRNA 254693 reduced HuR levels. Interestingly, silencing of HuR reduced the expression and stability of lncRNA 254693 and alleviate podocyte injury, apoptosis and inflammation (89). Therefore, lncRNA 254693 might be a predicted factor for DKD treatment.

Other lncRNAs in DM and DKD

LncRNA CASC2 expression in renal samples and serum was identified to be downregulated in type 2 DM patients with chronic renal failure (90). Low serum level of CASC2 was associated with higher incidence of kidney failure, indicating that serum lncRNA CASC2 could be a biomarker for prediction of the occurrence of kidney failure in type 2 DM patients (90). By RT-PCR analysis in 77 type 2 DM patients, 60 diabetic nephropathy and 60 healthy people, one group found that lncRNA PANDAR in the serum was upregulated compared with healthy people (91). PANDAR expression was linked to the level of proteinuria and glomerular filtration rate. PANDAR might serve as a biomarker for judgement of DKD prognosis (91). Yang et al. reported the differential expression profiles of circulating lncRNAs in DM and DKD patients. Compared with healthy persons, 245 lncRNAs were increased, while 680 lncRNAs were decreased in the serum of DM patients. Compared with diabetes patients, 45 and 813 lncRNAs were increased and decreased in the serum of DKD patients, respectively (92). LncRNA ARAP1-AS1 expression was elevated during DM and DKD progression, while lncRNA ARAP1-AS2 was decreased in DM and DKD progression (92). Hence, circulating lncRNA ARAP1-AS1 and ARAP1-AS2 might predict the progression of DM and DKD.

Another group identified that lncRNA KCNQ1OT1 was abnormally elevated in PBMCs of diabetic nephropathy, which was correlated with the activation of MEK/ERK pathway (83). LncRNA CASC2 modulated cell proliferation, oxidative stress and extracellular matrix promotion in human mesangial cells upon high glucose treatment through regulation of miR-133b and FOXP1 expressions (93). LncRNA CASC2 mitigated diabetic nephropathy development *via* sponging miR-144 and regulating SOCS2 expression (94). LncRNA CASC2 ablated cell inflammation, proliferation and fibrosis in glomerular mesangial cells upon high glucose exposures *via* targeting miR-135a-5p/TIMP3 pathway and JNK pathway (95).

LncRNAs regulate glucose metabolism in cancer

Competing endogenous RNAs (ceRNA) can compete for shared miRNAs to modulate the expression of other RNA transcripts. A ceRNA network profile has identified the several lncRNAs for classifying diabetic pancreatic cancer form non-diabetic pancreatic cancer, including HOTAIR, CECR7, UCA1, suggesting that lncRNAs are important predictors for diabetic pancreatic cancer (96). In the following paragraphs, we will discuss the association between lncRNAs and glucose metabolisms in human cancer (Figure 1).

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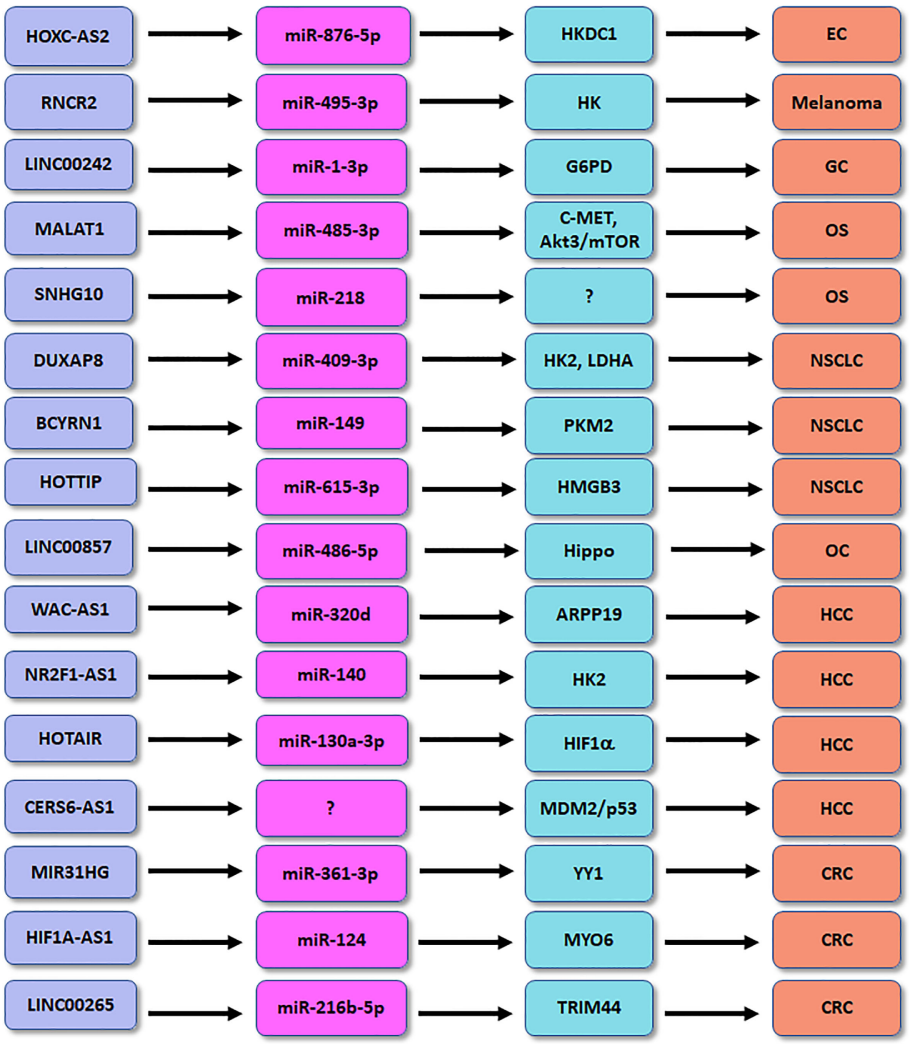

FIGURE 1
The role of lncRNAs in regulation of glucose metabolism in human cancers. EC, endometrial cancer; OS, osteosarcoma; HCC, hepatocellular carcinoma; CRC, colorectal cancer; GC, gastric cancer; NSCLC, non-small cell lung cancer.

LncRNAs regulate glucose metabolism in cancer

Evidence has dissected that lncRNA-associated genetic variants are shared between cancers and type 2 DM in human (97). LncRNA DRAIR has been known to involve in the development of type 2 DM (98). One study showed that the expression of lncRNA DRAIR was remarkably elevated in triple-negative breast cancer (TNBC) samples and plasma (99). High expression of DRAIR in plasma was associated with chemoresistance after therapy and tumor recurrence in TNBC patients. *In vitro* experiments showed that overexpression of DRAIR enhanced proliferation and viability of TNBC cells after doxorubicin treatment (99).

Accumulated evidence dissected that lncRNA HOXC-AS2 participated in the progression in high glucose-related endometrial cancer (EC) (100). EC patients with diabetes had the increased expression of HKDC1 compared with EC patients with normal glucose. HKDC1 governed pyroptosis, a highly inflammatory response of regulated cell death, *via* regulation of ROS and cytokine release in EC cells after high glucose stimulation (100). Moreover, miR-876-5p can inhibit the expression of HKDC1 in high glucose-related EC. LncRNA HOXC-AS2 was dissected to suppress the miR-876-5p/HKDC1 axis in high glucose-associated EC (100). HKDC1 affected the formation of TME *via* promotion of glycolysis, leading to accelerating EC progression. This work provided the new therapeutic strategy for EC patients with diabetes by targeting lncRNA HOXC-AS2 (100). LncRNA SNHG10

enhanced glucose uptake and increased proliferation of osteosarcoma cells *via* promotion of miR-218 methylation (101). LncRNA MALAT1 facilitated glycolysis and tumor metastasis *via* blocking miR-485-3p and upregulating c-MET and Akt3/mTOR pathways in osteosarcoma (102). LncRNA CERS6-AS1 regulated the MDM2/p53 axis and modulated glucose metabolism and progression of HCC (103). LncRNA WAC-AS1 sponged miR-320d and regulated the expression of ARPP19, which promoted glucose uptake and lactate production in HCC (104). LncRNA NR2F1-AS1 affected hypoxia-mediated glycolysis and migratory ability of HCC cells *via* targeting miR-140 and HK2 (105). Depletion of lncRNA HOTAIR reduced glycolysis *via* inhibition of miR-130a-3p and upregulation of HIF1α in HCC cells under hypoxia (106).

LncRNA MALAT1 modulated MYBL2/mTOR pathway and caused glucose metabolism changes in prostate cancer (107). LncRNA MIR31HG heightened glycolysis and tumor malignant progression *via* regulating miR-361-3p and YY1 transcription factor in colorectal cancer (108). LncRNA KCNQ1OT1 accelerated colorectal oncogenesis *via* promoting aerobic glycolysis by upregulation of HK2 (109). HNF1A-AS1 governed glycolysis, invasion and migration through targeting miR-124 and MYO6 in colorectal cancer (110). Similarly, LINC00265 enhanced glycolysis and lactate release *via* binding with miR-216b-5p and elevating the expression of TRIM44 in colorectal cancer (111). LncRNA RNCR2 promoted glycolysis and EMT and proliferation of melanoma cells *via* interacting with miR-495-3p and upregulating HK2 in melanoma (112). LINC00242 combined miR-1-3p and elevated the expression of G6PD, leading to enhancement of aerobic glycolysis and oncogenesis of gastric cancer (113). LncRNA MSC-AS1 increased glycolysis and cell growth *via* targeting PFKFB3 expression in gastric cancer cells (114). OIP5-AS1 heightened aerobic glycolysis and proliferation *via* miR-186 sponge in gastric cancer (115).

LINC00551 inhibited glycolysis and blocked tumor progression *via* modulation of c-Myc-induced PKM2 expression in lung cancer (116). LncRNA CRYBG3 potentiated glycolysis *via* interaction with lactate dehydrogenase A (LDHA) in lung cancer (117). LncRNA DUXAP8 accelerated glycolysis, viability and migratory capacities *via* suppression of miR-409-3p and upregulation of HK2 and LDHA in NSCLC cells (118). LncRNA BCYRN1 accelerated glycolysis *via* controlling the miR-149 expression and elevating PKM2 expression in NSCLC (119). HOTTIP enhanced hypoxia-mediated glycolysis *via* modulation of miR-615-3p and HMGB3 in NSCLC cells (120). LINC00857 was found to regulate glycolysis and tumor progression *via* governing the Hippo signaling pathway by binding to miR-486-5p in ovarian cancer (121). Downregulation of lncRNA UCA1 attenuated glycolysis pathway and led to suppression of growth of pituitary cancer cells (122). Overexpression of lncRNA PCED1B-AS1 resulted in upregulation of glucose uptake, proliferation and lactate production in glioblastoma by activation of HIF-1α pathway (123). LncRNA HNF4A-AS1 elevated aerobic glycolysis and tumor progression *via* modulating hnRNPU/CTCF axis in neuroblastoma (124).

High/low glucose regulates lncRNAs in cancer

Some studies have demonstrated that high glucose or glucose deprivation affected the expression of lncRNAs in cancer cells. For example, U87 and LN18 glioma cells after glucose deprivation had upregulation of lncRNA TP53TG1 and glucose metabolism-associated genes, including LDHA, IDH1 and GRP79 (125). Downregulation of TP53TG1 suppressed proliferation and migration of U87 cells after glucose deprivation, while overexpression of TP53TG1 displayed the opposite functions (125). Low glucose condition promoted the efficacy of TP53TG1 compared with high glucose condition. This study suggested that glucose metabolism dysregulation can affect the expression of TP53TG1 and tumor proliferation and migration in glioma (125).

High glucose increased the expression of miR-483-3p in hepatocellular carcinoma (HCC) cells. Moreover, upregulation of miR-483-3p inhibited the expression of ER protein 29 (ERp29), resulting in promotion of proliferation and migration of HCC cells (126). Furthermore, lncRNA MEG3 can bind with miR-483-3p in HCC cells. High glucose also reduced the expression of lncRNA MEG3 in HCC cells. Consistently, silencing of lncRNA MEG3 suppressed the expression of ERp29 in HCC cells (126). This study showed that high glucose could affect the expression of lncRNA MEG3 and govern the miR-483-3p/ERp29 proteins in HCC patients, suggesting that management of lncRNA MEG3 could be promising for the treatment of HCC patients with diabetes (126). Low glucose elevated the expression of lncRNA HOXC-AS3, leading to promotion of metabolic reprogramming of breast cancer *via* binding to SIRT6 and inactivating HIF1α (127).

Targeting lncRNAs for treating DKD and cancer

Klotho is often known as an antiaging protein to prevent of aging. Klotho has been identified to protect renal tubular EMT during the DKD development (52). Overexpression of Klotho reduced the lncRNA NEAT1 expression in HFD/STZ-mediated DKD mice. Moreover, overexpression of Klotho attenuated the expression levels of NEAT1 in BSA-treated HK2 cells (52). On the contrary, knockdown of Klotho increased the expression of lncRNA NEAT1 in HK2 cells. Thereby, knockdown of Klotho caused upregulation of NEAT1 and activation of EMT and fibrosis in a ERK1/2-dependent manner (52). Another study

showed that Klotho blocked EMT *via* downregulation of early growth response factor 1 (Egr-1) by suppression of the ERK1/2 pathway in DKD mice (128). Similarly, Klotho decreased Egr-1 expression *via* repressing TGF-β1/Smad3 pathway in human mesangial cells after high glucose exposures (129). Triptolide, a diterpenoid epoxide that is obtained from the thunder god vine, blocked renal tubular EMT *via* modulation of miR-188-5p-involved PI3K/Akt pathway in DKD (130). Several studies have showed that triptolide regulated the expression of multiple lncRNAs, including lncRNAs WAKMAR2, PACER, ENST00000619282, RP11-83J16.1 (131–135). Therefore, whether triptolide regulates the lncRNA expression in DKD needs to be further explored. Berberine, an isoquinoline alkaloid, has been reported to upregulate the expression of lncRNA GAS5 to reduce the mitochondrial ROS generation in HK-2 cells under high glucose environment through regulation of miR-18a-5p and C/EBPβ expression (136). The antisense oligonucleotide treatment by targeting specific lncRNAs could provide targeted medicine to cure DKD and cancer in the future.

Conclusion

In summary, burgeoning data demonstrate that lncRNAs play an essential role in the development of DKD and diabetes-associated cancer. LncRNAs could be diagnosis and prognosis biomarkers for DKD and diabetes-related cancer. Modulation of lncRNAs might be a promising strategy for treating DKD and diabetes-associated cancer. It is important to note that it is far from being fully clarified, although some studies have explored the role of lncRNAs in DKD and cancer patients with DM. A small number of lncRNAs are identified in regulation of DKD and cancer patients with abnormal glucose metabolism. Whether other lncRNAs also participate in DKD and diabetes-associated cancer need to be explored. Compared with other factors such as m6A and signaling pathways, it remains questionable whether lncRNAs are more important in modulation of DKD and diabetes-related cancers. Addressing these questions will help us understand the mechanism of lncRNAs-regulated DKD and cancers, which could provide the clues for discovering new therapeutic strategy for DKD and cancer patients with diabetes.

Author contributions

YC and XW wrote the manuscript. YX made the figures. YC and PW edited the manuscript and supervised this study. All authors read and approved the final manuscript.

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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.

Publisher's note

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Comprehensive analysis of lncRNA-mediated ceRNA networkfor hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is a high-burden cancer. The molecular mechanism of HCC has not been fully elucidated. Notably, current research has revealed a significant function for long non-coding RNAs (lncRNAs) in the prognosis of patients with HCC. Here, this study aims to construct a regulated lncRNA-mediated ceRNA network and find biological targets for the treatment of HCC.

Methods: Based on the RNA expression patterns from the TCGA, we did an analysis to determine which genes were expressed differently between liver tumor tissues and noncancerous tissues. Then, using bioinformatic tools, we built a lncRNA-miRNA-mRNA ceRNA network and used GO and KEGG functional analyses on the DEmRNAs connected to ceRNA networks. The main lncRNAs in the subnetwork were chosen, and we next looked at the relationships between these lncRNAs and the clinical characteristics of patients with HCC. The prognosis-related genes and immune cells were identified using Kaplan-Meier and Cox proportional hazard analyses, and CIBERSORT was utilized to separate the 22 immune cell types. CCK8 assay was performed to measure cell viability in HCC cells after lncRNA HOTTIP modulation.

Results: Differentially expressed mRNA and lncRNAs in HCC and paracancerous tissues were identified. There are 245 lncRNAs, 126 miRNAs, and 1980 mRNAs that are expressed differently in liver tumour tissues than in noncancerous cells. Function analysis showed that mRNAs in ceRNA network were significantly enriched in G1/S transition of mototiv cell cycle, positive regulation of cell cycle process, hepatocellular carcinoma, and cancer related pathways. CD8 T cells and T follicular helper cells had a favourable link with a 0.65 correlation coefficient. Additionally, there was a strong correlation between Eosinophils, activated NK cells, and B memory cells. Strikingly, depletion of lncRNA HOTTIP inhibited viability of HCC cells. In addition, miR-205 upregulation suppressed viability of HCC cells, while miR-205 downregulation repressed viability of HCC cells. Notably, miR-205 depletion rescued HOTTIP depletion-mediated suppression of cell viability in HCC.

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Conclusion: A ceRNA network was created by examining the lncRNA, miRNA, and mRNA expression profiles of liver tumours from the TCGA database. LncRNA HOTTIP promoted cell viability *via* inhibition of miR-205 in HCC cells.

KEYWORDS

HCC, lncRNA, HOTTIP, MiR-205, prognosis, immune microenvironment

Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors in the world (1). The epidemiology of HCC shows two main patterns: one in North America and Western Europe, and the other in non-Western countries, such as sub-Saharan Africa, Central and South-east Asia, and the Amazon Basin (2). Men are generally more affected than women, and most commonly between the ages of 30 and 50, and HCC causes 662,000 deaths worldwide each year, about half of them in China (3). By 2022, there will be 41,260 new cases and 30,520 new deaths according to cancer statistics (4).

The treatment of HCC can be divided into surgical treatment and non-surgical treatment (5). Surgical treatment includes resection, cryoablation and liver transplantation. Nonsurgical treatments include either liver-specific therapy (i.e., percutaneous ethanol injection, radiofrequency/microwave ablation, trans-arterial embolization, external radiation therapy) or systemic therapy (chemotherapy, molecular targeted therapy, immune checkpoint inhibitor therapy) (6). Surgical treatment is the main treatment for HCC, but the 5-year survival rate after surgical resection is only about 30% (7). Currently, the molecular mechanism of HCC occurrence has not been fully elucidated (8), so it is of great significance to explore the molecular mechanism of HCC occurrence and development and screen biological markers for the early diagnosis of HCC for the treatment of this deadly disease.

Long non-coding RNA (lncRNA) is a class of non-coding RNA with a length greater than 200bp, which has no protein-coding function, poor conserved between species, and strong tissue specificity and spatio-temporal specificity (9). Recently, more and more lncRNAs have been found to act as oncogenic or tumor suppressor factors, and many oncogenic genomic loci are mainly transcribed from those lncRNAs that play an important role in cancer induction (10–13). With the rapid development of cancer research, a large number of lncRNAs related to tumorigenesis have been identified (14–16). Studies have shown that lncRNA MEG3 can promote the proliferation of HCC cells (17, 18). At the same time, some studies have proved that lncRNAs HOTTIP and UCA1 can promote the deterioration of HCC. Epigenetic related molecules (such as SNRPC), noncoding RNAs (such as HSA-Mir-221) and immune-related molecules (such as DCK) can be used as potential biomarkers for diagnosis, treatment and prognosis of HCC (19–22).

Studies have shown that lncRNAs have a wide range of biological functions, such as participating in RNA generation and processing, transcription regulation and chromatin remodeling (23). However, the mechanism of action is complex, such as participating in transcriptional regulation by binding characteristic proteins or taking part in post-transcriptional regulation as ceRNA (24). According to the ceRNA hypothesis proposed in 2011, when circRNA and mRNA share the same miRNA response element (MRE), they will competitively bind MRE to regulate the expression level of related genes. The most common mechanism of action between lncRNAs and miRNAs is that lncRNAs directly complement and pair with miRNA seed sequences, adsorb miRNAs to form complexes, and reduce the number of miRNAs involved in downstream gene regulation in cells (25). Studies have shown that the abnormal expression of lncRNAs affects the expression of mRNAs through sponge miRNAs, leading to tumorigenesis and cancer progression (26, 27). Therefore, in order to better study the functions of lncRNAs, there is an urgent need to better understand the regulatory networks of lncRNAs. This article aimed to perform a comprehensive analysis of TCGA transcriptome data to identify differentially expressed lncRNAs, miRNAs, and mRNAs to generate a regulated ceRNA network.

Abbreviations: ceRNA, competing endogenous RNA; circRNA, circular RNAs; CSC, cancer stem cells; DSCR4, Down Syndrome critical region 4; EZH2, Enhancer of Zeste 2; GO, gene ontology; HCC, hepatocellular carcinoma; HOTTIP, HOXA distal transcript antisense RNA; HOXA13, homeobox A13; KEGG, The Kyoto Encyclopedia of Genes and Genomes; lncRNA, long noncoding RNA; MEG3, maternally expressed 3; miRNA, microRNA; mRNA, messenger RNA; SLC7A11, solute carrier family 7 member 11; TCGA, The Cancer Genome Atlas; TDRG1, testis development related 1; UCA1, urothelial cancer associated 1.

Methods:

Database

Data were downloaded from the TCGA database (https://portal.gdc.cancer.gov/) (28). The data were used to study the

differential expression of lncRNA, mRNA, miRNA among HCC patients. In addition, edgeR(3.28.0) (29) of R language was mainly used to identify differentially expressed RNA. Screening criteria is: |logFC| > 2, and P value < 0.01 for mRNA and miRNA, and |logFC| > 4, and P value < 0.01 for lncRNA.

Construction of ceRNA network and prognosis model

Using the mircode database, the correlation analysis of differentially expressed lncRNAs and mRNAs was carried out. Then, a ceRNA network was built using the miRDB, miRTarBase, and TargetScan databases. By adding edge and nodal gene information to the interaction network with Cytoscape 3.8.0, the HCC ceRNA network was displayed. The Kyoto Encyclopedia of Genes and Genomes and Gene Ontology (GO) (30) were then used to annotate the differentially expressed mRNAs in the ceRNA network. The R language's "clusterProfiler," (31) "org.Hs.eg.db," "enrichplot," and "ggplot2" (32) packages were used to carry out the aforementioned analysis. According to the median model risk score, all patients were separated into high-risk and low-risk groups, and Kaplan-Meier survival curves were created to analyze the variations in survival between the two groups (33). It can be regarded as statistically significant when P <0.05. Using the area under the curve (AUC), the model's predictive effectiveness was assessed (visualized by the "timeROC" package in R language). After that, the "rms" package of R was used to build the nomogram survival prediction map, and the calibration curve was used to assess the accuracy of the map in predicting the survival rate of HCC patients.

Assessing immune cells

Using the "CIBERSORT" software tool in the R language, the abundance of 22 immune cell subsets in 424 tumour specimens were estimated (34). The threshold used was P <0.05. The "Pheatmap" programme was used in the study to show immune cell infiltration in HCC. To examine the immune cells that are connected to survival, we employed univariate Cox regression (35). Lasso regression and multivariate Cox regression analysis were used together to create the prognosis related model (36), and the model's accuracy was assessed using the AUC value. We estimated the prognostic model's quantification using the multi-index AUC value, and we determined the nomogram's predictive power using the calibration curve. To investigate the connection between important genes in the ceRNA network and immune cells in the model, we employed Pearson correlation analysis (37).

Construction of nomogram model

For the derived independent prognostic indicators, a nomogram model was created and shown. The concordance index (C-index) of the independent prognostic factors and the Nomogram model, comprised of risk group fitting with the Coxph model, were determined to validate the nomogram's prediction abilities. The statistical test was then run with resampling, and the significant P < value was determined. The degree to which the independent prognostic factors and compound factors fitted the Coxph model was evaluated, and the factor with the lowest P value was chosen to draw the calibration curve. The model created by this component performed better when the calibration curve was closer to 45°.

Transfection

The HOTTIP siRNA and negative control siRNA (NC), miR-205 mimics and miR-205 inhibitors were obtained from GenePharm (Shanghai, China). The HOTTIP and miR-205 were transfected into HCC cells by lipofectamine 3000 (Invitrogen) based on the manufacturer's protocol.

Reverse transcription-quantitative PCR analysis

Total RNA was isolated from HCC cell lines using TRIzol reagent based on the manufacturer's protocol. RT-PCR was performed as described before. The primers are as follows: HOTTIP: forward primer: 5'-CCT AAA GCC ACG CTT CTT TG-3'; reverse primer: 5'-TGC AGG CTG GAG ATC CTA CT-3'; GAPDH: forward primer: 5'-GTC AAC GGA TTT GGT CTG TATT-3'; reverse primer: 5'-AGT CTT CTG GGT GGC AGT GAT-3'.

Cell counting assay Kit-8 assay

Huh-7 and HepG2 HCC cell lines were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin, at 37°C under a 5% CO_2 atmosphere. CCK-8 assay was performed to analyze cell proliferation based on the manufacture's protocol in HCC cell lines after 48 and 72 h transfection. The OD450nm was measured by a microplate reader.

Statistical analysis

Statistical analyses for CCK-8 assay were performed by GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA). The

results were presented as mean ± standard deviation (SD). Multiple groups were analyzed by one-way analysis of variance (ANOVA) test followed Turkey's posttest. $P < 0.05$ was indicated statistically significant.

Results:

Expression of DERNAs

In order to better understand the expression of RNAs in HCC, we conducted differential analysis on mRNAs, lncRNAs and miRNAs, and the results are shown in Figures 1A–F. The Volcano diagram and Heatmap demonstrated that 1980 DEmRNAs had 1770 up-regulated and 210 down-regulated mRNAs (Figures 1A, B). There were 245 DElncRNAs, including 242 up-regulated and 3 down-regulated lncRNAs (Figures 1C, D). Moreover, 126 DEmiRNAs had 123 up-regulated and 3 down-regulated miRNAs (Figures 1E, F).

Go and KEGG pathway analysis

The biofunctional analysis of the ceRNA co-modules is undertaken to further investigate the functional role of mRNAs/miRNAs/lncRNAs in carcinogenesis and if the ceRNA co-modules identified in the study are connected with HCC. Function analysis showed that mRNAs in ceRNA network were significantly enriched in G1/S transition of mototiv cell cycle, positive regulation of cell cycle process, hepatocellular carcinoma, and cancer related pathways (Figures 2A, B).

Construction of a lncRNA-miRNA-mRNA ceRNA network

Based on the several databases, including miRcode database, a ceRNA network was constructed by differentially expressed lncRNAs, miRNAs and mRNAs. 8 DElncRNAs, 11 DEmiRNAs, 98 DEmRNAs are shown as nodes, and 156 interactions are shown as edges. Visualization is achieved by Cytoscape (Figure 2C).

Prognosis model of hepatocellular carcinoma

Our data imply that 24 mRNAs may be protective factors (Figure 3). We screened 24 genes associated with HCC prognosis (Figure 3). The prognosis of 31 genes was determined by univariate Cox regression analysis. Then, the results of Lasso regression showed that ERVMER61-1, SLC7A11, CEP55, CBX2, EZH2, PBK, AP002478.1 and miR-137 were associated with the HCC prognosis (P <0.05) (Figure 4A). The median risk score was used to categorise patients into high-risk and low-risk groups. The high-risk samples had a lower survival duration than the low-risk samples (Figures 4B–D). The AUC at 1 years, 3 years, 5 years were 0.764,0.61 and 0.802 (Figure 4E). The low-risk group had a worse chance of survival than the high-risk group (Figure 4F).

The independent prognostic indicators were then used to create the Nomogram, which forecasts survival rates for the first, three to five years. There is a nomogram model made up of CBX2, PBK and AP002478.1 (Figures 5A, B). The nomogram model's calibration curves demonstrated good agreement between the anticipated 1/3/5-year overall survival rate and the actual survival rate, indicating that the model was accurate in its predictions (Figure 5C).

Immune infiltration in HCC

CD8 T cells were positively correlated with T follicular helper cells, and the correlation coefficient was 0.65. Additionally, there was a positive link between Eosinophils and NK activated cells and B memory cells with a correlation coefficient of 0.75 and 0.68, respectively (Figure 5D). The expression of dendritic resting cells was higher in G3/4 and lower in G1/2 (Figure 6A). Similarly, T cell expression was higher in the G3/4 than in the G1/2 (Figure 6A). Neutrophils was highly expressed in T3/4 (Figure 6A). Macrophage M2 was highly expressed in T3/4 and stage III/IV (Figure 6B).

To prevent overfitting and resolve severe collinearity, we performed lasso regression (Figures 6C, D). The AUC curve demonstrates that the nomogram survival prediction model had a high accuracy (Figure 6E). The AUC at 1 year, 3 years, and 5 years were 0.764, 0.61, and 0.812, respectively (Figure 6E). The high-risk group had worse survival than the low-risk group (Figure 6F). Figure 7A depicted that dendritic activated cells, and eosinophil were highly expressed in tumor cells. The Pearson correlation test was used to investigate the relationship between major genes in the ceRNA network and prognosis-related immune cells (Figure 7B).

LncRNA HOTTIP promotes cell viability *via* targeting miR-205

Our ceRNA network showed the role of lncRNA HOTTIP and miR-205 in HCC progression. To validate the function of lncRNA HOTTIP on cell viability of HCC, HOTTIP was downregulated by siRNA transfection in HCC cells (Figure 8A). CCK-8 data showed that downregulation of HOTTIP led to inhibition of cell viability in HepG2 and Huh7 cells (Figure 8B). Moreover, miR-205 expression level was increased in HCC cells after miR-205 mimic transfection,

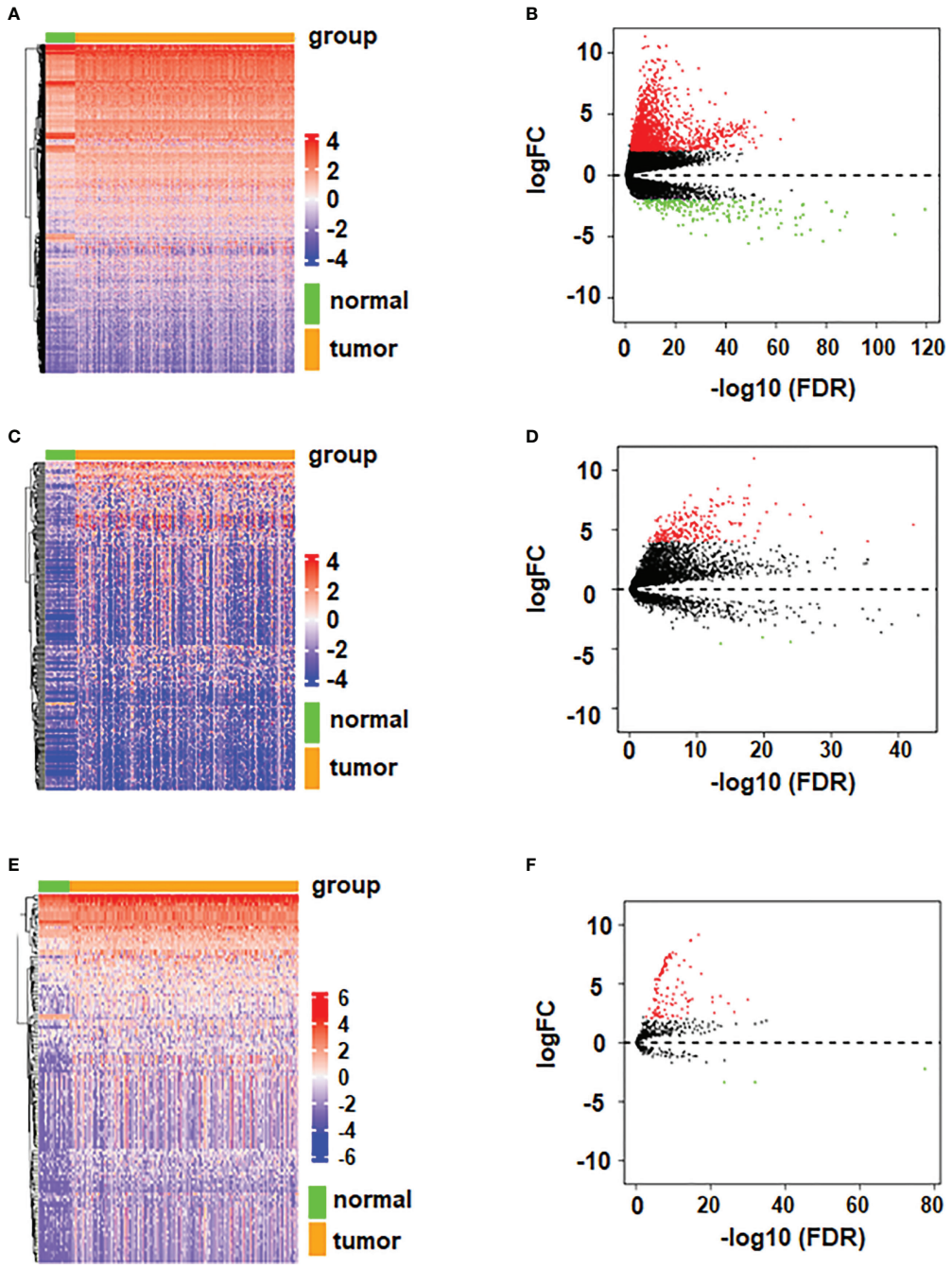

FIGURE 1
Volcano plots and heatmaps of differentially expressed mRNA, miRNA, and lncRNA. **(A)** Volcano plot of differentially expressed mRNA. **(B)** Heatmap of differentially expressed mRNA. **(C)** Volcano plot of differentially expressed lncRNA. **(D)** Heatmap of differentially expressed lncRNA. **(E)** Volcano plot of differentially expressed miRNA. **(F)** Heatmap of differentially expressed miRNA.

while miR-205 was downregulated in HCC cells after miR-205 inhibitor treatment (Figure 8C). Furthermore, inhibition of miR-205 increased viability of HCC cells, whereas upregulation of miR-205 decreased viability of HCC cells (Figures 8D, E). Notably, inhibition of miR-205 rescued HOTTIP siRNA-mediated suppression of cell viability in HCC (Figure 8F). Altogether, TOTTIP enhanced cell viability *via* targeting miR-205 in HCC cells.

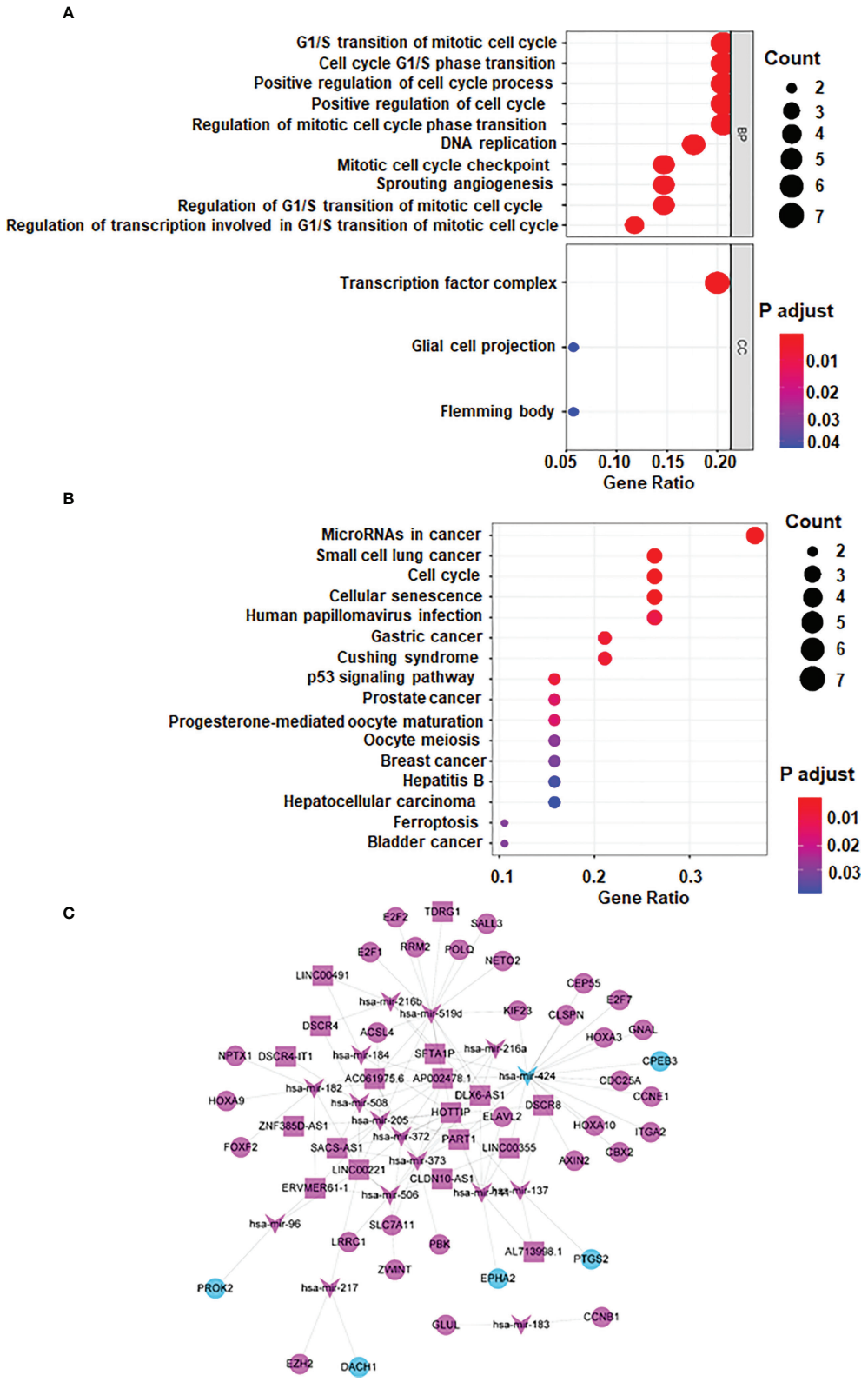

FIGURE 2
lncRNA-miRNA-mRNA ceRNA network. **(A)** GO analysis **(B)** KEGG Pathway Analysis **(C)** lncRNA-miRNA-mRNA ceRNA network generated by Cytoscape (version 3.6.1, https://cytoscape.org/). Red: upregulated RNAs. Green: downregulated RNAs. Triangle: miRNA. Diamond: lncRNA. Circular: mRNA.

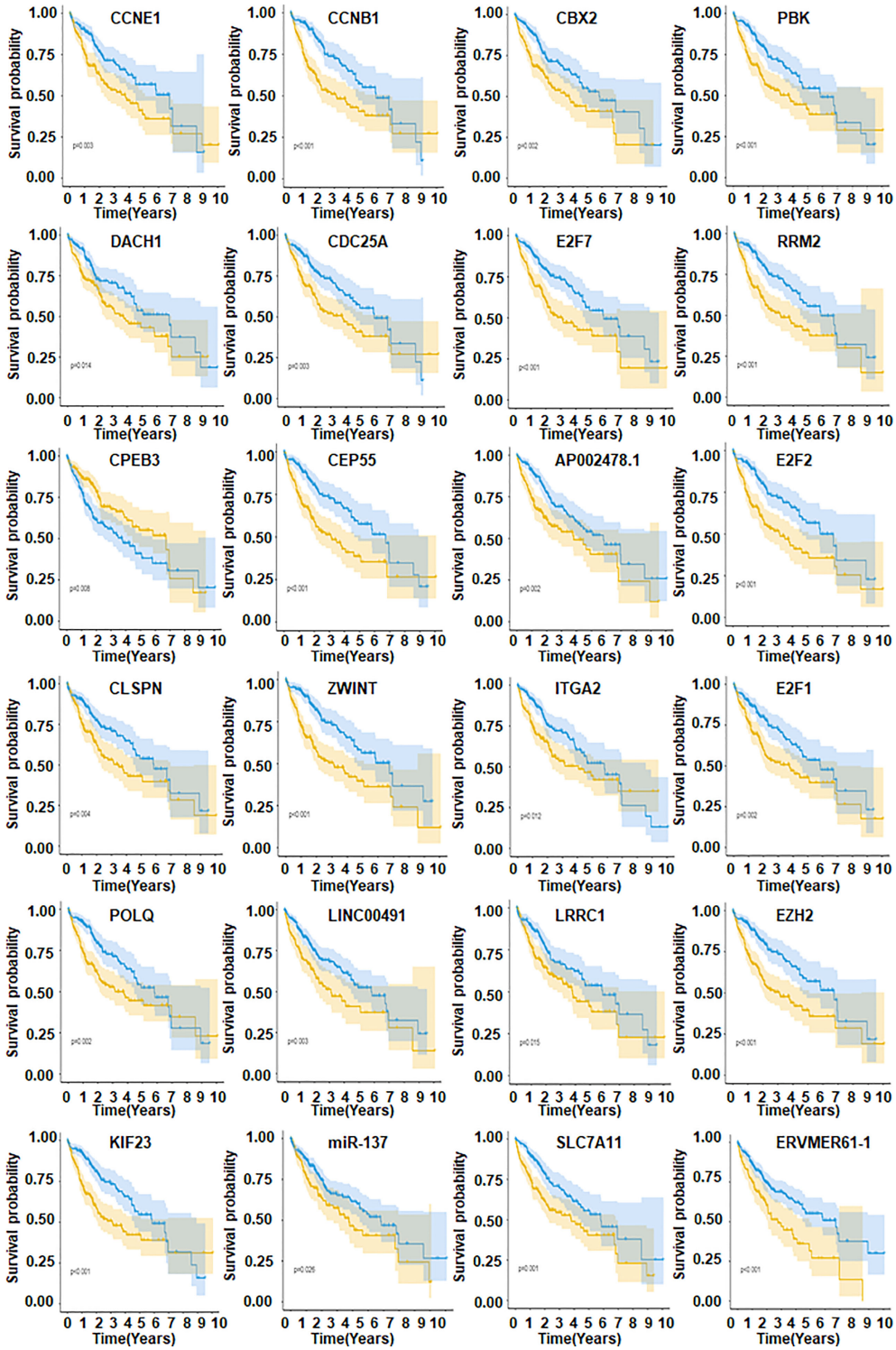

FIGURE 3
Kaplan-Meier method to find 24 genes.

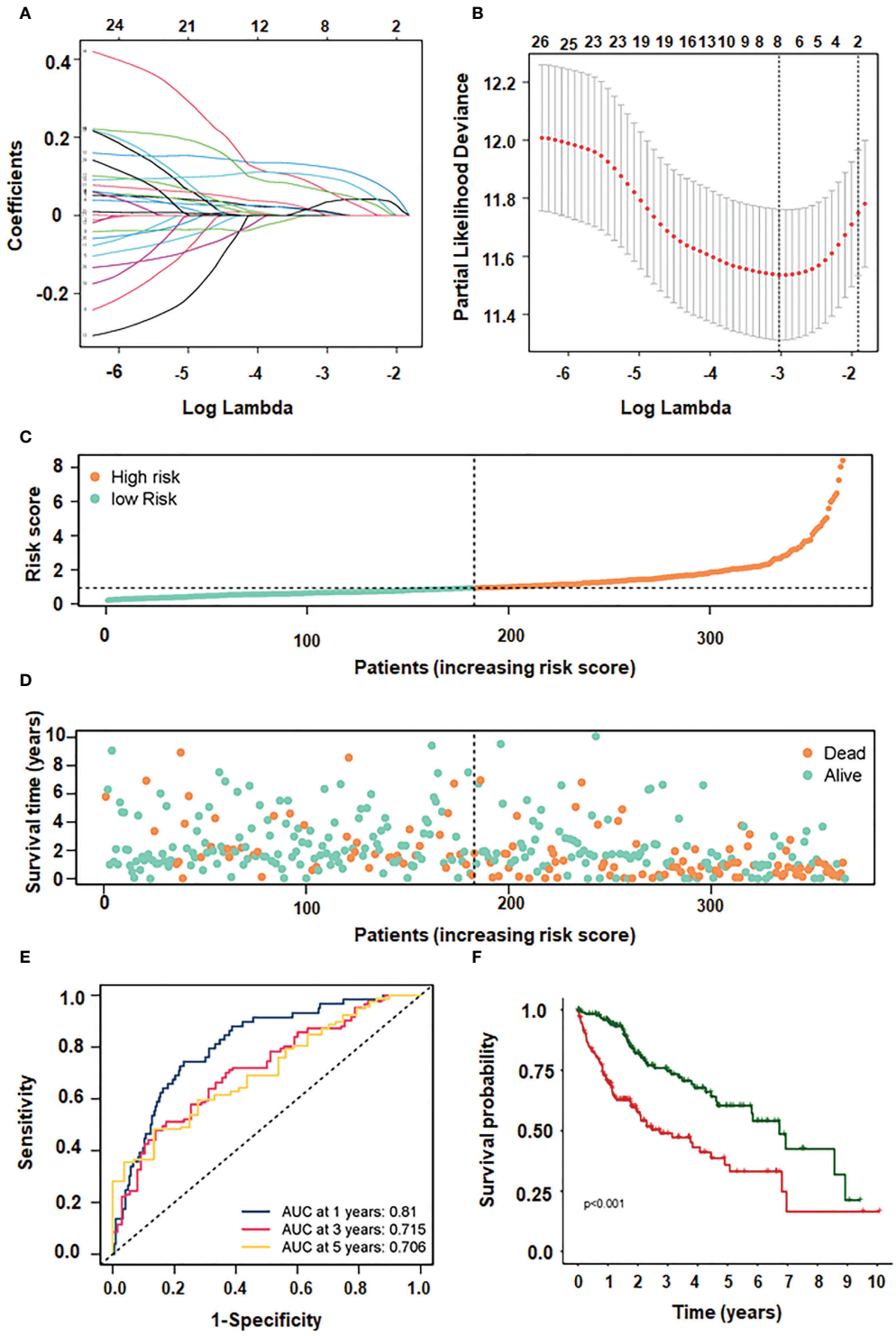

FIGURE 4
LASSO and multivariate regression models screening 4 genes. **(A)** different characteristics of genes and their corresponding coefficients. **(B)** cross-validation to build the model. **(C)** risk score with survival status cases. **(D)** risk score with survival time. **(E)** ROC curve plotted under the prediction model. **(F)** KM curve of this prediction model.

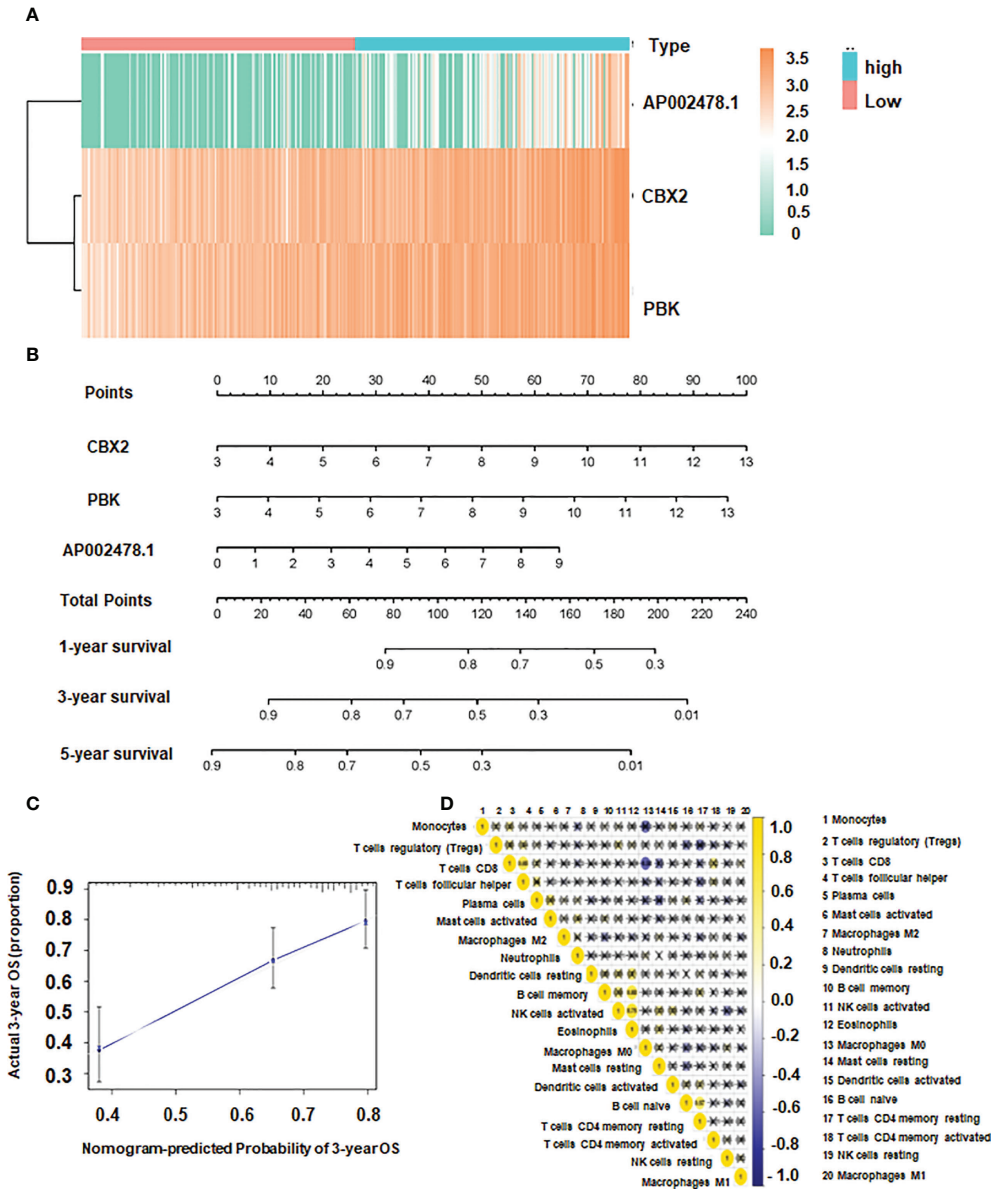

FIGURE 5
The association of CBX2, PBK, AP002478.1 and survival. **(A)** Heatmap of differentially expressed genes. **(B)** Nomogram based on multiple Cox regression. **(C)** Calibration curve for 3-year survival. The closer to the diagonal, the better the prediction effect. **(D)** Co-expression patterns among fractions of immune cells.

Discussion

HCC is one of the most common malignant tumors in the gastrointestinal system (38). At present, the molecular pathogenesis of HCC is still unclear (39). Therefore, it is very important to understand the molecular mechanism and process of HCC and identify new therapeutic targets to improve the clinical prognosis of patients. LncRNAs have been considered as an underappreciated novel therapeutic target (40). LncRNAs play important roles in gene regulation, including regulation of gene activation and silencing, X chromosome inactivation, alternative splicing, and post-translational regulation (41). Besides, lncRNAs have also been shown to be associated with tumor progression (12). Therefore, in this study, we studied the relationship between lncRNAs and liver cancer, and constructed a ceRNA regulatory network based on lncRNAs.

The current work used lncRNA-associated ceRNA to identify key biomarkers related to the prognosis of HCC. The

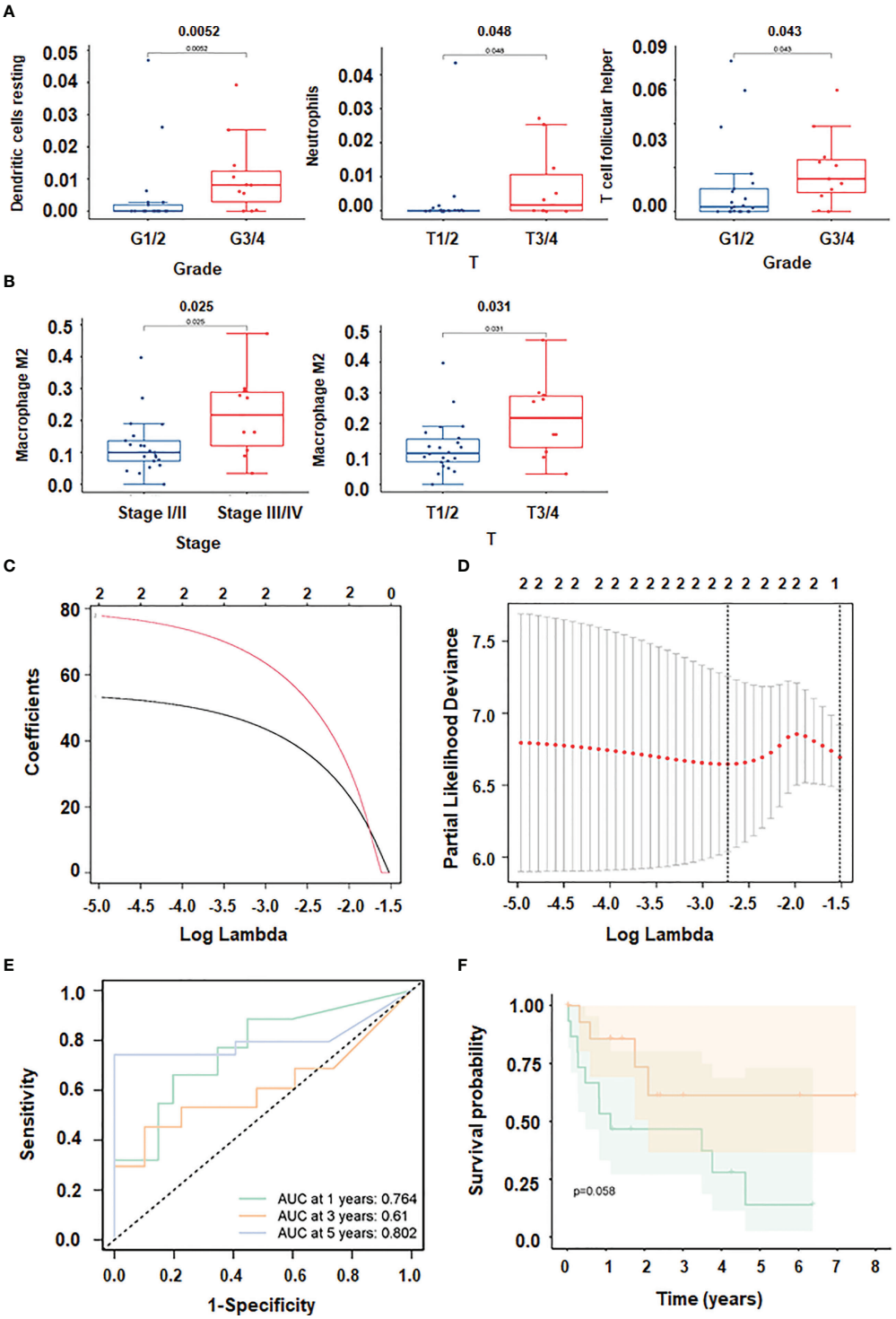

FIGURE 6
Immune cell infiltration in HCC. **(A, B)** Correlation between immune cells and clinical shape. **(C, D)**: different characteristics of immune cells and their corresponding coefficients; **(E)** ROC curve plotted under the prediction model). **(F)** KM curve of this prediction model.

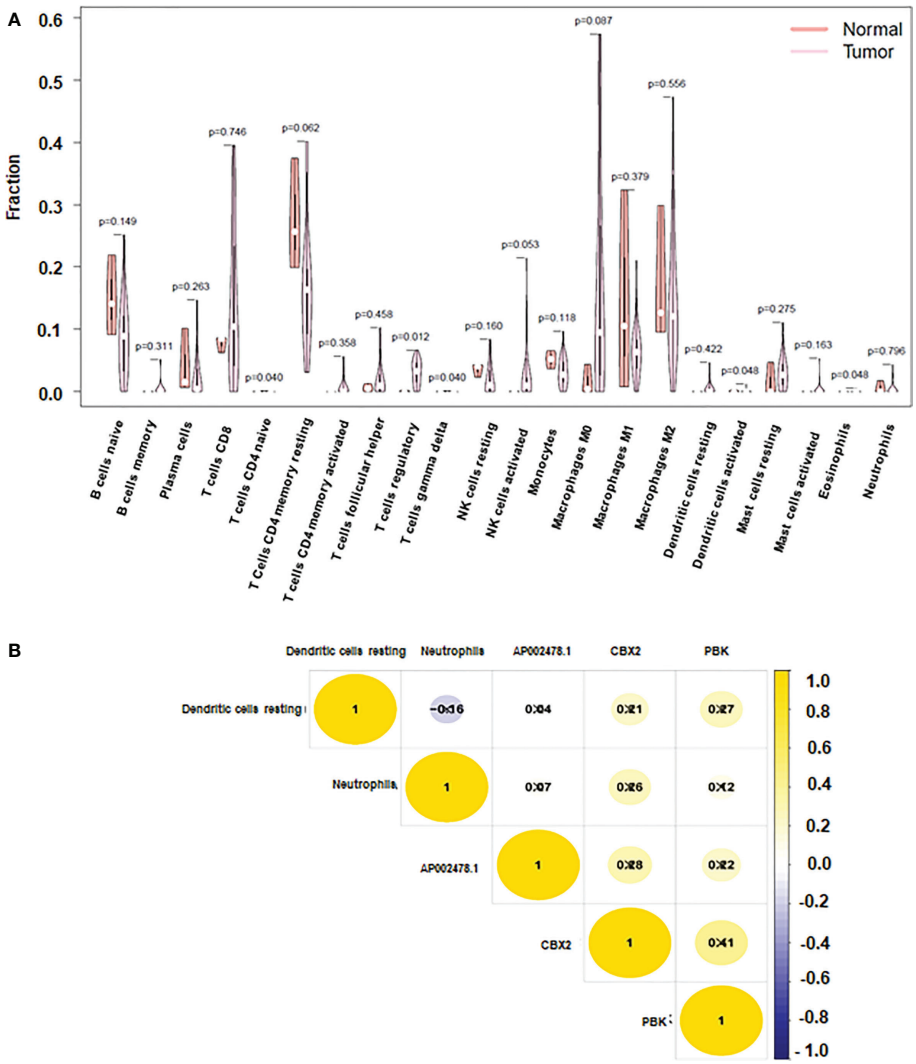

FIGURE 7
Immune cells and CBX2, PBK, AP002478.1 in HCC. **(A)** Heatmap of the three immune cells in the Cox regression model. **(B)** correlation between immune cells and genes.

lncRNAs LINC00491, DSCR4, AC061975.6, LINC00221, TDRG1 and HOTTIP served as hub nodes in the ceRNA network, which targeted other miRNAs and mRNAs. The ceRNA modulatory network of CBX2, PBK and AP002478.1 may impact HCC progression, according to KM survival and correlation study. Consistent with our study, one recent study suggested that LINC00491 promotes tumor growth and lung metastasis in mouse xenografts. LINC00491 is highly expressed in HCC patients and is associated with poor prognosis. More deeply, LINC00491 promotes HCC progression by sponging miR-324-5p/ROCK1 and may be a potential therapeutic target for HCC (42). In addition, overexpression of lncRNA TDRG1 promotes the viability, invasion and migration of endometrial cancer cells, inhibits cell apoptosis, and upregulates the expression of VEGF-A, PI3K, Bcl-2, MMP2 and surviving (43). Similarly, TDRG1 overexpression also promoted GC growth and metastasis *in vitro* and *in vivo*, which was regulated by the miR-873-5p/HDGF pathway (44).

LncRNA HOTTIP has been reported to regulate HCC development and progression (45). In our study, we found that HOTTIP can act as a node of ceRNA and is closely related to the development of HCC. Meanwhile, a study also demonstrated that HOTTIP acts as a molecular sponge for miR-148a-3p to increase WNT1 expression, thereby modulating the CSC-like properties of breast cancer, suggesting that HOTTIP is a new target for breast cancer treatment (46). LncRNA HOTTIP expression was correlated with tumor recurrence in HCC patients following liver transplantation (47). Similarly, lncRNA

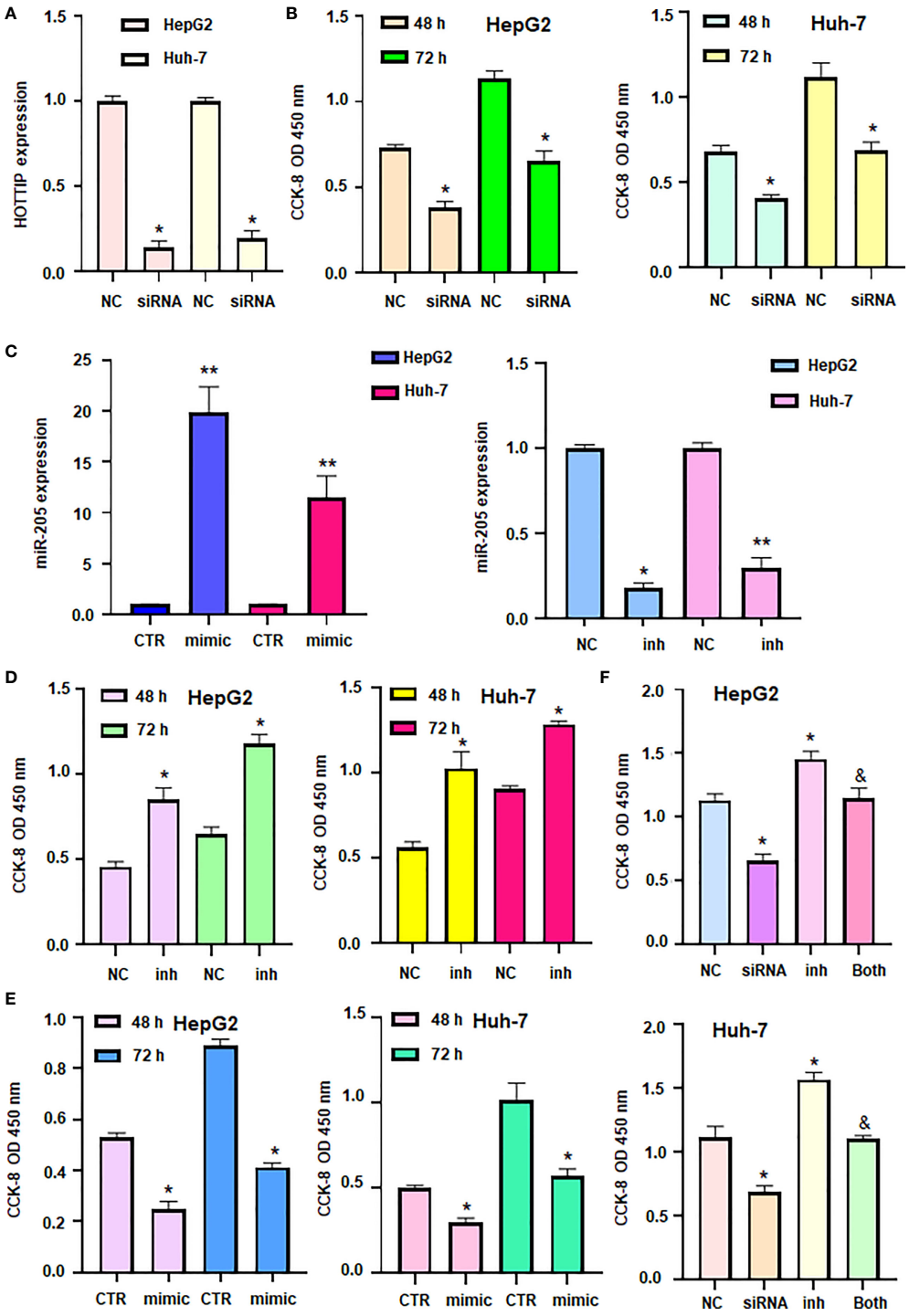

FIGURE 8
LncRNA HOTTIP promoted cell viability via miR-205 in HCC. **(A)** RT-PCR was done to measure the expression of HOTTIP in HCC cells after HOTTIP siRNA transfection. **(B)** CCK8 assay was done to measure cell viability in HCC cells after HOTTIP siRNA transfection. **(C)**: RT-PCR was done to measure the expression of miR-205 in HCC cells after miR-205 inhibitor or mimics transfection. **(D, E)** CCK8 assay was done to measure cell viability in HCC cells after miR-205 inhibitor or mimics transfection. **(F)** CCK8 assay was done to measure cell viability in HCC cells after HOTTIP siRNA transfection or miR-205 inhibitor or combination treatments. NC, negative control; inh, inhibitor; CTR, control. *P < 0.05; **P < 0.01 compared with NC or CTR; &P<0.05 compared with siRNA alone or inhibitor alone.

HOTTIP/HOXA13 expression was linked to tumor progression and therapeutic outcome in HCC patients (19). Additionally, lncRNA HOTTIP was elevated in HCC and was regulated by miR-125b (48). Ge et al. found that miR-192 and miR-204 reduced the expression of lncRNA HOTTIP and impaired GLS1-involved glutaminolysis in HCC (49). Tang et al. reported that solamargine inhibited the expression of lncRNA HOTTIP and TUG1, increased miR-4726-5p expression and inhibited MUC1 expression in HCC cells, leading to inhibition of cell growth and promotion of sorafenib efficacy (50). Our *in vitro* data further identified that HOTTIP promoted viability of HCC cells *via* regulation of miR-205. Dong et al. discovered that lncRNA HOTTIP promoted cisplatin resistance *via* targeting miR-205 and modulating ZEB2 in ovarian cancer cells (51). It is pivotal to determine whether lncRNA HOTTIP enhanced viability of HCC cells *via* regulation of miR-205/ZEB2 axis.

In our study, we found that dendritic activated cells and eosinophil are closely related to liver cancer. Dendritic cells are the most powerful professional antigen-presenting cells, widely distributed in peripheral lymphoid tissues, and play an important role in the immune regulation of the body. Liver DCs play an important role in the regulation of hepatic immune responses, and their precursor cells can induce antigen-specific immune tolerance in T cells that have not been stimulated by specific antigens, while for T memory cells, they are strong immune stimulators (52–54). *In vitro* studies have shown that once hepatic DC precursor cells are induced by antigen, they can also stimulate the proliferation of non-antigen-sensitized allogeneic T cells (55). There are some limitations in our study. First of all, the main disadvantage of this study is the lack of *in vivo* validation. Secondly, this study only included the TCGA database, and more databases should be included for research in the future.

Conclusion

The TCGA database's analysis of the lncRNA, miRNA, and mRNA expression profiles of HCC resulted in the creation of a ceRNA network. It was discovered that lncRNAs may play a role in carcinogenesis and may help predict the overall survival of patients with HCC. To ascertain their viability as therapeutic or diagnostic biomarkers, it is important to investigate the probable processes of these lncRNAs in HCC. However, further research on the clinical practice of this nomogram combined model and the exact molecular mechanism of the two lncRNAs identified in this study are required.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

WC, FC, MG, ZJ and LS performed the experiments, analyzed the data. WC and JW wrote the manuscript. Z-WW edited the manuscript. JW supervised this study. All authors contributed to the article and approved the submitted version.

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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