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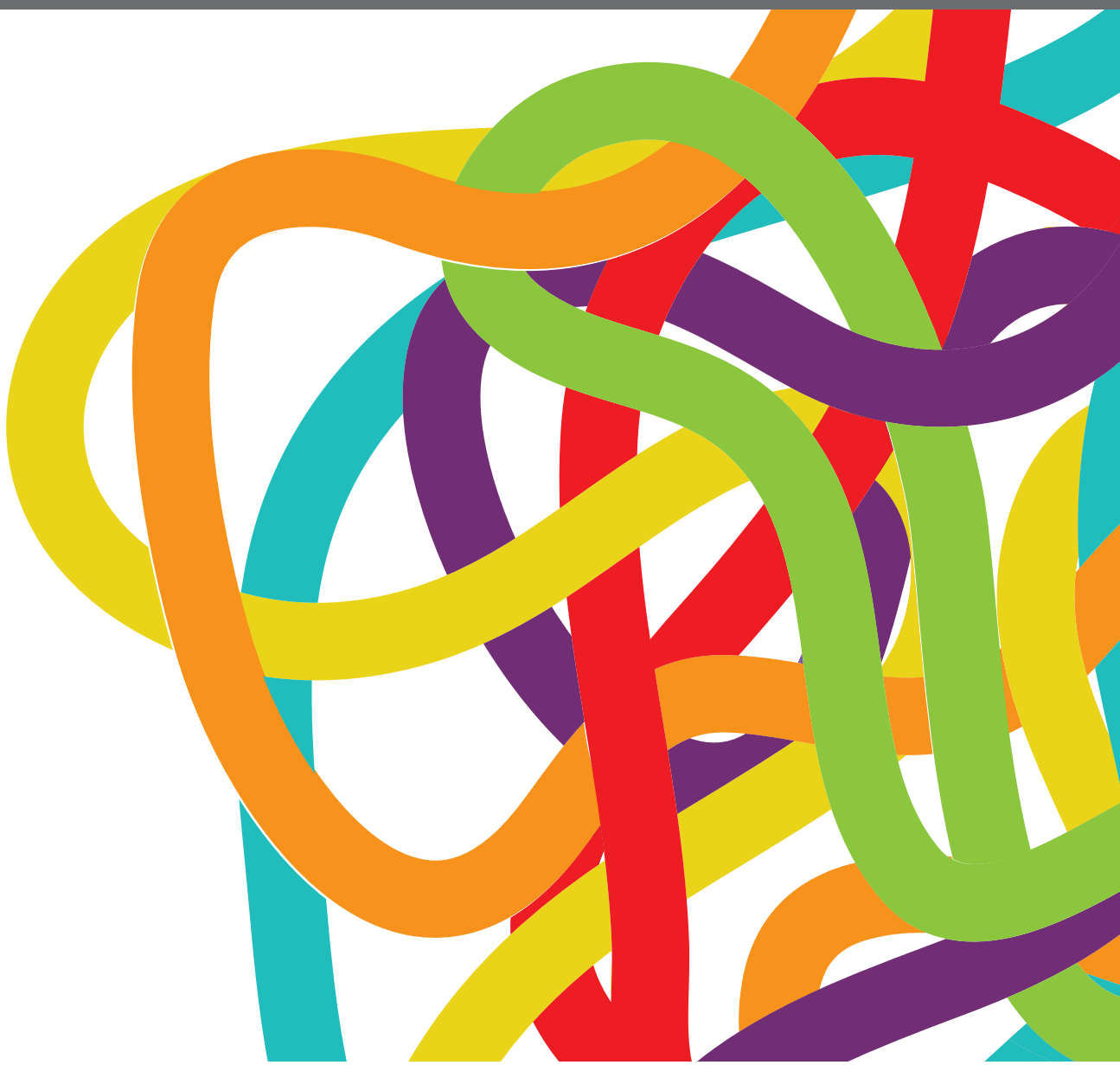

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RECENT ADVANCES IN THE UNDERSTANDING OF HEPATOCELLULAR CARCINOGENESIS, 2nd Edition

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Editorial: Recent advances in the understanding of hepatocellular carcinogenesis

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Editorial on the Research Topic
Recent advances in the understanding of hepatocellular carcinogenesis

Hepatocellular Carcinoma (HCC) is one of the deadliest cancers worldwide and a major health problem across the globe Suresh et al. (1). A better understanding of its multifactorial underpinnings and disease pathogenesis will aid in the design of novel and targeted therapeutic strategies for HCC. This special collection of original and review articles on Recent Advances in the Understanding of Hepatocellular Carcinogenesis provides new insights on the complexity of the disease.

The crucial role of miRNAs and associated RISC complex in the development and progression of HCC is highlighted (2–4). Several miRNAs (miR-631, miR-532-3p, miR-125b) showed tumor suppressor activities in HCC *via* targeting of various pathways, including receptor-type protein tyrosine phosphatase epsilon (PTPRE), WEE1 G2 checkpoint kinase, TGF-β1 signalling associated SMAD2 protein and MMP-2, MMP-9, and MMP-14 (Chen et al., Ma et al., Kim et al.). Previous work also demonstrated that TGF-β1 signalling and MMP9 were involved in HCC development (5, 6). A network meta-analysis showed that single nucleotide polymorphisms (SNPs) of miR-196a2 rs11614913 are significantly associated with the initiation and development of HCC (Zhang et al.). SNPs and epithelial mesenchymal transition (EMT)-related genes are associated with Hepatitis B virus (HBV)-related HCC (Liu et al.) (5). The tryptophan 2,3-dioxygenase (TDO2) enzyme promotes EMT of HCC through the Kyn-AhR pathway, with Kyn being the main product of Trp metabolism (Li et al.). Comprehensive analysis by Zhu et al. proposed a novel prognostic signature involving four differentially co-expressed hub genes CDCA8, KIF20A, KIF2C and CEP55 that associate with HCC (Zhu et al.). Bioinformatic analysis using the TCGA database identified methylation status of PDK4 and CTF1 in survival prediction and as treatment biomarkers for HCC (Liang et al.).

Circulating tumor cells and extracellular vesicles including exosomes are important in HCC metastasis (Luo et al.) (7–8). Ubiquitin-specific protease 1 (USP1) maintains survival of the circulating liver tumor cells (HCC) by deubiquitinating and stabilization of transducin β-like 1 X-linked receptor 1 (TBLR1) which plays a pivotal role in Wnt signalling (Li et al.). Circulating tumor-associated white blood cell clusters in peripheral blood signify poor disease prognosis in these patients (Luo et al.). Hepatic infiltration and metastasis of small cell neuroendocrine carcinoma cells led to a rare case of acute liver failure (Yan et al.). Upon partial hepatectomy for HCC, the liver induces a TNF-dependent Kupffer cell death pathway that favors cancer cell proliferation (Hastir et al.). Complement molecules regulate cancer associated stem cells (CSCs) and serve as a molecular and functional link between the innate and adaptive immune system, activating immune cells which are critical in driving hepatocarcinogenesis (Malik et al.). Delineation of these molecules and molecular pathways show the complexity of HCC and provide therapeutic opportunities for tumor specific targeted intervention and management of patients with HCC.

Chemotherapy is essential in current treatment paradigms for HCC. Metronomic celecoxib reduced tumor burden in HBVtg mice with implanted spontaneous hepatocarcinogenesis (Ye et al.). Hepatic artery infusion chemotherapy (HAIC) along with programmed cell death protein 1(PD-1) inhibitors plus lenvatinib improved treatment response and survival in patients with advanced HCC compared to PD-1 inhibitors plus lenvatinib. (Mei et al.). Along with small molecular targeted therapy, hepatic resection, trans-arterial chemoembolization (TACE), radiotherapy (RT) and various combinatorial therapies may be safe and effective in patients with HCC and portal vein tumor thrombosis. (Luo et al.). Attention should be paid to the possibility of acute kidney injury (AKI) in HCC patients with type 2 diabetes, as AKI during TACE treatment significantly increases patient mortality (Mou et al.). Surveillance after HCC treatment is essential in early detection of disease recurrence and can advise subsequent treatment strategies. Frequent and timely surveillance at intervals not exceeding 90 days appears effective in reducing the incidence of extra-Milan criteria relapse for HCC patients with stage B after attaining complete remission (Wu et al.).

Both nonalcoholic fatty liver disease (NAFLD) and alcohol associated fatty liver disease (AFLD) and related HCC have become major public health issues across the globe. Lifestyle modification through healthy dietary habits and routine physical activity, exercise and weight loss in NAFLD and avoiding alcohol consumption in AFLD serve as major preventive strategies (Suresh et al.). Due to the metabolic and genetic complexities underlying NAFLD and AFLD, precision and personalized treatment strategies could aid in the treatment of HCC associated with these conditions. Dietary natural compounds such as the phytochemical Withaferin A may be effective in HCC treatment (Suresh et al.) (9). Withaferin A activates LXR-α and negatively regulates NF-κB transcription factor, inhibiting several principal hallmarks of HCC cells and showing promise in the treatment of highly aggressive HCC (Shiragannavar et al.) (10).

Early-stage detection and surgical resection can prevent the development of advanced HCC. Discovering novel molecular and cellular targets for HCC therapy is essential to understanding disease progression and for developing new preventive strategies. In addition, creating global networks and collaborative registries with centralized pathology and radiology data can help to provide insights for treatment of HCC as well as combined Hepatocellular-Cholangiocarcinoma (Azizi et al.). Finally, consensus-based recommendations on the use of minimally invasive and multidisciplinary treatments will help in the detection of early-and intermediate-stages HCC amenable to curative therapy (Chen et al.).

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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USP1 Maintains the Survival of Liver Circulating Tumor Cells by Deubiquitinating and Stabilizing TBLR1

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The prognosis of hepatocellular carcinoma (HCC) is closely associated with the occurrence of distant metastases, which is likely due to circulating tumor cells (CTCs). However, the low number of CTCs is the main obstacle limiting research of the mechanism of CTC metastasis. Here, We evaluated the role of ubiquitin-specific protease 1 (USP1) in promoting CTC survival during blood-borne metastases. We observed that USP1 was frequently upregulated in CTCs and correlated with metastasis and a reduced overall survival rate of patients. Additionally, genetic knockout of *USP1* the survival rate of CTCs. Further analyses showed that USP1 mediates oncogenic activity by deubiquitinating and stabilizing transducin β-like 1 X-linked receptor 1 (TBLR1), which plays essential roles in regulating Wnt signaling. These results demonstrated that USP1 may act as an essential factor in promoting the survival of CTCs and suggest that inhibition of USP1 is a potential strategy for HCC treatment.

Keywords: circulating tumor cells, deubiquitination, hepatocellular carcinoma, USP1, Wnt pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most prevalent malignancies worldwide accounting for >90% of human liver cancer cases. The morbidity and mortality rates of HCC has increased in recent decades (1). HCC has a high risk of metastasis, especially intrahepatic metastasis, and recurrence, which are the primary causes of death (2). Dissemination of circulating tumor cells (CTCs) is highly correlated with cancer metastasis and recurrence (3). Enumeration of CTCs is currently performed to monitor the anticancer treatment response and guide the prognosis of patients (4). Further investigation of the CTC survival mechanism may improve our understanding of metastasis and lead to new cancer therapies targeting CTCs. However, the number of CTCs is very low (1–10 single CTCs per 7.5 mL blood) and the lack of CTCs to analyze is the main obstacle to studies of the survival mechanisms of CTCs in blood-borne metastasis (5, 6).

Deubiquitination, a highly regulated process, is essential for maintaining cellular homeostasis via the regulation of numerous cellular functions, including protein levels, apoptosis,

DNA repair, and cell motility (7–11). Ubiquitin-specific protease 1 (USP1), a sub-type of deubiquitinases, reportedly regulates DNA-repair processes by deubiquitinating proliferating cell nuclear antigen and Fanconi anemia group D2 and preserves cancer stem cells in osteosarcoma by stabilizing inhibitor of DNA binding (ID)1 and ID2 (12, 13). However, few studies have examined USP1-related function in HCC and/or the mechanism of CTC survival. We demonstrated that USP1 promotes the survival of liver CTCs in the bloodstream by regulating the ubiquitination of transducin β-like 1 X-linked receptor 1 (TBLR1), a critical regulator of the Wnt pathway, suggesting USP1 as a potential target for anticancer therapy.

MATERIALS AND METHODS

Patients and Specimens

For immunohistochemistry (IHC) assay, from 2002 to 2008, 217 tissue specimens from patients with HCC were collected at the Zhongshan Hospital (Shanghai, China). For CTC analytical assay, blood (7.5 mL) was obtained from the peripheral veins of patients from 2017 to 2018. This study was approved by the Research Ethics Committee of the Zhongshan Hospital (B2017-159R), and the procedures were in accordance with the ethical guidelines outlined in the 1975 Declaration of Helsinki.

Cell Culture and Construction of USP1-Knockout Cells

The PLC/PRF/5 human HCC cell line and 293T cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) and the MHCC-97H cell line was obtained from the Liver Cancer Institute of the Zhongshan Hospital, Fudan University (Shanghai, China). MHCC-97H-GFP and PLC/PRF/5-GFP cell lines were constructed by lentiviral transfection, and MHCC-97H and PLC/PRF/5 *USP1*-knockout (KO) cell lines were constructed using CRISPR technology as follows: MHCC-97H and PLC/PRF/5, wild-type cell lines, were transfected with a *USP1*-targeting KO plasmid. Following digestion, single cells were seeded into a well, and after reaching confluence, sequencing was performed to confirm the construction of the KO cell lines (14).

RNA Extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Ambion, Austin, TX, United States). cDNA of the target gene was reverse-transcribed from total RNA (1 μg) using the Transcriptor reverse transcriptase kit (RR036A; TaKaRa, Shiga, Japan). Single CTCs from each patient were transferred to individual PCR tubes containing lysis buffer by micromanipulation. Single CTCs from each patient were transferred individually to single PCR tubes containing lysate buffer. Single cell RNA was extracted from each CTC and reverse-transcribed by Single Cell-to-CT qRT-PCR kit (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocols. SYBR Green (Bio-Rad Laboratories, Hercules, CA, United States) and ABI Prism 7500 real-time PCR (Bio-Rad) were used for single-step qRT-PCR. Gene expression was calculated relative to that of β-*actin* expression using the $2^{-\Delta\Delta Ct}$ method.

Tissue Microarrays, Immunohistochemistry, and Evaluation

An immunohistochemistry assay was performed. Briefly, serial-sectioning of tissue samples was performed after fixation in paraffin using 4% paraformaldehyde, microwave antigen retrieval was performed and the samples were incubated overnight with primary antibody followed by 1 h incubation with secondary antibodies. All tissues were counterstained with hematoxylin. The antibodies used in IHC assay included anti-USP1 (1:300; Proteintech, Rosemont, IL, United States), anti-TBLR1 (1:300; Proteintech).

Circulating Tumor Cells Capture and Fluorescence-Activated Cell-Sorting

Circulating tumor cells were enriched from 7.5 ml blood samples by Ficoll solution (Sigma-Aldrich, St. Louis, MO, United States), incubated with fluorescent antibodies include anti-cytokeratin 19 (1:300; Cell Signaling Technology, Danvers, MA, United States), anti-EpCAM (1:300; Cell Signaling Technology), anti-CD45 (1:300; Cell Signaling Technology), and captured by flow cytometry (BD Biosciences, San Diego, CA, United States). The criteria for identifying the captured cells as the CTCs were: EpCAM positive; pan-cytokeratin-19 positive; CD45 negative; the presence of a nucleus, stained using 4',6-diamidino-2-phenylindole (DAPI) (15). GFP cells sorted from mouse blood after injection or cultured GFP cells harvested from plates, were evaluated by Annexin V-Allophycocyanin (APC)/7-Aminoactinomycin D (7-AAD) kit (BD Biosciences Pharmingen, San Diego, CA, United States) following the manufacturer's instructions and FlowJo software (TreeStar, Ashland, OR, United States) was used for data analysis.

Western Blot Analysis

Cells were lysed using RIPA buffer (Beyotime, Nantong, China) containing 1 mM phenylmethylsulfonyl fluoride (Beyotime). Protein was loaded and separated by 8% or 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany), and blocked with bovine serum albumin (5%; Sangon Biotech, Shanghai, China). The primary antibodies included anti-USP1 (1:1000; Cell Signaling Technology), anti-TBLR1 (1:1000; Abcam, Cambridge, United Kingdom), anti-HA (1:2000; Proteintech, Wuhan, China), anti-β-actin (1:5000; Cell Signaling Technology). Secondary antibodies included donkey anti-rabbit (1:2500; Cell Signaling Technology), rabbit anti-mouse (1:2500; Cell Signaling Technology), and anti-light chain (1:3000; Abcam).

Ubiquitination and CHX-Protein Stability Assays

For the ubiquitination assay, HA-ubiquitin plasmids were transfected into USP1-NC and USP1-KO cells. Following treated with the proteasome inhibitor MG-132 (5 μM) for 6 h, cells were harvested and lysed for immunoprecipitation of TBLR1 and

immunoblotting of HA. Western blot was performed as described above. For the CHX-protein stability assay, in order to inhibit protein synthesis, cells in each group were treated with CHX (100 μg/mL) for 0, 3, 6, and 9 h. MG-132 (5 μM) was added along with CHX (16). Cell lysates were collected and western blot was performed as described above.

Establishment of Mouse Tumor Xenograft Model

Twenty male BALB/c nude mice were divided into four groups randomly (n = 5/group). For the subcutaneous assay, 1×10^6 tumor cells were injected subcutaneously into each mouse. For the liver xenograft assay, 1×10^6 tumor cells were transplanted into the hepatic lobes of mice. All animal experiments were approved by the Research Ethics Committee of the Zhongshan Hospital (B2017-159R) and mice were sacrificed at 5 weeks post-injection.

Statistical Analysis

All experimental results were obtained from assays performed in triplicate and are shown as the mean ± standard deviation. Relationships between *USP1* expression and clinicopathological factors were analyzed using the Pearson χ^2 test. Differences between treated and control groups were determined using the Student's t-test and one-way analysis of variance. $P < 0.05$ was considered a statistically significant result.

RESULTS

USP1 Is Upregulated in HCC and Correlated With Metastasis

Ubiquitin-specific protease 1 is ubiquitously expressed in human tissues. We first assessed *USP1* mRNA levels in tumor and para-tumor tissues, which revealed higher *USP1* expression in tumor tissues than in para-tumor tissues (**Figure 1A**). IHC and western blot analysis also confirmed higher levels of USP1 in HCC samples relative to those in adjacent non-tumor tissues (**Figures 1B,C**). Next, a tissue microarray with 217 samples of HCC tissues was IHC stained to test the correlations between USP1 levels and overall survival (OS) of patients. According to the staining intensity, we observed elevated levels of USP1 (92/217) in patients with a short OS and low levels (125/217) in patients with a long OS (P = 0.0248) (**Figure 1D**). We then evaluated the relationship between the USP1 levels and the clinical characteristics of patients with HCC (**Supplementary Table S1**). Interestingly, the only clinical characteristics positively correlated with USP1 levels were serum α-fetoprotein level (P = 0.013) and tumor number (P = 0.028). USP1 level was not correlated with tumor size (P = 0.696). These results indicates that the short OS of HCC patients with high levels of USP1 is mainly caused by metastasis rather than proliferation.

We knocked down the expression of USP1 in MHCC-97H cells (**Supplementary Figure S1A**) and, as expected, found no significant difference in proliferation between USP1-knockdown cells and control cells (**Supplementary Figure S1B**). Further, we created USP1 knockout (USP1-KO) cells in MHCC-97H and PLC/PRF/5 cell lines by sgRNA-Cas9 system (**Supplementary Figure S1C**). The data showed USP1 level did not correlate with proliferation (**Figures 1E,F**). These results indicated that USP1 is involved in metastasis and may be the reason for poor patient prognoses. We found no significant difference in migration or invasion between USP1-knockdown cells and control cells (**Supplementary Figures S1D,E**). Thus, we hypothesized that USP1 contributed to cancer metastasis mainly by promoting cancer cell survival in the blood rather than promoting cancer cell invasion.

USP1 Maintains CTC Survival in Blood-Borne Metastasis

To identify the role of USP1 in cancer cell survival in the blood, we obtained single CTCs from peripheral vein blood and extracted RNA using a single-cell-to-CT quantitative reverse transcription-PCR (qRT-PCR) kit. We observed that *USP1* expression in CTCs was higher than in primary tumor cells (**Figure 2A**). Additionally, we injected PLC/PRF/5-GFP or MHCC-97H-GFP cells into the peripheral tail vein of nude mice and sorted GFP-positive cells by flow cytometry after 0, 12, 24, and 36 h, with results showing that *USP1* expression increased over time (**Figure 2B**). These results indicate USP1 is involved in the survival of CTCs.

To test the above hypothesis, cancer cells (1×10^6) were injected into mice via the peripheral tail vein, which simulated CTCs in the blood. FACS cell counting results showed that the number of CTCs in the *USP1*-KO group was less than in the control group (**Figure 2C**). Apoptotic assays showed that *USP1*-KO cells have a lower survival rate compared with control cells at 24 h post-injection (**Figure 2D**). Similar results were observed in USP1-knockdown cells and control cells (**Supplementary Figure S2A**). Individually *USP1* knockout did not affect cell apoptosis in cultured medium (**Supplementary Figure S2B**). These results indicated that USP1 depletion attenuated the survival ability of CTCs.

A Xenograft-formation assay was performed to determine the effects of USP1 *in vivo*. After 5 weeks of liver xenografting, the *USP1*-KO group showed a lower tumor number than the *USP1*-NC group ($P < 0.05$) (**Figure 2E**). Furthermore, we observed pulmonary tumor formation in the control group but not in the *USP1*-KO group according to computed tomography (**Figure 2F**) and hematoxylin-eosin staining (**Figures 2G,H**). Additionally, apoptotic cell usually exhibit the pattern with cell shrinkage or cell membrane rupture which can be detected by cell surface marker (17). We enriched the CTCs in mouse blood, labeled the CTCs with fluorescent EpCAM (green), detected CTCs with their apoptotic pattern[16] by microscope (**Figure 2I**, left) and found that knockout of USP1 increased apoptotic-like CTC patterns compared with the control group (USP1-NC 55% VS USP1-KO 73%) (**Figure 2I**, right). These observations demonstrate that USP1 modulates HCC CTC blood-borne metastasis *in vivo*. Moreover, the cell apoptotic rate (**Figure 2D**) and metastases number

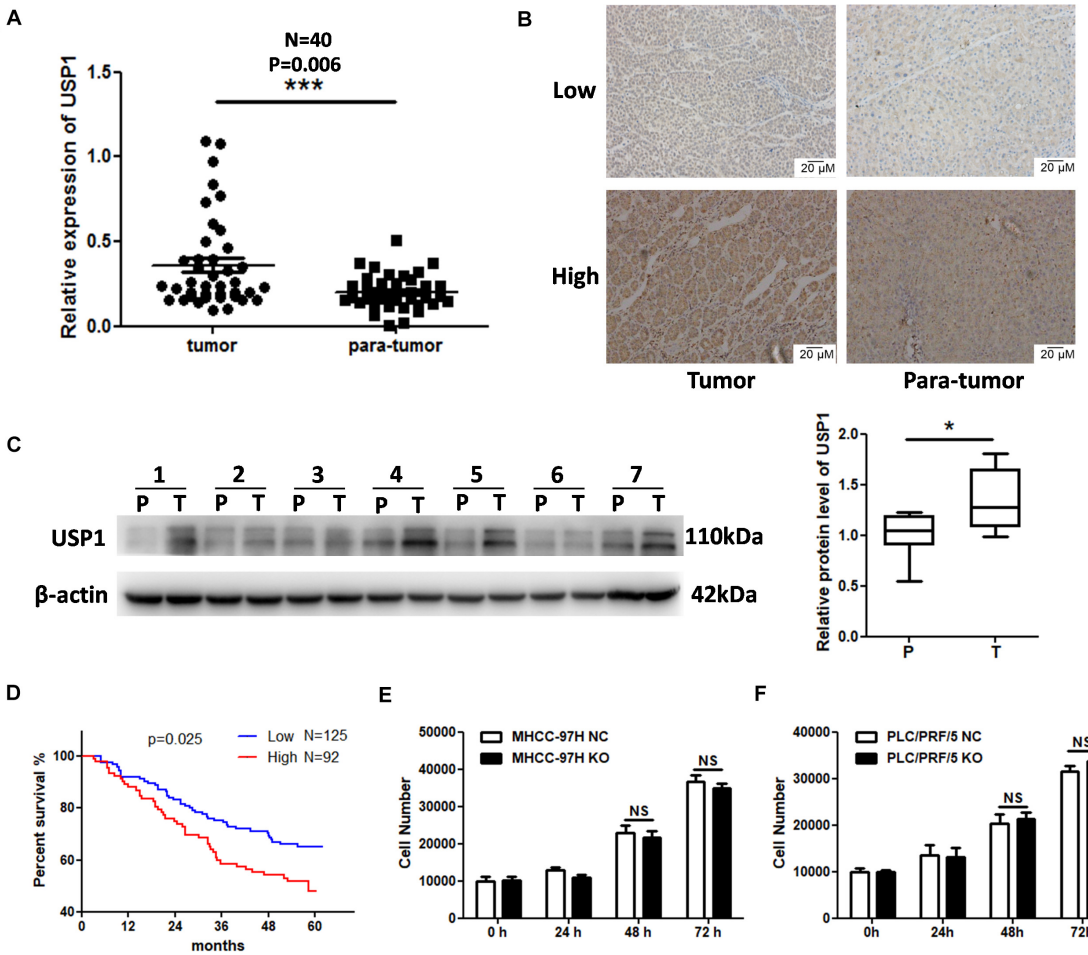

FIGURE 1 | Elevated expression of *USP1* in HCC. **(A)** USP1 mRNA levels in 40-paired specimens of HCC tissues and matched para-tumor liver tissues. **(B)** IHC staining of USP1 in 56 pairs of HCC and matching para-tumor tissues. Representative images are shown. **(C)** USP1 levels in seven paired samples. T, HCC tissues; P, para-tumor tissues. β-actin was used as a control. **(D)** Kaplan–Meier analysis with log-rank testing of survival was performed in 217 patients with HCC exhibiting different *USP1* expression levels. Error bars represent the standard deviation. *P = 0.025. **(E,F)** Cell numbers were count by cell counting instrument after 0, 24, 48, and 96 h of seeding.

(**Figure 2H**) confirmed that USP1 assists CTC survival in the bloodstream.

USP1 KO Impairs Wnt Targets in HCC

High-throughput sequencing (The Beijing Genomics Institute, Beijing, China) was used to identify USP1-regulated pathways in HCC and determine how USP1 affects cancer cell survival and metastasis. As expected, compared with those in the *USP1*-KO group, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed the enrichment of Wnt, Notch, and Hedgehog pathways, which play essential roles in cancer stem cell regulation (18) and may support the survival of CTCs (**Figure 3A**). In gene set enrichment analysis, Wnt signaling and Notch signaling showed significant associations with USP1 knockout (**Figure 3B**).

The inhibition of most Wnt targets by *USP1* KO was confirmed by real-time PCR (**Supplementary Figure S3**). These results suggest USP1 as an essential factor involved in Wnt-signaling. We performed a label-free ubiquitin quantitative assay using MHCC-97H cells to determine the USP1 target(s) in the Wnt signaling pathway. Ubiquitin is the substrate of deubiquitinases; therefore, immunoprecipitation (IP) with an antibody against ubiquitin chains can enrich ubiquitin-modified proteins. Moreover, subsequent high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Shanghai Applied Protein Technology Company, Shanghai, China) and quantitative proteomics analysis were performed to investigate how USP1 influences the ubiquitination levels of the target peptide-binding region in HCC cell lines (**Figure 3C**). Cells were divided into two groups: the MHCC-97H-*USP1*-NC cell line with endogenous USP1-deubiquitinating activity and MHCC-97H-*USP1*-KO cell line with no USP1-deubiquitinating activity. Among the peptide-matched proteins, 29 proteins were identified as part of the ubiquitin interactome only in MHCC-97H-*USP1*-NC cells (**Figure 3D**) and not in USP1-KO cells, whereas 275 highly ubiquitin modified proteins were specifically detected in *USP1*-KO cells, indicating that

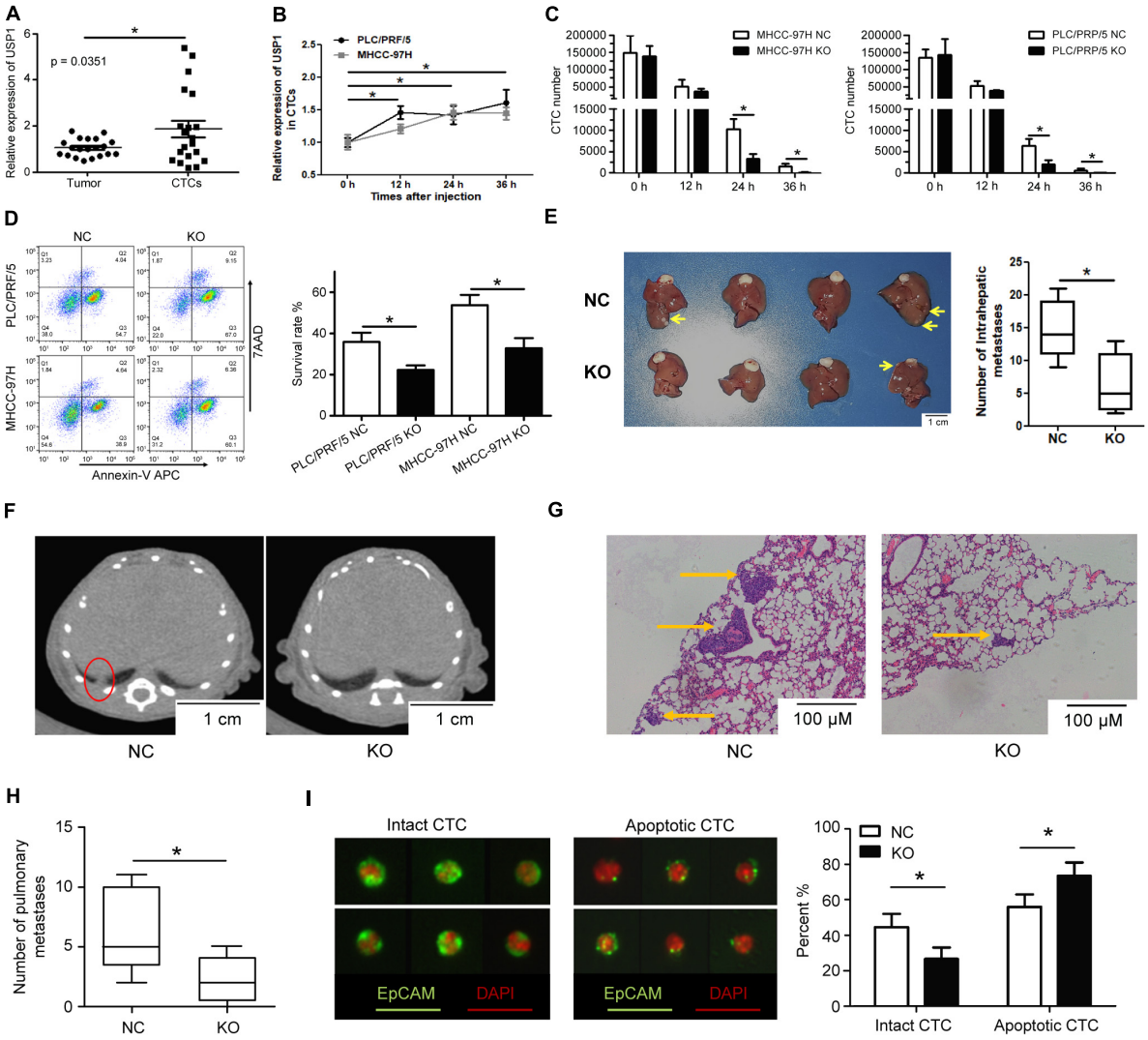

FIGURE 2 | USP1 promotes circulating tumor cell survival in blood. **(A)** *USP1* mRNA expression level in primary tumors and CTCs. **(B)** *USP1* expression at 0, 12, 24, and 36 h after tail injection. **(C)** After 0, 12, 24, and 36 h of tail injection, GFP-labeled cells were sorted and counted by FACS. **(D)** 24 h after injection, GFP-labeled cells were sorted and labeled with Annexin V-APC and 7AAD. **(E)** Intrahepatic metastatic tumors in hepatic lobes 5 weeks after liver xenografting. **(F,G)** Lung metastasis detected by computed tomography and IHC. The arrows were used to show lung metastasis. **(H)** Bar graph of pulmonary metastases tumor numbers. **(I)** CTCs detected in blood and labeled by EpCAM and pan-cytokeratin (green) and DAPI (red). *$P < 0.05$.

USP1 can modulate the cell state by deubiquitinating these proteins. KEGG analysis showed that among these 275 proteins, five participate in Wnt signaling, including TBLR1, Ras-related C3 botulinum toxin substrate 1, SMAD4, BMP4 and protein phosphatase 2B regulatory subunit 1 (**Figure 3E**).

USP1 Maintains the Survival of CTCs by Deubiquitinating and Stabilizing TBLR1

We then investigated the biochemical interaction between USP1 and these five proteins. We observed that USP1 interacted with TBLR1 in a Co-IP assay (**Figure 4A**). Reciprocal Co-IP assays using tag antibodies revealed similar results, showing that USP1 can co-interact with TBLR1 (**Supplementary Figure S4A**). In contrast, the other four proteins did not show a co-interaction with USP1 (**Supplementary Figure S4**B). *USP1* KO decreased TBLR1 protein levels in PLC/PRF/5 and MHCC-97H cells (**Supplementary Figure S4C**); however, *TBLR1* mRNA levels were unaffected by *USP1* KO (**Supplementary Figure S4D**). Additionally, in patient samples, we found that USP1 was positively correlated with the TBLR1 level at the protein but not the mRNA level (**Supplementary Figure S4E**). Moreover, we observed that PLC/PRF/5 and MHCC-97H cells degraded TBLR1 in a proteasome-dependent manner, as TBLR1 accumulated after treatment with the proteasome inhibitor MG-132 (**Supplementary Figure S4F**). These findings demonstrate that USP1 regulates TBLR1 at the protein level. We performed an *in vitro* ubiquitination assay to determine whether USP1

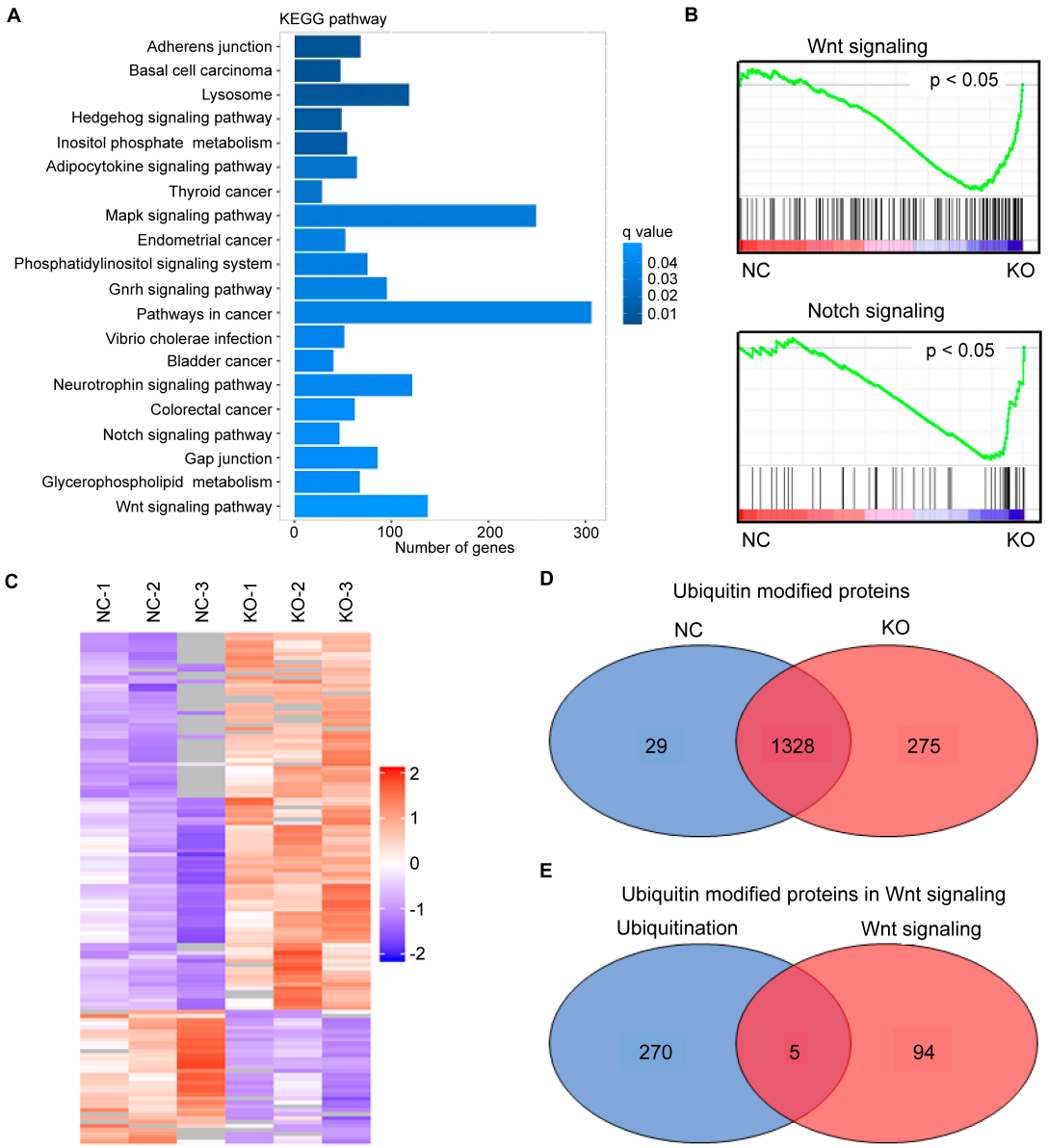

FIGURE 3 | *USP1* KO impairs Wnt targets. **(A)** KEGG pathway enrichment of differentially expressed genes (DEGs) in *USP1*-KO cells versus control cells (in MHCC-97H cell line). **(B)** GSEA enrichment plots of Wnt signaling and Notch signaling. **(C)** Identification of USP1-deubiquitinating targets using a combination of ubiquitin-chain-specific IP and label-free LC-MS/MS analysis. **(D)** Venn diagram showing the number of ubiquitin-modified proteins in the two groups. **(E)** Venn diagram showing five overlapping proteins between the *USP1*-KO group and Wnt signaling.

stabilizes TBLR1 in a deubiquitination-dependent manner. Using an anti-TBLR1 antibody for co-IP, we showed that in the absence of USP1, the HA-ubiquitin ligation level was enhanced (**Figure 4B**). Furthermore, we conducted a cycloheximide (CHX) chase assay to investigate the effects of USP1 on TBLR1 stability. After 0, 3, 6, and 9 h of CHX treatment, TBLR1 levels were quantified by western blot analysis (**Figure 4C**). As expected, TBLR1 degradation occurred faster in *USP1*-KO cells than in control cells. These results demonstrated that USP1 deubiquitinates and stabilizes TBLR1.

The depletion of TBL1X-TBLR1 significantly inhibited the expression of Wnt target genes (19, 20). To show that USP1 regulates Wnt signaling by deubiquitinating TBLR1 in HCC, we overexpressed *TBLR1* (TBLR1-OE). Compared with control cells, TBLR1-OE cells had increased levels of c-Myc, Met, MMP7, and CD44 (**Figure 5A**), and overexpression rescued the repression effect of USP1 depletion. After overexpressing TBLR1, USP1-NC-TBLR1-OE cells, and USP1-NC-TBLR1-OE cells showed the similar HA-ubiquitin ligation level, which means overexpressing TBLR1 rescued the ubiquitination effect of USP1

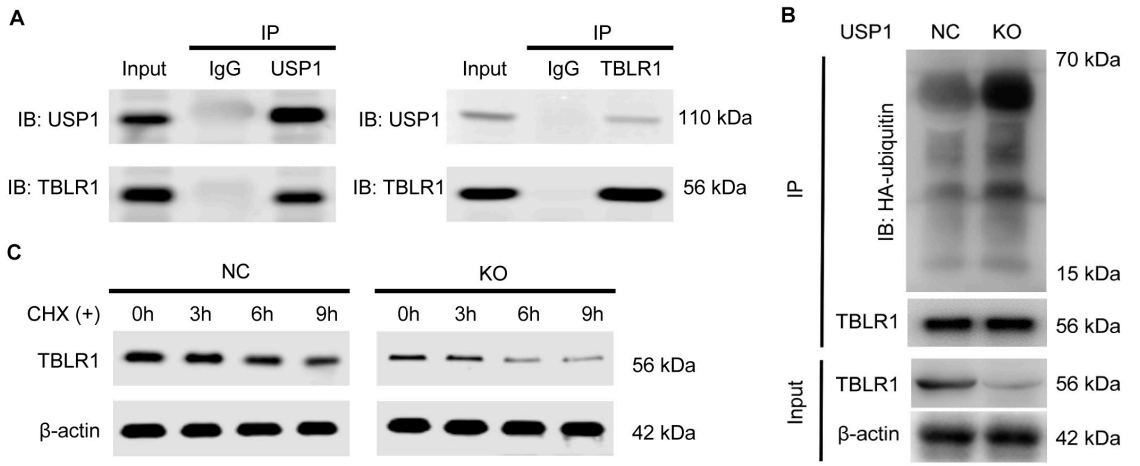

FIGURE 4 | USP1 interacts, deubiquitinates, and stabilizes TBLR1. **(A)** Co-IP assays of USP1 and TBLR1 in MHCC-97H cells. **(B)** Impact of USP1 on TBLR1 ubiquitination *in vivo*. Immunoblot using an HA-tag to detect poly-ubiquitination of TBLR1. **(C)** USP1 enhances TBLR1 stability; cells were treated with CHX (100 μg/mL) and collected at 0, 3, 6, and 9 h. TBLR1 levels were analyzed by western blotting.

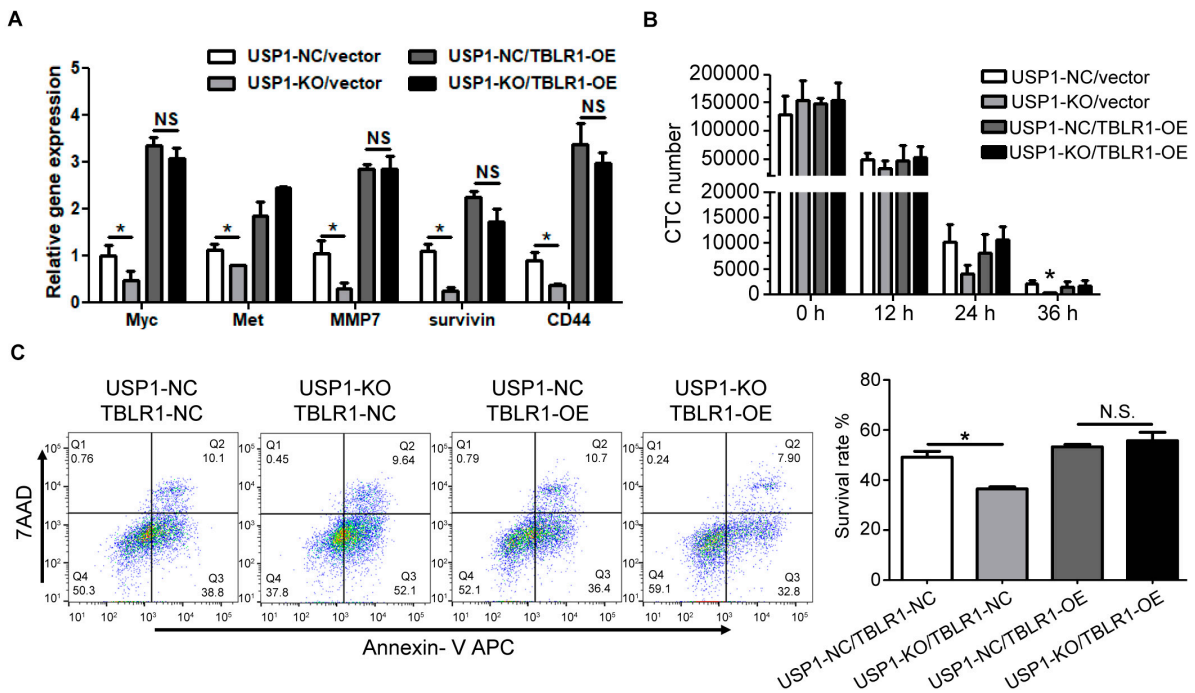

FIGURE 5 | Overexpressing TBLR1 rescues the survival of CTC by USP1 depletion. **(A)** TBLR1 overexpression rescued the expression of Wnt targets caused by USP1-knockout. **(B,C)** Constructed from the MHCC-97H cell line, USP1-NC-GFP-vector cells, USP1-KO-GFP-vector cells, USP1-NC-GFP-TBLR1-OE cells, and USP1-KO-GFP-TBLR1-OE cells were injected into the peripheral tail vein. After 24 h, the CTC number **(B)** and cell-survival rate **(C)** was detected by FACS. The CTC number **(B)** and cell-survival rate **(C)** of USP1-NC-GFP-vector cells, USP1-KO-GFP-vector cells, USP1-NC-GFP-TBLR1-OE cells, and USP1-KO-GFP-TBLR1-OE cells after 24 h of tail injection. $^*P < 0.05$.

depletion (**Supplementary Figure S5**). Besides, overexpressing *TBLR1*, *USP1*-KO and control cells showed a similar cell count number (**Figure 5B**) and survival rate (**Figure 5C**) at 24 h after injection via the tail vein. These results demonstrate that USP1 maintains the survival of CTCs by stabilizing TBLR1 (**Figure 6**).

DISCUSSION

Recent studies of CTCs have mostly focused on the relationship between CTC counts and clinical patterns (21); however, studies of the mechanisms underlying CTC survival are limited. We investigated the CTC survival mechanism by

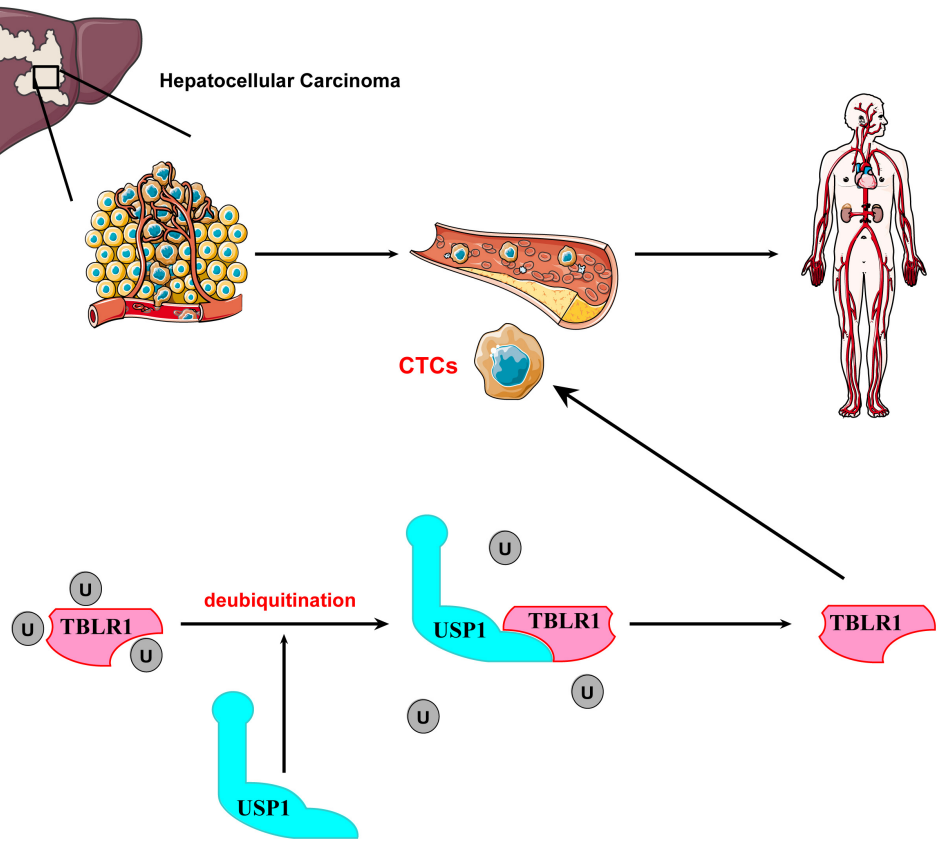

FIGURE 6 | The mechanism of USP1 maintaining the survival of CTCs by stabilizing TBLR1.

gene profiling, proteomics analysis, and analyzing changes in signaling pathways. The results suggested that USP1 promotes CTC survival, which may lead to metastasis and recurrence. We also prepared *USP1*-overexpressing PLC/PRF/5 cells and MHCC-97H cells; however, we found no significant changes in phenotypes, such as CTC apoptosis, proliferation, migration, colony formation, and spheroid formation, between *USP1*-NC and *USP1*-overexpressing cells. We hypothesized that USP1 is regulated by signal molecules when deubiquitination is required rather than diffused in the cytoplasm for random substrate deubiquitination.

Cancer metastasis is an inefficient process, with only a small proportion of tumor cells successfully surviving hematogenous spreading (22). CTCs should endure various forms of stress such as anoikis, reactive oxygen species, chemotherapy drugs, fluid shear stress, the immune system, and senescence during blood-borne metastasis. Adjusting to the specialized microenvironment, adult stem cells may regulate their state such as proliferation, quiescence, self-renewal, or differentiation (23, 24). Cancer stem cells arise from mutant stem cells, which may benefit from the transformation of suitable cell state to fit different microenvironments (24, 25). It has been reported that CTCs with stem cell characteristics are at higher risk for tumor recurrence and metastasis (26). In this study, RNA-seq results showed the enrichment of 3 stem cell-related signaling in the top 20 pathways, including Wnt signaling, Notch signaling, and Hedgehog signaling, indicating a critical role for USP1 in cancer cell stemness. As Notch signaling plays an essential role in cancer stem cells, we detected downregulation of *Notch1* and *Notch2* in *USP1*-KO cells by qRT-PCR; however, we did not detect any difference in ubiquitination of proteins involved in Notch signaling between *USP1*-KO and wild-type cells using label-free quantification LC-MS/MS. Therefore, the mechanism of action of USP1 involving Notch signaling requires further investigation. As an aspect of cancer stem cell and metastasis, a previous study, which reported that USP1 preserves osteosarcoma stem cells by deubiquitinating ID proteins (12) also observed a relationship between USP1 and circulating tumor stem cells. Thus, whether USP1 maintains the survival of CTCs by preserving CTCs in a stem cell-like state should be further investigated.

Ubiquitin-specific protease 1 could not directly affect the migration or invasion of cancer cells (**Supplementary Figures S1D,E**), whereas adherent junction pathways can still be enriched in RNA-seq. Moreover, we often observe evidence of anchorage dependence in our recent CTC research (15). We consider that an anchorage-dependent microenvironment plays a crucial role in CTC survival; however, the mechanism is unclear. Platelets are known to affect the CTC microenvironment (27). Platelets may be recruited and surrounded by CTCs to shield and provide an anchoring base for the CTCs, avoid anoikis, avoid damage caused

by fluid shear stress, and protect CTCs from immune cell attack (28, 29). Drugs that can neutralize the microenvironment of CTCs in the blood may be useful for attenuating metastases. We found that USP1-specific inhibitors, such as SJB3, could affect the survival of CTCs. Our future studies will focus on the mechanism of USP1 inhibitors in the blood-borne survival of CTCs.

Attack by the immune system is a major factor limiting the survival of CTCs. Some CTCs may enter a dormant state to evade immune surveillance, whereas others may upregulate their "do not eat me" signals to enable them to escape from the immune system (23, 30, 31). We co-cultured USP1-KO cells or USP1-NC cells with immune cells (CD8+ T cells or natural killer cells); however, our evidence is insufficient to demonstrate a relationship between USP1 and immune escape.

We also established a CTC blood-borne mouse model to investigate the relationship between visible CTC survival CTC genotype states; however, because of the limitations associated with CTC acquisition and culture, we were unable to investigate real-time changes in patient CTCs, which will prevent the development of personalized therapy. CTC survival is the primary cause of metastasis, suggesting that inhibition of USP1, a potential therapeutic target, can effectively induce the apoptosis of CTCs in the blood and reduce metastasis. Additionally, numerous chemicals and target-directed drugs are being used to induce apoptosis in cancer cells. Evaluation of the CTC survival rate and genotype after treatment with anticancer reagents may be a practical approach for determining disease prognosis, which may also accelerate the development of novel therapeutics.

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 human and animal study was approved by the Research Ethics Committee of the Zhongshan Hospital (B2017-159R). The patients/participants provided their written informed consent to participate in this study. The animal study was approved by the Research Ethics Committee of the Zhongshan Hospital (B2017-159R).

AUTHOR CONTRIBUTIONS

YL, YX, CG, and XY participated in the conception and design of the study. YL, CG, YS, KZ, PW, WG, and JF performed the experiments. YL, YX, CG, JC, CY, JF, and XY interpreted the data produced and edited the drafts of 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.2020.554809/full#supplementary-material

FIGURE S1 | Migration and invasion ability of USP1 knockdown cells. **(A)** USP1 knockdown cells were constructed in the MHCC-97H cell line. The *USP1* expression level was measured by qPCR. **(B)** 1×10^4 cells were seed in a 96 well plate. Cell numbers were count using a cell counting instrument after 0, 24, 48, and 96 h of seeding. **(C)** The *USP1* knockout efficiency in PLC/PRF/5 and MHCC-97H cell lines. **(D,E)** Wound healing assay and Transwell assay using *USP1*-NC cells and *USP1*-KO cells in the MHCC-97H cell line.

FIGURE S2 | USP1 deficiency inhibits CTC survive and tumor growth. **(A)** USP1-NC-GFP and USP1-KD-GFP cells were injected into the peripheral tail vein. After 24 h, GFP-cells were sorted by FACS and labeled by Annexin V-APC and 7AAD. **(B)** Apoptosis rate of USP1 knockout cells and control cells.

FIGURE S3 | mRNA levels of Wnt targets in NC cell lines and *USP1*-KO cell lines.

FIGURE S4 | USP1 interacts with TBLR1 and correlates with TBLR1 protein level. **(A)** Co-IP assays of USP1-HA and TBLR1-Flag in 293T cells. **(B)** Co-IP assays of USP1-HA and RAC1, SMAD4, BMP4 or PPP2R1 in 293T cells. **(C)** TBLR1 expression in USP1-NC and USP1-KO cell lines. **(D)** TBLR1 mRNA level in USP1-NC and USP1-KO cell lines. **(E)** Correlation of USP1 and TBLR1 in patient samples. **(F)** TBLR1 is degraded in a proteasome-dependent manner and inhibited by MG-132.

FIGURE S5 | Ub assay in overexpression system by using USP1, TBLR1, and Ub plasmid.

TABLE S1| Expression of USP1 and correlation with clinical characteristics of HCC patients (n = 217).

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MINI REVIEW
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Update on the Genetics of and Systemic Therapy Options for Combined Hepatocellular Cholangiocarcinoma

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Combined hepatocellular-cholangiocarcinoma (cHCC-ICC) is an uncommon and aggressive form of primary liver cancer. Currently, there are no international guidelines for optimal management. For localized tumors, radical resection represents the preferred treatment option, whereas for advanced tumors, systemic therapies recommended for intrahepatic cholangiocarcinoma (ICC) and hepatocellular carcinoma (HCC) are often selected. Emerging information from comparative cohort studies, genomic and transcriptomic data sets are starting to build a case for rationalized approaches to systemic treatment in the advanced setting specific to cHCC-ICC.

Keywords: Combined hepatocellular-cholangiocarcinoma, cHCC-ICC, cHCC-CCA, biphenotypic, primary liver cancer, genomics, systemic therapy

INTRODUCTION

Combined hepatocellular-cholangiocarcinoma (cHCC-ICC/ cHCC-CCA) or "biphenotypic" primary liver cancer is a form of primary liver carcinoma (PLC) with phenotypic characteristics of both hepatocytic and cholangiocytic differentiation (1, 2). Additional acceptable terminology for this form of PLC is mixed hepatocellular-cholangiocarcinoma (mixed HCC-CC), mixed hepatobiliary carcinoma, or hepatocholangiocarcinoma (3). At present, there are no accepted international management guidelines; there is no standard first line systemic therapy option for cHCC-ICC and it has a dismal prognosis, worse than that of either hepatocellular carcinoma (HCC) or cholangiocarcinoma (CCA) (1, 4, 5). This review focuses on the genetics of and current systemic treatment options for advanced, unresectable and metastatic cHCC-ICC in order to provide a platform for future trials.

Epidemiology

cHCC-ICC is likely to comprise between 0.4 and 4.7% of all PLCs, incidence ratio for male:female patients is 1.8–2.1:1 and median age at diagnosis is 62–65 years-old (2, 6–12). Data from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute reveals that patients tend to present with distant, metastatic disease (130/380, 34.2%) rather than localized (98/380, 25.8%) or regional disease (97/380, 25.5%) according to their generic staging system (*vida infra*) (11, 12). The risk factors remain unclear and retrospective case-control studies report conflicting associations; some Asian studies suggest similarities between the risk factors for hepatocellular carcinoma (HCC) and cHCC-ICC such as chronic liver disease caused by infection

with hepatotropic viruses such as hepatitis B (HBV) or hepatitis C (HBC) and alcohol. Western world datasets however propose closer similarities to the risk factors associated with intrahepatic cholangiocarcinoma (ICC) such as primary sclerosing cholangitis, chronic liver fluke infections, biliary-duct cysts, and hepatolithiasis (4, 10, 13–16).

Histological Characterization and classification

The 2019 World Health Organization (WHO) guidelines have streamlined previous histopathological classification systems (1, 3). The definition and diagnosis of cHCC-ICC now simply requires histopathological identification of unequivocal hepatocytic and cholangiocytic differentiation morphologically within the same tumor using routine hematoxylin and eosin (H&E) staining (**Figure 1**) (1, 3). There is no agreed proportion of each required for a diagnosis and no strict requirement to subtype the tumors (3). cHCC-ICC may or may not include cells with stem cell features, however the use of the category "cHCC-CCA with stem cell features" is no longer recommended (1, 3, 17, 18). Morphologically, the two components can be adjacent to each other or deeply intermingled, with a sharp or poorly defined transition. cHCC-ICC with a sharp, or a poorly defined transition, used to be known as type B and type C cHCC-ICC, respectively according to the 1949 Allen and Lisa classification (7). Some genomic studies still divide tumors morphologically using the Allen and Lisa classification and it is emerging that there may be significant genomic differences between them (*vide infra*) (**Figure 2**) (7). Rarely, cHCC-ICC may show homogenous features intermediate between hepatocytes and cholangiocytes throughout the tumor mass. This is known as "intermediate cell carcinoma of the liver" and is currently incorporated within the definition of cHCC-CCA, however there is a lack of consensus as to whether this is a distinct entity or not (1, 3, 7).

Within the cHCC-ICC tumor mass, the ICC component shows mucin-producing glandular structures within stroma, whereas HCC differentiation is characterized by Mallory-Denk bodies, bile canaliculi and a trabecular growth pattern. This can be further substantiated using a panel of immunohistochemical stains, although this is neither necessary nor sufficient for the diagnosis (**Figure 1**). Immunomarkers supporting cholangiocytic differentiation, include Ber EP4, MOC31, CK7, and CK19, whilst arginase-1, hep par 1 and canalicular expression of polyclonal CEA and CD10 is more supportive of hepatocellular differentiation. In the past, CK19, CD56, CD117 and nestin expression have been used to identify "stem cell" features (19). The cell of origin of at least classical cHCC-ICC could be a single form of bipotent hepatic progenitor cell capable of terminal differentiation into either hepatocytes or cholangiocytes (1, 4, 20–22).

Cholangiolocellular carcinoma (CLC) contains glandular epithelial cells consisting of thin, ductular-like structures within a dense hyalinized stroma and used to be classified as a subtype of cHCC-ICC (4, 7, 18, 23, 24). However, morphologically, this resembles ICC and CLC is now considered to be a subtype of ICC (in keeping with available genomic data), unless there is an admixed hepatocytic component (1, 3, 25).

Imaging Characterization

Cross-sectional imaging with Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are the mainstay in the characterization of liver malignancy (**Figure 3**) (26–31). Characteristic imaging features of HCC include arterial hyperenhancement with washout, delayed enhancing pseudocapsule, and intra-lesional fat (32–34); and those of ICC include progressive centripetal enhancement, capsular retraction, and bile duct dilatation (30). Appearances can overlap and cHCC-ICC can demonstrate features of both (30, 31, 35, 36).

The most widely adopted strategy for the diagnosis of PLC in high risk patients based on imaging alone is Liver Imaging-Reporting and Data System (LI-RADS) (34). This includes a "LR-M" category encompassing definitely or probably malignant observations which are not specifically HCC; atypical HCC, ICC and cHCC-ICC would fall into this category and a biopsy is needed (34). Features in favor of LR-M category include a targetoid mass appearance or other features such as infiltrative appearance, marked diffusion restriction, necrosis, or severe ischaemia. LI-RADS has been validated for high risk (e.g., cirrhotic) patients only and contemporary studies show the potential for misclassification of cHCC-ICC: diagnostic discordance between imaging and biopsy findings has been noted in 52% of cases of cHCC-ICC ($n = 42$) (37). Of 61 cases, 54.1% of cHCC-ICC could have been misclassified with LI-RADS using major criteria alone (35). Comparison of LI-RADS to MRI with gadolinium ethoxybenzyl diethylenetriamine (Gd-EOB) showed that ∼37% of cHCC-ICC were being wrongly categorized as HCC (36). Combining imaging and biopsy (including immunophenotypical markers) can improve diagnostic performance, with a 12% increase in sensitivity reported in certain series (37).

Circulating Tumor Markers

The diagnosis of and differentiation between HCC and ICC can be supported by circulating biomarkers (4, 38). Elevated serum Cancer Antigen 19.9 (CA19.9) is associated with ICC and elevated alpha-fetoprotein (AFP) is associated with HCC; the elevation of both or either can be seen in cHCC-ICC (4, 38). Concurrently elevated CA19.9 and AFP in a radiologically diagnosed PLC, or elevation in a biomarker discordant with the features on the imaging may indicate that the tumor is cHCC-ICC (38–40). There are several serum additional biomarkers associated with the diagnosis of HCC in particular including AFP-L3, des-γ-carboxyprothrombin (DCP), golgi protein 73 (GP73), and osteopontin (OPN), but these have not been studied robustly in appropriate series in cHCC-ICC (39, 41, 42).

GENETIC CHARACTERIZATION AND MOLECULAR BIOLOGY

Identification of genetic and molecular alterations in cHCC-ICC tumors may aid accurate diagnosis, define tumor etiology, support biomarker development, predict disease prognosis and

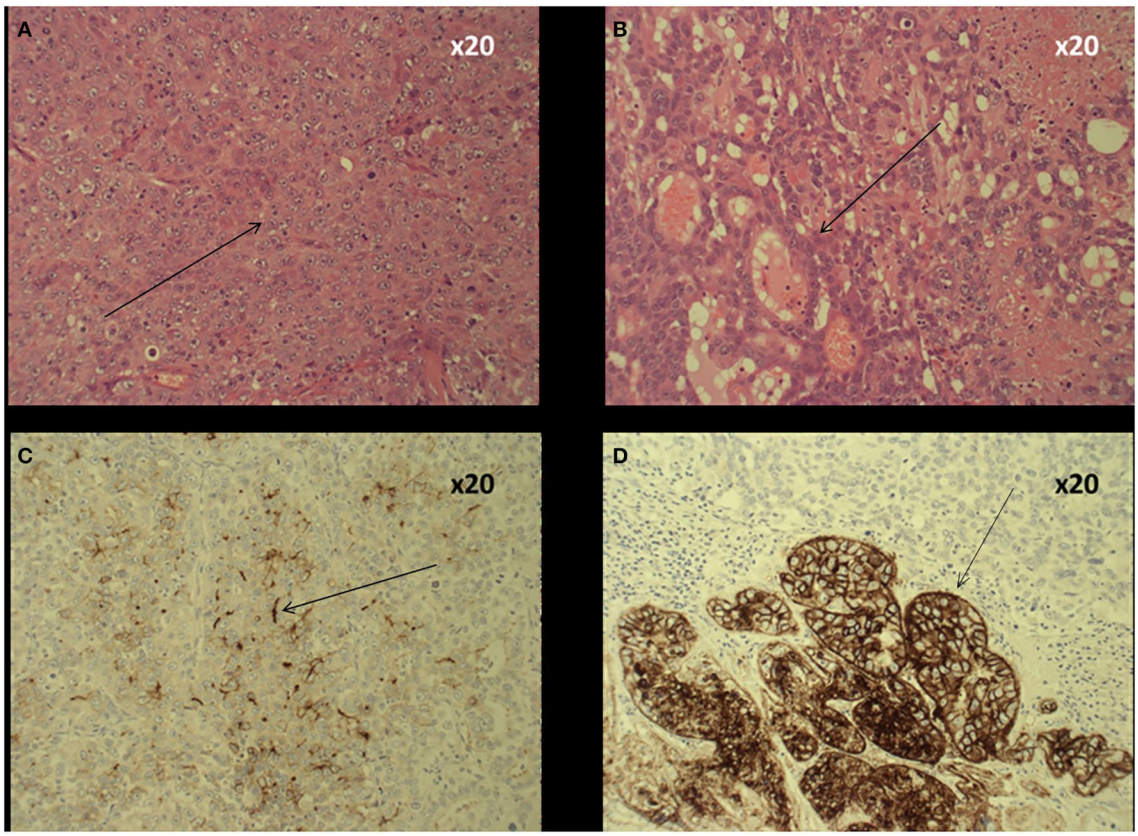

FIGURE 1 | Histology of cHCC-ICC. **(A)** Haematoxylin and eosin (H&E) slide shows an area of tumor with features of poorly differentiated hepatocellular carcinoma namely nuclear pleomorphism, hyperchromasia and coarse chromatin pattern. **(B)** H&E slide showing an area within the same tumor showing more prominent glandular architecture, morphologically consistent with cholangiocarcinoma. **(C)** The area with hepatocellular morphology shows a canalicular pattern of reactivity with polyclonal CEA, supportive of hepatocellular differentiation. This area does not react with BER EP4 polyclonal antibody. **(D)** The glandular area is immunoreactive for BER EP4 supportive of glandular epithelial differentiation consistent with the cholangiocellular component.

guide therapy. Most studies analyze the tumor mass as a whole. However, to begin, some studies *have* looked at the distinct histological elements which resemble HCC and ICC within the cHCC-ICC tumor mass. Concordant copy number changes and shared mutations on whole exome sequencing (WES) can show that these two areas of the tumor which appear different histologically are subclones from a monoclonal origin. However, there is notable intratumour heterogeneity even in these studies, for example, there can be marked differences in the magnitude of these copy number variations and there can be key differential gene expression leading to hepatocyte-like or cholangiocyte-like differentiation, notably in *VCAN, ACVR2A,* and *FCGBP* (19, 43, 44).

Genomic studies have shown that cHCC-ICC are genetically distinct from HCC and ICC with important differences in their molecular aberrations (4, 43, 45). As initial examples, cHCC-ICC shows increased frequency of genetic alternations in RYR3 and FBN2, and increased amplifications and gains of function in MYC compared to HCC and ICC (4, 19, 46, 47). Mutations in catenin beta-1 (CTNNB1) and KRAS, commonly found in HCC and ICC respectively, have been observed at almost insignificant rates in cHCC-ICC (19). In terms of tumor suppressors, tumor protein 53 (TP53) has been consistently reported as one of the most important genes mutated in cHCC-ICC; the largest comparative genomics study to date has shown that TP53 mutations were more frequent in cHCC-ICC compared to HCC and ICC alone [49.2 vs. 31% ($p < 0.001$) and 22% ($p < 0.0001$), respectively] (19, 47).

As in HCC and ICC, non-coding alterations are common in cHCC-ICC, for example large cohorts have shown 22.9% of cases with *TERT* promoter mutations and 29.7% of cases with *NEAT1* (an intergenic non-coding RNA gene for a long non-coding RNA) alterations, but detailed comparisons to HCC and ICC and how to target these changes therapeutically are not yet clear (19, 48–50).

cHCC-ICC studies integrating both genomics and transcriptomics using RNA-seq, WES and whole genome sequencing (WGS) find similar patterns in changes of key genes and tend to find more similarities between cHCC-ICC [especially Lisa and Allen type C (poorly defined transition) cHCC-ICC] and HCC, such as in TP53 and CTNNB1, rather than ICC (even ICCs arising in cirrhotic livers). Furthermore, molecular

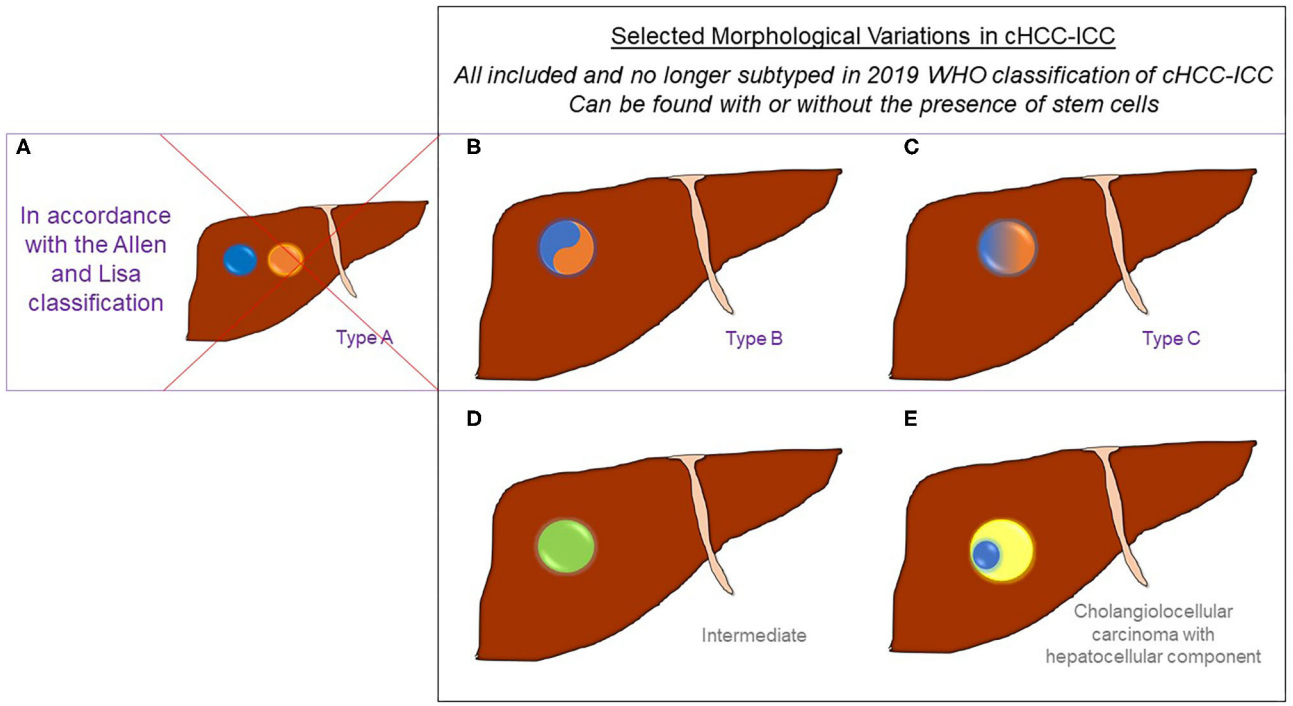

FIGURE 2 | Selected Morphological Variations in cHCC-ICC. cHCC-ICC tumors contain unequivocal cells of both hepatocytic (denoted in **blue**) and cholangiocytic differentiation (denoted in **orange**) within the same tumor mass using routine hematoxylin and eosin staining. All may contain variable levels of stem cell features. Prior classification systems used to differentiate between different morphological forms of cHCC-ICC. **(A)** Represents Allen and Lisa's Type A tumors which are HCC and ICC in the same lobe of the liver but not within the same tumor mass; these are no longer diagnosed as cHCC-ICC but are considered concomitant HCC and ICC tumors (included for completeness). **(B)** Represents Allen and Lisa's Type B tumors which contain HCC and ICC histological features within the same tumor mass with a sharp transition between them. **(C)** Represents Allen and Lisa's Type C tumors show HCC and ICC histological features blending into each other within the same tumor mass. **(D)** Intermediate cell carcinoma (in **green**) **(E)** and cholangiolocellular carcinoma (in **light yellow**) but only when it also contains a hepatocellular component (in **blue),** are also included within the definition of cHCC-ICC.

alterations characteristically seen in ICC, such as changes in PBRM1, IDH1, IDH2, FGFR2, and BAP1 were not present across cHCC-ICC (44, 47, 51).

Transcriptomic and molecular clusters have been described in cHCC-ICC using WES techniques (44, 52). The most detailed study to date on the complex molecular profile of cHCC-ICC has been provided by an integrative genomic analysis of 133 pan-Asian cases (19). This study concluded that Allen and Lisa type B (sharply defined transition) and type C (poorly defined transition) are distinct (based on their genetic and transcriptomic data) and hence the Allen and Lisa criteria is valid on a molecular level (**Figure 2**) (19). The transcriptomic profile clustering in this work showed that type B cHCC-ICC was genetically more similar to ICC, with enhanced expression of biliary markers (EpCAM, KRT19, and PRDM5) and frequent KRAS and IDH1 mutations. Whereas, using similar techniques, type C cHCC-ICC was associated more closely with poorly differentiated HCC features such as increased expression of liver cell markers (APOE, GPC3 and SALL4), more frequent TP53 mutations, enrichment in immune pathways within the tumor microenvironment and raised serum AFP levels (2, 19, 53). This correlates with clinicopathological data which has shown marked similarity between type C cHCC-ICC and HCC with regards to male/female ratio, hepatitis infection, serum AFP levels and non-tumor liver histology (14, 46).

This genetic study also identified both monoclonal and multiclonal origins of the tumors irrespective of the Allen and Lisa subtype of PLC. This finding which correlates with recent studies on the trans-differentiation of hepatocytes to cholangiocytes and HCC to cHCC-ICC-like tumors, supporting the theory of plasticity of hepatobiliary cells and a critical role of the tumor microenvironment (TME) in directing the differentiation of genetically identical liver cells into different lineages (2, 19, 54–56). The dependence of tumor development on the TME is supported by the identification of associations between clinical/environmental factors and patterns of mutations in cHCC-ICC (57). To date, no data has been published for either the immune component of the TME nor tumor mutational burden in cHCC-ICC (58).

Thus, former genomic and transcriptomic studies of all cHCC-ICC subtypes disagree on the separation from HCC and/or ICC, but recent studies suggest that Type C (poorly defined transition) subtype is genetically similar to HCC, and Type B (sharply defined transition) subtype is closer to ICC (19, 59). These findings could have potential implications for therapeutic approaches e.g., type C subtype could be treated

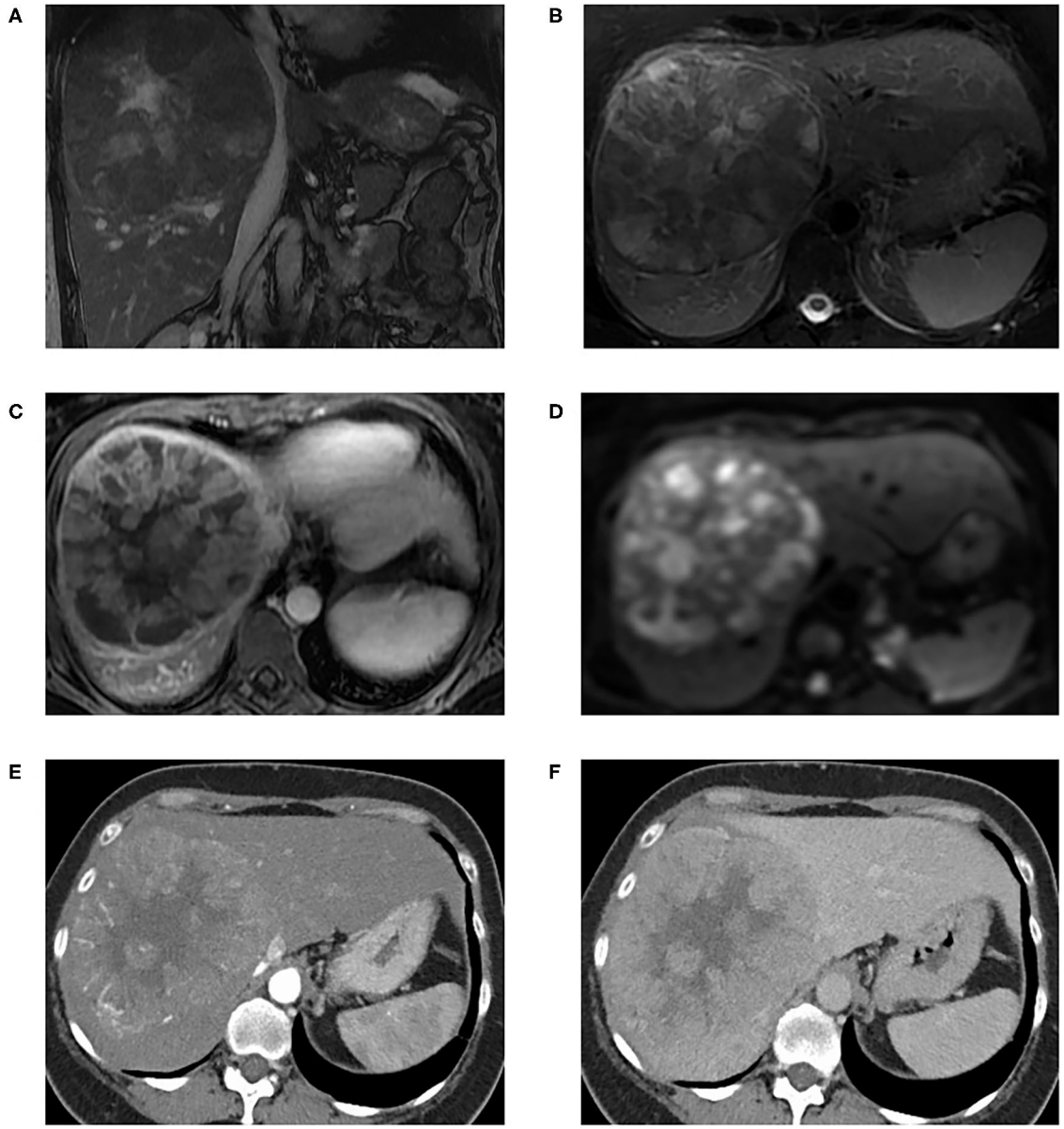

FIGURE 3 | MRI liver with extracellular gadolinium contrast agent from a patient with histologically confirmed neoplastic liver lesion with variable, moderate to poor, differentiation with areas of hepatocellular differentiation and other areas of immunohistochemical evidence of cholangiocellular differentiation. Coronal FIESTA **(A)** and axial T2-weighted Fat Saturated **(B)** images show an 11cm well-defined centrally necrotic heterogeneous liver mass at the right lobe of the liver. This is effacing the IVC although there is no definite venous tumor invasion. The middle and right hepatic veins were not appreciable, presumed completely effaced; the main and branch portal veins were patent (not shown). In addition, there is associated mild intrahepatic biliary duct dilatation. T1-weighted arterial phase axial image **(C)** shows heterogeneous peripheral enhancement. Diffusion weighted imaging ($B = 600$) **(D)** shows heterogeneously restricted diffusion on correlation with Apparent Diffusion Coefficient maps. Post-intravenous contrast CT in arterial phase **(E)** and portovenous phase **(F)** for the same patient shows a large vascular mass in the central aspect of the right lobe liver with arterial hyperenhancement and portovenous wash-out, and central necrotic areas.

more like HCC tumors and Type B subtype could be treated like ICC. Also, the inferences from these molecular studies may have repercussions for the new simplified WHO classification which had aimed to reduce the need for morphological subtyping. The recent finding that morphological subtypes of cHCC-ICC may correlate with genomics could explain discrepancies between some studies finding genomic similarities between cHCC-ICC and HCC, and others with ICC (19).

The genomic, transcriptomic and proteomic landscape of cHCC-ICC is reliant on a small number of disparate studies with different patient cohorts internationally, which do not perfectly agree. A summary of published aberrant genomic markers

TABLE 1 | Genomic and molecular differences between different subtypes of primary liver cancer.

cHCC-ICC (all forms)		HCC	ICC
Type B (sharply defined transition)	**Type C (poorly defined transition)**		
Mutations in TP53 and CTNNB1 (similar to HCC) (47)		Similar mutations in TP53 and CTNNB1 (47)	
TERT promotor mutations (similar to HCC) (60)		TERT promotor mutations (60)	
Altered spectrum of target genes in the TGFβ and Wnt/CTNNB1 cell signaling pathways, and increased LEF1 and SOX9 expression tending toward biliary differentiation (similar to ICC) (52)			Altered spectrum of target genes in the TGFβ and Wnt/CTNNB1 cell signaling pathways, and increased LEF1 and SOX9 expression tending toward biliary differentiation (52)
Increased mutations of RYR3, FBN2, KNCC3, and MYC (distinct from HCC) (47)		Fewer mutations in RYR3, FBN2 and MYC (47)	
Tendency for LoH at chromosomes 3p and 14q (distinct from HCC) (46)			Tendency for LoH at chromosomes 3p and 14q (46)
Increased TP53 mutations (predominately missense) (distinct from HCC and ICC) (19)		Fewer TP53 mutations than cHCC-ICC in this study (19)	Fewer TP53 mutations (19)
Rare to have mutations in CTNNB1 (distinct from HCC and ICC) (19)		Commonly mutated CTBBN1 (19)	Commonly mutated CTBBN1 (19)
Enhanced expression of EpCAM, KRT19, and PRDM5 (19)	Increased expression of APOE, GPC3, and SALL4 (19)	Increased expression of APOE, GPC3 and SALL4 (19)	Enhanced expression of EpCAM, KRT19 and PRDM5 (19)
Frequent KRAS and IDH1 mutations (19)	More frequent TP53 mutations (19)	More frequent TP53 mutations (19)	Frequent KRAS and IDH1 mutations (19)

Data from studies examining differences between cHCC-ICC taken as a whole tumor mass are in black, data from studies comparing Type B and C (Allen and Lisa classification) cHCC-ICC to HCC and ICC are in blue and purple, respectively (19, 45–47, 60). cHCC-ICC, combined hepatocellular-cholangiocarcinoma; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CK, Cytokeratin; CTNNB1, Catenin Beta 1; LoH, loss of heterozygosity; Rb-1, retinoblastoma (RB) Transcriptional Corepressor 1; RYR3, ryanodine receptor 3; FBN2, fibrillin 2; MYC, MYC Proto-Oncogene, BHLH Transcription Factor; EpCAM, epithelial cell adhesion molecule; KNCC3, calcium-activated potassium ion channel gene; ARID1A, AT-Rich Interaction Domain 1A; PBRM1, Polybromo 1; LEF1, lymphoid enhancer-binding factor-1; SOX9, SRY-Box Transcription Factor 9; KRT19, Keratin 19; PRDM5, PR/SET Domain 5; APOE, Apolipoprotein E; GPC3, glyican 3; SALL4, sal-like protein 4.

(**Table 1**) and possible molecular drivers and targets (**Table 2**) therefore should be interpreted with caution. The detailed roles of oncogenic driver mutations are still poorly understood in all forms of PLC, especially cHCC-ICC. However TGF-β, Wnt, AKT, N-RAS, Notch-Hedgehog pathway activation and NF-κB pathway inactivation have all been implicated in pathogenesis, as has signaling through AXIN1, KMT2D, RB1, PTEN, FGFR, nestin, ARID1A, KEAP1, IDH1, versican, EpCAM, Erbb2, and TERT (2, 19, 47, 53, 58, 61–63). A number of these are potential drug targets being evaluated in early phase clinical trials.

Staging and Prognosis

cHCC-ICC is staged by TNM in a clinical context (as opposed to SEER staging of epidemiological data) using the same staging algorithm as for ICC (**Table 3**) (64–66). It is difficult to get accurate measures of patient survival without treatment (i.e., the true prognosis) but two large epidemiological datasets from the United States provide some guidance (12, 67). Median overall survival (mOS) of patients stratified by the SEER stage for distant, regionalized, and localized cHCC-HCC was 4 months (95% CI, 3–6), 7 months (95% CI, 5–11), and 20 months (95% CI 16–28), respectively ($p < 0.001$), with the difference between regionalised and localized explained by suitability for resection (12). A similar pattern is seen using TNM staging data from the National Cancer Data Base (NCDB) where mOS was 28.6 m for patients with Stage I disease, 24.2 m for stage II, 7.5 m for stage III and 3.1 m for stage IV (67).

TREATMENT

In patients with localized cHCC-ICC and good performance status, surgical resection may provide the chance of long-term benefit, for example, 5 year survival rate of 30% has been reported (12, 68, 69). These tumors show locoregional spread in similar patterns to HCC (hepatic and portal venous invasion) and to ICC (lymph node dissemination). Therefore liver resection with hilar node dissection is attempted In suitable patients with satisfactory liver function, however for patients with underlying cirrhosis, resections are limited to avoid hepatic decompensation (70–72). Pre-operatively tools such as the Model for End-stage Liver Disease (MELD) score calculated from INR, bilirubin, and creatinine, can be utilized in the risk assessment to predict post-operative mortality following surgical resection (73).

The observed survival after surgery is similar to ICC, where transplant is not standard, and notably less than for HCC where transplantation may be offered (74, 75). Transplanted cHCC-ICC patients ($n = 19$) compared with transplanted HCC patients ($n = 1147$) had inferior 5-year OS rates of 48 vs. 78% ($p = 0.01$) (75). A meta-analysis

TABLE 2 | Summary table of potentially actionable molecular aberrations encountered in cHCC-ICC.

Gene	Function	Frequency	Alteration
Versican (VCAN)	Proteoglycan involved in cell growth, division, adhesion and migration, angiogenesis and aerobic glycolysis	21.4%	Increased frequency of mutations (usually missense) (44)
Activin A receptor type 2A (ACVR2A)	Receptor involved in cell growth and differentiation signaling	14.3%	Increased frequency of mutations (usually missense) (44)
Epithelial cell adhesion molecule (EpCAM)	Transmembrane oncogenic mediator of epithelial cell-cell adhesion, cell signaling, migration, proliferation and differentiation		Increased expression (19)
Tumor protein p53 (TP53)	Master tumor suppressor regulating cell cycle, apoptosis, senescence and DNA repair	46–57%	Higher rate of loss of function mutations (19, 44)
MYC	Oncogenic transcription factor promoting expression of factors driving cell proliferation, cell growth and cell stemness whilst inhibiting apoptosis and differentiation	73%	Higher rate of mutations and focal amplifications (19)
Telomerase reverse transcriptase (TERT)	Crucial enzymatic component of the telomerase complex that allows lengthening of DNA strand telomeres preventing apoptosis in senescent cells	19%	Higher rate of promoter mutations and focal amplifications (19)
Cyclin D1 (CCND1)	Cell cycle positive regulator with role in angiogenesis, cell migration and cell metabolism	30%	Higher rate of focal amplifications (19, 51)
Cyclin E1 (CCNE1)	Cell cycle regulator	5–24%	Higher rate of focal amplifications (19, 51)
CDK6	Cell cycle regulator	20%	Higher rate of focal amplifications (19)
Cyclin-dependent kinase N2A (CDKN2A)	Encodes for p16 and p14arf; tumor suppressor proteins that negatively regulate the cell cycle	37%	Deletions and loss of function (19)
MET	Tyrosine kinase with established oncogenic properties including activation of cancer pathways such as RAS and PI3K, cell proliferation and angiogenesis	15–24%	Higher rate of mutations and focal amplifications (19, 51)
K-RAS	GTPase protein with established oncogenic properties including activation of pathways such as MAP kinase and PI3K/mTOR pathways that promote cell growth, protein synthesis and cell division	5%	Higher rate of mutations (but lower when compared to ICC) and increased expression (19, 51)
Phosphatase and tensin homolog (PTEN)	Phosphatase acting a tumor suppressor factor via negative regulation of the Akt/PKB signaling pathway and inhibition of cell cycle and division.	10%	Higher rate of mutations (19, 51)
AT-rich interaction domain 1A (ARID1A)	Combined helicase and ATPase, part of an ATP-dependent chromatin-remodeling complex that acts as a tumor suppressor by regulating transcription of genes involved in oncogenesis	19.5%	Higher rate of mutations (19)
AT-rich interaction domain 1B (ARID1B)	Combined helicase and ATPase, part of an ATP-dependent chromatin-remodeling complex that acts as a tumor suppressor by regulating transcription of genes involved in oncogenesis	28.6%	Increased frequency of mutations (usually missense) (44)
AT-rich interaction domain 2 (ARID2)	Combined helicase and ATPase, part of an ATP-dependent chromatin-remodeling complex that acts as a tumor suppressor by regulating transcription of genes involved in oncogenesis	19.5%	Higher rate of mutations (19)
Adenomatous polyposis coli (APC)	A tumor suppressor protein regulating cell adhesion, invasion and cell proliferation by negatively regulating of beta-catenin via interaction with E-cadherin within the Wnt signaling pathway	7.2%	Increased frequency of mutations (usually missense) (44)
Retinoblastoma (RB1)	Multifunctional protein acting as a tumor suppressor by inhibiting cell cycle progression and inducing senescence thus regulating cell growth and proliferation and preventing metastasis	26%	Deletions and loss of function (19)
PTMS-AP1G1	Important component of clathrin-coated vesicles for intra-cellular transportation	11.7%	Fusion events (19)
Fibroblast growth factor receptor (FGFR)	Cell surface membrane receptor tyrosine kinase which activates secondary messanger systems key to processes such as proliferation, differentiation, cell migration, and survival	6.5%	Fusion events (19)
CTNNB1 (β-catenin 1)	Multifunctional protein involved in the regulation of gene transcription and cell-cell adhesion as part of the cadherin complex in the Wnt signaling pathway where it acts as an oncogene		Higher rate of mutations (but lower when compared to HCC) (19)
NFATC2/3	DNA-binding protein regulating cell invasiveness and migration.	7.2% (NFATC2), 28.6% (NFATC3)	Increased frequency of mutations (44)
AXIN1	Cytoplasmic protein that acts as negative regulator of the Wnt signaling pathway to induce apoptosis	25%	Deletions and loss of function
IDH1	Enzyme involved in metabolic processes that can inactivate tumor suppressor genes and activate oncogenes	21.2%	Higher rate of mutations (19)

MYC, MYC Proto-Oncogene; BHLH, Transcription Factor; MET, MET Proto-Oncogene, Receptor Tyrosine Kinase; K-RAS, KRAS proto-oncogene, GTPase; PTMS-AP1G1, Parathymosin-AP-1 complex subunit gamma-1; CTNNB1, Catenin Beta 1; NFAT2/3, Nuclear Factor Of Activated T Cells 2/3; IDH1 gene, isocitrate dehydrogenase [NADP(+)] 1, cytosolic (2, 19, 44, 51, 53, 61).

TABLE 3 | Summary table of staging systems.

TNM Stage	Tumor	Node	Metastasis	SEER General Staging System
IA	T1a	N0	M0	Localized
IB	T1b	N0	M0	
II	T2	N0	M0	
				Regional
IIIA	T3	N0	M0	
IIIB	T4	N0	M0	
	Any	N1	M0	
IV	Any	Any	M1	Distant

The tumor, node and metastasis scores of each tumor allow TNM staging. The primary tumor is classified as follows; T1 if it is a solitary tumor with no vascular invasion (T1a if ≤5 cm and T1b if >5 cm), T2 if it is either a solitary tumor with vascular invasion or there are multiple primary tumors (irrespective of vascular invasion), T3 if the primary tumor perforates the visceral peritoneum and, T4 if the tumor involves local extrahepatic structures by direct invasion. N1 denotes regional lymph node metastases and M1 denotes distant metastatic disease. The SEER general staging system for tumors such as cHCC-ICC is included and compared with the TNM system; some TNM stage II tumors may be classified as localized and others as regional. The majority of large epidemiological studies to date use the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute: localized cancer is limited to the anatomical site of origin without spread, regional cancer is limited to the nearby draining lymph nodes, tissues or organs by direct extension, and distant cancer has spread to distant non-continuous parts of the body (7, 10–12). SEER, Surveillance, Epidemiology, and End Results program; TNM, tumor node metastasis.

of NCDB cases indicates that transplantation does not result in improved outcome when compared with resection in cHCC-ICC, making a case for careful pre-operative diagnostic assessment to minimize the risk of misdiagnosis with HCC and for the limited supply of donor livers to be more beneficially applied for conditions with better post-transplant outcomes (67, 70).

Non-surgical treatment options in patients with localized disease include ablation procedures, transarterial (chemo)embolization (TA(C)E), hepatic arterial infusional chemotherapy, radioembolization, and systemic therapy (68, 71). The data for benefit of loco-regional therapies in cHCC-ICC is limited to small retrospective studies but there are recognizable partial response rates which may allow subsequent surgical resection and potentially survival benefit (4, 68, 76, 77).

Even following treatment for localized disease it is common for the disease to recur, often with unresectable regional or distant/metastatic disease; (4, 40, 76, 78) tumor recurrence rates at 1, 3, and 5 years were 60.8, 71.8, and 80.7%, respectively in one study, and median disease-free survival of 10 months has been reported (4, 5, 69, 78). Recurrence rates seem to be non-significantly different in comparison to HCC and ICC, but mOS after recurrence tends to be worse than HCC and possibly worse than ICC (4, 5, 78).

Systemic Treatment Options

There is no globally accepted standard first line therapy for advanced cHCC-ICC as the evidence base is limited, therefore clinicians offer first line treatments utilized for either advanced HCC or ICC to patients with Eastern Cooperative performance (ECOG) performance score (PS) 0–2. Systemic treatment planning for cHCC-ICC patients requires careful consideration of comorbid cirrhosis and compromised liver function.

Standard first line therapy for CCA is gemcitabine 1,000 mg/m^2 and cisplatin 25 mg/m^2 doublet chemotherapy administered on days 1 and 8 of a 21 day cycle, for patients with good ECOG PS of 0–1, based on the ABC-02 trial (79, 80). The dose of gemcitabine may be reduced to 800mg/m^2 if there is pre-existing liver dysfunction (81–83). In terms of second-line treatments upon progression, patients may get re-treated with gemcitabine/cisplatin depending on their initial outcome, or can be referred for clinical trials. A recent trial of oxaliplatin/5-FU (mFOLFOX) plus active symptom control given to advanced biliary tract cancer (including ICC) patients after first-line gemcitabine-cisplatin doublet chemotherapy showed modest extended mOS by just under a month (5.3 vs. 6.2 months) between study arms, however differences in OS rate at 6-months (35.5 vs. 50.6%) and 12-month (11.4 vs. 25.9%) were potentially clinically meaningful (84).

For advanced HCC, cytotoxic therapies are generally not used in standard practice due to lack of efficacy and toxicity concerns, particularly in cirrhotic patients, but there is significant data to support the role of small molecule multitargeted tyrosine kinase inhibitors (TKIs) sorafenib and lenvatinib in the first-line treatment setting (85–88). More recently there has been positive data in first line treatment setting for HCC patients using immune checkpoint inhibitor (ICPI) therapies in combination with other agents such as bevacizumab, or tyrosine kinase inhibitors such as lenvatinib which has led to approval by the Food and Drug Administration (FDA) in the United States of America (89, 90). Evidence for utility of TKIs in cHCC-ICC patients is generally in the form of case-reports and single-center retrospective studies with a very weak signal of efficacy, but in the absence of international guidance and concerns about toxicity of cytotoxic chemotherapy they are commonly offered to patients (77, 91–93).

The comparative data on systemic therapy in cHCC-ICC is sparse, but tends to favor the efficacy of chemotherapy over sorafenib (77, 92, 93). In small retrospective studies (n = 41, 28 and 17), cytotoxic regimens seem to achieve a reasonable response rate and modest mOS benefit (77, 92, 93). In the largest of these cohorts, there were no recorded objective responses for sorafenib monotherapy (n = 5 evaluable), the median progression free survival (mPFS) was 4.8 m (n = 7) and mOS was 9.6 m (n = 7), whereas for gemcitabine-cisplatin doublet chemotherapy, the partial response rate was 24% (9/37 evaluable), mPFS was 8.0 m (n = 41), and mOS was 11.5 m (n = 41) (77). Another showed that both mPFS [3.0 m (95% CI, 0.0–9.1)] and mOS [10.2 m (95% CI, 3.9–16.6)] tend to be larger than observed with sorafenib [PFS 1.6 m (95% CI, 1.2–2.0), mOS 3.5 m (95% CI: 0.0–7.6)] with a statistically significantly improved hazard ratio (HR) for mOS [HR: 5.50 (95% CI, 1.17–25.84)] (92). Furthermore, on multivariate analysis, sorafenib monotherapy remained an independent

poor prognostic factor for survival compared to first line gemcitabine-cisplatin chemotherapy [HR: 10.7, (95% CI, 1.4–80.7), $p = 0.022$] (92). cHCC-ICC management along the lines of ICC (chemotherapy as first line treatment) may be more effective than for HCC and should be the preferred option if safe (77, 92, 93).

Given the increasing evidence for ICPI efficacy in the management of both advanced ICC with microsatellite instability (MSI) and HCC, there is rationale to try this approach in cHCC-ICC (94, 95). A case report describing a near complete radiological response to ICPI in a cHCC-ICC patient showing no MSI but raised neoantigen burden in his tumor, has highlighted utility of this therapeutic approach in selected patients (96, 97).

Perspectives From Pre-Clinical and Translational Studies

Improved models of cHCC-ICC may provide valuable information on neoplastic development, progression and therapeutic strategies for this rare tumor. Currently, one mouse model of cHCC-ICC has been developed (56). It was created from a mouse model of HCC by inhibiting nuclear factor kappa-B (NF-κB) signaling by deleting NF-kappa-B essential modulator (NEMO)/ nuclear factor kappa-B kinase subunit gamma (IKKγ) selectively from hepatocytes; the effect of different treatments on this model have not yet been explored (56). Patient derived organoid models of cHCC-ICC from resected combined tumors have recently been described, which demonstrate preserved histological architecture, gene expression and genomic landscape of the original tumor, permitting discrimination between different subtypes, even following long-term expansion in culture (56, 98). Drug sensitivity assays of the organoids recapitulated sensitivity to each of gemcitabine and sorafenib in one of the two cHCC-ICC models and sensitivity to sorafenib in the other (98). Sensitivity was also shown across the two models to taselisib (a beta-isoform sparing PI3K inhibitor), LGK974 (PORCN inhibitor), deltarasin (reduces KRAS activity by inhibiting KRAS-PDEδ interactions), vorinostat (HDAC inhibitor Class I, IIa, IIb, IV), SCH772984 (ERK1/2 inhibitor) (98). These models may provide a platform for drug screening and validation of "actionable" therapeutic targets in cHCC-ICC patients.

DISCUSSION

Given the rarity of cHCC-ICC, there are extremely limited clinical trial options available specifically for this group of patients. Genomic, pre-clinical and clinical studies underline inconsistencies between these tumors and either HCC or ICC in genotype, phenotype and treatment response, therefore it is emerging that these tumors may need to be regarded as a separate entity for optimal management. Current data supports the use of cytotoxic chemotherapy where possible for cHCC-ICC, but different histological and molecular subtypes (which is a different emphasis to the recent WHO histological guidance) could form the basis for more nuanced strategies for empirical chemotherapy, molecularly targeted treatment or immunotherapy. However, it should be noted that the current genomic, proteomic and systemic therapy evidence is underdeveloped and predominately from small, retrospective studies and more rigorous prospective data is desirable to allow more definitive conclusions. Molecular profiling and enrolment into tumor-agnostic "basket" trials selecting for molecular alterations could be helpful in the short term, to gain an understanding of how responsiveness of potentially "actionable" phenotypes may be impacted by the biology and environment of these unusual tumors. In the longer term, better pre-clinical models and international collaborations and registries with centralized pathology and radiology are highly desirable to optimize the knowledge base, and rationalize management strategies (1).

AUTHOR CONTRIBUTIONS

All authors made (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and (3) final approval of the version to be published. AA and BB: conceptualization and project administration. AA, AH, CG, AD, and BB: data curation, resources, visualization, and writing–review and editing. AA, AH, and BB: formal analysis, investigation, and validation. AA: methodology. BB: supervision. AA and AH: writing–original draft.

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Surveillance Strategy for Patients With BCLC Stage B Hepatocellular Carcinoma After Achieving Complete Remission: Data From the Real World

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Purpose: There is a lack of consensus on the surveillance strategy for Barcelona Clinic liver cancer (BCLC) stage B hepatocellular carcinoma (HCC) patients with complete remission (CR). We performed a real-world, retrospective analysis of the surveillance strategy for BCLC stage B HCC patients after radical therapy with CR to support clinical decision-making.

Materials and Methods: We analyzed 546 BCLC stage B HCC patients with CR after radical treatments (surgery/ablation) at Sun Yat-sen University Cancer Center, from January 2007 to December 2019. The intensity of surveillance interval was defined as the mean of surveillance interval within 2 years. The primary endpoint of the study was overall survival (OS) and extra-Milan criteria relapse.

Results: During a median follow-up time of 23.9 months (range = 3.1–148.3 months), there were 11.9% of patients died, 56.6% of patients developed recurrence, the vast majority of patients experienced recurrence within 2 years, and 27.8% patients developed extra-Milan criteria recurrence. The median disease-free survival and OS were 33.6 and 60.0 months, respectively. Patients were divided into regular surveillance group (RS) ($\leq$4.3 months) and irregular surveillance (IRS) group (>4.3 months) based on the optimal cutoff value of the intensity of surveillance interval. The RS group owned a lower incident of extra-Milan criteria relapse and smaller and fewer tumors at recurrence than IRS group, which contributed to the prolonged OS. Besides, the cutoff values of surveillance interval that could lead to significant differences in the incidence of extra-Milan criteria relapse during 0–6, 6–12, and 12–18 months after CR were 2.6, 2.9, and 3 months, respectively.

Conclusions: The average surveillance interval for patients with BCLC stage B HCC achieved CR should not exceed 4.3 months during the first 2 years' follow-up. During three different phases of the initial 18 months after CR, individualized surveillance showed intervals no more than 3 months were required to reduce the incidence of extra-Milan criteria relapse.

Keywords: hepatocellular carcinoma, BCLC stage B, surveillance interval, complete remission, extra-Milan criteria relapse

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide in 2018 (1). Rates of both incidence and mortality are two to three times higher among men in most regions (1). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are considered to be the main pathogens for the development of HCC, especially in Asia (2). Recently, the number of patients with HCC originating from HCV has increased year by year, and the number of HCC patients owing to HBV has decreased (3).

The Barcelona Clinic liver cancer (BCLC) stage B (intermediate stage) (4) patients account for ∼19.4% of total HCC (5). Also, the BCLC stage B represents a heterogeneous group of patients (6), which were more complicated and experience relapses earlier than BCLC stage A. The main factors were the span of liver function score (Child–Pugh: 5–9), the difference of tumor size (diameter 3–10 cm or more), tumor number (2–20 or more), and the difference of tumor distribution (single lobe limited or double lobe diffused).

Untreated patients at BCLC stage B present a median survival of 16 months or a survival rate of 49% at 2 years (7, 8). Chemoembolization extends the survival of these patients to a median of up to 19–20 months (8, 9). Surgery and ablation comprise potentially curative treatment modalities for BCLC stage B HCC patients (10). Besides, patients in this stage achieving downstaging from combined treatments or TACE (transarterial chemoembolization) can be suitable for radical treatments (6, 11). Unfortunately, the median survival of BCLC stage B HCC patients after curative treatment was 45 months (12). Tumor recurrence after curative surgery occurs in 50–70% of patients, which constitutes either intrahepatic metastases (often within 2 years after surgery) or a new HCC in the remaining cirrhotic liver (10, 13). Factors of early or late recurrence or/and metastases were complex, including tumor size and history of rupture, etc. (11, 14, 15).

Patients with recurrence after radical therapies may still be candidates for curative therapies (10, 16, 17). Early diagnosis of recurrence is more likely to receive curative treatment and achieve better disease control and prolonged survival (18). Although recent guidelines recommend surveillance strategies (10, 19) for patients after curative treatment, there is a lack of specific consensus on surveillance regimen after curative treatment of HCC, especially for BCLC stage B HCC patients with complete remission (CR) after radical treatment. For HCC patients with BCLC stage B, whether the current surveillance strategies are sufficient remains unclear. Moreover, although patients are recommended for surveillance according to the guidelines in the clinic, in the real world, for various reasons, patients cannot fully follow the guidelines for surveillance strategies. Therefore, the impact of irregular surveillance (IRS) in the real world on patient survival is also unclear.

Based on this background, we performed a real-world, retrospective analysis of the surveillance strategy for BCLC stage B HCC patients after radical therapy with CR to support clinical decision-making.

Abbreviations: HCC, hepatocellular carcinoma; CR, complete remission; BCLC, Barcelona Clinic Liver Cancer; RS, regular surveillance; IRS, irregular surveillance; HBV, hepatitis B virus; HCV, hepatitis C virus; DFS, disease-free survival; OS, overall survival; TACE, transarterial chemoembolization; CT, multidetector computed tomography; MRI, magnetic resonance imaging; AFP, α-fetoprotein; HR, hazard ratio; CI, confidence interval.

MATERIALS AND METHODS

Patients

This study met the requirements of the Declaration of Helsinki and was approved by the Institutional Review Board of Sun Yat-sen University Cancer Center. We retrospectively analyzed BCLC stage B HCC patients who underwent radical therapy (surgery/ablation) from an institutional database at Sun Yat-sen University Cancer Center, from January 2007 to December 2019. A total of 2,193 consecutive patients were initially considered eligible. All cases were diagnosed as HCC according to pathology or clinical criteria (10, 19). This study included BCLC stage B HCC patients who received radical treatment (surgery/ablation) and achieved CR. Multidetector computed tomography (CT) and/or magnetic resonance imaging (MRI) were performed routinely to evaluate the local or distant extension of the primary tumors. Patients who visited our hospital at least 3 months after radical treatment were candidates for this study. CR is defined as no recurrence within 3 months after radical treatments. Patients were also excluded if they met any of the following criteria: age <18 or >75 years, non-HCC, mixed liver cancer, non-BCLC stage B, non-radical treatment, non-CR, died of postoperative complications. After excluding 1,637 patients according to the exclusion criteria, 546 patients were finally included in the study. All patients received radical treatment, including surgery and ablation. Some patients were treated with TACE before having undergone radical treatment, whereas others received a one-stage radical treatment.

Surveillance Strategy

After radical operation, patients were informed to perform multiphasic, high-quality, cross-sectional imaging of the chest, abdomen, and pelvis every 3–6 months for 2 years and then followed up every 6–12 months as recommended by the guideline (19). Recurrence was defined as radiological evidence of intra-abdominal or abdominal soft tissue around the surgical site, or else distant metastasis. Besides, the date of each surveillance was recorded, and the end point of the surveillance was the time of tumor extra-Milan criteria recurrence and death. Intensity of surveillance interval was defined as the mean of surveillance interval within 2 years. For patients who died, survival time after curative treatment and the result of death were recorded.

Statistical Analysis

Overall survival (OS) and extra-Milan criteria recurrence were measured from the date of CR to death or extra-Milan criteria recurrence or last follow-up evaluation. Continuous variables were presented as mean ± standard deviation and analyzed using the Student t-test. Categorical variables were analyzed using the χ^2 or Fisher exact test, as appropriate. Survival rates were

estimated by the Kaplan–Meier (K-M) method. Differences in OS were assessed for significance using the log-rank test. The Cox proportional hazards regression model was used to determine the factors associated with survival. As per initial design, all variables with a $P < 0.05$ by univariable analysis were entered in the multivariable analysis. Finally, only one variable was found to be associated with survival, and multivariable analysis could not be performed. Optimal cutoff for analysis was selected using X-Tile. Statistical analyses were performed using SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA). Pictures were drawn using GraphPad Prism version 6.0 and R-3.6.3 software. All *P*-values were two-sided, and $P < 0.05$ was considered significant.

RESULTS

Clinical and Pathological Characteristics of All HCC Patients

We enrolled 546 BCLC stage B HCC patients who achieved CR after radical treatment. The clinical and pathological characteristics of all the 546 patients are listed in **Table 1**. The 546 patients were followed 2,115 times, with an average of 4 times per person over 2 years. According to the best cutoff, regular surveillance group (RS) ($n = 441$) was defined as receipt of repeated CT/MRI with mean interval ≤4.3 months within 2 years. The IRS group ($n = 105$) was defined as receipt of repeated CT/MRI with mean interval >4.3 months within 2 years. Overall demographics were similar, but RS patients with a higher proportion of poor differentiation ($P < 0.001$).

Follow-Up and Assessment of Prognosis of All HCC Patients

Median follow-up time was 23.9 months (range = 3.1–148.3 months), and the median disease-free survival (DFS) and OS were 33.6 and 60.0 months, respectively; 11.9% of patients (65/546) died, with a 2-years OS rate of 88.0%, and the 5-years OS rate was 87.0%; 56.6% of patients (309/546) developed a recurrence, with 2-years DFS rate of 53.0% and 5-years DFS rate of 46.0% (**Figures 1A,B**). The 1-, 3-, and 5-years survival rates

TABLE 1 | Correlation between surveillance interval and clinicopathological characteristics in HCC patients.

Variable		RS ($n = 441$)		IRS ($n = 105$)		*P*
		%	No.	%	No.	
Gender	Male	87.5	386	89.5	94	0.573
	Female	12.5	55	11.5	11	
Age (years)	≤41	18.8	83	11.4	12	0.073
	>41	81.2	358	88.6	93	
HBV or HCV	No	12.9	57	16.2	17	0.380
	Yes	87.1	384	83.8	88	
Cirrhosis	No	34.7	153	34.3	36	0.937
	Yes	65.3	288	65.7	69	
AFP (μg/L)	≤400	53.1	234	59.0	62	0.359
	>400	46.9	207	41.0	43	
Tumor size (mm)	≤40	38.1	168	37.1	39	0.857
	>40	61.9	273	62.9	66	
Multinodular tumor (≥4)	No	82.5	364	89.5	94	0.080
	Yes	17.5	77	10.5	11	
One-stage radical treatment	No	56.5	249	60.0	63	0.419
	Yes	43.5	192	40.0	42	
Therapeutic modalities	Surgery	78.9	348	83.8	88	0.261
	Ablation	21.1	93	16.2	17	
Differentiation	Well	4.2	15	13.2	12	**0.001**
	Moderated	53.1	188	60.4	55	
	Poor	42.7	151	26.4	24	
Satellite nodules	No	90.0	316	93.4	85	0.319
	Yes	10.0	35	6.6	6	
Venous invasion	No	67.2	236	73.6	67	0.240
	Yes	32.8	115	26.4	24	
Perineural invasion	No	99.7	350	97.8	89	0.109
	Yes	0.3	1	2.2	2	

The meaning of the bold values provided was p < 0.05.

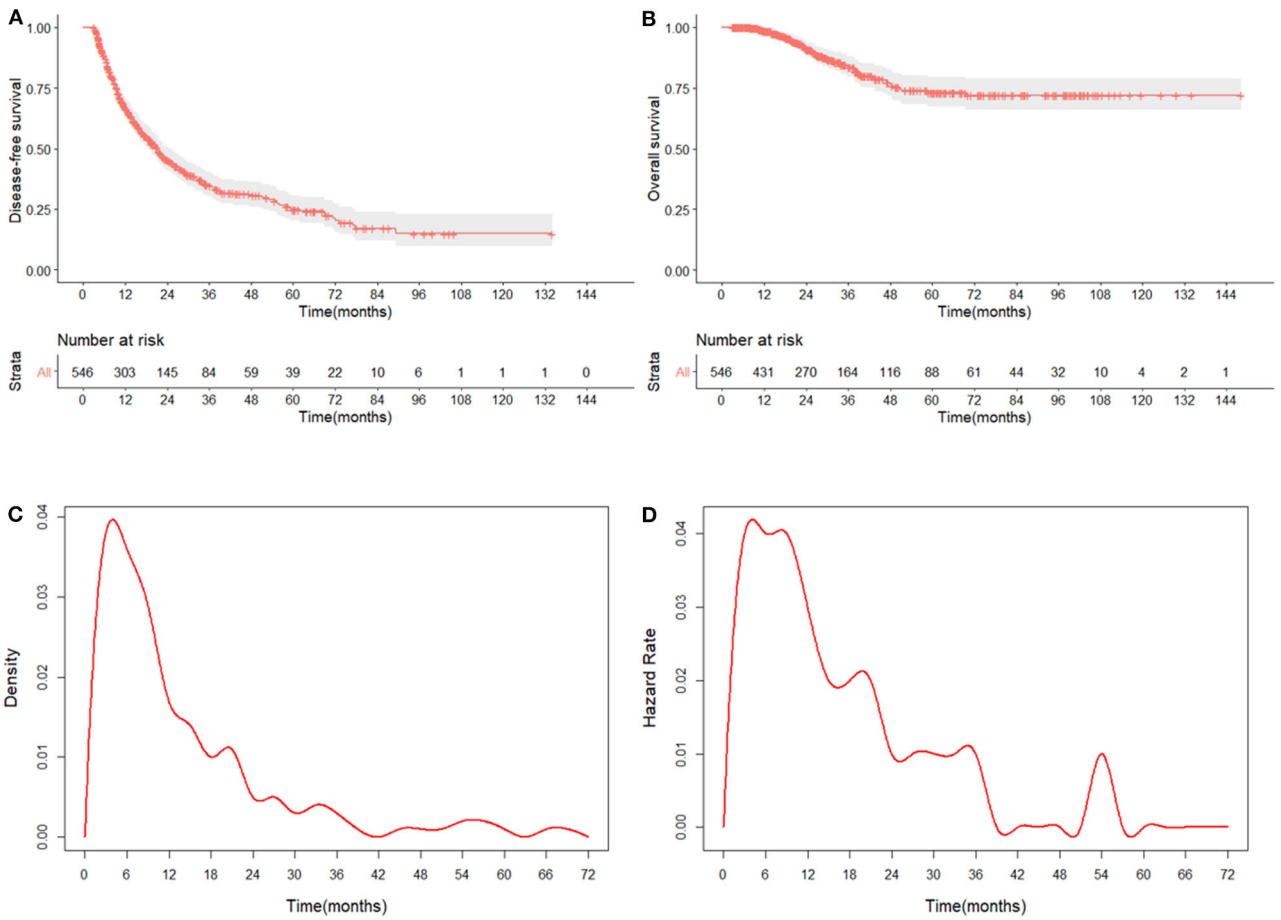

FIGURE 1 | The DFS and OS curves with 95% CIs and risk tables and the recurrence patterns for HCC patients. **(A)** The DFS of 546 patients. The 2- and 5-years DFS rates were 53.0 and 46.0%; **(B)** the OS of 546 patients. The 2- and 5-years OS rates were 88.0 and 87.0%, respectively; **(C)** the probability density plot of recurrence showed that relapse cases centered in the first 2 years after curative treatment; **(D)** the hazard rate of recurrence curve showed that the recurrence hazard peaked during the first 2 years after curative treatment.

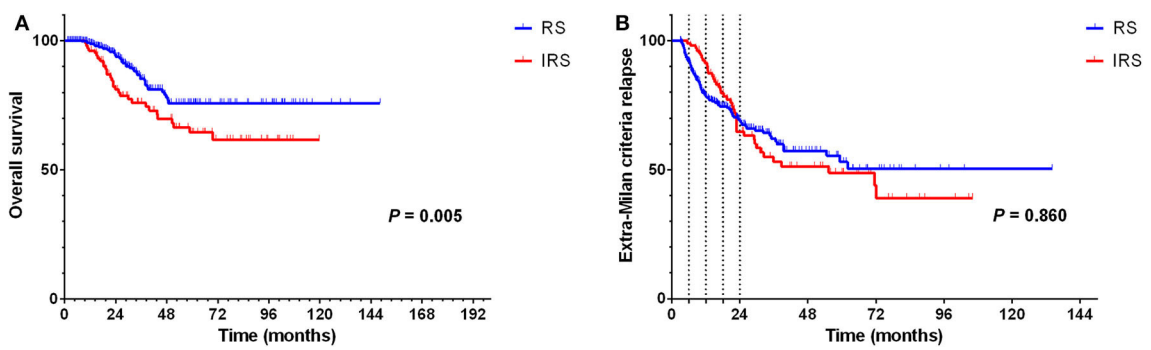

FIGURE 2 | In the RS group, significantly prolonged OS in BCLC stage B HCC patients with CR after radical treatment, but there was no difference between the two groups in terms of the extra-Milan criteria relapse. **(A)** Kaplan–Meier curve for OS of HCC patients stratified by surveillance interval; **(B)** Kaplan–Meier curve for extra-Milan criteria relapse of HCC patients stratified by surveillance interval.

were 99, 97, and 91% in the RS group, and 96, 79, and 72% in the IRS group. Besides, 27.8% of patients (152/546) developed extra-Milan criteria recurrence. In patients with recurrence, the IRS group owned a higher ratio of extra-Milan criteria recurrence than the RS group ($P = 0.004$), 64.6 and 44.7%, respectively. After recurrence, 75% of patients received further treatment, including

radical resection (41.5%), local treatment (55.0%), and systemic treatment (3.5%).

From the DFS curve and the probability density plot, we found that 90.0% of patients experienced recurrence within 1 year, and 97.0% of patients experienced recurrence within 2 years (**Figures 1A,C**). Moreover, the hazard of relapse reached its peak in the first 2 years (**Figure 1D**). Thus, it makes sense to focus on surveillance during the first 2-years after curative treatments to detect early recurrence at a potentially more treatable stage.

Univariate and Multivariate Analyses of Prognostic Factors for Recurrence and Survival of All HCC Patients

The result of univariate analysis revealed that surveillance interval [$P = 0.005$, HR = 1.981, 95% confidence interval (CI) = 1.227–3.198] (**Figure 2A**) was prognostic factors for OS, but not for extra-Milan criteria relapse ($P = 0.860$, HR = 0.968, 95% CI = 0.677–1.385) (**Figure 2B**). Besides, age ($P = 0.013$, HR = 0.498, 95% CI = 0.288–0.863), tumor size ($P = 0.019$, HR = 1.952, 95% CI = 1.116–3.414), and differentiation ($P = 0.044$, HR = 1.552, 95% CI = 1.011–2.381) were prognostic factors for OS (**Table 2**). In addition, univariate analysis revealed that age ($P = 0.006$, HR = 0.583, 95% CI = 0.398–0.854) was a prognostic factor only for extra-Milan criteria relapse (**Table 3**).

Multivariate analysis demonstrated that surveillance interval ($P = 0.037$, HR = 1.798, 95% CI = 1.037–3.117), age ($P = 0.008$, HR = 0.456, 95% CI = 0.256–0.811), and tumor size ($P = 0.018$, HR = 2.379, 95% CI = 1.160–4.876) were independent risk factors for OS (**Table 2**).

Univariate and Multivariate Analyses of Prognostic Factors for Survival of HCC Patients With Relapse

To further assess the association between surveillance interval and survival, further analysis was performed on relapsed patients. The correlation analysis demonstrated that patients in the IRS group owned a higher incidence of extra-Milan criteria recurrence ($P = 0.004$), a larger size of the recurrent tumor ($P = 0.011$), and a higher proportion of multinodular tumors ($P = 0.003$) (**Table 4**) and less likely to receive secondary treatments after recurrence ($P = 0.001$). Moreover, the violin plot also

TABLE 3 | Cox proportional hazard regression analysis of patients' extra-Milan criteria relapse.

Variable	Univariable		Multivariable	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Gender (male/female)	1.248 (0.787–1.978)	0.346		
Age (years) (≤41/>41)	0.583 (0.398–0.854)	**0.006**		
HBV or HCV (no/yes)	0.889 (0.579–1.366)	0.592		
Cirrhosis (no/yes)	1.326 (0.119–1.326)	0.119		
AFP (μg/L) (≤400/>400)	0.767 (0.555–1.061)	0.109		
Tumor size (mm) (≤40/>40)	1.167 (0.839–1.632)	0.359		
Multinodular tumor (no/yes)	1.418 (0.956–2.104)	0.083		
One-stage radical treatment (no/yes)	1.115 (0.809–1.538)	0.506		
Therapeutic modalities (surgery/ablation)	0.764 (0.494–1.181)	0.226		
Differentiation (well/moderated/poor)	0.987 (0.738–1.320)	0.928		
Satellite nodules (no/yes)	1.524 (0.879–2.643)	0.134		
Venous invasion (no/yes)	1.166 (0.812–1.674)	0.406		
Perineural invasion (no/yes)	0.899 (0.126–6.432)	0.916		
Surveillance interval (RS/IRS)	0.968 (0.677–1.385)	0.860		

The meaning of the bold values provided was p < 0.05.

TABLE 2 | Cox proportional hazard regression analysis of patients' overall survival.

Variable	Univariable		Multivariable	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Gender (male/female)	1.239 (0.592–2.589)	0.570		
Age (years) (≤41/>41)	0.498 (0.288–0.863)	**0.013**	0.456 (0.256–0.811)	**0.008**
HBV or HCV (no/yes)	2.150 (0.865–5.5346)	0.099		
Cirrhosis (no/yes)	1.288 (0.759–2.185)	0.348		
AFP (μg/L) (≤400/>400)	1.212 (0.756–1.943)	0.426		
Tumor size (mm) (≤40/>40)	1.952 (1.116–3.414)	**0.019**	2.379 (1.160–4.876)	**0.018**
Multinodular tumor (no/yes)	1.653 (0.965–2.829)	0.067		
One-stage radical treatment (no/yes)	1.381 (0.861–2.215)	0.180		
Therapeutic modalities (surgery/ablation)	0.822 (0.441–1.531)	0.536		
Differentiation (well/moderated/poor)	1.552 (1.011–2.381)	**0.044**	1.509 (0.977–2.331)	0.063
Satellite nodules (no/yes)	1.644 (0.744–3.632)	0.219		
Venous invasion (no/yes)	0.972 (0.530–1.786)	0.928		
Perineural invasion (no/yes)	2.623 (0.363–18.981)	0.339		
Surveillance interval (RS/IRS)	1.981 (1.227–3.198)	**0.005**	1.798 (1.037–3.117)	**0.037**

The meaning of the bold values provided was p < 0.05.

TABLE 4 | Correlation between surveillance interval and clinicopathological characteristics with relapsed HCC patients.

Variable		RS (n = 244)		IRS (n = 65)		P
		%	No.	%	No.	
Extra-Milan criteria relapse	No	55.3	135	35.4	23	**0.004**
	Yes	44.7	109	64.6	42	
AFP (μg/L)	≤400	63.5	155	56.9	37	0.330
	>400	36.5	89	43.1	28	
Relapse location	Local	86.1	210	80.0	52	0.226
	Distant	13.9	34	20.0	13	
Size of recurrent tumor (mm)	≤30	85.7	209	72.3	47	**0.011**
	>30	14.3	35	27.7	18	
Multinodular recurrence	No	79.5	192	61.5	39	**0.003**
	Yes	20.5	55	38.5	26	
Secondary treatment	No	20.6	50	41.5	27	**0.001**
	Yes	79.4	193	58.5	38	

The meaning of the bold values provided was p < 0.05.

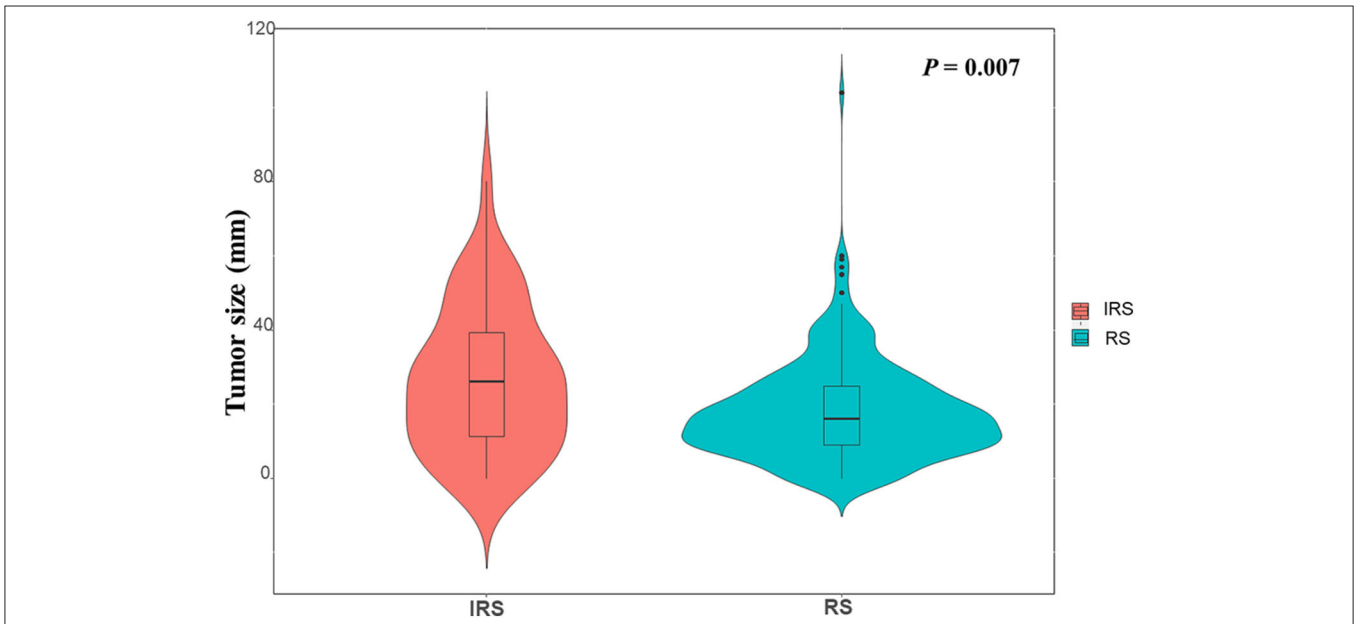

FIGURE 3 | The violin plot indicated that the RS group and the IRS group had significant difference in size of recurrent tumor (P = 0.007).

indicated that the IRS group owned a larger size of the recurrent tumor (**Figure 3**).

Besides, univariate analysis also revealed surveillance interval (P = 0.002, HR = 2.160, 95% CI = 1.338–3.488) (**Figure 5A**), extra-Milan criteria relapse (P < 0.001, HR = 2.638, 95% CI = 1.597–4.358) (**Figure 4A**), size of recurrent tumor (P < 0.001, HR = 2.758, 95% CI = 1.661–4.579) (**Figure 4B**), multinodular recurrence (P < 0.001, HR = 4.682, 95% CI = 2.903–7.552) (**Figure 4C**), and secondary treatment (P < 0.001, HR = 0.261, 95% CI = 0.155–0.439) (**Figure 4D**) were prognostic factors for OS in relapsed patients (**Table 5**). Multivariate analysis demonstrated that extra-Milan criteria relapse (P = 0.038, HR = 1.782, 95% CI = 1.032–3.077) and secondary treatment (P < 0.001, HR = 0.335, 95% CI = 0.193–0.581) were independent risk factors for OS (**Table 5**).

Comparison of Surveillance Interval and 0–18 Months Extra-Milan Criteria Relapse of All HCC Patients

According to the hazard rate curve that the recurrence risk of BCLC stage B patients with CR was still high at 0–24 months (**Figure 1D**). Moreover, although the K-M curve showed no difference between the RS and IRS groups for extra-Milan criteria

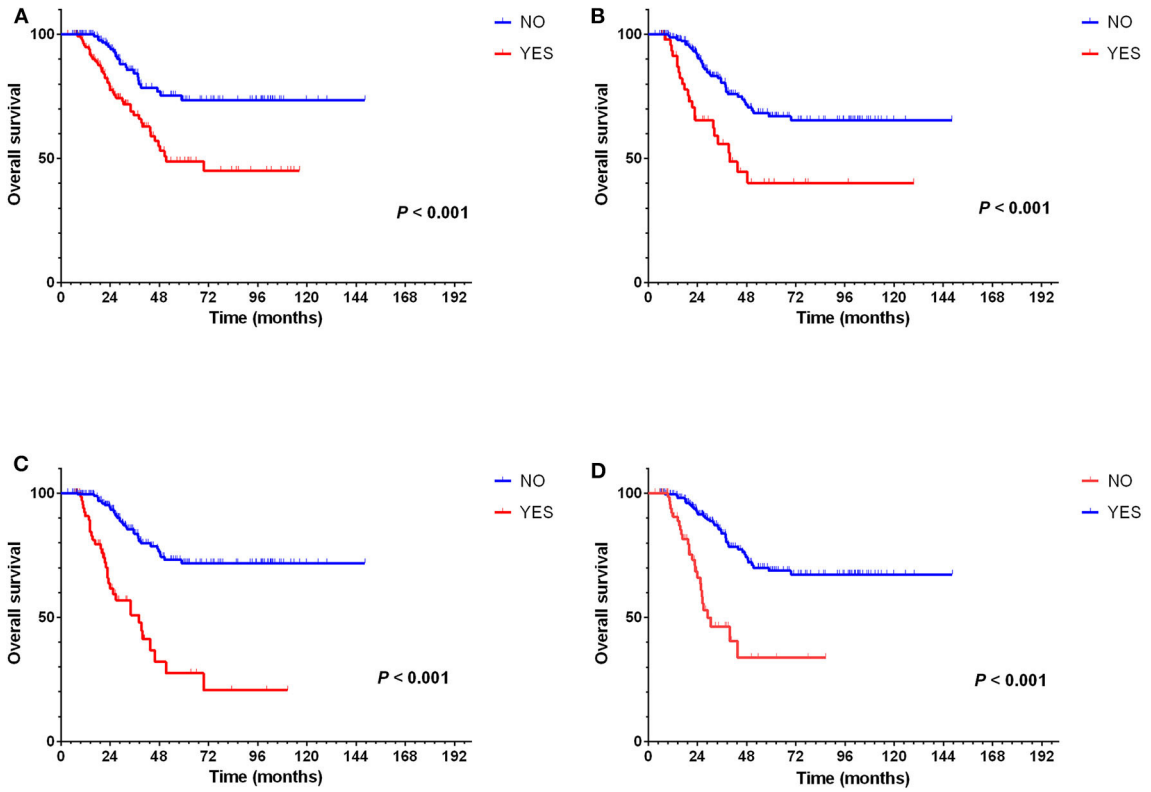

FIGURE 4 | Extra-Milan criteria relapse, size of recurrent tumor, multinodular recurrence, and secondary treatment were prognostic factors for OS but not extra-Milan criteria relapse in relapsed patients. **(A)** Kaplan–Meier curve for OS of relapsed HCC patients stratified by extra-Milan criteria recurrence; **(B)** Kaplan–Meier curve for OS of relapsed HCC patients stratified by size of recurrent tumor; **(C)** Kaplan–Meier curve for OS of relapsed HCC patients stratified by multinodular recurrence; **(D)** Kaplan–Meier curve for OS of relapsed HCC patients stratified by secondary treatment.

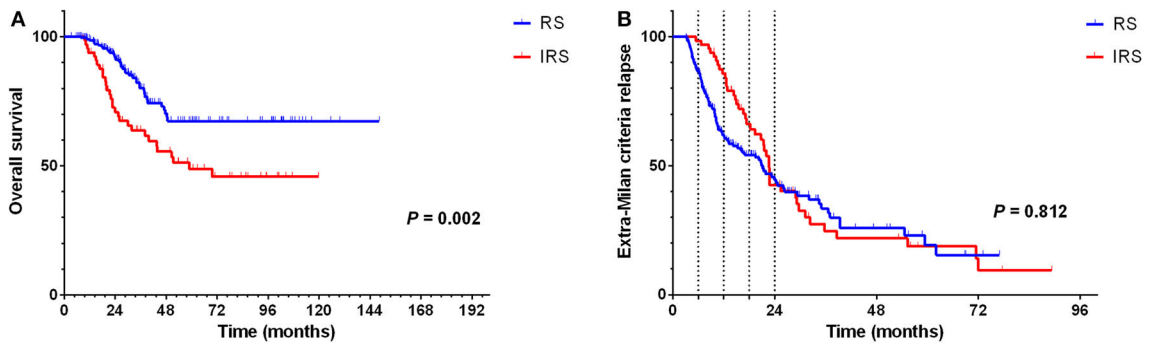

FIGURE 5 | In the RS group, recurrence was significantly prolonged OS in relapsed HCC patients, but there was no difference between the two groups in terms of the extra-Milan criteria relapse. **(A)** Kaplan–Meier curve for OS of relapsed HCC patients stratified by surveillance interval; **(B)** Kaplan–Meier curve for extra-Milan criteria recurrence of relapsed HCC patients stratified by surveillance interval.

relapse in HCC patients (**Figure 2B**) and HCC patients relapse (**Figure 5B**), the interval between 0 and 18 months of surveillance also appeared to be associated with extra-Milan criteria relapse (**Figure 5B**). In the further analysis of patients with extra-Milan criteria relapse in 0–18 months, we found that the RS group could earlier detect extra-Milan criteria relapse ($P = 0.046$, HR = 0.602, 95% CI = 0.366–0.991) (**Figure 6B**), which significantly prolonged OS ($P < 0.001$, HR = 2.893, 95% CI = 1.647–5.082) (**Figure 6A**). Based on this, we further analyzed the surveillance interval of 0–6, 6–12, 12–18, and 18–24 months.

We found that patients with an average surveillance interval ≤2.6 months within 0–6 months could earlier detect extra-Milan criteria relapse ($P = 0.042$, HR = 0.713, 95% CI = 0.515–0.988) (**Figure 7A**). In addition, patients with an average

TABLE 5 | Cox proportional hazard regression analysis of relapsed patients' overall survival.

Variable	Univariable		Multivariable	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Extra-Milan criteria relapse (no/yes)	2.638 (1.597–4.358)	**<0.001**	1.782 (1.032–3.077)	**0.038**
AFP (μg/L) (≤400/>400)	0.912 (0.562–1.480)	0.710		
Relapse location (local/distant)	1.055 (0.688–1.618)	0.805		
Size of recurrent tumor (mm) (≤30/>30)	2.758 (1.661–4.579)	**<0.001**		
Multinodular recurrence (no/yes)	4.682 (2.903–7.552)	**<0.001**		
Secondary treatment (no/yes)	0.261 (0.155–0.439)	**<0.001**	0.335 (0.193–0.581)	**<0.001**
Surveillance interval (RS/IRS)	2.160 (1.338–3.488)	**0.002**	1.309 (0.777–2.207)	0.312

The meaning of the bold values provided was p < 0.05.

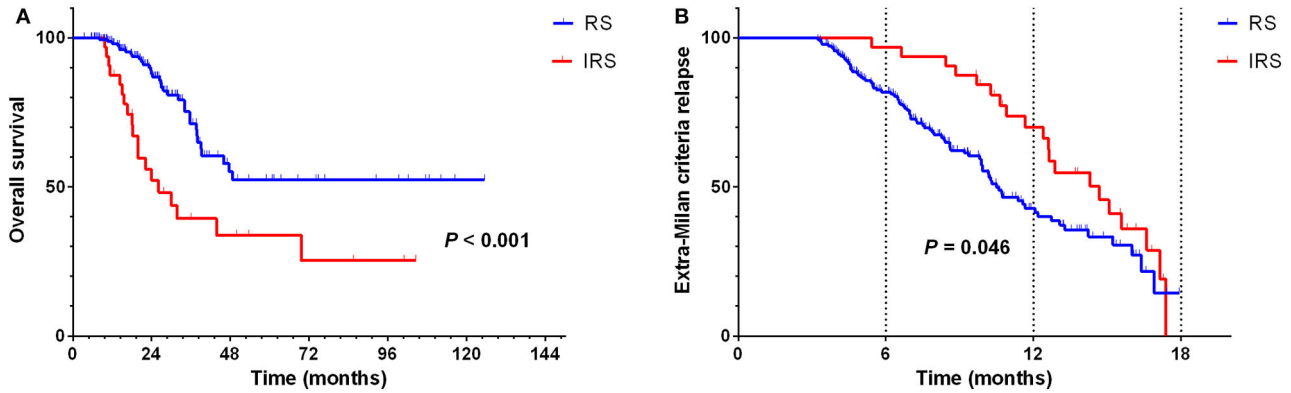

FIGURE 6 | In the RS group, extra-Milan criteria recurrence was detected earlier and significantly prolonged OS in 0–18 months relapsed HCC patients. **(A)** Kaplan–Meier curve for OS of 0–18 months relapsed HCC patients stratified by surveillance interval; **(B)** Kaplan–Meier curve for extra-Milan criteria recurrence of 0–18 months relapsed HCC patients stratified by surveillance interval.

surveillance interval ≤2.9 months within 6–12 months (P = 0.045, HR = 0.593, 95% CI = 0.356–0.989) and an average surveillance interval ≤3 months within 12–18 months (P = 0.002, HR = 0.299, 95% CI = 0.137–0.654) could earlier detect extra-Milan criteria relapse (**Figures 7B,C**). However, there was no significant difference between the average surveillance interval within 18–24 months (P = 0.271, HR = 0.038, 95% CI = 0.000–12.896) (**Figure 7D**).

DISCUSSION

Currently, the European Society for Medical Oncology proposes that follow-up of patients who underwent radical treatments (resection or ablation) should consist of the clinical evaluation of liver decompensation and the early detection of recurrence by dynamic CT or MRI studies every 3 months during the 1st year and surveillance every 6 months thereafter (10). But the National Comprehensive Cancer Network offers a different view, recommending continuous surveillance every 3–6 months, for 2 years, and then every 6–12 months (19). However, these two guidelines are not sufficient to guide clinical practice, in which the follow-up strategies of the two clinical guidelines are quite different in terms of the surveillance interval, not for specific patients. Also, the guidelines do not specifically recommend surveillance intervals for BCLC stage B HCC patients with CR, which were more complicated and had relapses earlier than did those of BCLC stage A. Although patients are recommended for surveillance according to clinical guidelines, in the real world, for a variety of reasons, patients cannot fully follow the guidelines for surveillance strategies. Therefore, the impact of IRS in the real world on patient survival is unclear.

Previous studies have indicated that earlier identification of disease may facilitate patient eligibility for investigational studies or other forms of treatment (19, 20). HY K et al. demonstrated that the detection of small HCC eligible for curative treatment is increased by frequent surveillance (16, 18). Besides, patients in the RS group were diagnosed at earlier stages than the IRS or non-surveillance groups, which had more chance for curative treatments (18). Moreover, AA M et al. also reported that a long surveillance interval compromises OS in high-risk patients who underwent curative thermal ablation for HCC within the Milan criteria (21). Besides, other tumors have the same results for which more intensive surveillance after surgery for esophagogastric adenocarcinoma, colorectal

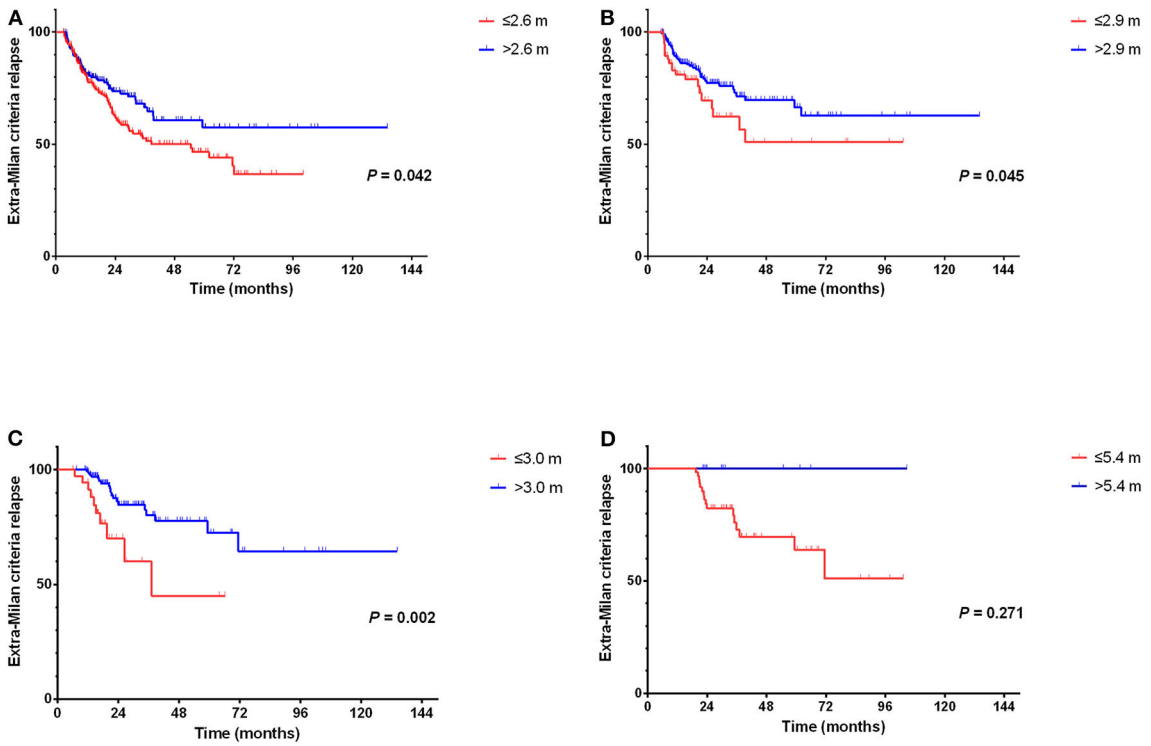

FIGURE 7 | Comparison of surveillance interval and extra-Milan criteria relapse. **(A)** Patients with an average surveillance interval ≤2.6 months within 0–6 months could earlier detect extra-Milan criteria relapse; **(B)** patients with an average surveillance interval ≤2.9 months within 6–12 months could earlier detect extra-Milan criteria relapse; **(C)** patients with an average surveillance interval ≤3.0 months within 6–12 months could earlier detect extra-Milan criteria relapse; **(D)** there was no significant difference between the average surveillance interval within 18–24 months.

cancer, and non–small cell lung cancer translates into improved survival (22–25). Although there is no high-level evidence, the cutoff of 2 years has been adopted to grossly classify early and late recurrences (14, 26). In our result, we also found that the vast majority of patients experienced recurrence within 2 years. Other than that, we also proved that RS owned a lower incidence of extra-Milan criteria relapse and smaller and fewer tumors at recurrence than those of IRS group, which contributed to the prolonged OS. Thereby, the average surveillance interval for patients with BCLC stage B HCC who achieved CR should not exceed 4.3 months during the first 2 years' follow-up.

Over the past 20 years, the Milan criteria have been highly successful in selecting patients for good long-term survival and remain the criteria for potential transplant candidates for HCC (27). It is important to identify the possible predictive factors of within and extra-Milan criteria recurrences after radical treatments (28). Early diagnosis of extra-Milan criteria recurrence can enable patients to receive a more timely intervention after recurrence and control the development of tumors. In our study, we also found that the RS group could earlier detect extra-Milan criteria relapse and significantly prolonged OS in 0–18 months relapsed patients. Moreover, during 0–6, 6–12, and 12–18 months of the initial 18 months after CR, individualized surveillance intervals that no more than 3 months were required to reduce the incidence of extra-Milan criteria relapse. The interval of surveillance according to current guidelines is therefore insufficient, especially 12–18 months after CR.

As mentioned above, despite this study having many clinical implications, we should be clear that it is a retrospective study with its limitations. First, our study was conducted in a single center. The collection of multicenter data to expand the sample size is the next step that needs to be done. Moreover, the follow-up strategy of patients in different stages after radical operation needs to be further explored. Finally, RS could detect tumor recurrence at an early stage and prolong the survival of patients, which requires further clinical trials to verify it.

In conclusion, our results demonstrated that the surveillance interval for BCLC stage B HCC patients with CR after curative treatment should not exceed 4.3 months during the first 2 years' follow-up. Besides, during 0–6, 6–12, and 12–18 months of the initial 18 months after CR, individualized surveillance intervals of no more than 3 months were required to reduce the incidence of extra-Milan criteria relapse.

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 Institutional Review Board of Sun Yat-sen University Cancer Center. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WF: study conception. YW, LS, and HQ: analysis and interpretation of data. All authors: acquisition of data, drafting of manuscript, and final approval.

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frontiers in Oncology

ORIGINAL RESEARCH
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Hepatocarcinoma Induces a Tumor Necrosis Factor-Dependent Kupffer Cell Death Pathway That Favors Its Proliferation Upon Partial Hepatectomy

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Partial hepatectomy (PH) is the main treatment for early-stage hepatocellular carcinoma (HCC). Yet, a significant number of patients undergo recursion of the disease that could be linked to the fate of innate immune cells during the liver regeneration process. In this study, using a murine model, we investigated the impact of PH on HCC development by bioluminescence imaging and flow cytometry. While non-resected mice were able to control and reject orthotopic implanted Hepa1-6 hepatocarcinoma cells, resected liver underwent an increased tumoral proliferation. This phenomenon was associated with a PH-induced reduction in the number of liver-resident macrophages, i.e., Kupffer cells (KC). Using a conditional ablation model, KC were proved to participate in Hepa1-6 rejection. We demonstrated that in the absence of Hepa1-6, PH-induced KC number reduction was dependent on tumor necrosis factor-alpha (TNF-α), receptor-interacting protein kinase (RIPK) 3, and caspase-8 activation, whereas interleukin (IL)-6 acted as a KC pro-survival signal. In mice with previous Hepa1-6 encounter, the KC reduction switched toward a TNF-α-RIPK3–caspase-1 activation. Moreover, KC disappearance associated with caspase-1 activity induced the recruitment of monocyte-derived cells that are beneficial for tumor growth, while caspase-8-dependent reduction did not. In conclusion, our study highlights the importance of the TNF-α-dependent death pathway induced in liver macrophages following partial hepatectomy in regulating the antitumoral immune responses.

Keywords: Kupffer cells, hepatocellular carcinoma, liver regeneration, partial hepatectomy, cell death, inflammation, tumor necrosis factor-alpha

INTRODUCTION

Primary liver malignancy constitutes one of the most common forms of cancers worldwide associated with a high mortality rate (1). Hepatocellular carcinoma (HCC) accounts for up to 90% of these malignancies (2) and, therefore, constitutes a major health issue. Partial hepatectomy (PH) is a commonly used curative therapy for HCC (3) with good results at early stage (4) and can even lead to better results than transcatheter arterial chemoembolization within patients carefully selected beyond the traditional Milan criteria (5). While hepatic resection is considered as a treatment of choice, a significant number of patients undergo recursion of the disease (6, 7). Recurrence can be either due to the formation of *de novo* tumoral site or to the presence of an ignored cryptic tumoral site not removed during surgery. Relapse constitutes a bad prognostic for the patient and the available therapeutic options might get limited depending on the anatomical location of the tumor, actual liver functions, and general status of the patient. Therefore, the development of strategies aimed at reducing the risk of recursion is a paramount element of the surgery-based approach. Following PH, liver regeneration (compensatory hypertrophy and hyperplasia without restoration of the original anatomical shape) occurs, aiming at re-establishing the numerous physiological functions of the organ. Various signaling molecules and pathways are activated during liver regeneration (including mitogen-activated protein kinases, phosphoinositide 3-kinases, insulin-like growth factor, and hepatocyte growth factor pathways) and participate in the process (8, 9). Yet, major alterations of these pathways are linked with the development and progression of liver cancers (10–12). Immune cells play a key role in driving and participating in the activation of the complex process leading to the compensatory hyperplasia of hepatocytes. Most of the studies on liver regeneration have focused on deciphering the mechanisms leading to hepatocyte proliferation in the absence of pathology. Therefore, the impact that PH has on immune cells and how it affects tumor recurrence are still not fully understood. In a normal context, Kupffer cells (KC) drive the early response to liver partial ablation by producing tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 that in turn stimulate hepatocyte proliferation through activation of nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathway, respectively (13–16). Both of these cytokines are associated with tumor aggressiveness and metastasis (10, 11). Phosphorylated STAT3 (i.e., activated) has been found in a majority of human HCC, and this activation was associated with tumor aggressiveness and poor prognosis (17). As for NF-κB, its inhibition in different mouse models of HCC was associated with limited tumor development (18). It is therefore expected that tumor cells would be able to use TNF-α and IL-6 signaling to their own advantage. On the other hand, NF-κB's ability to maintain antioxidant defenses can also contribute to reduce liver damage (18), and in diethylnitrosamine-induced HCC, NF-κB participates in the maintenance of hepatocyte survival resulting in limiting cancer development (19). TNF-α is also a cell death inducer and a pro-inflammatory cytokine that can activate immunity. Indeed, signaling through TNFR1 can lead, under specific conditions, to the formation of a protein complex containing RIPK1 and RIPK3, which can either lead to the phosphorylation of mixed lineage kinase-like (MLKL) and the induction of necroptosis or the activation of caspase-8 and subsequent induction of apoptosis (20). Finally, aggression of the liver can lead to monocyte and monocyte-derived cell recruitment. This is notably the case in other liver injury models such as acetaminophen-induced liver fibrosis model where monocyte-derived cells with a different phenotype than KC (referred here as $Ly6C^{low}$ macrophages) can be found and participate in the remodeling of the organ (21). Such population's recruitment following PH and impact on the recurrence phenomenon is also poorly described.

In the present study, we used an *in vivo* mouse model of HCC and PH combined with bioluminescence imaging to study the impact of PH on primary Hepa1-6 HCC development. We demonstrated the protective role of KC in this setup using conditional ablation and further analyzed the *in vivo* mechanisms modifying the innate immune response toward a tumor-favorable environment following PH.

Abbreviations: KC, Kupffer cells; TNF, Tumor necrosis factor-α; RIPK3, Receptor-interacting protein 3 kinase; PH, partial hepatectomy; H, hepatectomy.

MATERIALS AND METHODS

Mice

Eight- to 12-week-old male C57BL/6 mice were used (ENVIGO, Zeist, Netherlands). IL-6 KO and CCR2 KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). RIPK3 KO mice were provided by Peter Vandenabeele (Inflammation Research Center, VIB, Ghent, Belgium). Myeloid TNF KO mice (TNF^{M-KO} mice; TNFflox/flox LysMcre/cre mice) were provided by Sergei Nedospasov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences and Lomonosov Moscow State University, Moscow, Russia) and KC-DTR mice were mated in our specific pathogen-free animal facility (Gosselies, Belgium). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 86-23 revised 1985).

Hepa1-6 and Hepa1-6-Fluc Cell Lines

Mycoplasma-free Hepa1-6 cells (ATCC) and Hepa1-6-Fluc cells generated through transduction with lentiviral vectors encoding firefly luciferase (transfer plasmid pDUAL_SFFV-Fluc_Ub-puroR) were cultured in Dulbecco's modified Eagle's medium (DMEM/Lonza, BioWhittaker™) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM non-essential amino acids, 100 mM sodium pyruvate, penicillin (10 U/ml)–streptomycin (10 μg/ml), 10–5M 2-ME (Lonza Research Products, Basel, Switzerland), and puromycin (5 μg/ml, Sigma-Aldrich).

Surgical Procedure for Orthotopic Tumor Implantation and Partial Hepatectomy

Mice were injected with Hepa1-6-Fluc cells 1 week before partial hepatectomy (H+PH group). The control group did not undergo surgery (H group). For flow cytometry experiments, a third group of control mice underwent phantom operation (sham group). Mice were then used either for bioluminescence imaging or flow cytometry experiments. For experiments investigating partial hepatectomy, mice underwent 40% partial hepatectomy and their liver was collected at various time points following surgery.

Under anesthesia (xylazine 50 mg/kg and ketamine 100 mg/kg), a small midline laparotomy was performed on prehydrated (0.9% NaCl, 200 μl) mice. For tumor inoculation, the median lobe of the liver was exposed and injected under the Glisson's capsule with 10^6 Hepa1-6 cells suspended in 50 μl PBS. For partial hepatectomy, the left lobe of the liver was ligated and resected. Body temperature was maintained at 36.5–37°C during the surgical procedures. The abdominal wall and the skin were sutured separately. Sham-operated mice underwent the same procedure with 50 μl PBS injection and without ligation and resection of the left lobe of the liver.

KC Depletion

KC-DTR mice were intraperitoneally injected with 2 or 5 ng of diphtheria toxin (Sigma) 7 days after intrahepatic Hepa1-6-Fluc inoculation.

Bioluminescence Imaging

In vivo follow-up was performed after tumor inoculation and carried over the 4 weeks following surgery or phantom operation. Mice were anesthetized with 4% of isoflurane vaporized in 2 L/min O_2 and then maintained with 2% isoflurane in 0.3 L/min O_2 per mouse. Before imaging, mice were shaved to decrease the light absorption and scattering of animal fur. Each animal received s.c. 150 mg/kg body weight of a 20-mg/ml solution of D-luciferin in a 20-mg/ml solution in NaCl 0.9% (VivoGlo, Promega). Mice were imaged in a Photon Imager Optima (Biospace Lab, France) that dynamically counted the emitted photons for at least 25 min. Image analysis was performed with M3Vision software (Biospace Lab). ROIs were drawn on the mice abdomen in the liver area and signal intensities were quantified individually for a time lapse of 5 min corresponding to the maximum signal intensity plateau.

Flow Cytometry

Livers were collected at various time points following resection or phantom operation (24 h, 36 h, 2 days, or 7 days). The liver lobes were weighted and transferred into gentleMACS tubes (Miltenyi Biotec, Leiden, Netherlands) supplemented with RPMI 1640 medium and collagenase A (type III, Worthington Biochemicals, New Jersey, USA) and DNase I (Roche) for one round of the m_liver_01_03 protocol of the gentleMACS dissociator (Miltenyl Biotec). After 20 min at 37°C, tubes completed the m_liver_02_03 protocol of the same dissociator. The obtained suspension was diluted in FACS buffer and passed through a sterile 100-μm filter, centrifuged (1,400 rpm, 7 min at 4°C), and resuspended for 1 min in ammonium–chloride–potassium lysis buffer.

Caspase-8 and caspase-1 activity assays were performed following the manufacturer's protocol. Cells were incubated with FAM-FLICA (Bio-Rad AbD) for 1 h before proceeding to standard extracellular staining. Propidium iodide (2 μl) from the same kit (Bio-Rad AbD) was used for staining 15 min at room temperature prior to standard extracellular staining.

For standard extracellular staining, cells were resuspended and stained in the dark at 4°C for 20 min with polyclonal unconjugated anti-Clec4F antibodies (R&D Systems). Samples were then incubated in the dark at 4°C for 20 min with a mix of antibodies purchased from BD Biosciences (CD45, Ly6G, Ly6C, CD11b, CD11c), eBioscience (F4/80, Tim4), BioLegend (PDCA1), and Invitrogen (secondary antibody for Clec4F detection).

For intracellular staining of p-MLKL (Ser345), cells were fixed using the Foxp3 kit from eBioscience. Cell permeabilization and fixation were run in accordance with the manufacturer's protocol. After washing, cells were incubated at 4°C for 20 min in the dark with a primary p-MLKL (Ser345) (D6E3G) antibody (Cell Signaling Technology). After a washing step, a secondary detection antibody (anti-rabbit IgG FabAlexa Fluor® 488 Cell Signaling Technology) was incubated with the cells at 4°C for 20 min in the dark. For intracellular staining of IL-6 and TNF, cells were incubated with BD GolgiPlug™ (1 μl/ml), phorbol 12-myristate 13-acetate (5 ng/ml), and ionomycin (500 ng/ml) for 4 h at 37°C prior to staining of extracellular markers. After extracellular staining, cells were fixed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. Experiments were run in accordance with manufacturer's protocol. Cells were incubated at 4°C for 30 min in the dark with anti-TNF, anti-IL6, or control isotype (BD Biosciences). Samples were measured using the BD LSR Fortessa™ (BD Bioscience, Erembodegem, Belgium). The total amount of cells passed for each sample varies from 700,000 to 1,400,000 cells. Data were analyzed using the FlowJo V9.9.6 software (FlowJo, Ashland, USA).

RNA Purification and Real-Time Reverse Transcription Polymerase Chain Reaction

Liver was collected at various time points following partial hepatectomy or phantom operation (0 min, 30 min, 1 h, 2 h, overnight). RNA was extracted from liver lobes using an EZNA HP Total RNA Kit (Omega Bio-tek, Georgia, USA). Extracted RNA samples were quantified using the NanoDrop™ spectrophotometer and stored at −20°C before being used for reverse transcription quantitative polymerase chain reaction (qPCR). For the quantification of transcripts, reverse transcription and qPCR were performed in a single step using the TaqMan RNA Amplification (Roche Diagnostics) on a Lightcycler 480 apparatus (Roche Diagnostics) with the following conditions: 10 min at 50°C, 10 min at 45°C and 30 s at 95°C, and then 45 cycles of 5 s at 95°C and 30 s at 60°C. For the granulocyte macrophage-colony stimulating factor (GM-CSF) gene, RNA was reverse-transcribed with the transcriptor High fidelity cDNA

synthesis (Roche Diagnostics). cDNA was amplified using SYBR green. For individual samples, relative RNA levels (2–ΔΔCt) were determined by comparing a) the cycle thresholds (Ct) for the gene of interest and calibrator gene (ΔCt), Hprt, and b) 2–ΔCt values for the experimental group vs. the reference sample (H group). The sequences of primers and probes are presented in **Table 1**.

Histology

Formalin-fixed hepatic lobes of interest were embedded in paraffin. Five-micrometer liver sections were stained with hematoxylin/eosin (HE). Conversion of glass slides into digital data was performed using a NanoZoomer 9200S (Hamamatsu Photonics K.K.). Determination of tumor size was performed on a digital slide using the NDP.view2 software (Hamamatsu Photonics K.K.).

Statistical Analysis

Statistical comparison between experimental groups was made using GraphPad Prism (GraphPad Software, Inc.). The nature of the test used is described in the figure legends. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Partial Hepatectomy Favors the Proliferation of Hepa1-6 Cells in the Liver

In order to evaluate the impact of PH on tumoral development, we used a preclinical model based on the injection of a murine hepatocellular carcinoma cell line (Hepa1-6 cells) in the liver median lobe. One week later, the experimental group of mice underwent PH by resection of their left lobe, accounting for 40% of the total liver mass (H+PH group), and the control group underwent phantom operation (H group) (**Figure 1A**). Tumor implantation was confirmed by histological observations in wild-type animals. We observed that PH increases the tumoral burden as monitored by the increased median size of tumor foci and higher global size of the tumor (**Figures 1B,C**). We further quantified the impact of PH on tumoral development by inoculating Hepa1-6 cells expressing the firefly luciferase enzyme (Hepa1-6-Fluc cells) in the liver median lobe, which allowed for *in vivo* follow-up of tumor over time. As described by Sakai et al. (22), the tumoral development of Hepa1-6-Fluc cells in the control group of mice was halted after a first week period of proliferation (**Figures 1D,E**). Concerning the impact of PH on tumor growth, bioluminescence signal in the PH group was significantly increased 4 and 7 days after PH and underwent a delayed abrogation after 21 days instead of 7 days in the H group of mice (**Figures 1D,E**), indicating that tumor cell proliferation is uncontrolled during liver regeneration, a process that in a murine model takes about 1 week for completion [(8, 23) and **Supplementary Figure 1**].

Partial Hepatectomy Modifies Innate Immune Cell Composition in the Liver

We next sought to determine the possible causes of the increased tumor proliferation we observed following PH. Since the regeneration process is known to be relying on innate immune cells and that KC are important gatekeepers of liver physiology, we evaluated their absolute numbers and proportion in total leukocyte population (determined by the expression of CD45) using flow cytometry (gating strategies are described in **Figure 2A**). First, we observed that the number of KC ($CD45^+$ $CD11b^{int}$ $F4/80^+$ $Tim4^+$ $Clec4F^+$ $Ly6G^-$ cells) progressively increased and reached maximal value 9 days after tumor inoculation in the H group of mice compared with the sham group of mice (placebo treatment). A drastic decrease of KC number was observed at day 2 post PH in the H+PH group compared with the H group (**Figure 2B**). Then, both groups had significantly lowered KC number compared with the sham group 14 days post tumor inoculation (**Figure 2B**). Analysis of the proportion of KC in $CD45^+$ cells confirmed the reduction observed 2 days after PH (**Figure 2C**). Yet, the increased absolute number of cells observed in the H group at the same timing did not translate to an increased proportion in $CD45^+$ cells, indicating that other $CD45^+$ populations were recruited in this experimental condition at that time. The absolute number of monocytes ($CD45^+$ $CD11b^{high}$ $Ly6C^{high}$ $F4/80^-$ $Ly6G^-$ cells) also progressively increased and reached maximal value 9 days after tumor inoculation in the H group compared with the sham and H+PH groups of mice. As for KC on that day, this increased absolute number did not translate to an increase in the proportion in $CD45^+$ cells. Two significant increases of monocyte number were observed in the H+PH group at day 1 post PH when compared with the sham group and at day 7 post PH when compared with the H and sham groups. The same type of observations was made in the proportion of $CD45^+$ cells with a significant increase observed at day 1 and day 7 post PH (**Figures 2B,C**). Concerning the $Ly6C^{low}$ macrophages ($CD45^+$ $CD11b^+$ $Ly6C^{int}$ $Ly6G^-$ $CD11c^-$ $pDCA1^-$), their number increased significantly at day 7 post PH in the H+PH group compared with the H group of mice (**Figure 2B**). This observation corroborated the one made when analyzing the proportion of these cells in $CD45^+$ population. Moreover, a significantly higher proportion of $Ly6C^{low}$ macrophages were also observed as early as day 2 post PH (**Figure 2C**). In contrast to KC, both numbers of monocytes and $Ly6C^{low}$ macrophages were increased compared with the sham group 7 days post PH.

Taken together, our results demonstrate that PH induced a drop in KC number in tumor-bearing liver and an "earlier" monocyte influx as well as another "delayed" monocyte recruitment that correlates with a late increased number and proportion in $CD45^+$ cells of $Ly6C^{low}$ macrophages in the organ.

KC Depletion Results in an Increased Hepa1-6 Proliferation

The reduction of KC observed in mice undergoing PH associated with the increased tumor proliferation raised the possibility that they would be important agents in the antitumoral process. Macrophages are known for their dual role during cancer development, and while resident cells can limit its progression in early stages, tumor-associated macrophages derived from monocytes found at a later time point have an anti-inflammatory

TABLE 1 | The sequences of primers and probes.

Gene	Forward	Reverse	Probe	PCR product
MCP1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA	CTCGCCAGATGCAGTTAACGCCCC	127 bp
GM-CSF	ACCCGCCTGAAGATATTCG	AGCTGGCTGTCATGTTCAAG	/	69 bp
IL6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	CAGAATTGCCATTGCACAACTCTTTTCTCA	140 bp
TNFα	CAGACCCTCACACTCAGATCA	CACTTGGTGGTTTGCTACGA	TCGAGTGACAAGCCTGTAGCCCA	78 bp
HPRT	GGACCTCTCGAAGTGTTGGAT	CCAACAACAAACTTGTCTGGAA	CAGGCCAGACTTTGTTGGATTTGAA	70 bp

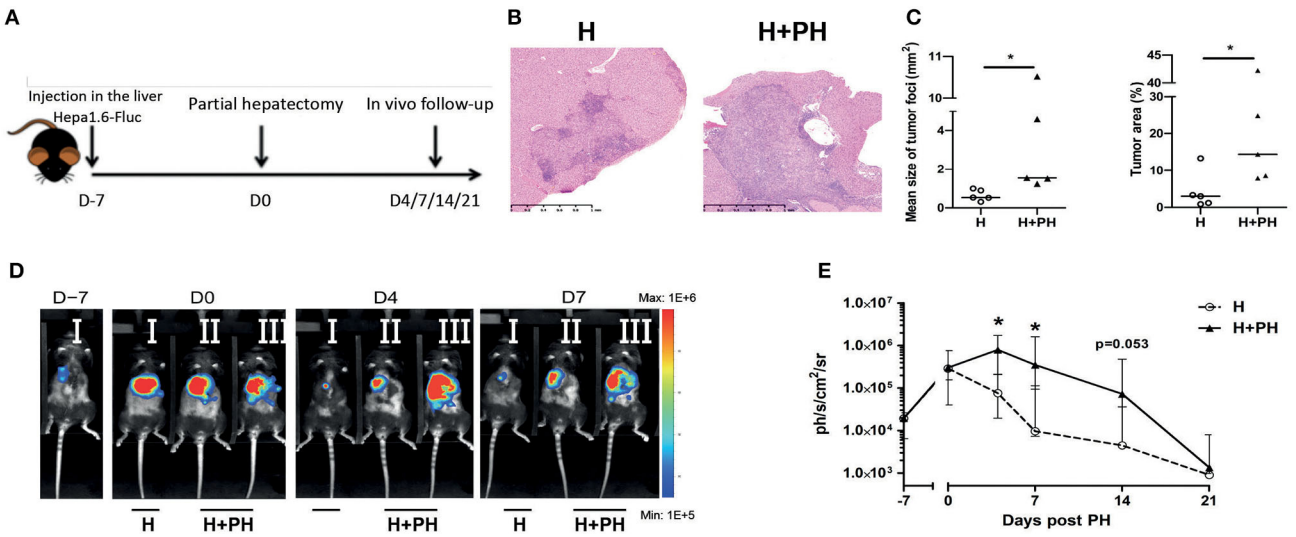

FIGURE 1 | Proliferation of Hepa1-6 cells is increased following partial hepatectomy. **(A)** Experimental procedure used. Mice were injected with Hepa1-6-Fluc cells 1 week before partial hepatectomy (H+PH group). The control group did not undergo surgery (H group). **(B)** Liver histology visualized by HE staining. Representative pictures of the nonresected (H) and resected (H+PH) experimental groups. **(C)** Quantification of tumor burden by mean size of tumor foci (left panel) and percentage of tumor on a slide (right panel) in wild-type animals. **(D)** Representative image of the bioluminescence data generated until 7 days post PH. **(E)** Bioluminescence signal detection over time following partial hepatectomy. Two-tailed Mann–Whitney test, $*p < 0.05$. Results presented as median and interquartile range ($n = 7$/group) from at least three independent experiments.

"alternatively activated" (M2) phenotype. This is notably known in humans as HCC where later-stage patients with poor prognosis have increased M2 macrophages infiltrating the tumor (24). Nevertheless, PH is a treatment reserved for early-stage patients and it is thus expected that liver-resident KC would limit cancer development. To evaluate the impact of the decreased KC number on tumor growth, we mimic the effect of PH-induced KC disappearance by using KC-DTR mice previously described by Scott et al. (25). This strain has the unique characteristic of being specifically depleted in the KC compartment after diphtheria toxin (DT) injection in a dose-dependent manner (25). We used a single 2-ng injection of DT (**Figure 3A**) that does not cause side effects on the liver regeneration post PH as evaluated by the liver to body weight ratio in WT mice (**Supplementary Figure 2A**). That DT dose caused a partial decrease of KC numbers (**Supplementary Figure 2B**), which went back to pre-injection level 1 week following the injection (**Figure 3B**). This recovery was associated with a decreased number of monocytes in the organ 2 days post DT injection. No significant modifications in the Ly6C^{low} macrophage compartment could be observed at tested timing (**Figure 3B**), demonstrating that our model specifically induces modifications in the KC compartment.

Next, we observed a significantly increased proliferation of Hepa1-6-Fluc cells during the first week following the 2-ng DT injection, while PBS-injected mice naturally rejected the tumor (**Figures 3C,D**), effectively recapitulating the previous observations in mice undergoing liver regeneration (**Figure 1**). Taken together, our results indicate that the reduction of the absolute number of KC observed in mice undergoing liver regeneration might, at least partially, explain the increased tumoral proliferation observed in this condition, raising the question on the mechanisms leading to KC disappearance.

TNF and IL-6 Influence KC Survival During Liver Regeneration and Impact the Tumor Proliferation

We next wanted to decipher the mechanism linking the KC number reduction and the increased tumor growth induced upon PH. We observed that the transcript levels of IL-6 and TNF, two major actors of liver regeneration, were significantly increased

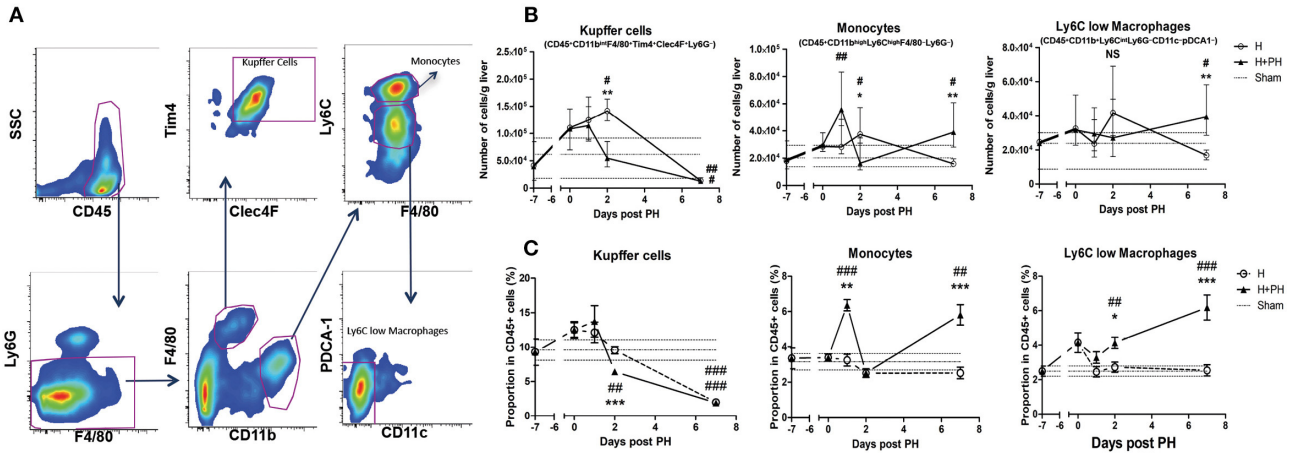

FIGURE 2 | Absolute number and proportion of Kupffer cells among CD45$^+$ cells are reduced following PH, while monocytes and Ly6C^{low} macrophages increase after surgery. **(A)** Representative picture of the gating strategies used for the discrimination of Kupffer cells, monocytes, and Ly6C^{low} macrophages. **(B)** Kinetics of the number of Kupffer cells, monocytes, and Ly6C^{low} macrophages following PH in C57BL/6 mice liver. Flow cytometry analyses were done 0, 1, 2, and 7 days following PH (H+PH group) or phantom operation (H group). The sham group was injected with physiological serum and received placebo surgery 7 days later. $^*p < 0.05$, $^{**}p < 0.01$, Kruskal–Wallis/one-way ANOVA followed by Dunn's *post hoc* test/Bonferroni's multiple comparison test between the H and H+PH groups/$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, Kruskal–Wallis/one-way ANOVA followed by Dunn's *post hoc* test/Bonferroni's multiple comparison test between the sham and H and H+PH groups (n = 4–9/time point). Choice of the test was based on the result of the Shapiro–Wilk normality test. Results presented as median and interquartile range from at least three independent experiments. **(C)** Kinetics of the proportion among leukocytes (CD45$^+$ cells) of Kupffer cells, monocytes, and Ly6C^{low} macrophages following PH in C57BL/6 mice liver. Flow cytometry analyses were done 0, 1, 2, and 7 days following PH (H+PH group) or phantom operation (H group). The sham group was injected with physiological serum and received placebo surgery 7 days later. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Kruskal–Wallis/one-way ANOVA followed by Dunn's *post hoc* test/Bonferroni's multiple comparison test between the H and H+PH groups/$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, Kruskal–Wallis/one-way ANOVA followed by Dunn's *post hoc* test/Bonferroni's multiple comparison test between the sham and H and H+PH groups (n = 4–9/time point). Choice of the test was based on the result of the Shapiro–Wilk normality test. NS, not significant.

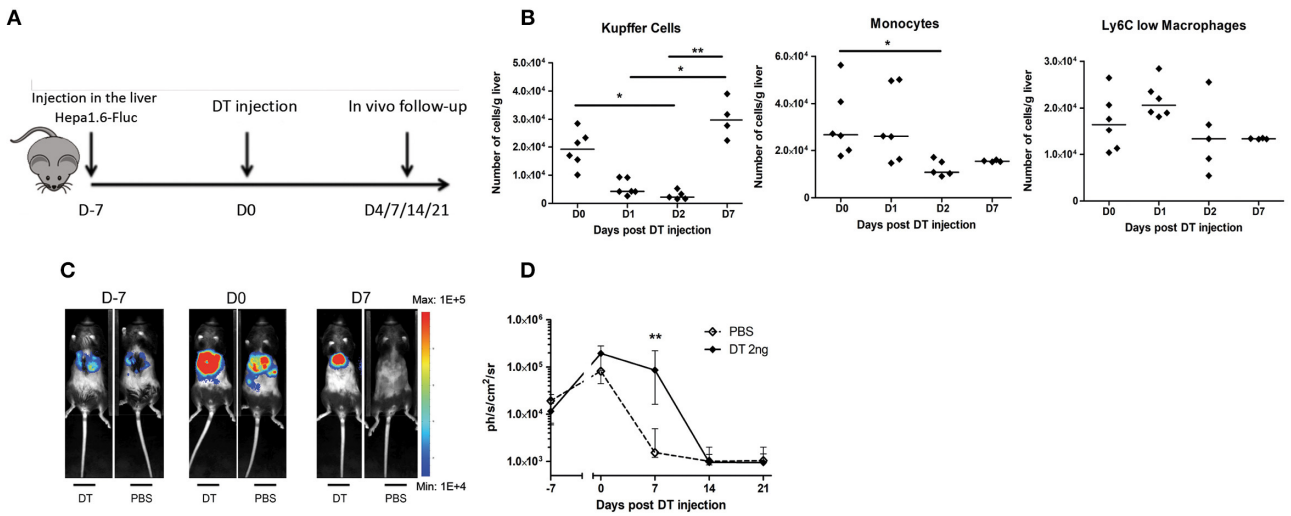

FIGURE 3 | Proliferation of Hepa1-6 cells is increased following Kupffer cell depletion. **(A)** Experimental procedure used. Mice were injected with Hepa1-6-Fluc cells 1 week before intraperitoneal injection of diphtheria toxin (DT 2 ng group) or PBS. **(B)** Kinetics of the absolute number of KC, monocytes, and Ly6C^{low} macrophages in KC-DTR mice after 2-ng DT injection. $^*p < 0.05$, $^{**}p < 0.01$, Kruskal–Wallis followed by Dunn's *post hoc* test. Results presented as median. **(C)** Representative image of the bioluminescence data generated until 7 days post injection. **(D)** Bioluminescence signal detection over time following injection. Two-tailed Mann–Whitney test, $^{**}p < 0.001$. Results presented as median and interquartile range (n = 7–8/group) from at least three independent experiments.

in the partially resected liver 1 h post surgery (**Figure 4A**). This increase was also observed at protein level 24 h after surgery. Intracellular staining of IL-6 and TNF revealed a significant increase of the median fluorescence intensity and the proportion of CD45$^+$ cells positively stained following PH as compared with the control group (**Figures 4B,C**). We also observed that following PH, the myeloid compartment of CD45$^+$ cells (discriminated on the basis of CD11b expression) had a significantly higher proportion of cells positively stained for both cytokines than the CD11b$^-$ cells (**Figure 4D**).

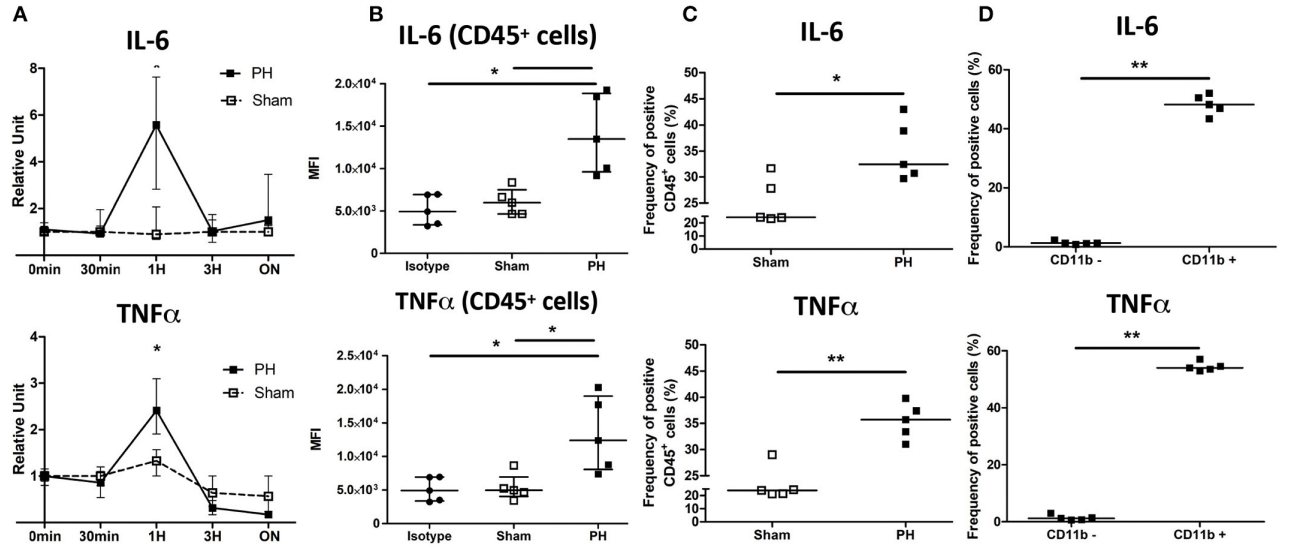

FIGURE 4 | IL-6 and TNF are induced following partial hepatectomy. **(A)** Relative expression in total liver of C57BL/6 mice of IL-6 and TNF over time following PH or phantom sham operation. *$p < 0.05$, two-tailed Mann–Whitney test ($n = 3$–7/time point). **(B)** Median fluorescence intensity (MFI) of C57BL/6 CD45$^+$ cells stained for TNF, IL-6, or control isotype (isotype group) 24 h following partial hepatectomy (PH group) or phantom operation (sham group). *$p < 0.05$, Kruskal–Wallis followed by Dunn's *post hoc* test. Results presented as median and interquartile range. **(C)** Frequency of CD45$^+$ cells producing TNF or IL-6 24 h following partial hepatectomy (PH group) or phantom operation (sham group). Determination of positive gate was based on isotype control staining. Results presented as median. *$p < 0.05$, **$p < 0.01$, two-tailed Mann–Whitney test. Results presented as median. **(D)** Frequency of CD11b$^-$ cells and CD11b$^+$ producing TNF or IL-6 24 h following partial hepatectomy. Cells were firstly gated for CD45 expression. Determination of positive gate was based on isotype control staining. **$p < 0.01$, two-tailed Mann–Whitney test. Results presented as median.

First, we evaluated the role of IL-6 in the outcome of KC post PH. We observed that IL-6 KO mice displayed a faster decrease in KC numbers 24 h following resection (**Figure 5A**), whereas WT mice underwent a decrease 2 days post PH. This indicates that IL-6 would act as a KC cytoprotective factor. Moreover, tumoral proliferation was strongly increased in the H+PH group of IL-6 KO mice compared with the H group 7, 14, and 21 days post PH (**Figure 5B**). Of note, in this strain compared with WT mice, both the H and H+PH groups failed at rejecting the tumor even at day 21, strengthening the role of IL-6 as a major early contributor to KC survival and to the liver protection against tumor proliferation.

Next, we evaluated the role of TNF and the RIPK3 cell death-associated signaling molecule. To do this, we ran PH experiments in $TNF^{flox/flox}LysM^{Cre/WT}$ mice (TNF^{M-KO} mice with exclusive *Tnf* gene deletion in lysozyme M-expressing myeloid cells like monocytes, neutrophils, and macrophages) and in RIPK3 KO mice. We observed that the PH-induced KC disappearance observed in C57BL/6 mice was abrogated in TNF^{M-KO} mice and in RIPK3 KO mice (**Figure 5B**). Interestingly, we observed that the TNF^{M-KO} and RIPK3 KO strains showed no increased Hepa1-6-Fluc cell proliferation after PH (**Figure 5C**).

Liver Resection Induces a RIPK3-Dependent Activation of Caspase-8 in KC

We next sought to determine the mechanism responsible for the TNF-dependent KC reduction following PH (i.e., KC apoptosis via caspase-8, necroptosis via phosphorylation of MLKL, or pyroptosis via caspase-1). In assays determining the functional ability of the protein, we detected an increased caspase-8 activity but no increased levels of caspase-1 and p-MLKL (Ser345) in WT mice KC following PH (**Figure 6A**). Neither increased caspase-8 activation nor increased level of p-MLKL (Ser345) was observed in RIPK3 KO KC under the same condition (**Figure 6B**). Taken together, our results support the idea that following PH, KC undergo a TNF/RIPK3-dependent apoptosis resulting in a reduction of their number in the organ.

Tumor Encounter Switches TNF/RIPK3-Dependent Activation of Caspase-8 to TNF/RIPK3-Dependent Activation of Caspase-1 in KC Following PH

We further evaluated the role of TNF/RIPK3 in the fate of KC post PH in tumor-inoculated mice. While PH-induced KC disappearance was still abrogated in H+PH TNF^{M-KO} mouse, we observed a sharp drop of KC in H+PH RIPK3 KO mice 2 days post PH (**Figure 7A**), in opposition with KC survival in tumor-free RIPK3 KO mice after PH (**Figure 5A**). This suggested that Hepa1-6 had an impact on the cell death signaling pathway. Indeed, in contrast with KC from WT mice undergoing PH (**Figure 6A**), KC from the H+PH group of WT mice were no longer associated with an increase in caspase-8 activity nor increased level of p-MLKL (Ser345) post PH (**Figures 7B,C**). Yet, an increased fraction of KC was positively stained with propidium iodide at the same timing (**Figure 7D**), indicating

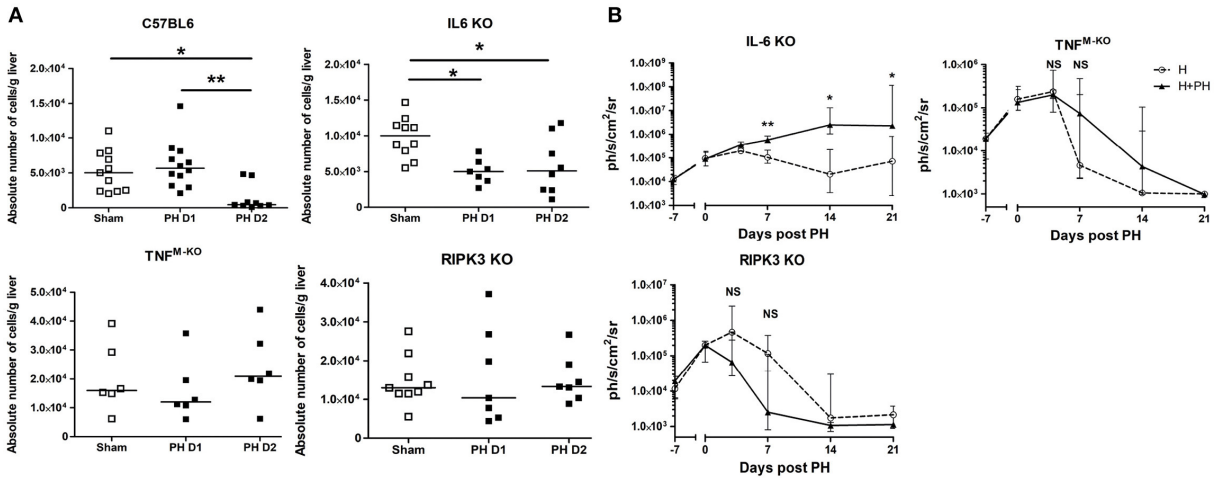

FIGURE 5 | PH-induced Kupffer cell number reduction is regulated by TNF-α and IL-6. **(A)** Absolute number of KC in C57BL/6, IL-6 KO, TNF^{M-KO}, and RIPK3 KO mice. Analyses were run at day 1 or 2 following PH. The control sham group underwent phantom operation. **(B)** Bioluminescence signal detection over time following partial hepatectomy in IL-6 KO, TNF^{M-KO}, and RIPK3 KO mice. Two-tailed Mann–Whitney test, $*p < 0.05$, $**p < 0.01$, NS: non-significant difference ($n = 7$–8/group). All results presented as median and interquartile range from at least three independent experiments.

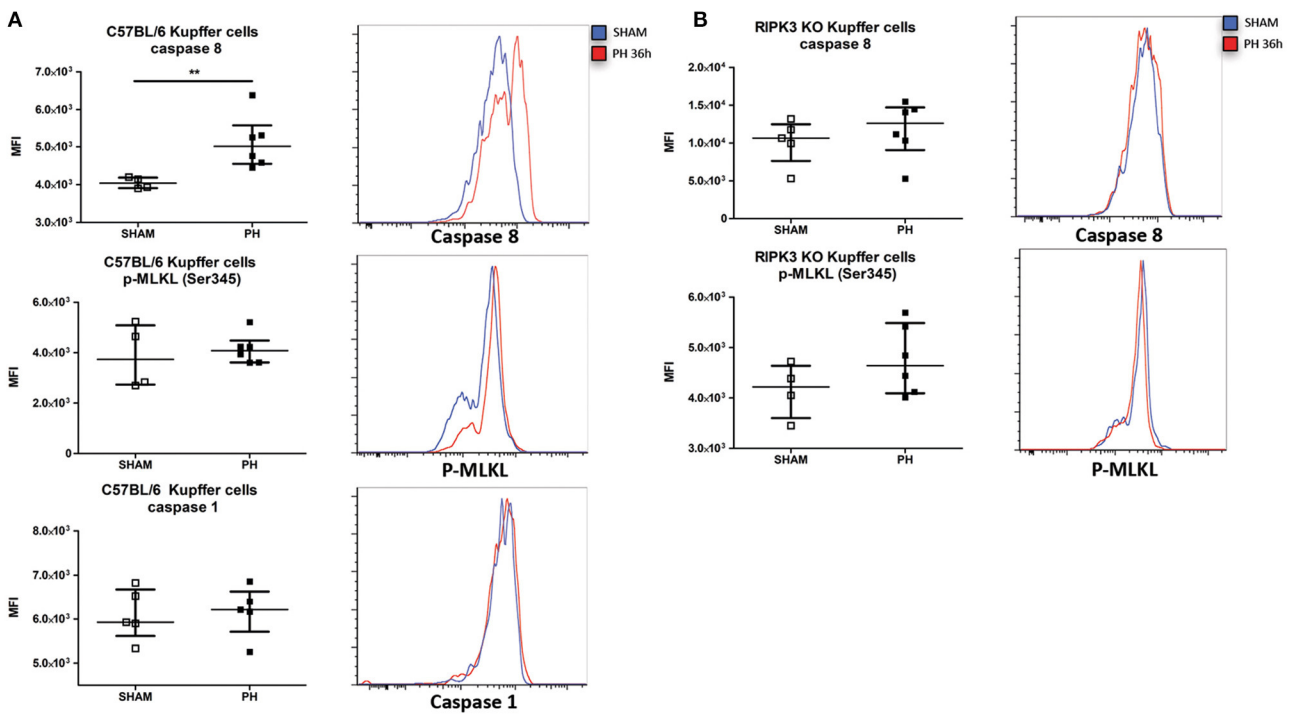

FIGURE 6 | PH induces a RIPK3-dependent apoptosis of Kupffer cells. (**A**, upper panel) Median fluorescence intensity (MFI) of C57BL/6 KC stained with fluorescent-labeled inhibitor of caspase-8 36 h post PH and representative histogram of the fluorescence intensity. $**p < 0.01$, two-tailed Mann–Whitney test. (**A**, middle panel) MFI of C57BL/6 KC stained for Ser345 phosphorylated MLKL (p-MLKL Ser345) 36 h post PH and representative histogram of the fluorescence intensity. (**A**, lower panel) MFI of C57BL/6 KC stained with labeled inhibitor of caspase-1 36 h post PH and representative histogram of the fluorescence intensity. (**B**, upper panel) MFI of RIPK3 KO KC stained with fluorescent-labeled inhibitor of caspase-8 36 h post PH and representative histogram of the fluorescence intensity. Results presented median and interquartile range. (**B**, lower panel) MFI of RIPK3 KO KC stained for p-MLKL Ser345 36 h post PH and representative histogram of the fluorescence intensity. All results presented as median and interquartile range from at least three independent experiments.

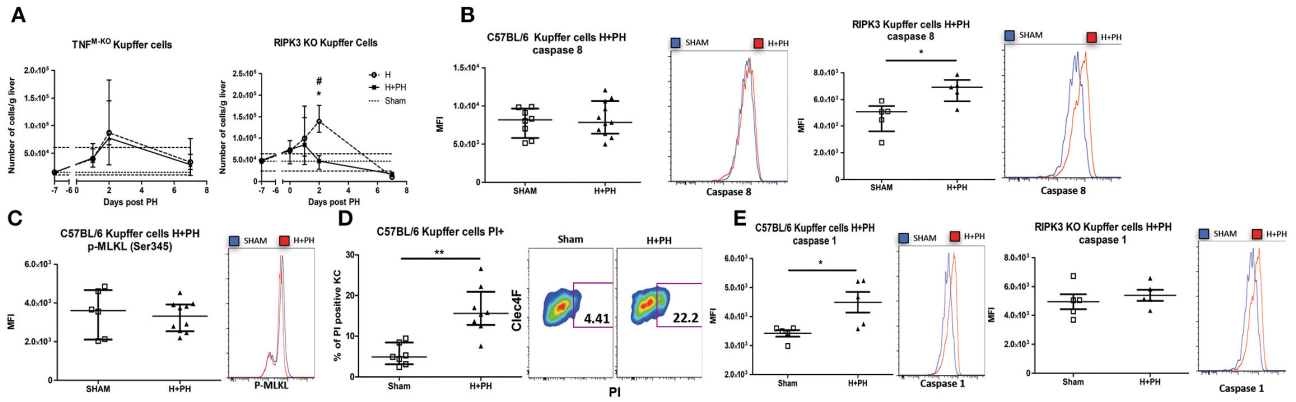

FIGURE 7 | Tumor encounter switches caspase-8 activation to caspase-1 activation in KC following partial hepatectomy. **(A)** Kinetics of KC following PH in TNF^{M-KO} and RIPK3 KO mice liver. Flow cytometry analyses were done 0, 1, 2, and 7 days following PH (H+PH group) or phantom operation (H group). The sham group was injected with physiological serum and received placebo surgery 7 days later. $^{*}p < 0.05$, Kruskal–Wallis followed by Dunn's *post hoc* test between the H and H+PH groups/$\#p < 0.05$, Kruskal–Wallis followed by Dunn's *post hoc* test between the sham and other experimental groups ($n = 5$–9/time point). Test chosen based on the result of the Shapiro–Wilk normality test. **(B)** MFI of C57BL/6 or RIPK3 KO KC from mice injected with Hepa1-6 cells and stained with fluorescent-labeled inhibitor of caspase-8 36 h post PH or phantom operation (sham) and representative histogram of the fluorescence intensity. $^{*}p < 0.05$, two-tailed Mann–Whitney test. **(C)** MFI of KC of C57BL/6 from mice injected with Hepa1-6 cells, stained for p-MLKL Ser345 36 h post PH and representative histogram of the fluorescence intensity. **(D)** Percentage of positive KC for propidium staining 36 h post PH and representative dot plot of the staining. $^{**}p < 0.01$, two-tailed Mann–Whitney test. **(E)** MFI of C57BL/6 or RIPK3 KO KC from mice injected with Hepa1-6 cells and stained with fluorescent-labeled inhibitor of caspase-1 36 h post PH and representative histogram of the fluorescence intensity. $^{*}p < 0.05$, two-tailed Mann–Whitney test. All results presented as median and interquartile range from at least three independent experiments.

that they were undergoing cell death. We further observed that KC from H+PH WT mice displayed an increased caspase-1 activity, a hallmark of pyroptosis (**Figure 7E**). We further linked inflammasome activation with TNF/RIPK3 signaling by demonstrating that KC from RIPK3 KO mice were, in contrast to WT mice, positive for caspase-8 but not for caspase-1 activity under the same conditions (**Figures 7B,E**). Taken together, our results support that tumor encounter modifies the TNF/RIPK3-dependent induction of caspase-8 activity to TNF/RIPK3-dependent caspase-1 activity after PH. This suggests an effective switch from apoptosis to pyroptosis induction in KC.

KC Death Pathway Influences the Recruitment of Ly6C^{low} Macrophages and Monocytes Promoting Tumor Growth

The protection against the PH-induced tumoral proliferation in RIPK3 KO mice remained unexplained from the previous experiments. We noticed that in contrast to WT mice, RIPK3 KO mice and TNF^{M-KO} mice did not display any increase in monocytes nor Ly6C^{low} macrophages 7 days post PH (**Figures 8A,B**). Taking into account the previously shown correlation between the presence of monocytes and Ly6C^{low} macrophages, we first demonstrated that PH induces the expression of CCL2 (a chemoattractant for monocyte) and GM-CSF in the early hours following PH in tumor-free wild-type mice (**Supplementary Figure 3A**). We then followed the kinetics of Ly6C^{low} macrophages and monocytes in PH wild-type mice (**Supplementary Figure 3B**). Recruitment of Ly6C^{low} macrophages in wild-type strain was especially striking on day 2 post PH. Moreover, CCR2 KO liver had an effective reduction of both monocytes and Ly6C^{low} macrophages (**Supplementary Figure 3B**) with no modifications of the KC compartment kinetics post PH.

Zigmond et al. (21) described the restorative and remodeling aspect of similar Ly6C^{low} macrophages, which raised the hypothesis that these cells might be beneficial for tumor development. This idea was strengthened by the finding of a strong correlation (Pearson correlation, $R = 0.8909/p = 0.0011$) between the amount of Ly6C^{low} macrophages in the liver and the size of the tumor assessed by histological analysis at the same timing (**Figure 8C**). Moreover, the CCR2 KO strain showed no significantly increased tumor proliferation following PH (**Figures 8D,E**), and the protection observed in RIPK3 KO strain can, at least partially, be attributed to the absence of recruitment of these cells.

DISCUSSION

Partial hepatectomy gives a good overall survival chance in patients carefully selected (5, 26). Yet, tumor recurrence constitutes a major problem for this approach with complications in nearly 70% of the cases at 5 years (7, 26). Complications can arise notably from occulted tumor sites, a scenario that our model reproduces. Clinical and experimental studies have suggested that liver regeneration following surgical resection facilitates tumor growth following a surgery procedure (27–29), a concept that our results support.

While the importance of T-cell immunity in the rejection of the Hepa1-6 cell line was described previously (22), an observation that we confirmed in our model (data not shown),

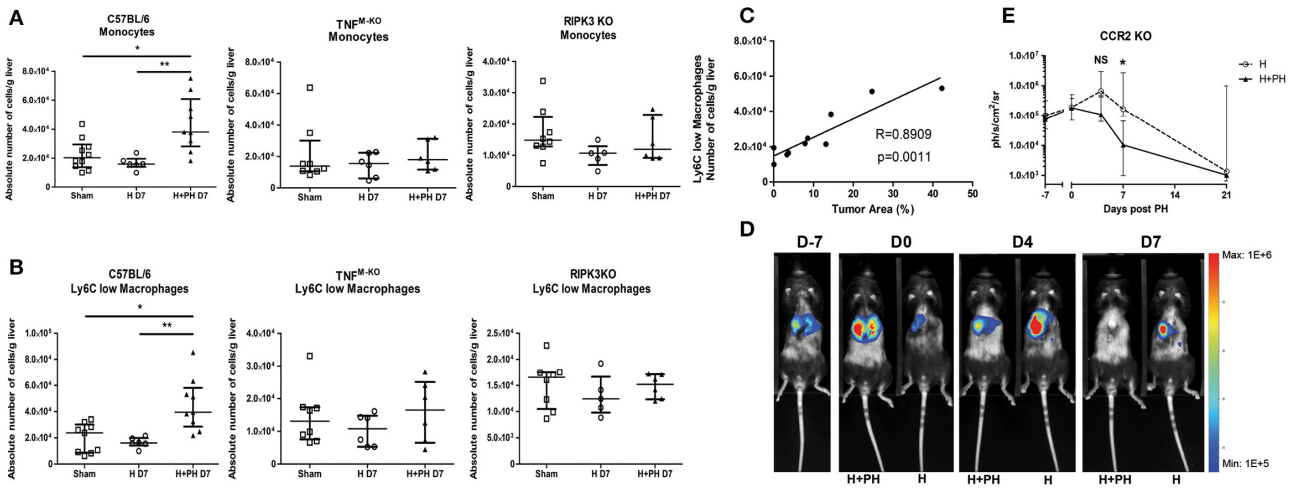

FIGURE 8 | PH induces a recruitment of monocyte-derived cells favorable for tumor proliferation that correlates with the differential death pathway activated in KC. **(A**, **B)** Absolute number of monocytes and Ly6C^{low} macrophages in C57BL/6, TNF^{M-KO}, and RIPK3 KO mice injected with Hepa1-6. Flow cytometry analyses were done 7 days following PH (H+PH group) or phantom operation (H group). The sham group was injected with physiological serum and received placebo surgery 7 days later. *$p < 0.05$, **$p < 0.01$, Kruskal–Wallis followed by Dunn's *post hoc* test. **(C)** Pearson correlation analysis between absolute number of Ly6C^{low} macrophages and size of the tumor. Absolute number of cell was determined by flow cytometry and tumor size by histological analysis (percentage of tumor on a slide) from wild-type animals at day 7. Pearson correlation (*R*-value) and statistical significance (*p*-value) are displayed. **(D)** Representative image of the bioluminescence data generated until 7 days post PH in CCR2 KO mice. **(E)** Bioluminescence signal detection over time following partial hepatectomy. Two-tailed Mann–Whitney test, *$p < 0.05$. Results presented as median and interquartile range ($n = 5$/group) from at least three independent experiments. NS, not significant.

our results clearly demonstrate a role for KC, and therefore the innate immunity component, in tumor rejection. This places KC at the intersection between the induction of liver regeneration and antitumoral responses.

Based on our flow cytometry observations and KC-DTR bioluminescence experiments, we suggest that during the early stages of tumoral development, the absence of KC induced by PH participates in increased tumoral proliferation, while maintenance of their number (and presumably their inflammatory factors) allows for accelerated rejection of the tumor cells. Interestingly, we could observe a reduction in the amount of monocytes in KC-DTR mice 2 days following DT injection. As already demonstrated in the KC-DTR strain, following DT injection, monocytes are recruited and differentiate in KC. Stellate cells and endothelial cells orchestrate this phenomenon (30). We therefore expect the same differentiation mechanism to be responsible for our observation.

The reduction in KC number following PH seemed to replicate the diminution observed in bacterial and ischemia–reperfusion injury models. In particular, the importance of KC necroptosis induced by phagocytosis of bacteria, in generating antimicrobial response production of CCL2 and IL-1β, has underlined the death pathway induced in macrophages as an element regulating inflammation (31). While our results ruled out necroptosis as the reason for KC reduction since no increase of phosphorylated MLKL level could be seen, we firstly described the TNF-α/RIPK3-dependent activation of caspase-8 occurring in KC upon PH. Presumably, activation of the complex IIb downstream of TNFR1 mediates KC apoptosis between days 1 and 2 following PH (**Figure 8**). TNF induces IL-6 production by KC and triggers hepatocyte proliferation via STAT3 activation during the regenerative process (8). While our results demonstrate that IL-6 is also a crucial factor for KC maintenance and survival in early stages of the regenerative process as well as for tumor clearance, complex IIb formation and subsequent apoptosis might be seen as a way to regulate KC activity following PH and avoid oversignalization via complex I, NF-κB activation, continuous inflammation, and damages in the regenerating organ.

Our study also shows for the first time that the concomitant growth of tumoral cells effectively switches caspase-8 to caspase-1 activation in KC. While these results suggest an induction of pyroptosis in these cells after PH, detection of cleaved Gasdermin D (a pore-forming protein that is activated after cleavage by caspase-1) within KC would be necessary to confirm this idea. Nevertheless, this activation of cell death mechanisms within KC following PH alongside the effective switch we observed and the importance this switch has on the recruitment of monocytes and monocyte-derived cells beneficial for the tumor had up until now never been documented in this context. Based on our results and published literature, we therefore propose the model presented in **Figure 9**.

The switch we observed was fully dependent on TNF and RIPK3 signaling since TNF^{M-KO} mice did not show a reduction in KC number and RIPK3 KO KC exhibited increased caspase-8 over caspase-1 activity. RIPK3 is able to activate NLR family pyrin domain containing 3 (NLRP3) inflammasome independently of MLKL presence in bone marrow-derived macrophages (32), and a similar mechanism might be occurring in KC. In dendritic cells, caspase-8 activity was shown to regulate RIPK3-dependent

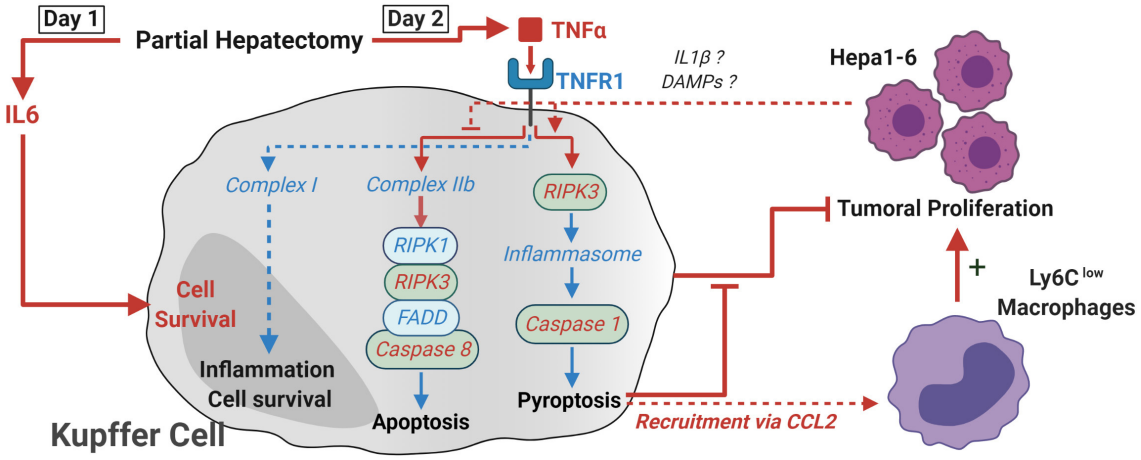

FIGURE 9 | Proposed model of how TNF-α and RIPK3 induce KC death following PH. Following PH, IL-6 acts on KC as a pro-survival signal. In the absence of tumor, TNF signaling leads to the activation of complex IIb and the apoptosis of KC through activation of caspase-8 between day 1 and day 2. The presence of tumor cells within the organ leads to a switch in the activation of cell death pathways and the RIPK3-dependent activation of caspase-1, probably through the activation of inflammasome macromolecular complexes. This KC death was linked with a CCL2-dependent recruitment of Ly6C^{low} macrophages. This latter population was shown to favor tumor development, while KC maintenance accelerated antitumoral responses. Critical elements demonstrated in the paper are described with red arrows and fonts, while deduction from the literature is in blue.

activation of the NLRP3 inflammasome, and suppression of caspase-8 activity favored pyroptosis induction (33). The fact that we observed caspase-8 activity but no caspase-1 induction in KC following PH in animals without tumor might hint toward an analogous regulation in KC. We can only speculate on how the switch from apoptosis to pyroptosis is made. Inflammasome assembly and activation mechanism is still a subject of debate especially for NLRP3. Yet, the current model proposes two distinct signals to be necessary (34): the priming signal being transmitted via TLRs, IL-1R, TNFR, or NOD2 and the second signal depending on $Ca2^{+}$ signaling, K^{+} efflux, changes in cell volume, or rupture of lysosomes or reactive oxygen species (ROS). Tumor proliferation, the inflammation generated against it via IL-1β secretion or liberation of DAMPs from dying cells, which are potent TLR ligands, might be participating in the process. Nevertheless, differential death pathway activation in KC leads to different recruitment of monocytes and monocyte-derived macrophages beneficial for tumor proliferation as the data from CCR2 KO mice confirmed. Even though the first clinical trials using carlumab (CNTO888), a human anti-CCL2 antibody, had disappointing results (35, 36) (despite overall low toxicity), modulation of the CCL2–CCR2 axis seems to be a promising way to alleviate the risk of recurrence following PH.

Monocytes are known for their vascular remodeling abilities, and in acute APAP-induced liver injury, monocyte-derived Ly6C^{low} macrophages, with a similar phenotype to the cells we observed, were shown to be important elements for the resolution of inflammation and to have wound healing and tissue remodeling abilities (21). Moreover, in the subcutaneous tumor development model, monocyte-derived CD11b^{+} MHCII^{-} Ly6C^{int} cells infiltrating the tumor were shown to suppress T-cell proliferation and to have important proangiogenic abilities (37). While those models differ from ours, the close phenotype and the same cellular origin of those cells might be giving a hint toward their protumoral activity in our observations. Since RIPK3 KO KC died from apoptosis and no monocyte nor Ly6C^{low} macrophage recruitment was observed, it is reasonable to think that upon inflammation induced by pyroptosis of KC, circulating monocytes are recruited and differentiate into the organ in cells phenotypically different from KC, while a less inflammatory cell death (apoptosis) would not lead to such recruitment and differentiation. These observations might help in understanding the clinical problems faced in tumor embolization protocol and the re-revascularization of the tumor site and overall tumor growth (38).

The dependence of KC death on TNF signaling raises the possibility for the development of strategies aiming at enhancing tumor rejection. The use of anti-TNF antibody treatment is standard nowadays for rheumatoid arthritis and is also of interest for a variety of other autoimmune disease such as Crohn's disease, psoriasis, ulcerative colitis, and ankylosing spondylitis (39). Yet, this approach has not always been efficient, as shown in multiple sclerosis (40), or even dangerous for patients, as demonstrated in clinical trials for heart failure (41, 42). Based on the protection against tumor proliferation observed in TNF^{M-KO} mice and since TNF signalization is also associated with ischemia–reperfusion injury (another liver-damaging reaction occurring in humans following PH due to a surgical procedure), it seems rational to hypothesize a potential beneficial impact of anti-TNF treatment in patients following PH. Yet, caution should be taken when transposing our results in humans, and choosing the right balance between liver regeneration and tumor rejection might be complex. This would be probably achieved by careful selection of the timing at which the treatment would be implemented. The

background on which tumor has developed might also be of importance when assessing the effect of anti-TNF treatment since HCC develops mainly in the liver with a previous inflammatory environment due to pathogen (HBV/HCV) or excessive alcohol consumption (alcoholic liver cirrhosis) (43). Other than anti-TNF treatment, specific macrophage inhibitor of pyroptosis might be just as relevant in our specific case. In conclusion, our work highlights the necessity for a comprehensive multidisciplinary treatment approach following PH in order to reduce the risk of complications occurring after surgery.

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 animal study was reviewed and approved by Ethics committee of Biopole ULB Charleroi.

AUTHOR CONTRIBUTIONS

J-FH and VF contributed to the concept, design of the research contributed, and writing of the manuscript. J-FH and SD performed the experiments and procedures. LL and SL performed the bioluminescence imaging. DG provided expertise to set up the PH model. CG and KB generated the Hepa1-6-Fluc cell line. JA performed the histology. MG and AB provided the genetically modified mice. 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.2020.547013/full#supplementary-material

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Metronomic Celecoxib Therapy in Clinically Available Dosage Ablates Hepatocellular Carcinoma via Suppressing Cell Invasion, Growth, and Stemness in Pre-Clinical Models

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Objective: To investigate the anti-carcinogenic effect of metronomic Celecoxib (i.e., frequent administration in clinically available doses) against hepatocellular carcinoma (HCC) in the perspective of metastasis, spontaneous hepatocarcinogenesis, cancer invasion, proliferation, and stemness in vivo and in vitro.

Background: Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is known to cause anti-carcinogenic effects for HCC in suprapharmacological doses. However, the effects of metronomic Celecoxib treatment on HCC cells remain unclear.

Methods: The *in vivo* chemopreventive effect of metronomic Celecoxib (10mg/kg/d) was investigated by the syngeneic HCC implantation model and spontaneous hepatocarcinogenesis in HBV-transgenic(HBVtg) mice individually. HCC cell lines were treated by either suprapharmacological (100 μM) or metronomic (4 μM) Celecoxib therapy. Anti-carcinogenic effects were evaluated using cell invasion, cancer proliferation, angiogenesis, and phenotype of cancer stem/progenitor cells (CSPC). The molecular mechanism of metronomic Celecoxib on HCC was dissected using Luciferase assay.

Results: In vivo metronomic Celecoxib exerted its chemopreventive effect by significantly reducing tumor growth of implanted syngeneic HCC and spontaneous hepatocarcinogenesis in HBVtg mice. Unlike suprapharmacological dose, metronomic Celecoxib can only inhibit HCC cell invasion after a 7-day course of treatment via NF-κB/MMP9 dependent, COX2/PGE2 independent pathway. Metronomic Celecoxib also significantly suppressed HCC cell proliferation after a 7-day or 30-day culture. Besides, metronomic Celecoxib reduced CSPC phenotype by diminishing sphere formation, percentage of CD90+ population in sphere cells, and expression of CSPC markers.

Conclusions: Metronomic Celecoxib should be investigated clinically as a chemopreventive agent for selected high-risk HCC patients (e.g., HCC patients after curative treatments).

Keywords: NSAID (nonsteroidal anti-inflammatory drug), Celecoxib, hepatocellular carcinoma (HCC), NFκB, metronomic, chemoprevention

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the 3rd common cause of cancer-related mortality in the world (1). Preventive strategies for HCC are clinically relevant. They can focus on different levels, such as prevention of hepatitis B virus (HBV) related spontaneous hepatocarcinogenesis (secondary chemoprevention) and prevention of relapse or metastasis of HCC after curative treatments (tertiary chemoprevention) (2). Nearly 40% of HCC patients suffered tumor relapse within two years after curative therapies, which means a strong need for effective chemopreventive modalities (3). Prognostic factors of recurrent HCC after surgery include vascular invasion, tumor size, and expression of cancer stem/progenitor cells (CSPC) markers such as CD90 (i.e., recurrence-related CSPC marker) and CD133 (4). Thus, potential targets of chemoprevention may include cancer invasion, cell proliferation, and phenotype of cancer stem cells.

Metronomic use of chemotherapy (i.e., long-term administration at low doses without long drug-free intervals) is well known to reduce the drug-related adverse effect and the risk of developing acquired drug resistance in cancer therapy (5). Similarly, metronomic use of Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) (i.e., long-term administration at clinically available dose) are also in association with reduced risk of various cancers, including recurrent HCC after curative liver resection (6, 7). NSAIDs, particularly selective cyclooxygenase-2 (COX-2) inhibitors such as Celecoxib, could effectively inhibit cell proliferation, restore cell apoptosis, and reduce angiogenesis in various cancer cell lines (6, 8, 9). However, most of the studies were performed in the setting of using Celecoxib at suprapharmacological doses (i.e., more than 5 μmol/L) (8–11). In contrast, the anti-carcinogenic effect and relevant molecular mechanism of metronomic Celecoxib were less investigated and remained elusive.

Increased expression of COX-2 or nuclear factor-kappa B (NFκB) was in association with carcinogenesis in HCC clinically (12, 13). Celecoxib could inhibit carcinogenesis via COX-2/PGE2 dependent and independent mechanisms (6, 8). Accordingly, Celecoxib was reported to inhibit growth and induce apoptosis in HCC cells, which can be partially reversed by COX-2 and prostaglandin E2 (PGE2) treatment (14). Also, Celecoxib could reduce angiogenesis, cell division, and metastasis via nuclear factor-kappa B (NFκB)/COX-2/prostaglandins pathway or other NFκB dependent signaling pathways (e.g., NFκB/matrix metalloproteinase 9 (MMP9) or cyclin D) (8, 10). However, all these mechanisms were mainly discovered while administrating Celecoxib at supra-pharmacologic doses (8, 10, 11). By contrast, molecular mechanisms underlying metronomic Celecoxib-mediated chemoprevention against HCC recurrence remain unclear and need to be investigated. In this study, we evaluated the effects and mechanism of metronomic Celecoxib treatment in preventing recurrent HCC. We found that metronomic Celecoxib therapy suppressed tumor regrowth of implanted syngeneic HCC, spontaneous hepatocarcinogenesis in HBV transgenic (HBVtg) mice, cell invasion, proliferation, and CSPC phenotype of HCC cells in vitro. The present study filled gaps between basic and clinical studies. Moreover, metronomic Celecoxib treatment should be investigated clinically as a chemopreventive modality for selective high-risk HCC patients after curative treatments.

MATERIALS AND METHODS

Metronomic Celecoxib Therapy on Syngeneic HCC Implantation Tumor Model and Spontaneous HBVtg-HCC Model

We followed the Guidelines for the Care and Use of Laboratory Animals (Ministry of Sciences and Technology, Taiwan) in animal experiments, which were approved by the China Medical University Committee of Laboratory Animal Welfare. We purchased Hepa1-6 cells for the syngeneic HCC model from ATCC (CRL-1830; Taipei, Taiwan), and modified the tumor development protocol from the previous report (15). We fed the mice by either placebo or metronomic Celecoxib therapy (10 mg/kg/d) 7 days earlier before Hepa1-6 (10^6/implantation site) cells were implanted into bilateral flanks of C57BL/6 mice (n = 18 sites in metronomic Celecoxib group; n = 16 sites in placebo groups). Then, the mice received therapy consecutively for 36 days. During the treatments, we measured the body weight and subcutaneously implanted tumor size by the previous protocol (16). We sacrificed the mice on post-implant day 37 and photographed and collected the tumors.

For the spontaneous HBVtg-HCC model, we obtained the HBVtg mouse and modified HBVtg-HCC protocol from Professor James Ou at the University of Southern California (17). The HBVtg-HCC mouse model was established and characterized as described earlier. (18–20) In brief, the HBVtg mice were intra-peritoneally (i.p.) injected with a carcinogen (diethylnitroasamine; DEN; 20 mg/kg) on the 14th days of pup mice. After genotyping to confirm HBVtg genotype, the mice were randomly assigned to two groups (18). For the metronomic Celecoxib group, we treated HBVtg-HCC mice with Celecoxib (10 mg/kg/daily) since the age of 20 weeks (i.e., the time of liver

tumor initiation) for consecutive 16 weeks, and then sacrificed the mice at the age of 36 weeks (i.e., fast-growing phase of liver tumor) (18). During the therapy period, we recorded the bodyweight of the mice daily. The mice in the metronomic Celecoxib group (n = 6) whose body weight was comparable to those in the placebo group (n = 9) were taken to record liver weight, tumor size, and tumor number at the time of sacrifice.

Histology Diagnosis and Immunohistochemistry

The subcutaneously implanted liver tumors from the syngeneic HCC model and whole livers from the HBVtg-HCC mice were collected and embedded in paraffine block for histology exam. The histological studies were performed with modifications as described in previous studies (16, 21). For histologic inspection, we treated tissue sections (2 μM) with hematoxylin and eosin or stained sections with antibodies specific for CD34 (abcam, ab81289) immunohistochemical (IHC) staining while using an ABC kit (Vector Laboratories) to enhance the staining signals. The slides were scanned with the Aperio ScanScope CS system (Leica Biosystems, Buffalo Grove, IL, United States) at 200× (objective lens) for further image analysis using ImageJ (NIH). The staining distributions were graded using a five-point scale according to the percentage of positive staining in whole scanned area (positive area/total area × %).

Statistical Analysis

Statistical analyses were performed using Student's t test. All experiments were repeated at least three times, and *P* values less than 0.05 were considered to indicate statistical significance.

The detailed materials and methods related cell culture, tube formation assay, and gene expression measurements were described in supplemental text.

RESULTS

Metronomic Celecoxib Reduced *In Vivo* Tumor Regrowth of Implanted Syngeneic HCC and Spontaneous Hepatocarcinogenesis in HBVtg-HCC Models

To test the *in vivo* chemopreventive effect of metronomic Celecoxib on seeded cancer, we implanted syngeneic HCC cells into bilateral flanks of C57BL/6 mice that were fed by either metronomic Celecoxib (n = 18 sites) or placebo (n = 16 sites) as protocol (**Figure 1A**). The bodyweight of both groups was comparable that may imply metronomic Celecoxib therapy did not impair the general physiologic status of mice (e.g., growth and intake) (**Figure 1B**). However, tumor size of implanted syngeneic HCC was significantly reduced in the "metronomic Celecoxib" group compared to the placebo group (tumor volume on post-implant day 37 [mean ± SEM] = 539.8 ± 135.8 mm^3 vs. 1138.0 ± 175.0 mm^3, P < 0.05) (**Figures 1C, D**). H&E stating at comparable-sized HCCs showed a significant central necrosis in the "metronomic Celecoxib" group compared to the placebo group (**Figure 1E**)

To investigate the chemopreventive effect of metronomic Celecoxib on spontaneous hepatocarcinogenesis, we compared tumor number and size of HBVtg-HCC mice that were fed by either metronomic Celecoxib (n = 6) or placebo (n = 9) as protocol and harvested liver for measurement after sacrificing them (**Figures 1F, G**). The body weight of mice was comparable between both groups (**Figure 1H**). The tumor numbers were significantly reduced in the "metronomic Celecoxib" group compared to the placebo group (Mean ± SEM= 9.3 ± 2.2 vs. 18.0± 2.4, P < 0.05) (**Figure 1I**). In addition, the tumor size was also smaller in the "metronomic Celecoxib" group compared to the placebo group (tumor largest diameter [Mean ± SEM] = 3.3± 0.4 mm vs. 5.3± 0.6 mm, P < 0.05) (**Figure 1J**). H&E staining at comparable-sized HCCs showed less eosinophilic staining in the "metronomic Celecoxib" group compared to the placebo group that may imply less intracellular protein component in the metronomic group (**Figure 1K**).

Metronomic Celecoxib Treatment During Long-Term Therapy Significantly Attenuated Cell Invasion Capability of HCC Cells

Several studies have highlighted the anticarcinogenic effect of Celecoxib on HCC cells; however, studies about mechanisms underlying the risk of HCC recurrence are limited. Therefore, we tested the effect of clinically available and suprapharmacological doses of Celecoxib on HCC cells and determined its effect in a chronic treatment module that mimicked long-term therapies. A cell invasion assay was employed to ascertain the oncogenic behavior of Tong, Huh 7, and HepG2 cells after treatment with suprapharmacological (100 μM, high-dose treatment) and clinically available (4 μM, low-dose treatment) doses of Celecoxib for 2 or 7 days. As shown in **Figure 2A**, exposure to a high-dose Celecoxib significantly reduced the cell invasion capability of HCC cells compared with vehicle-treated control cells in the 2-day treatment scheme. In a similar experiment, we did not observe any significant modulation in cell invasiveness in low-dose 2-day treated cells compared with controls (**Figure 2B**). However, metronomic Celecoxib treatment (4 μM, 7 days) significantly reduced the cell invasion capability of HCC cells (**Figure 2C**). These data indicated that low-dose Celecoxib treatment needs a longer time (i.e., metronomic therapy) to exert its effect against cell invasion of HCC.

Celecoxib is a selective inhibitor of COX-2, which generates PGE2 that stimulates cell invasion, proliferation, and migration behavior in hepatoma cells (22). Therefore, we tested the effect of metronomic Celecoxib treatment (4 μM, 7 days) on the invasive properties of HCC cells in the presence or absence of PGE2 (1μM, a supra-physiological concentration in the portal vein of the human) (23). As expected, PGE2 treatment significantly increased the invasiveness of HCC cells compared with vehicle-treated cells (**Figures 3A, B**). By contrast, a decline was observed

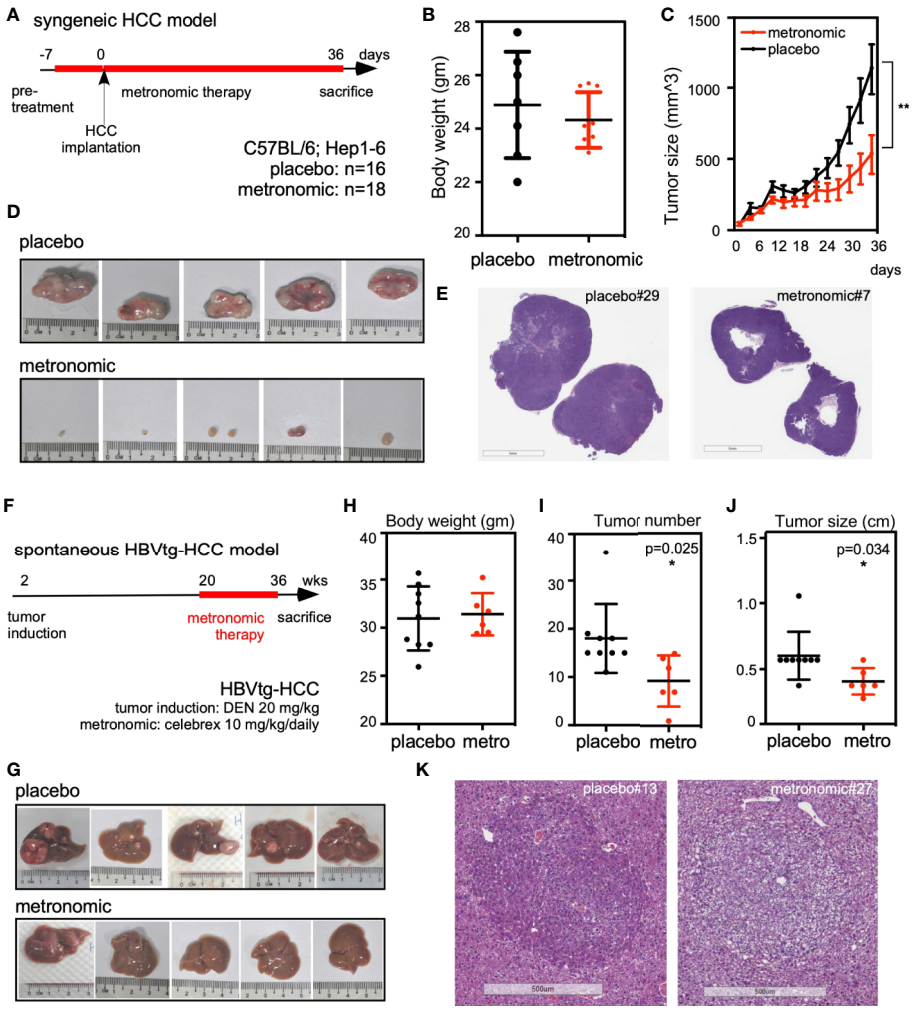

FIGURE 1 | Metronomic Celecoxib significantly suppressed *in vivo* tumor regrowth of seeded syngeneic HCC and spontaneous hepatocarcinogenesis in the HBVtg-HCC model. **(A)** Protocol of metronomic Celecoxib on the syngeneic HCC implantation model. C57BL/6 mice were pretreated with metronomic Celecoxib (10 mg/kg/d) orally before implanting Hepa1-6 cells (10^6/implantation site) into bilateral flanks. After implantation, these mice were treated with either metronomic Celecoxib or placebo for another 36 days and sacrificed on the 37th day for measurement. **(B)** The bodyweight of mice was comparable between the placebo and the "metronomic Celecoxib" group. **(C, D)** The implanted Hepa1-6 HCC tumor size was significantly suppressed in the "metronomic Celecoxib" group when compared to the placebo group (day-37 tumor size [mean ± SEM] = 539.8 ± 135.8 mm^3 vs. 1138.0 ± 175.0 mm^3, P < 0.01). **(E)** H&E stain showed significant central necrotic portion of HCC in the "metronomic Celecoxib" group at the syngeneic HCC model. **(F)** Protocol for spontaneous hepatocarcinogenesis in the HBVtg-HCC model. HBV transgenic mice (HBVtg) mice were given Diethylnitroasamine (DEN; 20 mg/kg) intraperitoneally at the age of 14th day. Metronomic Celecoxib (10 mg/kg/d) or placebo was fed from the age of 20th week to 36th week. Then, the mice were sacrificed for the measurement of liver tumors. **(G)** Spontaneous hepatocarcinogenesis in the harvested liver from the "metronomic Celecoxib" group was grossly less than that in the placebo group. **(H–J)** Bodyweight of mice was also comparable between the "metronomic Celecoxib" group and the placebo group. Tumor number and tumor size were significantly reduced in "metronomic Celecoxib" group compared to placebo group (tumor number [Mean ± SEM] = 9.3 ± 2.2 vs. 18.0± 2.4, P < 0.05; tumor largest diameter [Mean ± SEM] = 3.3 ± 0.4 mm vs. 5.3 ± 0.6 mm, P < 0.05). **(K)** H&E staining at comparable-sized HCCs showed less eosinophilic staining in the "metronomic Celecoxib" group compared to the placebo group in HBVtg-HCC model. * Indicates *P* < 0.05 and ** indicates *P* < 0.01.

in the invasion capability of HCC cells upon metronomic Celecoxib treatment when compared with vehicle-treated cells. However, stimulation with PGE2 did not significantly abrogate the anti-invasive effect of Celecoxib in HCC cells. These data indicated that metronomic Celecoxib inhibited basal as well as PGE2-stimulated cellular invasion, implicating the involvement of COX-2/PGE2-independent mechanisms in the suppression of invasive properties of HCC cells.

Metronomic Celecoxib Suppressed the Invasive Properties of HCCs by Inhibiting MMP9 Through Perturbation of NFκB Activity

To obtain more profound insights into the role of metronomic Celecoxib in NFκB-mediated invasiveness of HCCs, we assessed NFκB luciferase reporter activity after 7-day Celecoxib treatment. Results showed that low-dose (4 μM) Celecoxib

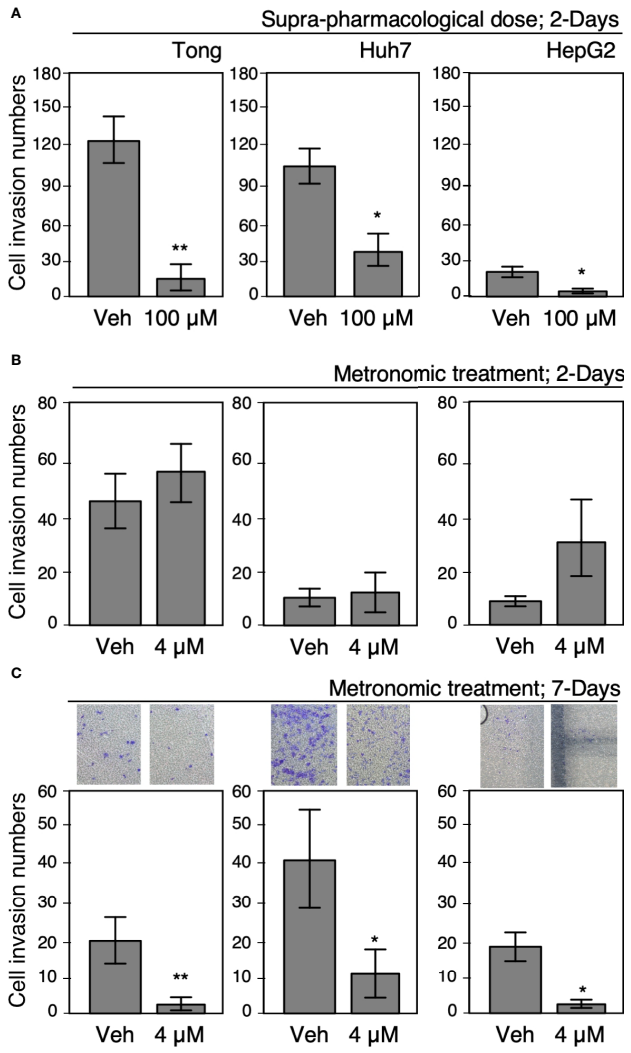

FIGURE 2 | Metronomic Celecoxib treatments reduced HCC cell invasion. **(A)** Suprapharmacological Celecoxib treatments (100 μM) on human HCC cells (Tong, Huh7, and HepG2) for 2 days suppressed cell invasion. **(B)** Clinically available Celecoxib treatments (4 μM) for 2 days did not suppress cell invasion. **(C)** Metronomic Celecoxib treatment (4 μM, 7 days) could suppress cell invasion in the HCC cells. The HCC cells treated with or without Celecoxib were plated onto Matrigel-coated transwells, incubated for 18 h to observe cell invasion, and recorded as corresponding photos. The data were from at least three reproducible independent experiments in which the raw invasive cell numbers were counted, and mean values with standard errors were plotted graphically. * Indicates $P < 0.05$ and ** indicates $P < 0.01$.

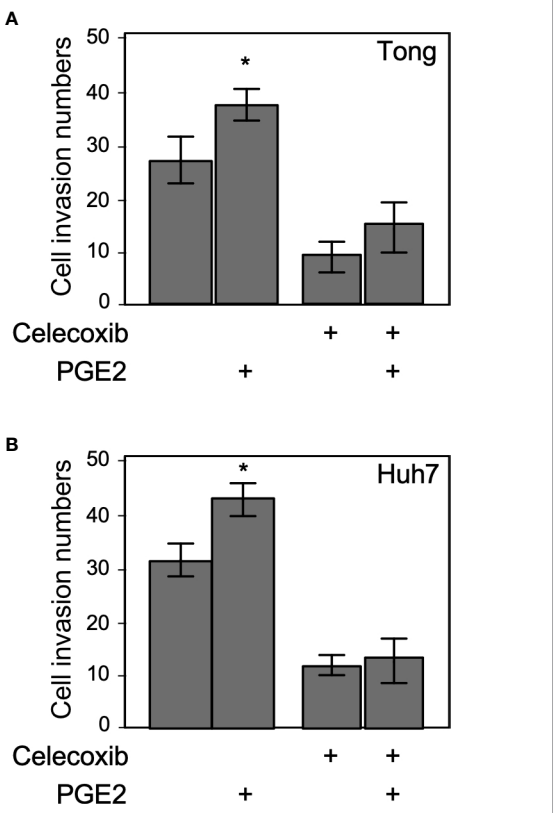

FIGURE 3 | Suppression of invasion by metronomic Celecoxib treatments is a PGE2-independent event. **(A)** The cell invasion capacity of the Tong cells increased slightly by supra-physiological doses of PGE2 treatments (1 μM; lane 1 vs. 2). However, PGE2 co-treated with metronomic Celecoxib (4 μM, 7 days) did not reverse the Celecoxib suppression effect on cell invasion. **(B)** The cell invasion capacity of the Huh7 cells was increased by PGE2 treatments (1 μM; lane 1 vs. 2). However, PGE2 and metronomic Celecoxib cotreatment did not reverse Celecoxib-mediated suppression effect on cell invasion. The data were from at least three reproducible independent experiments in which the raw invasive cell number was counted, and mean values with standard errors were plotted on the graph. * Indicates $P < 0.05$.

treatment significantly suppressed the NFκB promoter activity (**Figure 4A**), whereas we observed a similar result upon analyzing CM for NFκB reporter activity (**Figure 4B**). Next, HCC cells were treated with Celecoxib in the presence or absence of PGE2 as performed previously and analyzed for NFκB promoter activity. As expected, PGE2 stimulation enhanced NFκB luciferase activity. However, Celecoxib inhibited both basal and PGE2-stimulated NFκB promoter activity (**Figure 4C**). These results suggest that metronomic Celecoxib treatment inhibited the invasive behavior of HCC cells through the suppression of NFκB transcriptional activity, and the mechanisms involved were independent of the COX-2/PGE2 pathway. Increased MMP9 expression is associated with enhanced tumor invasion properties; therefore, we ascertained the effect of Celecoxib on MMP9 promoter activity in HCC cells. We found that low-dose Celecoxib treatment significantly reduced MMP9 luciferase activity (**Figure 4D**). Because Celecoxib inhibited both NFκB and MMP9 activity, we speculated that the invasive properties of HCCs are mediated through NFκB transcriptional activity on the MMP9 promoter. To examine this possibility, we used an MMP9 luciferase reporter plasmid with a mutation at the NFκB binding site. Notably, MMP9 promoter activity with the mutated NFκB binding site was not affected by Celecoxib treatment (**Figure 4E**). Together, these data indicated that metronomic Celecoxib treatment exerted an inhibitory effect on the invasive property of

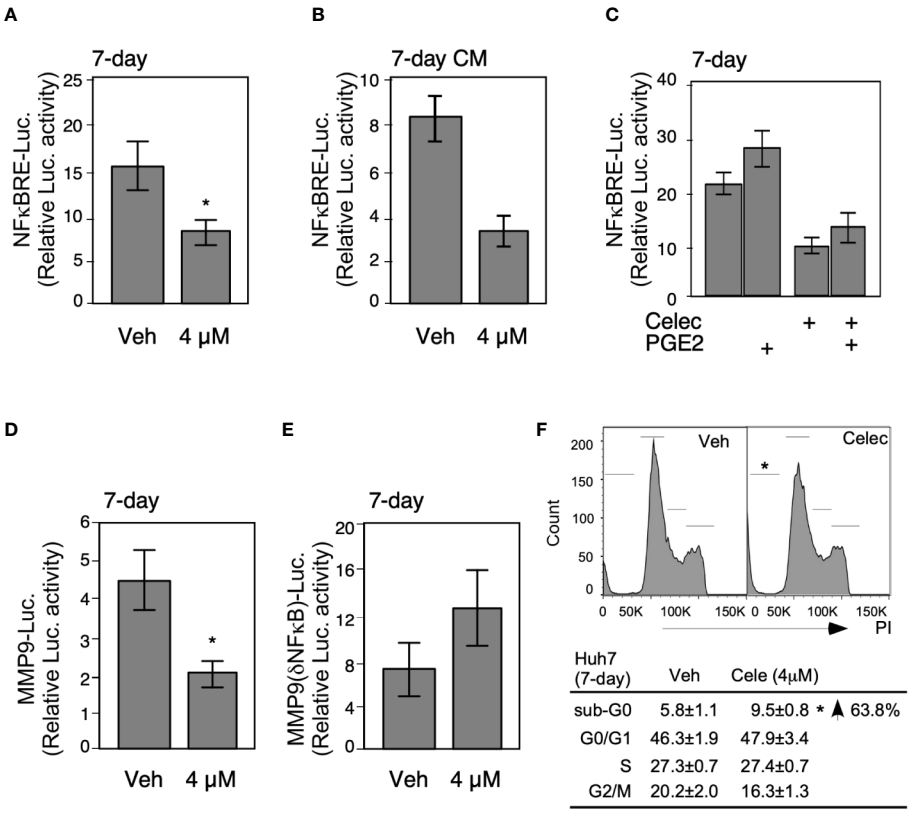

FIGURE 4 | Metronomic Celecoxib treatments suppress HCC cell invasion through NFκB-MMP9 pathway. **(A)** The metronomic Celecoxib treatment suppressed NFκB response element (NFκBRE) activity in HepG2 cells. The HepG2 cells were treated with Celecoxib 4 μM for 4 days and transfected with a NFκBRE–luciferase construct; then, treatment was continued for another 3 days. The dual-luciferase activity was measured on the seventh day of treatments. **(B)** The conditioned medium from the HepG2 cells treated with metronomic Celecoxib suppressed NFκBRE activity. The conditioned medium obtained from the HepG2 cells treated with celecoxib (4 μM) for 7 days were used to treat the HepG2 cells containing the NFκBRE–luciferase construct to measure dual-luciferase activity. **(C)** PGE2 cotreatment did not rescue NFκBRE–luciferase activity inhibition caused by metronomic Celecoxib treatment. The HepG2 cells were treated with either Celecoxib 4 μM or PGE2 1 μM for 4 days, transfected with NFκBRE–luciferase construct, and treated again with Celecoxib or PGE2 for another 3 days. The dual-luciferase activity was measured at the seventh day of treatments. **(D, E)** The suppression of invasiveness by metronomic Celecoxib treatment could partially go through the NFκB-MMP9 pathway. Similar treatments [as **(A)**] were applied on the HepG2 cells, but transfected with MMP9 wild-type promoter (MMP9-luciferase; **(D)** construct, or NFκBRE deletion mutant of MMP9 promoter (MMP9–(ΔNFκBRE)–luciferase; **(E)** constructs to measure luciferase activity. The data were from at least four reproducible independent experiments in which the mean values with standard errors were plotted on graph. **(F)** The cell-cycle and sub-G0 population were measured in Huh7 cells treated w/wo metronomic celecoxib regimen. * Indicates $P < 0.05$.

HCC cells by reducing COX-2/PGE2 independent, NFκB-dependent MMP-9 expression. In addition to verify the metronomic cell growth inhibition effect through altering cell cycle or cell death, we performed PI staining following flow cytometry assay. As showed in **Figure 4F**, the G0/G1, S, and G2/M phases are comparable between vehicle or celecoxib treatment group (7 days). In terms of sub-G0 phase (represent as dead cells), the death population was increased in metronic celecoxib treated cells (**Figure 4F**).

We examined tumor related angiogenesis by using the tube formation assay and CD34 IHC staining. We obtained CM from Tong, Huh 7, and HepG2 cells treated with a high dose (**Figure 5A**; 100 μM, 2 days) and low dose (**Figure 5B**; 4 μM, 7 days) of Celecoxib. Each CM was then applied onto umbilical cord-derived endothelial cells to observe the angiogenic capacity of the CM. Our results showed that CM obtained from cells treated with either high-dose or low-dose celecoxib could not significantly affect degree of angiogenesis compared to the placebo groups (**Figures 5A, B**). Similarly, CD34 IHC staining did not show significant difference of angiogenesis between the "metronomic Celecoxib" group and the placebo group in both syngeneic HCC and spontaneous HCC *in vivo* models (**Figures 5C–H**). All these findings indicated that the micro-environment of HCC treated by metronomic Celecoxib could not significantly affect HCC related angiogenesis.

Metronomic Celecoxib Inhibited Cell Viability and Proliferation Capability of HCC Cells

To further delineate the effect of suprapharmacological and clinically available doses of Celecoxib treatment on HCC cell viability, we performed a series of colorimetric assays, cell

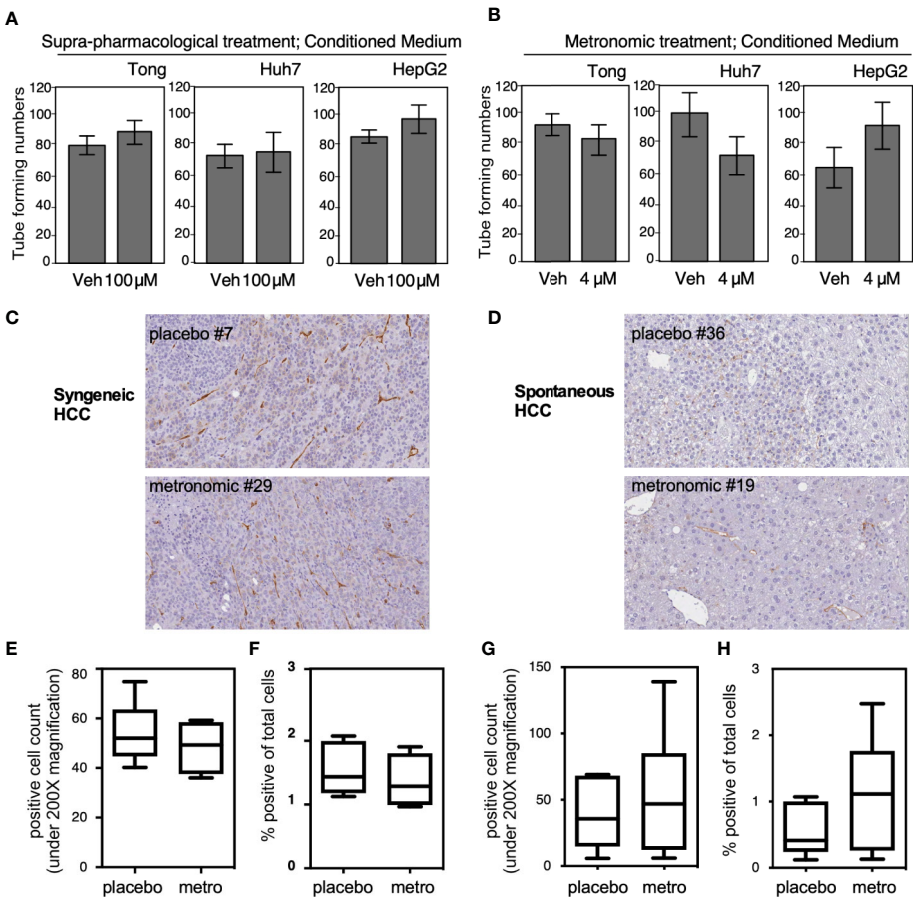

FIGURE 5 | Microenvironmental influence from the HCC cells treated with metronomic Celecoxib did not alter the angiogenenesis phenotype. **(A, B)** Three HCC cell types (Tong, Huh7, and HepG2) were treated with suprapharmacological [**(A)** 100 μM for 2 days] or metronomic Celecoxib [**(B)** 4 μM, 7 days] and the conditioned medium (CM) was harvested. Each CM was then applied onto umbilical cord-derived endothelial cells to observe the angiogenic capacity of the CM. The tube-forming number was counted as described in materials & methods section, and quantitation result was plotted with standard error from three independent experiments. **(C, D)** Micro-vessel densities determined by CD34 IHC staining in comparable-sized HCCs from either syngeneic HCC models or spontaneous HCC models. Micro-vessel densities expressed by CD34+ cell counts **(E)** and percentage of CD34+ area to total scanned area **(F)** were comparable between the "metronomic Celecoxib" group and the placebo group in syngeneic HCC model. **(G, H)** a similar finding was also noticed in the spontaneous HCC model.

viability assays, and colony formation assays for an incubation period of 2, 7, or 30 days, respectively. We found that a suprapharmacological dose (100 μM) of Celecoxib significantly inhibited HCC cell viability compared with control cells for a 2-day incubation period (**Figure 6A**). However, a similar treatment module at a clinically available dose (4 μM) did not elicit a significant suppression effect on HCC cell viability (**Figure 6B**). Next, we treated plated HCC cells with metronomic Celecoxib (4 μM, 7 days), and ascertained HCC cell numbers after treatment. Celecoxib-treated cells exhibited more significant suppression of HCC cell counts than did vehicle-treated control cells (**Figure 6C**). Next, we evaluated the effects of long-term metronomic Celecoxib treatment (4 μM, 30 days) on HCC cell proliferation potential that mimicked chronic HCC treatment modalities. The HCC cell colony formation ability was significantly attenuated over a long-term treatment duration (**Figure 6D**). Similar to metronomic Celecoxib against cell invasiveness, these data suggested the effects of Celecoxib at a clinically available dose in inhibiting HCC cell viability and proliferation may only be present when it is given for a longer time.

Metronomic Celecoxib Inhibited the Cancer Stem/Progenitor Cells Phenotype in HCCs

To test the effect of metronomic Celecoxib on the self-renewal potential of CSPCs, we examined its effect on the sphere formation ability of HCC cells and the marker expression of CSPCs. In the sphere formation assay performed using long-term metronomic Celecoxib treatment (4 μM, 21 days), Celecoxib significantly attenuated sphere formation in HCC cells (**Figure 7A**). Next, we assessed the expression level of the recurrence-associated stem cell marker CD90 in HCC sphere cells after metronomic Celecoxib treatment, as performed in previous experiments. We found that the number of CD90+ cells

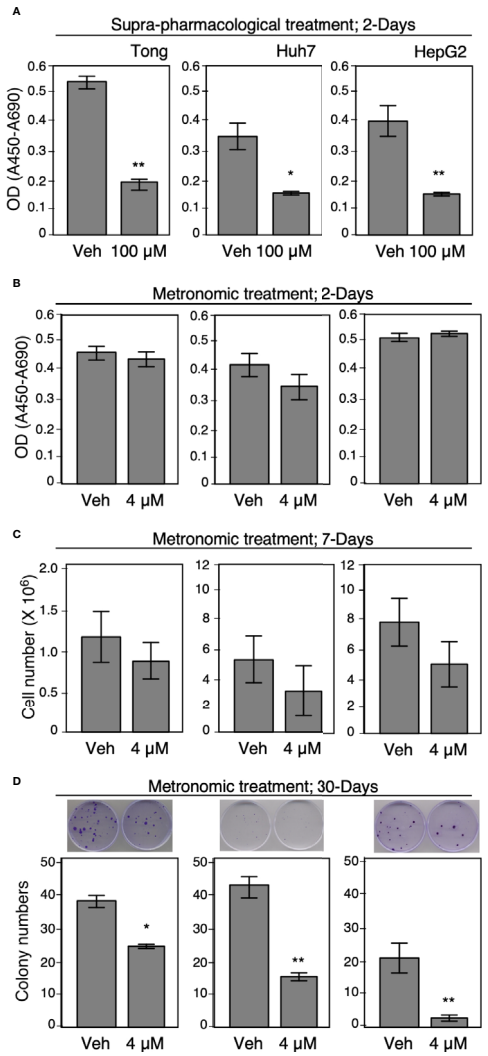

FIGURE 6 | Metronomic Celecoxib treatments reduced HCC cell growth. **(A, B)** Suprapharmacological (100 μM) treatment, or clinically available Celecoxib treatment (4 μM) for 2 days affect human HCC cell growth (Tong, Huh7, and HepG2). The cells were seeded on 96-well plates, and the cell growth was measured by adding WST-1 reagent into the culture medium. After 1 h of incubation, the optical density or absorbance (OD or Å450-Å690) was recorded and the readings were plotted on graph. Unlike supra-pharmacological treatment, short-term clinically available Celecoxib (4 μM, 2 days) could not cause significant suppression on cell growth. **(C)** Metronomic Celecoxib (4 μM, 7 days) treatments on HCC cells could exhibit a greater suppression of HCC cell counts than did vehicle-treated control cells. The cells were plated onto 60-mm dish (2×10^5 cells/plate) then treated with or without 4 μM Celecoxib, cultured for 7 days. The cell number was counted on day 7 by using plate cytometer, the total cell number was calculated, and the numbers were plotted on graph. **(D)** Long-term metronomic Celecoxib treatment (4 μM; 30 days) could reduce colony formation among the HCC cells. The HCC cells (500 cell/plate) were plated onto 60-mm dishes, treated with or without Celecoxib (4 μM), and were cultured for 30 days. The cells were fixed with 4% buffered formalin, stained with trypan blue, and recorded as corresponding photos. The colony numbers were counted, and the values were plotted on the graph. The data were obtained from at least three reproducible independent experiments, and the mean values with standard errors were plotted. * Indicates $P < 0.05$ and ** indicates $P < 0.01$.

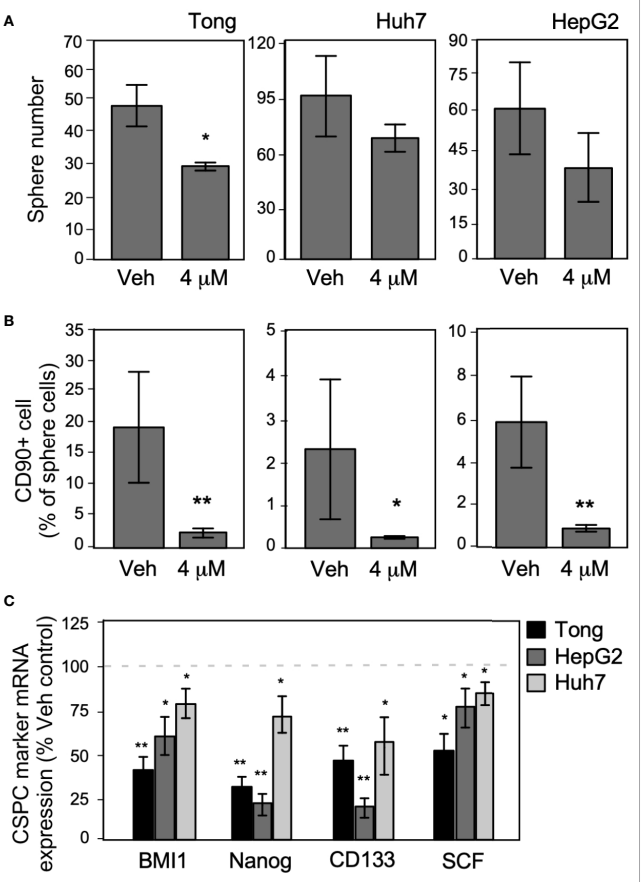

FIGURE 7 | Long-term metronomic Celecoxib treatments could reduce CSPC self-renew capacity and CD90+ cell population in CSPC sphere cells. **(A)** Long-term metronomic Celecoxib treatments reduce HCC cells (Tong, Huh7, and HepG2) CSPC self-renewal. The HCC cells (500 cell/plate) were plated onto low-attachment 60-mm dishes with sphere-forming medium (low serum), treated with or without Celecoxib (4 μM; 21 days). The sphere number was counted, and the values were plotted on the graph. **(B)** Long-term metronomic Celecoxib treatments reduce CD90+ populations in the sphere cells. The cells from **(A)** were harvested, fixed with cold-methanol, stained with CD90 antibody, then observed CD90+ population by flow cytometry. The CD90+ percentage was plotted on the graph. **(C)** Long-term metronomic Celecoxib treatments reduce CSPC maker genes of the sphere cells. The cells from **(A)** were harvested, and the total RNA was extracted. CSPC marker genes expression (BMI1, Nanog, CD133, and SCF) was measured using real-time RT-PCR. The value was compared with vehicle treatment and plotted on the graph as % of Veh. control. The actin expressions were used as loading control of each set of experiments. The data were from at least three reproducible independent sets of the experiment, and the mean values with standard errors were plotted graphically. * Indicates $P < 0.05$ and ** indicates $P < 0.01$.

in the spheres was considerably lower among Celecoxib-treated cells than among vehicle-treated cells (**Figure 7B**). Finally, we determined the expression level of CSPC markers using Q-RT-PCR after metronomic Celecoxib treatment. The mRNA expression levels of BMI1, Nanog, CD133, and SCF were significantly lower in Celecoxib-treated HCC cells than in vehicle-treated HCC cells (p-values of CSPS markers: BMI1, Nanog, CD133 < 0.01, SCF < 0.05 in Tong cells; Nanog, CD133 < 0.01, and BMI1, SCF < 0.05 in

HepG2 cells; BMI1, Nanog, CD133, SCF < 0.05 in Huh7 cells) (**Figure 7C**). By contrast, when we repeated similar exams while treating HCC cells at suprapharmacological concentrations, no viable cells could be detected after 21-day incubation time (data not shown).

DISCUSSION

To our best knowledge, this is the first study to provide pre-clinical *in vivo* and *in vitro* evidence that metronomic Celecoxib at clinically available dosage significantly reduce HCC cell invasion, proliferation, stemness, and suppress tumor regrowth of seeded HCC (i.e., tertiary chemoprevention) and primary hepatocarcinogenesis (i.e., secondary chemoprevention). The mechanistic model of metronomic celecoxib on HCC suppression to prevent post hepatectomy surgery recurrence is illustrated in **Figure 8**. Besides, metronomic Celecoxib treatment mainly reduced HCC cell invasion via COX-2/PGE2 independent NF-kB/MMP9 dependent pathway. Based on these results, metronomic Celecoxib should be tried clinically as chemopreventive agents in selected high-risk HCC patients, such as HCC patients following curative treatments.

NSAIDs, regardless of selective or non-selective agents, are limited in clinically long-term usage due to increased risk of cardiovascular events (24). However, considering a significant risk of recurrent HCC after curative liver resection, some safer NSAIDs, such as selective COX-2 inhibitors, applied as chemopreventive agents in this high-risk population might be justified. Though some specific selective COX-2 inhibitors (e.g., Celecoxib) is a relatively safer medication than others (e.g., Rofecoxib) due to less risk of serious cardiac events, the cardiovascular risk still cannot be ignored and is significantly related to dose and dosing interval (24, 25). The cardiovascular risk in Celecoxib users was lowest for the 400-mg-QD dose compared to 200-mg-BID and 400-mg-BID (26). A pharmacokinetic study in a group of healthy subjects showed Cmax (705 ng/ml, equal to 1.85 μM) in those taking Celecoxib at a single dose of 200 mg (27). Therefore, we considered 4uM concentration of Celecoxib as a clinically available concentration while patients take Celecoxib at recommended doses (i.e., 400-mg-QD or 200-mg-BID). Regarding the dose of Celecoxib used at *in vivo* mice models, the conversion rate of drugs between human and mice is around 1 to 12 (28). Considering the risk of cardiovascular events in proportion to the dose of Celecoxib, we tried a dosage of Celecoxib (i.e., 10 mg/kg/d) at *in vivo* studies, and it is around 50-mg-QD Celecoxib in a 60-kg adult (24, 25). We considered that the reduced dose of Celecoxib should be safer for long-term application clinically as a chemopreventive medication. Hence, the chemopreventive effect and molecular mechanism of Celecoxib on HCC cells at a clinically available concentration is the most central and clinically relevant finding in this study.

Considering significant cardiovascular risk in high-dose Celecoxib use, we mainly exam the effect of metronomic Celecoxib (i.e., frequent administrating at a clinically available dose) on tumor invasion, proliferation, angiogenesis, and metastatic potential. Under metronomic Celecoxib treatment, tumor invasion, proliferation, and metastatic potential were significantly reduced. Our results corresponded well to the previous researches, where Celecoxib suppresses cell viability by inhibiting cell proliferation and colony formation, although previous researches mainly investigated Celecoxib at supra-pharmacological concentration (8). Unlike previous studies,

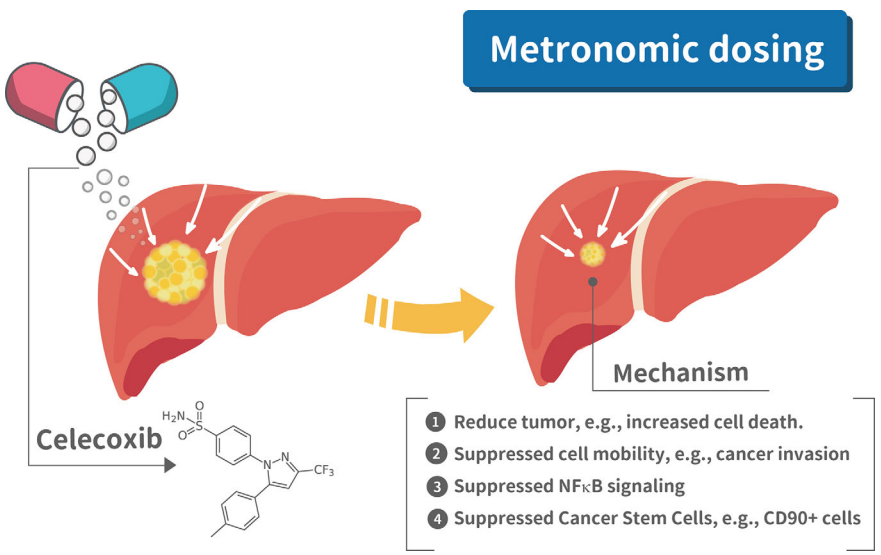

FIGURE 8 | Mechanistic illustration of metronomic celecoxib effects on suppressing HCC prognosis. There are four mechanism for metronomic dosing in suppressing HCC prognosis, e.g., 1. Reduced tumor growth; 2. Suppressed cell mobility; 3. Suppressed NFkB signaling; 4. Suppressed cancer stem cells.

angiogenesis was not significantly attenuated under metronomic Celecoxib treatment (8). A similar finding was also noticed by measuring micro-vessel density by CD34 IHC staining at *in vivo* models. The results indicated that anti-carcinogenic effect of metronomic Celecoxib may not rely on anti-angiogenesis effect.

In the pre-clinical *in vivo* study, we investigated the effect of metronomic Celecoxib on *in vivo* tumor growth of HCC with either homogenous or heterogeneous genetic backgrounds using two different animal models. We found a significant reduction in tumor regrowth of seeded syngeneic HCC while treating with metronomic Celecoxib compared to placebo. The implanted Hepa1-6 HCC cell line is derived from C57L mice with homogenous genetic background and widely accepted for studying *in vivo* tumor growth and metastasis of HCC in immunocompetent environment (29). However, syngeneic implanted HCC model using an established HCC cell line after multiple passages may not truly reflect clinically relevant situations. Thus, we used the other animal model (i.e., HBVtg-HCC model) to investigate spontaneous hepatocarcinogenesis that comes from freshly developed HCC tumor cells with heterogeneous genetic backgrounds. Noteworthy, Celecoxib had been proven effective in chemoprevention in the DEN-induced HCC animal model if it is given before or along with DEN (200mg/Kg) because Celecoxib may upregulate cytochrome-P450 activity and reduce the toxicity of DEN sequentially (30). However, this model is not the case related to the clinical situation that exposure to a carcinogen (e.g., hepatitis virus B or aflatoxin) usually precedes the usage of chemopreventive drugs. In our study, metronomic Celecoxib was given long (at the age of 20th week) after low-dose DEN (20mg/Kg, at the age of 2nd week) administrated to HBVtg mice. This model is more clinically relevant and more like secondary chemoprevention to reduce progression to HCC from underlying chronic viral hepatitis (2).

NF-κB has been well known as a cancer promoter, particularly in inflammation-associated tumor such as HCC (31). The mechanism of anti-carcinogenic effect by Celecoxib (e.g., inhibition on NFκB) were extensively investigated (8). However, most studies investigated the interaction between Celecoxib and NFκB at clinically irrelevant conditions, such as supra-pharmacologic dosage of Celecoxib (i.e., more than five μM) or short-term treatment (e.g., hours) (8). To determine the exact mechanism under clinically relevant situations, we particularly treat the Luciferase system using metronomic Celecoxib (4 μM, 7 days). Consistent with previous reports, we found that NFκB transcriptional activity could also be suppressed in HCC cells by metronomic Celecoxib treatment (8). Furthermore, we could not abrogate the inhibitory effect of metronomic Celecoxib on NF-κB even by applying supra-physiological dosage of PGE2 (1μM) (23), and it implied that metronomic Celecoxib mainly exerts its inhibitory effect via NFκB dependent and COX2/PGE2 independent pathway.

We examined whether metronomic Celecoxib treatment could suppress the more resistant subpopulations of HCCs by reducing the numbers of sphere-forming cells or CSPCs. CSPCs have extensive self-renewal ability, tumorigenesis, and differentiation potential; consequently, they give rise to new anaplastic tumor cells that exhibit resistance to cytotoxic chemotherapy and ionizing radiation (32, 33). This resistance may be attributed to their presumably slow cell cycle and overexpression of efflux pumps (34), which gives rise to CSPC subpopulations within each tumor (19, 32, 33, 35). Given the essential role of CSPC in metastasis, recurrence, and therapeutic resistance, it becomes imperative to identify novel therapies, specifically targeting CSPCs, which can potentially eradicate the renewal capacity of the tumor (36). In this study, we found that a metronomic Celecoxib therapy could significantly reduce sphere formation in HCCs, CD90+ population in sphere cells, and expression of the CSPC markers (BMI1, Nanog, CD133, and SDF). The finding suggested that metronomic Celecoxib treatment could reduce the formation and phenotype of CSPC in HCC that also corresponded to the previous study that Celecoxib could suppress HCC stemness at a higher-than-normal concentration (10μM) (9).

This study evaluated the invasiveness, cell proliferation, metastatic potential, and tumor growth of HCC cells under metronomic Celecoxib treatment using *in vivo* and *in vitro* system. Because of cardiovascular risk and effective anti-carcinogenesis of selective COX-2 inhibitors, we considered metronomic Celecoxib therapy might be a potentially effective chemopreventive agent for reducing the risk of tumor recurrence, progression, and metastasis in selected high-risk HCC patients such as HCC patients after curative treatments. Based on this pre-clinical *in vivo* and *in vitro* study, further pharmacokinetic studies and clinical studies are warranted to validate the effective dose and chemopreventive potential of metronomic Celecoxib against HCC.

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 animal study was reviewed and approved by China Medical University Committee of Laboratory Animal Welfare.

AUTHOR CONTRIBUTIONS

C-C Y performed the experiments, developed the concept, and manuscript editing. SP, P-Y L, and S-Y Y conducted experiments and interpreted data and drafted the manuscript. H-C L, L-B J, and W-C C were responsible for clinical consultation and participated in manuscript editing. W-L M developed the concept, supported the entire study, and edited and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Circulating Tumor-Cell-Associated White Blood Cell Clusters in Peripheral Blood Indicate Poor Prognosis in Patients With Hepatocellular Carcinoma

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Aim: Circulating tumor cells (CTC) are a precursor to metastasis in several types of cancer and are occasionally found in the bloodstream in association with immune cells, such as white blood cells (WBCs). CTC-associated WBC (CTC-WBC) clusters can promote CTC appreciation and metastasis, suggesting that patients with CTC-WBC clusters found in the peripheral blood may have a worse prognosis. However, it is unclear whether CTC-WBC clusters are present in the peripheral blood of patients with hepatocellular carcinoma (HCC) and suggest a poor prognosis for HCC.

Methods: We collected peripheral blood from 214 patients with HCC from January 2014 to December 2016. CanPatrol^{TM} CTC analysis technology was used to isolate and count CTCs and CTC-WBC clusters in the patients' peripheral blood. Chi-squared analysis was used to calculate the correlation between the CTC-WBC clusters and clinicopathological characteristics. Kaplan–Meier survival analysis and Cox regression analysis were used to assess patient prognosis.

Results: We used CanPatrol^{TM} CTC analysis technology to count different types of CTCs and CTC-WBC clusters. The results showed that CTC-WBC clusters and tumor size ($P = 0.001$), tumor number ($P = 0.005$), portal vein tumor thrombus ($P = 0.026$), BCLC stage ($P < 0.001$), AFP level ($P = 0.002$), and total number of CTCs ($P < 0.001$) were statistically related. Cox regression analysis revealed that CTC-WBC clusters are an independent prognostic indicator of DFS (HR = 1.951, 95%CI:1.348–2.824, $P < 0.001$) and OS (HR = 3.026, 95%CI:1.906–4.802, $P < 0.001$) in HCC patients. Using Kaplan–Meier analysis, we found that positive CTC-WBC cluster patients had significantly shorter DFS and OS than patients with negative CTC-WBC ($P < 0.001$ and $P < 0.001$, respectively).

Conclusions: CTC-WBC clusters in the peripheral blood are an independent predictor of DFS and OS, and their presence indicates poor prognosis in patients with HCC.

Keywords: CTC-WBC cluster, hepatocellular carcinoma, prognosis, Kaplan-Meier plot, circulating tumor cells

INTRODUCTION

Liver cancer is predicted to be the sixth most commonly diagnosed cancer and fourth leading cause of cancer-related death worldwide in 2018, with ∼841,000 new cases and 782,000 deaths annually (1). China alone accounts for about 50% of the total number of cases and deaths (2). In the past two decades, despite various advances in the treatment of hepatocellular carcinoma (HCC), such as molecular-targeted therapy, radiofrequency ablation, and interventional embolization, surgery remains the most important treatment (3, 4). The high metastasis and recurrence rate of HCC indicates that its overall prognosis is still unsatisfactory (5–7). Metastasis and recurrence are the leading causes of death in patients diagnosed with invasive cancer. Tumor cells that leave the site of the primary tumor and are transported by circulation to distant organs are called circulating tumor cells (CTCs). CTCs are considered the source of tumor metastasis and recurrence (8–10).

The "seed and soil" hypothesis states that tumor cells (seeds) and stromal cells (soil) are involved in metastasis and clump together to form tumor microemboli (11, 12). It is a widely held view that the presence of tumor microemboli in the circulation is associated with a poor prognosis (13, 14). CTCs are precursors to metastasis in several types of cancer, occasionally appearing in clusters in the blood or found associated with immune-related cells, such as white blood cells (WBCs) (15). In some cases, the fact that CTCs clusters are "the soil with seeds" may help further transfer them to distant organs and continue to grow. However, it is unclear whether there are circulating tumor cell-associated white blood cell (CTC-WBC) clusters in the peripheral blood of patients with HCC and whether the presence of such CTC-WBC clusters is related to the prognosis of HCC.

In this study, we evaluated CTCs and CTC-WBC clusters in the peripheral blood of 214 preoperative HCC patients, starting in 2014. First, we used CanPatrol^{TM} CTC analysis technology to label CTCs and WBCs with different markers and then counted CTCs and CTC-WBC clusters using fluorescence microscopy. The main purpose of this retrospective study was to determine the influence of CTC-WBC clusters on the risk of recurrence and metastasis, and thus determine whether CTC-WBC clusters are potential biomarkers for tumor recurrence and metastasis.

MATERIALS AND METHODS

Study Design

From January 2014 to December 2016, 374 patients with HCC who underwent radical resection at the Zhujiang Hospital of Southern Medical University participated in this retrospective cohort study. The final 214 patients were screened according to inclusion criteria. The inclusion criteria were as follows (**Figure 1**): (a) HCC was diagnosed according to the World Health Organization's pathological standard (16); (b) patients did not have a relapsed or ruptured HCC, or cholangiocarcinoma; (c) patients underwent radical resection defined as R0 liver resection [patients who were microscopically positive (R1 liver resection), grossly positive (R2 liver resection), or whose margins were uncertain were excluded]; (d) patients did not die during the perioperative period or were not lost to follow-up after resection; and (e) patients did not receive anti-cancer treatment before surgery. The tumor stage was determined according to the Barcelona Clinical Liver Cancer (BCLC) staging system, while tumor differentiation was determined according to the Edmondson classification system.

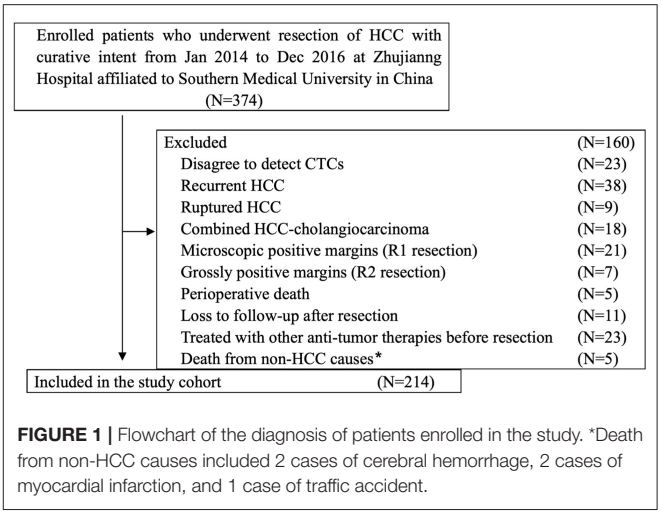

FIGURE 1 | Flowchart of the diagnosis of patients enrolled in the study. *Death from non-HCC causes included 2 cases of cerebral hemorrhage, 2 cases of myocardial infarction, and 1 case of traffic accident.

Patient Follow-Up

After collecting peripheral blood samples at admission (7 days before surgery), the patient entered a clinical follow-up period to monitor recurrence and death. Patients underwent various follow-up examinations and treatments according to a routine clinical schedule after surgery. Recurrence was determined based on the results of serum alpha-fetoprotein (AFP) levels, color Doppler ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), digital subtraction angiography (DSA), and positron emission tomography (PET). Recurrence was defined as intrahepatic recurrence and extrahepatic metastases. Recurrence or death was considered as the end point. Follow-up period was from January 1, 2014 to December 31, 2019. The median follow-up time was 52 months. All 214 resectable HCC patients have complete follow-up information.

CTCs and CTC-WBC Clusters Test

The CanPatrol^{TM} CTC analysis system (SurExam, China) was used to detect the number of CTCs and CTC-WBC clusters in 7.5 ml of whole samples of peripheral blood, similar to previous studies (17, 18). RNA-ISH was used to detect the following target sequences: white blood cells were labeled with CD45 and visible as white fluorescence, epithelial cells were labeled with EpCAM and CK8/18/19 and visible as red fluorescence, and mesenchymal cells were labeled with vimentin/twist and visible as green fluorescence. Nuclei were labeled with 40,6-diamidino-2-phenylindole (DAPI) and visible as blue fluorescence. CTC-WBC clusters are seen as a white dot of WBCs around a red, green, or red/green mixture of CTCs (**Figure 2**). After being labeled, the cells were analyzed with a fluorescence microscope.

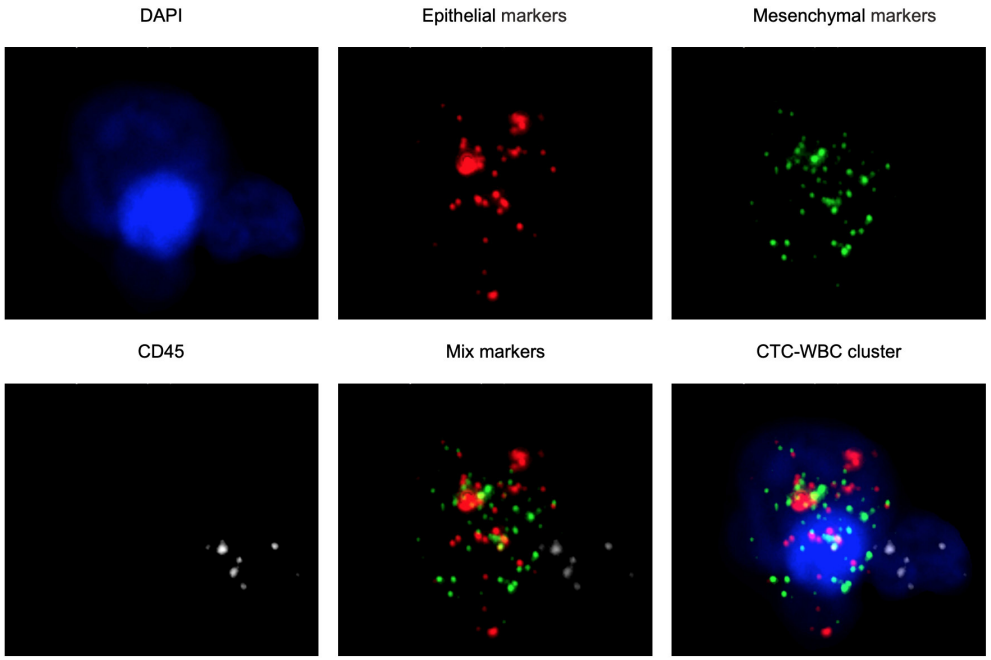

FIGURE 2 | Examples of CTCs, WBCs, and CTC-WBC clusters under automated fluorescent microscope imaging. Epithelial CTCs stained with EpCAM or CK8/18/19 (red). Mesenchymal CTCs stained with Vimentin or Twist (green). WBCs stained with CD45 (white). Nuclei stained with DAPI (blue). DAPI 40,6-diamidino-2-phenylindole.

Statistical Analysis

PASS version 11 is used to estimate the sample size of survival data 1-year survival rate of patients with CTC-WBC clusters positive and negative hepatocellular carcinoma in our department are estimated to be 72.6 and 84.5%, respectively. The time of all patients enrolled is estimated to be 36 months, and the follow-up time is planned to be 36 months, set $\alpha = 0.05$ (two-sided), $\beta = 0.2$, the ratio between the positive group and negative group is 1:1, and the loss to follow-up rate is 10%. Finally, the estimated total sample size is 142 cases. Categorized data were compared by the Chi-squared or Fisher's exact probability test. The OS and DFS were assessed by Kaplan-Meier analysis using the log-rank test. The Cox proportion hazard regression model was used for the multivariable survival analysis to determine prognostic factors that were significant in univariate analysis for either DFS or OS. All statistical analyses were two-tailed and a $P < 0.05$ was considered to be statistically significant. The data were analyzed using IBM SPSS Statistics 25.0.

RESULTS

Clinical and Pathological Characteristics

In the present study, peripheral blood was collected from 214 patients from Zhujiang Hospital for analysis, which is affiliated to Southern Medical University, including 28 women and 186 men, with a median age of 53 (range: 18–78) years. In total, 111 (51.9%) subjects had a tumor with a diameter >5 cm, while the remaining 103 (48.1%) had a tumor with a diameter ≤5 cm. While 185 (86.4%) patients had hepatitis B, 110 (51.4%) patients had liver cirrhosis. Preoperative examination showed portal vein tumor thrombosis in 19 (8.9%) patients. Regarding BCLC staging, there were 13 (6.1%) cases of stage 0, 72 (33.6%) cases of stage A, 110 (51.4%) cases of stage B, and 19 (8.9%) cases of stage C. Edmondson staging was performed postoperatively; there were 48 (22.4%) cases of stage I, 67 (31.3%) of stage II, 55 (25.7%) of stage III, and 44 (20.6%) of stage IV. There were 65 (30.4%) cases of encapsulation invasion and 58 (27.1%) cases of microvascular invasion. The cutoff values for total CTCs and CTC-WBC clusters were determined via ROC curve analysis, and the cutoff was considered positive for total CTCs ≥3, CTC-WBC clusters ≥2 (**Extended Data Table 1**, **Figure 1**). CTC-WBC clusters and different phenotypic CTC counts showed 141 (65.9%) positive CTCs and 89 (41.6%) positive CTC-WBC clusters. The follow-up period ended on December 31, 2019. There were 156 (72.9%) recurrences and 98 (45.8%) deaths. **Table 1** shows the relationship between CTC-WBC clusters and clinicopathological characteristics of HCC. Statistical analysis showed that CTC-WBC clusters were significantly correlated with tumor size ($P = 0.001$), tumor number ($P = 0.005$), portal vein tumor thrombus ($P = 0.026$), BCLC stage ($P < 0.001$), AFP level ($P = 0.002$), and total number of CTCs ($P < 0.001$). However, it was not related to gender, age, liver cirrhosis, Edmondson stage, Tumor encapsulation, Microvascular invasion, and HBsAg.

Survival Analysis

A Cox regression univariate analysis revealed that some factors were associated with the DFS of HCC patients, including

TABLE 1 | Relationship between CTC-WBC cluster and the clinicopathological characteristics of HCC patients.

Groups		CTC-WBC cluster		χ^2	*P*
		Negative	Positive		
Total	214	125	89		
Gender	Male	111	75	0.938	0.333
	Female	14	14		
Age (years)	<60	89	68	1.262	0.261
	≥60	36	21		
Tumor size (cm)	≤5	72	31	10.795	0.001*
	>5	53	58		
Tumor number	Solitary	92	49	7.954	0.005*
	Multiple	33	40		
Liver cirrhosis	No	61	43	0.005	0.944
	Yes	64	46		
Portal vein tumor thrombus	No	118	76	4.978	0.026*
	Yes	7	13		
BCLC stage	0+A	62	23	12.255	<0.001*
	B+C	63	66		
Edmondson stage	I+II	73	42	2.627	0.105
	III+IV	52	47		
Tumor encapsulation	Complete	93	56	3.239	0.072
	None	32	33		
Microvascular invasion	No	97	59	3.365	0.067
	Yes	28	30		
HBsAg	Negative	19	10	0.697	0.404
	Positive	106	79		
AFP (μg/L)	<400	85	42	9.330	0.002*
	≥400	40	47		
Total CTCs	Negative	66	7	46.702	<0.001*
	Positive	59	82		

*$P < 0.05$.

BCLC, Barcelona Clinical Liver Cancer; SD, standard deviation; HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; CTCs, circulating tumor cells; CTC-WBC, circulating tumor cell-white blood cells.

CTC-WBC clusters, tumor size, portal vein tumor thrombus, BCLC stage, Edmondson stage, microvascular invasion, AFP level, and total CTCs (**Table 2**). Some factors were associated with the OS of HCC patients, including CTC-WBC cluster, tumor size, portal vein tumor thrombus, BCLC stage, AFP, and total CTCs (**Table 3**). A multivariable analysis was performed and the results showed that CTC-WBC clusters (HR = 1.951, 95% CI: 1.348–2.824, $P < 0.001$), tumor size, portal vein tumor thrombus, BCLC stage, AFP and total CTC number were independent predictors of DFS (**Table 2**). CTC-WBC clusters (HR = 3.026, 95% CI: 1.906–4.802, $P < 0.001$), tumor size, portal vein tumor thrombus, and total CTC number were independent predictors of OS (**Table 3**). In addition, the Kaplan-Meier curve showed that the DFS ($P < 0.001$) and OS ($P < 0.001$) of HCC patients in the CTC-WBC cluster positive group were shorter than those in the negative group. The 3-year survival rate of the CTC-WBC cluster-positive group was 34.8% and the 5-year survival rate was 17.9%. The 3-year survival rate of the CTC-WBC cluster-negative group was 81.5% and the 5-year survival rate was 70.0% (**Figures 3A,B**).

DISCUSSION

We used CanPatrol™ CTC analysis technology to count different types of CTCs and CTC-WBC clusters. CTCs can be divided into different subtypes, including epithelial CTCs, mesenchymal CTCs, and mixed (epithelial/mesenchymal) CTCs. Different subtypes can form CTC-WBC clusters with WBCs. A large number of studies have shown that epithelial-mesenchymal transition (EMT) plays a key role in tumor recurrence and

TABLE 2 | Univariate and multivariable analyses of the predictors of disease-free survival in HCC patients.

	Univariate analysis			Multivariate analysis		
	HR	***P***	**95% CI**	**HR**	***P***	**95% CI**
Gender						
Female vs. Male	1.098	0.684	0.699–1.724			
Age (years)						
≥60 vs. <60	1.037	0.840	0.728–1.478			
Tumor size (cm)						
>5 vs. ≤5	4.785	0.000*	3.346–6.841	1.880	0.036*	1.041–3.394
Tumor number						
Multiple vs. Solitary	1.261	0.163	0.910–1.748			
Liver cirrhosis						
Yes vs. No	0.908	0.548	0.664–1.243			
Portal vein tumor thrombus						
Yes vs. No	3.998	0.000*	2.451–6.522	1.950	0.010*	1.176–3.234
BCLC stage						
B+C vs. 0+A	4.466	0.000*	3.069–6.499	2.078	0.022*	1.112–3.882
Edmondson stage						
III+IV vs. I+II	1.419	0.029*	1.036–1.944	0.926	0.661	0.657–1.305
Tumor encapsulation						
None vs. Complete	1.306	0.120	0.932–1.830			
Microscopic vascular invasion						
Yes vs. No	1.459	0.033*	1.030–2.066	0.832	0.335	0.572–1.209
HBsAg						
Yes vs. No	0.851	0.474	0.546–1.325			
AFP (μg/L)						
≥400 vs. <400	2.276	0.000*	1.655–3.129	1.438	0.045*	1.008–2.051
Total CTCs						
Positive vs. Negative	3.164	0.000*	2.159–4.638	1.675	0.018*	1.094–2.563
CTC-WBC cluster						
Positive vs. Negative	3.147	0.000*	2.276–4.351	1.951	<0.001*	1.348–2.824

**P < 0.05.*

HR, Hazard ratio; CI, confidence interval; BCLC, Barcelona Clinical Liver Cancer; SD, Standard Deviation; HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; CTCs, circulating tumor cells; CTC-WBC, circulating tumor cell-white blood cells.

metastasis (19). Our team's previous research also confirmed that mesenchymal CTCs are more ideal early predictors of HCC recurrence (17, 20). However, we found in the study that CTCs in the CTC-WBC clusters were almost all mixed CTCs. This study explored the relationship between CTC-WBC clusters and prognosis in patients with HCC before radical resection. The results showed that CTC-WBC clusters are independent prognostic indicators of DFS and OS in HCC patients. The presence of CTC-WBC clusters in the peripheral blood and CTC-WBC cluster-positive patients have worse prognosis.

CTCs are relatively safe and readily available "liquid biopsy" specimens (21, 22). In fact, CTCs can serve as both an indicator of diagnosis and prognosis and provide molecular information to guide treatment decisions (17, 22, 23). Several studies have shown that CTCs are independent risk factors for HCC, and patients with higher CTC counts have a poorer prognosis (23, 24). The circulating CTCs usually exist in the form of single cells and multiple CTCs can also be clustered together (25). In addition, previous studies have shown that CTC clusters in the peripheral blood have a survival advantage and have enhanced tumor cell metastasis and colonization capacity in both mouse models and patients (25, 26). Do CTCs form clusters with other cells? We first detected CTC-WBC clusters in the peripheral blood of HCC patients using CanPatrol^{TM} CTC analysis technology in 2014. The finding supports the role of tumor-associated immune cells in the development of cancer. It has potential value because the application of immune checkpoint blocking in the treatment of many different types of cancer is ever increasing. However, what role and significance do WBCs in the peripheral blood have for CTCs? It is clear that in a tumor microenvironment, tumor-associated neutrophils (TANs) promote the growth and

TABLE 3 | Univariate and multivariable analyses of the predictors of overall survival in HCC patients.

	Univariate analysis			Multivariate analysis		
	HR	***P***	**95% CI**	**HR**	***P***	**95% CI**
Gender						
Female vs. Male	0.615	0.165	0.310–1.221			
Age (years)						
≥60 vs. <60	1.447	0.089	0.945–2.216			
Tumor size (cm)						
>5 vs. ≤5	7.757	<0.001*	4.514–13.331	2.714	0.016*	1.202–6.128
Tumor number						
Multiple vs. Solitary	1.461	0.065	0.976–2.186			
Liver cirrhosis						
Yes vs. No	1.081	0.700	0.727–1.609			
Portal vein tumor thrombus						
Yes vs. No	8.167	<0.001*	4.722–14.123	3.744	<0.001*	2.117–6.622
BCLC stage						
B+C vs. 0+A	8.240	<0.001*	4.386–15.481	2.374	0.075	0.916–6.153
Edmondson stage						
III+IVvs. I+II	1.361	0.127	0.916–2.024			
Tumor encapsulation						
None vs. Complete	1.218	0.356	0.801–1.852			
Microscopic vascular invasion						
Yes vs. No	1.427	0.102	0.932–2.184			
HBsAg						
Yes vs. No	1.002	0.995	0.558–1.798			
AFP (μg/L)						
≥400 vs. <400	2.101	<0.001*	1.411–3.127	1.039	0.858	0.681–1.586
Total CTCs						
Positive vs. Negative	6.697	<0.001*	3.559–12.602	2.805	0.003*	1.432–5.495
CTC-WBC cluster						
Positive vs. Negative	5.347	<0.001*	3.471–8.236	3.026	<0.001*	1.906–4.802

*$P < 0.05$.

HR, hazard ratio; CI, confidence interval; BCLC, Barcelona Clinical Liver Cancer; SD, Standard Deviation; HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; CTCs, circulating tumor cells; CTC-WBC, circulating tumor cell-white blood cells.

metastasis of cancer cells through direct effects on cancer cells and indirect effects on tumor cells by changing the tumor microenvironment (27). Zhou et al. found that TANs play a crucial role in tumor development and progression in the tumor microenvironment (28). However, these studies only confirmed that TANs in the primary tumor microenvironment promoted the growth of HCC. It is not clear whether TANs are present in the peripheral blood, and if so, whether it also promotes CTC proliferation. Neutrophils are part of the natural immune system and form the largest proportion of white blood cells (WBC) in the human circulation. In this study, we found that CTC-WBC clusters in the peripheral blood are associated with portal vein tumor thrombi and microvascular invasion. Nonetheless, we do not know whether CTC-WBC clusters are contained in portal vein tumor thrombi and microvessel invasion nests. However, as we know portal vein tumor thrombus and microvascular invasion are independent prognostic factors of hepatocellular carcinoma, this correlation between CTC-WBC cluster and portal vein tumor thrombus or microvascular invasion also suggest that HCC patients with positive CTC-WBC cluster have a poor prognosis. In a recent publication, Szczerba et al. (29) tested for CTCs in blood samples from 70 breast cancer patients. It was found that most CTCs in the circulation were single CTCs and a small number of CTC clusters (8.6%) were CTC-WBC clusters (3.4%). It was confirmed that compared with a single CTCs or CTC cluster, the presence of CTC-neutrophil clusters is related to the poor prognosis of breast cancer patients. We identified the presence of CTC-WBC clusters in the peripheral blood of patients with HCC in 2014 and counted CTC-WBC clusters when classifying patients' peripheral blood CTCs. The 5-year follow-up confirmed that CTC-WBC clusters were related to DFS and OS in HCC patients and

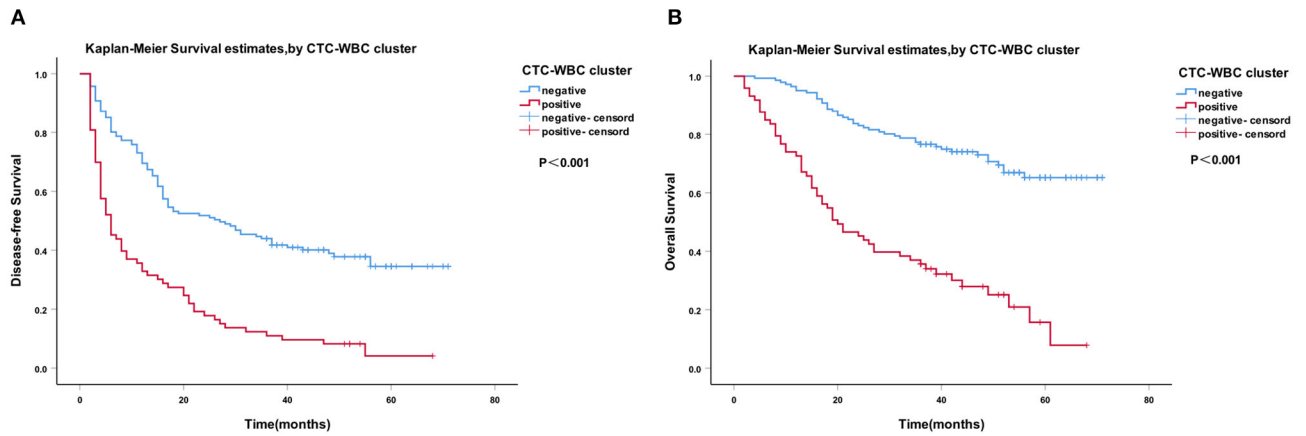

FIGURE 3 | Survival analysis of hepatocellular carcinoma (HCC) patients. CTC-WBC cluster-positive status is associated with a poorer outcome in patients with HCC. Kaplan-Meier analysis revealed significant differences in disease-free survival ($P < 0.001$) **(A)** and overall survival ($P < 0.001$) **(B)** between preoperative CTC-WBC cluster-positive and -negative patients in an HCC cohort. Survival analysis of HCC patients using the Kaplan-Meier method. *P*-values were determined using the log-rank test. DFS, disease-free survival; OS, overall survival. **Extended Data Figure 1** ROC curve showing the effectiveness of the use of total CTCs and CTC-WBC clusters in the diagnosis of HCC.

were an independent predictor of DFS and OS. In addition, the Kaplan–Meier analysis also showed that CTC-WBC cluster-positive patients lived for a shorter time than the CTC-WBC cluster-negative patients.

CONCLUSIONS

In summary, we found that there are a certain number of CTC-WBC clusters in the peripheral blood of patients with HCC. CTC-WBC clusters are associated with common risk factors such as AFP, total CTC count, portal vein tumor thrombus, and microvascular invasion, and CTC-WBC clusters in the peripheral blood are an independent predictor of DFS and OS and their presence indicate poor prognosis in patients with HCC. This phenomenon gives us a hint that circulating CTCs may have their own immune microenvironment and both the "seed" and "soil" are involved in metastasis. This may open the door to new therapeutic targets directed against cell-cell junctions and associated survival pathways. In addition, we found in the study that CTCs in the CTC-WBC cluster were almost all mixed CTCs; why this was the case is not clear at this time, and is a direction worthy of future research. The results of this study can provide evidence for CTC-WBC cluster as a potential biomarker for the prognosis of HCC. However, The present study had several limitations. First, retrospective cohort study and limited sample sizes have affected statistical power to draw clear conclusions. If this conclusion can be further verified in a follow-up prospective multicenter study, it will be more reliable. Second, it included only chinese patients recruited from a single institution, the achieved results cannot be generalized to other patient populations, especially to non-Asian patients. Finally, we mainly focused on patients with resectable hepatocellular carcinoma after surgery, and data on advanced patients is worthy of further study and discussion.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Second Affiliated Hospital of Southern Medical University (2020-KY-003-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MP and YC: conception and design. QL and BP: development of methodology. BP, LC, HL, KC, and CZ: acquisition of data. QL and CW: analysis and interpretation of data and writing, review, and/or revision of the manuscript. MP and XP: administrative, technical, or material support. MP and YC: study supervision. 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.2020.01758/full#supplementary-material

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MINI REVIEW
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Etiology of Hepatocellular Carcinoma: Special Focus on Fatty Liver Disease

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Hepatocellular Carcinoma (HCC) is a highly aggressive cancer with mortality running parallel to its incidence and has limited therapeutic options. Chronic liver inflammation and injury contribute significantly to the development and progression of HCC. Several factors such as gender, age, ethnicity, and demographic regions increase the HCC incidence rates and the major risk factors are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), carcinogens (food contaminants, tobacco smoking, and environmental toxins), and inherited diseases. In recent years evidence highlights the association of metabolic syndrome (diabetes and obesity), excessive alcohol consumption (alcoholic fatty liver disease), and high-calorie intake (nonalcoholic fatty liver disease) to be the prime causes for HCC in countries with a westernized sedentary lifestyle. HCC predominantly occurs in the setting of chronic liver disease and cirrhosis (80%), however, 20% of the cases have been known in patients with non-cirrhotic liver. It is widely believed that there exist possible interactions between different etiological agents leading to the involvement of diverse mechanisms in the pathogenesis of HCC. Understanding the molecular mechanisms of HCC development and progression is imperative in developing effective targeted therapies to combat this deadly disease. Noteworthy, a detailed understanding of the risk factors is also critical to improve the screening, early detection, prevention, and management of HCC. Thus, this review recapitulates the etiology of HCC focusing especially on the nonalcoholic fatty liver disease (NAFLD)- and alcoholic fatty liver disease (AFLD)-associated HCC.

Keywords: hepatocellular carcinoma, alcoholic fatty liver disease, non-alcoholic fatty liver disease, etiology, metabolic syndrome, hepatitis viruses

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INTRODUCTION

Hepatocellular Carcinoma (HCC) is a serious public health issue and the fourth leading cause of cancer mortality worldwide (1, 2). HCC accounts for about 80% of the primary liver cancer while the other types include cholangiocarcinoma (10–20%) and angiosarcoma (1%) (3). There is a striking variation in HCC incidence rates across geographic regions and at the global level, each year over 800,000 people are diagnosed with liver cancer (4, 5). HCC cases are highest in Eastern Asia and sub-Saharan Africa, followed by intermediate rates in Southern and Western European

countries, North and Central America, and the lowest incidence rates are observed in and Northern Europe and South Central Asia (6, 7). HCC predominantly affects men more than women (two to four times higher in men) with its highest incidence in the age group of 45–65 years (8, 9). According to Globocan 2018, HCC is the fifth most common cancer in men and the ninth most commonly occurring cancer in women (10). The overall ratio of mortality to incidence is 0.95 and reflects the poor prognosis of HCC (11).

HCC is an extremely complex condition and there are multiple factors involved in the etiology of HCC. The major risk factors for HCC include hepatitis B virus (HBV) and hepatitis C virus (HCV), diabetes, obesity, alcoholic fatty liver disease (AFLD), and non-alcoholic fatty liver disease (NAFLD). Additional risk factors that are also known to increase the incidence of HCC are tobacco smoking, food contaminants such as aflatoxins, familial or genetic factors, and various environmental toxins that act as carcinogens (12–14) (**Figure 1**). The development of HCC is initiated by hepatic injury involving inflammation leading to necrosis of hepatocytes and regeneration. This chronic liver disease sequentially transitions to fibrosis, cirrhosis, and hepatocellular carcinoma (15, 16). HCC that often occurs in the setting of chronic liver disease and cirrhosis is diagnosed late in its course and liver transplantation is the best option for patients at this stage (12, 17). Multiple treatment options are available to treat HCC including surgical resection, local ablation with radiofrequency, transcatheter arterial chemoembolization (TACE), radioembolization, and systemic targeted agents like sorafenib depending on the tumor extent or underlying liver dysfunction (12, 14, 18). Furthermore, the viable treatment options offered to the patients also depend on the causative agent of HCC as they define the disease course and patient characteristics. However, with the improved treatment for HCC, the demographic landscape has changed (6, 19). In this mini-review, we aim to describe the traditional risk factors in brief and highlight on fatty liver disease, which is the emerging etiological risk factor contributing to the increasing incidences of HCC.

VIRUS AND HCC

The chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) are the traditional risk factors that are associated with HCC for 33,600 years and 1,000 years, respectively (20, 21).

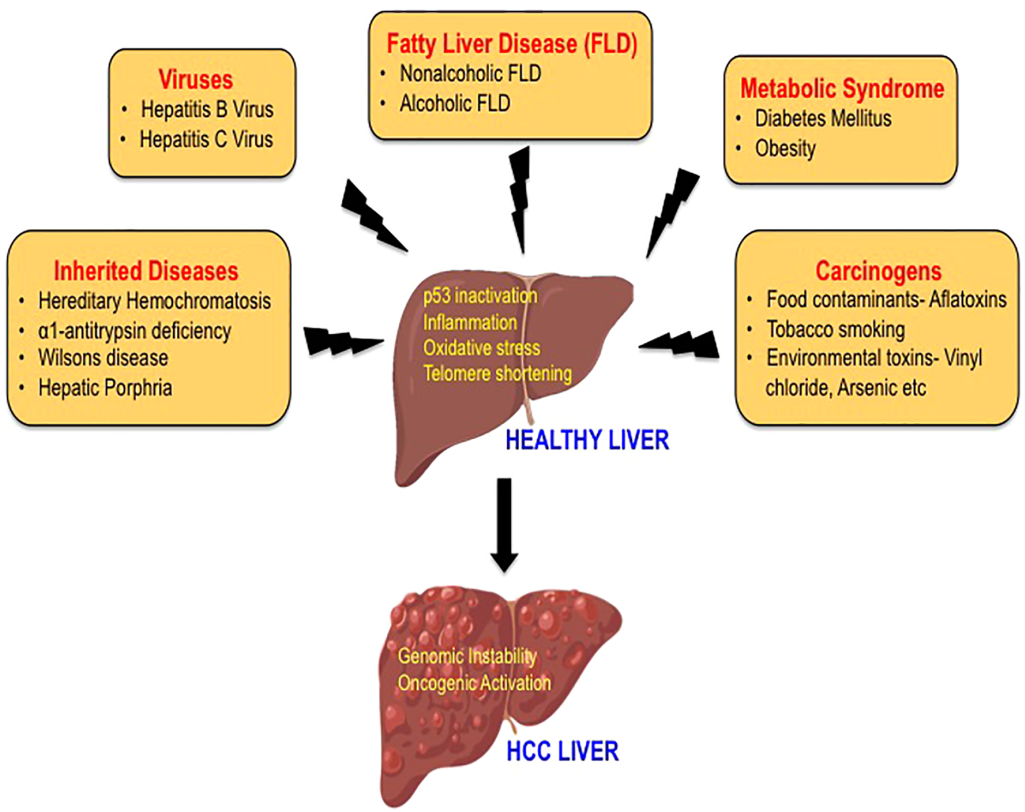

FIGURE 1 | The etiology of hepatocellular carcinoma. A variety of risk factors have been associated with the development of HCC, including hepatitis viruses, carcinogens, heredity diseases, metabolic syndrome, and fatty liver disease. The mechanisms by which these etiological factors may induce hepatocarcinogenesis mainly include p53 inactivation, inflammation, oxidative stress, and telomere shortening leading to genomic instability and activation of multiple oncogenic signaling pathways.

The virus-associated mechanisms driving hepatocarcinogenesis are complex and cause liver cirrhosis, which progresses to HCC in about 80–90% of the cases (15, 22).

HBV is partially a double-stranded circular DNA virus, which belongs to the genus *Avihepadnavirus* of the Hepadnaviridae family. HBV infection accounts for 75–80% of virus-associated HCC and infects over 240 million people around the world (23). The incorporation of the genetic material of this virus into the human genome causes p53 inactivation, inflammation, or oxidative stress, which causes hepatocarcinogenesis (24, 25). HBV-induced HCC can be both cirrhotic and non-cirrhotic and involves an array of processes such as proliferation and loss of growth control (caused by p53 inactivation), sustained cycles of necrosis and regeneration (resultant of inflammation), and activation of various oncogenic pathways such PI3K/Akt/STAT3 pathway and Wnt/β-catenin (induction of oxidative stress), all of which leads to genomic instability (26, 27).

Contrary to HBV, the Hepatitis C virus (HCV) is a non-integrating, single-stranded RNA virus belonging to the genus *Hepacivirus* of the Flaviviridae family. HCV infects over 57 million people worldwide and accounts for 10–20% of virus-associated HCC (28, 29). Unlike HBV infection, there is no integration of genetic material into the host's genome by the HCV virus. It is the HCV proteins (structural and non-structural proteins) that play a critical role in the development of HCC (30). HCV-induced hepatocarcinogenesis is highly complex involving the activation of multiple cellular pathways and gets initiated by the establishment of HCV infection leading to chronic hepatic inflammation, which further progresses to liver cirrhosis and HCC development (31). HCV proteins either directly or indirectly modulate a wide range of host cellular activities, including transcriptional regulation, cytokine modulation, hepatocyte growth regulation, and lipid metabolism that lead to chronic liver injury. In addition to inducing oxidative stress and endoplasmic reticulum (ER) stress, HCV proteins are also known to cause epigenetic alterations by modulating micro RNA (miRNA) and long noncoding RNA (lncRNA) in the host cells (32). Thus, HCV shows a high propensity (60–80%) to induce chronic infection and promotes liver cirrhosis 10–20 fold higher than HBV. The angiogenic and metastatic pathways activated by HCV further promote hepatocytes' malignant transformation and accelerate HCC development (33). Hepatitis D virus (HDV) and human immunodeficiency virus (HIV) are also considered as modulators of HCC (14).

CARCINOGENS AND HCC

In addition to hepatitis viruses, chemical carcinogens also play important roles in the etiology of HCC (34). Exposure to carcinogens including aflatoxins, tobacco smoking, vinyl chloride, arsenic, and various other chemicals act either independently or in combination with viruses to cause DNA damage, induce liver cirrhosis, and contribute to HCC (35).

Aflatoxin is a potent liver carcinogen produced by the Aspergillus fungus, which is found to contaminate foodstuffs such as peanuts, corn, soya beans stored in damp conditions. This mycotoxin induces mutation in the p53 tumor suppressor gene and causes uninhibited growth of liver cells leading to the development of HCC (36, 37). It is reported that the chemicals in tobacco smoke (4-aminobiphenyl and polycyclic aromatic hydrocarbons), areca nut (nitrosamines), and betel leaves (safrole) cause hepatotoxicity (13, 35). Besides, studies have demonstrated that the human exposure to groundwater contaminants (chemicals such as cadmium, lead, nickel, arsenic), organic solvents (toluene, dioxin, xylene), and chemicals such as vinyl chloride and dichlorodiphenyltrichloroethane (DDT) have shown to increase the risk of HCC as they exert hepatocarcinogenic effect *via* induction of oxidative stress and telomere shortening (34, 38).

INHERITED DISEASES AND HCC

Certain metabolic disorders such as hereditary hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, and hepatic porphyria are associated with high risk for the development of HCC. These hereditary diseases are known to promote hepatocarcinogenesis as a result of increased inflammation and hepatocellular damage (39–41).

METABOLIC SYNDROME AND HCC

Diabetes mellitus, a component of the metabolic syndrome has been shown to attribute about 7% of the HCC cases worldwide (5, 42). Meta-analyses have shown that diabetes is associated with HCC independent of viral hepatitis in which diabetic patients show 2-3 fold greater risks in developing HCC compared with non-diabetic controls (43). The pathophysiological conditions such as hyperglycemia, hyperinsulinemia, insulin resistance, and activation of insulin-like growth factor signaling pathways provide a strong association for diabetes to be the risk factor in the pathogenesis of HCC (5, 44). Obesity, a pathological state characterized by insulin resistance, hyperinsulinemia, and inflammation is also closely associated with HCC (45). It is demonstrated that increased reactive oxygen species, dysregulated adipokines, and adipose tissue remodeling, alteration of gut microbiota, and dysregulated microRNA increases the relative risk of HCC in obese patients (46–48). Accordingly, obesity is one of the common causes of NAFLD, which is also an underlying risk factor of HCC (46).

FATTY LIVER DISEASE AND HCC

Over the last decade, fatty liver disease is emerging as the leading etiologies for chronic liver disease progressing to HCC (49). The changing scenario is attributed to improved antiviral therapy for virus-related HCC (50). With the growing inclination towards western dietary pattern, sociocultural changes and the lifestyle with limited or no physical activity

has sharply increased the incidence rates of NAFLD- and AFLD-associated HCC across the continents (51, 52). The pathological spectra of liver injury in promoting HCC development are similar in these two fatty liver diseases despite having divergent pathogenic origin with yet some key distinct features (**Figure 2**). Furthermore, a high-calorie diet and ethanol act synergistically at multiple levels potentiating hepatocarcinogenesis (53).

Non-Alcoholic Fatty Liver Disease (NAFLD)-Associated HCC

NAFLD is characterized by excessive hepatic lipid accumulation (steatosis), which further transitions to steatohepatitis upon the inflammatory insult, to cirrhosis and HCC (54, 55). It's a pathophysiological condition that is not associated with excess alcohol consumption or other secondary causes such as viral infection and heredity liver diseases (56). NAFLD is classically associated with metabolic disorders such as obesity, hypertension, dyslipidemia, insulin resistance, and type 2 diabetes (57, 58).

A meta-analysis by Younossi et al. (86 studies from 22 countries carried out between 1989 and 2015) reported that the worldwide prevalence of NAFLD is 25.24% (59). The prevalence of NAFLD varies across the continent with the highest in the Middle East (31.79%) followed by South America (30.45%), Asia (27.37%), North America (24.13%), Europe (23.71%), and Africa (13.48%) (51, 60). Studies also indicate that NAFLD is more common in men (42% for white males *vs.* 24% for white females) and the prevalence of NAFLD increases with age (61, 62). However, as obesity increases in children and adolescents, there is an increasing prevalence of NAFLD and NAFLD-associated HCC compared to adults (63, 64). While studies have shown that NAFLD accounts for about 13% of HCC cases, Wong et al., have reported that NAFLD is the fastest-growing etiology, which is indicative of liver transplantation in HCC patients (65). Studies from long term follow up of non-alcoholic fatty liver patients have shown the prevalence of HCC to be 0.5 and 2.8% in NAFLD and NASH respectively (66, 67). It is interesting to note that 80% of HCC patients have cirrhosis (68). However, HCC is also reported in non-cirrhotic NASH (69). Thus, with the rise in the incidence of NAFLD-associated HCC in recent years, the contribution of NAFLD is underscored among the risk factors that induce HCC (70).

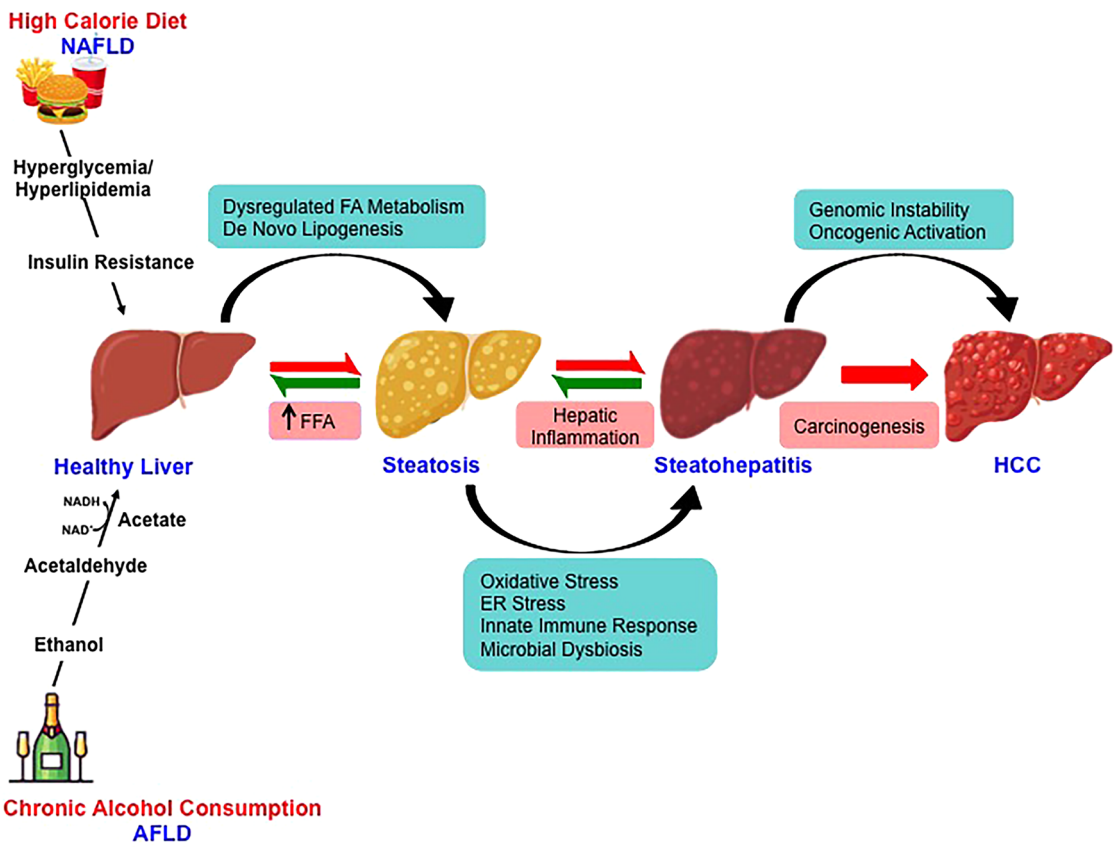

FIGURE 2 | Molecular mechanisms involved in nonalcoholic- and alcoholic-associated HCC. High-calorie diet and excessive alcohol consumption is the major risk factor for the development of NAFLD and AFLD respectively. Despite the divergent pathogenic origin, the pathological spectra of liver injury in promoting HCC development in NAFLD and AFLD share common molecular pathways.

Emerging evidence has established multiple risk factors for NAFLD-associated HCC including obesity, diabetes, iron deposition, genetic and epigenetic factors, microRNA, and gut microbiota (49, 71). In the modern era with a sedentary lifestyle and unhealthy dietary habits, obesity is rapidly increasing and has been established as a risk factor for HCC (56). It is been reported to increase the risk by 1.5–4 times either by contributing to the development of NAFLD or by directly exerting carcinogenic effect leading to HCC (72). Albeit most patients with NAFLD are obese in the western countries, lean NAFLD has also been reported from Asian countries (73). Furthermore, large population-based cohort studies have found that diabetes mellitus is associated with 1.8–4 fold increased risk of HCC (74). Along the same line, a study by Turati et al. reported that the combined effect of diabetes and obesity among the metabolic syndrome was positively associated with HCC risk (75). Excessive iron deposit in the liver is thought to be a risk factor for NAFLD-HCC (76). Indeed, experimental studies by Paola et al., demonstrated that hepatic iron overload might be associated with HCC development in NASH patients (77). Additionally, genetic factors are known to increase the risk of HCC in NAFLD such as the PNPLA3 I148M variant and rs58542926 (E167K) variant in TM6SF2 (78, 79). Studies carried out in mouse models of NAFLD and also in patients with NAFLD or HCC have identified epigenetic-mediated gene regulation involved in the development and progression of the disease (80, 81). Among the various risk factors, the gut microbiota has emerged as an important contributor to NAFLD-associated HCC (82).

The mechanism of NAFLD-associated HCC progression is complex. Hepatic lipid accumulation as a result of high-calorie intake (high carbohydrate and high dietary fat) and low physical activity in the absence of excessive alcohol consumption is a major contributor to the onset of NAFLD development (56). Steatosis progresses to necroinflammation leading to hepatocarcinogenesis as a consequence of multiple parallel acting conditions such as insulin resistance, hyperinsulinemia, dyslipidemia, adipose tissue remodeling, oxidative/endoplasmic reticulum (ER) stress, altered immune system, genetic alterations, and dysbiosis in the gut microbiome. These modifications in association with genetic factors and epigenetic changes activate oncogenic signaling and promote HCC development (83). Insulin resistance leads to increased release of free fatty acids (FFA) and release of various inflammatory cytokines including tumor necrosis factor- α (TNF-α), interleukin 6 (IL-6), leptin, and resistin. This is also accompanied by decreased amounts of adiponectin (84). Insulin resistance along with hyperinsulinemia up-regulates insulin and insulin-like growth factor (IGF-1), a growth stimulator aiding hepatocyte proliferation and apoptosis inhibition (85, 86).

Furthermore, hepatic lipotoxicity due to insulin resistance leads to imbalanced energy metabolism. Elevated FFAs β-oxidation induces oxidative stress through the release of reactive oxygen species (ROS) eventually leading to mitochondrial dysfunction accompanied by ER stress (87, 88). There exists a potent cross talk between oxidative/endoplasmic reticulum (ER) stress, and apoptotic pathways along with inflammatory cytokines, innate and adaptive immune responses that significantly contribute to NASH progression to HCC (83). Further, the oxidative stress promotes tumorigenesis by activation of c-Jun amino-terminal kinase 1 (JNK1), a mitogen-activated protein kinase, and by suppressing the action of p53 tumor suppressor gene and nuclear respiratory factor 1 (Nrf1) (89). Interestingly, studies have confirmed the potential role of immune cells such as $CD8^+$, $CD4^+$ T lymphocytes, and Kupffer cells in NASH progression with altered intestinal gut microbiome being one of the contributors (90, 91). Thus, the molecular connection between regulations of hepatocyte cell cycle and energy balance is the key driving force of NAFLD-associated HCC.

Unfortunately, there is yet no FDA-approved drug for the effective treatment of NAFLD and NAFLD-HCC. A better understanding of the cellular and molecular mechanisms will open up treatment options for HCC subjects with NAFLD etiology. Dietary and lifestyle modifications being the mainstay of disease management need to be tailored to meet individual patients' needs. Furthermore, knowing the co-morbidities of NAFLD-HCC will aid in designing effective treatment strategies that can be employed in clinical practice.

Alcoholic Fatty Liver Disease (AFLD)-Associated HCC

As the name suggests, AFLD is attributed to excessive alcohol consumption that causes hepatic injury by the build-up of fats, inflammation, and scarring leading to HCC, which could be fatal (92). Globally, the prevalence of AFLD is increasing and has become a significant contributor to the liver disease burden accounting for 30% of HCC related deaths (93). The "safe" levels of drinking as defined in the dietary guidelines in the United States is two drinks for men and one drink for women per day as one alcoholic drink (12 ounces of beer, 5 ounces of wine, or 1 ounce of hard liquor) accounts for about 14 g of alcohol (defined as standard drink by WHO) (53). By contrast, excessive alcohol consumption (more than 14 drinks/week and 7 drinks/week for men and women respectively) is considered to cause AFLD (51). The threshold level of alcohol intake causing hepatotoxic effect varies and it depends on a variety of factors such as gender, ethnicity, and genetics (94).

A large population-based prospective study conducted by Becker et al., for 12 years have provided evidence that females are more susceptible to the toxic effects of alcohol than male for any given level of alcohol intake (95). The possible mechanisms include lower gastric alcohol dehydrogenase (ADH) activity in females and estrogen levels that activate Kupffer cells due to increased gut permeability and portal endotoxin levels leading to alcohol-induced liver injury (96, 97). Furthermore, studies have demonstrated that in the United States, compared to Whites, Blacks, and Hispanics drinkers have a two-fold increase in liver enzymes (98). Since there is no significant difference among

other ethnic groups, factors such as polymorphism of genes associated with alcohol metabolism (*ADH*, *CYP2E1*) and antioxidant enzymes and genes coding for cytokines are also investigated in association with alcoholic liver disease (99). However, it remains critical to consider factors such as amount and type of alcohol consumption and socioeconomic status with the development of AFLD.

As per the global status report on alcohol and health, 2018, there are 2.3 billion active drinkers worldwide (100). In America, Europe, and Western Pacific more than half of the population account for active alcoholics. Though the percentage of drinkers has decreased in Africa and America, there is an increase observed in the Western Pacific region and has remained stable in the regions of Southeast Asia (101). Alcohol is one of the commonest causes of chronic liver disease with nearly 75 million diagnosed for the risk of AFLD and contributes to 50% of mortality related to cirrhosis (102). According to the global health report on alcohol and health, 2018 by World Health Organization (WHO), the alcohol-attributable deaths (AAD) from liver cirrhosis varies across the countries. The top five in the list includes India (Safe limits: ≤16 g/day for men and ≤8 g/day for women, Comparison of international alcohol drinking guidelines, 2019), China (Safe limits: ≤25 g/day for men and ≤15 g/day for women, Chinese Dietary Guidelines, 2016), Nigeria (Safe limits: no written national policy, WHO, 2018), United States (Safe limits: ≤24 g/day for men and ≤14 g/day for women, Dietary Guidelines for Americans 2015–2020), and Russia (Safe limits: ≤30 g/day for men and ≤20 g/day for women, Prevention of alcohol and drug use, National Medicine Research Center for Therapy and Preventive Medicine). It is also reported that liver cancer (22.5%) is the largest contributor to the burden of alcohol-attributable cancer DALY (disability-adjusted life year), followed by colorectal (20.6%) and esophageal (18.5%) cancers (100). The global HCC BRIDGE study by Park et al. reported that AFLD contributes to HCC development to a large portion in Europe (37%) and North America (21%) compared to East Asia (4–13%) (103). Furthermore, progression to cirrhosis and mortality is higher in patients with AFLD (36%) compared to NAFLD (7%) (104) and studies have reported that AFLD accounts for 10.3% of HCC in liver transplantation candidates (105). It is noteworthy that there is a synergy between excessive alcohol consumption with other risk factors including diabetes mellitus and viral hepatitis (106).

Despite the differences in the epidemiological and clinical characteristics, AFLD-associated HCC shares a similar mechanism of HCC pathogenesis with that of NAFLD. Acetaldehyde, an oxidation product of ethanol is a potent carcinogen driving the tumorigenesis by the formation of DNA adducts (106). Although the major pathway of metabolizing ethanol involves CYP2E1 in microsomes, acetaldehyde, and reactive oxygen species (ROS) are formed nevertheless (107). Interestingly, ethanol also induces steatosis by elevating the enzyme levels of *de novo* lipogenesis (DNL) and by suppressing the oxidation of fatty acid by downregulating PPARα (108, 109). In addition, progressive alterations in PNPLA3 and TM6SF2 genes, and micro RNA are known to promote steatosis, fibrosis, and cirrhosis in AFLD (110, 111). Thus similar to NAFLD-associated HCC, alcohol induces cirrhosis and promotes HCC development *via* the production of ROS, induction of chronic inflammation, activation of the immune response, leaky gut, and alteration of gene expression. However, the infiltration of inflammatory cells is found to be higher in AFLD (105, 112).

CONCLUSION AND FUTURE PERSPECTIVES

HCC is a highly fatal cancer driven by multiple etiological factors, among which, fatty liver disease is emerging as a major cause worldwide. Based on the pathogenic origin, NAFLD has been strongly associated with glucose and lipid metabolism, whereas AFLD has been associated with a strong inflammatory response. NAFLD and AFLD share common molecular mechanisms in promoting HCC development, which involves vicious interplay between various pathways including immunological pathways, endocrine pathways, and metabolic pathways. However, there still exists a gap in the knowledge in understanding the molecular mechanisms of inflammation, genetic and epigenetic regulations, and genomic instability leading to hepatocarcinogenesis. Indeed, a comprehensive understanding of these diseases would aid in the identification of biomarkers and therapeutic targets leading to early detection and management.

Albeit, NAFLD- and AFLD-associated HCC are major challenging public health issues, it is preventable. The widely implemented curative approach is lifestyle alteration involving modifications in dietary habits and improving physical activity in case of NAFLD and alcohol abstinence in AFLD. Further personalized treatment strategies could improve healthcare and quality of patient care, thereby reducing the mortality rate. Alternatively, strategies like pharmacological treatment and bariatric surgery are also considered in patients unresponsive to lifestyle changes. Conclusively, it is important to develop diagnostic tests for the detection of early stages of HCC.

AUTHOR CONTRIBUTIONS

DS and DPK devised and wrote the manuscript. AS and DPK made the figures. All authors contributed to the article and approved the submitted version.

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miR-631 Inhibits Intrahepatic Metastasis of Hepatocellular Carcinoma by Targeting PTPRE

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MicroRNAs (miRNAs) have been reported to play critical roles in the pathological development of hepatocellular carcinoma (HCC), one of the most common cancers in the world. Our study aims to explore the expression, function and mechanism of miR-631 in HCC. Our findings are that expression of miR-631 is significantly down-regulated in HCC tissue compared with that in adjacent non-cancerous tissue, and low expression of miR-631 in HCC tissue is associated with cirrhosis, multiple tumors, incomplete tumor encapsulation, poor tumor differentiation, and high TNM stage. Our test results showed that miR-631 could inhibit migration, invasion, epithelial–mesenchymal transition (EMT) and intrahepatic metastasis of HCC. Receptor-type protein tyrosine phosphatase epsilon (PTPRE) as a downstream target of miR-631 could promote migration, invasion and EMT of HCC cells. Besides, the expression of PTPRE had a negative correlation with the expression of miR-631 both *in vivo* and *in vitro*, and increasing expression of PTPRE could reverse inhibitory effects of miR-631 in HCC cells. In sum, our study first demonstrated that miR-631 targeted PTPRE to inhibit intrahepatic metastasis in HCC. We gain insights from these findings into the mechanism of miRNAs regulation in HCC metastasis and further introduce a novel therapeutic target for HCC treatment.

Keywords: miR-631, receptor-type protein tyrosine phosphatase epsilon, tumor suppressor, hepatocellular carcinoma, intrahepatic metastasis

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, and it is the fourth driver of cancer-related mortality (1). Most of the HCC patients are diagnosed at advanced stages because they have few symptoms early, many of whom have suffered intrahepatic metastasis and lung metastasis, meaning some of them hardly have the chance to accept radical operation. For advanced HCC cases, moreover, the recurrence rate is nearly 80% with the patients, whose 5-year survival rate is only 25–39% (2). In conclusion, metastasis, recurrence and lack of more effective therapy constitute obstacles against HCC treatment, thus it is critical to find some new therapeutic targets from exploring molecular mechanisms of HCC metastasis.

microRNAs (miRNAs) are short (20–24 nt) and conservative non-coding RNAs, which can play big roles in regulating the post-transcriptional level of gene expression by binding the 3′-untranslated regions (3′-UTRs) of mRNAs and consequently interfering with both stability and translation of mRNAs (3). Over the decades, many studies have proved that miRNAs were dysregulated in HCC and could contribute to tumorigenesis and metastasis of HCC. For instance, some miRNAs, such as miR-221 and miR-25, were up-regulated in HCC tissue and could induce tumorigenesis of HCC (4, 5). On the contrary, let-7 and miR-214 were reported functioning as suppressive factors in HCC (6, 7). We searched miRNA profiles of HCC metastasis in dbDEMC 2.0, and we found GSE26323 of Gene Expression Omnibus (GEO) had compared the expression of miRNAs between primary HCC tissue and metastasis tissue (8, 9). Then we selected miR-631, which was down-regulated in HCC metastasis tissue compared with that in primary HCC tissue (LogFC = −2.67, P = 0.005), as the research target in our following efforts. Some studies have shown that miR-631 could inhibit migration and invasion of prostate cancer cells, resensitize bortezomib-resistant multiple myeloma cell lines, and increase bovine embryo development (10–12). However, miR-631 has never been reported in literature specific to HCC.

In our study, we selected miR-631 to be the research target through bioinformatics tools and aimed to explore the function and mechanism of miR-631 in HCC treatment. We detected the expression of miR-631 in HCC tissue and non-cancerous tissue (ANT) and analyzed their clinicopathologic characteristics and prognosis, respectively. Then we explored the function of miR-631 in HCC from *in vitro* and *in vivo* experiments and searched the mechanism of it. Our study aims to explore the expression, function and mechanism of miR-631 in HCC treatment and unveil the potential value of miR-631 as a new therapeutic target in HCC treatment.

MATERIALS AND METHODOLOGY

Patients and Specimens

Some 64 liver tissue samples were taken from HCC patients who underwent hepatectomy at the Hepatic Surgery Center, Tongji Hospital of Huazhong University of Science and Technology (Wuhan, China) during June 2014 and January 2015. These samples were stored at −80°C. The *in vivo* sampling was approved by the Ethics Committee of Tongji Hospital, and the study was arranged following our vow of the Declaration of Helsinki Principles. We kept following patients up until December 31, 2019.

Cell Lines, Media and Culturing Environment

Huh7, MHCC97-L and HLF cells were received from the China Center for Type Culture Collection (CCTCC, Wuhan, China). BEL-7402 and HEK293T cells were received from the Hepatic Surgery Center of Tongji Hospital and identified by using the STR genotyping test (Genechem Co., Ltd, Shanghai, China).

These cells were cultured using Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA, USA) with 10% fetal bovine serum (Life Technologies Inc., Gibco/Brl Division, Grand Island, NY, USA) in a humid culture room (5% CO_2/37°C).

Plasmid Construction

Using psiCHECK-2, we constructed vectors which participated in a luciferase reporter assay. PTPRE WT1, PTPRE MUT1, PTPRE WT2 and PTPRE MUT2 were synthesized by TsingKe (Wuhan, China). pLenti-CMV-puro was used to establish stably overexpressed miR-631. The coding sequence of the PTPRE gene was amplified by PCR and then subcloned into pCDNA3.1 to establish pCDNA3.1-PTPRE, while pCDNA3.1 was used as control. These sequences are summarized in **Supplementary Table 1**.

Cell Transfection and Transduction

miR-631 mimic, negative control mimic, miR-631 inhibitor, negative control inhibitor and PTPRE siRNA were brought from RiboBio (Guangzhou, China). All oligonucleotides and plasmids were transfected into cells using Lipofectamine 3000 (Invitrogen). To obtain stable cell lines that could overexpress miR-631, BEL-7402 cells were transduced with lentivirus for 24h and then selected from culture media containing 5 μg/ml puromycin (Cayman Chemical Company, Ann Arbor, MI, USA) for 14 days.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Tissues stored in liquid nitrogen was ground into powders and added with TRIzol solution (Thermo Fisher Scientific, MA, USA), or added TRIzol solution into cells rinsed with 4 °C PBS, then pipetted the mixture to homogenize it. We used miRcute miRNA Isolation Kit (Tiangen, Beijing, China) to isolate total miRNA. For total RNA, after adding TRIzol solution, we incubated the mixture for 10 min and added chloroform into it to further mix, and then incubated the mixture for 5 min. We centrifuged the mixture for 15 min at 12,000*g*/4°C. We transferred the aqueous phase to a blank test tube and mixed it with isopropanol. After incubating for 10 min, we centrifuged the mixture for 10 min at 12,000*g*/4°C and discarded the supernatant. We used 75% ethanol to wash the sediment before centrifuging for 5 min at 12,000*g*/4°C. We discarded the supernatant and air-dried the sediment for 5 min. After adding RNase-free water to resuspend the pellet, we derived total RNA.

For miRNA, miRcute Plus miRNA First-Strand cDNA Synthesis kits (Tiangen, Beijing, China) were used for reverse transcription. The second step was completed using miRcute Plus miRNA qPCR Detection Kits (Tiangen, Beijing, China). For mRNA, reverse-transcription system kits (Toyobo, Osaka, Japan) were used to complete reverse transcription. qPCR analysis could be made with standard SYBR Green PCR kits (Toyobo, Osaka, Japan). Small RNA RNU6B (U6) (RiboBio, Guangzhou, China) was used as a control for the expression of miRNA and the GAPDH (RiboBio, Guangzhou, China) was used for the mRNA. The miDETECT a trackTM miR-631 forward primer was brought from RiboBio (Guangzhou, China). PTPRE mRNA primer sequences are summarized in **Supplementary Table 1**.

Wound Healing Assays

Wound healing assays were conducted in 6-well plates with 1×10^6 cells per plate. After the cells grown to 95% confluence, we used a pipette tip to scratch the plate, and observed the wound at 0 and 48 h, respectively. Transwell assays including migration and invasion tests were conducted in 24-well plates.

Transwell Assays

For migration assays, we added DMEM to incubate the upper chamber of a Transwell for 0.5 h before plated cells. After that, we re-suspended cells with DMEM to 1×10^5 cells/ml, and added 200 μl in the upper chamber, while adding DMEM with 5% fetal bovine serum in the nether layer. After cell penetration for 24 h, we scrubbed the cells on the upper chamber membrane, then fixed the chamber in 4% paraformaldehyde for 10min and dyed the chamber in 0.1% crystal violet for 10 min. The invasion assays were conducted by pre-coating with 20% Matrigel (BD Biosciences, NJ, USA) diluted with DMEM in the upper chamber of a Transwell 2h earlier before plated cells and adjusted the concentration of resuspension to 2×10^5 cells/ml. Other steps were the same as the migration assays. Cell counts are the average of cells per visual field.

Western Blot Assay

Tissues stored in liquid nitrogen were ground into powder or discarded the growth media and washed the cells using 4 °C PBS. After removing PBS, we added 4 °C lysis buffer containing RIPA buffer, aprotinin and leupeptin to lyse cells for 30 min in ice. We scraped the cell culture dish and transferred the mixture into a test tube, then centrifuged it for 15 min at 12,000*g*/4°C. The supernatant was total protein.

Briefly, BCA protein assay kits (Bio-Rad, Hercules, CA, USA) were used to measure protein concentrations. Proteins of equal total amounts were separated electrophoretically in 10% SDS-PAGE. Then the proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) from gels. The membranes were soaked into Tris-buffered saline containing 0.1% Tween-20 (TBST) with 5% non-fat milk for blocking 2 h. After that, we incubated the PVDF membranes at 4 °C for more than 8 h with primary antibodies of PTPRE (Proteintech Group inc. CHI, USA) and GAPDH (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were used to incubate the membranes the next day for 2 h and then we used an enhanced chemiluminescence system (EMD Millipore, Darmstadt, Germany) to get the results.

Luciferase Reporter Assay

psiCHECK-2-vectors were constructed. 1×10^5 of HEK293T cells per well were added into 24-well plates and cultured for 24 h before being transfected. Then cells were co-transfected with 0.4 μg psiCHECK-2 vector named PTPRE WT1, PTPRE MUT1, PTPRE WT2, or PTPRE MUT2, and 50 nM miR-631 or control mimic using Lipofectamine 3000 (Invitrogen). After being transfected for 48 h, Firefly and Renilla luciferase activities were measured with DualGlo Luciferase Assay System (Promega, USA).

HCC Orthotopic Implantation

Four-week-old male nude mice purchased from HFK BioScience (Beijing, China) were housed under specific pathogen-free (SPF) conditions, and then bred as per Institutional Laboratory Guidelines for Animal Care. BEL-7402-control and BEL-7402-overexpress miR-631 cells (1×10^6) were suspended with 100 μl DMEM and injected subcutaneously into the flanks of nude mice. After 4 weeks, we anatomized the mice and removed the tumors, cut the tumors into small tissues of approximately 1 mm^3, then transplanted them into the livers of nude mice (six mice per group) (13). Some 7 weeks later, liver tissues of the nude mice were dissected and fixed. All animal experiments complied with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

Statistical Analyses

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS 22.0 (SPSS Inc., Chicago, IL, USA) software were used for statistical analyses. Quantitative data were analyzed by two-tailed paired or unpaired Student's t-test. Categorical data were analyzed by Chi-square or Correction Chi-square test. The log-rank test was conducted for survival analysis, and univariate and multivariate Cox hazard analyses were conducted to evaluate the risk factors of mortality. $P < 0.05$ was assumed as a statistically significant difference.

RESULTS

miR-631 Is Down-Regulated in HCC Tissues

To explore the valuable miRNA in HCC, we searched HCC metastasis miRNAs profiles in the database dbDEMC 2.0, and found the data in GEO serial number was GSE26323, indicating that miR-631 was down-regulated in HCC metastasis tissue compared with primary HCC tissue (LogFC = −2.67, P = 0.005). This finding meant miR-631 might contribute to the metastasis of HCC. Some studies have shown that miR-631 could inhibit migration and invasion of prostate cancer cells, indicating miR-631 might be a suppressor in other cancers, but it had not been reported in literature specific to HCC.

In order to make clear miR-631 expression in HCC tissue, we detected its expression in 64 HCC patients, including HCC tissue and adjacent non-cancerous tissue (ANT) by means of qRT-PCR. First, the expression of miR-631 was normalized with that of U6, and we calculated relative miR-631 expression in HCC tissue and ANT in a logarithmic scale of 64 HCC patients. The results indicated that miR-631 expression in HCC tissue was significantly different from that in ANT (**Figure 1A**). Then we normalized miR-631 expression of HCC tissue with that of ANT and derived the ratio in a logarithmic scale of 64 HCC patients. The results showed that miR-631 expression of HCC tissue was lower than that of ANT in 44 HCC patients, and 20 patients had high miR-631 expression in HCC tissue, meaning that miR-631 expression in HCC tissue was significantly lower than that in ANT (**Figure 1B**).

miR-631 Expression Is Associated With Intrahepatic Metastasis and Prognosis of HCC

The 64 HCC patients were separated into two groups by the median of miR-631 expression in HCC tissues. The low expression group included 32 patients who had low miR-631

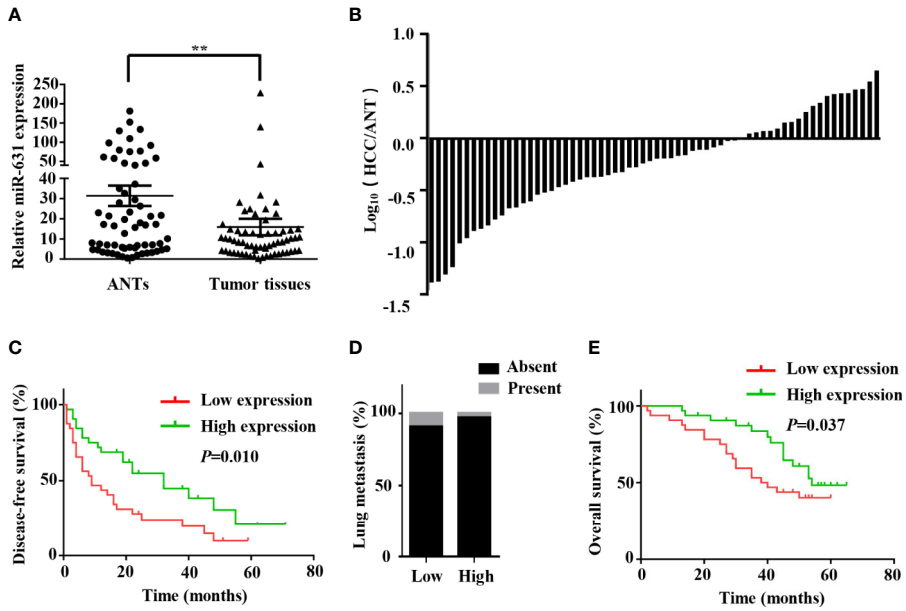

FIGURE 1 | miR-631 is downregulated in tumor tissues of HCC. **(A)** miR-631 expressions in 64 paired HCC tissues and ANTs, measured by qRT-PCR and unit of U6. The data were analyzed by the delta Ct method on a logarithmic scale and compared by paired Student's t-test. **(B)** The bars represent relative miR-631 expression with the ratio of its level in HCC tissue versus ANT in a logarithmic scale of 64 paired HCC patients. **(C)** Kaplan–Meier survival curves showed that the expression of miR-631 was associated with the disease-free survival of HCC. **(D)** There is no significant difference between the expression of miR-631 and lung metastasis. **(E)** The expression of miR-631 was associated with the overall survival of HCC analyzed by Kaplan–Meier survival curves. **P < 0.01.

levels in HCC tissues. And other 32 patients were separated into high expression group. The clinicopathological analysis showed that patients of both groups had no significant difference in gender, age, alpha-fetoprotein (AFP) expression, Child–Pugh class, and tumor size, but low expression of miR-631 in the tumor was significantly associated with cirrhosis, multiple tumors, incomplete tumor encapsulation, poor tumor differentiation and high TNM stage (**Table 1**).

Since multiple tumors, incomplete tumor encapsulation, poor tumor differentiation and high TNM stage were associated with HCC metastasis while tumor size was associated with HCC growth, we speculated that miR-631 is strongly associated with HCC metastasis instead of HCC growth. After analyzing the follow-up data, we found that in the low expression group, the 1-year disease-free survival (DFS) was 43.75%, the 3-year DFS was 24.11% and the 5-year DFS was 9.64%, but in the high expression group, the 1-year DFS was 68.75%, the 3-year DFS was 44.91% and the 5-year DFS was 20.53%, meaning that low expression group had lower DFS than that of high expression group (**Figure 1C**). Moreover, miR-631 expression was not statistically associated with lung metastasis (**Figure 1D**), meaning it might have an important function in intrahepatic metastasis.

Kaplan–Meier log-rank analysis was conducted to explore the correlation between expression of miR-631 and prognosis of HCC patients. The results showed that in the low expression group, the 1-year overall survival (OS) was 90.63%, the 3-year OS was 53.13% and the 5-year OS was 40.1%, but in the high expression group, the 1-year OS was 96.88%, the 3-year OS was 83.53%, and the 5-year OS was 48.26%, meaning HCC patients with the low level of miR-631 expression had lower OS than that of patients with high miR-631 expression (**Figure 1E**).

Since cirrhosis, tumor number, tumor encapsulation, tumor differentiation, and TNM stage were also correlated with HCC

TABLE 1 | Correlations between miR-631 expression and clinicopathologic characteristics in 64 HCC patients.

Variables		miR-631^{low}	miR-631^{high}	χ^2	P-value
Gender	Male	27	30	0.642	0.423
	Female	5	2		
Age (years)	≤50	14	20	2.259	0.133
	>50	18	12		
AFP (g/L)	≤20	9	10	0.075	0.784
	>20	23	22		
Cirrhosis	Absent	9	17	4.146	**0.042***
	Present	23	15		
Child-Pugh Class	A	24	27	0.869	0.351
	B	8	5		
Tumor number	1	20	27	3.925	**0.048***
	≥2	12	5		
Tumor size (cm)	≤5	12	14	0.259	0.611
	>5	20	18		
Tumor encapsulation	Complete	13	22	5.107	**0.024***
	Incomplete	19	10		
Tumor differentiation	I–II	18	26	4.655	**0.031***
	III–IV	14	6		
TNM stage	I	13	23	6.349	**0.021***
	II-IV	19	9		

In bold: The value is statistically significant.

prognosis. We stratified these clinicopathologic characteristics to explore whether miR-631 was a prognostic factor of HCC. The results in **Table 2** showed that the expression of miR-631 in patients with cirrhosis, multiple tumor number, and incomplete tumor encapsulation displayed a significant correlation with HCC prognosis. Then we gathered all individual prognostic factors for multivariate analysis (**Table 3**). We found that after considering effects of these prognostic factors, miR-631 was still an independent prognostic factor for OS.

TABLE 3 | Multivariate analysis of individual prognostic factors.

Variables	P-value	HR	95.0% CI for HR	
			Lower	Upper
miR-631	**0.025***	2.401	1.116	5.164
Cirrhosis	**0.034***	2.741	1.082	6.947
Tumor number	**0.024***	3.220	1.167	8.884
Tumor size (cm)	0.715	1.178	0.490	2.829
Tumor differentiation	0.575	0.764	0.298	1.958
TNM stage	0.135	2.344	0.766	7.170

In bold: The value is statistically significant.

miR-631 Inhibits Migration and Invasion of HCC Cells

We detected the expression of miR-631 in human HCC cell lines including Huh7, MHCC97-L, HLF and BEL-7402 cells. The results showed that Huh7 and MHCC97-L cells had high expression of miR-631 while HLF and BEL-7402 cells had a low expression (**Figure 2A**), which might suggest that the level of miR-631 was potentially related to metastasis since it had high expression in cells of low motility and low expression in cells of high motility. We chose Huh7 and BEL-7402 cells to explore the biological function of miR-631 in HCC cells for further study.

To make the role of miR-631 in HCC migration clear, we carried out cell wound healing assays and Transwell assays. BEL-7402 cells were transfected with miR-Control mimic and miR-631 mimic, while Huh7 cells were transfected with miR-Control inhibitor and miR-631 inhibitor (**Figure 2B**). The images of wound healing assays were shown (**Figures 2C, D**) and the percent of wound closure was calculated, indicating that upregulating miR-631 could decrease the speed of wound closure of BEL-7402 cells, while Huh7 cells with decreased miR-631 expression had a faster wound closure speed than that of control cells (**Figure 2E**).

Then we carried out the Transwell migration assay and invasion assay. The results revealed that after overexpressing miR-631 by transfecting miR-631 mimic, migration and invasion capacities of BEL-7402 cells decreased (**Figures 2F, G**). In contrast, down-regulation of miR-631 expression in Huh7 cells significantly increased the invasion capacities (**Figures 2H, I**). Besides, after regulating the expression of miR-631, we found changes in the expression of epithelial marker (E-cadherin), mesenchymal marker (Vimentin) and transcriptional factor (Snail) as well. The expression of E-cadherin had a positive correlation with the expression of miR-631, while expressions of Vimentin and Snail had negative correlations with miR-631, meaning that miR-631 could inhibit the process of epithelial–mesenchymal transition (EMT), which is widely considered to be crucial to the invasion-metastasis cascade during cancer progression (**Figures 2J–L**). These results proved that miR-631 could inhibit the action of migration and invasion in HCC cells.

PTPRE Is a Direct Downstream Target of miR-631

We collected the data of predictive miR-631 targets from five independent databases: DIANA (275 candidate targets) (14), CoMeTa (512 candidate targets) (15), mirDIP (105 candidate targets) (16), miRWalk (15218 candidate targets) (17) and TargetScan (3388 candidate targets) (18), and drew a Venn diagram from them (**Figure 3A**). As shown in the Venn diagram, 29 candidate targets overlapped in the five databases. By analyzing the characteristics and functions of 29 genes, we chose PTPRE for

TABLE 2 | Univariate stratified cox analysis of prognostic factors.

Variables	Death number (%)		P-value	HR	95.0% CI for HR	
	miR-631^{low}	miR-631^{high}			Lower	Upper
All cases	21 (66%)	14 (44%)	**0.044***	2.012	1.020	3.968
Cirrhosis						
Absent	1 (11%)	7 (41%)	0.106	0.177	0.022	1.447
Present	20 (87%)	7 (47%)	**0.001***	5.013	1.933	13.004
Tumor number (>1)						
1	9 (45%)	11 (41%)	0.924	1.044	0.432	2.523
≥2	12 (100%)	3 (60%)	**0.019***	6.587	1.372	31.625
Tumor encapsulation						
Complete	6 (46%)	10 (45%)	0.982	0.988	0.358	2.724
Incomplete	15 (79%)	4 (40%)	**0.018***	3.841	1.255	11.759
Tumor differentiation						
I–II	9 (50%)	10 (38%)	0.418	1.452	0.589	3.577
III–IV	12 (86%)	4 (67%)	0.199	2.130	0.672	6.748
TNM stage						
I	4 (31%)	9 (39%)	0.520	0.679	0.209	2.210
II-IV	17 (89%)	5 (56%)	0.050	2.795	1.000	7.811

In bold: The value is statistically significant.

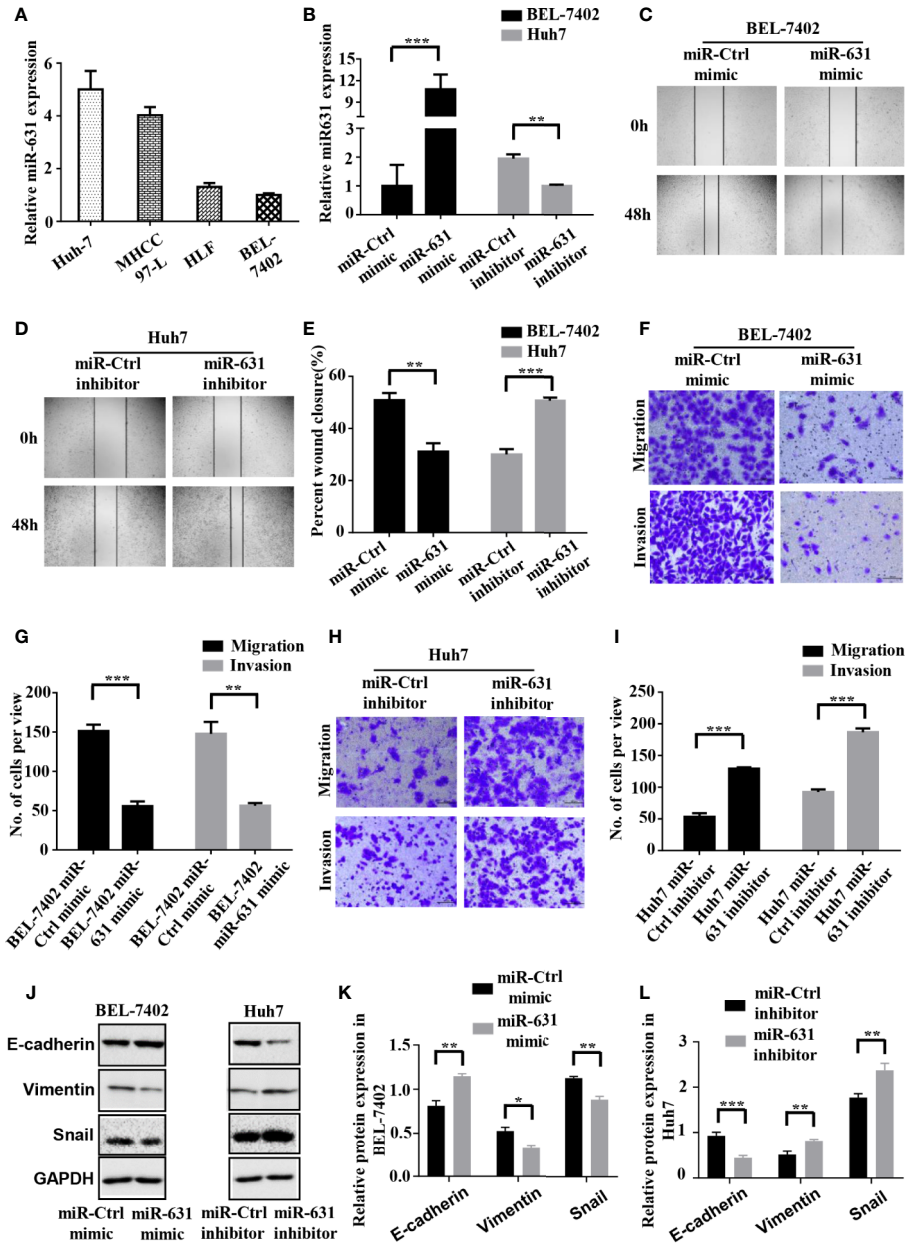

FIGURE 2 | miR-631 inhibits migration and invasion of HCC cells. **(A)** Expression of miR-631 in *in vivo* HCC cell lines. **(B)** Relative expression of miR-631 detected by qRT-PCR in BEL-7402 cells transfected by miR-Control or miR-631 mimic and Huh7 cells transfected by miR-Control or miR-631 inhibitor. The concentration of mimic and inhibitor is 50 nM. **(C–E)** Representative images of wound healing assays and percentages of wound closure were calculated. **(F, G)** Transwell migration and invasion assays of BEL-7402. **(H, I)** Transwell migration and invasion assays of Huh7. **(J–L)** Western blot assay of protein in EMT. Results were represented as mean ± S.E.M. (n = 3) *P < 0.05; **P < 0.01; ***P < 0.001.

the later study. PTPRE is an isoform of a subfamily of the protein tyrosine phosphatases (PTPs), which plays a role in controlling the reversible phosphorylation of tyrosine residues (19–21). In addition, it had been reported that PTPRE could act as an oncogene in some kind of cancers (22–24), indicating it had an opposite function with miR-631.

To make clear whether PTPRE was a direct target of miR-631, we carried out a dual-luciferase reporter assay. The binding sites of miR-631 and PTPRE were predicted on Targetscan (**Figure 3B**). The reporter vectors contained wild-type or mutated binding sequences, we transfected them into HEK293T cells with miR-control or miR-631 mimic respectively. Data showed that there was no significant difference in the PTPRE-WT1 group after being transfected by miR-631 mimic (**Figure 3C**). However, after increasing miR-631 expression, the relative luciferase activity of PTPRE-WT2 group was down-regulated

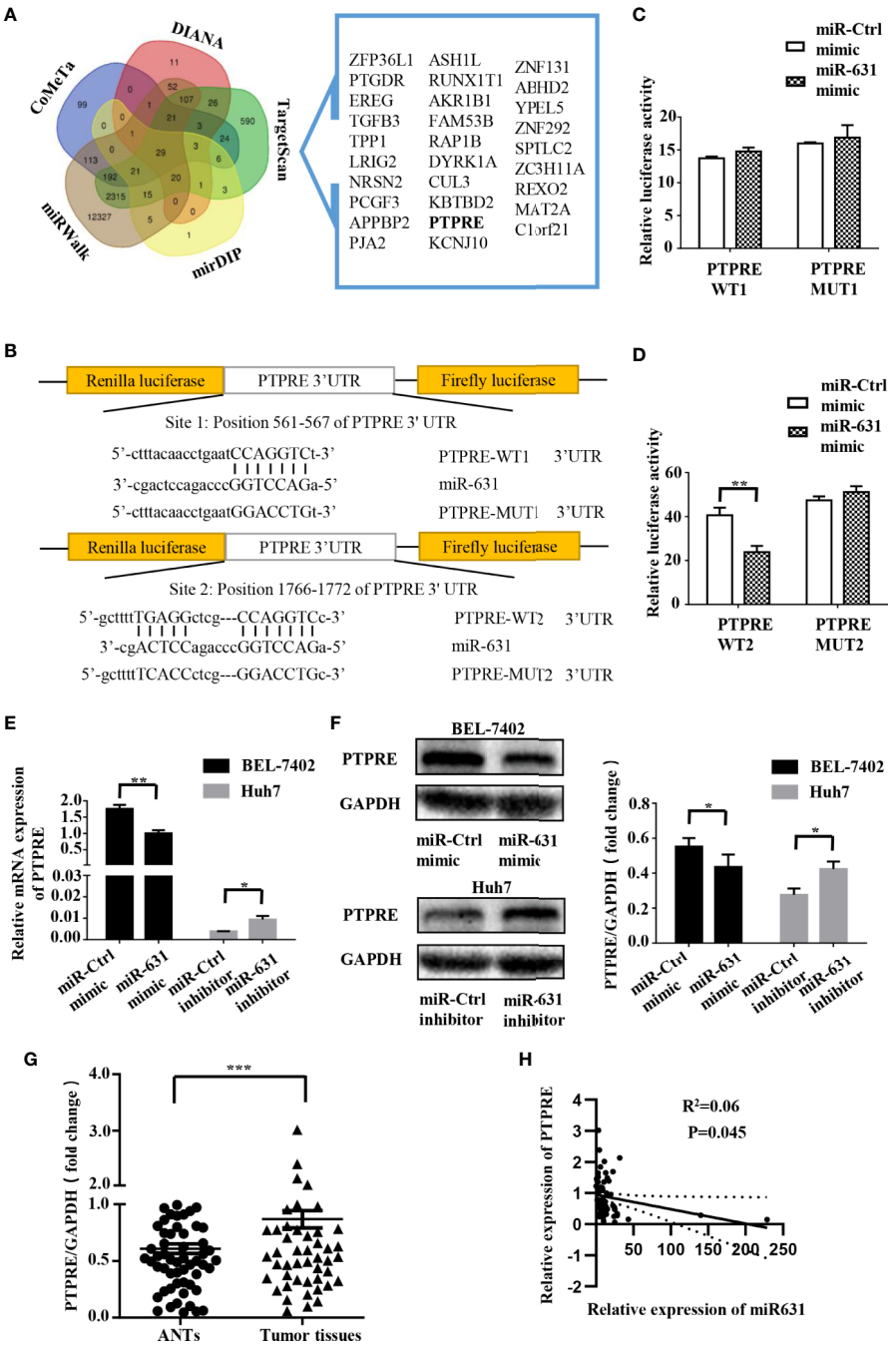

FIGURE 3 | PTPRE is a direct downstream target of miR-631 in HCC cell lines. **(A)** Venn diagram of five databases that predicted downstream target of miR-631. The overlaps are shown. **(B)** The putative binding sites of miR-631 and the corresponding mutant sites in PTPRE 3′-UTR. The diagram showed the positions of sites in psiCHECK-2-vector. **(C, D)** Relative luciferase assay in HEK293 cells where miR-631 mimic was co-transfected with psiCHECK-PTPRE wild-type or psiCHECK-PTPRE mutant vector of sites 1 and 2. **(E, F)** The mRNA and protein expression levels of PTPRE in BEL-7402 and Huh7 cells after being transfected by miR-631 mimic and miR-631 inhibitor, respectively. **(G)** The protein expression of PTPRE in 64 HCC tissues. **(H)** The correlation between the expression of PTPRE and miR-631 in HCC tissues. The concentration of mimic and inhibitor is 50 nM. *P < 0.05; **P < 0.01; ***P < 0.001.

(**Figure 3D**). These results suggested that miR-631 could target mRNA of PTPRE on the binding site 2 directly.

By increasing miR-631 expression in BEL-7402, the level of mRNA of PTPRE was decreased. And the expression of PTPRE mRNA was upregulated after downregulated miR-631 in Huh7 cells (**Figure 3E**). The change of PTPRE protein level followed the expression of PTPRE mRNA (**Figure 3F**). Besides, we detected PTPRE expression in 64 HCC patients, including

HCC tissues and ANTs by Western Blot. The results showed that the PTPRE expression in HCC tissue was significantly higher than that in ANT (**Figure 3G**). By comparing the expression of PTPRE with miR-631 in HCC tissues, we found a negative correlation between them (**Figure 3H**).

These findings revealed that miR-631 expression had a negative correlation with mRNA and protein of PTPRE, suggesting that miR-631 did have a certain impact on the PTPRE translation process.

PTPRE Promotes Migration and Invasion of HCC Cells

To investigate the function of PTPRE, we used siRNA to knockdown PTPRE expression in BEL-7402 cells. Huh7 cells, meanwhile, were overexpressed PTPRE from being transfected by pcDNA3.1-PTPRE (**Figure 4A**). Then we proceeded to cell wound healing assays and Transwell assays. The wound healing assay showed cells with a high level of PTPRE had higher percent of wound closure area than cells with a low PTPRE level (**Figures 4B–D**). The Transwell chamber migration and invasion assays showed that the mobility of BEL-7402 cells was decreased after down-regulating PTPRE (**Figures 4E, F**), and high expression of PTPRE could promote migration and invasion of Huh7 (**Figures 4G, H**). Besides, the expression of PTPRE had a negative correlation with the expression of E-cadherin and had positive correlations with expressions of Vimentin and Snail, suggesting PTPRE might induce EMT (**Figures 4I–K**). These findings indicated that PTPRE promoted migration and invasion of HCC cells.

miR-631 Inhibits Migration and Invasion in HCC Cells Through PTPRE

We carried out a rescue experiment to further demonstrate that miR-631 inhibited HCC migration and invasion by targeting PTPRE. We separated BEL-7402 cells into three groups. Cells of the control group were transfected by miR-Control mimic and pcDNA3.1-Control. Cells of the miR-631 overexpression group were transfected by miR-631 mimic and pcDNA3.1-Control. And cells of the high expression of miR-631 and PTPRE group were transfected by pcDNA3.1-PTPRE and miR-631 mimic (**Figure 5A**). The cell wound healing assays showed that the reduced percentage of cells wound closure area was reversed by up-regulating PTPRE (**Figure 5B**). And Transwell migration and invasion assays showed that after increasing expression of PTPRE, the inhibitory effect caused by miR-631 in migration and invasion of BEL-7402 cells was partially reversed (**Figures 5C, D**). These results provided evidence that miR-631 could act as a tumor suppressor by inhibiting PTPRE-enhanced migration and invasion in HCC cells.

miR-631 Could Inhibit Intrahepatic Metastasis of HCC

To test *in vivo* function of miR-631 in HCC metastasis, we used lentivirus to construct BEL-7402 cells that could stably overexpress miR-631 (**Figure 6A**) and a mouse model. First, we conducted an *in vivo* tumorigenesis assay. After the tumor diameter was near 1 cm, we cut the tumor tissue into pieces approximate 1 mm^3 and transplanted them into livers of nude mice. Some 7 weeks later, mice were anatomized (**Figure 6B**), and the liver tissues showed that those with low expression of miR-631 were easier to have intrahepatic metastasis than those of high miR-631 level (**Figures 6C, D**), meaning that miR-631 was able to inhibit intrahepatic metastasis.

We detected the expression of miR-631 and the mRNA level of PTPRE in liver tissues of nude mice (**Figures 6E, F**). The results indicated a negative correlation between the expression of miR-631 and PTPRE mRNA in HCC tissues of mice models (**Figure 6G**).

DISCUSSION

Since the first miRNA was discovered in 1993 (25), a myriad of miRNAs had been researched and extensive studies revealed that miRNAs could contribute to the progression of a lot of cancers including HCC. In our study, we found that the expression of miR-631 was lower in HCC tissue than that in ANT. The analysis of clinicopathological and prognostic features revealed that patients with low expression of miR-631 were significantly associated with cirrhosis, multiple tumors, incomplete tumor encapsulation, poor tumor differentiation, high TNM stage, short disease-free survival time and short overall survival time, and had no significant difference in gender, age, alpha-fetoprotein (AFP) expression, Child–Pugh class, tumor size, and lung metastasis. From these results, we found that miR-631 was strongly associated with HCC metastasis, especially intrahepatic metastasis. Kaplan–Meier log-rank analysis showed that HCC patients with a low level of miR-631 expression had lower OS than that of patients with high miR-631 expression. Univariate stratified cox hazard analysis was used to evaluate prognostic factors while excluding the impact of some clinicopathologic characteristics (**Table 2**). We found that the expression of miR-631 was correlated with prognosis in HCC patients with cirrhosis, multiple tumor number, and incomplete tumor encapsulation. Multivariate analysis was then used to evaluate the influence of individual prognostic factors, including miR-631, cirrhosis, tumor number, tumor size, tumor differentiation, and TNM stage. The results indicated that the expression of miR-631 was still an independent prognostic factor for HCC OS.

Some studies had shown that miR-631 could inhibit the mobility of migration and invasion of prostate cancer cells by targeting Zeta chain of T cell receptor-associated protein kinase 70, meaning miR-631 might be a biomarker to reveal the capacity of tumor metastasis. However, more clinical supports are still needed.

Our speculations above were also confirmed in terms of expression levels of miR-631 in several HCC cell lines. miR-631 had low expressions in cells with high invasion ability and had high expressions in cells with low invasion ability, meaning the level of miR-631 was potentially associated with HCC metastasis. And the cell wound healing assays and Transwell assays showed that miR-631 played an important role in the motion, migration, and invasion of HCC cell lines. Western blot assay indicated that the expression of miR-631 had a positive

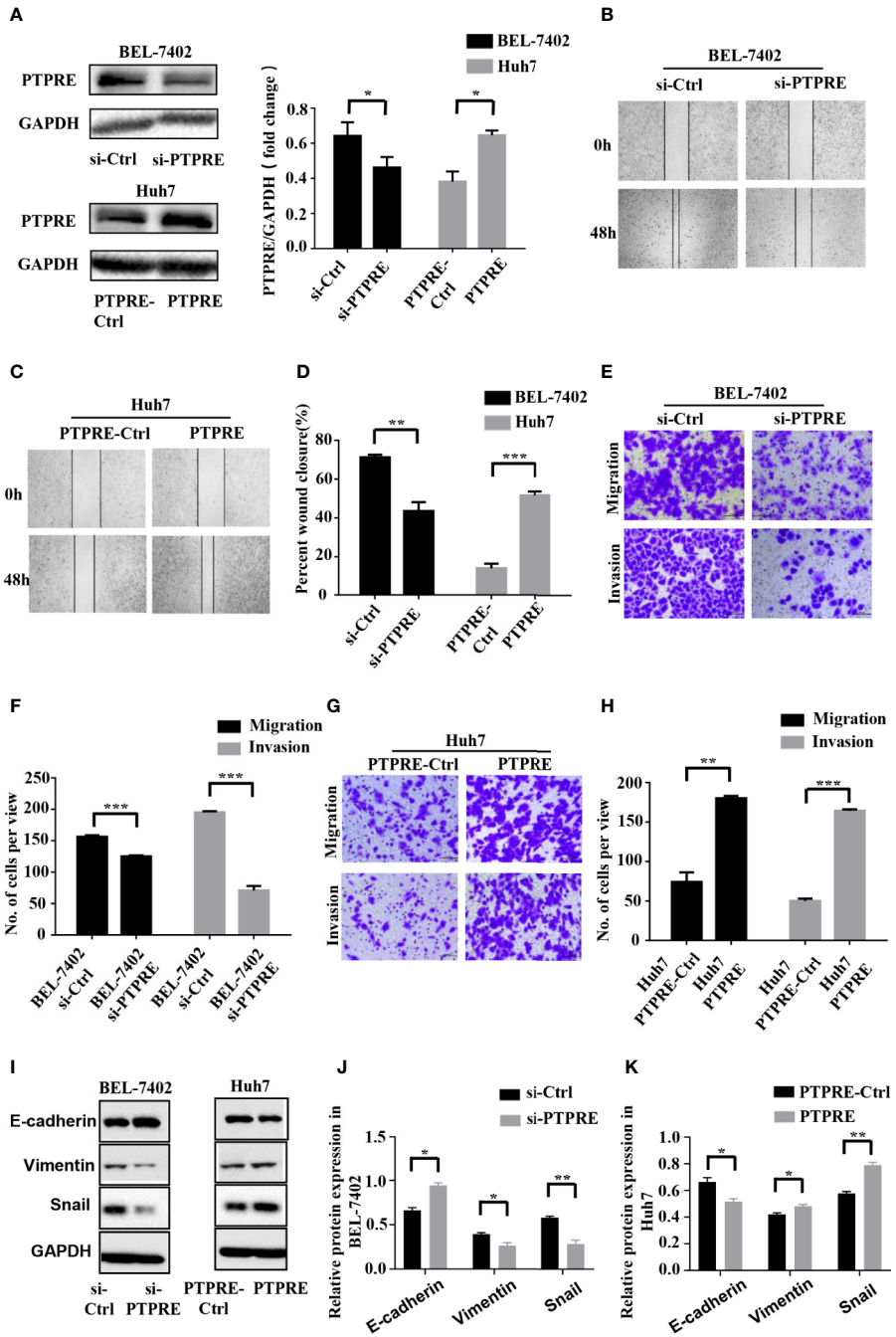

FIGURE 4 | PTPRE induces migration and invasion of HCC cells. **(A)** Western blot assays of PTPRE in BEL-7402 and Huh7 cells after knocking down and overexpressing PTPRE separately. **(B–D)** The representative images of wound healing assay were obtained and the percentages of wound closure were calculated. **(E, F)** Transwell migration and invasion assays of BEL-7402 cells. **(G, H)** Transwell migration and invasion assays of Huh7 cells. **(I–K)** Western blot assay of protein in EMT. *P < 0.05; **P < 0.01; ***P < 0.001.

correlation with E-cadherin level and had negative correlations with expressions of Vimentin and Snail, suggesting miR-631 might inhibit the process of EMT.

Next, we analyzed the data on predicted miR-631 targets from five independent databases. And we proved that miR-631 could bound with the second predicted binding site of PTPRE mRNA by Dual-luciferase reporter assay. Changing miR-631 expression in HCC cells could make a reverse effect on the expression level of PTPRE mRNA and protein both in HCC cell lines and HCC tissues. Inhibiting translation is the most important function of miRNAs acting in biological processes and it includes two parts: initiation step and post-initiation step. At the initiation step,

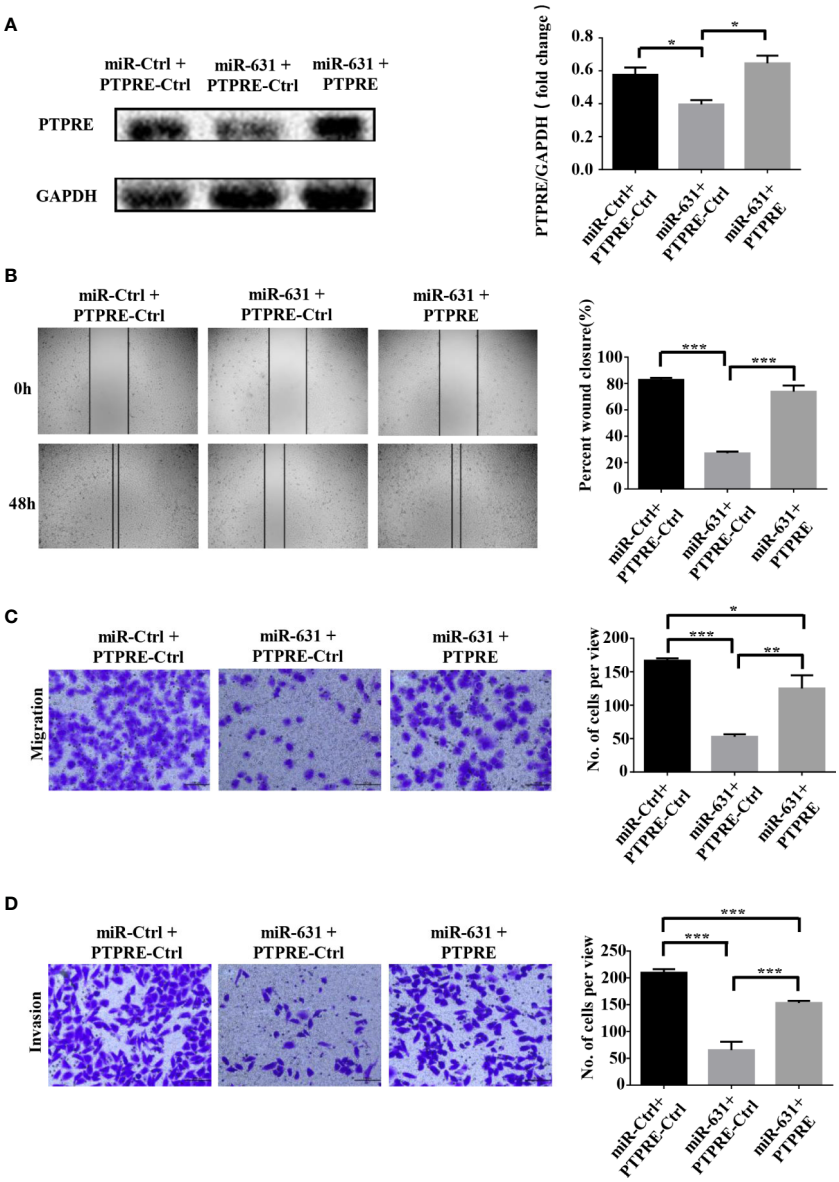

FIGURE 5 | miR-631 inhibits migration and invasion in HCC cells through PTPRE. **(A)** BEL-7402 cells were separated into three groups for carrying out a rescue experiment. **(B)** The representative images of wound healing assay were obtained and the percentages of wound closure were calculated. **(C, D)** Transwell migration and invasion assays of rescue experiment. *P < 0.05; **P < 0.01; ***P < 0.001.

miRNAs restrained ribosomes from binding to the 5′-cap structure of mRNAs (26–28). And miRNAs could target mRNA in the polysome fraction at the post-initiation stage (29–32). The two interactions might be the reason why miR-631 could regulate the expression of PTPRE mRNA PTPRE proteins. However, more efforts are still needed to explore the in-depth mechanism of the interaction between miR-631 and PTPRE mRNA.

It's reported that in hepatocytes and liver, PTPRE inactivates insulin receptor signaling (33), which might influence both risk and prognosis in many kinds of cancers (34, 35). And our laboratory had discovered that PTPRE could activate the transforming growth factor-β (TGF-β) β signaling pathway, meaning it could stimulate the EMT and promote migration and invasion of HCC cells (36–39). In our study, PTPRE was proved to have the ability of promoting cell migration and invasion by wound healing assays and Transwell assays. Western blot assay also suggested that PTPRE might induce EMT. And increasing PTPRE in HCC cells could partially reverse the effects caused by miR-631, meaning other target proteins or signal pathways may need to be explored.

The animal study showed that miR-631 could inhibit intrahepatic metastasis of HCC *in vivo*. Same with the results *in vitro*, the expressions of miR-631 and PTPRE in HCC tissues

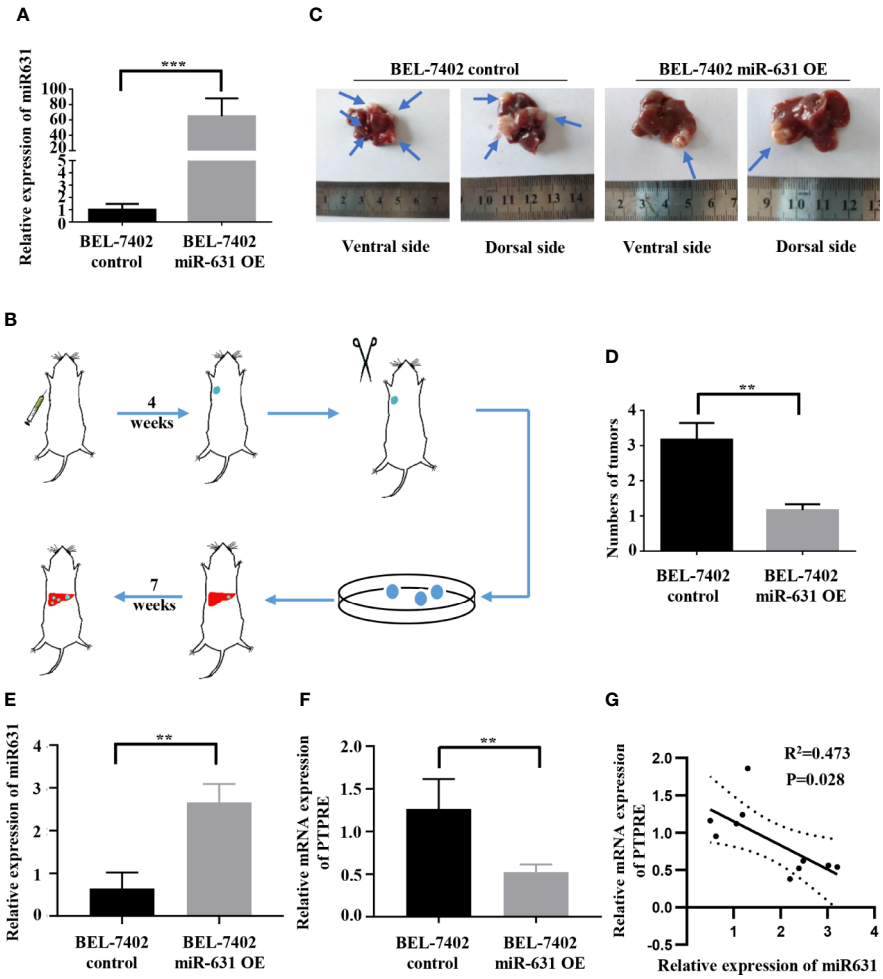

FIGURE 6 | miR-631 inhibits tumor intrahepatic metastasis *in vivo*. **(A)** Relative expression of miR-631 detected by qRT-PCR in BEL-7402 control and BEL-7402 miR-631 OE cells which stably overexpressed miR-631 (n = 3). **(B)** Schematic illustration of *in vivo* metastasis mouse model. **(C)** Images of intrahepatic metastasis tumors of BEL-7402 control and BEL-7402 miR-631 OE. **(D)** The tumor count in livers of BEL-7402 control and BEL-7402 miR-631 OE (n = 6). **(E)** The relative expression of miR-631 in HCC tissues of mice models. **(F)** The relative mRNA expression of PTPRE in HCC tissues of mice models. **(G)** The correlation between miR-631 and mRNA of PTPRE in HCC tissues of mice models. **P < 0.01; ***P < 0.001.

of mice models were negatively related, meaning that our speculations *in vitro* were confirmed *in vivo* by the animal study.

Our study indicated that low expression of miR-631 in HCC was related to the aggressive tumor and proved that miR-631 participated in the process of EMT and could inhibit HCC migration, invasion and intrahepatic metastasis. Besides, PTPRE, which could induce HCC cell migration, invasion and EMT, was demonstrated to be a direct target of miR-631. The expression of PTPRE had a negative correlation with miR-631 level and upregulating PTPRE could partially reverse the effects caused by a high level of miR-631. To our knowledge, our study for the first time showed that miR-631 had a low expression in HCC tissue and explored the miR-631/PTPRE axis in the progression of HCC. But the rescue experiment revealed that more efforts are still needed to explore other downstream targets. Further studies are required to investigate whether miR-631 can serve as a potential prognostic biomarker of HCC and a new therapeutic target in HCC treatment.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethic Committee of Tongji Hospital, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this

study. The animal study was reviewed and approved by the Ethic Committee of Tongji Hospital, Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

BC and ZL carried on the experiments and analysis of this study. BC, ZL and YQ designed the research. HZ and CS provided administrative supports. BC wrote the manuscript. XC, BZ, and HL revised 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.2020.565266/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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MiRNA Polymorphisms and Hepatocellular Carcinoma Susceptibility: A Systematic Review and Network Meta-Analysis

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Background: Hepatocellular carcinoma (HCC) is an intractable public health threat worldwide, representing the second leading cause of cancer-related mortality, with limited early detection and therapeutic options. Recent findings have revealed that the susceptibility of HCC is closely related to microRNA (miRNA). We performed this systematic review with a network meta-analysis to investigated four single nucleotide polymorphisms (SNPs) that most regularly reported in miRNAs, exploring their involvement in HCC susceptibility and interaction with hepatitis B virus (HBV).

Methods: Databases were reviewed for related studies published up to May 2019 to identify all studies that compared genotypes of miR-146a rs2910164, miR-149 rs2292832, miR-196a2 rs11614913, and miR-499 rs3746444 with no language and date restrictions. A pairwise meta-analysis was performed to estimate pooled odds ratios and 95% confidence intervals incorporating heterogeneity to assess the relationship between four miRNA polymorphisms and HCC. To further clarify the effect of polymorphisms on HCC, a Bayesian network meta-analysis was conducted to combine the effective sizes of direct and indirect comparisons. Calculations were performed by R version 3.6.1 and STATA 14.0. All steps were performed according to PRISMA guidelines.

Results: A total of 20 studies were enrolled in this network meta-analysis, providing 5,337 hepatocellular carcinoma cases and 6,585 controls. All included studies had an acceptable quality. Pairwise meta-analysis demonstrated that miR-196a2 rs11614913 was significantly associated with the susceptibility of HCC, while the other three SNPs were not found to have a significant association. In the analysis of HCC patients under different HBV infection status, only miR-196a2 revealed correlation of threefold risk. The network results showed no significant difference in the distribution of genotype frequencies except for miR-196a2, which appeared to have the highest superiority index when comparing and ranking four SNPs.

Conclusion: MiR-196a2 rs11614913 was significantly associated with the susceptibility of HCC, especially for HBV- related HCC, and that individuals with TC/CC were more susceptible. No significant association was found in the other three miRNA genes. MiR-196a2 could serve as the best predictor of susceptibility in HCC.

Keywords: hepatocellular carcinoma, microRNA, polymorphism, network meta-analysis, susceptibility, hepatitis B virus

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, has been an intractable public health threat worldwide (1). Approximately 700,000 new cases and 600,000 deaths are attributable to HCC annually (2), representing the sixth leading cause of cancer and the second leading cause of cancer-related mortality (3). Asia, Sub-Saharan Africa and the Middle East are high-risk regions with high incidence rates of HCC (4), and in some of these regions HCC ranks as the leading cause of death due to cancer (5). But, it is worth noting that the incidence and mortality have been increasing in North America and some areas of Europe (6, 7). The incidence in the United States has tripled over the past three decades (8). In the European Union, estimated by WHO, about 47,000 people die of liver cancer each year (9). In Canada, HCC has become the only cancer whose mortality rate is still on the rise. The incidence has been increasing rapidly and is projected to continue beyond 2020 (3). Multiple factors are responsible for its development including inborn diseases, chemicals, and viruses, of which Hepatitis B virus (HBV) is widely acknowledged. HCC has become a tremendous global burden (10), with the characteristics of high incidence, short duration, poor prognosis, high degree of malignancy, and five-year survival rate of 7% (11), yet remains one of the most ill-informed cancers and compounds by limited early detection and therapeutic options (12, 13). Therefore, exploring and clarifying the disease mechanism of HCC is conducive for effective prevention and treatment (14).

Genetic association studies are of great significance for epidemiological analyses, as they can identify candidate genome regions associated to specific diseases (15). Many findings have revealed that the presence of single nucleotide polymorphisms (SNPs) in some miRNA genes can alter the expression or maturation of miRNAs, making individuals more susceptible to certain types of cancer (16, 17). Several SNPs in miRNA genes can influence the development of HCC (18), providing a novel perspective of pathophysiological mechanism for the etiology of HCC (19).

MiR-146a (rs2910164), miR149 (rs2292832), miR-196a2 (rs11614913), and miR-499 (rs3746444) are well-established functional miRNAs (20–24). Researches have demonstrated that they can participate in essential regulatory processes related to cellular senescence, inflammation, immune response thus have potential value as biomarkers for many diseases (25, 26). The results on the association between genetic polymorphisms and HCC susceptibility remain inconsistent due to differences in race, disease stage, sample size, or other uncertainties. To further explore whether polymorphisms in these four SNPs might predispose to HCC, additional research and quantitative statistical studies are required to resolve discrepancies (27). Network meta-analyses (NMA) can be used to summarize and compare studies on multiple interventions (28), and combine direct and indirect evidence thus produce a result more precise (29). We conducted this systematic review with a network meta-analysis to provide more comprehensive information on the polymorphisms of four selected miRNAs and their involvement in HCC susceptibility and interaction with HBV.

MATERIALS AND METHODS

Literature Search

In this systematic review and meta-analysis, we searched the database of PubMed, EMbase and the Cochrane Central Register to identify all eligible case-controlled trials that compared genotypes of the four selected miRNA genes in HCC patients with non-cancer control groups. All searches were performed in May 2019 and no language and date restrictions were set. The searching items were: "hepatocellular carcinoma", "hepatoma", "liver cancer", "HCC", and "microRNAs", "miRNA", and "polymorphism", "allele", "variation", "SNP".

Selection Criteria

Eligible studies met the following criteria: (1) Case-controlled trials of subjects with HCC and healthy participants without HCC; (2) Evaluate the relationship between four common SNPs of miRNA (miR-146a rs2910164, miR-149 rs2292832, miR-196a2 rs11614913, miR-499 rs3746444) and HCC risk; (3) Investigate at least two selected SNPs at the same time; (4) Either DNA sequencing or PCR is used as a genotypic method for detection.

Articles were excluded based on the criteria: (1) Duplicated articles or data; (2) Irrelevant cancers or SNPs; (3) Functional studies; (4) Lack of available genotype frequency.

Data Abstraction and Assessment of Bias

Two investigators independently abstracted the data on the studies. Discrepancies were resolved by consensus, referring back to the original study, or consulting a third reviewer. Besides genotype and frequency, the following data were also extracted from original studies: first author, year of publication, country, ethnicity, genotyping method, study design, case-control matching, sample size (cases/controls), and HBV infection status. To reduce the risk of bias due to individual studies, the Newcastle-Ottawa scale (NOS) score was applied to

evaluate the methodological quality. The scale assesses three domains (selection bias, group comparability, and cohort exposure), based on "yes" or "no" answers to the following questions: (1) Is the case definition adequate; (2) Is there representativeness of the cases; (3) Is there selection of controls; (4) Is there a definition of controls; (5) Is there comparability of cases and controls; (6) Is there ascertainment of exposure; (7) Is the same method of ascertainment used for cases and controls; (8) Is there a non-response rate. The total score of NOS ranges from 0 to 9. A systematic analysis of the included studies was performed, and those with scores less than 5 were excluded. Two investigators independently performed the risk of bias assessments, with disagreement resolved by a third researcher when needed.

Statistical Analysis

A traditional pairwise meta-analysis was performed to estimate pooled odds ratios (ORs) and 95% confidence intervals (CIs) incorporating heterogeneity within and between studies. Statistical heterogeneity between each study was assessed with using the Chi-square tests and the inconsistency index I-square, with the values of 25%, 50%, and 75% denoting low, moderate and high heterogeneity, respectively. A random effects model was applied when I^2 are over 50% (30). We went a step further and analyzed whether different genotypes might predispose to HCC under different HBV infection status. Meta-regression analysis was performed on the basis of the ethnicity, HWE, case-control match, and sample size to assess the heterogeneity that may have influences on the association between miRNA polymorphisms and HCC. The Begg' s and Egger' s tests were conducted to detect potential publication bias. Calculations and plotting were implemented by STATA 14.0 software.

To further clarify the effect between four polymorphisms of miRNA on HCC, we conducted a Bayesian network meta-analysis. First a network plot depicting the connection within four SNPs was drawn. Every SNP was represented by a node, and the node size represented the number of studies of a corresponding SNP, the line thickness between two nodes represented the number of paired studies. Then, the analysis of variance (ANOVA) model was applied to combine the effective sizes of direct and indirect comparisons. The ability to rank interventions is an attractive feature of NMA compared to traditional analysis. The superiority index was calculated to rank competing polymorphisms. The superiority index ranges from 0 to ∞, which tends toward ∞ as the genetic model has a higher likelihood of predicting the risk of HCC and tends toward 1 indicating equal effect. Calculations were performed by R version 3.6.1 and STATA 14.0 was used to assist graphical functions.

RESULTS

Characteristics and Bias of Enrolled Studies

Overall, 985 citations were identified using the search strategy. Among them, 269 citations were duplicates and 623 were excluded due to inappropriate tumor, functional studies, meta-analysis, reviews after assessing titles and abstracts. In the remaining 93 articles, there are two unhealthy controls, seven lack of sufficient data, 62 articles of irrelevant polymorphisms were removed. In Akkiz's study, three articles investigating the same population and separately reporting three miRNA genes were considered as one. Therefore, 20 studies were enrolled providing a total of 5,337 HCC cases and 6,585 controls (**Figure 1**). The publication date of enrolled studies was from 2011 to 2019. The publications were mostly conducted in Asia, and two from Africa. In terms of ethnicity, 16 of the studies had Asian subjects and four studies had Caucasian subjects. Characteristics of included studies were presented in **Table 1**. The assessments of study quality were presented in **Figure 2**, and the NOS scale score result showed that all included studies had an acceptable quality.

Pairwise Meta-Analysis

The forest plots of four miRNAs and their involvement in HCC susceptibility and relationship with HBV were explored and compared in **Figures 3** and **4**, respectively. The results indicated that miR-196a2 rs11614913 polymorphism was significantly associated with the susceptibility of HCC (miR-196a2 rs11614913: TC+CC vs. TT: OR=1.232, 95%CI=1.028–1.476), while the other three SNPs were not found to have a significant association (miR-146a rs2910164: GC+CC vs. GG: OR=1.003, 95%CI=0.904–1.113; miR-149 rs2292832: TC+CC vs. TT: OR=0.898, 95%CI=0.756–1.068; miR-499 rs3746444: TC +CC vs. TT: OR=1.197, 95%CI= 0.973–1.472, respectively). In the analysis of HCC patients under different HBV infection status, only miR-196a2 rs11614913 revealed significant correlation of threefold risk (miR-146a rs2910164: GC+CC vs. GG: OR=1.687, 95%CI=0.667–4.263; miR-149 rs2292832: TC +CC vs. TT: OR=2.435, 95%CI=0.116–51.063; miR-196a2 rs11614913: TC+CC vs. TT: OR=3.005, 95%CI=1.239–7.287; miR-499 rs3746444: TC+CC vs. TT: OR=0.690, 95%CI= 0.211–2.261, respectively). The results of meta-regression demonstrated that no overall significant heterogeneity was found in ethnicity, case-control match, and whether genotype distribution of controls was consistent with HWE or the sample size larger than 500 (**Table 2**). The results of Begg's and Egger' s tests were shown in **Table 3**, with the symmetrical distribution of effect sizes inside the Begg's funnel plots (**Figure 5**), suggesting no significant publication bias among the included studies. Meta-regression and publication bias on miR-149 rs2292832 was not performed on account of insufficient studies.

Network Meta-Analysis

The current study contained four SNPs: miR-146a rs2910164, miR-149 rs2292832, miR-196a2 rs11614913, miR-499 rs3746444. It was observed from the network evidence that the number of direct comparisons of miR-146a vs. miR-499 was the largest, followed by miR-146a vs. miR-196a2, miR-196a2 vs. miR-499 (**Figure 6**). The predictive value of pairwise and network results of four miRNAs were explored and compared in **Table 4**. The NMA results showed no significant difference in the distribution of genotype frequencies except for miR-196a2 rs11614913, which appeared to have the highest superiority

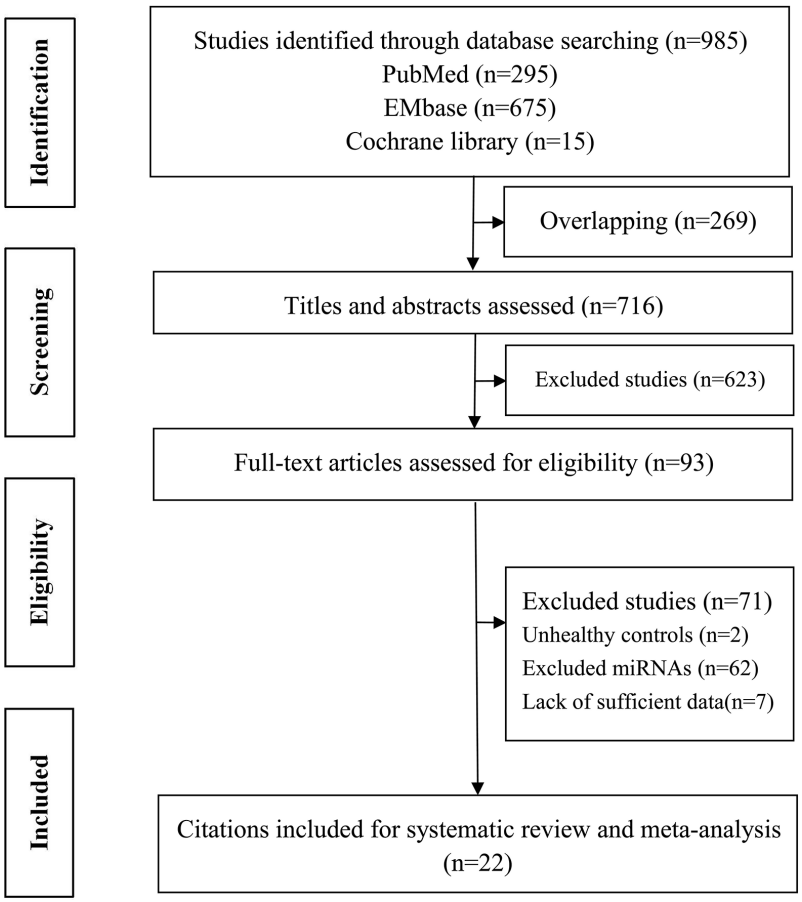
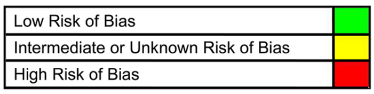


FIGURE 1 | PRISMA Flow diagram of the literature during the review process for the systematic review and meta-analysis.

TABLE 1 | Main characteristics and methodological quality of eligible studies.

Author	Year	Country	Continent	Ethnicity	Genotyping method	Source of controls	Match	Case/Control
Farokhizadeh et al. (31)	2019	Iran	Asia	Caucasian	PCR-RFLP	PB	Y	100/120
Abdel-Hamid et al. (32)	2018	Egypt	Africa	Caucasian	PCR-RFLP	PB	Y	50/50
Zhang et al. (33)	2016	China	Asia	Asian	PCR-RFLP	HB	N	175/302
Toraih et al. (34)	2016	Egypt	Africa	Caucasian	Real-time PCR	PB	Y	60/150
Yan et al. (35)	2015	China	Asia	Asian	PCR-RFLP	HB	N	274/328
Li et al. (36)	2015	China	Asia	Asian	PCR-RFLP	HB	N	266/266
Li et al. (37)	2015	China	Asia	Asian	PCR-RFLP	HB	Y	184/184
Qi et al. (38)	2014	China	Asia	Asian	Sequenom	PB	N	314/407
Kou et al. (39)	2014	China	Asia	Asian	PCR-RFLP	HB	N	271/532
Wang et al. (40)	2014	China	Asia	Asian	PCR-RFLP	HB	N	152/304
Zhou et al. (41)	2014	China	Asia	Asian	Sequenom	HB	N	266/281
Chu et al. (42)	2014	China	Asia	Asian	PCR-RFLP	HB	Y	188/337
Hao et al. (43)	2014	China	Asia	Asian	PCR-RFLP	HB	Y	235/281
Zhang et al. (44)	2013	China	Asia	Asian	Sequenom	HB	N	1,000/1,000
Shan et al. (45)	2013	China	Asia	Asian	PCR-RFLP	HB	Y	172/185
Kim et al. (46)	2012	Korea	Asia	Asian	PCR-RFLP	PB	N	159/201
Xiang et al. (47)	2012	China	Asia	Asian	PCR-RFLP	HB	N	100/100
Zhou et al. (48)	2012	China	Asia	Asian	PCR-RFLP	HB	Y	186/483
Akkiz et al. (49–51)	2011	Turkey	Asia	Caucasian	PCR-RFLP	HB	Y	222/222
Zhang et al. (52)	2011	China	Asia	Asian	PIRA-PCR	HB	N	963/852

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism assay; PB, population-based; HB: hospital-based.



Study	Is the case definition adequate	Representativeness of the cases	Selection of Controls	Definition of Controls	Comparability of cases and controls (/2)	Ascertainment of exposure(/2)	Same method of ascertainment for cases and controls	Non-Response rate	Overall rating and TOTAL SCORE / 10
Farokhizadeh Z,2019	1	0	1	1	2	0	1	1	7
Abdel-Hamid M,2018	1	0	0	1	2	0	1	1	6
Zhang LH,2016	1	0	0	1	2	0	1	1	6
Toraih EA,2016	1	0	1	1	2	1	1	1	8
Yan P,2015	1	0	0	1	2	0	1	1	6
Li X,2015	1	0	0	1	2	0	1	1	6
Li D,2015	1	0	0	1	2	0	1	1	6
Qi JH,2014	1	0	1	1	2	0	1	1	7
Kou JT,2014	1	0	0	1	2	0	1	1	6
Wang XH,2014	1	0	0	1	2	0	1	1	6
Zhou B,2014	1	0	0	1	2	0	1	1	6
Chu YH,2014	1	0	0	1	2	0	1	1	6
Hao YX,2014	1	0	0	1	2	0	1	0	5
Zhang J,2013	1	0	0	1	2	0	1	0	5
Shan YF,2013	1	0	0	1	2	0	1	1	6
Kim WH,2012	1	0	1	1	2	0	1	1	7
Xiang Y,2012	1	0	0	1	2	0	1	1	6
Zhou J,2012	1	0	0	1	2	0	1	1	6
Akkiz H,2011	1	1	0	1	2	0	1	1	7
Zhang XW,2011	1	0	0	1	2	0	1	1	6

FIGURE 2 | Risk of bias assessment using the Newcastle-Ottawa Scale for case-control studies.

index when comparing and ranking four SNPs, further suggesting that it could be an effective indicator of the occurrence of HCC.

DISCUSSION

MiRNAs play an important role in gene regulation of diseases (53), and have been proved to be tumor-suppressor genes as wells as oncogenes (54, 55). The dysregualtion of miRNA and its associated gene expression are involved in the occurrence and prognosis of HCC (56). The discovery of polymorphisms in miRNA genes has potential as new biomarkers for early diagnosis and prognosis in high-risk population, opening up new prospects for individualized treatment of HCC (57).

Of the 20 studies included in our research, the vast majority came from Asia, only two from Africa. Despite the rising incidence in North America and Europe, no enrolled studies came from either continent. The incidence of HCC varies widely within geographic locations. It is more common in low- and middle-income countries than in developed countries (58). It's worth noting that, HCC incidence rates have been increasing in the United States, Europe and other developed areas (59). Obesity, smoking, diabetes, alcoholic cirrhosis and non-alcoholic steatosis are main causes of the increasing incidence of HCC (60–62). Currently, studies on the relationship between polymorphisms of miRNA and HCC are still lacking in relatively low-incidence areas. Our study illustrated the need for multi-ethnic, large-sample case-control studies that include data from a broad range of ethnic groups to obtain more stable and reliable results.

The pairwise results indicated that among the polymorphisms of miR-146a rs2910164, miR-149 rs2292832, miR-196a2 rs11614913, miR-499 rs3746444, only miR-196a2 was significantly associated with the susceptibility of HCC. When compared with TT genotype, CT or TT genotype in miR-196a2 carried a 1.232-fold increased risk of HCC. The network results were consistent with the direct results, with slight difference which was acceptable, indicating that our network evidence were robust. When comparing and ranking four SNPs, miR-196a2

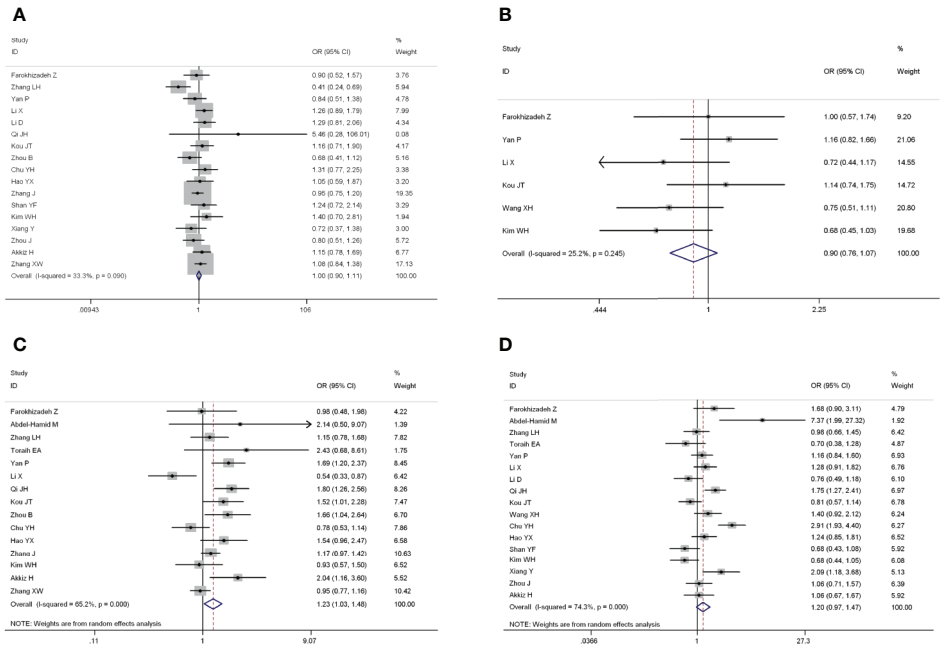


FIGURE 3 | Forest plots of the association of four single nucleotide polymorphisms (SNPs) and hepatocellular carcinoma (HCC) risk. **(A)** miR-146a rs2910164; **(B)** miR-149 rs2292832; **(C)** miR-196a2 rs11614913; **(D)** miR-499 rs3746444.

rs11614913 appeared to have the highest superiority index. All of the above might come to the conclusion that miR-196a2 could serve as the best predictor of susceptibility in HCC.

HBV infection has been well established as one of the leading causes for the carcinogenesis of HCC (63). When comparing HBV-positive with HBV-negative HCC patients, a significant 3-fold increase in the frequencies of TC+CC versus TT was observed in miR-196a2 rs11614913. This indicated that the miR-196a2 rs11614913 polymorphism could be associated with the risk of HBV-related HCC. There have been studies that miRNAs could be involved in the development of HBV-related HCC. Previous reports have indicated that compared to normal liver, miRNA expression profiles were altered in chronic hepatitis B tissues (64). Wang et al. speculated that cellular miRNAs might function in HBV-related HCC, which affected HBV gene expression by binding to HBV transcripts or targeted cellular transcriptions factors that were necessary for HCC development (65). HBV infection could affect miRNA expression and contribute to enhanced viral replication and pathogenesis, and could ultimately lead to HCC (66).

MiR-196a2 rs11614913 is reported to be an important SNP associated with the etiology, progression and prognosis of several kinds of cancer. MiR-196a2 is located in the 3'passenger strand mature sequence of miR-196a2 (67), whose C to T mutation results in a G:C mutation to a G:U mismatch, leading to a decrease in the processing efficiency of the precursor of miRNAs to its mature form and ability to regulate target genes (68). The impacted expression level of the mature miR-196a2 can lead to genetic susceptibility and affect the survival of certain types of tumor. A number of studies has supported the proposition that the polymorphism of miR-196a2 rs11614913 may contribute to the susceptibility of several cancers (69–72). In the updated meta-analysis of Liu et al. (69), the link between miR-196a2 rs11614913 and a variety of cancers was explored and found that it was associated with HCC and lung cancer susceptibility. Hoffman's research suggested that miR-196a2 had potential carcinogenic effects during the development of breast cancer (70). Hu et al. provided evidence that miR-196a2 variant homozygote was associated with a 1.76-fold-elevated HR, which was unfavorable to the overall survival of non-small cell lung cancer (71). In a case-control study conducted by Dikaiakos et al. (72), no significant association was found between miRNA-196a2 and colorectal cancer. Research has shown that the C allele of miRNA-196a2 increased the expression of mature miRNA-196a2 in HCC tissues (73). It is biologically plausible that miR-196a2 rs11614913 polymorphism may contribute to genetic susceptibility of HCC.

Although our results indicated that only miR-196a2 was associated with the susceptibility of HCC, and there was not enough evidence to support the association in miR-146, miR-149 or miR-499, the negative results still could not be ignored and should be interpreted cautiously. This is due to the occurrence of HCC is the result of multiple factors, in addition to complex genetic factors, there are hepatitis, aflatoxin exposure, Hepatitis C virus infection and other factors (74–76). Instead the incidence of HCC, gene variation may only cause increased susceptibility in a certain extent (77). Geography and ethnicity also need to be

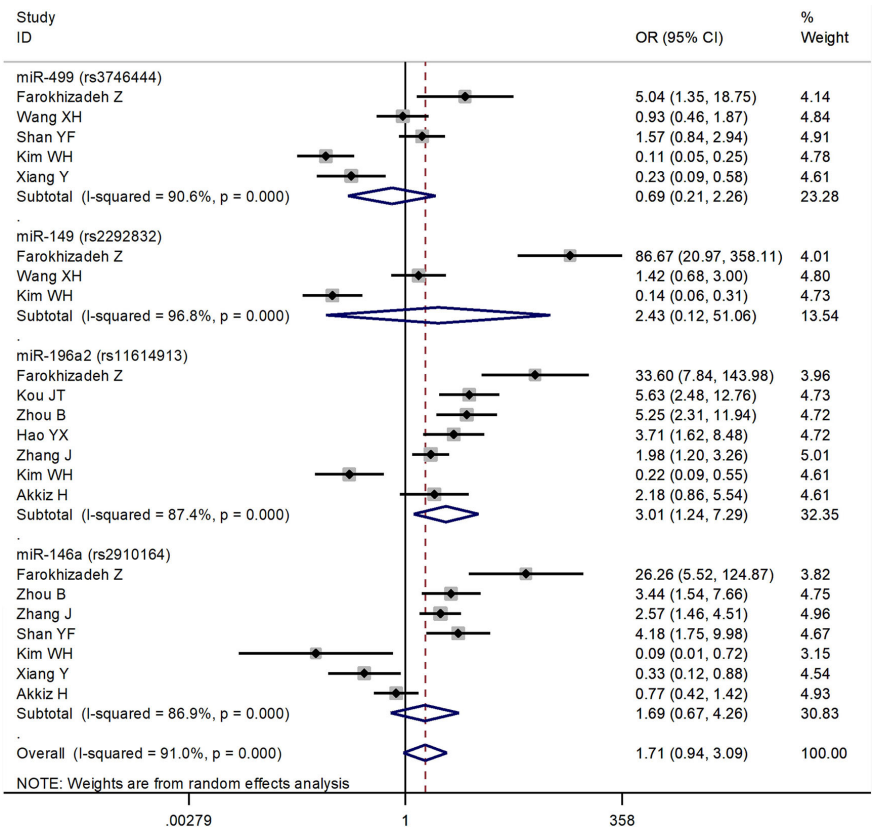


FIGURE 4 | Forest plots of the association of four single nucleotide polymorphisms (SNPs) under different hepatitis B virus (HBV) infection status in hepatocellular carcinoma (HCC) patients.

TABLE 2 | Summary of meta-regression analyses for heterogeneity ascertainment.

Covariate	miR-146a rs2910164				miR-196a2 rs11614913				miR-499 rs3746444			
	Coefficient	Std.Err	*t*	*P*	Coefficient	Std.Err	*t*	*P*	Coefficient	Std.Err	*t*	*P*
Ethnicity	-0.361	0.303	-0.12	0.907	-0.654	0.430	-1.52	0.159	-0.370	0.420	-0.73	0.480
HWE	-0.085	0.182	-0.47	0.650	-0.477	0.210	-2.27	0.047	0.275	0.286	0.96	0.355
Match	0.211	0.191	1.10	0.291	-0.196	0.259	-0.76	0.467	0.051	0.311	0.16	0.873
Sample size	0.119	0.204	0.58	0.572	-0.002	0.271	-0.01	0.992	0.240	0.296	0.81	0.434

Std.Err, Standard error.

TABLE 3 | Summary of Begg' s and Egger' s tests.

SNP	Begg's test		Egger's test	
	Z	*P*	Z	*P*
miR-146a (rs2910164)	0.08	0.934	0.01	0.994
miR-149 (rs2292832)	-0.19	0.851	-1.79	0.655
miR-196a2 (rs11614913)	-0.15	0.882	0.95	0.352
miR-499 (rs3746444)	0.16	0.869	1.39	0.493

taken into account. Differences in populations are an important consideration in genetic association studies which may lead to inconsistent outcomes and difficulties in repetition (78, 79).

There are some limitations in our study. Only published studies were included, and those studies with negative results that could not be published were likely to be omitted, leading to incomplete studies. Secondly, selection bias could exist and

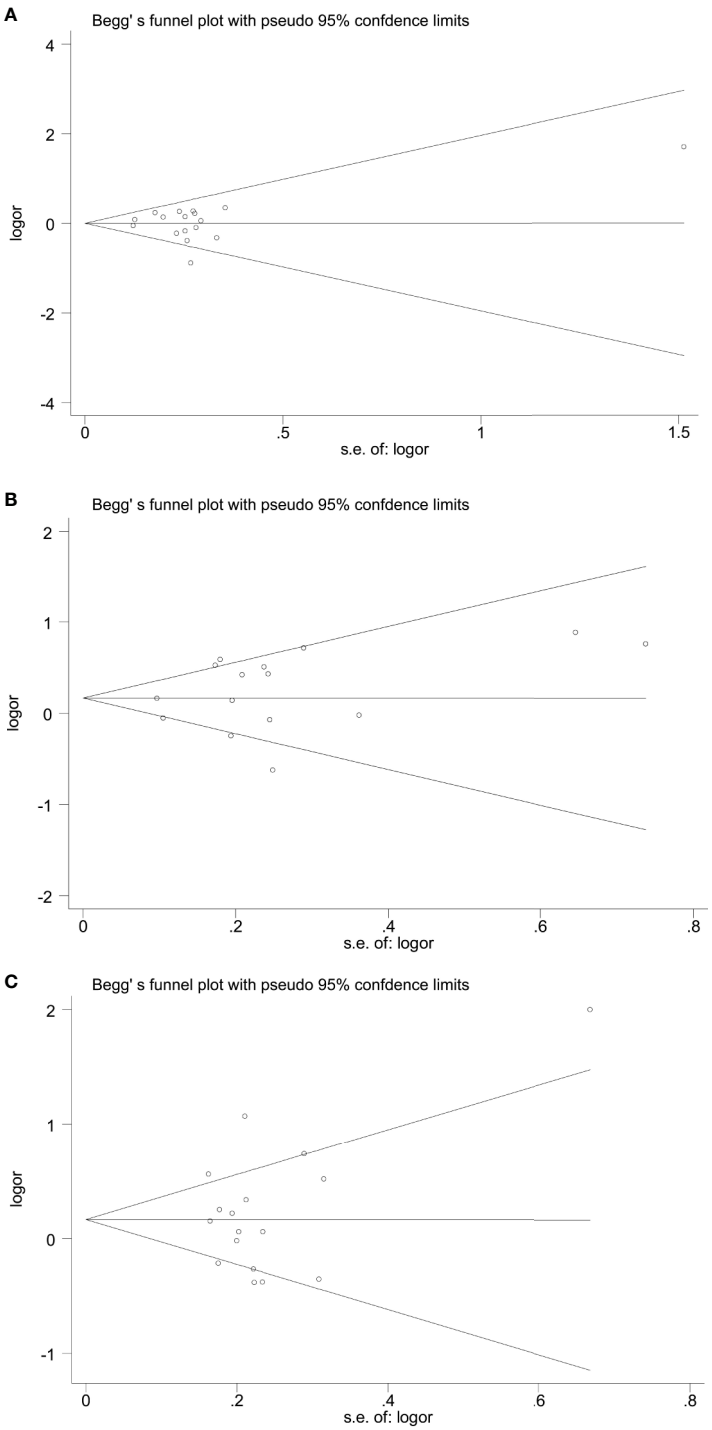


FIGURE 5 | Begg' funnel plots of publication bias. **(A)** miR-146a rs2910164; **(B)** miR-196a2 rs11614913; **(C)** miR-499 rs3746444.

impact on the results since the control group in most studies are hospital-based rather than population-based. Finally, although we found that miR-196a2 could be a potential indicator, how this might predispose to HCC are unclear and further functional studies are needed to clarify the mechanisms.

CONCLUSION

In conclusion, we found that the genetic polymorphism of miR-196a2 rs11614913 is significantly associated with the occurrence of HCC, especially for HBV- related HCC, and that individuals

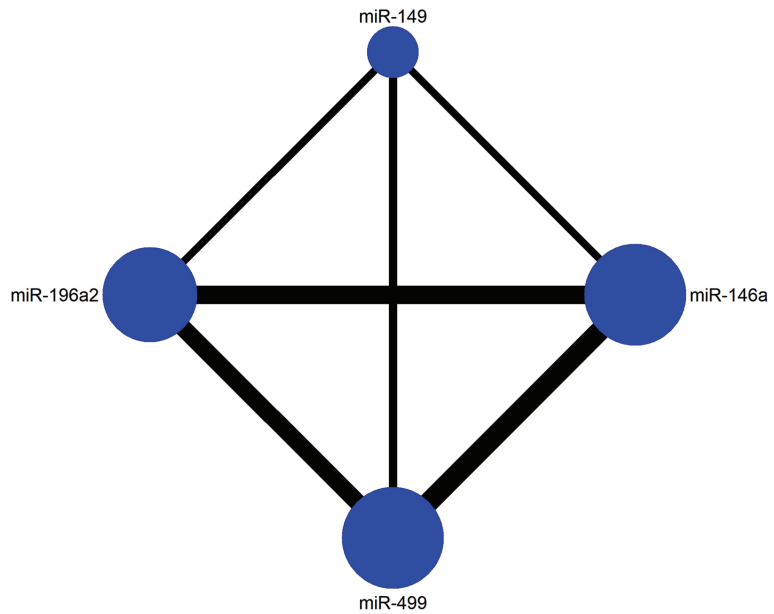


FIGURE 6 | The network evidence plot of four single nucleotide polymorphisms (SNPs).

TABLE 4 | The comparisons of pairwise and network results.

SNP	Comparisons	Pairwise meta-analysis		Network meta-analysis		S
		Direct OR	95% CI	Network OR	95% CI	
Case vs. control group						
miR-146a (rs2910164)	GC+CC vs.GG	1.003	0.904–1.113	1.02	0.73–1.38	0.89
miR-149 (rs2292832)	TC+CC vs.TT	0.898	0.756–1.068	0.96	0.47–1.79	0.96
miR-196a2 (rs11614913)	TC+CC vs.TT	1.232	1.028–1.476	1.20	1.01–1.43	2.31
miR-499 (rs3746444)	TC+CC vs.TT	1.197	0.973–1.472	1.20	0.96–1.47	1.00
HBV-positive vs. HBV-negative in case group						
miR-146a (rs2910164)	GC+CC vs.GG	1.687	0.667–4.263	1.62	0.77–2.87	1.40
miR-149 (rs2292832)	TC+CC vs.TT	2.435	0.116–51.063	2.45	0.51–7.53	2.65
miR-196a2 (rs11614913)	TC+CC vs.TT	3.005	1.239–7.287	2.46	1.31–4.09	2.34
miR-499 (rs3746444)	TC+CC vs.TT	0.690	0.211–2.261	0.96	0.38–2.01	0.54

OR, odd ratios; 95%CI, 95% confidence intervals.

with TC/CC allele were more susceptible. No significant association was found in miR-146a rs2910164, miR-149 rs2292832, or miR-499 rs3746444. Our work could provide important information on the relationship between these four miRNAs and the susceptibility of HCC, suggesting potential novel diagnostic options. This would contribute to the reduction of mortality through early screening and diagnosis and improve the efficacy in HCC management.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

QZ conducted the design of study, extracted the data, performed statistical analysis, and wrote the initial manuscript after consultation with the other authors. XX and HL improved the design, revised the manuscript, and approved the final version. TQ and SW checked the preliminary data. MW participated in the quality assessment adjudication. All authors contributed to the article and approved the submitted version.

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TDO2 Promotes the EMT of Hepatocellular Carcinoma Through Kyn-AhR Pathway

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Tryptophan 2,3-dioxygenase (TDO2), an enzyme involved in tryptophan (Trp) metabolism has been linked with some malignant traits of various cancers. Kyn, the main product of Trp metabolism pathway catalyzed by TDO2 and indoleamine 2,3-dioxygenase (IDO) in tumor cells, was also demonstrated to activate aryl hydrocarbon receptor (AhR), which may regulate cancer growth and invasion in some malignancies. However, whether TDO2 participates in the metastasis and invasion of HCC has not been explored before. The underlying mechanism played by TDO2 in this process still requires further investigation. Here, we demonstrated that overexpression of TDO2 correlates with advanced stage or malignant traits in HCC patients. Knockdown or inhibition of TDO2 suppressed the migration and invasion of HCC cells *in vitro* and *in vivo*. Epithelial to mesenchymal transition (EMT) is an essential program happened in the initial phase of cancer metastasis. We found that in HCC cells, TDO2 promoted the EMT process evidenced by altered levels of biomarkers for EMT. Mechanically, TDO2 regulated the Kyn production in HCC cell *via* activated aryl hydrocarbon receptor (AhR). Together, these results indicate that TDO2 promotes the EMT of hepatocellular carcinoma through activating Kyn-AhR pathway, thereby participating in the metastasis and invasion of HCC.

Keywords: hepatocellular carcinoma, metastasis, epithelial to mesenchymal transition (EMT), Tryptophan 2,3-dioxygenase (TDO2), aryl hydrocarbon receptor (AhR)

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INTRODUCTION

Liver cancer, the fifth most common cancer, ranks the second leading cause of cancer-related death worldwide (1, 2). Hepatocellular carcinoma (HCC) is the major forms of primary liver cancer. Overall prognosis for HCC patients remains poor due to the highly metastatic and aggressive biological features of HCC, which leading to advanced clinical stages and high recurrence rate of HCC patients (3, 4). Although molecular mechanisms underlying HCC metastasis has drawn a great deal of attention, it still remains unclear and requires further investigation.

Tryptophan 2,3-dioxygenase (TDO2), encoded by gene Tdo2, is expressed normally at high levels in the liver. It acts as the first and rate-limiting step of tryptophan (Trp) metabolism along kynurenine (Kyn) pathway and maintains systemic tryptophan levels (5). Kyn, the main product of Trp metabolism pathway catalyzed by TDO2 and indoleamine 2,3-dioxygenase (IDO) in tumor cells, was demonstrated

to activate aryl hydrocarbon receptor (AhR), suppressing antitumor immune responses and promoting tumor-cell survival and motility through AhR in an autocrine/paracrine fashion (6). AhR is identified as a ligand-activated transcription factor of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family and also plays an essential role in a wide range of physical and pathological condition (7). TDO2 is constitutively expressed in various cancer cells, such as hepatocarcinoma, bladder carcinoma, breast carcinoma, colorectal carcinoma, lung carcinoma, and glioblastoma, playing a role in immune surveillance and tumor biology (6, 8, 9). Recent studies revealed that TDO2 affects biological features directly in different cancers (6, 9, 10). TDO2 is highly expressed in HCC, however, whether TDO2 participates in the metastasis and invasion of HCC has not been explored before. Further, the underlying mechanism played by TDO2 in this process in HCC still requires further investigation.

Epithelial to mesenchymal transition (EMT), a process that epithelial cells lose their polarized organization and acquire migratory and invasive capabilities, is considered to contribute to cancer metastasis (11, 12). Therapeutics targeted to EMT pathway show a great potential for preventing tumor dissemination or sweeping off metastatic cancer cells in patients in advanced stage (13, 14). EMT biomarkers, such as Vimentin, N-Cadherin and MMP9 are overexpressed on HCC and participate in facilitating the metastasis of HCC (15–18). These suggest that induction EMT contributes to the acquisition of high metastatic trait of HCC. Many studies revealed that AhR activity contributed to the loss of cell contact-inhibition and altering extracellular matrix remodel (19). Considerable evidence has been piled up supporting the critical role of AhR activation in the induction of EMT (19–22). Previous study showed that AhR was overexpressed in HCC and associated with its tumorigenesis and invasion (23, 24). These results prompted us to hypothesis that TDO2 may contribute to tumorigenesis and metastasis and invasion of HCC *via* activation of AhR leading to increased EMT.

Here, we report our studies of the role of TDO2 in the metastasis and invasion of HCC, we searched TCGA database and mined data of the expression of TDO2 in different cancers. We found that the expression level varied in HCC and stomach adenocarcinoma according to different stages. The TDO2 expression was relatively high in HCC with vascular invasion and so was it in stomach adenocarcinoma with advanced stages in clinical samples. The effect of knockdown or inhibition of TDO2 on the EMT associated metastasis ability of HCC cell lines was investigated by *in vitro* and vivo experiments. Mechanistically, we demonstrated that TDO2 was responsible for the metabolism of Trp along Kyn pathway in HCC cells, and regulated the EMT process at least partly through Kyn-AhR pathway. Together, our results indicate that the overexpression of TDO2 promotes HCC metastasis capability through Kyn-AhR mediated induction of EMT.

MATERIALS AND METHODS

Materials

Huh7 and LM3 HCC cell lines were transfected with two puro plasmid expressing sh-Tdo2 and scrambled control using transfection reagent (provided by Haro Life, Shanghai, China). Two shRNAs were designed to knockdown of TDO2, shown as following.

TDO2 inhibitor 680C91, Tryptophan and Kynurenine were purchased from Sigma-Aldrich, AhR inhibitor CH-223191 were purchased from Selleck Chemicals (Houston, TX, USA).

pLKO.1puro-shhTDO2-AF

C C G G G G A A A G A A C T C C A G
GTTTACTCGAGTAAACCTGGAGTTCTTTCCTTTTT

pLKO.1puro-shhTDO2-AR

A A T T A A A A A G G A A A G A A C T C
CAGGTTTACTCGAGTAAACCTGGAGTTCTTTCC

pLKO.1puro-shhTDO2-BF

C C G G T C A T A A G G A T T C A
GGCTAACTCGAGTTAGCCTGAATCCTTATGATTTTT

pLKO.1puro-shhTDO2-BR

A A T T A A A A A T C A T A A G G A T
TCAGGCTAACTCGAGTTAGCCTGAATCCTTATGA

Specimens and Cell Culture

All clinical specimens were obtained with informed consent of patients in Shanghai general hospital and confirmed by pathologists. Twenty-three cases of paired specimens, HCC tissue and the adjacent normal tissue, and 16 cases of gastric cancer tissue were collected and stored in liquid nitrogen. Another 28 cases of HCC specimens were collected and stored in formalin. Human liver cancer cell lines (Huh7, LM3, Hep3B, HepG2, 97H-GFP-LC3) and normal human liver cell (LO2) were obtained from Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cell lines except for HepG2 were cultured in DMEM medium and HepG2 in MEM medium, with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin under a humidified atmosphere containing 5% CO2 at 37°C.

Migration and Invasion Assays

Scratch wound assays and Transwell chamber assays were applied to test the migration and invasion capabilities of HCC cells. For scratch wound healing assay, 5×10^5 cells were firstly seeded per well in 6-well plates and cultured for 24 h, after which scratching with a 200 μl micropipette tip in the center of the well were performed. Then, the cells were cultured with serum-free medium and corresponding treatment. Images were captured at 0 and 48 h after scratch. The width of wound healing was measured, and migration rate was calculated.

Transwell chamber assays with and without Matrigel-coated were performed to show cell migration and invasiveness. Cells were seeded at 20,000 cells or 40,000 per well in DMEM medium in the upper chamber without or with Matrigel coated, respectively. Six hundred μl Medium containing 10% FBS was added to the bottom chamber. Forty-eight hours later, cells in the upper surface of transwell chamber were erased with swab and cells transferred to the lower surface of the chamber were fixed in 4% paraformaldehyde and then stained with aniline violet for visualization and photography.

Three independent experiments were carried out. Mean ± standard error of mean (SEM) was calculated for each of the experiments.

Western Blot Analysis

Specimens and Cells were lysed in RIPA with 1% PMSF. Nuclear and cytoplasmic separation were guided according to the manual of Nucleo-cytoplasmic protein extraction kit (Thermo Scientific, USA). Cell protein extracts (50 μg) were denatured by boiling, separated on SDS-PAGE gels, and electrotransferred to polyvinylidene fluoride membranes (Millipore 0.45 um, USA). After the membranes were blocked with 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween20, they were incubated with primary antibodies overnight at 4°C, including anti-TDO2 (NOVUS, USA), anti-AhR (Abcam, UK), anti-Cyp1b1(Abcam, UK), anti-E Cadherin(CST, USA), anti-N Cadherin(CST, USA), anti-MMP9 (CST, USA), or anti-Vimentin antibodies(CST, USA). The antibodies above were applied at the concentration of 1:500. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG was used as secondary antibodies, at the concentration of 1:2,000. Immunocomplexes were visualized with an ECL luminescence reagent (Absin, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1,000 dilution, Proteintech, USA) was used as an internal control. Densitometry quantification was performed using Image J.

Quantitative RT-PCR

Total RNA was isolated using the traditional Trizol methods. cDNA was synthesized with ProFlex™ PCR system using PrimeScript™ RT Master Mix reagent kit (TaKaRa, Shiga, Japan). Quantitative reverse transcription PCR (qRT-PCR) was performed in Roche LightCycler 96 using SYBR Premix Ex Taq™ (TaKaRa, Shiga, Japan). All fold-change data were normalized to GAPDH. The 2-ΔΔCt method was used to calculate relative expression levels.

Immunohistochemistry

The HCC tissue paraffin sections were subjected to de-paraffinization in xylene, rehydration through graded ethanol (100, 95, 85, 80, 75%) and distilled water, prior to boiling in 10 mM citrate buffer solution (pH 6.0) for 15 min for antigen retrieval. Three percent H_2O_2 was applied to incubate the tissue arrays for 10 min to quench endogenous peroxidase activity. After blocked with 1% bovine serum albumin for 20 min, tissue arrays were incubated with primary antibody, including TDO2 antibody, anti-N cadherin or anti-E cadherin, with 1:100 dilution overnight at 4°C, prior to incubation with biotinylated secondary antibody for 30 min at 37°C. Coloration lasted for 1 min in DAB (Invitrogen, USA). Images were recorded using Lax software under the unified parameters.

Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.3% TritonX-100 in PBS for 20 min and then blocked with 1% BSA in PBS containing 0.3M glycine. Subsequently, the primary anti-AhR antibody (1:100 dilution) was added to the cells and incubated overnight at 4°C. The secondary FITC combined antibody (Solarbio, China) was diluted in 1:100 and added to cells for 30 min at room temperature. 40,6-diamidino-2-phenylindole (DAPI) was last added for 5 min to visualize the nuclear of cells. Immunostaining was observed at 400 magnification using the Leica TCS SP8 confocal microscope and images were captured using the Leica LAS-AF software (Leica Microsystems, Germany).

Analysis of Tryptophan and Kynurenine

High performance liquid chromatography (HPLC) was used to analyze the concentration of Trp and Kyn. Cell culture supernatant was collected, centrifuged, and transferred to fresh tubes and frozen until subjected to analysis. Two hundred μL sample was precisely pipetted, and 200 μM perchloric acid was added for purification. The sample was mixed with vortex for 30 s and placed at room temperature for 5–10 min, followed by centrifugation for 5 min at 10,000 r/min. The supernatant was collected for test. VWD C18 column (250*4.6mm;5μl) was used as detector. Twenty μl sample was injected with the speed of 1.0 ml/min and measured at 225 nm wavelength. Fifteen mmol/L acetic acid: sodium acetate (containing 2.7% acetonitrile, PH = 3.6) was applied as mobile phase. The concentrations were calculated based on standard solutions.

In Vivo Models

A total of 20 BALB/c nude mice were used for the orthotopic mouse model of HCC according to previous study (25). Mice were anesthetized by isoflurane, and 2×10^6 cells in 25μl PBS containing 25% Matrigel were injected into the subcostal region of the left lobe. Mice were sacrificed 6 weeks later, and the livers were removed, imaged, and embedded in paraffin. Hematoxylin and eosin staining were performed to confirm tumor metastasis. All animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai General Hospital.

Statistical Methods

Statistical analyses were carried out and graphics were generated using GraphPad Prism 7.00. Results are shown as representative images or as mean ± SEM of at least three independent experiments. Data according with Gaussian distribution were analyzed using the unpaired t-test. The basic information parameters of patients were analyzed using Fisher's exact test. Data shown in graphical format represented as means (± SEM) or medians with interquartile range. P value <0.05 is considered statistically significant.

RESULTS

Overexpression of TDO2 Was Associated With Advanced Stage or Malignant Traits in Patients With HCC and Stomach Carcinoma

To investigate whether the expression of TDO2 correlates with HCC progress and other digestive maliganancies, we utilized the

public data available in TCGA (The Cancer Genome Atlas) database. We found that stomach adenocarcinoma and esophageal carcinoma in advanced stages had higher TDO2 expression than that in early stages (**Supplementary Figure 1A**). As for HCC, the public data also showed an upregulated expression of TDO2 in cancer of metastasis compared with that without metastasis (**Figure 1A**). We used qRT-PCR and IHC to analyze TDO2 mRNA and protein levels in 23 pairs of HCC samples and adjacent normal tissues. We demonstrated that HCC with vascular invasion had higher TDO2 expression at transcriptional and translational level than HCC without vascular invasion (**Figures 1B, C**). Besides, by Western Blot analysis of clinical gastric carcinoma samples (n = 8), we also found that the expression level of TDO2 was relatively higher in gastric carcinoma in stage III–IV than in stage I–II (**Supplementary Figure 1B**). And the analysis of co-relationship of TDO2 expression level and overall survival of HCC patients showed a shorter survival time in high TDO2 group than in low TDO2 group, while no statistical significance reached (**Figure 1D**). These results indicate that the upregulated expression of TDO2 is related to malignancy grade, which may contribute to the invasion and metastasis of HCC, and further influencing the prognosis of patients.

Knockdown or Inhibition of TDO2 Decreased the Migration and Invasion Capabilities of HCC Cell Lines *In Vitro* and *Vivo*

To examine the role of TDO2 in promoting the development of HCC, we used both knockdown and inhibitory approaches to suppress the TDO2 activity in 5 HCC cell lines. The level of TDO2 expression level was upregulated in LM3, Huh7 and Hep3B cell lines as compared with that in the immortalized normal human liver cell line LO2, at both protein and mRNA levels (**Figure 2A**). Then two shRNA sequences packed with effective lentivirus were designed and utilized to knockdown Tdo2 gene in Huh7 and LM3 cells, while only one of them showed knockdown effects (**Figure 2B**). Thus, cells transferred with sh-Tdo2-B were used for the following experiments as a knockdown group (or sh-Tdo2 group). Scratch wound assays and Transwell assay showed that sh-Tdo2 groups in both Huh7 and LM3 cells has significantly reduced capabilities of the migration and invasion than sh-scramble groups ($P < 0.01$, **Figures 2C, D**). In addition, to inhibit the TDO2 in these cells, we applied 680C91, a specific inhibitor used for suppressing TDO2 activity (5) to these cells at 10 or 20 μM concentrations. Consistently, the inhibition of TDO2 also suppressed the migration and invasion capabilities of Huh7 and LM3 ($P < 0.01$, **Figures 2E, F**).

We further used HCC orthotopic model in nude mice to evaluate the effect of TDO2 on metastasis of HCC cells *in vivo*. Huh7 cell line was utilized for developing the *in vivo* model since it developed the satisfactory characteristics of tumorigenesis in this model. Both knockdown of TDO2 in Huh7sh-Tdo2 cells and inhibition of TDO2 in the Huh7 cell lines treated with 680C91 group developed less metastatic nodules than their respective control groups (**Figures 3A, B**). The above results indicate that TDO2 participates in the migration and invasion of HCC cells both *in vitro* and vivo.

TDO2 Promoted the Epithelial-To-Mesenchymal Transition Process in Hepatocellular Carcinoma Cells

We further investigated whether TDO2 overexpression promoted metastasis by modulating EMT of these HCC cell lines. EMT occurs during tumor progression to the metastatic phenotype. E-cadherin, a key marker of the epithelial phenotype, is a transmembrane protein responsible for cell-cell contact and adherence junction, the loss of which is considered as a key step for metastasis (27). N-cadherin and Vimentin, two proteins considered to be markers of a mesenchymal phenotype and crucial for cellular migration, are upregulated during EMT (15, 16). MMPs, such as MMP2 and MMP9, are upregulated markers during EMT that are capable of helping migratory cells to invade neighboring tissues and break through the basement membrane by cleaving cell-surface proteins and degrading components of extracellular matrix (28). Therefore, we utilized these EMT-associated markers (E and N-cadherin, MMP9 and vimentin) to assess EMT status of these HCC cells. We documented that in sh-Tdo2 and inhibitory groups of Huh7 and LM3 cells, an increased expression of E-cadherin, as well as decreased expressions of N-cadherin, MMP9, and Vimentin, was observed compared to negative control groups (**Figures 4A, B**). This result indicates that knockdown or inhibition of TDO2 impeded EMT process in HCC cells. IHC assay was performed to compare the expression levels of E-cadherin, N-cadherin, and Vimentin in HCC samples with different levels of TDO2. HCC tissue with low TDO2 expression level showed higher level of E-cadherin and lower level of N-cadherin and Vimentin, whereas samples with high TDO2 expression level showed relatively lower level of E-cadherin and higher level of N-cadherin and Vimentin (**Figure 4C**). These data suggested that TDO2 overexpression promoted EMT to facilitate metastasis in HCC.

TDO2 Promoted Epithelial-To-Mesenchymal Transition Process *via* Kyn-AhR Pathway

We further explored the molecular mechanism underlying TDO2-promoted EMT process. TDO2 was the main enzyme catalyzing Tryptophan in HCC cell lines, as the expression of IDO was relatively low (**Figure 5A**). Therefore, we further investigate whether TDO2 promoted EMT process *via* Kyn-AhR pathway. We tested whether TDO2 knockdown or inhibition affected Kyn production in Huh7 cell line. TDO2 knockdown increased Trp accumulation and decreased Kyn level. Consistently, Kyn/Trp ratio, a marker routinely used for measuring the activity of Trp metabolic enzymes, TDO2 and IDO (29), was decreased while TDO2 inhibitor was applied to the cells (**Figure 5B**). TDO2 knockdown or inhibition abrogated the abundance of CYP1b1, which indicated weakened activity of AhR (**Figure 5C**). Treating Huh7 cells with 50 μM exogenous

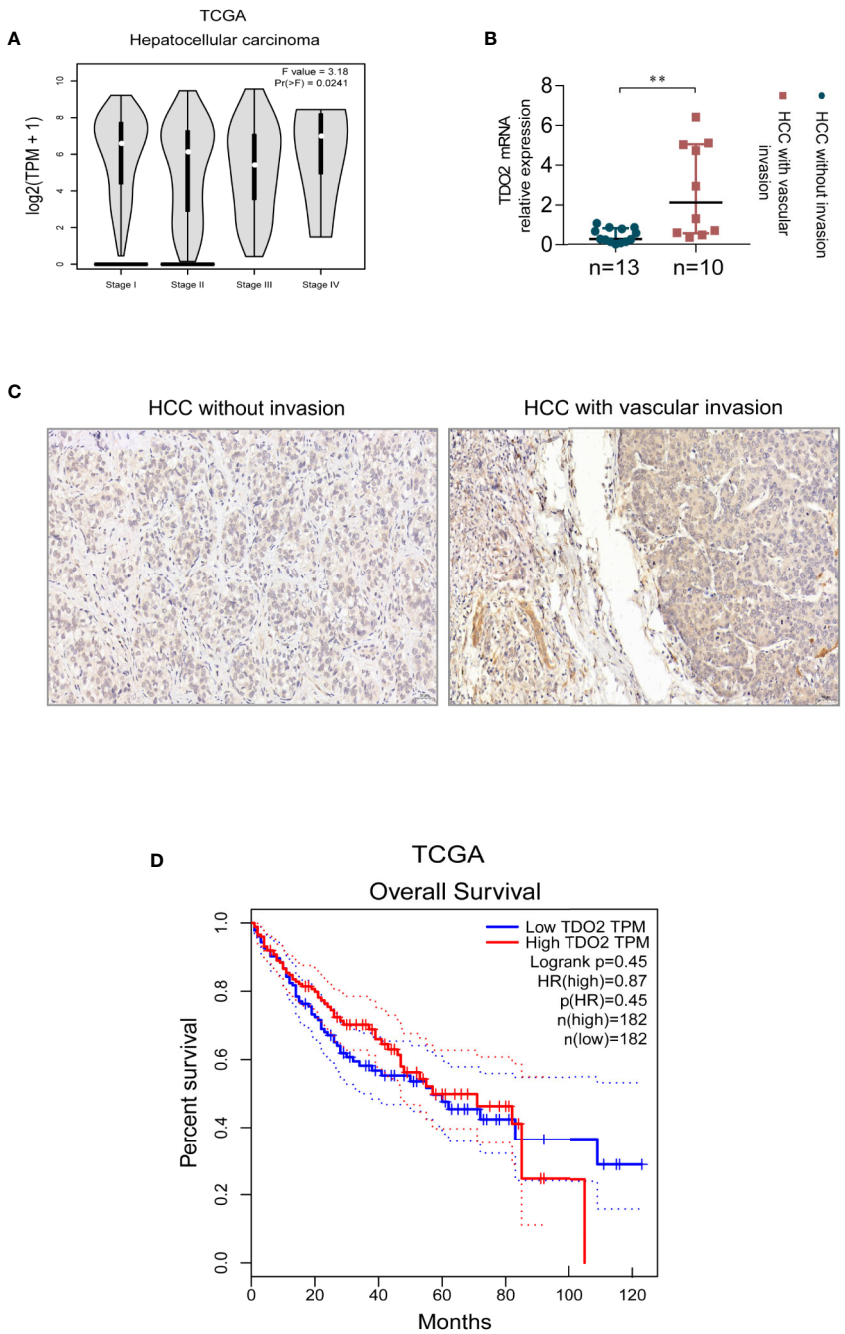

FIGURE 1 | The expression level of TDO2 correlates with advanced stage or malignant traits of carcinoma. **(A)** TDO2 expression in HCC of different stages shown by data from TCGA. **(B, C)** TDO2 mRNA and protein level in HCC samples with and without vascular invasion measured by qRT-PCR and IHC, respectively. **(D)** The relationship between TDO2 expression and overall survival of HCC patients according to the data obtained from TCGA database. **P < 0.01. Scale bar = 50 μm. The graphs were derived from the website GEPIA (26).

Kyn, AhR was observed to be activated and transferred to the nuclei by Western Blot (**Figure 5D**) and further verified by fluorescence confocal microscope (**Figure 5E**), demonstrating that Kyn mediated the activation of AhR in Huh7 cells.

Besides, public data showed a positive correlation between the level of TDO2 and that of AhR in malignant tumors, including colon (Spearman r = 0.33, P = 3.2e-08) and rectum adenocarcinoma (Spearman r = 0.32, P = 0.0019), thymoma (Spearman r = 0.51, P = 3.4e-09), testicular Germ Cell Tumors (Spearman r = 0.53, P = 4.4e-11), and Uveal melanoma (UM) (Spearman r = 0.50, P = 2.2e-06) (**Supplementary Figure 1C**). Further Western Blot examining the expression of AhR and its downstream target gene CYP1b1 in LO2

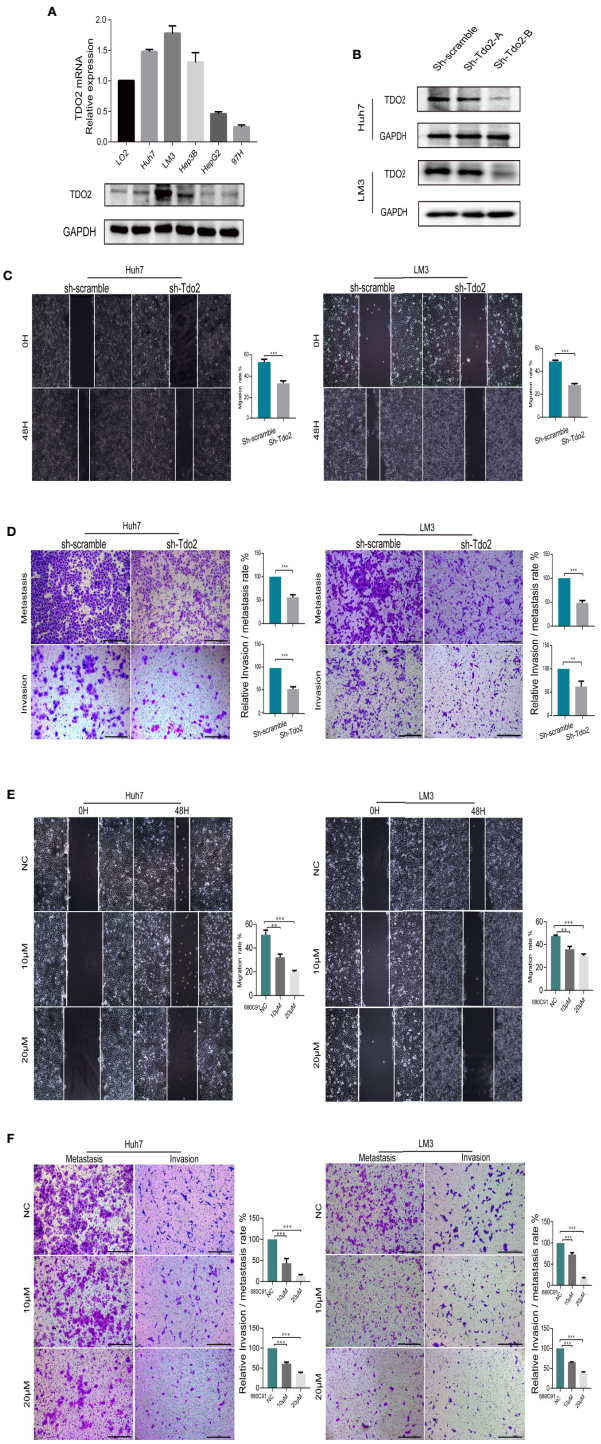

FIGURE 2 | TDO2 enhanced the metastasis of HCC cells *in vitro* and vivo. **(A)** Relative expression of TDO2 in HCC cell lines compared to normal liver cell LO2, as shown by qRT-PCR and Western Blot. **(B)** Establishment of TDO2 knockdown cell lines in Huh7 and LM3 cells, confirmed by Western Blot. **(C)** Representative data from Scratch wound assays performed with sh-Tdo2 and sh-scramble groups in Huh7 and LM3 cells. The migration distance, that is the difference between the width of wound at 0 h and that at 48 h measured using Adobe illustrator software, was recorded, and the migration rate, namely the ratio of migration distance to the initial wound width, was calculated. **(D)** Representative data from Transwell migration and Matrigel invasion assays with indicated cells. **(E)** Representative data from Scratch wound assays performed with the Huh7 and LM3 cell lines treated with TDO2 inhibitor 680C91 at different concentration. **(F)** Representative data from Transwell migration and Matrigel invasion assays with Huh7 and LM3 cells treated with TDO2 inhibitor 680C91 at different concentration. All data were recorded as means ± SEM of three independent experiments. Scale bar = 200 μm. $^{**}P < 0.01$; $^{***}P < 0.001$.

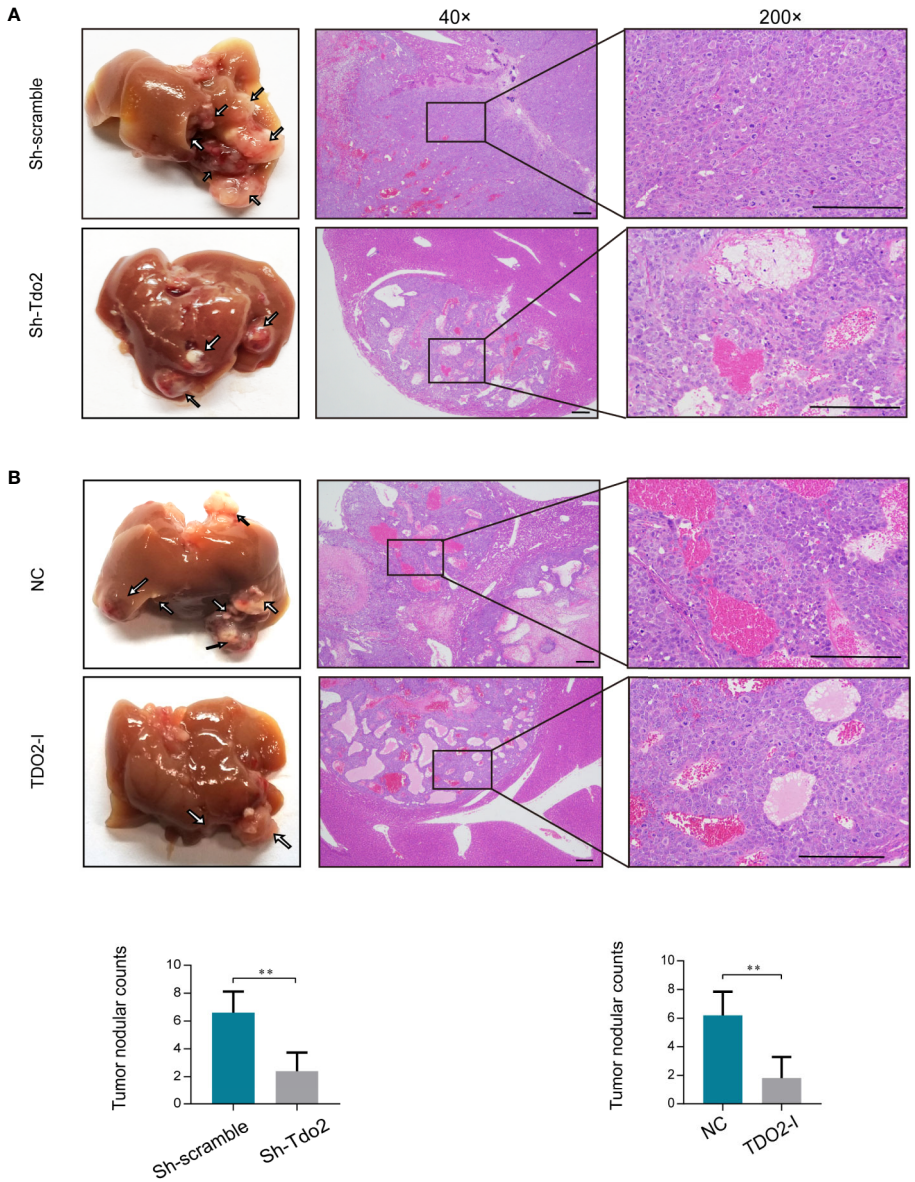

FIGURE 3 | **(A)** Knockdown of TDO2 decreased intrahepatic tumor metastasis of Huh7 cells in mice. **(B)** Inhibition of TDO2 by inhibitor 680C91 decreased intrahepatic tumor metastasis of Huh7 cells in mice. Photo micrograph of HE-stained tissues that showed metastasized HCC cell masses (magnification×4 and ×200, Scale bar = 200 mm). **P < 0.01.

and HCC cell lines showed that cell lines with TDO2 overexpression express relative higher level of AhR and CYP1b1 than the cell line with low expression of TDO2 (**Supplementary Figure 1D**), providing a clue that TDO2 probably regulated AhR in a translational level, which needs further work to explore.

Furthermore, addition of 50 μM exogenous Kyn resulted in a significant restore in the migration and invasion abilities of sh-Tdo2 Huh7 cells, which was counteracted by 10μM AhR antagonist CH-223191 (**Figure 5F**). Sh-Tdo2 Huh7 cells treated with exogenous Kyn showed decreased expression of E-cadherin and increased expression of N-Cadherin, MMP9 and Vimentin to different levels. These changes were reversed by AhR antagonist (**Figure 5G**). These data strongly suggested that TDO2 promoted HCC EMT through Kyn-AhR pathway.

DISCUSSION

HCC is one of the top life-threaten cancers worldwide, with a fearsome rate of recurrence that reaches 60–70% with 5 years and impedes the long survival of patients, despite comprehensive therapies have been applied to treat advanced HCC in clinic (3). Therefore, the fundamental mechanism of the malignant biological feature of HCC underlying the metastasis and invasion requires

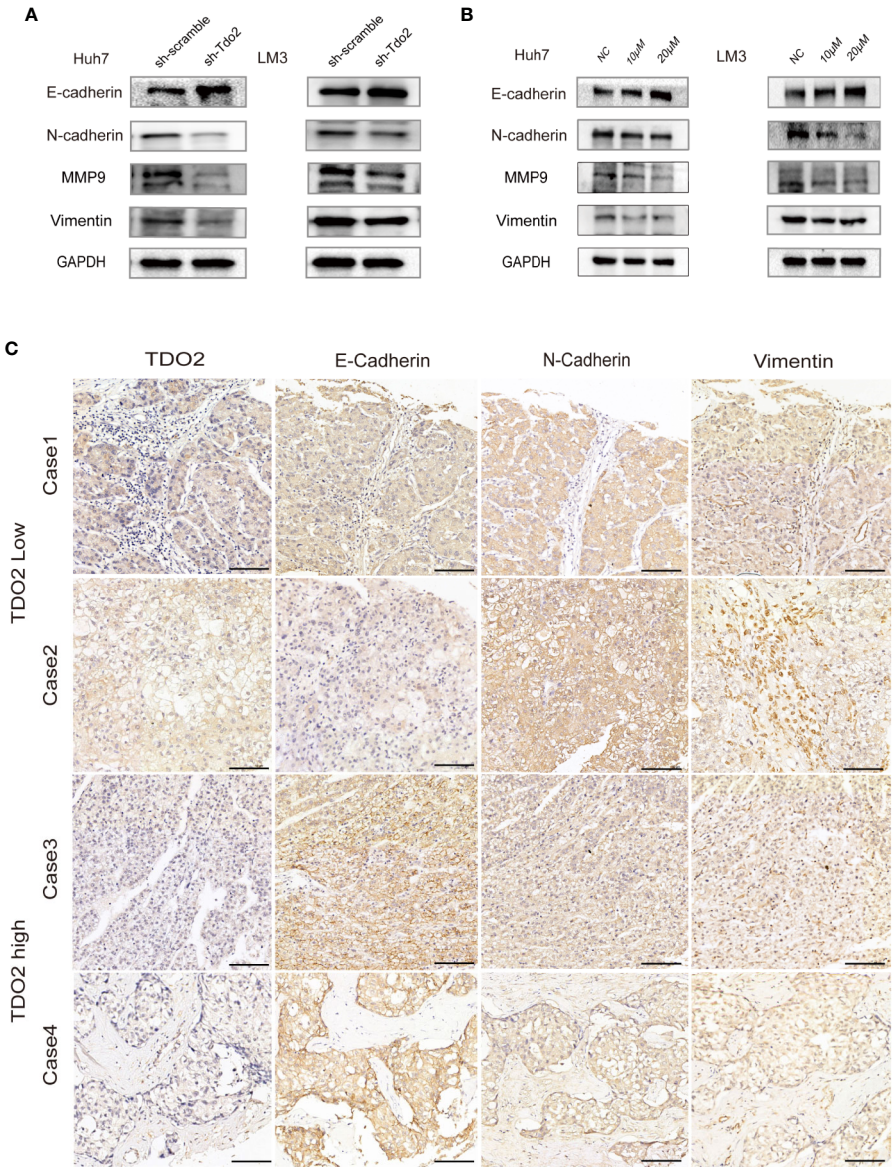

FIGURE 4 | TDO2 could promote EMT process in HCC cells. **(A)** Relative expression levels of E-cadherin, N-cadherin, Vimentin, and MMP9 in sh-Tdo2 and sh-scramble groups in Huh7 and LM3 cells. **(B)** Relative expression levels of E-cadherin, N-cadherin, Vimentin, and MMP9 in Huh7 and LM3 cells treated with TDO2 inhibitor 680C91. **(C)** Representative pictures of IHC of E-cadherin, N-cadherin, and Vimentin comparing tissues with high TDO2 levels and those with low TDO2 levels. Scale bar = 100 μm.

further exploration. TDO2 has been demonstrated to have immunomodulatory functions in promoting tumor immune resistance, which drew increasing attention to target this pathway for cancer immunotherapy (8, 30, 31). Some data have revealed that cancer cells can escape immune surveillance by overexpressing TDO2 and activating AhR in a range of cells of both the innate and adaptive immune system—dendritic cells, macrophages, natural killer cells, innate lymphoid cells, cytotoxic T cells and regulatory T cells (32, 33). Recently, TDO2 has been verified to strongly expressed in various cancers, including glioma, breast cancer, lung cancer, esophageal squamous cell carcinoma (ESCC), and could affect cancer biological features, including proliferation and metastasis, directly (9, 10, 34–36). Overexpression of TDO2 in triple negative breast cancer facilitated anoikis resistance and enhanced the metastatic capability of breast cancer cells *in vivo* (9). TDO2 was overexpressed in tumor tissue specimens obtained from UM hepatic metastasis and could be associated with the development and growth of metastasis in UM (34). TDO2 was demonstrated positively expressed in HCC (8), but there was no study for defining the role of TDO2 played in HCC. Here, we examined the effect of TDO2 on the metastasis of HCC and found that highly expression of TDO2 was related to advanced stage or invasion capabilities in

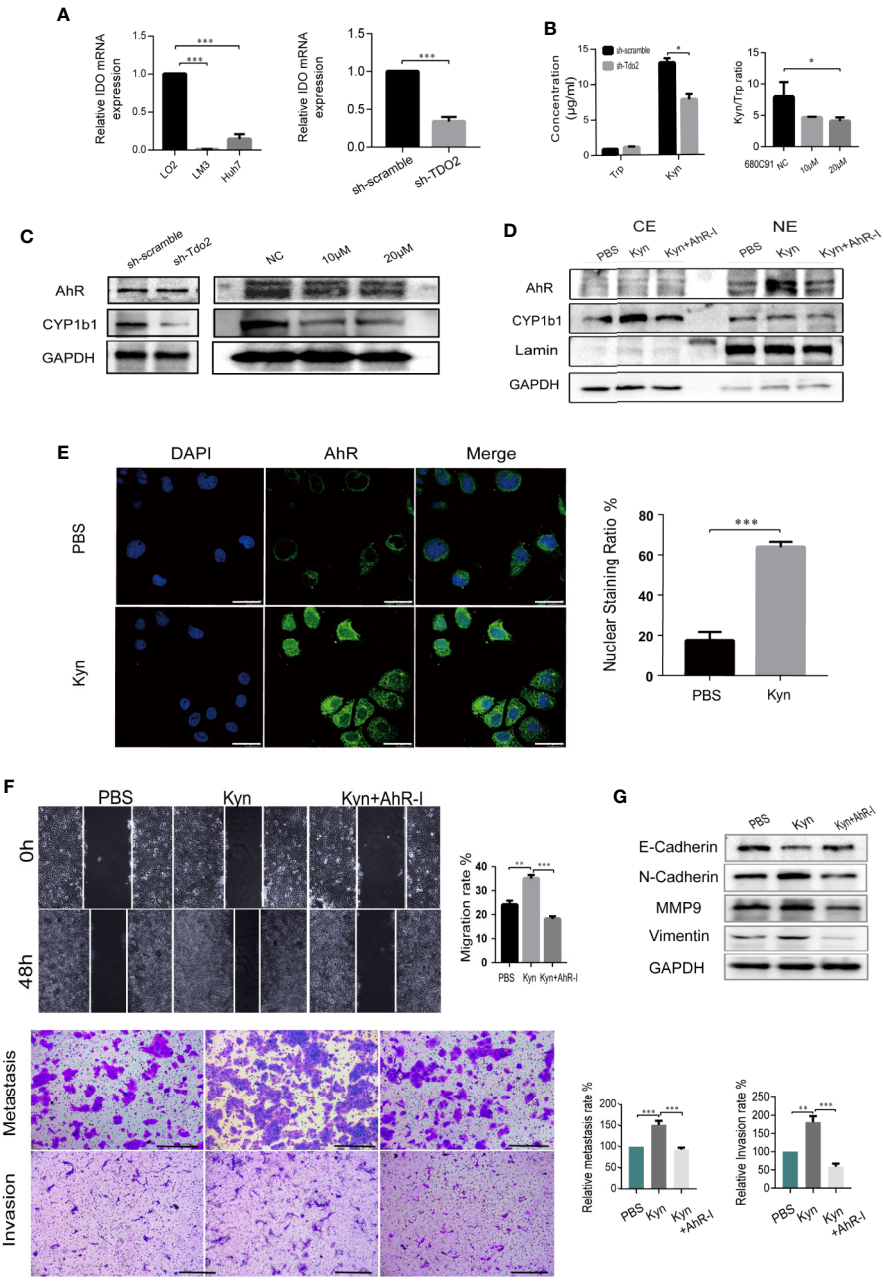

FIGURE 5 | TDO2 promoted EMT process *via* Kyn-AhR pathway. **(A)** The relative expression of IDO in LM3 andHuh7 compared to normal liver cell LO2, and in sh-Tdo2 Huh7 cells compared to sh-Scramble Huh7 cells, verified by qRT-PCR. **(B)** The concentration of Trp and Kyn in the supernatant of sh-scramble and sh-TDO2 Huh7 cells and the ratio of Kyn/Trp in the supernatant of Huh7 cells treated with TDO2 inhibitor 680C91. The concentration of Trp and Kyn was measured by HPLC. **(C)** Relative expression levels of AhR and its target gene CYP1b1 in the indicated cells. **(D, E)** Translocation of AhR activated by exogenous Kyn observed by Western blot and laser confocal fluorescence microscopy, Scale bar = 25 μm. **(F)** Scramble cell assay and Transwell metastasis and invasion assay of sh-Tdo2 Huh7 cells treated with PBS, Kyn (50 μM) and Kyn (50 μM) combined with AhR inhibitor CH-223191 (10 μM), Scale bar = 200 μm. **(G)** Relative expression levels of E-cadherin, N-cadherin, Vimentin and MMP9 in the sh-Tdo2 Huh7 cells treated with Kyn and AhR inhibitor measured by Western Blot. *P < 0.05; **P < 0.01; ***P < 0.001.

cancers and enhanced migration and invasion capabilities of HCC cells both *in vitro* and vivo. The study in ESCC also corroborates with our findings in that inhibition or knockdown of TDO2 suppressed ESCC cell line proliferation and invasion (10).

EMT has been commonly considered as an important mechanism of migration and invasion for most cancer cells and related to prognosis and treatment of metastatic cancers. An altered expression of EMT markers, in particular low E-

cadherin, is involved in an aggressive, malignant phenotype and early disease recurrence in HCC (11). Four EMT genes, including E-cadherin and MMP9, were found to be predictive of clinical overall survival and disease-free survival in a cohort of 128 HCC patients (37), and this was further confirmed in studies involving different centers and cohorts (38). Sorafenib, which inhibits STAT3 and phosphorylates TGF-β that are both up-regulated in EMT, is being considered as a potential therapeutic agent in HCC, but adverse events and resistance limited the therapeutic effectiveness (4). In our study, upregulated E-cadherin and downregulated N-cadherin, Vimentin and MMP9 induced by knockdown or inhibition of TDO2 were observed in HCC cells, as well as a negative correlation between TDO2 and E-cadherin and a positive correlation between TDO2 and N-cadherin in HCC samples, suggesting that TDO2 overexpression promoted HCC metastasis through inducing EMT in HCC cells. This result shed a new light on TDO2 on the development of EMT for the metastasis in HCC.

Considerable evidence supports the critical role of AhR in induction of EMT (19–22). AhR participates in the induction of Slug expression, which represses E-cadherin expression. The expression of MMPs is also a target of AhR pathway. TCDD exposure up-regulated the expression and activity of MMP9 in various malignancies including melanoma cells (39), urothelial cancer cell (40), prostate cancer cell (41), and gastric cancer cell (42). AhR was involved in the induction of EMT by Polychlorinated biphenyls in HCC cells (21). Kyn-AhR pathway has been in intensive focus recent years. Kyn has been considered to be a potent agonist of AhR, which can regulate the differentiation and activity of immune cells and thus suppress the immune response against tumors, leading to tumor immune tolerance (30). Besides, Kyn activating AhR regulated cancer growth and invasion in some malignancies (6, 22, 43). The study carried by Venkateswaran N showed that Kyn was elevated and functioned as an oncometabolite in colon cancer by promoting proliferation of colon cancer cells (43). Our *in vitro* results revealed that TDO2 was the main enzyme catalyzing Trp to Kyn in HCC cell lines, and Kyn activated AhR promoted migration and invasion capabilities through regulating EMT of HCC cell lines. Our finding is comparable with the observation published previously showing that kyn induced AhR activation enhanced invasiveness in thyroid cancer cells (22). The underlying molecular mechanism of the interaction between AhR and EMT markers and clinical relevance of this pathway remains unclear and warrants further investigation.

In conclusion, our study shows for the first time that highly expression of TDO2 is related to advanced stage and malignant traits in HCC and promotes migration and invasion capabilities of HCC cells by Kyn- AhR mediated induction of EMT. Further exploration of this pathway will provide a novel perspective into the mechanism of HCC metastasis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repositories can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Shanghai General hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the ethics committee of Shanghai General Hospital.

AUTHOR CONTRIBUTIONS

LL and TW carried out the studies, participated in collecting data, performed the statistical analysis, and drafted the manuscript. SL helped with the cell experiments. ZC and QW contributed to the animal experiments. JW and WC participated in collecting the clinical samples. XQ polished the manuscript. JX designed and supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

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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.2020.562823/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | (A) TDO2 expression in stomach adenocarcinoma and esophageal carcinoma in different stages shown by data from TCGA. **(B)** The expression level of TDO2 in gastric carcinoma in stage I–II and stage III–IV tested by Western Blot. **(C)** The correlationship of TDO2 and AhR in several cancers analyzed by data from TCGA database. **(D)** AhR and CYP1b1 expression in LO2 and 5 HCC cell lines tested by Western Blot.

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Withaferin A Acts as a Novel Regulator of Liver X Receptor-α in HCC

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Withaferin A, a steroidal lactone derived from the *Withania somnifera* plant has been known for its anti-cancerous effects on various types of cancer cells. However, its effect on the hallmarks of cancer such as proliferation, migration, invasion, and angiogenesis is still poorly understood. The antitumor property of Withaferin A and its molecular mechanism of action on hepatocellular carcinoma (HCC) cells is not yet completely established. In this study, we aimed to elucidate the novel molecular function of Withaferin A on HCC cells and its effect on various gene expression. Our results clearly showed that Withaferin A treatment to HCC cells inhibited proliferation, migration, invasion, and anchorage-independent growth. Further, we explored the Withaferin A target genes by blotting human angiogenesis, and cytokine arrays using conditioned media of Withaferin A treated QGY-7703 cells. We found that many of Nuclear factor kappa B (NF-κB), angiogenesis and inflammation associated proteins secretion is downregulated upon Withaferin A treatment. Interestingly, all these genes expression is also negatively regulated by nuclear receptor Liver X receptor-α (LXR-α). Here, we explored a novel mechanism that Withaferin-A activated LXR-α inhibits NF-κB transcriptional activity and suppressed the proliferation, migration, invasion, and anchorage-independent growth of these HCC cells. All these data strongly confirmed that Withaferin A is a potent anticancer compound and suppresses various angiogenesis and inflammatory markers which are associated with the development and progression of HCC. This beneficial and potential therapeutic property of Withaferin A will be very useful for the treatment of HCC.

Keywords: Withaferin A, hepatocellular carcinoma, migration, invasion, angiogenesis, proliferation

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INTRODUCTION

Hepatocellular Carcinoma is one of the menacing and most common types of primary liver cancers and it is the third most leading cause of cancer-related deaths across the globe (1, 2). Commonly known HCC causes include Hepatitis B Virus, Hepatitis C Virus, exposure to dietary and environmental toxins, and carcinogens such as Aflatoxins and aristolochic acid, also chronic and excess alcoholism. Recently, due to lifestyle modifications, lack of physical activity or exercise is

leading to obesity, type 2 diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD) associated HCC (3). HCC has a direct link with excess intake of high calorie diet, dyslipidemia, insulin resistance, endoplasmic reticulum stress, oxidative stress, and adiposity (4). There are various signaling pathways associated with the initiation, development, and progression of hepatocellular carcinoma (5). Some of these signaling pathways are involved in proliferation, invasion, migration, anchorage-independent growth, and resistance to apoptotic stimuli (6). Targeting these pathways with suitable and specific drugs to treat HCC is the urgent need of the hour.

Angiogenesis is one of the important hallmarks of all types of cancer and is also involved in growth, development, and metastasis of HCC (7). There are many angiogenic factors involved in this HCC associated tumor angiogenesis (8). Along with these angiogenic factors many inflammatory cytokines are also known to play a major role in this disease progression (9). It is also known that many natural compounds have exhibited their inhibitory effect on the secretion of angiogenic factors and inflammatory cytokines in various types of cancers including HCC (10, 11).

Withaferin A, a natural steroidal lactone and dietary phytochemical from Indian medicinal plant Ashwagandha (*Withania Somnifera*) are very well studied for its antiangiogenic potential and anti-inflammatory properties (12). Withaferin A inhibits NF-κB, Specificity protein 1 (Sp1) transcription factors, and downregulates Vascular Endothelial Growth Factor (VEGF) gene expression (13, 14). It also acts as a ligand for nuclear receptor LXR-α and activates and regulates LXR-α mediated metabolic functions (15, 16). A recent study showed the leptin sensitizing property of Withaferin-A with strong antidiabetic properties on diet induced obesity mice (17). All these studies have demonstrated the anti-metabolic syndrome effect of Withaferin A (18, 19). However, the exact molecular mechanism behind its role in the inhibition of important hallmarks of hepatocellular carcinoma is not well established and is yet to be explored.

To explore and elucidate the molecular mechanism of action of Withaferin A on HCC cells, we examined the effect of Withaferin A on proliferation, anchorage-independent growth ability, migration, invasion using HCC cells. Here, we established a very strong link between angiogenic factors and inflammatory cytokines secretion and their role in controlling cancer hallmarks upon Withaferin A treatment. We found that Withaferin A modulates the secretion of angiogenic factors and inflammatory cytokines and also inhibits proliferation, migration, invasion, and anchorage-independent growth of these cells through the activation of LXR-α and LXR-α mediated suppression of NF-κB transcription factor. Based on all these beneficial effects along with the multifaceted function of this wonder compound (19), it can also be used as a therapeutic drug in the treatment of hepatocellular carcinoma.

MATERIALS AND METHODS

Cell Culture

HepG2 cells, Hep3B cells, Huh-7 cells, QGY-7703 cells, which are very well studied human hepatoma and hepatocellular carcinoma cell lines are used in this study. HepG2 and Hep3B were obtained from the American Type Culture Collection, Manassas, VA, USA. Huh-7 and QGY-7703 cells were a kind gift from Dr. Devanand Sarkar, Virginia Commonwealth University, Richmond, VA, USA. HepG2 cells, Huh-7 cells, QGY-7703 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum supplemented with 10% FBS and 1% penicillin/streptomycin, and Hep3B cells were grown in MEM alpha with 10% FBS, 5% Sodium Pyruvate, 5% Non-essential amino acids, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 18% O_2. When cells reached 80–90% confluence of growth, they were trypsinized and seeded in different culture plates or flasks based on our experimental needs.

Proliferation Assay

Cell proliferation was evaluated by Water Soluble Tetrazolium-1 (WST-1) Cell Proliferation Assay System (Roche Diagnostics, Rotkreuz, Switzerland). HCC cells were seeded in 96-well plates at 5×10^3 cells per well and treated with Withaferin A (5 μM) at 37°C under 5% CO_2 for 24 h. At the end of the 24 h period, 10 μl premixed WST-1 reagent was added to each well, and the plates were incubated further for 2 h at 37°C under 5% CO_2. Thereafter, absorbance was measured at 450 nm using a Turner-Biosystems microplate reader.

Colony Formation Assay

Colony formation assay was carried out using Huh-7 and QGY-7703 cells. The cells were seeded in 6 cm dishes at a density of 500 cells per plate and treated with Withaferin A (5 μM) and cultured for about 14–16 days until the colonies were visible. The cells were fixed in formaldehyde for 20 mins and washed with running tap water and stained with 10% Giemsa (Sigma-Aldrich, St. Louis, MO, USA). After rinsing and washing with running tap water, the plates were air dried, visualized under the microscope, and photographed. The images were analyzed using NIH ImageJ software and colonies counted and numbers showed in the bar graph.

Wound Healing Assay

Wound healing assay was carried out using Huh-7 and QGY-7703 cells (2×10^5 cells/3 ml). The cells were seeded in a six-well plate and incubated at 37°C until cells were 90% confluent. A scratch was made using a 100–200 ml pipette tip, followed by washing with PBS to remove cell debris, and then treated with 5 μM Withaferin A in a complete medium. After 24 h of incubation, the cells were observed under a light microscope and randomly chosen fields were photographed at 20× objective. The percentage of Huh-7 and QGY-7703 cells migrated into the scratched area was calculated using ImageJ software.

Transwell Invasion Assay

Transwell Invasion assay was conducted using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA) as suggested in the manufacturer's instructions. Pre warmed serum free media was added to the bottom side of the transwell as well as the upper chamber above the matrigel for 2 h

at room temperature for rehydration. Huh-7 and QGY-7703 cells (5×10^4 cells) were seeded in the upper chamber in serum free medium (with or without 5μM Withaferin A) while the wells of the lower chamber were filled with complete medium (5% FBS). After 22 h of incubation at 37°C and 5% CO_2, the cells on the upper surface of the transwell filters were removed by gentle wiping with a cotton swab and the cells attached on the lower surface of the filters were fixed and stained with Diff-Quick stain (IMEB Inc., San Marcos, CA, USA). After staining the invaded cells on the transwell filter were photographed using a microscope and invasion was determined by counting the cells using ImageJ software (6).

Soft Agar Colony Formation Assay

Anchorage-independent growth ability of HCC cells was measured by conducting soft agar colony formation assay using highly aggressive QGY-7703 cells. These cells were pretreated with vehicle control and Withaferin A for 4 h and cells were trypsinized, counted, and seeded at 10^5 cells/plate in 6 cm dishes with culture media containing 0.4% noble agar (Sigma-Aldrich, St. Louis, MO, USA) over a 0.8% agar base layer at 37°C with 5% CO_2 for 15 days. The colonies formed were counted manually under the microscope and photographed.

Human Angiogenesis and Cytokine Arrays

Human Angiogenesis and Cytokine Arrays were carried out to measure the secreted angiogenic and cytokine markers. The QGY-7703 cells were cultured up to 70% confluence and Withaferin A was treated for 24 h and the media was changed to serum free media for further 24 h. Supernatants of cells cultured in serum free media (conditioned media) were collected, centrifuged, cell debris was separated, and the only supernatant was used to check the expression and secretion of angiogenesis associated growth factors, cytokines, and other related molecules using commercially available human angiogenesis antibody array and Human Cytokine Array kit following the manufacturer's instructions sheets (R&D Systems, Minneapolis, MN, USA).

Quantitative Real-Time PCR

Total RNA was extracted from HepG2 cells treated with or without Withaferin A using TRIzol reagent (Thermos Scientific, Waltham, MA, USA). The experimental procedure was followed as described previously (6) and the primer sequences for the selected and validated LXR-α target genes are given in **Table 1**.

Statistical Analysis

All the data are presented as means ± SEM (n = 3). Statistical significance was analyzed using a two-tailed unpaired Student's t-test. GraphPad Prism software (version 6) was used for all statistical analyses and p values <0.05 were considered significant.

RESULTS

Withaferin A Inhibits Proliferation, Migration, and Invasion of HCC Cells

In this study, we explored the therapeutic potential of Withaferin A on proliferation, migration, and invasion of HCC cells. HCC cells (Hep3B, HepG2, Huh-7, and QGY-7703) were treated with various doses (1, 5, and 10 μM) of Withaferin A for 24 h. The results of the WST-1 cell proliferation assay conducted at the end of the treatment period, showed that Withaferin A significantly inhibited the proliferation of HCC cells (**Figure 1A**) and the images were photographed under the microscope after the treatment of 5 μM Withaferin A to these cells (**Figure 1B**). Further, we validated the effect of Withaferin A on the colony formation ability of these cells and the results showed that more than 50% inhibition of colony formation was observed in Withaferin A treated cells compared to control cells. Colony formation assay (**Figure 1D**) and Soft agar colony formation assay (**Figures 1C, E**). Next, we determined the effects of Withaferin A (2.5 μM) on migration and invasion of QGY-7703 and Huh-7 cells by employing scratch wound-healing assay and transwell invasion assay. As shown, both the assays demonstrated that Withaferin A attenuated the migration (**Figures 2A–C**) and invasion (**Figure 2D**) of QGY-7703 and Huh-7 cells.

Withaferin A Activates LXR-α and Inhibits NF-κB Signaling in QGY-7703 Cells

Here, we evaluated the effect of Withaferin A on the secretion of various angiogenesis markers and cytokines by QGY-7703 cells. Recently, few studies have shown that Withaferin A has LXR-α agonist property and it acts as a specific ligand for LXR-α (16–19). However, the significance of this property of Withaferin A and its molecular action is not studied in cancer cells. Withaferin A is also known for its anti-inflammatory properties *via* inhibiting the NF-κB transcription factor (20). LXR-α, a nuclear receptor family member is known to play a pivotal role in the various biological process which includes inflammation,

TABLE 1 | Primer sequences for the selected LXR-α target genes were used for validation after Withaferin A treatment to HepG2 cells.

SL	Gene	Primer sequence
1	hABCA1	5′-TTCCCGCATTATCTGGAAAGC-3′ (Forward primer) 5′-CAAGGTCCATTTCTTGGCTGT-3′ (Reverse primer)
2	hABCG1	5′-ATTCAGGGACCTTTCCTATTCGG-3′ (Forward primer) 5′-CTCACCACTATTGAACTTCCCG-3′ (Reverse primer)
3	hApoE	5′-GTTGCTGGTCACATTCCTGG-3′ (Forward primer) 5′-GCAGGTAATCCCAAAAGCGAC-3′ (Reverse primer)

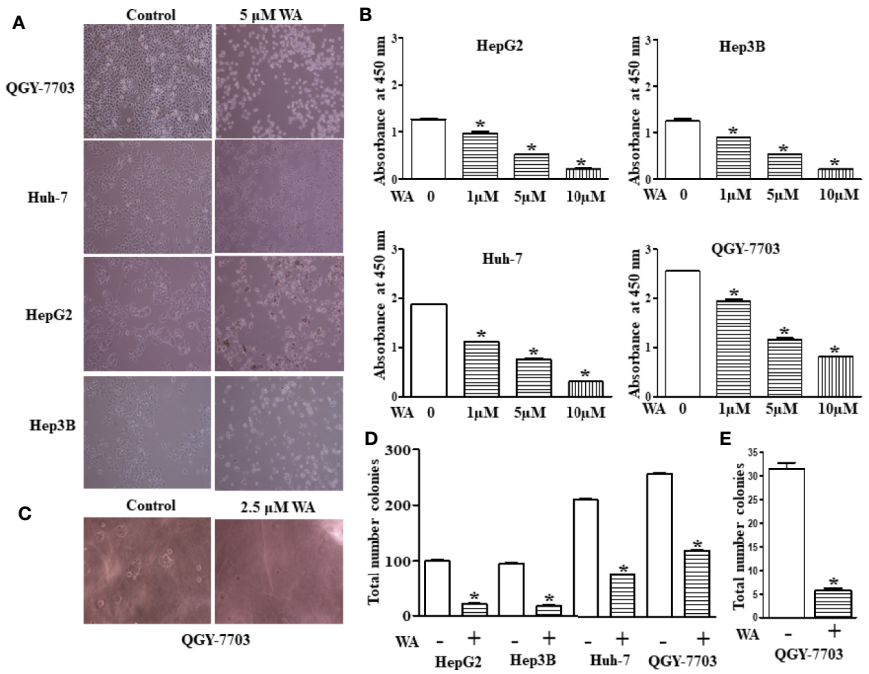


FIGURE 1 | Withaferin A inhibits proliferation of HCC cells. Cell death was induced in HCC cells by Withaferin A Cells (HepG2, Huh7, Hep3B, and QGY-7703 cells) were treated with/without Withaferin A (5 μM) for 24 h and then observed under inverted microscope (n = 3) **(A)**. Withaferin A suppressed the proliferation of HCC cells, absorbance was measured at 48 h (n = 3) **(B)**. Withaferin A (2.5 μM) inhibited the anchorage-independent growth of QGY-7703 cells (n = 3) **(C, E)** and colony formation ability (n = 3) **(D)**.

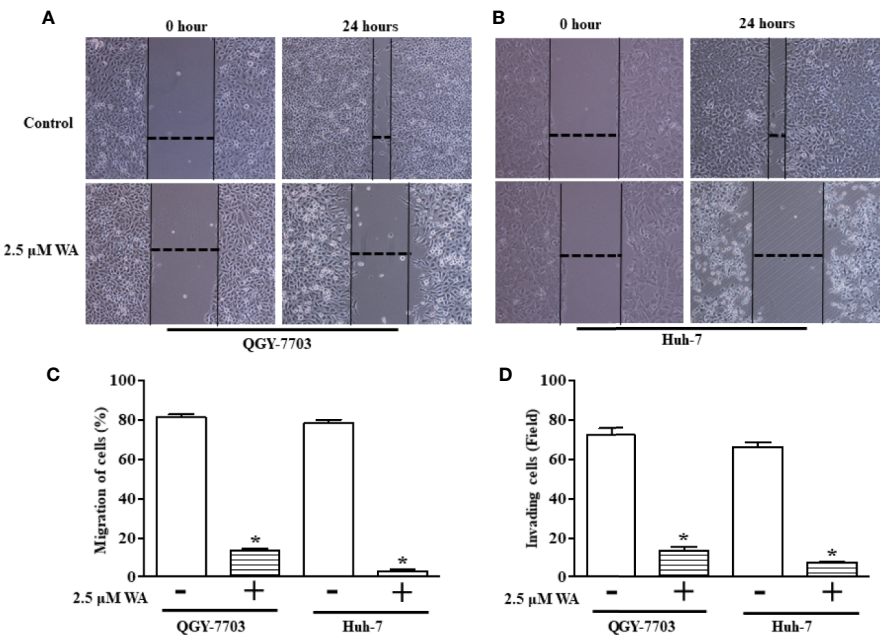


FIGURE 2 | Withaferin A inhibits migration and invasion of QGY-7703 and Huh-7 cells. Cells were treated with Withaferin A (2.5 μM) for 24 h and pictures were taken before and after the treatment and the migration distance was measured using ImageJ software and percentage inhibition was measured (n = 3) **(A–C)** and transwell invasion was measured by staining and counting the number of invaded QGY-7703 and Huh-7 cells (n = 3) **(D)**. *p value is less than 0.05.

cholesterol homeostasis, lipogenesis, cellular reprogramming, and decisions (16). Therefore, we focused our study on LXR-α/NF-κB signaling pathway, and the data supported our hypothesis. Withaferin A (2.5 μM) treatment decreased the secretion of various angiogenesis-related markers, growth factors, and cytokines (Serpin F1(PEDF), uPA, PDGF-AA, Angiogenin, Endothelin-1, Macrophage migration inhibitory factor (MIF), PAI-1, MCP1, ICAM-1 in QGY-7703 cells (**Figures 3A, B**). These factors are very well known for their pivotal role in proliferation, migration, invasion, angiogenesis, inflammation, and metastasis (21–23). It is also a known fact that NF-κB is a master regulator of various inflammatory signaling pathways (24). All these factors are directly or indirectly regulated by both NF-κB and LXR-α (25, 26). LXR-α is a negative regulator of NF-κB signaling and in this study activation of LXR-α by Withaferin A may downregulate the secretion of all these molecules *via* suppressing NF-κB activity.

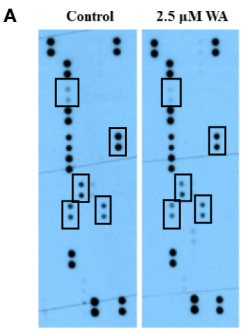
Withaferin A Induces LXR-α Target Genes in HepG2 Cells

Further, to confirm the agonistic role of Withaferin A we thought of validating some of the LXR-α target genes in HCC cells. Therefore, we treated HepG2 cells with Withaferin A (2.5 μM) for 4 h and isolated total RNA from these cells, and measured the expression of ATP-binding cassette sub-family A member 1 (ABCA1), ATP-binding cassette sub-family G member 1 (ABCG1), and Apolipoprotein E(ApoE). These three genes are commonly known LXR-α target genes and were found to be significantly increased in Withaferin A treated cells in comparison with vehicle controls cells (**Figure 4**).

DISCUSSION

Natural compounds are gaining increasing popularity in recent years as pharmaceutical drugs due to their pleiotropic effects and multifaceted beneficial properties (17, 27, 28). Dietary natural compounds are even more popular, and they lack toxic side effects, and also, they can be consumed very easily as a tonic or oral pill (29). In this study, we demonstrated the novel function of Withaferin A, a natural compound from the roots and leaves of Indian winter cherry, on the growth and aggressive behavior of HCC cells and their reprogramming *via* LXR-α activation (16, 17). Many previous studies have documented the medicinal properties of this miracle compound including anti-cancer activity (12, 14, 16). Withaferin A induces apoptosis by generating reactive oxygen species and down-regulating B-cell lymphoma 2 (Bcl-2) protein in human melanoma cells and breast cancer cells (30). Withaferin A suppressed human endothelial cells proliferation and tube forming ability (12, 14). It also upregulates the Nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor and protects from Acetaminophen-induced hepatotoxicity and liver injury (31). In this work, we showed that Withaferin A significantly inhibited hepatic cancer cell proliferation, migration, invasion, colony formation, and induced apoptosis as well as suppressed the secretion of angiogenic markers and inflammatory cytokines suggesting its beneficial effects on HCC cells.

Here, we tried to explore the molecular mechanism behind the inhibitory action of Withaferin-A on proliferation, migration, invasion, and anchorage-independent growth of HCC cells. The possible action of Withaferin A and its



Reference	Serpin B5	MIP-1α	GDNF	CF III	Reference
	Serpin E1	MMP-8	GM-CSF	CXCL16	
	Serpin F1	MMP-9	HB-EGF	DPPIV	Activin A
	TIMP-1	NRG1-β1	HGF	EGF	ADAMTS-1
	TIMP-4	PTX-3	IGFBP-1	EG-VEGF	ANG
	TSP-1	PD-ECGF	IGFBP-2	Endoglin	Ang-1
	TSP-2	PDGF-AA	IGFBP-3	Endostatin	Ang-2
	uPA	PDGF-BB	IL-1β	Endothelin-1	Angiostatin
	Vasohibin	Persephin	IL-8	FGF acidic	Amphiregulin
	VEGF	PF4	TGF-β1	FGF basic	Artemin
	VEGF-C	PIGF	Leptin	FGF-4	
NC		Prolactin	MCP-1	FGF-7	Reference

Reference				Reference
MIF	IL-13	IL-1 ra	CXCL11	CCL1
PAI-1	IL-16	IL-2	CXCL12	MCP1
TNF-α	IL-17A	IL-4	G-CSF	MIP-1α /β
TREM-1	IL-17E	IL-5	GM-CSF	CCL5
NC	IL-18	IL-6	ICAM-1	CD40 ligand
	IL-21	IL-8	IFN-γ	C5/C5a
	IL-27	IL-10	IL-1α	CXCL1
	IL-32α	IL-12p70	IL-1β	CXCL10
				Reference

FIGURE 3 | Withaferin A inhibited the secretion of angiogenesis factors and cytokines production in HCC cells. QGY-7703 cells were grown in serum free media for 24 h along with or without Withaferin A (2.5 μM). The conditioned media was used to detect the secretary angiogenic factors and cytokines (n = 3) **(A, B)**.

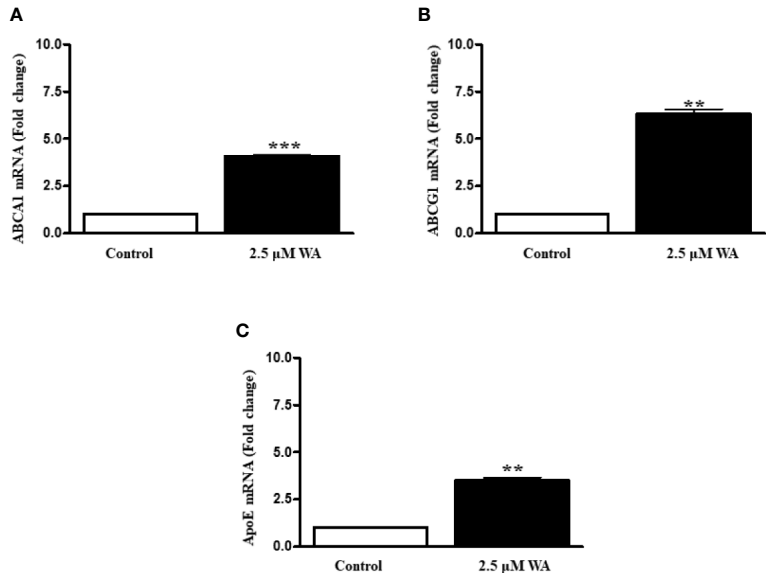


FIGURE 4 | Withaferin A activates LXR-α and induces its target genes. HepG2 cells were grown in regular media for 4 h along with or without Withaferin A (2.5 μM). The gene expression of ABCA1, ABCG1, and ApoE were measured (n = 3) **(A–C)**. **p value is less than 0.005 and ***p value is less than 0.001.

mechanism of inhibition may be by suppressing the NF-κB pathway. Inhibition of NF-κB by Withaferin A also suppressed the anchorage-independent growth, invasion, and migration (**Figures 1C, E** and **Figures 2A–D**).

Based on our angiogenesis and cytokine arrays data, we found that many LXR-α and NF-κB target genes secretion were downregulated. Some of the important angiogenic factors which are downregulated include Angiogenin, Serpin F1, or pigment epithelium-derived factor (PEDF), Platelet-Derived Growth Factor-AA (PDGF-AA), Endothelin-1, and Urokinase-type plasminogen activator (uPA). All these factors are known to be directly regulated by NF-κB signaling (32). LXR-α was known to inhibit the expression of Endothelin-1 and also suppresses the PDGF-induced proliferation and regulates uPA gene expression (33–35). Also, a previously reported study on gene regulation by LXR agonist treatment shows that synthetic LXR-ligands downregulates Angiogenin expression in the liver (36). Our Bioinformatics analysis using Champion ChiP Transcription Factor Search Portal of SA Biosciences database known as DECODE (DECipherment of DNA Elements) revealed that

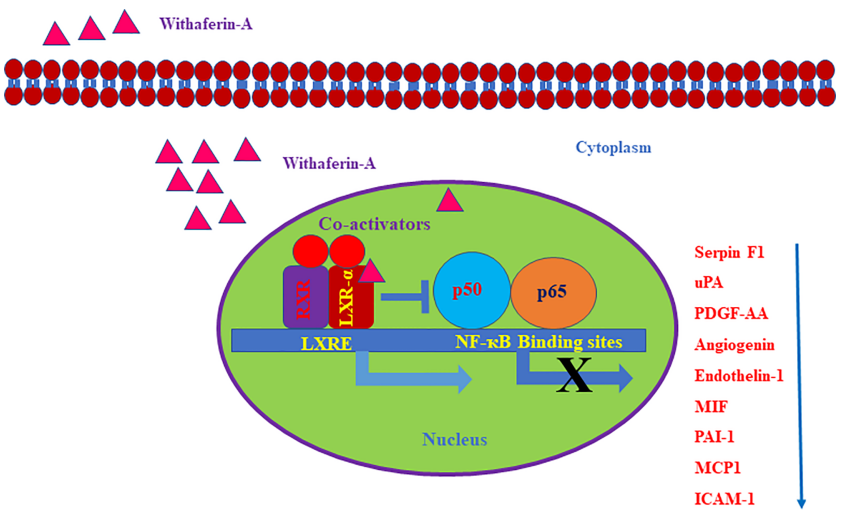


FIGURE 5 | Schematic representation of withaferin A mediated regulation of LXR-α and NF-κB signaling in HCC. The negative regulatory role of LXR-α on NF-κB activation and Withaferin A mediated expression of LXR-α target genes.

human Endothelin-1, Angiogenin, uPA, PDGFA, CCL2 (MCP-1), ICAM1(CD54), Serpin E1(PAI-1), and macrophage migration inhibitory factor (MIF) gene promoter regions have NF-κB binding sites. Many LXR-α agonists were also known for their effective inhibitory action on MCP-1, ICAM1, PAI-1, and other inflammatory markers (37). To confirm our experimental evidence, we further validated some of the LXR-α target genes and found that these target genes were significantly increased after Withaferin A treatment. Based on this strong and convincing evidence from our data and already known information from few reports on LXR-α and its negative regulatory role on NF-κB signaling (26), we are proposing the possible novel mechanistic model that Withaferin A may negatively regulate NF-κB transcription factor *via* activating LXR-α (**Figure 5**). There are many elegant studies, which support our evidence-based claim and have shown that activation of LXR-α results in suppression of HCC growth and development (38, 39).

In conclusion, Withaferin A inhibited the secretion of various angiogenic factors and cytokines secreted from human HCC cells. In this study, we also showed that Withaferin A inhibited principal hallmarks of HCC cells, such as proliferation, invasion, migration, and anchorage independent growth. Our findings provide additional evidence that this well-known dietary phytochemical has a novel function and it can be used as a promising anticancer compound in the treatment of highly aggressive HCC.

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

VS and NS: Contributed experimentally to this article. DK and FM: Contributed intellectually to this article. PS: Contributed experimentally and intellectually and wrote this article. All authors contributed to the article and approved the submitted version.

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REVIEW
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"Complimenting the Complement": Mechanistic Insights and Opportunities for Therapeutics in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and a leading cause of death in the US and worldwide. HCC remains a global health problem and is highly aggressive with unfavorable prognosis. Even with surgical interventions and newer medical treatment regimens, patients with HCC have poor survival rates. These limited therapeutic strategies and mechanistic understandings of HCC immunopathogenesis urgently warrant non-palliative treatment measures. Irrespective of the multitude etiologies, the liver microenvironment in HCC is intricately associated with chronic necroinflammation, progressive fibrosis, and cirrhosis as precedent events along with dysregulated innate and adaptive immune responses. Central to these immunological networks is the complement cascade (CC), a fundamental defense system inherent to the liver which tightly regulates humoral and cellular responses to noxious stimuli. Importantly, the liver is the primary source for biosynthesis of >80% of complement components and expresses a variety of complement receptors. Recent studies implicate the complement system in liver inflammation, abnormal regenerative responses, fibrosis, carcinogenesis, and development of HCC. Although complement activation differentially promotes immunosuppressive, stimulant, and angiogenic microenvironments conducive to HCC development, it remains under-investigated. Here, we review derangement of specific complement proteins in HCC in the context of altered complement regulatory factors, immune-activating components, and their implications in disease pathogenesis. We also summarize how complement molecules regulate cancer stem cells (CSCs), interact with complement-coagulation cascades, and provide therapeutic opportunities for targeted intervention in HCC.

Keywords: hepatocellular carcinoma, HCC and COVID-19, complement activation, complement proteins, prognostic markers, inflammatory factors, complement-based therapeutics, immunotherapy

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HCC: INCIDENCE, ETIOLOGY, AND TREATMENT

Hepatocellular Carcinoma (HCC) is the major form of primary malignancy of the liver, derived mostly from hepatocytes in more than 80% of the cases. HCC ranks as the fifth most common cancer in men and the seventh in women, representing a third of all cancer-related deaths (1) and centralizing mostly in developing countries. Globally, the incidence of HCC continues to rise, with rates increasing from 2.7/100,000 in 1997 to 8.8/100,000 in 2016 in men and from 0.8/100,000 to 2.2/100,000 in women. HCC is associated with unfavorable trends in North America, Northern and Central Europe, and Latin America. Development of HCC with enhanced tumor burden is highly prevalent in patients with liver cirrhosis as the single-most important etiology (2, 3). While HCC uniformly results in high mortality, the etiology and epidemiology differ widely in their geographical distributions. In western countries, including USA, and in Japan, chronic hepatitis C virus (HCV) infection is the primary risk factor (4, 5) and hepatitis B virus (HBV) infection is more prevalent in Southeast Asia, China, and sub-Saharan Africa (6). Since liver cirrhosis underpins the fundamental cause of HCC, patients with chronic liver diseases and a predisposition to cirrhosis are at substantial risk (7). However, contributions from nonalcoholic steatohepatitis (NASH), diabetes mellitus, obesity, and autoimmune and cholestatic diseases as predisposing factors in the onset of HCC are relatively minor (8). In contrast, an alarming rise in HCV, alcohol-related, and post-NASH HCC has been found in the United States, Canada, areas of Europe, Australia, and New Zealand (9, 10).

As an aggressive disease typified frequently by late diagnosis, the prognosis for HCC remains very poor (7), with median survival following diagnosis ranging from 6 to 20 months (11) and a 5-year relative survival rate of 18.4%. Cirrhosis and portal vein occlusion define the length of survival and severely limit therapeutic options, resulting in liver failure, tumor progression, and death. The existence of underlying advanced chronic liver disease, tumor stage, and portal hypertension in most of patients with HCC dictates and complicates the adoption of treatment strategies and prognosis. Treatment options including medical and transplantation for large non-resectable HCC patients, unfortunately, share high tumor recurrence rates due to persistent cirrhosis that confers a preneoplastic environment (12). The only curative treatment strategies involve orthotopic liver transplantation (OLT) and surgical liver resections (LR). OLT, however, is limited by organ shortage, resulting in increased utilization of extended-criteria donor (ECD) allografts (13). Other surgical interventions include but are not limited to the locoregional tumor ablation therapies including TACE (14), trans-arterial radioembolization with Yttrium-90 (Y-90) (15), stereotactic body radiotherapy (SBRT) (16), percutaneous ethanol injection (PEI) (17), high-intensity focused ultrasound (HIFU) (18), irreversible electroporation (IRE) (19), and radiofrequency-, microwave-, and cryo-ablations (19, 20). While surgical therapy remains the mainstay of treating HCC, systemic treatments targeting the molecular signaling pathways are frequently implemented for patients with unresectable and/or advanced HCC. Taking advantage of the molecular signaling pathways, systemic therapies involving Sorafenib (21), Lenvatinib (22), Nivolumab (23), Regorafenib (24), and Cabozantinib (25) have shown survival benefits in HCC patient cohorts. However, the surgical, systemic, and locoregional therapies currently advocated and in practice for treating HCC are associated with several adverse events summarized in **Table 1**. The ability to systemically treat, albeit partially, the highly chemotherapy resistant HCC tumors and increased understanding of disease pathogenesis are expected to pave way for future therapeutics.

Since systemic therapies target proliferative and angiogenic pathways involving tyrosine kinases, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR)-β tyrosine kinases, fibrosarcoma kinases, etc (26), and HCC is characterized as an immunogenic cancer, greater opportunities can be envisaged for specific and more effective treatment strategies. In general, cancer-associated inflammation, present at different stages of tumorigenesis, contributes to genomic instability, stimulation of angiogenesis, epigenetic modifications, aggressive cancer cell proliferation, enhanced anti-apoptotic pathways, and cancer dissemination (27). Studies in the last two decades have implicated inflammatory pathways in cancer with emphasis on understanding how immune cells impact tumor fate in different stages of disease: early neoplastic transformation, clinically detected tumors, metastatic dissemination, and therapeutic intervention. Despite the significant advances in our understandings of the immunological basis of cancer (28), the immunopathogenesis of HCC remains underexplored.

IMMUNOPATHOGENESIS OF HCC

While the liver is highly tolerogenic and prevents hostile immune responses, organ homeostasis is maintained by natural killer (NK), natural killer T (NKT) cells, γδT cells, Kupffer cells (KCs), etc (29). However, breakdown of this tight regulation by virus infection, alcohol abuse, and lipid accumulation results in chronic inflammation and destruction of hepatocyte and cholangiocyte epithelial cells, leading to cirrhosis (30). Inflammation-associated cellular proliferation, genomic DNA mutations, and reactive oxygen species (ROS) production further enhance malignant transformation (31). In this environment, cancer cells evade immune surveillance and are associated with increased tumor infiltration by immune cells and, amplification of pro-tumorigenic cytokines, etc (32). Several cell-death pathways linked to TNFα, IL-6, NF-κB, STAT3, and JNK, and innate and adaptive immunity are activated in HCC, attesting to the dominant roles of immune mechanisms in hepatocarcinogenesis (30, 33). In particular, innate immune responses involving NK, NKT cells, dendritic cells (DCs), tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid derived suppressor cells

TABLE 1 | Adverse events associated with systemic and surgical hepatocellular carcinoma (HCC) treatment approaches.

Therapeutic approach	Target	Adverse events
SYSTEMIC TREATMENT: **1) Tyrosine Kinase Inhibitors:** Sorafenib, Lenvatinib, Regorafenib, Cabozantinib, Imatinib	PDGFR, FLT3 VEGFR 1-3, FGFR 1-4, PDGFA, Tyrosine kinase receptor, VEGF, MET, AXT, RET, TYRO3, MER-ABL, BCR-ABL, c-KIT	Diarrhea, hand-foot syndrome, hypertension, decreased appetite and weight loss, fatigue, hypothyroidism.
2) Immune-Checkpoint Inhibitors: Nivolumab, Pembrolizumab, Durvalumab, Atezolizumab, Tremelimumab, Iplimumab	PD-1 Ab PD-L1 Ab CTLA-4 Ab	Skin rash, fatigue, diarrhea, pruritis, decreased appetite and weight loss, joints and musculoskeletal pain, constipation, dyspnea.
SURGICAL TREATMENT: **3) Surgical approach:** Liver resection Liver Transplantation	Surgical resection of liver tumor and/or liver transplantation	Pain, fatigue, hypovolemic shock, risk of intrahepatic recurrence, high mortality rate, hepatic failure.
4) Locoregional therapy: Radiofrequency Ablation (RFA) Transarterial chemoembolization (TACE) Percutaneous ethanol injection (PEI) Radioembolization Cryotherapy	Tumor targeted therapies	Liver failure, thoracic complications, bile duct injury, intraperitoneal bleeding, hepatic abscess, gastrointestinal perforation, tumor seeding, nausea, pain, fever, loss of appetite, hair loss, low white blood cells and platelets counts, ascites, obstructive jaundice, portal hypertension, radiation pneumonitis, hepatic dysfunction, vascular injury, hematoma, hepatic decompensation.

(MDSCs), regulatory T cells (Tregs), and cytokines/chemokines derived from these cells form the first-line events in either dampening or promoting tumor initiation and progression within the tumor microenvironment (TME) (34).

In HCC, NK cells are activated by NKT, DC, and KCs, and suppressed by Tregs and hepatic stellate cells (HSCs) (35). However, NK cell numbers are reduced in HCC lesions, with reduced levels of IFNγ and cytotoxic potentials (36), possibly due to hypoxic stress and/or transitory behavior of activating/inhibitory NK receptors. In addition, α-fetoprotein (AFP), MDSCs, and TAMs dampen activating NKG2D receptors and block NK cell cytotoxicities (37). The role of NKT cells, however, remains less understood, with Th2 cytokine-producing tumor-promoting and anti-tumor $CD4^+$ NKT cells that accumulate in the TME. Another important component of innate immunity involves DCs that serve as professional antigen-presenting cells (APCs), priming T cells against tumor associated antigens (TAAs) in HCC. However, DCs in patients with HCC remain refractory to high inflammatory cytokine maturation stimuli and show defective antigen presentations due to decreased HLA class-I expressions and a weakened T cell response (38). Furthermore, the frequencies of activated $CD83^+$ DCs are lower in HCC livers and absent in tumor nodules, denoting impaired cytotoxic responses.

In parallel with the DC phenotypes, the alternatively activated $CD163^+$ M2 TAMs promote tumor initiation, progression, and metastatic malignancy, and are considered as negative prognostic markers associated with low survival rates (39). In HCC, this M2 polarization is sustained by high levels of colony stimulating factor-1 (CSF-1) and reduced innate and adaptive immunity *via* IL4 (40). TAM-derived IL-10 and interactions with MDSCs result in decreased IL-6, IL-12, and MHCII, and increased anti-inflammatory IL-10, TGF-β1, and $Foxp3^+$ Treg frequencies to facilitate tumor growth and immune tolerance (41, 42). Similar to TAMs, the recently described TANs recruit macrophages and Tregs to the TME, promoting tumorigenesis and resistance to sorafenib in preclinical studies (43). In patients with HCC, $CD66B^+$ neutrophils colocalized with CCL2 and CCL17, infiltrating the liver stroma (44). In experimental models, TANs secrete BMP2 and TGF-β2, trigger miR-301-3p expression in HCC cells, suppress LSAMP and CYLD expressions, and enhance HCC stemness (44). In patient specimens, increased TANs were associated with increased CXCL5 expression and miR-301b-3p levels, decreased LSAMP and CYLD expressions, and nuclear p65 accumulation, collectively contributing to immunosuppression and HCC patient prognosis (45).

The immunosuppressive TME is further elevated by MDSCs, a heterogeneous inhibitory cell population with increased arginase-1, nitric oxide, ROS, and TGF-β activities that promote induction of Tregs (46). While $CD14^+$/HLA-$DR^{-/low}$ MDSCs populate HCC livers and block T-cell responses, circulating MDSCs have been negatively correlated with reduced HCC recurrence-free survival (47). Furthermore, MDSCs in the TME suppress IFN-γ production by NKT cells, express Galectin-9 to interact with and induce T-cell apoptosis, and inhibit NK cell cytotoxicity *via* interactions with Nkp30 receptor (48, 49). In HCC, increased intratumoral Treg activity is always associated with defective anti-tumor responses and poor prognosis. Higher frequencies of Tregs were found to be intricately associated with lower $CD8^+$ T cell responses, absent tumor encapsulation, and increased tumor vascular invasion (50). A concerted interaction between Amphiregulin (AR)-expressing HCC cells and Tregs triggered mTORC1 expression in Tregs, suppressing $CD8^+$ T cell mediated anti-tumor responses. Similarly, inhibiting mTORC1 *via* rapamycin or blocking AR/EGFR signaling using Gefitinib enhanced anti-tumor $CD8^+$ T-cell functions, highlighting the importance of Treg-driven processes in HCC TME (51). Similarly, increased accumulation of Tregs in HCC tumors correlated with reduced $CD8^+$ T-cell infiltrations and reduced Granzyme A, Granzyme B, and Perforin expressions. Importantly, these events are associated with significantly reduced survival times and increased mortality of HCC patients. Such intratumoral inverse correlations of Tregs and $CD8^+$ T-cells also contribute to the prognostic value of HCC patients by facilitating angiogenesis and

substantially modulating anti-tumor $CD8^+$ T-cell functions (52). An immunosuppressive functional role has also been identified for IL-35 in HCC, a cytokine expressed primarily by $Foxp3^+$ Tregs. IL-35 induces conversion of naïve T cells and B cells into Tregs and Bregs, respectively, and is involved in negative regulation of autoimmune diseases (53, 54). Patients with elevated IL-35 were at a higher risk of postoperative recurrence after curative HCC resection and correlated with increased infiltration of a new $CD39^+Foxp3^+$ Treg subset (55). Meta-analysis of 23 studies with a total of 1,279 patients with HCC and 547 healthy controls revealed that a) the frequency of circulating Tregs was 87% higher than in healthy controls and b) intratumoral Treg levels were higher than the surrounding tissue and healthy controls (56).

CYTOKINES AND CHEMOKINES IN HCC

In conjunction with the suppressive functions and escape mechanisms of the immune-cell compartments, several proinflammatory and immunomodulatory Th1 and Th2 cytokines and chemokines define the outcomes of tumorigenesis in HCC (57, 58). The sustained and permissive cytokine and chemokine synthesis in the TME promotes a maladaptive immune response, amplifying dysplastic cellular responses. Immune and epithelial cells within the hepatobiliary system elaborate a range of cytokines with simultaneous expression of receptors.

PRO- AND ANTI-INFLAMMATORY CYTOKINES

In patients with cirrhotic livers, high levels of Kupffer cell derived IL-6 are associated with poor disease prognosis (59). TAMs also utilize IL-6/STAT3 axis to promote expansion of liver cancer stem cells (CSCs) *via* autocrine IL-6 signaling (60). High levels of IL-4 and IL-5 in the TME are also associated with increased HCC metastasis and a polarized Th2 phenotype (61). IL-22, a member of the immunosuppressive IL-10 family, is also elevated in the TME, promoting HCC tumorigenesis, metastasis, and inhibition of apoptosis *via* activation of STAT3 (62). IL-10 itself is upregulated in HCC TME, defining risk of progression after tumor resection (63).

IL-1, IL-18, and IL-36, members of the IL-1 family of the cytokines, are pro-inflammatory and mostly associated with tumor growth. IL-1 induces synthesis of DC-derived CCL22 to recruit immunosuppressive Tregs and further enhance HCC. However, antitumor activity is shown by the presence of IL-36a in HCC, decreased levels of which predict poor prognosis and survival (64). Similarly, IL-37 inhibits HCC growth *via* $CD57^+$ NK cells (65), limiting G2/M cell cycle arrest and decreasing cell proliferation (66). The pro-inflammatory cytokines TNF-α and IL-1β are robustly involved in HCC tumor invasion, angiogenesis, and metastasis. IL-1β has been found to increase soluble MHC Class I Polypeptide-Related Sequence A (MICA) thereby blocking NK activity and enhancing HCC (67). TNF-α suppresses anti-tumor $CD8^+$ T-cell responses by upregulating macrophage cell surface expressions of the negative co-stimulatory molecules B7H1 or PDL1 (68). IL-1β, while promoting increased synthesis of IL-2, IL-6, and TNF-α, also acts as a tumor growth promoting molecule in conditions of chronic inflammation. TAM-derived IL-1β in the TME is known to drive metastatic potentials of HCC (69). A recent study showed that HCC patients with necrotic tumors harbored significantly higher levels of $CD68^+$ TAMs and were associated with elevated levels of serum IL-1β and poor prognosis. Importantly, areas with TAMs showed high expressions of IL-1 receptor, HIF-1α and Vimentin suggesting epithelial mesenchymal transition (EMT). In a Huh-7 xenograft mouse model, the authors showed that IL-1β-induced EMT was mediated through HIF-1α resulting in metastatic lesions (70). Higher levels of TNF-α and IL-1β are also found in tumor-independent areas of tissue metastases (71). In association with TNF-α and IL-1β, increased levels of IL-17A predict poor prognosis (72). IL-17A also induces EMT *via* AKT signaling, promotes invasion/metastasis and HCC cell colonization (73), and increases cell motility by upregulating MMP-2 and MMP-9 and activating NF-κβ (74). IL-17 acts directly on HCC cells, inducing AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression (75). In contrast, increased infiltration of $IL\text{-}33^+$ cells derived mostly from $CD8^+$ T-cells was associated with better prognosis in patients undergoing surgical resection (76). Key cytokines of the IL-2 family, including IL-2 and IL-15, potently stimulate lymphocyte activity and proliferation of cytotoxic T lymphocytes (CTLs) and NK cells. IL-2 enhances CTL activity and IFN-γ production and modulates HCC progression in mice (71). Similarly, an increase in Th1 IL-2 expression is associated with enhanced $CD8^+$ T-cell activity, increased IFN-γ production, and improved prognosis (77). IL-15, which positively upregulates proliferation and activation of NK, NK-T, and $CD8^+$ T-cells, corrects NK cell dysfunction (78), controls HCC tumorigenesis (79), and promotes tumor-specific $CD8^+$ T-cell responses (80).

This cytokine milieu not only regulates developmental and regenerative responses in the liver but also contributes to pathogenesis of hepatic cirrhosis, fibro-inflammation, and HCC. In particular, altered levels of proinflammatory IL-1α, IL-1β, IL-2, TNF-α, and Th2-like IL-4, IL-5, IL-8, and IL-10 cytokines have been associated with HCC phenotypes. In general, the cytokine milieu in HCC is skewed towards an anti-inflammatory over pro-inflammatory environment.

ROLE OF CHEMOKINES IN HCC

Aligned with the pro-tumorigenic roles of cytokines, the chemokines and their receptors promote extravasation of immune cells and migration along a chemotactic gradient towards areas of fibroinflammation. The most relevant chemokine-dependent immunoregulatory pathways in HCC include the CXCL12–CXCR4, CXCL5/8–CXCR2, CCL2–CCR2,

and CCL3/5–CCR1/5 axes. The CXCL12-CXCR4 axis represents the most extensively investigated system in HCC, which regulates angiogenesis and promotes tumorigenesis. In liver specimens from HCC patients, CXCL12–CXCR4 signals are more selectively localized to tumors than the adjacent normal or cirrhotic areas (81). In HCC cell lines, this chemokine axis promoted and enhanced the growth, invasion and metastatic potentials (82), and migration of tumor cells (83). Associations of the CXCL12–CXCR4 pathway in supporting metastasis and disease severity have also been demonstrated using HCC cell lines, showing increased MMP2 and MMP9 secretion (84) and decreased 3-year-survival rates in patients (85). Importantly, the CXCL12–CXCR4 axis interacts with MMP10 (86), further supporting tumor development, angiogenesis, and metastasis. The importance of MMPs in early invasion of HCC is further exemplified by the interactions of CXCL12 and CXCR4 with MMP2, MMP7, and MMP9. In this context, the CXCL12–CXCR4 axis provides avenues for development of novel therapeutics (87). In the aforementioned pathway, TGFβ interactions with CXCR4 shift HCC cells towards a mesenchymal phenotype (88) and increase invasiveness when treated with exogenous CXCL12 (89). High levels of CXCR4-expressing OV6+ tumor-initiating cells in HCC patient livers are associated with aggressive pathobiology, increased invasion, metastasis, and poor prognosis (90). Signaling pathways linked to EGF-EGFR in concert with CXCL5 regulate development of HCC (91), while the CXCL5-CXCR2 axis contributes to EMT of HCC cells *via* PI3K/Akt/GSK-3β/Snail signaling (92). CXCL5 also influences the development of an inflammatory TME by regulating the infiltration of MDSCs in HCC tumor sites *via* elaboration of IL-17A in γδ T cells (93). In conjunction with CXCL5, high serum levels of CXCL8 in HCC have been associated with increased tumor burden, aggressiveness, and poor patient prognosis (94). Additionally, epithelial cell derived CXCL8 chemoattracts peritumoral neutrophils and regulates disease progression by stimulating angiogenesis *via* secretion of MMP9 (95) and *via* VEGF–VEGFR2 axis in endothelial cells (96).

Similar to the CXC chemokines, the CC chemokines CCL2 and CCL5 interact with their receptors CCR2 and CCR1/5 respectively and are primarily involved in driving pro-tumorigenic and pro-fibrogenic responses. HSC, hepatocyte, macrophage, and endothelial cell derived CCL2 drives hepatic macrophage infiltrations (97) and provides pro-angiogenic signals *via* VEGF and MMP9 (98). Activation of CCL2–CCR2 also promotes migration, invasion, epithelial-mesenchymal transition, and metastasis of HCC *via* endothelial progenitor cells (99). Correspondingly, CCL5 promotes fibrogenic responses *via* resident Kupffer cells, bone marrow-derived macrophages, and HSCs (100) necessary for development of HCC. Investigations into other CCL chemokines CCL19, CCL20, and CCL21 showed specific upregulation of CCL20 in HCC tissues, together with increased expressions of the cognate receptor CCR6 (101). The authors demonstrated that CCL20-CCR6 axis regulates tumorigenicity in HCC, with increased CCL20 and CCR6 expressions in grade III tumors. Elevated expressions of CCR6 also correlate with formation of pseudopodia in HCC cell lines, increased metastasis, and poor survival in patients (102). Recent studies have also identified Fractalkine–CX3CR1 interactions in HCC cell cycle and CX3CL1 dependent cytotoxic T cell, IL-2, and IFN-γ responses that block tumor development (103).

In summary, the immune cells, soluble effector molecules and the chemokine receptors have been a subject of intense research and investigations as potential therapeutic targets to treat the chronic inflammatory states in HCC.

TARGETING IMMUNITY IN HCC: CURRENT STRATEGIES, LIMITATIONS, AND NEW MECHANISMS

As discussed above, the complex interplay of the immune cells with soluble effector molecules in chronic inflammatory states of HCC alters the immune system, either suppressing or facilitating tumor growth. Harnessing these multimodal mechanisms *via* immunotherapeutics is therefore expected to be beneficial in early and advanced stages of HCC. Utilizing the differential responses, systemic therapies to perturb VEGF-dependent angiogenesis, WNT, PI3K/AKT/mTOR, AMPK, and c-MET pathways in the TME are either approved or in clinical trials (104). However, Sorafenib, an oral tyrosine kinase inhibitor (TKI), remains the only FDA-approved treatment with survival benefits for HCC. It inhibits VEGFR, Raf-1, B-Raf, platelet-derived growth factor receptor (PDGFR), c-KIT receptor, and p38 signaling pathways involved in angiogenesis and tumor proliferation (105). Notwithstanding the survival benefits, therapeutic efficacy of Sorafenib is limited, with patients experiencing severe adverse effects and disease progression; prognosis is poor in patients discontinuing Sorafenib, with no additional available therapies (106). Similarly, Lenvatinib is a first-line TKI for unresectable HCC currently in clinical trials (107). Second-line therapies for advanced HCC that are intolerant to first-line treatment include Regorafenib, Cabozantinib, Sunitinib, Linifanib, Brivanib, Tivantinib, Donafenib, etc. which target tyrosine kinases, HGF-MET axis, and related pathways (108). Though many of these newer therapies show improved survival with robust and durable responses, development of drug resistance, severe adverse events, and cytostatic properties limit therapeutic benefits and patient acceptability. In this context, several indirect and direct immunotherapies that target adaptive and innate immune cells have been developed. The immune checkpoint inhibitors (ICIs) block T cell activation and promote T cell exhaustion by primarily targeting either CTLA-4 (cytotoxic T lymphocyte antigen-4; CD152) or PD-1/PD-L1, some currently approved or under clinical trials for HCC (109). Anti-PD-1 ICIs (Nivolumab, Pembrolizumab, Tislelizumab, Camrelizumab, Cemiplimab, Sintilimab) block the co-inhibitory receptor PD-1 on T cells, activating antitumor T cell responses, durable response, and improved survival. Alternatively, anti-PD-L1 ICIs (Durvalumab, Atezolizumab, Avelumab) target increased

PD-L1 expression on DCs, macrophages, T and B cells, and tumor and endothelial cells. Mono or combination therapies have demonstrated reasonable response rates, improving progression-free survival (110). Similarly, in an immunosuppressive environment, CTLA-4 inhibits T cell activation, promotes Treg differentiation, and deregulates antigen-presenting functions of DCs (111). The anti-CTLA-4 ICIs (Tremelimumab, Ipilimumab) enhance anti-tumor immunity by reducing Treg frequencies, increasing activation threshold, and preventing anergy of T cells (112). Adoptive Cell Therapies (ACTs) that form the other arm of direct immunotherapy target HCC *via ex vivo* genetic modifications of autologous immune cells (113).

It is known that HCC-associated inflammation contributes to genomic instability, epigenetic modification, induction of cancer cell proliferation, enhancement of anti-apoptotic pathways, stimulation of angiogenesis, and eventually, cancer dissemination (30). Since immune cells are an essential player of HCC-related inflammation, efforts have focused on understanding how these cells impact tumor fate in different stages of disease: early neoplastic transformation, clinically detected tumors, metastatic dissemination, and therapeutic intervention (28). The aforementioned approaches on modulating the immune environment to treat HCC demonstrate limited feasibility of available therapies and offer opportunities for mechanistic explorations and development of effective HCC treatment measures. Monotherapy with ICIs, ACT, etc. have largely failed to meet the primary clinical endpoints of antitumor responses and decreased tumor size (114). Development of resistance, heterogeneity of tumors, circumvention of inhibitory mechanisms that prevent anti-tumor responses, altered TME, hypervascularity, hypoxia, severe adverse events, potential transplant rejection, etc. further complicate the use of ICIs in effectively managing HCC (115). Furthermore, these strategies mostly temper a singular population of cells and oversimplify the complex and multifaceted immune responses in the TME. As the current immunotherapies rely mostly on modulating adaptive immune responses, deciphering novel mechanisms involving innate immunity can improve therapeutic efficacy and reduce HCC burden. Newer treatment protocols may therefore take advantage of combining novel therapeutic agents with existing first and second-line therapies. One such area of mechanistic investigation and approach garnering significant attention is modulation of the multiple components of the Complement cascade ("CC" or "C"). CC is a critical and integral arm of the innate immune response involving the Complement system (C) that not only enhances the effects of antibodies and eliminates cellular debris, foreign intruders, and dead cells but also tightly regulates liver injury, inflammation, and regenerative responses (116).

COMPLEMENT SYSTEM: REGULATION OF IMMUNE RESPONSES

The complement system is an integral part of the innate immune response with abilities to discriminate self from non-self, and rapidly eliminate invasive pathogens while causing minimal injury to the host (117). It is an intricate system with broader functions in immune surveillance and homeostasis, controlled through a balance of activating and regulatory proteins (118). Complement activation occurs *via* three major pathways: classical pathway (CP), lectin pathway (LP), and alternative pathway (AP), which merge into a common terminal pathway to activate C3. CP and LP are activated by antibodies and other pattern recognition molecules whereas AP is continually activated in plasma through a process called tick-over *via* continuous formation of C3b (119). Activation of C3 leads to formation of C5 convertase which cleaves component 5 (C5) into C5a and C5b. C5b then binds to C6 and C7 to form the C5b–C6–C7 complex. This complex interacts with C8 and C9 to form the membrane attack complex (MAC), resulting in antibody-mediated complement-dependent cytotoxicity (CDC) when inserted into a membrane. These activated proteins can then be deposited on cell surfaces or released into body fluids to interact with specific receptors, leading to lysis of foreign cells *via* cytoplasmic swelling and rupture of cell membranes, which are classical characteristics of necrosis (120). However, recent studies provide new perspectives on the immunosuppressive functions of complement components. Over the last decade, studies have demonstrated that these complement components could contribute to regulating the functions and tumor-suppressing immune responses (121). Recent findings suggest an insidious relationship between complement and cancer, tumorigenic competency of the complement system, cellular proliferation, and regeneration. Complement principally plays a protective role against tumor formation in humans (122) while also contributing to a large variety of divergent inflammatory and immune processes (123). Since HCC has underlying origins of chronic and ectopic inflammatory states, premature complement activation can be envisaged as a potential driver of onco-inflammatory processes. Indeed, altered or enhanced complement activation underlies a wide spectrum of inflammatory diseases including asthma (124), kidney and cardiac diseases (125, 126), multiple sclerosis (127), and rheumatoid arthritis (128). In addition, complement regulates several key biological processes including liver injury and regeneration (116), cellular proliferation (129), angiogenesis (130), epithelial mesenchymal transition (131), and metastasis (132). An overview of the etiopathogenic events in HCC triggering complement activation is shown in **Figure 1**.

COMPLEMENT PROTEINS IN HCC

Despite substantial research on the role of inflammatory cells and their immunosurveillance within the TME, little attention has been given to the tumor propagating properties of the complement cascade. Although increased levels of complement proteins in malignant tumors promote proliferative tumorigenesis, the exact role of complement in HCC remains unclear. The relevance of the complement system is further underscored by its ability to principally regulate the cellular

and molecular events in HCC including TAMs, TANs, Tregs, MDSCs, DCs, NK cells, and cytokine (IL-1, IL-2, TNF-α, IL-4, IL-10, etc.) and chemokine axes (CXCL12–CXCR4, CCL2–CCR2, etc). Complement activation therefore can promote HCC *via* enhanced angiogenesis, protection of tumor cells from immunosurveillance, increased mitogenic signaling, activation of anti-apoptotic mechanisms and aberrant cell proliferation, invasion, and migration (133). It is only recently that the complement proteins have garnered interests in cancer through immunosuppression and their roles in promoting HCC are being discovered. Complement activation has also been linked with the development and spread of several cancers, raising the possibility that impaired complement regulation could be a risk factor for oncogenesis (119). In this context, recent work by Mittal et al., discussed the ability of the immune system to act against tumor progression in an "immune-editing process" composed of three distinct phases: elimination, equilibrium, and escape (134). The authors showed that the immunological responses were able to prevent tumor progression in elimination & equilibrium phase whereas the acquired adaptations of malignant cells and the host immune system allowed for expansion of the tumor cell population during the escape phase. The complement system, an integral component of the antitumor immune response, acts as an

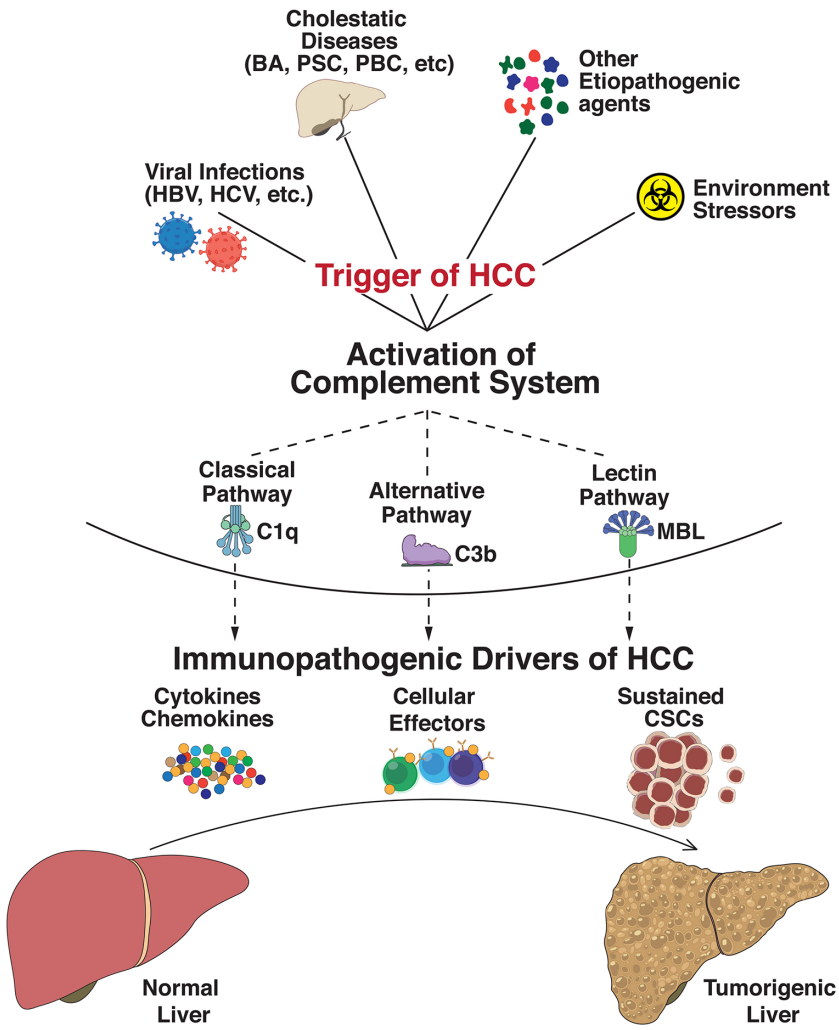

FIGURE 1 | Immunopathogenic complement activation regulates progression to hepatocellular carcinoma. Exposure of the hepatic milieu to several triggers linked either to viral infections (Hepatitis B virus, Hepatitis C virus, etc.), severe obstructive and cholestatic diseases (Biliary atresia, Primary Sclerosing Cholangitis, Primary Biliary Cirrhosis, etc.), environmental stressors/toxin exposures (polychlorinated biphenyls, arsenic, androgenic steroids, etc.), and other etiopathogenic agents (aflatoxins, oral contraceptives, vinyl chloride, etc.) dictate the evolution of hepatocellular carcinoma (HCC). These triggers activate the innate immune complement cascade *via* classical (involving C1q complex), alternative (C3b-dependent activation), or lectin (triggered by carbohydrates) pathways. Abnormal activation of these complement pathways modulates functional effects of intrahepatic immune and epithelial cell compartments and disseminates significant perturbation of effector innate and adaptive cells, cytokine and chemokine expressions, and sustained cancer stem cell (CSC) activities. The collective net result of these processes defines the progression of HCC tumorigenesis.

intrinsic effector mechanism to form a functional bridge between the innate and the adaptive immune system thereby promoting or suppressing tumor development. Complement activation within the liver may therefore contribute to the development of HCC by several mechanisms, for example, *via* activation of NF-κB in Kupffer cells and STAT3 in hepatocytes. While these events facilitate recovery of liver after acute injury, the sustained chronic activation promotes hepatocyte proliferation and development of HCC (135–137). **Figure 2** depicts the loss of homeostasis resulting in dysregulated complement activation, immune responses and biological processes promoting amplified hepatic oncogenic responses in HCC. In the following section, the roles of several components of the complement system in the etiology, pathogenesis, and therapeutic modulation of HCC are discussed.

Complement Factor H

CFH is a soluble complement protein expressed constitutively in the liver (138) by epithelial (139) and endothelial (140) cells, platelets (141), etc. CFH regulates the activation of AP by accelerating the decay of AP C3 convertase and inactivating C3b (142). A recent study using CFH-deficient mice demonstrated the importance of CFH in controlling hepatobiliary complement activation, absence of which resulted in chronic inflammation and development of HCC (119). CFH-deficient mice showed extensive complement activation and hepatocellular inflammation as early as 3 months of age and developed liver steatosis and chronic hepatic injury followed by HCC in >50% of mice by 15 months of age, confirming the role of activated AP in HCC (119). The tumor-suppressive effects of CFH in liver carcinogenesis were further confirmed by analyzing gene expression and methylation profiles in patients with HCC (143). Bioinformatic analysis by Laskowski et al., revealed that patients with mutations in the CFH gene were reported to have a median disease/progression-free time of less than a year compared to almost 2 years for those without mutations (119). In addition to rendering the hepatic environment susceptible to carcinogenesis, Seol et al. reported the co-operativity of CFH and complement

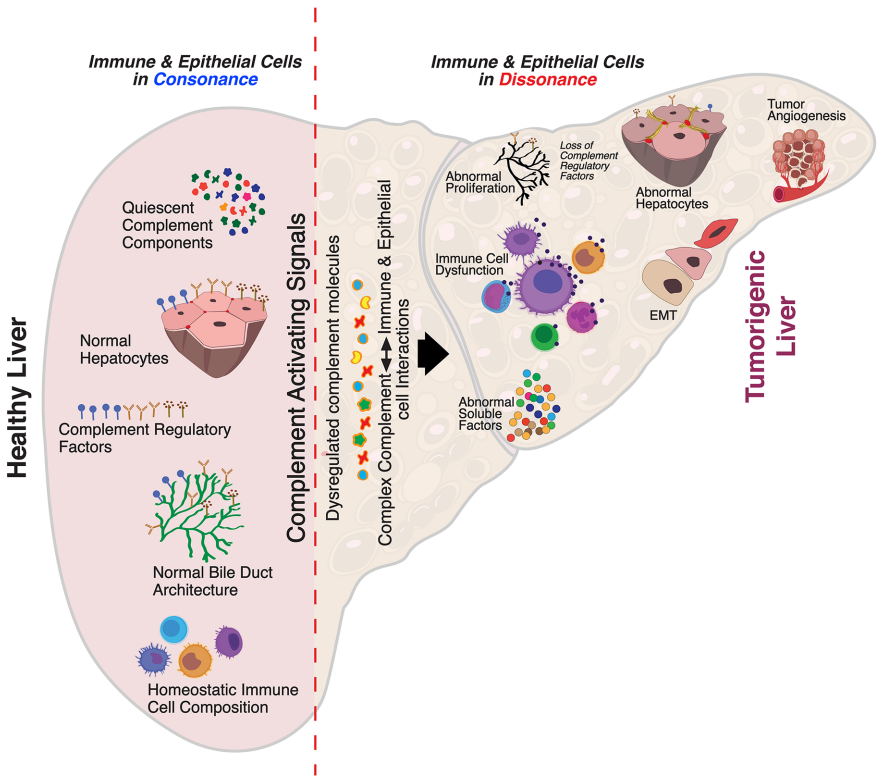

FIGURE 2 | Aberrant complement activation: a driver for disease progression in hepatocellular carcinoma. A schematic of the liver microenvironment depicting the transition of a healthy immunologically quiescent intrahepatic microenvironment to dysregulated immune status following activation cues to the complement system. In a healthy liver, immune and epithelial cells function in synergy to preserve normal architecture of bile ducts, quiescence of complement molecules, and homeostasis of immune cells. Complement activating disease-triggering signals orchestrate the evolution of dysregulated complement molecules (increased C3, C5, etc) and altered complement regulatory factors (CFH, etc). These acute and/or chronic sustenance of dysregulated complement molecules and their complex interaction with the immune and epithelial cell compartments drive the progression of hepatocellular carcinoma (HCC). Loss of complement regulatory factors and divergent activation of the complement system leads to abnormal hepatocyte architecture, deranged cellular and effector functions, and reactive bile duct profiles. Cumulatively, these events lead to epithelial to mesenchymal transition (EMT) and tumor angiogenesis which worsen the disease, resulting in poor clinical outcomes and death.

component C7 in maintaining the stemness and replication competency of tumor-initiating hepatocarcinoma cells (144). Using tumor-sphere cultures, the authors showed that absence of C7 and CFH abrogated tumor-sphere formation with restored stem cell proliferation in liver cells that overexpress these components. The ability of CFH and C7 to maintain cancer cell stemness was mediated through the induction of Late SV-40 factor (LSF-1) which plays a prominent oncogenic role in HCC and is overexpressed in >90% of patients with HCC (145). Inhibition of LSF significantly attenuated metastasis of HCC in nude mice while overexpression promoted aggressiveness and angiogenic and metastatic potentials of HCC tumors. It is important to note that LSF regulates a string of proteins involved in hepatocarcinogenesis, including osteopontin (OPN) (146). OPN sequesters CFH on the surface of the tumor cells and hinders the formation of membrane attack complex (MAC), effectively preventing complement-mediated lysis and enabling tumor cells to escape immune surveillance (146). Several recent studies have also investigated the role of Complement factor H-related 3 (CFHR3) in HCC, which until recently, remained unexplored. CFHR3, a member of the human factor H family, exhibited significantly lower mRNA and protein levels in HCC tumor tissue. Studies by Liu et al. showed overexpression of CFHR3 blocked cellular proliferation and viability, and enhanced apoptosis (147). In patients with HCC, differential expression levels of CFHR3 correlated with better prognosis (148). Gene enrichment analysis showed decreased CFHR3 expressions with pathways upregulated in tumorigenesis including regulation of cell activation cycle and WNT and NOTCH signaling pathways. Searching for novel prognostic biomarkers of HCC, Pan et al. identified a panel of 10 differentially expressed genes between cohorts of patients with high and low infiltrations of immune and stromal cells (149). The 10-gene signature predicted a favorable outcome of overall survival in HCC patients. The presence of CFHR3 in conjunction with other markers in the TME may therefore serve as a prognostic predictor for targeted therapeutics in HCC. Similarly, RNASeq data analysis of HCC patients identified 17 genes with significant effect on HCC prognosis (150). Of these, a set of seven genes that contained CFHR1 defined a clinical prognostic signature that predicts the survival of HCC patients. Collectively, these data point towards treatment options that enhance CFH/CFHR levels either by gene therapies or by CFH reconstitution to lower tumor burden in HCC.

CD59

CD59 is another mCRP that is involved in restricting initiation and progression of complement activation on cell surfaces. In general, downregulation of CD59 promotes the activation of complement-mediated cell lysis while increased expression can confer resistance to cancer cells (151). Low levels of CD59 are also linked to several autoimmune diseases including rheumatic diseases (152), autoimmune thrombocytopenia (153), diabetes (154), and multiple sclerosis (155). In some instances, increased expression of CD59 correlates with overall decreased survival rates in patients with colorectal cancer (156), prostate cancer (157), and B cell lymphoma (158) while low CD59 expression in breast tumors correlates with increased invasiveness and poor survival (159). Regardless of the spectrum of expression, extreme variations in the levels of CD59 result in pathologic outcomes of oncogenesis or cancer progression (160). Recent studies have linked CD59 to pathogenesis of HCC by prevention of complement mediated apoptosis. Abdel-Latif et al. showed increased mRNA and protein expressions of CD59 in a DEN-induced rat model of HCC that promoted enhanced tumor growth (161). In rats with HCC, increased levels of serum CD59 were not linked to phospholipase D (PLD)-dependent cleavage of CD59 (162) but rather to inflammation driven shedding from membrane lipid rafts (163, 164). Treatment with CoenzymeQ10 resulted in decreased CD59 and proinflammatory responses, providing protection against HCC. Using ChIP assays to study the role of the Hepatitis B virus (HBV) X protein (HBx) in HCC development, Shan et al. reported the upregulation of CD59 levels and protection from complement-dependent cytotoxicity (CDC) (165). siRNA mediated downregulation of CD59 sensitized the HBx-positive tumor cells and rendered them susceptible to CDC, suggesting new therapeutic avenues in HBV–HCC patients (165). Recent work by Lan et al. has shown that CD59 can function as a potential oncogenic driver in HCC and metastasis. Liver specimens from HCC patients showed high expressions of CD59 that correlated with poor overall and disease-free survival. Mechanistically, the authors showed that loss of CD59 impaired *in vitro* and *in vivo* tumorigenic and metastatic capacities *via* excessive Smad7 formation and abolishment of Smad2/3 phosphorylation. Therefore, CD59 facilitates HCC pathogenesis *via* suppression of CDC and modulation of TGF-β signaling; it may serve as an effective prognostic biomarker and potential therapeutic target in HCC (166).

C1q

C1q forms the recognition element of complement component C1 as a complex with the proteases C1r and C1s involved in activation of the classical pathway (167). The C1q complex is not only involved in recognition of complement activating elements, but also in regulation of autoimmune diseases (168) and in prostate cancer *via* the activation of tumor suppressor molecule WOX1 (WW-domain containing oxidoreductase) (169). Unlike other complement proteins, C1q is synthesized by several cell types relevant to the pathophysiology of oncogenesis, including epithelial and mesenchymal cells (170), monocytes/macrophages (171), dendritic cells (172), fibroblasts (173), and endothelial cells (174). In addition, the human hepatoma-derived cell line HepG2 secretes functional complement proteins C1r, C1s, C2, C3, C4, C5, etc. (175). Emerging data shows the involvement of C1q in progression and survival of cancer cells. Similar to requirements of increased expressions in preventing autoimmune diseases (176), C1q sustains WOX1 in blocking cell proliferation and hyperplasia in prostate cancer (169). C1q interacts with cell surface binding proteins cC1q-R and gC1q-R (177) that show divergent roles in cancer, with cC1q-R showing tumor suppressive activity (178) and gC1q-R promoting tumor cell progression and metastasis (179). Earlier studies have shown measurement of C1q-binding serum factors as a useful method

in monitoring tumor growth in experimental animals (180) and enhanced C1q inhibition activity in sera of patients with HCC (181). In a similar approach, C1q solid phase assays were used to detect hepatitis B surface antigen (HBsAg) in HBsAg+ and HBsAg– patients with primary HCC (182) and to detect increased levels of CICs in HCC patients (183). Similar to C1q, Yao et al. showed the ability of HCV core/gC1qR interactions to suppress T cell immune responses, resulting in persistent infection (184). Takeuchi et al. further showed that C1qTNF6 is overexpressed in HCC tissue specimens and contributes to tumor angiogenesis by activating Akt pathway (185). A direct functional role for C1q in the tumor microenvironment was demonstrated in wild-type mice that showed early C1q deposition, high vascular density, and increased lung metastasis compared to C1qa-deficient mice. Results showed that C1q directly regulates complement activation, cancer cell adhesion, migration, and proliferation (186). Recent seminal work by Lee et al. showed that the collagen-like portion of C1q induces activation and upregulation of discoidin domain receptor 1 (DDR1), a collagen receptor, resulting in enhanced migration and invasion of HepG2 cells. C1q induced activation of MAPKs and PI3K/Akt signaling, and increased MMP2 and MMP9 expressions, strongly suggesting C1q–DDR1 interactions in the progression of HCC (187). In this context, MMP2 and MMP9 have been shown to regulate the migrative and invasive capacities of HepG2 cells (188). Independent of these primary functions of C1q, pioneering work by Ho et al. showed that C1q released from macrophages provided an unconventional signal that activated the β–catenin pathway and induced expansion and de-differentiation of periportal hepatic progenitor cells (HPCs). Treatment with C1q inhibitors blocked the β–catenin pathway and expansion of liver tumors, identifying a hitherto unknown pathway of hepatocarcinogenesis (189). Recognition of these novel regulatory pathways for C1q is expected to further expedite mechanistic understandings and design of new approaches for HCC treatment.

Complement Component C2 (C2)

Complement C2 is an important component of the complement cascade necessary for the formation of C3 convertase, a serine protease significantly associated with HCC. Analogous to CFH, Ning et al. reported that higher expressions of C2 were associated significantly with better prognosis in HCC patients, implicating a protective role for C2. Investigations showed that C2 influenced HCC prognosis *via* several mechanisms, including higher levels of tumor infiltrating CD4+ T and M0 macrophage cells in HCC patients with higher and lower levels of C2, respectively (190). These findings are important considering the association of high mortality rates and reduced survival time in HCC patients with loss of $CD4^+$ cytotoxic T cells (191) and M2 polarization of TAMs that promote tumorigenesis, angiogenesis, and metastasis (40). The ability of C2 to suppress HCC and regulate multiple biological processes was supported by the identification of pathways linked to cell cycle, complement and coagulation cascades, AMPK, and PPAR signaling pathways in patients with elevated C2 expressions. The importance of C2 is further exemplified by associations of single nucleotide polymorphisms (SNP) with disease severity of HCC. While C2 SNP rs9267665 is associated with increased risk of developing HCC, the SNP rs10947223 affords protection from HCC (192) (193). Higher expressions of C2 are therefore beneficial for HCC prognosis and modulating complement C2 levels can afford novel therapeutic avenues.

Complement Component C3 (C3)

C3, the central component of the complement system is also activated in the milieu of oncogenic development. Under normal physiologic and homeostatic conditions, C3 is primarily produced by hepatocytes and restricted mostly to the extracellular space. Several lines of evidence have now confirmed that C3 is generated locally as well as intracellularly by almost all cell types including myeloid, lymphocytic, fibroblastic, and epithelial cells (194). Within the TME, C3 is produced either systematically by tumors (195), or by tumor infiltrating $CD8^+$ T cells (196). This tumor cell-derived C3 imparts an immunosuppressive TME by regulating the activity of TAMs *via* C3a–C3AR–PI3Kγ signaling and suppressing antitumor responses (197). Increased activation of intracellular C3 significantly suppressed anti-tumor activity of $CD8^+$ T cells, enhanced T-cell exhaustion, promoted an environment rich in immunosuppressive M2 macrophages, and provided resistance to cell lines against anti-PD-L1 treatment. However, blocking tumor cell derived complement C3 enhanced antitumor functions by enhancing the efficacy of anti–PD-L1 treatment, suggesting C3 in combination with ICIs as a potential target for HCC therapy. In HCC, hepatic stellate cells (HSCs) promote complement C3 mediated immunosuppression by restricting proliferation and enhancing T-cell apoptosis, decreasing DC maturation, and amplifying expansion of MDSCs (198). Blocking or modulating C3 functions may not only augment existing treatments, but also dampen cellular responses promoting fibrosis. Equally important is HSC-driven maturation of DCs into MDSCs, a function critically dependent on the presence of C3; complement C3-deficient HSCs, however, fail to induce MDSCs. This immunosuppressive function was linked to HSC derived factor B and factor D, resulting in C3 cleavage to iC3b and C3d; addition of iC3b also promoted differentiation of immunosuppressive MDSCs (199). In HCC, MDSCs promote angiogenesis and immunosuppression. Several clinical studies show the translational importance of MDSC activities (200), providing rationale for future studies that simultaneously target C3 and MDSCs. Furthermore, levels of immunosuppressive iNOS, Arg-1, and IL-4Ra were augmented in HSC-induced MDSCs *via* activation of the COX2–PGE_2–EP4 signaling pathway. Inhibition of PGE_2 blocked HCC growth by decreasing HSC-induced MDSC accumulation. Complement C3 and PGE_2 may also participate in M2 polarization of macrophages in the TME to enhance anti-inflammatory effects (201). The biological roles of C3, however, precede its identification in serum of patients with HCC of HCV origin by MALDI-TOF and complement component C4 as potential biomarkers (202). In addition, other studies identified the

diagnostic roles of serum complement C3a in HCC. Using proteomics analysis, Leung et al. identified lower levels of C3a C-terminal truncated fragment in HCC serum *via* SELDI technology, suggesting its value as a serum biomarker for HCC (203). Using a related technology of SELDI-TOF MS analysis, Lee et al. identified complement C3a to be specifically and differentially elevated in patients with chronic hepatitis C and HCV-related HCC (204). These findings were further corroborated by a recent study by Kanmura et al. who aimed to identify novel diagnostic markers for HCC using ProteinChip SELDI system (205). Results showed that a combination of complement C3a fragment, AFP, and des-gamma-carboxy prothrombin (DCP) resulted in 98% positive identification rate. These recent advances in complement-based diagnostic markers are of clinical significance since AFP is the only diagnostic marker indicative of HCC, albeit in about 60% of cases. Complement C3, therefore, plays a central role in biological functions and as a potential biomarker and therapeutic modality.

Complement Component C5 (C5)

Complement component C5 forms the terminal and an integral component of the complement cascade (206) and is expressed by and interacts with C5AR1 on several cells including lymphocytes, macrophages/monocytes, myeloid cells, hematopoietic stem cells, epithelial cells, and more importantly cells undergoing oncogenic transformations (207). In the context of cancer, the C5-C5AR1 signaling modulates proliferative, anti-apoptotic and prosurvival pathways (208). Upon activation, complement component C5 generates C5a, an anaphylatoxin and a leukocyte chemoattractant, and plays a crucial role in TME by promoting metastasis of cancer cells. In patients with chronic HBV infection, serum complement component C5a is upregulated, predisposing the patients to develop HCC. Tumor cells from HCC patients as well as HCC cell lines show significant upregulation of the complement C5a receptor, C5AR1 (209). Activation of C5aR by C5a enhances the dissemination of circulating tumor cells (CTCs) in HCC *via* upregulation of INHBA/Activin and induction of EMT/MMP by phosphorylation of Smad2/3 (210). Hu et al. demonstrated that C5a ligation of C5aR resulted in activation of the ERK1/2 pathway and induced EMT by increasing Snail expression and downregulating E-cadherin and Claudin-1 expressions (209). While not much of the pathobiological role of complement C5/C5a–C5AR1 axis is known in HCC, its ability to critically influence and control signaling processes relevant to HCC is largely evident from several studies. Activation of the C5a-C5AR1 axis mediates tumorigenic polarization of TAMs *via* NF-κB pathway in metastatic liver lesions (211), while suppressing IL-12 production (212) and promoting immunosuppressive TME *via* $C5aR1^+$ macrophages (213). Increased C5ar1 expression also facilitates recruitment of other myeloid cells like neutrophils *via* IL-1 production (214) and leukotriene B4 (LTB4) (215), while C5a stimulates neutrophil derived tissue factor (TF) synthesis, enhancing tumor growth and metastasis formation (216). As a potent chemoattractant of MDSCs to primary tumors (217), C5a can augment disease severity in HCC by suppressing $CD8^+$ T cell function *via* immunosuppressive MDSCs (218). In Lewis lung cancer model, blockade of C5aR reduced MDSCs and inhibited tumor growth (219). Additionally, signaling *via* C5a–C5aR promotes Treg expansion and suppresses T cell responses in breast cancer metastasis (220), and increases expression of MCP-1, IL-10, Arg-1, and TGF-β1 in colon cancer tumor metastasis (221). Progressive HCC is typified by EMT with matrix metalloproteinases (MMPs) expressed in the TME predominating an important role in this process. C5a expressed by tumor cells triggers expression of MMPs, enhancing tumor invasiveness, release of pro-angiogenic factors, and cell migration (222). Collectively, complement C5a–C5AR1 axis plays a central role as a regulator of innate and adaptive immunity in the TME and a plausible target for development of novel therapeutics for HCC.

Complement Receptor 1 (CR1)

Complement receptor 1 (CR1, CD35) is a glycoprotein expressed either on the membrane or in soluble form on erythrocytes, DCs, monocytes, neutrophils, and B and T cells (223). CR1 inhibits both classical and alternative pathways of complement activation by binding C1q, cleaved C3b and C4b, MBL-2, collectins, and ficolins (224–226) on altered cell surfaces to prevent the formation of terminal membrane attack complex (MAC). Erythrocyte CR1 (E-CR1) is important for processing and removal of circulating immune complexes (CICs) to prevent tissue deposition (227). In HCC, serum CIC levels are abnormally high, with pathological implications (183). The ability of CR1 to bind CICs is particularly important in HCC, with underlying viral etiologies where free and IC-associated HCV binds to E-CR1, differentially driving HCV-IC related features of the disease (228). Kanto et al. showed an inverse correlation between low E-CR1 levels and higher C3d immune complexes. Incidentally, low E-CR1 correlated with severe liver inflammation, cirrhosis, and HCC than those with mild inflammation, demonstrating the relationship between IC and HCV disease severity (229). Similarly, low E-CR1 and high levels of IC were observed in patients with chronic hepatitis and liver cirrhosis (230), emphasizing the importance of defective CIC clearance by altered CR1 functions. A recent study by Luo et al. analyzed genetic polymorphisms and found that two SNPs in CR1 gene (rs3811381 and rs2274567) can potentially predispose subgroups of males, alcohol drinkers, and nonsmokers to HBV-HCC and HBV-chronic hepatitis B risks, while decreasing the risk to HBV-liver cirrhosis in females (231). In contrast, soluble sCR1 levels are increased in liver cirrhosis, end-stage renal failure, and hematologic malignancies (232). In addition, increased levels of sCR1 have been found in patients with increasing grades of cirrhosis and decreased liver functions (233). Since sCR1 levels are elevated in these inflammatory conditions, it is envisaged to play important regulatory and anti-inflammatory roles and act as a potential therapeutic target. Preclinical efficacies of a recombinant form of sCR1 with binding sites for C3b and C4b have been assessed in autoimmune and inflammatory disorders with a potential clinical use in HCC (234).

Mannose Binding Lectin (MBL)

Mannose-binding lectin is an important component involved in the lectin pathway of complement activation (235). MBL functions as a pattern recognition molecule in senescent fibroblast sensing (236), autoimmunity (237), and apoptotic/necrotic cell clearing (238). Further, MBL regulates anti-cancer immunity, plays a diverse role in TME, and contributes to either development or inhibition of tumor growth, depending on the type of cancer (239). MBL2 is primarily produced and secreted by liver cells with significantly elevated levels found in HCC and in HepG2 cell lines (240). Proteomic analysis of serum in patients with pancreatic cancer showed increased levels of MBL2 as a marker of potential diagnostic value (241). Using seven publicly available protein and gene databases, Awan et al. performed enrichment analysis and identified 6 proteins, including MBL2, that defined the biomarkers of HCC (242). This study also identified MBL2 to be a target of 11 circulating and 48 deregulated miRNAs, suggesting MBL2 as a strong candidate for biomarker discovery in HCC (242). Exploring the little-known roles of MBL in TME, Li et al. showed that the genetic loss of MBL in a murine model of HCC triggered enhanced tumorigenesis compared to wild-type mice (243). MBL-deficient mice showed increased accumulation of MDSCs, Treg induction, impaired $CD8^+$ T cell function, COX-2 expression, and PGE_2 production in tumor tissues. Mechanistically, MBL inhibited hepatic stellate cell activation *via* downregulation of ERK/COX-2/PGE_2 signaling pathway. Restoring MBL in these mice significantly reduced HCC progression by inhibiting HSC activation, suggesting MBL to be a potential therapeutic option for HCC. Jalal et al. explored circulating liver-derived lectins and found elevated serum binding activities of ficolin-2 and MBL as potential biomarkers of HCC development in chronic HCV infection (244). Interestingly, dysregulation of miRNAs has been associated with progressive HCC. miR-942-3p was found to be increased in HCC tissue and cell lines and was associated with tumor metastasis and poor patient prognosis. In cell lines, ectopic expression of miR-942-3p resulted in enhanced proliferation and invasiveness while restoration of MBL2 blocked progression of HCC and tumorigenic responses (245). Several studies have also investigated associations of genetic polymorphisms in MBL and altered functionality with HCC. MBL rs7096206 polymorphism was associated with polymorphisms in VDR/VEGF and IL-18 which collectively conferred susceptibility to HCC in Asian populations (246), while MBL2 polymorphisms tended to influence the outcomes of HCC susceptibility, progressive tumor development, and clinical outcomes in patients infected with HBV (247). Mutations in MBL2 are also proposed to predispose patients to elevated HCC risk with significantly reduced serum MBL2 and increased IL-6 and IL-1β levels in HCC (248). Similar analysis in HBV-related cirrhotic patients with HCC suggested that MBL2 SNP rs11003123 was a potential risk factor for HCC development in the Chinese population (249). The importance of MBL gene polymorphisms in progressive forms of severe hepatitis B and liver cirrhosis was further supported by a larger meta-analysis study (250). While some patients with chronic hepatitis B or C infection showed lowered levels of MBL (251), studies by Segat et al. showed no significant associations of MBL2 and MASP2 polymorphisms with either HBV/HCV infection dependent HCC or for HCC alone (252).

Mannose-Binding Lectin (MBL)-Associated Serine Protease-2 (MASP-2)

The MBL-associated protease MASP-2 predominantly promotes activation of the lectin complement pathway. While MASP-2 and lectin pathway components are highly conserved in immune defenses, loss of MASP-2 regulates infectious or autoimmune diseases, immunodeficiency of which are significantly associated with pyogenic bacterial infections, inflammatory lung disease, and autoimmunity (253). In oncogenic environments, increased levels of serum MASP and related lectin pathway molecules have been found to be associated with poor overall survival, disease progression, recurrence, and worse disease prognosis in patients with colorectal (254–256), ovarian (257), and cervical (258) cancers. Similar increases in MASP-2 protein were also associated with advanced clinical stages and nodal metastasis in esophageal squamous cell carcinoma (259). In agreement with these findings, serum MASP-2 levels were higher in pediatric patients with acute lymphoblastic leukemia, non-Hodgkin lymphoma, and CNS tumors (260). However, significant variations between pediatric and adult patients have been documented. In contrast to the severe disease pathogenesis defined by elevated levels of MASP-2, MASP-2 deficiency in leukemic children on chemotherapy was associated with increased risk of febrile neutropenia (FN), antimicrobial therapy, and prolonged duration of hospitalization (261). Similarly, higher serum levels of MASP-2 were associated with impaired event-free survival in pediatric patients with lymphoma (262). Schlapbach et al. further showed that MASP-2 deficient children had significantly increased risk of developing FN in children on chemotherapy (263). These studies show MASP-2 deficiency as a potential risk factor for infections. In the context of HCC, analysis of patient secretomes derived from cancer and adjacent normal tissues using integrative transcriptomics and proteomics identified chitinase-3-like protein 1 (CHI3L1) and MASP2 as biomarkers in HCC diagnosis. However, when diagnosed in combination, the detection for HCC was further enhanced (264). Analyzing patients with moderate and severe chronic hepatitis C, Tulio et al. identified five SNPs in regions critical for formation of MBL/MASP-2 complexes and C4 cleavage of MASP2 gene that resulted in high plasma levels of MASP-2 in hepatitis C patients (265). Mechanistic investigations into the determinants regulating MASP-2 expression *via in silico* analysis of the *MASP2* promoter regions revealed conservation of two putative Stat binding sites, StatA and StatB. *In vitro* analysis of hepatoma cell line HepG2 revealed double stranded StatB oligonucleotides, suggesting interaction of lectin and STAT signaling in liver diseases including fatty liver, fibrosis, and HCC (266). The diverse roles of MASP-2 documented in malignancies other than HCC warrant detailed further analysis into the roles of MASP-2 in adult and pediatric HCC.

C4b-Binding Protein (C4BP)

C4BP is a fluid-phase regulatory component with potent inhibitory properties of the classical and lectin pathways of complement system (267) by providing cofactor activity for factor I-dependent degradation of C4b and C3b (268) (269) and accelerating decay of C3-convertases (269, 270). C4BP is synthesized primarily by hepatocytes (271) and activated monocytes (272). Synthesis of C4BP is enhanced in the presence of inflammatory cytokines such as IFN-γ, IL-1, IL-6, and TNF-α (273), with increased levels of C4BP in inflammatory diseases (274–277). Searching for biomarkers of colorectal cancer (CRC), especially the asymptomatic nascent tumors, Kopylov et al. (278) identified increased levels of C4BP as a potential biomarker in patients with CRC. In patients with non-metastatic CRC, C4BP levels correlated with several coagulation factors, suggesting risk factors for intravascular coagulation activation (279). Elevated levels of fully sialylated C4BP are also found in patients with epithelial ovarian cancer and can distinguish early cases of ovarian clear cell carcinoma from endometriomas (280). Profiling the pre-therapy serum proteome of patients with non-small cell lung cancer (NSCLC) to discover biomarkers and for patient-tailored therapeutics, Liu et al. applied shotgun and targeted proteomic analysis to identify relapse-related gene signatures. Results from the analysis identified a combination of C4BP, LRG1, and SAA or C4BP alone as determinants of disease prognosis, treatment optimization, and overtreatment prevention in patients with NSCLC (281). Indeed, NSCLC cells produce C4BP and provide significant protection from complement mediated tumor cell death (282). Another study also found increased serum C4BP levels in patients with NSCLC and showed strong associations with clinical staging (283). Thus, the ability of C4BP to regulate tumorigenesis in multiple organs and the liver as a primary source strongly suggests a role for C4BP in HCC. The proinflammatory cytokines IL-1, IL-6, and Oncostatin M all significantly upregulated C4BP expressions in the HepG-2 hepatoma cell line (284), suggesting an interplay between inflammation-driven regulation of complement components shielding the tumor from cytotoxic effectors. Tomes et al. further showed that C4BP not only binds strongly to necrotic cells but also limits DNA release from necrotic cells, inhibiting complement activation in both events. Persistence of necrotic core due to C4BP binding may have serious implications in cancer patients, manifesting with poor prognosis, enhanced tumorigenesis, progressive metastases, and emergence of chemoresistance (285). In keeping with the protective and tumor-augmenting roles of C4BP, Williams et al. showed that C4BP binds to CD154 and prevents CD40 mediated cholangiocyte apoptosis. Livers of patients with HCC showed enhanced expression and co-localization of C4BP and CD40, suggesting modulation of cholangiocyte survival in conditions of chronic inflammation and malignancy (286). Similarly, the hepatitis B virus X protein (HBx) affords protection of hepatoma cells from complement attack by upregulating C4BPα *via* activation of the transcription factor Sp1 (287). In tissues from patients with HCC, C4BPα expressions positively correlated with HBx, suggesting tumor-enhancing properties. Using protein-protein interaction networks and gene expression data from patient populations, Ardakani, et al. (288) identified C4BP as an important component of a common molecular relationship between HCC and liver cirrhosis. Identification of such networks and associated molecular connections are expected to serve as novel biomarkers and/or aid in the development of novel treatment strategies. The integral roles played by C4BP in regulating processes critical to tumor growth and progression make it an attractive target for developing interventional therapeutics.

Complement Component C4 (C4)

C4 is the fourth component of the complement cascade, vital to several key roles in defense mechanisms, innate immune function, clearance of CICs, regulation of apoptotic bodies, and autoimmune processes (289, 290). Differentially altered levels of C4 are linked to inflammation in chronic liver diseases (291), metabolic syndrome (292), chronic urticaria (293), and autoimmune processes (294). Serum C4 levels can be used in early detection of HCC, particularly in HCV-infected patients with liver cirrhosis. Serum levels of complement C4 were detected at notably higher levels in the HCC group than in controls. Further analysis showed that a combination of AFP and C4 significantly improved the detection of HCC in HCV-related liver cirrhosis patients (295). More importantly, HCV proteins transcriptionally repress complement C4 expression in liver biopsy specimens from patients with HCV infection. mRNA levels of the two C4 isoforms C4a and C4b are also decreased in hepatocytes transfected with HCV RNA and in HCV core transgenic mice. Thus, the suppression of complement mediated immune responses promotes chronic HCV infection, fibrosis, and HCC (296). Investigating the impact of HBV infection on expression of serum C4 levels, Zhu et al. found that HBV similarly inhibits the expression of complement C3 and C4 *in vitro* and *in vivo* (297). Since AFP alone is used in clinical practice as a biomarker of HCC, Kim et al. performed global data mining using HCC proteomic databases to identify novel biomarkers. Alongside AFP, the data analysis revealed a set of other biomarkers including C4a (with ANLN and FLNB) that were proposed to further improve the screening of patients with HCC (298). Serum C4a/C4b also constitute clinically relevant candidate biomarkers in association with KNG1 and HPX, distinguishing patients with HCC and liver cirrhosis (299). Complement C4 also represents a component that can distinguish HCC and liver cirrhosis with the highest accuracy (300, 301). Increased levels of serum C4a were also found in HCV-infected alcoholic patients with progressive cirrhosis and HCC (302). As a precursor to development of HCC, patients with HBV or HCV infection are at greater risk, necessitating a specific biomarker with increased sensitivity. Dalal et al. identified increased C4a/C4b levels as a reliable marker in patients with HCV related end-stage liver disease (303). Thus, the direct participations in biological regulation of immune responses in HCC and the ability of differential expressions to distinguish patient populations as biomarkers signify complement C4/C4a/C4b as important targets for disease modulation and therapeutic targeting.

Complement Factor H-Related Protein 1 (CFHL1)

Similar to CFH, CFHL1 is an immunoregulatory complement component produced primarily in the liver (304). CFHL1 is derived *via* alternative splicing of the N-terminal domain and shares negative regulatory functions of the alternative complement pathway similar to N terminus of CFH (305). Along with CFHL1, the complement factor H-related protein 1 (CFHR1) functions as a complement regulator by blocking C5 convertase activity and C5b surface deposition (306). CFHR1 also competes with CFHL1 for binding to C3b during CFH-regulation of immune processes (307). The role of CFHR in bladder cancer has been documented, showing the importance of the CFH family of proteins in oncogenesis (308, 309). In surgically resected tissues from HCC patients, decreased CFH mRNA expressions correlated with increased CpG site methylations (143). Furthermore, reduced CFHR3 expression was associated with tumorigenesis, cell proliferation, and activation of WNT and NOTCH signaling pathways (148). In this context, Feng et al. recently demonstrated that CFHL1 can be used as a potential prognostic biomarker in HCC. Analysis of tumor and peritumor specimens from patients with HCC showed downregulation of CFHL1 that was associated with worse time-to-recurrence of the cancer and reduced patient survival rates. This signifies the high prognostic value and potential biomarker capacity of CFHL1 in postoperative patients with HCC (310). The importance of CFHL1 in tumor biology, particularly in HCC, has recently been explored. Future studies will expectedly investigate the clinical efficacies of restoring CFHL1 levels to counter progressive oncogenesis.

Complement Component 8A (C8A)

Complement component 8 alpha (C8A) is a late-phase component of the complement cascade and, along with C5, is involved in the formation of membrane attack complex (MAC). C8A is a liver-specific protein whose expression is regulated by hepatocyte nuclear factor 1α (HNF1α) (311). With relevance to HCC, C8A has been identified in the secretome of an HCC cell line, HEP3B. C8A was also identified as a putative biomarker in a study that investigated HCC–specific proteins enriched for cancer secretome followed by interactome analysis (242). Using genome-wide transcriptional profiling of patient specimens, 439 differentially expressed mRNAs (DEGs) and 214 long non-coding RNAs (lncRNAs; DELs) were identified in HCC. Multiple DELs correlated with tumor cell differentiation, thrombosis, AFP levels, and co-expressions of DEGs of complement cascade, including complement C8A (312). Similarly, Zhe L. et al. (313) utilized publicly available gene expression profiling datasets from the gene expression omnibus (GEO) to identify differentially expressed genes between tumor and adjacent healthy tissue, and found significant enrichment of genes involved in complement activation and coagulation cascade including C8a, C8b, and C6, in HCC specimens. Performing Ingenuity Pathway Analysis (IPA) of HCC gene expression data sets, Yin et al. identified uniquely decreased expression of C8A. Corresponding decreases in expression levels of other complement components including C1S, C2, C5, C6, C7, C8B, C8G, C9 were identified, strongly suggesting downregulation of key complement molecules during early stages of HCC (314). Mu Di et al. used ONCOMINE and TIMER to identify C6 as a candidate gene in diagnosis and prognosis that was associated with significantly decreased overall survival in patients with HCC (315). The regulatory roles of C8A participating in key functions of MAC formation and governing the fate of the tumor cell death can potentially be harnessed in understanding terminal complement processes and/or design of targeted therapeutics.

CD46

CD46 is a membrane-bound complement regulatory protein (mCRP) expressed on the cell surfaces that restrains over-activation of the complement system and protects tissues from injury. CD46 primarily controls the alternative over classical pathway of complement activation. Besides its role as an mCRP, CD46 uniquely functions as a regulator of T cell mediated immune responses that may be relevant in the pathophysiology of HCC invasion and progression. Binding of CD46 on $CD4^+$ T cells promotes differentiation to T regulatory phenotype (316) and dysregulated IL-10 production (317). While expression of CD46 on unconventional γδ T cells suppresses the production of IFN-γ and TNFα (318), $CD4^+$ T cell ligation of CD46 results in production of IFN-γ (319). Thus, the duality of CD46 signaling in anti- and pro-tumoral functions necessitates a careful evaluation of its function in oncogenesis. In patients with ovarian and breast cancer, expression of CD46 is linked to shorter relapse periods and worse prognosis (320) (321); similar outcomes are observed in patients with CRC (322) and multiple myeloma (320). In patients with HBV-HCC, the HBx protein upregulates CD46 in hepatoma and human immortalized liver cells and affords protection from complement mediated cell lysis mechanisms (323). Investigations into CD46 distribution and expression patterns in HCC specimens showed a non-polarized membrane localization of CD46 in contrast to the basolateral expression in non-cancerous livers. This divergent expression pattern may allow HCC cells to escape complement-dependent cytotoxicity (324). In this regard, intratumoral and IV therapies that utilize the nonpathogenic oncolytic measles virus Emonston strain (MV-Edm) showed significant inhibition of tumor growth, survival benefits, and tumor regression in susceptible mice *via* CD46 targeting. This approach, therefore, represents a novel HCC gene therapy system (325). A similar approach using a fiber chimeric oncolytic adenovirus that targets CD46, SG635-p53, showed antitumor activity in Hep3B subcutaneous xenograft tumor models. Intratumoral injections of the adenovirus resulted in significant inhibition of tumor growth and survival of animals, suggesting a safe approach for HCC treatment (326). CD46 was also targeted using another oncolytic adenovirus, SG511, which was fused to the human RANTES/CCL5 gene and regulated by oxygen-dependent degradation domain (ODD). The chimeric SG511-CCL5-ODD showed significantly enhanced antitumor efficacy in HCC xenograft models in nude mice (327). The importance of the CD46 signaling pathway association with miRNA signatures in HCC was demonstrated *via* bioinformatic analysis. The authors performed complement-related gene expression profiling in tissue samples and found a

total of 37 differentially regulated miRNA. Unsupervised hierarchical clustering analysis identified high CD46 expressions in HCC tissues, which negatively correlated with let-7b and miR-17 expression in HepG2 cells, suggesting important regulatory roles of CD46 in HCC *via* modulation of miRNA activities (328). Of note, upregulation of let-7 (329) and miR17 (330) has been associated with progressive carcinogenesis and poor prognosis of HCC. More importantly, the CD46 SNP rs2796267 was recently found to contribute to susceptibility and disease outcomes in HCC by modifying promoter activity. The rs2796267 AG/GG genotype was found to be associated with worse prognosis of resected patients with HCC (331). **Table 2** summarizes the various SNPs found in complement proteins relevant to the pathogenesis of HCC. To overcome the limitations associated with using monoclonal antibodies in cancer immunotherapies due to increased expressions of mCRPs, Geis et al. designed siRNAs for posttranscriptional gene knock down of CD46, CD55, and CD59 in tumor cell lines. The approach successfully reduced CD46 protein expression by 80% with a corresponding increase in CDC by 20%–30%, demonstrating sensitization of malignant cells to complement attack *via* siRNA mediated inhibition of mCRP as a means of cancer therapy (332). A concise summary of the complement proteins together with their biological functions and clinical implications is provided in **Table 3**.

COMPLEMENT PROTEINS AS REGULATORS OF LIVER METASTASES

The aforementioned components and receptors of the complement cascade not only regulate hepatic neoplasia but promote early events of metastases involving increased tumor cell motility, invasiveness, and intravasation. The extra- and intra-hepatic metastatic spread remains one of the major hurdles in improving health related quality of life and long-term survival in patients with metastatic HCC and therefore is one of the most prevalent form of cancers with poor prognosis. HCC cells that survive immune-mediated clearance continue to proliferate and reserve the capacity to generate secondary tumors. Within this framework, perturbation of the complement cascade facilitates dissemination of the tumor cells *via* triggering intracellular EMT pathways and transition to a highly motile cellular phenotype. Recent studies have correlated the C5a/C5AR1 axis with increased angiogenesis and metastasis promoting factors that induce EMT (333) and liver metastasis (334). In HCC, C5AR1 increases cell invasiveness by enhancing Snail and decreasing E-cadherin and Claudin-1 expressions (209). The ability of C5AR1 to facilitate metastasis was also linked to suppression of $CD8^+$ and $CD4^+$ T-cell responses *via* recruitment of immature myeloid cells and generation of Tregs. This study also showed that pharmacologic blockade or genetic ablation of C5AR1 prevented metastatic potential of cancer cells (220). Expression of C5AR1 on TAMs conferred M2 polarization in colon cancer and enhanced liver metastatic lesions affirming a central role for C5AR1 in metastatic spread; importantly, genetic loss of C5ar1 severely impaired the metastatic ability of colon cancer cells (211). Genetic ablation of other complement proteins such as C3 was also shown to have profound inhibitory effects on primary tumor growth and metastasis correlating to increased numbers of $IFN\gamma^+/TNF\alpha^+/IL10^+$ $CD4^+$ and $CD8^+$ T-cells (335). The ability of complement C3 to function in conjunction with EMT contributing towards metastasis is shown by the ability of TWIST1 to regulate C3 expression in tumor cells (209). Cumulatively, the complement components work in synchrony

TABLE 2 | Summary of complement protein single nucleotide polymorphisms (SNPs) in hepatocellular carcinoma (HCC).

Complement protein	SNP	Biological function	Reference
C2	rs9267665	Increases the risk of HCC and Liver Cirrhosis (LC). Alters transcriptional activity of C2	Clifford et al. (192)
	rs2647073	Associated with HCC and LC	
	rs3997872	Associated with HCC but not with LC	
	rs10947223	Protects from HCC. Alters transcriptional activity of C2	Namgoong et al. (193)
	rs9279450	Protective effects against HCC and chronic hepatitis B (CHB)	
CR1	rs3811381	Increases risk of HBV-HCC and HBV-Chronic hepatitis B in males	Luo et al. (231)
	rs2274567	Increases risk of CHB, HBV-HCC in males	
MBL2	rs7096206	Influences the outcomes of HCC susceptibility, progressive tumor development, and clinical outcomes in HBV-HCC	Gu et al. (247)
	rs1800450	Modifies disease in patients after HBV infection, and affects the prognosis of patients with HBV-HCC	
	rs11003123	Risk factor for HCC development in the Chinese population	Wang et al. (196)
MBL	rs7096206	Associated with polymorphisms in VDR/VEGF and IL-18 which collectively confer susceptibility to HCC in the Asian population	Quan et al. (246)
	Codon 52, Codon 54, Codon 57	Associated with disease prognosis in patients with HBV, severe hepatitis B (SHB) or LC. Not associated with HCC prognosis	Xu et al. (250)
	Codon 54	Associated with symptomatic hepatitis B cirrhosis and in patients with spontaneous bacterial peritonitis (SBP)	Yuen et al. (251)
MBL/MASP-2 complex	p.D371Y	Involved in C4 cleavage of MASP2 gene and susceptibility to HCV infection. High levels of plasma MASP-2 are found in Hep-C patients	Tulio et al. (265)
CD46	rs2796267	Associated with susceptibility and disease outcomes in HCC by modifying promoter activity. Also defines grave prognosis of resected patients with HCC	Liu et al. (148)

TABLE 3 | Summary of clinical and biological roles of complement proteins in hepatocellular carcinoma (HCC).

Complement proteins	Expression in HCC	Clinical implications in HCC	Biological functions	References
CFH	**Decreased**	Increases hepatocellular inflammation and injury Promotes enhanced tumorigenesis Poor overall and disease-free survival	Inactivates C3b and regulates activation of AP. Produced by epithelial endothelial cells	Weiler et al. (142) Laskowski et al. (119) Yang et al. (143)
CFHR3	**Decreased**	Increases cell proliferation and tumor burden Decreased cell apoptosis Poor overall and disease-free survival	Regulates WNT & NOTCH pathways. Prognostic predictor for targeted therapeutics in HCC	Liu et al. (147) Liu et al. (331) Pan et al. (149)
C2	**Decreased**	Worse prognosis time-to-recurrence of HCC Increases mortality and reduced survival times Promotes tumorigenesis and metastasis	Increases cytotoxicity of $CD4^+$ T cells. Reduces M2 macrophage polarization. Regulates multiple signaling pathways.	Ning et al. (190) Fu et al. (191) Tian et al. (40)
CR1	**Decreased**	Increases hepatocellular inflammation and injury Increases grades of cirrhosis and HCC Contributes to decreased disease-free survival	Inhibits classical and AP pathways. Defective clearance of CICs. Potential therapeutic target in HCC	Chen et al. (183) Kanto et al. (229) Weisman et al. (234)
MBL/MBL2	**Decreased**	Enhances tumorigenesis and cancer burden Enhances PGE_2 production and HCC progression Increases HSC activation and tumorigenesis Potential biomarker of diagnostic value	Promotes accumulation of MDSCs. Increases Treg function and activity Impairs $CD8^+$ T cell cytotoxicity Enhances activation of HSCs	Rong et al. (241) Li et al. (243) Yoshino et al. (245) Gu et al. (247)
CFHL1	**Decreased**	Worse time-to-recurrence of HCC Increased cell proliferation and tumorigenesis Reduced overall and disease-free patient survival	Negatively regulates AP of C activation. Regulates C5b deposition & immunity. Correlates with CpG site methylations. Regulates WNT/NOTCH pathways	Zipfel & Skerka. (305) Heinen et al. (306) Yang et al. (143) Liu et al. (331)
C8A	**Decreased**	Worse time-to-recurrence of HCC. Increased cell proliferation and tumorigenesis. Reduced overall and disease-free patient survival	Promotes differentiation & thrombosis. Decreased levels of C8A correlate with early HCC	Yao et al. (312) Yin et al. (313) Mu et al. (315)
C4	**Decreased**	Contributes to augmented liver inflammation Diagnostic marker for HCV related HCC. Biomarker for HCV infection, fibrosis and HCC	Distinguishes HCC & cirrhosis with highest accuracy Low C4 levels promote fibrosis & HCC	Potter et al. (291) Ali et al. (295) Banerjee et al. (296)
CD59	**Increased**	Decreases complement-mediated cell lysis Decreases apoptosis and increased tumor burden Worse overall and disease-free survival	Increased resistance of cancer cells Regulates Smad7 formation and Smad2/3 phosphorylation. Modulates TGF-β signaling	Fishelson et al. (151) Watson et al. (156) Abdel-Latif et al. (161) Lan & Wu (166)
CD46	**Increased**	Increases tumor growth & decreases regression Shorter relapse periods and worse prognosis Decreases overall and disease-free survival	Decreases complement cytotoxicity. Promotes differentiation of Tregs. Modulates HCC via miRNA activities	Sherbenou et al. (320) Kinugasa et al. (324) Lu et al. (328)
C1q	**Increased**	Increases cancer cell migration and proliferation Increases tumorigenesis and tumor burden. Poor overall and disease-free survival	Contributes to tumor angiogenesis Promotes cancer cell metastasis Enhances invasiveness of cancer cells	Hong et al. (169) Bulla et al. (186) Hoffken et al. (180) Ho et al. (189)
C3	**Increased**	Increases chemoresistance to therapeutics Promotes angiogenesis and metastasis Potential biomarker of diagnosis & prognosis	Promotes immunosuppressive. TME Suppresses anti-tumor $CD8^+$ T cells. Increases M2 macrophages & MDSCs	Pio et al. (195) Wang et al. (249) Leung et al. (203)
C5	**Increased**	Increases metastasis and EMT of cancer cells Modulates proliferative & apoptosis pathways Enhances dissemination of cancer tumor cells	Promotes immunosuppressive TME. Decreases $CD8^+$ T cell cytotoxicity. Enhances functions of MDSCs	Dai et al. (210) Medler et al. (213) Kusmartsev et al. (218)
C4BP	**Increased**	Promotes progressive metastases & tumor burden Positively correlates with HCC and liver cirrhosis Poor prognosis and HCC chemoresistance	Persistently maintains necrotic core. Modulates cholangiocyte survival. Shields tumors from cytotoxic cells	Phillips et al. (284) Tomes et al. (285) Williams et al. (286)
MASP-2	**Variable**	Diagnostic marker for HCC No clear role defined for MASP-2	Potentially regulates fibrosis and HCC	Ding et al. (264) Unterberger et al. (266)

as a "dark knight–a watchful protector" offering immune surveillance and regulating tumorigenesis and metastatic potential of the transformed oncogenic cells.

Juxtaposing these components are the membrane-bound and soluble complement regulatory factors that protect tumor cells from immune mediated cytotoxicity. Incidentally, high expressions of CD46, CD55, and CD59 are homogenously expressed and positively correlate with increased metastatic tumor cells in the liver of patients with colorectal (336) and other cancers with poor prognosis (321, 337). Inhibitory factors such as CFH were also shown to be highly expressed in exosomes of the metastatic cells (EV-CFH) resulting in increased migratory and invasive capacity of liver cancer cells. Blocking EV-CFH with a tumor specific anti-CFH antibody showed reduction in liver tumor promoting potentials and a potential therapeutic target (338). Directly "complementing" these pro-oncogenic functions, the complement cascade also interacts with the coagulation system resulting in a hyper-coagulable state and survival of tumor cells. In this context, C5a stimulates neutrophils to release tissue factor (339) while C3a induces platelet aggregation and activation (340), both processes culminating in a prothrombotic environment. Furthermore, the ability of neutrophil derived C3AR1 to form neutrophil extracellular trap (NET) drives tumorigenesis (341) and potentially enhances

metastatic capacities. It is therefore important to consider these interactive mutually synergistic pathways in the design of novel therapeutics targeting HCC.

THERAPEUTIC TARGETING OF COMPLEMENT SYSTEM: A REALITY?

The myriad effects of complement molecules in regulating the TME and molecular and cellular effectors of immunopathogenic mechanisms driving HCC may offer new avenues to develop complement-based therapeutics. **Figure 3** depicts the influence of differential complement protein expressions in regulating key pathobiological functions promoting oncogenesis in HCC and provides a platform for therapeutic interventions. In particular, the immune-based therapies have raised concerns and skepticism over failures to produce clinically meaningful disease-modulating effects in cancers. Some anticancer immunotherapies that inhibit PD-1 and/or PD-L1, such as Nivolumab and Pembrolizumab, are currently used to treat unresectable HCC. They can also induce complement activation due to their affinities for C1q and Fc receptors (342, 343); increased C1q levels have been shown to augment liver damage. In this context, C1-INH, approved by the FDA for treatment of hereditary angioedema, has been shown to block the classical activation pathway *via* C1q inhibition (344). Blocking C1q activity may thus represent a beneficial approach in regulating tumorigenesis in HCC while preserving the functions of other complement pathways. In addition to C1q, the roles of other downstream complement molecules such as C5a have been extensively studied in HCC. Inhibition of C5a within the TME without deleterious effects on additional complement dependent defenses has previously been proposed (344). In HCC, C5a activation was shown to induce EMT *via* inhibition of claudin-1 and activation of ERK1/2 pathway. Therefore, targeting C5a generation *via* anti-C5 antibodies (Eculizumab) or blocking C5a-C5aR interaction using a receptor antagonist (PMX-53) that are currently in clinical trials for acute myocardial infarction or rheumatoid arthritis respectively, may serve as promising therapeutic candidates for HCC. Other components of the complement cascade such as C3, C3a/C3b could also be targeted using inhibitors such as the Compostatin/POT-4, currently in clinical trials to treat age related macular degeneration (123). These studies highlight the importance of therapeutic targeting of complement as a novel therapeutic strategy for HCC. In parallel, complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) are the leading cause of cell death when treating tumors/cancer cells with monoclonal antibodies. Several factors and/or etiopathogenic agents have been associated with tumor progression in HCC. Circulating apoptosis inhibitor of macrophage (AIM) is one such element that was recently

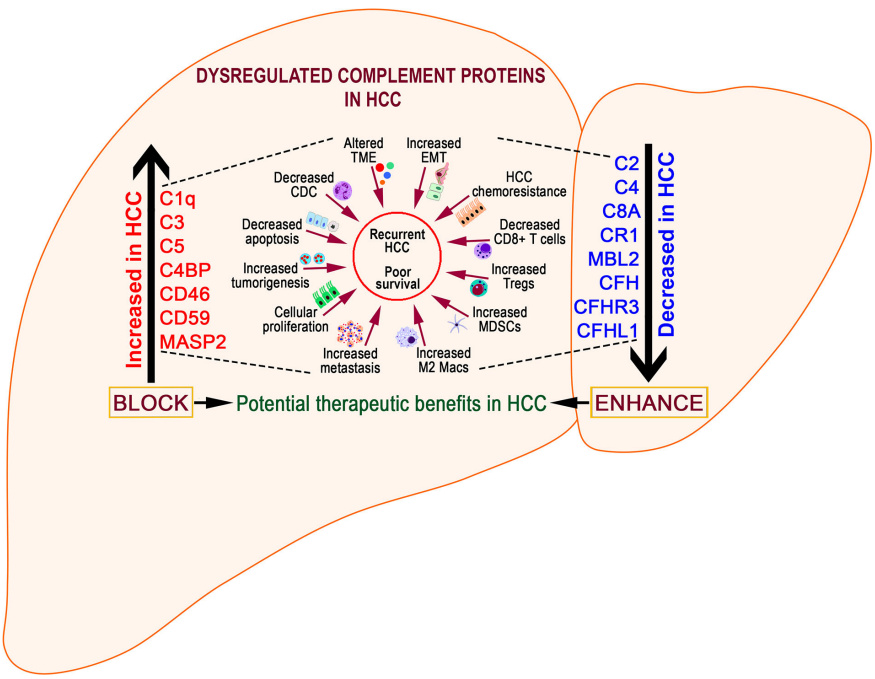

FIGURE 3 | Dysregulated expressions of complement components orchestrate the pathobiology of hepatocellular carcinoma. Breakdown of the tightly controlled activation and regulatory component signals of the complement system results in dysregulation of the normal homeostatic cellular processes within the liver microenvironment. Increased levels of C1q, C3, C5, C4BP, CD46, CD59, and MASP2 and decreased levels of C2, C4, C8A, CR1, MBL2, CFH, CFHR3, and CFHL1 with potential immunoregulatory functions contribute to increased cell proliferation, metastasis, EMT, altered immune cell functions, etc. resulting in recurrent episodes and/or poor overall or disease-free survival in patients with HCC. Images from Motifolio drawing toolkit software (http://motifolio.com) were used for rendering the figure.

described by Maehara et al. to play a role in activation of the complement cascade on the cell surface of tumorigenic, not normal, hepatocytes due to defective endocytosis (345). The authors showed that membrane bound AIM accumulation resulted in C3 activation *in vivo* and was detrimental for viability of cancer cells in HCC *via* CDC cascade. The fundamental dogma in complement biology is a skewing towards enhanced inflammation, with therapeutic approaches designed primarily towards inactivating the complement cascade; however, in situations of tumorigenesis, local stimulation of complement may be advantageous. In this regard the recently described anti-CD20 mAb currently in development: HuMax CD20, HuMax CD38, and HuMaX ZP3, have been demonstrated to increase CDC potency. Anti-CD20 mAb such as Rituximab may therefore prove beneficial in patients with HCC (346–348).

As discussed in the previous section, several factors contribute to the etiopathogenesis of HCC. One such factor, the oncolytic viruses, have been associated with tumorigenesis in HCC and are known to activate complement cascade. Recently, Kim et al., utilized Pexastimogene devacirepvec (Pexa-vec), an oncolytic virus, and showed its ability to induce complement-mediated cancer cell cytotoxicity in rabbits, resulting in improved survival in tumor bearing animals (349). Survival benefits were also achieved in patients with advanced hepatocellular carcinoma that were treated with Pexa-Vec (350). Due to significant success and promising clinical activity of the oncolytic virus, Pexa-vec has recently been tested along with Nivolumab to target tumor cells in HCC (348, 351). In conjunction with our review on the factors of complement cascade and the role of activated components in promoting HCC oncogenesis, we provide a platform identifying various molecules of the complement pathway as potential therapeutic targets in treating patients with HCC and fostering improved survival.

CONCLUDING REMARKS AND THE IMPACT OF COVID-19

HCC continues to be a grave prognostic feature for patients with advanced liver disease of varying etiologies. While early diagnosis remains the mainstay of appropriate medical and surgical approaches, the mostly uncharacteristic features of HCC circumvent early disease diagnosis. The worldwide prevalence, lack of available therapeutic modalities, and rapid progression to severely compromised liver functions urgently necessitate identification and interrogation of newer mechanisms towards better treatment approaches. This urgent need is also dictated by the only available treatment of HCC that relies on liver transplantation, which inherently suffers from shortage of donor livers, higher costs, risk of tumor recurrence, etc.

The demand and need for non-surgical systemic therapies to effectively manage and treat HCC are greater than ever due to the evolving COVID-19 pandemic that has greatly overwhelmed the healthcare system. HCC patients are especially vulnerable due to the decreased allocation of healthcare resources including limited access to operating rooms, deferrals and delays in curative surgery and ablation therapies. The ever-changing scenario of the pandemic, disparity amongst nations in infection rates and limited data of COVID-19 infected HCC patients dictates ongoing efforts in liver oncology. HCC patients require repeated hospital visits, experience social and nosocomial contacts, risks posed by the prevalence of asymptomatic COVID-19 carriers in the community, treatment-related immunosuppression and more importantly treatment delays (352). A recent study reported 21.5% of patients with HCC experienced a significant treatment delay: longer than 1 month in 2020 compared to 2019 (353) as well as a significant drop in number of follow up patients visits (354). Moreover, the significant burden on healthcare providers and resource-intensive protocols have offered little guidance in addressing treatment strategies (352). Therefore, care providers must ensure appropriate surveillance, treatment, and monitoring of patients with HCC and continue to provide therapeutic avenues as in non-COVID-19 pandemic. A system to triage HCC patients where resources are limited should be adapted along with efforts to eliminate the virus in patients with confirmed COVID-19 infection (355). Amongst the many etiopathogenic factors known to cause or promote HCC, the real impact of COVID-19 pandemic or the SARS-CoV-2 virus itself in HCC patients remains unknown. Recent reports have indicated about 15%–54% of patients infected with the virus have hepatic injury and elevated levels of transaminases (356). It is therefore plausible that HCC patients infected with COVID-19 may experience exacerbated disease symptoms and predisposed to increased risk of secondary infections leading to significant morbidities or early mortality. Indeed, the risk factors that predict higher overall mortality in patients with chronic liver disease and COVID-19 are alcohol-related liver disease, decompensated cirrhosis and HCC (357). Using retrospective cohorts, many studies have associated increased biomarkers of liver injury (ALT, AST, GGT) to SARS-CoV-2 infection (358–360) with worsened disease responses in HCC and other cancers. Although the fundamental and intrinsic regulators remain unknown, increased injury responses have been ascribed to direct cytopathic effects of the SARS-CoV-2 virus on hepatocytes and/or cholangiocytes, hypoxia, immune-mediated hepatitis, etc. (356, 361). Standard care treatments such as antivirals and antibiotics prescribed to treat COVID-19 infection have also been linked with increased risk for hepatotoxicity and elevated liver enzymes. To overcome these new challenges and design effective treatment strategies, combination therapies that utilize existing or newly designed immunomodulators targeting complement cascade proteins described herein with immune checkpoint inhibitors may hold significant promise and provide novel therapeutic strategies to treat HCC patients with superimposed COVID-19 infection.

HCC is an immunogenic cancer characterized by chronic inflammation, fibrosis, and cirrhosis. Dysregulated immune responses constitute a major risk factor for HCC. The chronic inflammation, secondary to persistent liver damage, promotes

immune cell activation and increased apoptosis. These events enhance tumorigenesis *via* cell stress, epigenetic modifications, altered mitochondrial metabolism, and activation of cellular senescence pathways. Combined, these biological phenomena directly regulate the high density of liver resident macrophages, NK cells, innate lymphocytes, etc. constituting the immune system as a potential target for managing and treating HCC. Central to these processes, recent studies have assigned multifaceted roles for complement molecules in the immunoregulation of HCC-TME. In this review, we have aimed to decipher the mechanistic roles of complement system in immune dysregulation and oncogenesis in HCC. Our review also describes several components of the complement cascade as potential targets for development of therapeutics. This is highly relevant in the context of limited chemotherapeutic measures that include Sorafenib, Lenvatinib and Regorafenib which currently form the standard care for advanced non-resectable HCC.

We have made an effort to elucidate how complement system regulates the activation of cellular and molecular responses including NK cells, DCs, MDSCs, TAMs, TANs, cytokines, and chemokines that form the first line of defense. Impaired complement activation alters the anti-tumorigenic immunological responses involved in halting the progression and expansion of the tumor, leading to HCC. Efforts to treat HCC *via* modulation of the pro-tumorigenic immune response in TME have been explored, but failure of these therapies in producing clinically meaningful effects limits their use. Elevated expressions of various complement components including C1q, C8a, and anaphylatoxins have been demonstrated in HCC tumors. Targeting these components either by specific inhibitors or antagonists in synergism with existing therapies has a great potential to treat HCC. In contrast to promoting tumor progression, components such as C2 and CFH exhibit tumor-suppressive effects and better prognosis in HCC patients. Furthermore, complement C3, C3a, C4, C4a, and C7 have been identified as biomarkers in HCC diagnosis while C7 and CFH are recognized for their critical roles in mediating stemness of tumor-initiating cells. Similarly, complement C5 regulates EMT, cell migration, and invasion, MBL activates stellate cells, CR1 clears complement CICs, C4BP acts as a complement inhibitor, and CD59 suppresses C3 activation and MAC formation while loss of CFHL1 corresponds to poor time-to-recurrence and overall survival rates. Given these divergent roles, future efforts should be directed toward developing strategies that selectively target or inhibit the tumorigenic effects of complement components while promoting or retaining their anti-tumorigenic effects. Development of novel and targeted therapeutics also benefits from an array of integrated bioinformatics analysis using either a biologically validated known set of genes or a discovery module that employs either large-scale transcriptomic or proteomic techniques. As an initial approach to identify molecular partners and biological processes linked to Complement, we subjected complement components found in HCC (C1q, C2, C3, C4BP, C5, C7, C8A, CD46, etc.) to bioinformatics analysis. Analyzing the genes using ToppGene database (http://toppgene.cchmc.org) with a threshold of $P<0.05$, we identified several biological processes that were further clustered using CIMminer (https://discover.nci.nih.gov/cimminer/home.do). A significant number of complement molecules defined several dominant pathways linked to activation and regulation of innate and adaptive immune systems emphasizing the importance of complement-driven immunopathogenesis (**Figure 4**). Protein-protein interaction network (**Figure 5**) generated using ToppGenet (https://toppgene.cchmc.org) and complement genes showed close associations with several molecules that either positively (ADM, APOA1, ATP12A, BIRC5, CFB, CHD1, DLG4, GRB2, ITGA2, ITGB1, KRAS, LCK) or negatively (APCS, BRCA2, CASK, CD27, CD81, CD82, DMP1, FCN2, GRK2, LLGL1) regulate the pathogenesis of HCC. Regulation of these diverse processes by complement molecules may further propel identification of novel

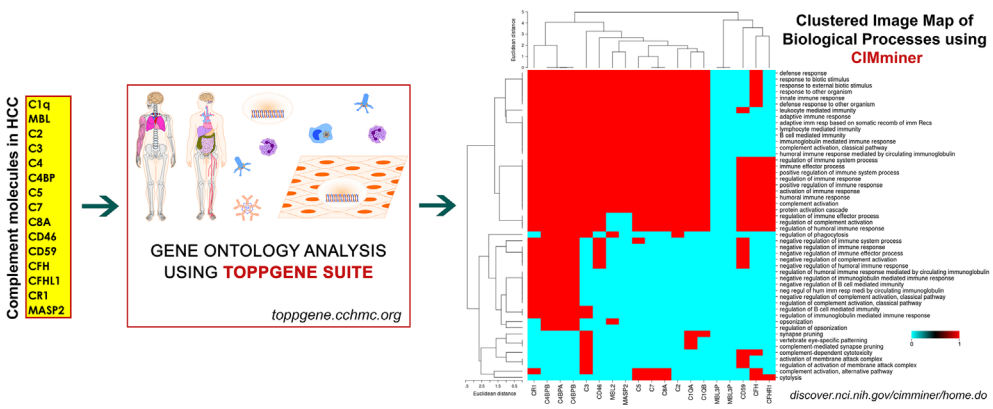

FIGURE 4 | Bioinformatics analysis identifies dominant signatures of immune–mediated biological processes. Supervised gene ontology (GO) annotation analysis of complement components in hepatocellular carcinoma (HCC) was performed *via* ToppGene Suite portal (http://toppgene.cchmc.org) for *in-silico* enrichment of biological processes with a threshold False Discovery Rate (FDR) corrected *P* value of <0.05. ToppGene Suite is a freely available online tool used for functional enrichment, prioritization of candidate genes using transcriptome, ontology, phenotype, proteome, and functional annotations. GO: biological processes identified by ToppGene were further subjected to functional enrichment using CIMminer (https://discover.nci.nih.gov/cimminer/home.do). Red areas depicted in the heatmap show closely related biological processes linked to immunity that are shared by a major group of complement molecules shown on the horizontal axis.

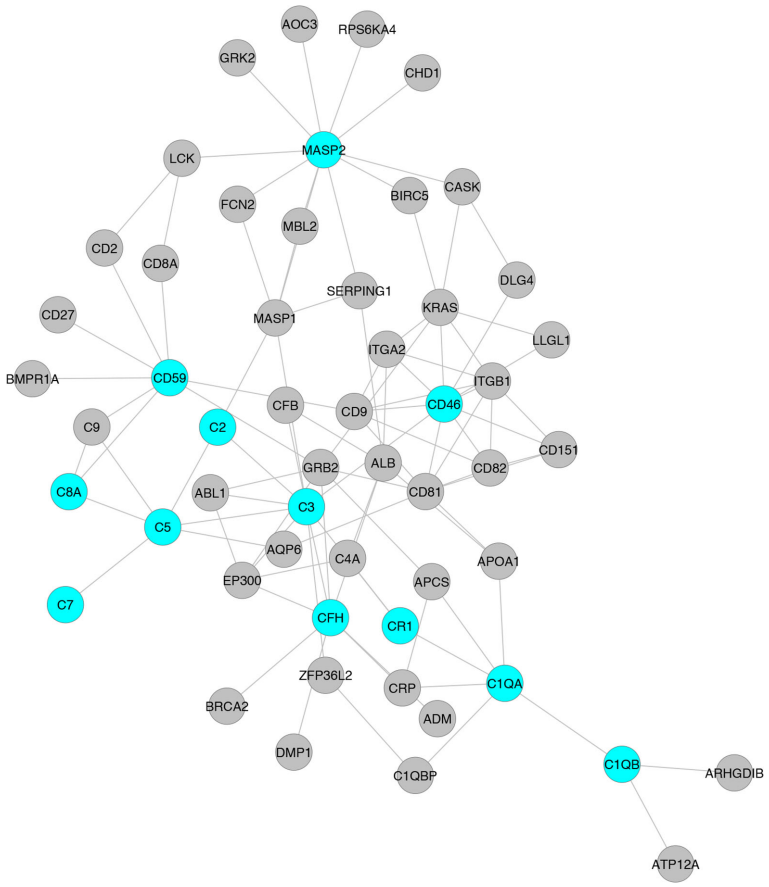

FIGURE 5 | Protein-protein interaction network analysis identifies biological relatedness of complement components to regulators of hepatocellular carcinoma (HCC) pathogenesis. Complement molecules of relevance to HCC were subjected to protein-protein interaction (PPI) network analysis using the network-based gene prioritization algorithm, ToppGenet of the ToppGene Suite (http://toppgene.cchmc.org). ToppGenet identifies and prioritizes candidate genes based on functional annotations, similar expressions, and network and topographical features. A Step Size of 6 and the Prioritization method of k-Step Markov were used as default analytical parameters. The Cytoscape-compatible ToppGenet output file was used to generate the graphical network. The first shell of 41 interacting proteins (grey color) associated directly with the input complement proteins (blue) in the PPI were generated by Cytoscape.

interventional targets for drug development and therapeutic interventions. Thus, the complement cascade serves as a link between the innate and adaptive immune system, activating immune cells critical to drive HCC pathogenesis. With the current understandings of complement molecules as oncogenic drivers, targeted therapies could be developed independently or in combination with existing first and second line of HCC therapies. In summary, a deeper understanding of the mechanistic role of tumor complement components in these pro- and anti-tumorigenic pathways supplemented by advanced bioinformatics approaches are expected to foster the design and development of effective clinical treatments for HCC.

AUTHOR CONTRIBUTIONS

AM and UT equally contributed towards design and manuscript drafting. SA, RM, SN, and AB compiled the literature and assisted in drafting the manuscript. RM analyzed complement genes using bioinformatics tools. PS conceived the original idea and completed the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Hepatic Arterial Infusion Chemotherapy Combined With PD-1 Inhibitors Plus Lenvatinib *Versus* PD-1 Inhibitors Plus Lenvatinib for Advanced Hepatocellular Carcinoma

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Background: Lenvatinib combined with programmed cell death protein-1 (PD-1) inhibitors has resulted in good survival outcomes in the treatment of unresectable hepatocellular carcinoma (HCC). Hepatic artery infusion chemotherapy (HAIC) has also attracted attention due to its high response rates and favorable survival for advanced HCC patients. The present study aimed to compare the efficacy of HAIC combined with PD-1 inhibitors plus lenvatinib (HPL) and PD-1 inhibitors plus lenvatinib (PL) in patients with advanced HCC.

Methods: Between July 2018 and December 2019, patients diagnosed with advanced HCC who initially received HPL or PL treatment were reviewed for eligibility. Efficacy was evaluated according to tumor response and survival.

Results: In total, 70 patients met the criteria and were included in the present study, and they were divided into the HPL group (n = 45) and PL group (n = 25). The overall response rate (40.0 *vs*. 16.0%, respectively; p = 0.038) and disease control rate (77.6 *vs*. 44.0%, respectively; p < 0.001) were higher in the HPL group than in the PL group. The median overall survival was 15.9 months in the HPL group and 8.6 months in the PL group (p = 0.0015; HR = 0.6; 95% CI 0.43–0.83). The median progression-free survival was 8.8 months in the HPL group and 5.4 months in the PL group (p = 0.0320; HR = 0.74; 95% CI 0.55–0.98).

Conclusion: Compared to PL, HPL was associated with a significantly better treatment response and survival benefits for patients with advanced HCC.

Keywords: hepatocellular carcinoma, hepatic artery infusion chemotherapy, programmed cell death protein-1, lenvatinib, FOLFOX

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the fourth leading cause of cancer-related deaths worldwide (1). For advanced HCC, surgical resection is inapplicable, and locoregional approaches bring little benefit (2). Lenvatinib and programmed cell death protein-1 (PD-1) inhibitors are currently well-studied and proven to bring survival benefit as first- and second-line treatment of advanced HCC (3–5). In an open-label multicenter study, lenvatinib plus pembrolizumab surprisingly showed a median overall survival (OS) of 22 months and a median progression-free survival (PFS) of 8.6 months in patients with unresectable hepatocellular carcinoma (6). In recent years, hepatic artery infusion chemotherapy (HAIC) has attracted attention due to high response rates and favorable survival for advanced HCC (7). Several randomized clinical trials have shown that HAIC combined with sorafenib yields significantly better survival compared to sorafenib monotherapy (8, 9). These findings imply that HAIC may have potential when combined with targeted drug therapy.

To date, no research has studied the efficacy of HAIC in combination with lenvatinib and PD-1 inhibitors. Therefore, we designed this retrospective study to compare the survival of patients with advanced HCC who received HAIC combined with lenvatinib plus PD-1 inhibitors (HPL) *versus* those who received lenvatinib plus PD-1 inhibitors (PL), aiming to provide a reference for the treatment of advanced HCC.

METHODS

This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. The analysis of the patient data was reviewed and approved by the Institutional Review Board and Human Ethics Committee at the Sun Yat-Sen University Cancer Center (SYSUCC; Guangzhou, China).

Patients

Between July 2018 and December 2019, the medical records of patients diagnosed with HCC who received HPL and PL treatment at the Department of Liver Surgery of SYSUCC were reviewed for eligibility. Patients were included based on the following specific criteria: a) patients were diagnosed with HCC through imaging or pathology according to the AASLD practice guidelines (10); b) no cancer-related therapies were involved before or during HPL or PL; c) patients had a tumor classification of Barcelona Clínic Liver Cancer (BCLC) B or C; d) Child-Pugh (CP) was classified as A or B; e) patients had at least two cycles of HPL or PL; f) no other malignant tumors were diagnosed; and g) complete medical and follow-up data were available. All laboratory serum test data were collected within 3 days before the initial treatment. Imaging evaluation included enhanced computed tomography (CT) or magnetic resonance imaging (MRI) examination within a week before the initial treatment.

Treatment Procedure

Lenvatinib (The UK, Eisai Europe Co. Ltd.) (8 to 12 mg according to bodyweight) was taken orally. PD-1 inhibitors were used intravenously at the standard dose (**Table S1**). The first use of PD-1 inhibitors was within 7 days of initiation of lenvatinib. For the HPL group, HAIC was administered according to previously described procedures (11). Femoral artery puncture and catheterization were performed in every cycle of treatment. The FOLFOX regimen was administered *via* the hepatic artery as follows: 85 or 135 mg/m^2 oxaliplatin, 400 mg/m^2 leucovorin, and 400 mg/m^2 fluorouracil on the first day; and 2400 mg/m^2 fluorouracil over 46 h. Patients received PD-1 inhibitors and lenvatinib within 3 days before or after the start of HAIC. The discontinuation of treatment depended on disease progression,

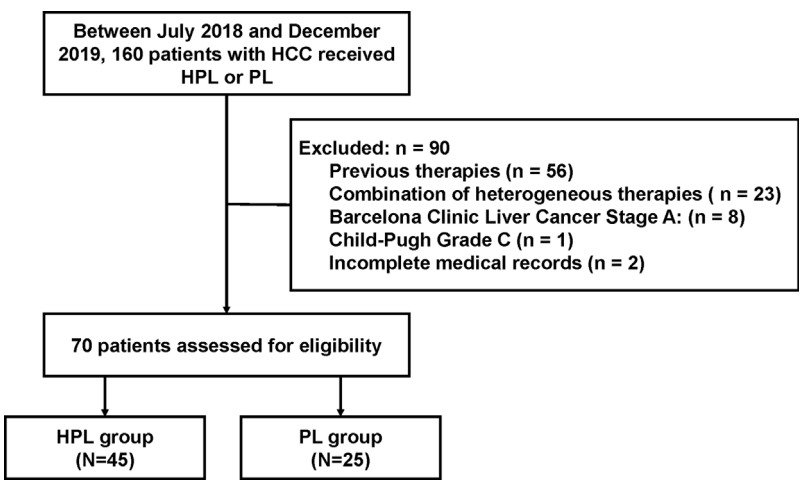

FIGURE 1 | Flow diagram summarizing the disposition process of patients.

unacceptable toxicity, patient withdrawal of consent, or changes of treatment plan. The final follow-up ended on September 30, 2020. Enhanced CT or MRI was performed every 2 or 3 months. Routine follow-up intervals were 2 to 4 months.

Diagnosis and Definitions

Tumor response was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to the modified Response Evaluation Criteria in Solid Tumors 1.1 (mRECIST) (12). Overall response rate (ORR) was calculated as the sum of CR and PR. Disease control rate (DCR) was calculated as the sum of CR, PR, and SD. Overall survival (OS) was defined as the time interval from treatment initiation to cancer-related death. Progression-free survival (PFS) was defined as the time interval from treatment initiation to progression or death. Treatment-related adverse events (AEs) were evaluated by National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0.

Statistical Analysis

Categorical variables in the baseline characteristics were compared using the Pearson's $\chi 2$ test or Fisher's exact test. Variable distribution was described using mean ± standard error (SE) for normally distributed values, and median and range were used for non-normally distributed values. Survival analysis was calculated using the Kaplan-Meier method, and differences in the survival curves were analyzed with a log-rank test. All variables with a P value < 0.05 in univariate analyses were used in multivariate analyses using Cox regression models. The hazard ratio (HR) and confidence intervals (CI) were calculated. A two-tailed P value < 0.05 was considered statistically significant. All data analyses were performed using SPSS 25.0 software (SPSS Inc., Chicago, IL) and GraphPad Prism (version 8.0; GraphPad, Inc.).

RESULTS

Identification and Characteristics of Study Patients

From July 2018 to December 2019, 160 patients with HCC who received HPL or PL were screened: 56 patients received previous surgery, interventional therapies, tyrosine kinase inhibitors or immune-targeted therapies; 23 patients participated in other treatments during HPL or PL; 8 patients were classified with a tumor grade of BCLC/A; 1 patient was classified as CP C; and 2 patients had missing sections in their medical records. Finally, a total of 70 patients who met the criteria were included in the study, and the patients were divided into the HPL group (n = 45) and PL group (n = 25). The patient characterization process is shown in **Figure 1**. Of note, the treatment of PD-1 inhibitors plus lenvatinib was available since July 2018 at our center.

The clinical characteristics and treatment of patients are summarized in **Table 1**. Most patients were classified into CP A (97.8% in the HPL group and 88% in the PL group) and BCLC/C (88.9% in the HPL group and 88.0% in the PL group). Two groups were comparable in the clinical characteristics, liver function, and tumor characteristics. A higher proportion of patients in the PL group had extrahepatic metastasis compared to the HPL group (52.0 *vs.* 33.3%), but the difference was not statistically significant (p = 0.127). In the HPL group, the cycles of PD-1 inhibitors plus lenvatinib ranged from 2 to 12, with a median of 5. While in the PL group, the cycles of PD-1 inhibitors plus lenvatinib ranged from 2 to 9, with a median

TABLE 1 | Baseline clinical characteristics of patients.

Characteristic*	HPL (n=45)	PL (n=25)	P value
Age	49.1 ± 10.6	50.1 ± 12.3	0.366
Gender*			0.212
Male	38 (84.4)	18 (72.0)	
Female	7 (15.6)	7 (28.0)	
HBV			0.533
Negative	8 (17.8)	6 (24.0)	
Positive	37 (82.2)	19 (76.0)	
HCV			1.000
Negative	44 (97.8)	25 (100.0)	
Positive	1 (2.2)	0 (0)	
WBC (10E9/L)	6.7 (5.6, 8.0)	7.4 (5.7, 9.7)	0.420
NEU (10E9/L)	4.78 (3.66, 5.92)	4.86 (3.20, 7.20)	0.876
LYM (10E9/L)	1.2 (0.9, 1.6)	1.4 (1.1, 1.8)	0.210
HB (g/L)	138.4 ± 20.7	137.9 ± 29.6	0.944
PLT (10E9/L)	227.0 (184.0, 328.0)	201.0 (149.8, 281.5)	0.351
ALT (U/L)	45.5 (34.0, 74.4)	57.0 (37.6, 89.8)	0.329
AST (U/L)	76.2 (55.6, 152.1)	96.7 (56.7, 173.4)	0.655
AFP (ng/ml)	4106.0 (72.8, 121000.0)	767.6 (23.3, 21940.5)	0.193
PIVKA-II (mAU/ml)	9929.0 (1672.0, 51343.0)	11794.5 (252.3, 75000.0)	0.952
Liver cirrhosis			0.143
Absent	5 (11.1)	7 (28.0)	
Present	40 (88.9)	18 (72.0)	
Child-Pugh			0.127
A	44 (97.8)	22 (88.0)	
B	1 (2.2)	3 (12.0)	
Barcelona Clínic Liver Cancer			1.000
B	5 (11.1)	3 (12.0)	
C	40 (88.9)	22 (88.0)	
Size of largest nodule (cm)	11.2 ± 3.9	10.9 ± 4.2	0.754
Tumor number			1.000
Solitary	9 (20.0)	5 (20.0)	
Multiple	36 (80.0)	20 (80.0)	
Tumor distribution			0.409
Uni-lobar	17 (37.8)	7 (28.0)	
Bi-lobar	28 (62.2)	18 (72.0)	
Tumor thrombus			0.237
Absent	9 (20.0)	7 (28.0)	
Branch of portal vein	20 (44.4)	6 (24.0)	
Main portal vein	16 (35.6)	12 (48.0)	
Extrahepatic metastasis			0.127
Absent	30 (66.7)	12 (48.0)	
Present	15 (33.3)	13 (52.0)	

**No (%).*

PD-1, programmed cell death protein-1; HPL, hepatic artery infusion chemotherapy combined with PD-1 inhibitors plus lenvatinb; PL, PD-1 inhibitors plus lenvatinib; HBV, hepatitis B virus; HCV, hepatitis C virus; WBC, white blood cell; NEU, neutrophil; LYM, lymphocyte; HB, haemoglobin; PLT, blood platelet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-I.

of 4. The PD-1 inhibitor categories in each group are summarized in **Table S1**.

Survival

The median follow-up time was 15.1 months. Patients in the HPL group had significantly better survival outcomes than those in the PL group. The 3-, 6-, and 12-month OS was 97.8, 86.7, and 67.4%, respectively, in the HPL group, and 83.6, 61.8, and 29.8%, respectively, in the PL group. The median OS was 15.9 months in the HPL group and 8.6 months in the PL group (p = 0.0015; HR = 0.6; 95% CI 0.43–0.83). The 3-, 6-, and 12-PFS was 86.7, 68.9, and 43.2%, respectively, in the HPL group, and 75.8, 49.2, and 15.7%,

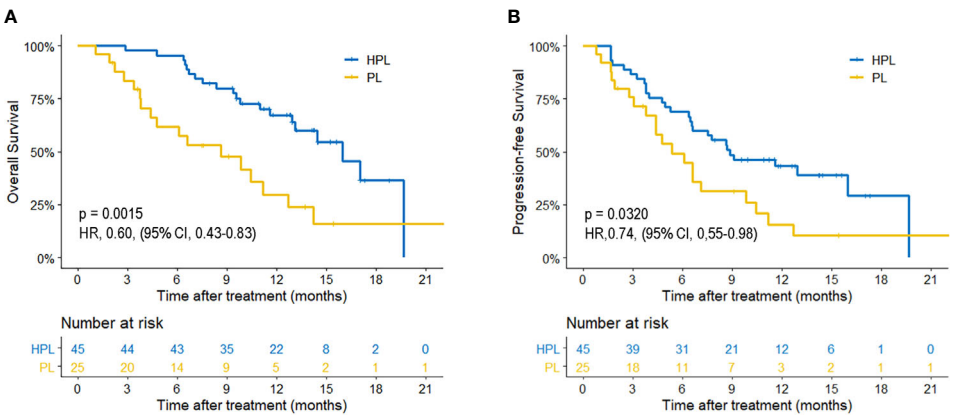

FIGURE 2 | Kaplan-Meier curves of survival outcomes of patients in the two groups. **(A)** Overall survival. **(B)** Progression-free survival. HPL, hepatic artery infusion chemotherapy combined with PD-1 inhibitors plus lenvatinib; PL, PD-1 inhibitors plus lenvatinib.

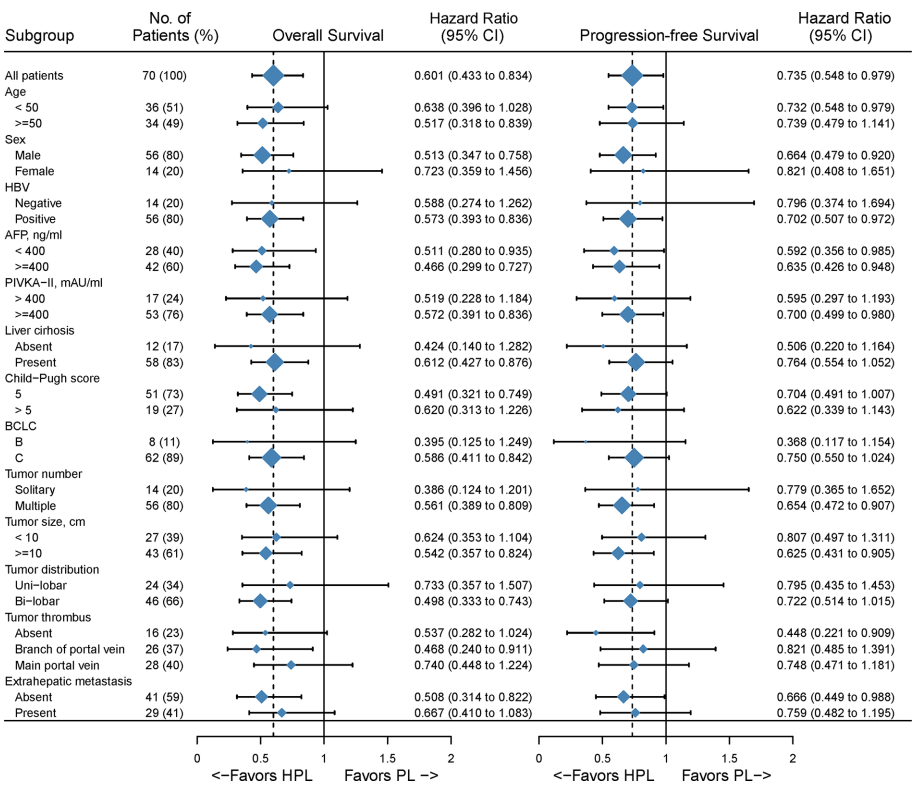

FIGURE 3 | Forest plot for overall survival of the matched cohorts of patients. HPL, hepatic artery infusion chemotherapy combined with PD-1 inhibitors plus lenvatinib; PL, PD-1 inhibitors plus lenvatinib.

respectively, in the PL group. The median PFS was 8.8 months in the HPL group and 5.4 months in the PL group (p = 0.0320; HR = 0.74; 95% CI 0.55–0.98). The survival curves are shown in **Figure 2**. The forest plot analysis of factors associated with OS and PFS is shown in **Figure 3**. HPL provided a clinical benefit in patients with large, multiple HCCs, but it failed to have a survival benefit in patients with main portal vein tumor thrombus or extrahepatic metastasis.

TABLE 2 | Summary of best response.

Variable	HPL (n=45) No. (%)[c]	PL (n=25) No. (%)[c]	P value
Overall response[a]			
Complete response	0 (0.0)	0 (0.0)	1.000
Partial response	18 (40.0)	4 (16.0)	0.038
Stable response	20 (44.4)	7 (28.0)	0.176
Progressive response	5 (11.1)	6 (24.0)	0.156
Not assessable	2 (4.4)	8 (32.0)	0.002
Overall response rate	18 (40.0)	4 (16.0)	0.038
Disease control rate	38 (77.6)	11 (44.0)	<0.001
Intrahepatic response[b]			
Complete response	0 (0.0)	0 (0.0)	1.000
Partial response	18 (40.0)	4 (16.0)	0.038
Stable response	22 (48.9)	9 (36.0)	0.298
Progressive response	3 (6.7)	4 (16.0)	0.212
Not assessable	2 (4.4)	8 (32.0)	0.002
Overall response rate	18 (40.0)	4 (16.0)	0.038
Disease control rate	40 (88.9)	13 (52.0)	0.001

HPL, hepatic artery infusion chemotherapy combined with programmed cell death protein-1 inhibitors plus lenvatinb; PL, programmed cell death protein-1 inhibitors plus lenvatinib.

[a]Overall response included assessment of the change in tumor burden inside and outside the liver.

[b]fimmu.2021.619776Intrahepatic response only included assessment of the change in tumor burden inside the liver.

[c]Treatment response was assessed in evaluable patients.

Tumor Response

The treatment response is summarized in **Table 2**. Based on mRESIST, the ORR was higher in the HPL group (40.0%) than in the PL group (16.0%) (p = 0.038). A higher DCR in both overall response (77.6 *vs.* 44.0%; p < 0.001) and intrahepatic response (88.9 *vs.* 52.0%; p = 0.001) was present in the HPL group compared to the PL group.

Safety Analysis

All AEs were evaluated as mild and manageable, and no toxicity-associated deaths occurred in the follow-up. More patients in the HPL group experienced grade 1–2 neutropenia and increased alanine aminotransferase. Only one patient experienced Grade 3 pain in the PL group. The details of the events were summarized in **Table 3**.

Prognostic Factor Analysis

The prognostic factors for survival are shown in **Table 4**. The comparison of PL to HPL was identified as an independent risk factor for both OS (HR = 3.180; 95% CI 1.608–6.290; p = 0.001) and PFS (HR = 2.702; 95% CI 1.440–5.070; p = 0.002). In addition, multivariate analyses identified that CP B and multiple tumors were risk factors for OS and that AFP ≥ 400 ng/ml was a risk factor for PFS.

TABLE 3 | Treatment-related adverse events.

Adverse events	Any grade			Grade 3/4		
	HPL (n=45)	PL (n=25)	P value	HPL (n=45)	PL (n=25)	P value
Treatment-related AEs, n (%)				0 (0)	1 (4.0)	0.357
Rash	3 (6.7)	2 (8.0)	1.000	0 (0)	0 (0)	1.000
Pruritus	3 (6.7)	2 (8.0)	1.000	0 (0)	0 (0)	1.000
Pain	7 (15.6)	7 (28.0)	0.212	0 (0)	1 (4.0)	0.357
Fever	12 (26.7)	5 (20.0)	0.500	0 (0)	0 (0)	1.000
Diarrhea	5 (11.1)	3 (12.0)	1.000	0 (0)	0 (0)	1.000
Fatigue	8 (17.8)	5 (20.0)	1.000	0 (0)	0 (0)	1.000
Nausea	6 (13.3)	1 (4.0)	0.408	0 (0)	0 (0)	1.000
Decreased appetite	8 (17.8)	2 (8.0)	0.314	0 (0)	0 (0)	1.000
Cough	4 (8.9)	3 (12.0)	0.694	0 (0)	0 (0)	1.000
Edema peripheral	2 (4.4)	1 (4.0)	1.000	0 (0)	0 (0)	1.000
Hypothyroidism	1 (2.2)	1 (4.0)	1.000	0 (0)	0 (0)	1.000
Hyperthyroidism	0 (0)	0 (0)	1.000	0 (0)	0 (0)	1.000
Laboratory-related AEs, n (%)				4 (8.9)	3 (12.0)	0.694
White blood cell count decreased	1 (2.2)	0 (0)	1.000	0 (0)	0 (0)	1.000
Hemoglobin decreased	2 (4.4)	0 (0)	0.534	0 (0)	0 (0)	1.000
Platelet count decreased	5 (11.1)	1 (4.0)	0.410	1 (2.2)	0 (0)	1.000
Neutropenia	7 (15.6)	0 (0)	0.045	0 (0)	0 (0)	1.000
Alanine aminotransferase increased	20 (44.5)	4 (16.0)	0.016	3 (6.7)	1 (4.0)	1.000
Aspertate aminotransferase increased	15 (33.3)	3 (12.0)	0.050	2 (4.4)	1 (4.0)	1.000
Total bilirubin increased	5 (11.1)	5 (20.0)	0.477	0 (0)	2 (8.0)	0.124
Albumin decreased	4 (8.9)	1 (4.0)	0.648	0 (0)	0 (0)	1.000
Creatinine increased	1 (2.2)	0 (0)	1.000	0 (0)	0 (0)	1.000

AEs, adverse events; HPL, hepatic artery infusion chemotherapy combined with programmed cell death protein-1 inhibitors plus lenvatinib; PL, programmed cell death protein-1 inhibitors plus lenvatinib.

TABLE 4 | Univariate and multivariate analysis of risk factors for overall survival and progression-free survival.

Variables	Overall survival						Progression-free survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P* value	HR	95% CI	*P*	HR	95% CI	*P*
Age (y), (</≥50)	1.039	0.539–2.003	0.908				0.754	0.423–1.343	0.338			
Gender, (female/male)	0.413	0.413–2.007	0.816				1.509	0.702–3.240	0.292			
Hepatitis B, (no/yes)	1.194	0.516–2.762	0.679				1.438	0.669–3.089	0.352			
AFP (ng/ml), (</≥400)	1.788	0.896–3.569	0.099				2.096	1.144–3.840	0.017	2.896	1.507–5.568	0.001
PIVKA-II, (mAU/ml), (</≥400)	1.353	0.591–3.097	0.474				1.481	0.714–3.071	0.291			
Liver cirrhosis (no/yes)	1.225	0.476–3.153	0.674				1.188	0.531–2.660	0.674			
Child-Pugh (A/B)	4.309	1.501–12.373	0.007	3.709	1.239–11.099	0.019	2.109	0.754–5.897	0.155			
BCLC (B/C)	1.163	0.475–2.848	0.741				1.537	0.638–3.701	0.337			
Largest tumor size (cm),(<10/≥10)	1.670	0.837–3.333	0.146				1.531	0.845–2.773	0.160			
Tumor number (1/>1)	3.127	1.097–8.915	0.033	3.193	1.093-9.327	0.034	1.938	0.865–4.346	0.108			
Tumor distribution (uni-/bi-lobar)	1.337	1.029–1.737	0.030				1.322	1.062–1.647	0.013			
Tumor thrombus												
Absent												
Branch of portal vein	0.465	0.194–1.114	0.086				1.054	0.487–2.234	0.891			
Main portal vein	0.933	0.440–1.979	0.857				1.182	0.570–2.451	0.653			
Extrahepatic metastasis (no/yes)	1.569	0.819–3.003	0.174				1.033	0.578–1.846	0.912			
Treatment (HPL/PL)	2.770	1.437–5.340	0.002	3.180	1.608–6.290	0.001	1.865	1.044–3.330	0.035	2.702	1.440–5.070	0.002

AFP, alpha fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; BCLC, Barcelona Clínic Liver Cancer; PD-1, programmed cell death protein-1; HPL, hepatic artery infusion chemotherapy combined with PD-1 inhibitors plus lemvatinb; PL, PD-1 inhibitors plus lenva; HR, hazard rate; CI, confidence interval.

DISCUSSION

Treatment strategies for advanced HCC have progressed with the emergence of new tyrosine kinase inhibitors (TKIs) and immune-targeted therapy. Lenvatinib plus pembrolizumab has recently become a potent systemic combination therapy for unresectable HCC (6). In clinical practice, locoregional-systemic combinations are widely applied due to the overall control of tumor conditions (13). The result of a randomized clinical trial conducted by Ming Shi et al. demonstrated that a combination of sorafenib plus HAIC using FOLFOX agents extends overall survival by 87.5% or 6.24 months compared to sorafenib alone in HCC patients with portal vein invasion (9). Thus, HAIC may play a role in PL treatment. However, no research has reported the efficacy of HPL *versus* PL. Our retrospective study demonstrated that in advanced HCC, HPL results in a significantly better survival benefit than PL.

The efficacy benefit observed in the present study may be attributed to the synergistic antitumor effect of PD-1 inhibitors, lenvatinib, and FOLFOX agents. Oxaliplatin induces immunogenic cell death in HCC cells and synergizes with PD-1 targeted immunotherapy (14). Lenvatinib inhibits multiple receptor tyrosine kinases (RTKs) targeting VEGFR1-3, FGFR1-4, PDGFR α, RET, and KIT (15). On the one hand, inhibition of VEGFR and FGFR elicits antitumor immunity and enhances PD-1 checkpoint blockade in HCC (16). On the other hand, antiangiogenesis normalizes tumor vessels and breaks the hypoxic microenvironment of tumors, thereby attenuating the activity of chemoresistance (17–19).

In this study, the median OS and PFS were 8.6 months and 5.4 months in the PL group, respectively, which were better than those observed in a sorafenib monotherapy trial in the Asia-Pacific region (20). However, the survival outcomes were far worse than those in the Keynote-524 trial (6). Worldwide trials of PD-1 inhibitors or lenvatinib monotherapy in advanced HCC have shown a better OS over 1 year (21, 22). Compared to these studies, the patients included in our study were relatively more late-staged with the majority of the patients in the PL group classified with BCLC stage C (88%), major (48%) or branch (24%) of portal vein tumor thrombus, extrahepatic metastases (52%) and tumor burden over 10 cm (60%). In contrast, the median OS and PFS were significantly better in the homogeneous patients in the HPL group, implying efficacy for the HPL therapy.

The treatment response showed significantly higher ORR and DCR in the HPL group compared to the PL group. Of note, eight patients were unable to assess tumor response in the PL group. One unavoidable reason was that patients treated with systemic medications were not hospitalized, causing the relatively high rate of missed imaging examinations during the treatment, which affected the accurate assessment of tumor response rates. Thus, this variable needs to be further controlled in prospective studies.

In the subgroup analysis, significant differences were not reached in certain subgroups with small proportional cohorts due to limitations in the number of cases. In general, HPL *versus* PL provided a survival advantage in patients with multiple tumors and tumor diameters greater than 10 cm, but HPL was less effective in patients with main portal vein tumor thrombus and extrahepatic metastases. These findings suggested that HAIC, as a locoregional approach, has a great ability to control intrahepatic lesions but that it may fail to manage extrahepatic metastases. Univariate and multivariate Cox regression analyses showed different factors associated with OS and PFS. This may be partly due to the incongruity between progression and

survival in the combination therapy of advanced HCC. Patients with the progressive disease could receive more treatment and get inconsistent survival benefit. Of note, HPL was an independent prognostic indicator for both OS and PFS, which confirmed the positive efficacy of HAIC in the combination therapy of PL.

The present study had some limitations. First, the study was a retrospective study in a single center, resulting in inevitable selection bias. Second, the PD-1 inhibitors were varied, which influenced the uniformity of the treatment procedure. Third, the number of cases was relatively small. Findings from this study should be further expanded to a multicenter study to obtain higher-level medical evidence.

Based on our results, HPL is associated with a significantly better treatment response and survival benefits compared to PL. Thus, HPL may be a potential new treatment option for advanced HCC.

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.

ETHICS STATEMENT

This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. The analysis of the patient data was reviewed and approved by the Institutional Review Board and Human Ethics Committee at the Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China).

AUTHOR CONTRIBUTIONS

R-PG, WW, and S-HL designed the study. JM and Y-HT collected the data. JM, Y-HT, MS, WW, S-HL, and R-PG analyzed and interpreted the data. LZ performed the radiological evaluation. JM, Y-HT, S-HL, and R-PG prepared the final draft. 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.2021.618206/full#supplementary-material

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OPINION
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A Rare Case of Acute Liver Failure Secondary to Diffuse Hepatic Infiltration of Small Cell Neuroendocrine Carcinoma

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Keywords: acute liver failure, biopsy, pulmonary neuroendocrine tumor, small cell lung cancer, case report

BACKGROUND

Acute liver failure (ALF) is defined as acute liver dysfunction manifesting as encephalopathy and coagulopathy [INR (international normalized ratio)] ≥ 1.5) of less than 26 weeks duration, without preexisting liver disease (1). Of the known etiologies of ALF in adults, drug toxicity (50%), viral hepatitis (9%), and autoimmune hepatitis (7%) are most common (2, 3). Although the liver is a common target for metastasis, a significant number of patients are asymptomatic with mildly abnormal liver function tests. There are very few reports of ALF resulting from malignancy (0.44–1.4%) (3, 4). Hematologic malignancies are the leading cause of ALF, especially non-Hodgkin lymphoma (3, 5).

The diagnosis of widespread infiltration of the liver can be challenging, as imaging and clinical presentations often do not reveal this type of hidden infiltration pattern (6). However, most cases have a poor prognosis with liver failure occurring within several days (2). Early liver biopsy in unexplained cases must be carried out, as the findings can provide information on appropriate treatment.

We report a case of ALF associated with malignant infiltration of small cell neuroendocrine carcinoma without a history of primary malignant tumors. We evaluated the clinical and laboratory data, treatment and prognosis.

CASE PRESENTATION

Chief Complaints

A 69-year-old man was admitted to a community hospital with abdominal pain, bloating, and burning under the xiphoid of 1 week duration. His symptoms worsened following the discovery of liver dysfunction 5 days later and he was then transferred to our hospital. He complained of intolerable abdominal distension and decreased appetite on admission.

Abbreviations: ALF, acute liver failure; CT, computed tomography; SCLC, small cell lung cancer; PNET, pulmonary neuroendocrine tumor.

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History of Past Illness

His medical history included hypertension treated with antihypertensive drugs for 14 years. He had also undergone laparoscopic appendectomy 20 years previously. There was no history of excessive alcohol consumption or hepatitis. It was noted that he took several types of Chinese traditional drugs (Qinghao Biejia Decoction) for a cold.

Physical Examination

The patient had jaundice. The lungs were clear, and his heart rate was normal. His upper abdomen was tender, but there was no organomegaly. The bowel sound was weakened with negative shifting dullness.

Laboratory Examinations

Blood tests showed leukocytosis of 13.73×10^9/L (normal range: 3.5–9.5×10^9/L) and neutrophilia of 12.07×10^9/L (1.8–6.3×10^9/L) with a normal red cell count and platelet count. Liver function tests demonstrated an anomalous pattern, with elevated aminotransferases/aspartate aminotransferase (ALT/AST) 285.3 U/L (15–40 U/L), ALT 481.8 U/L (9–50 U/L), alkaline phosphatase 471.4 U/L (45–125 U/L), γ-glutamyl transferase 1,424.1 U/L (10–60 U/L), total bilirubin (TB) 90.5 μmol/L (0–23 μmol/L), direct bilirubin (DB) 75 μmol/L (0–7 μmol/L), prolonged prothrombin time of 16.1 s, and INR of 1.19 (0.8–1.2). IgE level was 123.8 IU/ml, and the levels of serum amylase, autoantibody profile, and viral serology (HAV, HBV, HDV, HEV, EBV, CMV, HSV) were normal. Gastrointestinal marker carbohydrate antigen-199 was 90.92 U/mL (0–37 U/ml) and FER was 802.5 ng/ml (25–280 ng/ml).

Imaging Examinations

Abdominal X-ray showed incomplete small bowel obstruction. A computed tomography (CT) scan of the chest and abdomen revealed exudation of the bilateral lungs, and small pleural effusion with enlarged lymph nodes in the mediastinum (**Figure 2A**), peritonitis, and massive pelvic fluid (**Figure 2B**). The biliary ducts were not dilated.

FINAL DIAGNOSIS AND TREATMENT

An extensive workup including abdominal CT, viral serology, and autoimmune studies failed to show an etiology in this patient. A presumed diagnosis of liver dysfunction due to drug-induced hepatitis was made as the patient had taken Chinese traditional drugs before admission, which had nothing to do with ALF or carcinoma infiltration according to current literatures. The patient was treated with liver protective agents and diuretics. On the 8th day, the patient reported aggravated abdominal distension and jaundice. Liver function tests had also deteriorated with increased serum bilirubin level and reduced serum aminotransferase level. Laboratory indices were as follows: TB 201 μmol/L, DB 163.5 μmol/L, ALT 232.7 U/L, AST 213 U/L, and ALB 33.3 g/L, respectively (**Figure 1**). However, subsequent abdominal CT (**Figure 2C**) revealed diffuse regenerative nodules, cholecystitis, peritonitis, and ascites. No splenomegaly, biliary obstruction, or pulmonary nodules were observed (**Figure 2C**). Liver biopsy in multiple sections was performed after patient permission. On the 11th day, the patient developed sleep disorder, abnormal behavior with a decrease in calculation ability, and no fever, digestive tract hemorrhage, or other significant clinical findings were noted. Laboratory indices were TB 307.2 μmol/L, DB 256.1 μmol/L, ALT 173.4 U/L, AST 231.2 U/L, and ALB 32.4 g/L (**Figure 1**), while psychometric and serum ammonia 56 umol/L was normal. The patient then received N-methyl-D-aspartate (NMDA) ornithine, arginine, branched chain amino acid injection, and vinegar for clyster. The use of artificial liver treatment was also considered for this patient. But the liver biopsy results revealed high-grade neuroendocrine carcinoma originating from the lung, classified as small cell type, accompanied with cell necrosis and eosinophilic infiltration in the adjacent liver parenchyma. Immunohistochemical staining was positive for CgA, Syn, Ki-67, TTF-1, CD56, P53, CK19 showed dense positive brown nuclear immunoreactions and the distribution of which positive cells was not uniform in one slide or a few of cancer cells (>10%), while CK7, CK20, P40, GPC-3, HepPar1 were negative without cytoplasmic immunoreactions (**Figure 3**).

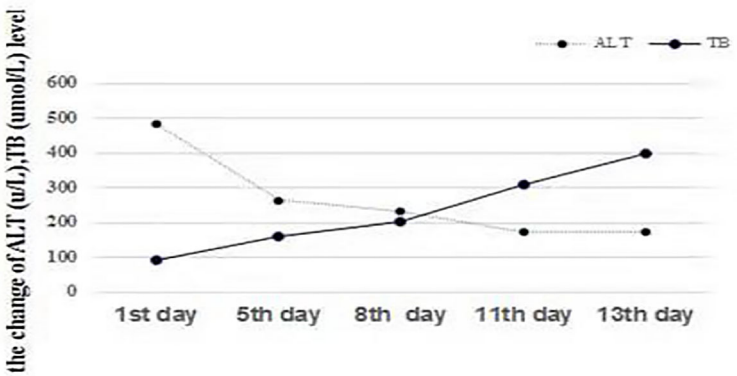

FIGURE 1 | Changes in ALT (U/L) and TB (μmol/L). The level of TB consistently increased with a decrease in ALT, indicating the dissociation of bilirubin and aminotransferases, which represents liver function deterioration.

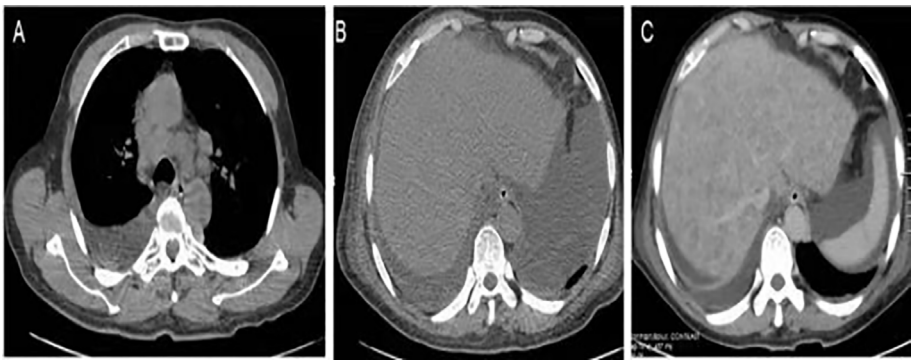

FIGURE 2 | Imaging findings. **(A)** Chest CT revealed enlarged lymph nodes in the mediastinum. **(B)** Abdominal CT showed ascites with no hepatic focal lesions. **(C)** Contrast-enhanced CT of the abdomen revealed diffuse regenerative nodules, cholecystitis, peritonitis, and ascites. No splenomegaly, biliary obstruction, or pulmonary nodules were observed.

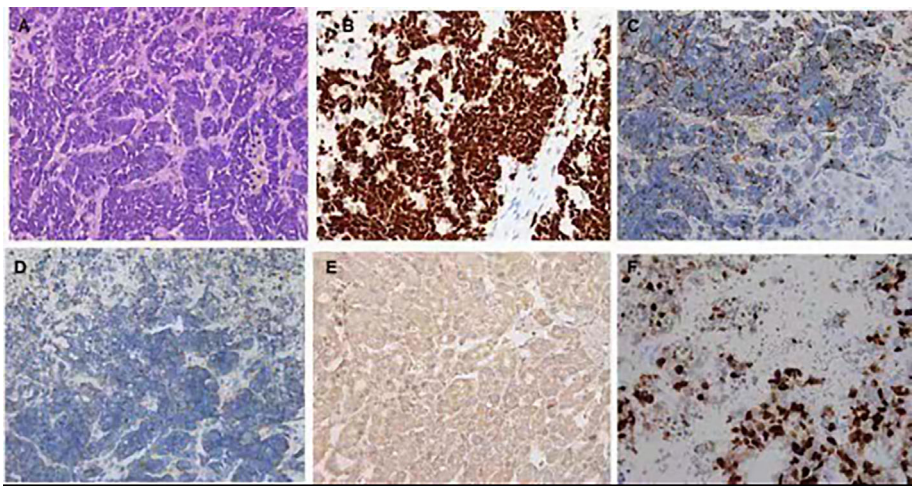

FIGURE 3 | Hematoxylin and eosin and immunohistochemistry staining of the liver biopsy specimen. **(A)** In the center of the field, diffuse infiltration by a poorly differentiated neuroendocrine carcinoma and arranged singly, in small clusters (hematoxylin and eosin ×400). Positive immunohistochemistry staining for TIF **(B)**, CgA **(C)**, Syn **(D)**, CD56 **(E)**, Ki-67 **(F)**.

OUTCOME AND FOLLOW-UP

The patient's clinical course continued to worsen. He refused further examination and treatment, and left the hospital on the 13th day. The patient died 10 days after hospital discharge.

DISCUSSION

We report a rare case of ALF due to infiltration by a pulmonary neuroendocrine tumor (PNET). To our knowledge, ALF secondary to malignant infiltration of the liver is unusual (3). The diagnosis of ALF secondary to malignancy can be difficult as standard laboratory values are not helpful in identifying the presence of malignancy. As in our patient, only a common clinical presentation was observed (1). Chest CT did not show a lung nodule and only revealed enlarged lymph nodes in the mediastinum. Abdominal CT revealed a nodular liver that has previously been described as "pseudocirrhosis" (6) (**Figure 2B**). The final diagnosis required liver biopsy, in the absence of previous tumors. Our patient underwent a confirmatory biopsy, highlighting the importance of early tissue sampling. It is also important, in terms of both early diagnosis and prompt initiation of treatment, to differentiate between related prodromal symptoms associated with the underlying disease and those associated with ALF. The patient had malaise and nausea for 2 weeks before ALF was diagnosed, and these symptoms are often neglected by patients as they were in our case.

The underlying etiology of ALF with malignant infiltration includes mainly non-Hodgkin's lymphoma and Hodgkin's

disease (3, 4). Scattered reports show that metastatic carcinoma from lungs (3, 7) and breast represents a rare cause. Our case is distinct from previous reports of ALF due to malignant infiltration of the liver from PNET. PNETs are divided into four major types: small cell lung cancer (SCLC), large cell neuroendocrine carcinoma, atypical carcinoid, and typical carcinoid. The liver biopsy in our case confirmed that the PNET responsible for ALF was the SCLC type, which has never been reported before. PNET has been described to metastasize to other organs and cause the secretion of various hormones, but it is unusual to manifest as ALF. Early liver biopsy with prompt immunostaining is necessary to determinate the diagnosis to ensure appropriate treatment. Lung NETs of the SCLC subtype at stage IV have a poor prognosis and are associated with shorter survival time (8). The Ki-67 level is associated with the degree of differentiation, prognosis, and survival rate (9). Our patient showed 90% Ki-67 staining. Surgery remains the only choice for cure (10), although most patients are diagnosed with metastatic disease, and curative surgery is usually not possible. Long-term systemic treatment with somatostatin analogs and peptide receptor radionuclide therapy alone or in combination can be given to patients with advanced disease who are unsuitable for surgery. Although thorough evaluation of the patient, ideal timing of treatment initiation, and the administration of various regimens are difficult (11), successful diagnosis and prompt treatment have been shown to increase survival and, to a certain extent, be beneficial for symptomatic relief.

CONCLUSION

Most patients with ALF due to neoplastic invasion have a dismal prognosis. The mortality rate of diffuse hepatic tumor infiltration varies from 3 days to 6 months after presentation (12). It should be noted that only accurate histological diagnosis following liver biopsy and early initiation of specific therapy in such patients will provide the best chance of recovery.

AUTHOR CONTRIBUTIONS

PY reviewed the literature and contributed to manuscript drafting, analyzed and interpreted the imaging findings. XC contributed to manuscript drafting and was responsible for revision of the manuscript for important intellectual content. YL and QW contributed to the analysis, interpretation, critical revision, and final approval of the manuscript. All authors contributed to the article and approved the submitted version. These authors (PY and YL) contributed equally to this work.

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ORIGINAL RESEARCH
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Identification of a Novel Four-Gene Signature Correlated With the Prognosis of Patients With Hepatocellular Carcinoma: A Comprehensive Analysis

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Purpose: Hepatocellular carcinoma (HCC) is a common solid-tumor malignancy with high heterogeneity, and accurate prognostic prediction in HCC remains difficult. This analysis was performed to find a novel prognostic multigene signature.

Methods: The TCGA-LIHC dataset was analyzed for differentially coexpressed genes through weighted gene coexpression network analysis (WGCNA) and differential gene expression analysis. A protein-protein interaction (PPI) network and univariate Cox regression analysis of overall survival (OS) were utilized to identify their prognostic value. Next, we used least absolute shrinkage and selection operator (LASSO) Cox regression to establish a prognostic module. Subsequently, the ICGC-LIRI-JP dataset was applied for further validation. Based on this module, HCC cases were stratified into high-risk and low-risk groups, and differentially expressed genes (DEGs) were identified. Functional enrichment analyses of these DEGs were conducted. Finally, single-sample gene set enrichment analysis (ssGSEA) was performed to explore the correlation between the prognostic signature and immune status.

Results: A total of 393 differentially coexpressed genes were obtained. Forty differentially coexpressed hub genes were identified using the CytoHubba plugin, and 38 of them were closely correlated with OS. Afterward, we established the four-gene prognostic signature with an acceptable accuracy (area under the curve [AUC] of 1-year survival: 0.739). The ICGC-LIRI-JP dataset also supported the acceptable accuracy (AUC of 1-year survival:0.752). Compared with low-risk cohort, HCC cases in the high-risk cohort had shorter OS, higher tumor grades, and higher T stages. The risk scores of this signature still act as independent predictors of OS (P<0.001). Functional enrichment analyses suggest that it was mainly organelle fission and nuclear division that were enriched. Finally, ssGSEA revealed that this signature is strongly associated with the immune status of HCC patients.

Conclusions: The proposed prognostic signature of four differentially coexpressed hub genes has satisfactory prognostic ability, providing important insight into the prediction of HCC prognosis.

Keywords: hepatocellular carcinoma, least absolute shrinkage and selection operator Cox regression, weighted gene coexpression network analysis, prognostic signature, single-sample gene set enrichment analysis, immune status

INTRODUCTION

It is estimated that nearly 42,810 new cases and 30,160 estimated deaths of hepatocellular carcinoma (HCC) will occur in 2020, leading to enormous socioeconomic pressure for HCC patients and their families (1). HCC accounts for 85%–90% of all primary liver cancer patients, and its occurrence is strongly associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcohol consumption, and nonalcoholic steatohepatitis (2). HCC has high interpatient, intertumoral and intratumoral heterogeneity (3). Patients with localized HCC usually have poor survival (with a 5-year overall survival [OS] rate of 30%), and this rate is less than 5% for HCC patients with distant metastasis (4). Currently, due to the complicated etiologic factors and the high heterogeneity of HCC, it remains difficult to accurately predict the prognosis of HCC patients. Although there were some similar studies published previously, they usually required many genes in their gene signatures, which may cause some difficulties in real-world practice (5, 6). Therefore, it is urgent to find the gene signature involved with less genes for the convenience of real-world practice.

With the rapid development of genome technology, bioinformatics analysis has been adopted for microarray datasets to further explore the underlying molecular mechanisms of diseases and detect disease-specific biomarkers (7). Weighted gene coexpression network analysis (WGCNA) is utilized to further understand gene coexpression networks and gene functions (8). WGCNA detects modules of closely correlated genes among samples to relate modules to external traits, providing significant insights into predicting possible functions of coexpressed genes (9). Additionally, differential gene expression analysis is often utilized in transcriptomic datasets to investigate potential biological and molecular mechanisms and quantify differences between the gene expression levels of experimental and control cohorts (10).

To increase the reliability of screening highly related genes, both methods mentioned above were used in our analysis. First, the RNA-Seq dataset and HCC clinical information were downloaded from The Cancer Genome Atlas (TCGA) database. Second, WGCNA and differential gene expression analysis were performed to obtain differentially coexpressed genes. Then, a protein-protein interaction (PPI) network was constructed, and 38 differential coexpression hub genes with prognostic value were detected. Afterward, we built a prognostic four-gene signature and verified it in the International Cancer Genome Consortium (ICGC) database. Ultimately, functional enrichment analysis was conducted to investigate the underlying biological mechanisms.

MATERIALS AND METHODS

The detailed process of data downloading, prognostic signature construction and external validation is presented in **Figure 1**. The details of each step are illustrated in the following subsections.

Datasets Downloaded From the TCGA and ICGC Databases

First, RNA-Seq and corresponding clinical data for liver hepatocellular carcinoma (LIHC) were obtained from the TCGA database (https://portal.gdc.cancer.gov/). A list of 424 samples was obtained, including 374 LIHC and 50 normal liver tissues, and RNA-seq count data on 19645 genes were obtained. The Illumina HiSeq 2000 platform was used to generate and annotate all data to a reference transcript set of the human hg38 gene standard track. The edgeR package tutorial suggested that genes with low read counts do not merit further analysis (11). Hence, genes with a count per million (CPM) <1 were omitted from this analysis. Next, the function rpkm in the edgeR package was adapted for further filtering. Consequently, 13,924 genes were acquired for subsequent analysis. Second, the RNA-Seq data and clinical data of HCC patients were acquired from the ICGC database (https://dcc.icgc.org/). A total of 260 HCC samples, which mainly originated from the Japanese population with HBV or HCV infection, were acquired (12).

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; WGCNA, Weighed Gene Co-expression Network Analysis; DEGs, differentially expressed genes; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; ssGSEA, single-sample Gene Set Enrichment Analysis; LIHC, liver hepatocellular carcinoma; PPI, protein-protein interaction network; FDR, false discovery Rate; BH, Benjamini–Hochberg; TOM, topological overlap matrix; CPM, count per million; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes pathway; STRING, Search Tool for the Retrieval of Interacting Genes; OS, overall survival; HR, hazard ratio; CI, confidence interval; LASSO, the least absolute shrinkage and selection operator analysis; ROC, receiver operating characteristic curves; PCA, principal component analysis; PC1, the first principal component; PC2, the second principal component; t-SNE, t-distributed stochastic neighbor embedding; tSNE1, the first component of t-SNE; tSNE2, the second component of t-SNE; AUC, Area Under Curve; aDCs, activated dendritic cells; IFN, interferon; DCs, dendritic cells; iDCs, immature Dendritic Cells; pDCs, plasmacytoid dendritic cells; NK cells, natural killer cells; Tfhs, follicular helper cells; Th1 cells, helper one T-cells; Th2 cells, helper two T-cells; TIL, tumor infiltrating lymphocytes; Treg, regulatory T cells; APC, antigen presenting cell; CCR, chemokine receptor type; HLA, human leukocyte antigen; MHC, major histocompatibility complex; RT-qPCR, real-time quantitative PCR; DFS, disease-free survival; RFS, recurrence-free survival; mTORC1, mammalian target of rapamycin complex1 signaling.

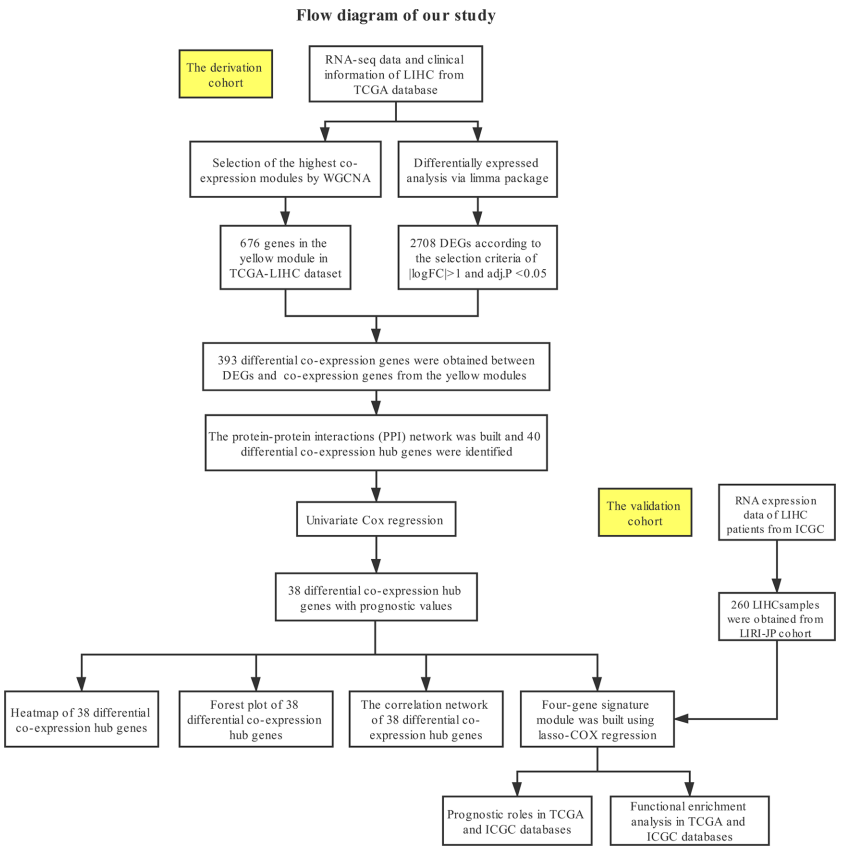

FIGURE 1 | Study design and workflow of this study.

We chose the normalized read count values of the ICGC-LIRI-JP cohort. As a result, 22,913 genes were obtained for the next analysis.

Identification of Key Coexpression Modules Using WGCNA

The gene coexpression network of the TCGA-LIHC dataset was built through the WGCNA package (8). To build a scale-free network, a soft-power $\beta = 7$ (**Figures 2A, B**) was used in the TCGA-LIHC dataset. Next, the adjacency matrix was created according to the formula $aij = |Sij|^{\beta}$ (aij: adjacency matrix between gene i and gene j, Sij: similarity matrix made by Pearson's correlation coefficient of all gene pairs, as well as β: soft-power value). Subsequently, we converted this matrix into a topological overlap matrix (TOM) and the corresponding dissimilarity (1-TOM). The hierarchical clustering dendrogram of the 1-TOM matrix was established to aggregate the genes with similar expression patterns into the same coexpression module. Afterward, the module-trait relations between modules and external traits were analyzed to identify functional modules from the coexpression network. Hence, the modules with the largest correlation coefficients were regarded as modules that highly correlated with clinical traits. We chose the module that was positively associated with LIHC for our subsequent analysis.

Identification of Differentially Coexpressed Genes

The limma package is often used to perform differential gene expression analysis of gene expression profiles and RNA-Seq datasets (13). Here, we applied the limma package in the differential expression analysis of the TCGA-LIHC dataset to identify differentially expressed genes (DEGs) between LIHC and nontumorous tissues. To minimize the false discovery rate (FDR) to the greatest extent possible, we adjusted the P-value with the Benjamini–Hochberg (BH) method. The filtering criteria for DEGs were $|logFC|>1$ and adj. $P<0.05$. Afterward, we took the intersection of genes between DEGs and coexpressed genes to improve the reliability of screening closely related genes, and these differentially coexpressed genes were used for the next analysis.

PPI Network Construction and Hub Gene Identification

The PPI network of differentially coexpressed genes was built through the Search Tool for the Retrieval of Interacting Genes (STRING) database (14). Then, we established a visual network of molecular interactions with combined scores ≥0.7 using Cytoscape (15). In addition, the degree values of all nodes in the PPI network were calculated using the CytoHubba plugin (16).

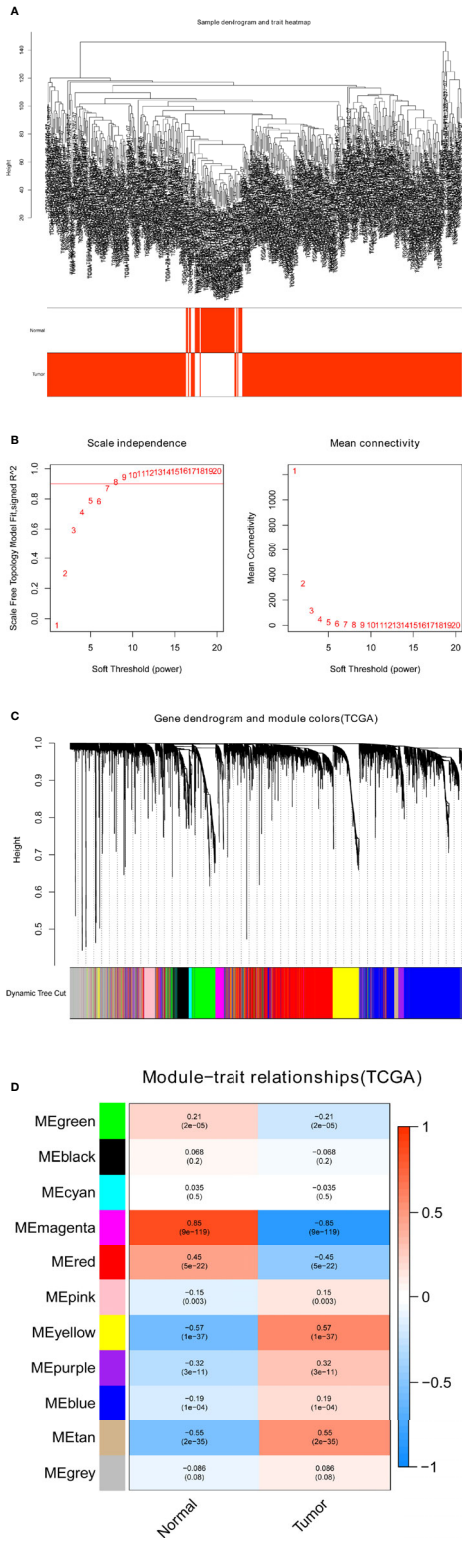

FIGURE 2 | Identification of modules related to the clinical traits in the TCGA-LIHC dataset. **(A)** Sample dendrogram and trait heatmap. **(B)** Scale independence and Mean connectivity. **(C)** The cluster dendrogram of co-expression network modules is ordered by a hierarchical clustering of genes based on the 1-TOM matrix. Different colors represent different modules. **(D)** Module-trait relationships. Each row represents a color module and every column represents a clinical trait (normal and tumor). Each cell contains the corresponding correlation and P-value.

The top 40 nodes with the highest degree scores were selected and regarded as hub genes associated with LIHC. The forty hub genes related to LIHC were displayed using the CytoHubba plug-in. In addition, we conducted gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the 40 hub genes to explore their biological functions. Adj. P values <0.05 were considered significant.

Survival Analysis of Hub Genes and the Correlation Network

To analyze the prognostic roles of the differentially coexpressed hub genes in LIHC, we performed univariate Cox regression analysis of OS using the survival package based on the TCGA-LIHC dataset. LIHC patients without follow-up information or a survival time=0 days were excluded from our analysis, and the other patients in the TCGA-LIHC dataset were classified into two groups considering the median expression levels of the differentially coexpressed hub genes. Log-rank P<0.01 was considered significant. Additionally, the correlation network of these differentially coexpressed hub genes was established through the igraph package. The filtering criterion was a cutoff >0.75.

Construction of the Gene Signature in the TCGA Database

To decrease the risk of overfitting to the greatest extent possible, we used least absolute shrinkage and selection operator (LASSO) Cox regression analysis to build the prognostic module of LIHC (17, 18). The LASSO algorithm is widely utilized to select and shrink variables using the glmnet package. We used the expression matrix of the differentially coexpressed hub genes with prognostic value as the independent variable, while the OS and status of patients in the TCGA-LIHC dataset were used as the response variables. Then, we determined the penalty parameter (λ) of this module using tenfold cross-validation following the minimum criteria, namely, the λ value corresponding to the minimum partial likelihood deviance.

Nomogram and Validation of the Expression Patterns of the Gene Signature

We calculated the risk scores of all LIHC patients using the expression level of every gene and the corresponding regression coefficient. The following formula was used: score= $e^{\text{sum (every gene's expression} \times \text{corresponding coefficient)}}$. Then, LIHC patients were divided into high- and low-risk cohorts based on the median value of the risk score. Subsequently, we constructed a nomogram of the prognostic signature to predict the survival of LIHC patients. Furthermore, we built calibration curves and time-dependent receiver operating characteristic (ROC) curves to evaluate the discrimination and accuracy of the prognostic multigene signature. The GSE112790 dataset was used to validate the expression patterns of the genes in the signature between LIHC and nontumorous tissues.

Distribution and Prognostic Value of the Gene Signature

To analyze the prognostic value of the gene signature, we performed Kaplan-Meier survival analysis between the low- and high-risk groups using the survminer package based on the TCGA-LIHC and ICGC-LIRI-JP datasets. Additionally, to explore distribution in the low- and high-risk cohorts, we performed principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) on the TCGA-LIHC and ICGC-LIRI-JP datasets using the stats and Rtsne packages, respectively. To determine whether the risk score acts as an independent indicator of the prognosis of LIHC patients, we performed univariate and multivariate Cox regression analyses among all available variables using the TCGA-LIHC and ICGC-LIRI-JP datasets.

Differential Gene Expression Analysis and Functional Enrichment Analysis

To acquire the DEGs between the low- and high-risk groups, we performed differential gene expression analysis using the limma package in the TCGA-LIHC and ICGC-LIRI-JP datasets. The P-value was adjusted using the BH method. The filtering criteria for DEGs were |logFC|>2 and adj. P <0.05. Afterward, we conducted GO and KEGG pathway analyses of the DEGs between the low- and high-risk groups in the TCGA-LIHC and ICGC-LIRI-JP datasets. To further analyze the relationship between the risk score and immune status, we calculated the infiltrating scores of 16 immune cells and 13 immune-related functions or pathways using single-sample gene set enrichment analysis (ssGSEA) (19).

RESULTS

Identification of Key Coexpression Modules Using WGCNA

To find the pivotal module in LIHC, the gene coexpression network was established in the TCGA-LIHC dataset. A list of 11 modules was generated (**Figure 2C**). Next, the heatmap revealed the correlations between the modules and clinical traits (normal and LIHC) in the TCGA-LIHC dataset (**Figure 2D**). Furthermore, the yellow module of the TCGA-LIHC dataset positively correlated with LIHC tissues (r=0.57, P=1e-37) and was used for our next analysis.

Selection of Differentially Coexpressed Genes

The heatmap displayed the expression patterns of fifty upregulated and fifty downregulated genes in the TCGA-LIHC dataset (**Figure 3A**). The volcano plot indicated that 2708 DEGs had a conspicuous dysregulation between LIHC and nontumorous tissues in the TCGA-LIHC dataset (**Figure 3B**). The Venn diagram showed the intersection of coexpressed genes (**Table S1**) and DEGs (**Table S2**); namely, 393 differentially coexpressed genes were identified (**Figure 3C**).

PPI Network Construction and Hub Gene Analysis

Figure 4A displays the PPI network of the differentially coexpressed genes with 241 nodes and 4792 edges. Subsequently, we quantified the degree scores of all nodes in this PPI network through the

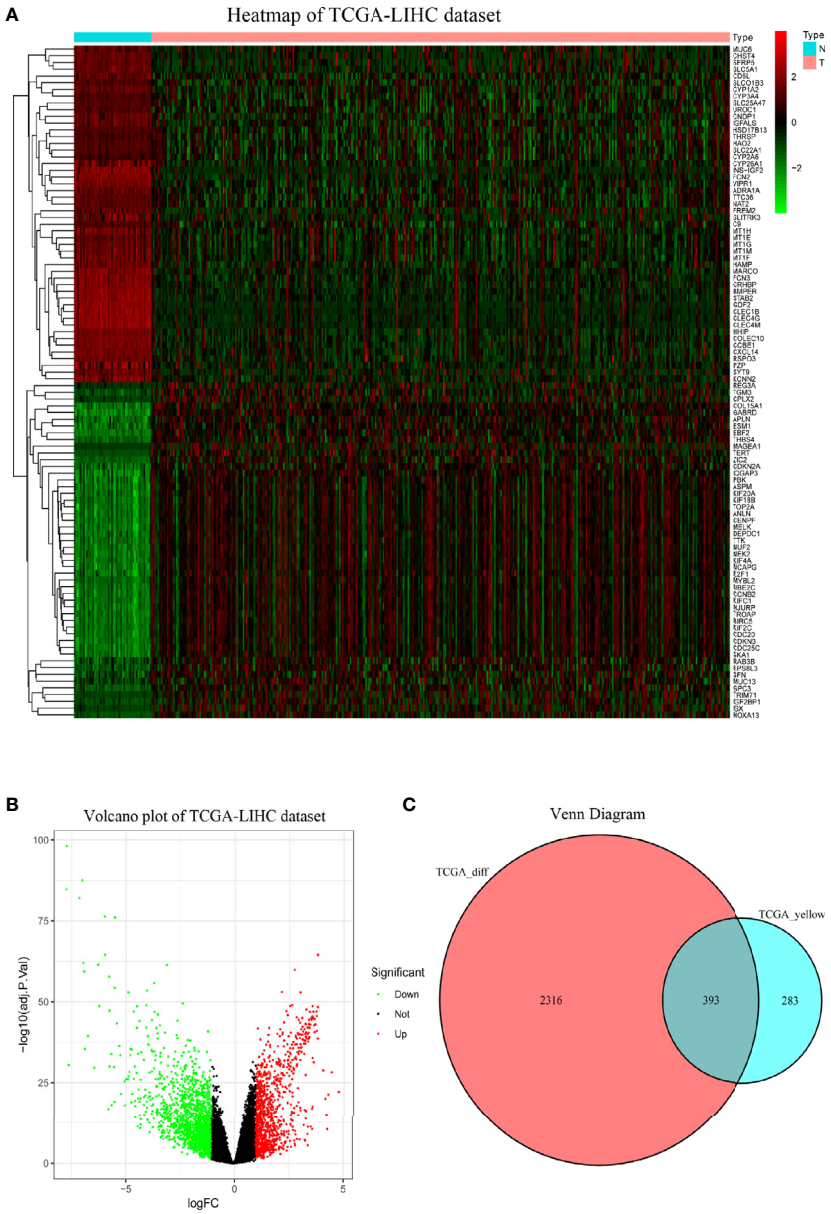

FIGURE 3 | Identification of differentially expressed genes (DEGs) in TCGA-LIHC dataset with the cut-off criteria of |logFC|>1 and adj.P <0.05. **(A)** Heatmap of top 50 upregulated and 50 downregulated DEGs of TCGA-LIHC dataset. **(B)** Volcano plot of DEGs in the TCGA-LIHC dataset. **(C)** The Venn diagram of genes between DEGs and co-expression genes. A total of 393 overlapping differential co-expression genes are detected.

CytoHubba plugin (**Table S3**) and chose the top 40 nodes as hub genes that are closely correlated with LIHC (**Figure 4B**). In addition, GO analysis showed significant enrichment in the mitotic nuclear division, organelle fission and spindles terms (**Figure S1A**). KEGG pathway analysis showed enrichment in the cell cycle and oocyte meiosis pathways (**Figure S1B**).

Survival Analysis and Correlation Network of the Differentially Coexpressed Hub Genes

Univariate Cox regression analysis of the differentially coexpressed hub genes demonstrated that 38 hub genes were closely associated with the survival of LIHC patients (**Figure 5A**). The heatmap revealed that the 38 hub genes with prognostic value were significantly overexpressed in LIHC tissues (**Figure 5B**). Additionally, the correlation network suggested that the differentially coexpressed hub genes closely interact with each other (**Figure 5C**).

Construction of the Gene Signature and Nomogram in the TCGA Database

We used the LASSO Cox regression module to build a prognostic signature based on the expression matrix of the 38 differentially

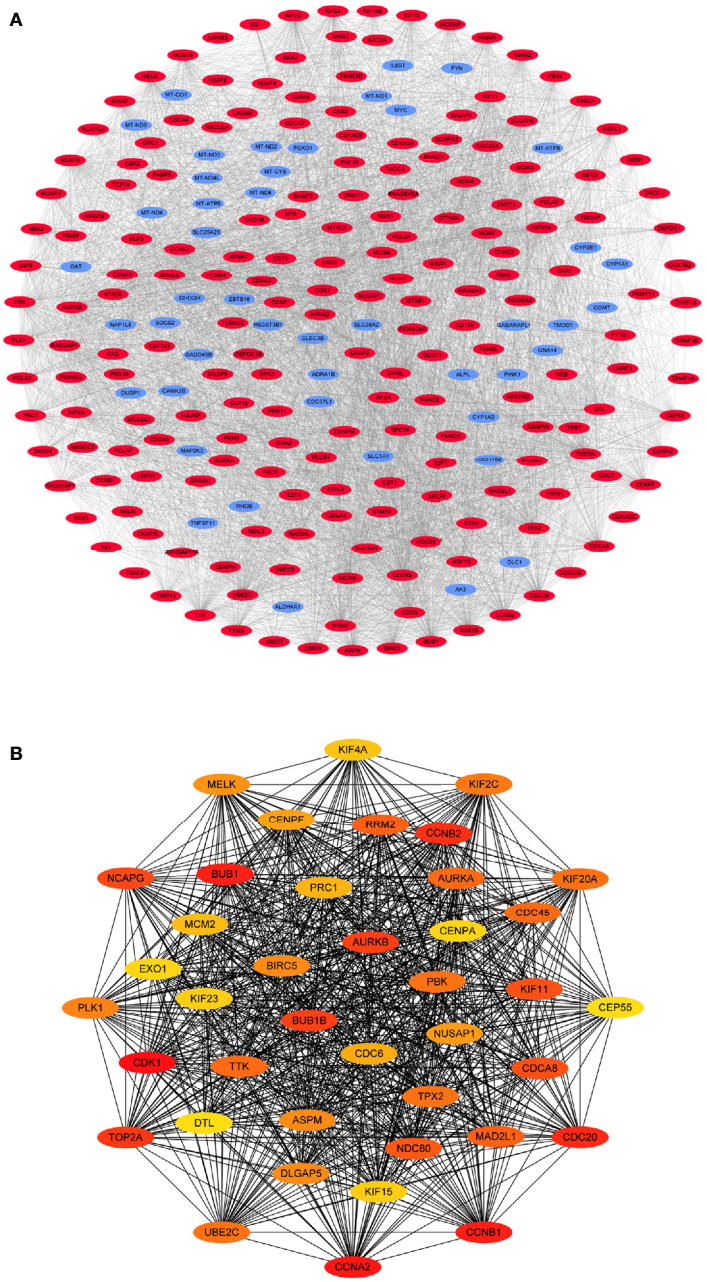

FIGURE 4 | Visualization of the protein-protein interaction (PPI) network and hub genes. **(A)** PPI network of differential co-expression genes. **(B)** The identification of 40 differential co-expressed hub genes using the degree algorithm.

coexpressed hub genes. Consequently, we identified a four-gene signature module according to the optimal λ value (**Figures 6A, B**). In addition, we calculated the risk scores of LIHC patients using the following formula: score= e $^{(0.225*\text{expression value of CDCA8}+0.124*\text{expression value of KIF20A}+0.012*\text{expression value of KIF2C}+0.144*\text{expression value of CEP55})}$.

Then, we established a nomogram to predict the 1-, 2-, and 3-year OS probability of LIHC patients (**Figure 6C**). The calibration curves of 1-, 2-, and 3-year OS probability showed satisfactory calibration of this nomogram (**Figures 6D–F**). Moreover, based on GSE112790, we confirmed that CDCA8, KIF20A, KIF2C and CEP55 were significantly overexpressed in LIHC tissues compared with nontumorous tissues (**Figure S2**). Furthermore, the ROC curves suggested acceptable accuracy of this nomogram (area under the curve [AUC] of 1-year survival: 0.739; AUC of 2-year survival: 0.714; and AUC of 3-year survival: 0.673) (**Figure 7A**). Afterward, all LIHC patients were divided into a low-risk cohort (n=183) and a high-risk cohort (n=182) based on the median risk score (**Figure 7B**). The high-risk cohort in the TCGA-LIHC dataset

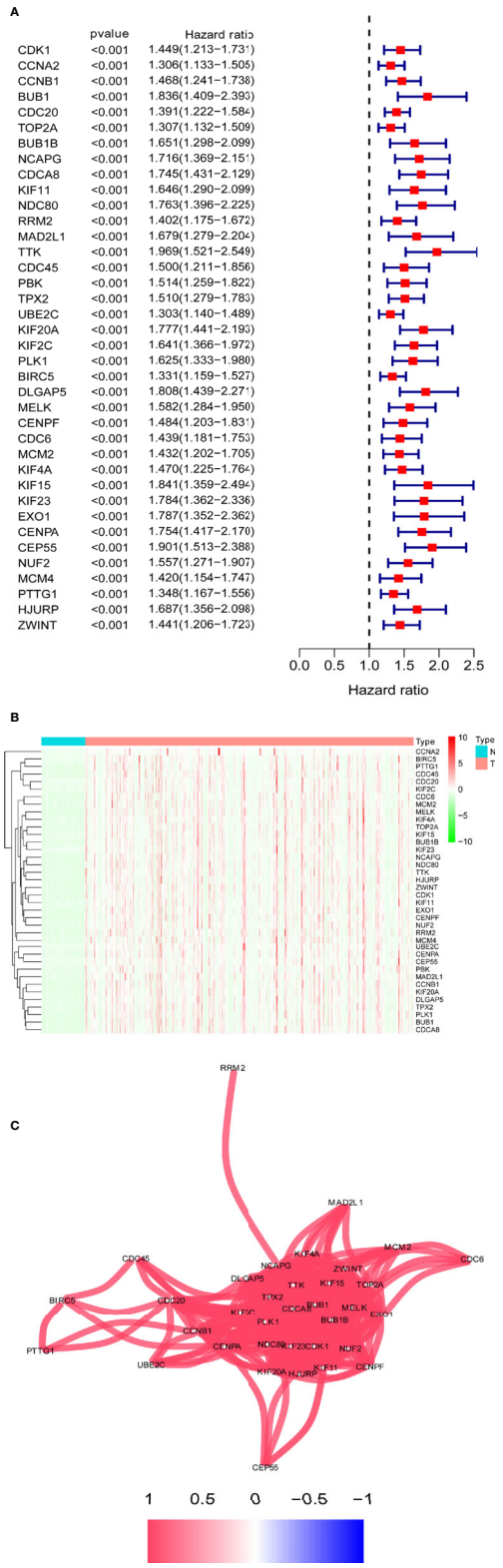

FIGURE 5 | Identification differential co-expressed hub genes with prognostic values. **(A)** Univariate Cox analysis for overall survival (OS) of 38 differential co-expressed hub genes with prognostic values. **(B)** 38 differential co-expressed hub genes with prognostic values are significantly upregulated in HCC tissues. **(C)** The correlation network of candidate genes. The correlation coefficients are represented by different colors.

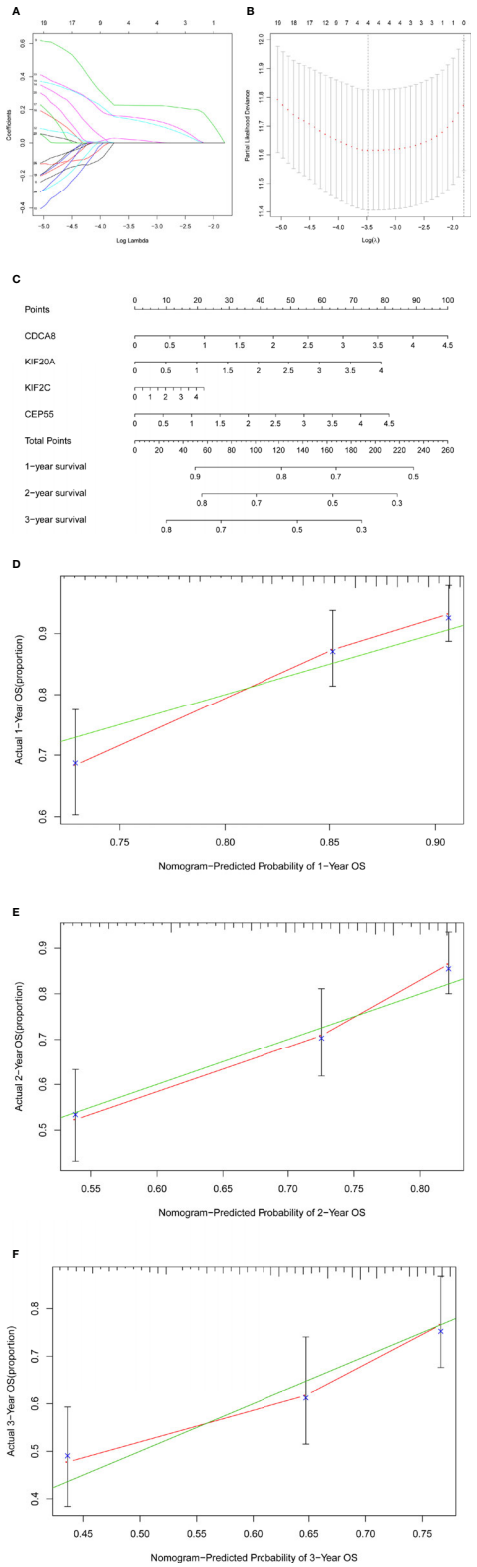

FIGURE 6 | |Construction of the gene signature and nomogram in TCGA-LIHC dataset. **(A, B)** The construction of the four-gene signature module. **(C)** The construction of the nomogram of this module. **(D–F)** The calibration curves of 1-, 2-, and 3-year overall survival probability.

had more deaths (**Figure 7C**), a poorer tumor grade, a higher clinical stage and a higher T stage (**Table 1**). Consistently, Kaplan-Meier survival analysis showed that LIHC patients in the high-risk cohort experienced shorter survival than those in the low-risk cohort (**Figure 7D**, P=1.14e-4). In the PCA of the TCGA-LIHC dataset, the first principal component (PC1) could explain 88.6% of total variance, and the PC1 scores were negatively correlated with the risk scores of patients (**Figure 7E**), while the second principal component (PC2) could explain 5.4% total variance (**Figure S3A**). Moreover, PCA and t-SNE analysis revealed that most LIHC patients in the high- and low-risk cohorts were distributed in two different directions (**Figure 7F**).

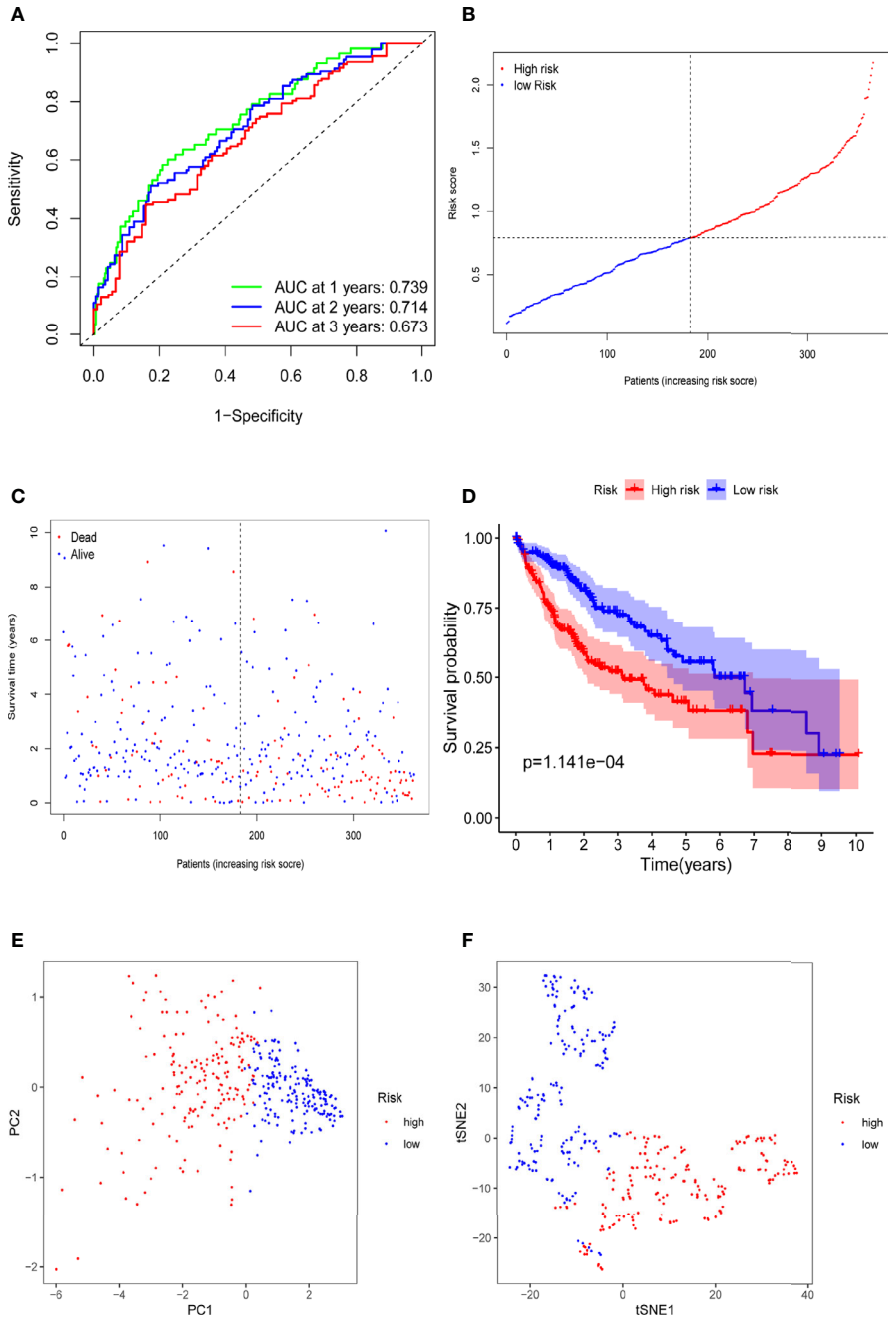

FIGURE 7 | Prognostic analysis of the four-gene signature model in TCGA-LIHC dataset. **(A)** AUC of time-dependent ROC curves verifies the prognostic performance of the risk score in TCGA-LIHC dataset. **(B)** The distribution and the median value of the risk scores in TCGA-LIHC dataset. **(C)** The distributions of OS status, OS and risk score in the TCGA-LIHC dataset. **(D)** Kaplan-Meier curves for the OS of patients in the high-risk group and low-risk group in TCGA-LIHC dataset. **(E)** PCA plot of TCGA-LIHC dataset. **(F)** t-SNE analysis of TCGA-LIHC dataset.

TABLE 1 | Baseline characteristics of LIHC patients in high-risk and low-risk cohorts.

Baseline characteristics		TCGA-LIHC dataset			ICGC-LIRP-JI dataset		
		High-risk	Low-risk	P-value	High-risk	Low-risk	P-value
Age (%)	≤60 year	97 (53.3)	76 (41.5)	**0.024**	35 (21.5)	15 (21.7)	0.964
	>60 year	85 (46.7)	105 (58.5)		128 (78.5)	54 (78.3)	
Gender (%)	Female	66 (36.3)	53 (29.0)	0.137	44 (27.2)	17 (24.6)	0.691
	Male	116 (63.7)	130 (71.0)		118 (72.8)	52 (75.4)	
Tumor grade (%)	G1 + G2	94 (51.6)	136 (74.3)	**<0.001**	–	–	–
	G3 + G4	85 (46.7)	45 (24.6)		–	–	
	unknown	3 (1.6)	2 (1.1)		–	–	
Clinical stage (%)	I + II	116 (63.7)	138 (75.4)	**0.008**	–	–	–
	III + IV	54 (29.7)	33 (18.0)		–	–	
	unknown	12 (6.6)	12 (6.6)		–	–	
T stage (%)	T1 + T2	124 (68.1)	147 (80.3)	**0.003**	96 (59.3)	45 (65.2)	0.395
	T3 + T4	58 (31.9)	33 (18.0)		66 (40.7)	24 (34.8)	
	unknown	0 (0.0)	3 (1.7)		0 (0.0)	0 (0.0)	
N stage (%)	N0	127 (69.8)	121 (66.1)	0.962	–	–	–
	N1 + N2 + N3	2 (1.1)	3 (1.6)		–	–	
	unknown	53 (29.1)	53 (29.0)		–	–	
M stage (%)	M0	136 (74.7)	127 (69.4)	0.230	–	–	–
	M1	0 (0.0)	3 (1.6)		–	–	
	unknown	46 (25.3)	53 (29.0)		–	–	

LIHC, liver hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium.
The bold P values means P < 0.05.

Verification of the Four-Gene Signature Module in the ICGC Database

To validate the robustness of the four-gene signature module from the TCGA-LIHC dataset, we chose the ICGC-LIRI-JP dataset for further verification. First, we stratified LIHC patients from the ICGC-LIRI-JP dataset into high-risk and low-risk cohorts according to the median value of the risk score, which was calculated using the formula mentioned above. Consistent with the outcomes from the TCGA-LIHC dataset, the four-gene signature had an excellent AUC (**Figure 8A**, 1-year survival: 0.752; 2-year survival: 0.751; and 3-year survival: 0.782). Moreover, the high-risk group correlated with a higher rate of mortality (**Figures 8B, C**). Additionally, patients from the high-risk cohort experienced significantly shorter survival than those in the low-risk cohort (**Figure 8D**, P=1.24e-3). In the PCA of the ICGC-LIRI-JP dataset, the PC1 could explain 79% of total variance, and the PC1 scores were positively correlated with the risk scores of patients (**Figure 8E**), whereas the PC2 could explain 13% total variance (**Figure S3B**). In addition, t-SNE analysis validated that most patients in the high- and low-risk cohorts were distributed in two different directions (**Figure 8F**). In general, these outcomes in the ICGC-LIRI-JP dataset were similar to those in the TCGA-LIHC dataset.

Independent Prognostic Role of the Four-Gene Signature

To determine whether the risk score plays an independent prognostic role, we performed univariate and multivariate Cox regression analyses of the survival of LIHC patients. The univariate Cox regression analysis indicated that a higher risk score was closely correlated with worse survival in LIHC patients using the TCGA-LIHC (**Figure 9A**, hazard ratio [HR]=3.324, 95% confidence interval [CI]: 2.181–5.066, P<0.001) and ICGC-LIRI-JP (**Figure 9B**, HR=1.413, 95% CI: 1.243–1.607, P<0.001) datasets. Similar to the results of the univariate Cox regression analysis, the multivariate Cox regression analysis still suggested the risk score as an independent indicator for the survival of LIHC patients using the TCGA-LIHC (**Figure 9C**, HR=3.041, 95% CI: 1.930–4.790, P<0.001) and ICGC-LIRI-JP (**Figure 9D**, HR=1.378, 95% CI: 1.210–1.569, P<0.001) datasets.

Differential Gene Expression Analysis and Functional Enrichment Analysis

Differential gene expression analyses were conducted in the TCGA-LIHC and ICGC-LIRI-JP datasets, and 499 and 185 DEGs between the high- and low-risk groups were obtained (**Tables S4**, **S5**), respectively. To explore the biological functions of the DEGs in the high- and low-risk groups, we again performed GO enrichment and KEGG pathway analyses. In the TCGA-LIHC dataset, GO enrichment analysis indicated significant enrichment in the organelle fission, nuclear division, chromosomal region and ATPase activity terms (**Figure 10A**). GO enrichment analysis of the ICGC-LIRI-JP dataset showed similar outcomes to the TCGA-LIHC dataset (**Figure 10B**). Additionally, KEGG pathway analysis of the TCGA-LIHC dataset showed significant enrichment in the cell cycle, oocyte meiosis and progesterone-medicated oocyte maturation pathways (**Figure 10C**). In the ICGC-LIRI-JP dataset, KEGG pathway analysis also demonstrated the analogical outcomes of the TCGA-LIHC dataset (**Figure 10D**).

To explore the correlation between the risk score and immune status, we calculated the infiltrating scores of 16 immune cells and 13 immune-related functions or pathways using ssGSEA. The scores of activated dendritic cells (aDCs), mast cells and follicular helper cells (Tfhs) were notably different between the high- and low-risk groups in the TCGA-LIHC dataset (all adj. P<0.001, **Figure 11A**). In the TCGA-LIHC dataset, the scores of cytolytic activity, type I interferon (IFN) response and type II

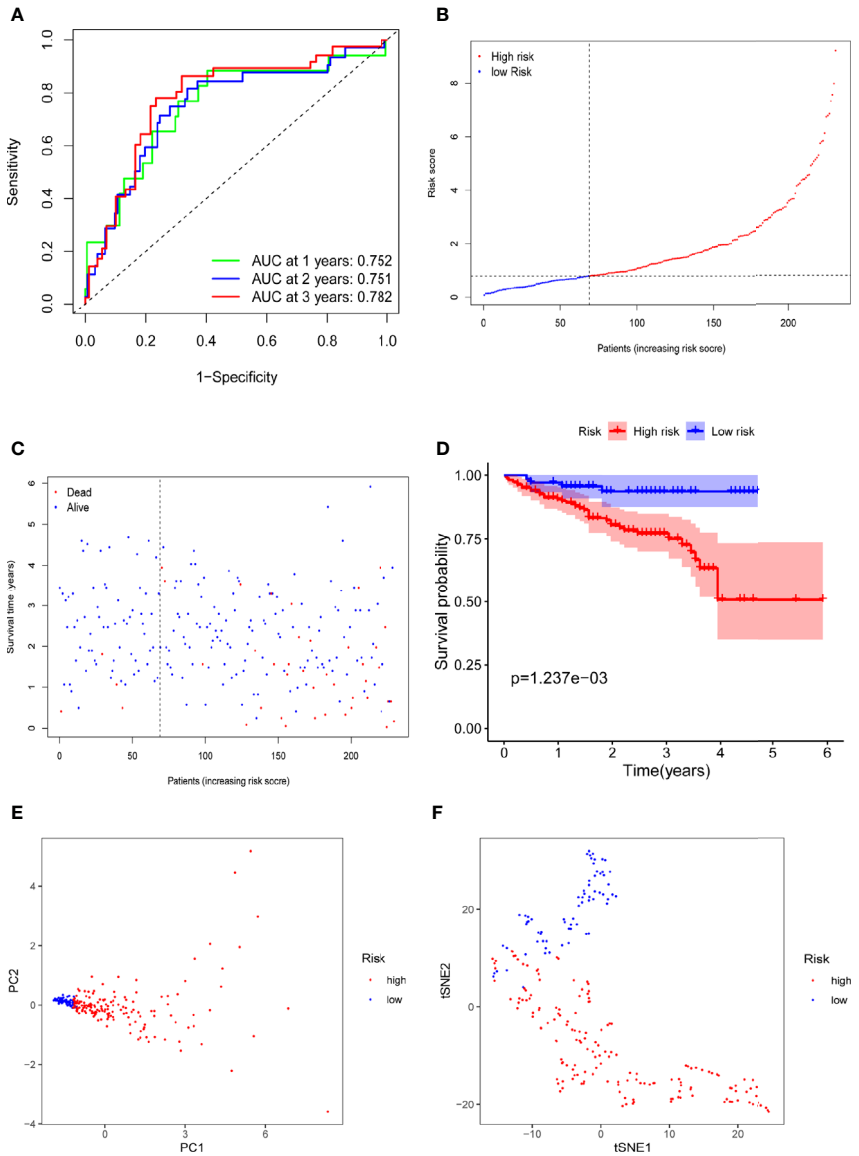

FIGURE 8 | Validation of the 10-gene signature in ICGC-LIRI-JP dataset. **(A)** AUC of time-dependent ROC curves verifies the prognostic performance of the risk score in ICGC-LIRI-JP dataset. **(B)** The distribution and the median value of the risk scores in ICGC-LIRI-JP dataset. **(C)** The distributions of OS status, OS and risk scores in ICGC-LIRI-JP dataset. **(D)** Kaplan-Meier curves for the OS of patients in the high-risk group and low-risk group in ICGC-LIRI-JP dataset. **(E)** PCA plot of ICGC-LIRI-JP dataset. **(F)** t-SNE analysis of ICGC-LIRI-JP dataset.

IFN response were obviously higher in the low-risk group, while the score of MHC class I was lower in the low-risk group (all adj. P<0.01, **Figure 11B**). Moreover, the ICGC-LIRI-JP dataset showed that aDCs, mast cells, MHC class I and type II IFN responses were significantly different between the two risk cohorts (**Figures 11C, D**), which is consistent with the results of the TCGA-LIHC dataset.

DISCUSSION

As a common solid-tumor malignancy with high mortality, HCC has brought great socioeconomic pressure to HCC patients and their families. Owing to the complex etiological factors and high heterogeneity of HCC, it remains difficult to accurately predict the survival of HCC patients. Thus, it is urgent to detect effective prognostic biomarkers to monitor the progression and predict the prognosis of HCC patients. In this study, 393 differentially coexpressed genes were obtained through WGCNA and differential gene expression analysis. Then, these genes were used to construct a PPI network, and 38 hub genes were observed to be closely correlated with OS. Subsequently, we established a novel four-gene prognostic signature in the TCGA-LIHC dataset and built a nomogram based on this novel module, which showed acceptable accuracy

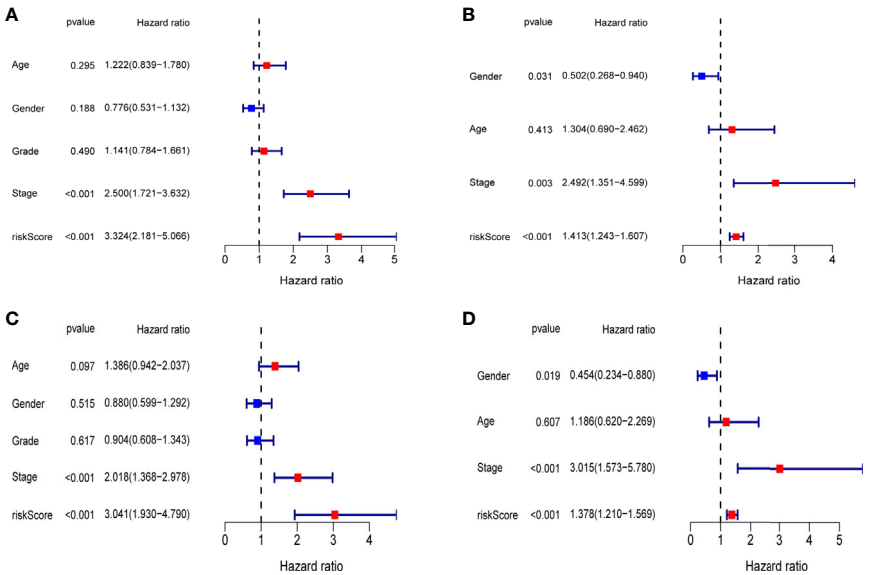

FIGURE 9 | Independent prognostic role of the four-gene signature. **(A)**The univariate Cox regression analysis in TCGA-LIHC dataset. **(B)**The univariate Cox regression analysis in ICGC-RI-JP dataset. **(C)** The multivariate Cox regression analysis in TCGA-LIHC dataset. **(D)** The multivariate Cox regression analysis in ICGC-LIRI-JP dataset.

and calibration. Afterward, the four-gene signature module was verified in the TCGA-LIHC dataset using the LASSO algorithm. To improve the robustness of the signature, we used the ICGC-LIRI-JP dataset for further validation. The four-gene signature was still found to have independent prognostic value. Finally, ssGSEA revealed significant differences in aDCs, mast cells, MHC class I and type II IFN responses between the two risk cohorts.

Several prior analyses have also shown that certain gene signatures may predict patient survival (20–26); however, our study has some differences and/or advantages compared with similar analyses. First, the gene signatures built in previous studies require many genes (20–23), which possibly leads to some difficulties in real-world practice. Our novel signature requires only 4 genes, and the predictive ability of our signature is acceptable, which increases the feasibility of the use of our signature in real-world practice. Second, in our study, we simultaneously used WGCNA, differential gene expression analysis, PPI network construction, univariate Cox regression analysis and LASSO Cox regression analysis, and these methods were rarely used together in one study for the construction of a prognostic module of HCC, which is a novel point of our study. Third, some previous studies did not verify their gene signature (24–26) using other datasets; however, we used two datasets (the ICGC-LIRI-JP dataset and GSE112790) for external validation, which is helpful to enhance the reliability of our findings. Interestingly, we observed that most differentially coexpressed hub genes (38/40) were significantly associated with survival time according to the results of the univariate Cox regression analysis. This finding suggests the possibility of establishing a prognostic signature using these differentially coexpressed hub genes.

The prognostic module proposed in our analysis was composed of CDCA8, KIF20A, KIF2C and CEP55, all of which are often reported as being dysregulated in HCC tissues (27–30). First, cell division cycle associated 8 (CDCA8) is regarded as a significant oncogene that is involved in the pathological development of various cancers, including HCC (27) and pancreatic ductal adenocarcinoma (31). Wu et al. reported that CDCA8 is obviously overexpressed at the mRNA and protein levels in HCC tissues, and the authors validated this finding at the mRNA level using real-time quantitative PCR (RT-qPCR) (32). Similarly, CDCA8 is closely correlated with cell division and growth in HCC, and CDCA8 is strongly associated with the pathological grades and T stages of HCC (33). Second, kinesin family member 20A (KIF20A) and KIF2C are the members of the kinesin superfamily proteins, both of which are closely regulated by E2F1. The depletion of KIF20A or KIF2C results in deforming microtubule structures, influencing cell motility and inhibiting cancer metastasis (34). A recent study suggested that KIF20A and KIF2C are obviously upregulated in HCC tissues, and higher expression of KIF20A and KIF2C correlates with worse survival (including OS and disease-free survival [DFS]), higher tumor stages and poorer pathological grades (35). Moreover, by conducting basic experiments, this study also showed that the downregulation of KIF20A and KIF2C can effectively inhibit the proliferation of HCC cells and increase G1 arrest in HCC cells (35). In addition, Lu et al. observed that high KIF20A expression was associated with more high-grade

FIGURE 10 | Functional enrichment analysis of differentially expressed genes (DEGs) between high-risk and low-risk groups. **(A)** Gene ontology (GO) enrichment analysis of DEGs of TCGA-LIHC dataset. **(B)** Gene ontology (GO) enrichment analysis of DEGs of ICGC-RI-JP dataset. **(C)** Kyoto encyclopedia of genes and genomes pathway analysis of DEGs of TCGA-LIHC dataset. **(D)** KEGG pathway analysis of DEGs of ICGC-RI-JP dataset.

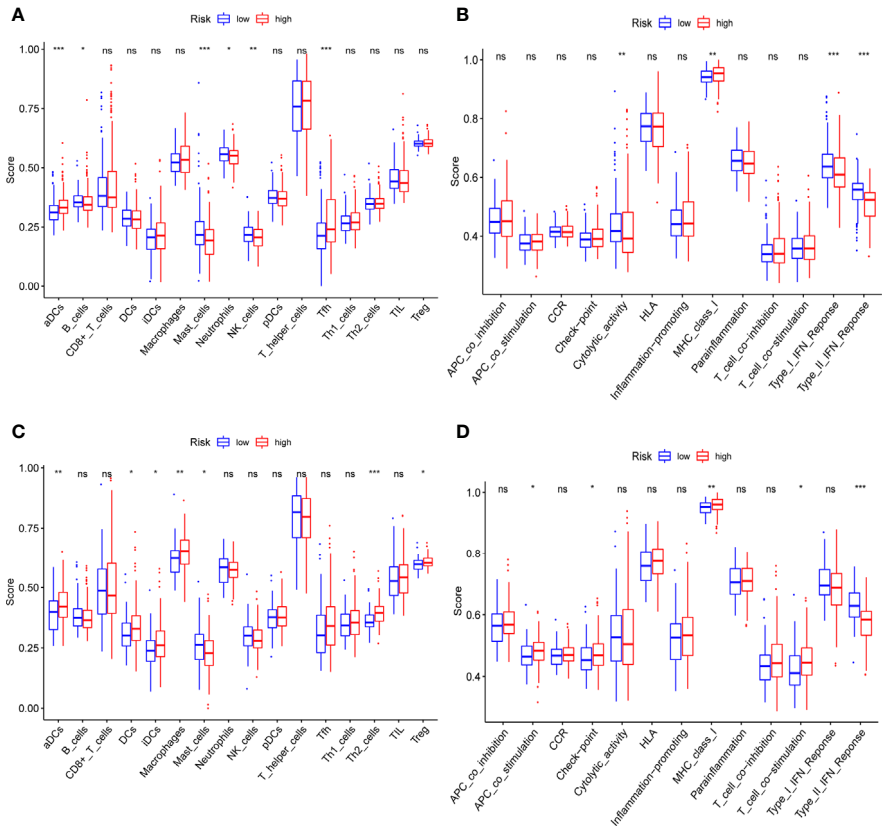

FIGURE 11 | Comparison of single-sample gene set enrichment (ssGSEA) scores between high-risk and low-risk groups in TCGA-LIHC and ICGC-LIRI-JP datasets. **(A, B)** The scores of 16 immune cells and 13 immune-related functions are displayed in boxplots in TCGA-LIHC dataset. **(C, D)** The scores of 16 immune cells and 13 immune-related functions are displayed in boxplots in ICGC-RI-JP dataset. Adjusted P values are showed as: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

HCC (52.3% vs. 32.5%, P=0.003), more advanced HCC (45.9% vs. 21.1%, P<0.0001), and more deaths (65.7% vs. 28.9%, P<0.0001) than low KIF20A expression, and the authors also reported that KIF20A could act as an independent prognostic indicator for poor OS (HR=1.30, 95% CI: 1.16–1.47, P<0.001) and recurrence-free survival (RFS) (HR=1.14, 95% CI: 1.03–1.27, P < 0.001) (36). KIF2C contributed to cell proliferation, adverse invasion, and metastasis *in vitro* and *in vivo* by performing both gain- and loss-of-function assays, and the authors further suggested that KIF2C plays an important role in mediating the crosstalk between Wnt/β-catenin and mammalian target of rapamycin complex 1 (mTORC1) signaling in the pathogenesis of HCC (37). Third, centrosomal protein 55 (CEP55) contributes to the carcinogenesis of many cancers and regulates PI3K/AKT signaling (38). Yang et al. showed that CEP55 is upregulated in HCC tissues, and CEP55 overexpression correlates with poor tumor grades and high T stages; the authors also showed that CEP55 acts as an independent predictor of the OS of HCC patients using multivariate analysis (39). In addition, CEP55 was found to promote cell migration and adverse invasion *via* the regulation of the JAK2-STAT3-MMP signaling pathway in HCC, and the knockdown of CEP55 strongly suppressed HCC cell migration and invasion (40).

Several limitations to our analysis exist. 1) The TCGA-LIHC dataset provides multiple HCC tissue samples, and the ICGC-LIRI-JP dataset and GSE112790 were applied for external validation. However, these datasets were obtained from public databases, and additional real-world datasets are required to validate the clinical utility of the four-gene prognostic signature. 2) Although we utilized comprehensive bioinformatics approaches to construct and validate this prognostic signature in HCC, it may not be very accurate for HCC patients with different grades and stages. 3) We did not verify the correlation between the risk score and immune status by conducting basic experiments, which is a significant issue that deserves further investigation in the future.

CONCLUSION

This comprehensive analysis proposes a novel prognostic signature of four differentially coexpressed hub genes that has

satisfactory prognostic value. This model was an independent predictor of OS in the TCGA-LIHC and ICGC-LIRI-JP datasets, providing insight into the prediction of HCC prognosis. Nevertheless, additional studies are required to further explore the underlying mechanisms of these differentially coexpressed hub genes and tumor immunity.

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

ZM had full access to all of the data in the manuscript and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: All authors. Acquisition, analysis, and interpretation of data: ZM. Drafting of the manuscript: ZM. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: All authors. Supervision: All authors. 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.2021.626654/full#supplementary-material

Supplementary Figure 1 | Functional enrichment analysis of 40 differentially co-expressed genes. **(A)** Gene ontology (GO) enrichment analysis of 40 differentially co-expressed genes. **(B)** Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of 40 differentially co-expressed genes.

Supplementary Figure 2 | Validation of the expression patterns of the four genes in the prognostic signature between LIHC and non-tumorous tissues based on GSE112790. The expression pattern of **(A)** CDCA8, **(B)** KIF20A, **(C)** KIF2C, and **(D)** CEP55 between LIHC and non-tumorous tissues.

Supplementary Figure 3 | Scree plot of the principal component analysis (PCA) in TCGA-LIHC and ICGC-LIRI-JP datasets. **(A)** In the PCA of the TCGA-LIHC dataset, the first principal component (PC1) could explain 88.6% of total variance, while the second principal component (PC2) could explain 5.4% total variance. **(B)** In the PCA of the ICGC-LIRI-JP dataset, the PC1 could explain 79% of total variance, whereas the PC2 could explain 13% total variance.

Supplementary Table 1 | The co-expression genes in the yellow module of TCGA-LIHC dataset.

Supplementary Table 2 | The differentially expressed genes (DEGs) in TCGA-LIHC dataset.

Supplementary Table 3 | Degree scores of all differential co-expressed genes.

Supplementary Table 4 | DEGs between high-risk and low-risk cohorts in TCGA-LIHC dataset.

Supplementary Table 5 | DEGs between high-risk and low-risk cohorts in ICGC-LIRI-JP dataset.

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Identification of Methylation-Regulated Differentially Expressed Genes and Related Pathways in Hepatocellular Carcinoma: A Study Based on TCGA Database and Bioinformatics Analysis

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Background: In recent years, DNA methylation modification has been shown to be a critical mechanism in the field of epigenetics.

Methods: Hepatocellular carcinoma (HCC) data were obtained from The Cancer Genome Atlas project, including RNA expression profiles, Illumina Human Methylation 450K BeadChip data, clinical information, and pathological features. Then, differentially expressed genes (DEGs) and differentially methylated genes were identified using R software. Methylation-regulated DEGs (MeDEGs) were further analyzed using Spearman's correlation analysis. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the DAVID database and ClueGO in Cytoscape software. Kaplan–Meier survival analysis explored the relationship between methylation, expression of MeDEGs, and survival time. Gene set enrichment analysis (GSEA) was conducted to predict the function of prognosis-related MeDEGs.

Results: A total of nine up-regulated and 72 down-regulated MeDEGs were identified. GO and KEGG pathway analyses results indicated that multiple cancer-related terms were enriched. Kaplan–Meier survival analysis showed that the methylation status of four MeDEGs (CTF1, FZD8, PDK4, and ZNF334) was negatively associated with overall survival. Moreover, the methylation status of CDF1 and PDK4 was identified as an independent prognostic factor. According to GSEA, hypermethylation of prognosis-related MeDEGs was enriched in pathways that included "Spliceosome", "Cell cycle", "RNA degradation", "RNA polymerase", "DNA replication", "Mismatch repair", "Base excision repair", "Nucleotide excision repair", "Homologous recombination", "Protein export", and "Pyrimidine metabolism".

Conclusions: Aberrant DNA methylation plays a critical role in malignant progression of HCC. Prognosis-related MeDEGs identified in this research may be potential biomarkers and targets in diagnosis and treatment.

Keywords: hepatocellular carcinoma, DNA methylation, overall survival, bioinformatic analysis, methylation-regulated differentially expressed genes

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common histopathological type of liver cancer, which ranked seventh in incidence and third in mortality among tumors worldwide in 2018 (1). Epidemiological studies have confirmed that HCC occurrence is associated with chronic hepatitis B/C virus infection, liver cirrhosis, environmental toxins, non-alcoholic fatty liver disease, metabolic disease, and lifestyle factors (2, 3). Although surgery combined with chemotherapy, radiotherapy, and immunotherapy can improve patient prognosis, the five-year survival rate in advanced-stage patients is still < 15% (4). Hence, a study into crucial biomarkers and molecular therapeutic pathways is of great significance for improving HCC patient prognosis.

DNA methylation modification has been considered to be a critical gene regulation mechanism in epigenetics and has been verified to be a reversible process. In the genome of normal cells, promoter cytosine-phosphate-guanine (CpG)-islands are typically hypomethylated. However, tumor cell hypermethylation of the CpG-island in the tumor suppressor promoter region is associated with malignant formation and progression. For instance, zinc finger protein 382 (ZNF382) is a potent tumor-suppressor and is down-regulated in hepatitis B-related HCC due to promoter methylation (5). However, research into DNA methylation of individual genes and pathways remains insufficient. Screening methylation-regulated differentially expressed genes (MeDEGs) with high-throughput data is of profound significance for clarifying the role of methylation and identifying future research directions.

In recent years, diverse gene-sequencing platforms have been utilized in basic and clinical HCC research. In addition, these techniques provide evidence for accurate tumor therapy. For instance, Illumina Human Methylation 450K BeadChip has been employed to detect genome-wide aberrant DNA methylation profiles between HCC cell line Huh7 and normal cell line L02. As a result, 62,702 (61.3%) CpG-island sites were hypermethylated and 39,552 (38.7%) CpG-island sites were hypomethylated (6). Zhang et al. have indicated that distinct DNA methylation differences emerge in the host immune system at an early stage based on the Illumina Human Methylation 450K BeadChip data, which may serve as noninvasive diagnostic HCC markers (7). The Illumina Methylation 450K BeadChip has been shown to play a critical role in the field of tumor epigenetics, but there is still a lack of conjoint correlation analysis of methylation, gene expression, and patient prognosis in large cohorts.

The present study applied bioinformatics analysis to identify MeDEGs based on *in silico* and clinical data from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) project (8). Then, MeDEG enrichment analysis was performed using an online database. Methylation of four genes was associated with prognosis in HCC patients. Gene set enrichment analysis (GSEA) was also performed.

MATERIALS AND METHODS

Data Collection and MeDEG Identification

TCGA database included the expression profiles of 374 HCC and 50 normal tissues (level 3) derived using RNA-seq and methylation data from 380 HCC and 50 normal tissues analyzed with the Illumina Human Methylation 450K BeadChip platform up to March 2020. Genomic Data Commons Data Transfer Tool 1.3.0 (8) was used to download the above profiles and clinical information data for further analysis. This research conformed to the guidelines published by TCGA on December 2015 (https://cancergenome.nih.gov/publications/publicationguidelines) and approval from an ethics committee was not required.

An RNA matrix that included 50 normal hepatic tissues and corresponding HCC tissues was constructed using PERL software. Methylation data matrix including 50 paired HCC and normal tissue samples was constructed using the same method. Differentially expressed genes (DEGs) and differentially methylated genes (DMGs) were identified using the "edgeR" and "limma" packages in R software with a threshold log2 fold change (FC) > 1.0 and $P < 0.01$. After a total of 42 normal and 374 HCC tissues were analyzed using RNA-seq and Illumina Human Methylation 450K BeadChip platform, expression and methylation data were merged together for Spearman's correlation analysis. The hypermethylated down-regulated and hypomethylated up-regulated genes that satisfied the cut-off criteria, including correlation coefficient < 0.2 and $P < 0.01$, were identified as MeDEGs. Furthermore, a heat map of the top 100

Abbreviations: HCC, Hepatocellular carcinoma; CpG, Cytosine-phosphate-guanine; MeDEGs, Methylation-regulated differentially expressed genes; TCGA, The Cancer Genome Atlas; GSEA, Gene set enrichment analysis; DEGs, Differentially expressed genes; DMGs, Differentially methylated genes; FC, Fold change; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Hyper-LG, hypermethylation and low-expression MeDEG; Hypo-HG, hypomethylation and high-expression MeDEG; ZNF334, Zinc finger protein 334; CTF1, Cardiotrophin-1; FZD8, Frizzled-8; PDK4, Pyruvate dehydrogenase kinase 4; KLF4, Kruppel-like factor 4; TACSTD2, Tumor-associated calcium signal transducer 2; RAC2, Ras-related C3 botulinum toxin substrate 2; GNB4, Guanine nucleotide-binding protein subunit beta-4; PTGER4, Prostaglandin E2 receptor EP4 subtype; CCNE2, G1/S-specific cyclin-E2; TGFB3, transforming growth factor beta-3; PRSAKA6, Ribosomal protein S6 kinase alpha-6; LAMA2, Laminin subunit alpha-2; GSTM1, Glutathione S-transferase Mu 1; MSI, Microsatellite-instability.

differentially expressed and methylated genes in 50 paired tissues were mapped using the "heatmap" package in R software.

MeDEG Enrichment Analyses

To further clarify the function of MeDEGs in HCC carcinogenesis and progression, gene ontology (GO) (9) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (10) analyses were performed using the DAVID database (https://david.ncifcrf.gov/) and ClueGO (11, 12) in Cytoscape 3.7.1. The enrichment results of GO and KEGG analyses were visualized as a bubble chart and network diagram, respectively. Differences with $P < 0.05$ were regarded as statistically significant.

Association Analysis of MeDEGs and Patient Prognosis

A total of 353 enrolled HCC patients were followed up for 80 months and had complete clinical data for the survival analysis. The 353 HCC patients were sorted into two groups according to the MeDEG median methylation value. In addition, a hypermethylation and low-expression MeDEG (Hyper-LG) group and a hypomethylation and high-expression MeDEG (Hypo-HG) group were established according to the median value of MeDEG methylation and expression. Kaplan–Meier method and log-rank test were used to compare the overall survival between the two groups using the "survival" package in R software. Differences with $P < 0.05$ were regarded as statistically significant.

GSEA of Prognosis-Related MeDEGs

GSEA of prognosis-related MeDEGs was conducted using GSEA 3.0 software with gene set c2 (cp.kegg.v.6.2.symbols.gmt). RNA expression profiles for 374 HCC tissues were selected as the dataset. The sample was marked as either "Hypermethylation" or "Hypomethylation" based on the median methylation value of prognosis-related MeDEGs. The enrichment score > 0.4 and $P < 0.05$ were regarded as statistically significant.

Statistical Analysis

All statistical analyses were performed using SPSS V18.0 (SPSS, Inc., Chicago, IL, USA). The association between methylation of prognosis-related MeDEGs and clinicopathological characteristics was analyzed using the chi-squared test. Cox proportional hazards model was applied to evaluate the influence of clinical data and methylation on prognosis. Differences with $P < 0.05$ were regarded as statistically significant.

RESULTS

Identification of MeDEGs in HCC

A total of 3157 up-regulated and 1080 down-regulated genes were screened as DEGs from 50 paired HCC and normal tissue samples. The top 100 DEGs with the highest and most significant differences are represented on a heat map in **Figure 1A**. Moreover, 1061 hypermethylated and 1401 hypomethylated DMGs were identified

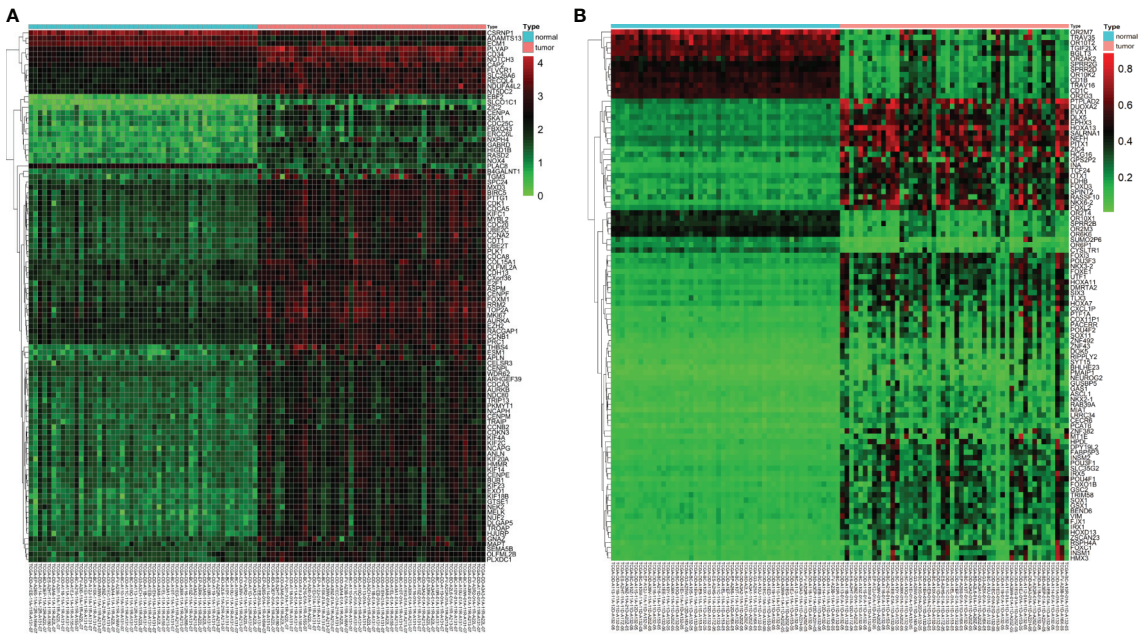

FIGURE 1 | Differentially expressed genes (DEGs) and differentially methylated genes (DMGs) identified from The Cancer Genome Atlas (TCGA) database. **(A)** Heat map of the top 100 DEGs (log_2 FC > 2, $P < 0.01$). Lower horizontal axis marks sample names, left vertical axis shows clusters of DEGs, and right vertical axis represents gene names. Red represents up-regulated genes and green represents down-regulated genes. **(B)** Heat map of the top 100 DMGs (log_2 FC > 1, $P < 0.01$). Lower horizontal axis marks sample names, left vertical axis shows clusters of DMGs, and right vertical axis represents gene names. Red represents hypermethylated genes and green represents hypomethylated genes.

and represented as a heat map of the top 100 DMGs (**Figure 1B**). According to the Spearman's correlation analysis results, 359 genes had a negative correlation between expression and methylation. Nine up-regulated and 72 down-regulated MeDEGs that satisfied the three conditions were obtained and gene lists were also identified (**Figure 2**). The top ten MeDEGs with the highest Spearman's correlation coefficient are shown in **Figure 3**.

Functional Enrichment Analyses of MeDEGs

GO analysis was used to clarify the function of 81 MeDEGs using DAVID 6.8 software (**Figure 4**). The biological process and molecular function terms were mainly associated with transcription regulation. Moreover, negative regulation of cell proliferation and motility was also enriched. In addition, KEGG pathway analysis results indicated that "Pathways in cancer", "Inflammatory bowel disease", "Transcriptional misregulation in cancer", and "Malaria" were significantly involved in MeDEGs. "Hepatocellular carcinoma" was also enriched and KEGG network enrichment diagram was mapped in **Figure 5**.

Prognosis-Related MeDEGs in HCC

Kaplan–Meier curve analysis revealed a relationship between MeDEG methylation value and overall survival in HCC patients. Hypermethylation of cardiotrophin-1 (CTF1), Frizzled-8 (FZD8), pyruvate dehydrogenase kinase 4 (PDK4), and zinc finger protein 334 (ZNF334) was negatively correlated with the overall survival (**Figures 6A–D**). Then, prognosis of the above four MeDEGs was further compared with the Hyper-LG and Hypo-HG groups. As compared with patients in the Hyper-LG group, Hypo-HG patients had a significant better survival (**Figures 6E–H**).

Identification of Methylation-Based Biomarkers

A total of 353 patients were divided into "Low" and "High" groups according to the median methylation of CTF1, FZD8, PDK4, and ZNF334. CTF1 methylation status significantly correlated with gender and T stage. In addition, PDK4 hypermethylation was associated with gender, T stage, and pathologic stage (**Table 1**). Univariate and multivariate Cox regression analyses were then conducted to evaluate the prognostic role of the above four genes' methylation status. The samples were divided into high or low methylation groups according to the median gene methylation status. Advanced T stage, pathologic stage, and high methylation of prognosis-related MeDEGs were associated with poor HCC patient prognosis (**Table 2**). Multivariate analysis results identified T stage and methylation status of CTF1 and PDK4 as independent factors in the overall survival.

GSEA of Prognosis-Related MeDEGs

GSEA results revealed the potential mechanisms of prognosis-related MeDEGs. A total of 11 consensus terms were obtained from the enriched KEGG terms and included "Spliceosome", "Cell cycle", "RNA degradation", "RNA polymerase", "DNA replication", "Mismatch repair", "Base excision repair", "Nucleotide excision repair", "Homologous recombination", "Protein export", and "Pyrimidine metabolism" (**Figure 7A**). PDK4 enrichment is represented as an example in **Figure 7B**.

DISCUSSION

Accumulating evidence has indicated that aberrant DNA methylation modification is a critical molecular event in HCC progression. Hypermethylated status in the promoter of tumor suppressor genes (13), cyclin (14), and DNA mismatch genes (15) has been studied in depth. HCC cell methylation profiles have become a new field of tumor biomarker study (16). Furthermore, DNA methylation has been recognized as a potential therapeutic target due to its reversibility (17). Therefore, MeDEG identification will provide more information on the role of methylation in HCC.

Bioinformatics analysis in the present study resulted in 81 MeDEGs. GO analysis indicated that the main terms are related

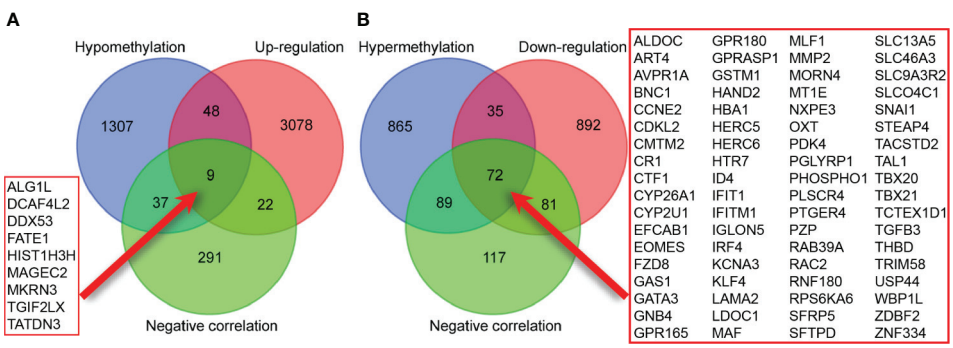

FIGURE 2 | Identification of methylation-regulated differentially expressed genes (MeDEGs). **(A)** A total of nine genes were identified as MeDEGs by intersecting three gene sets (hypomethylation, up-regulation, and negative correlation). **(B)** A total of 72 genes were identified as MeDEGs by intersecting three gene sets (hypermethylation, down-regulation, and negative correlation).

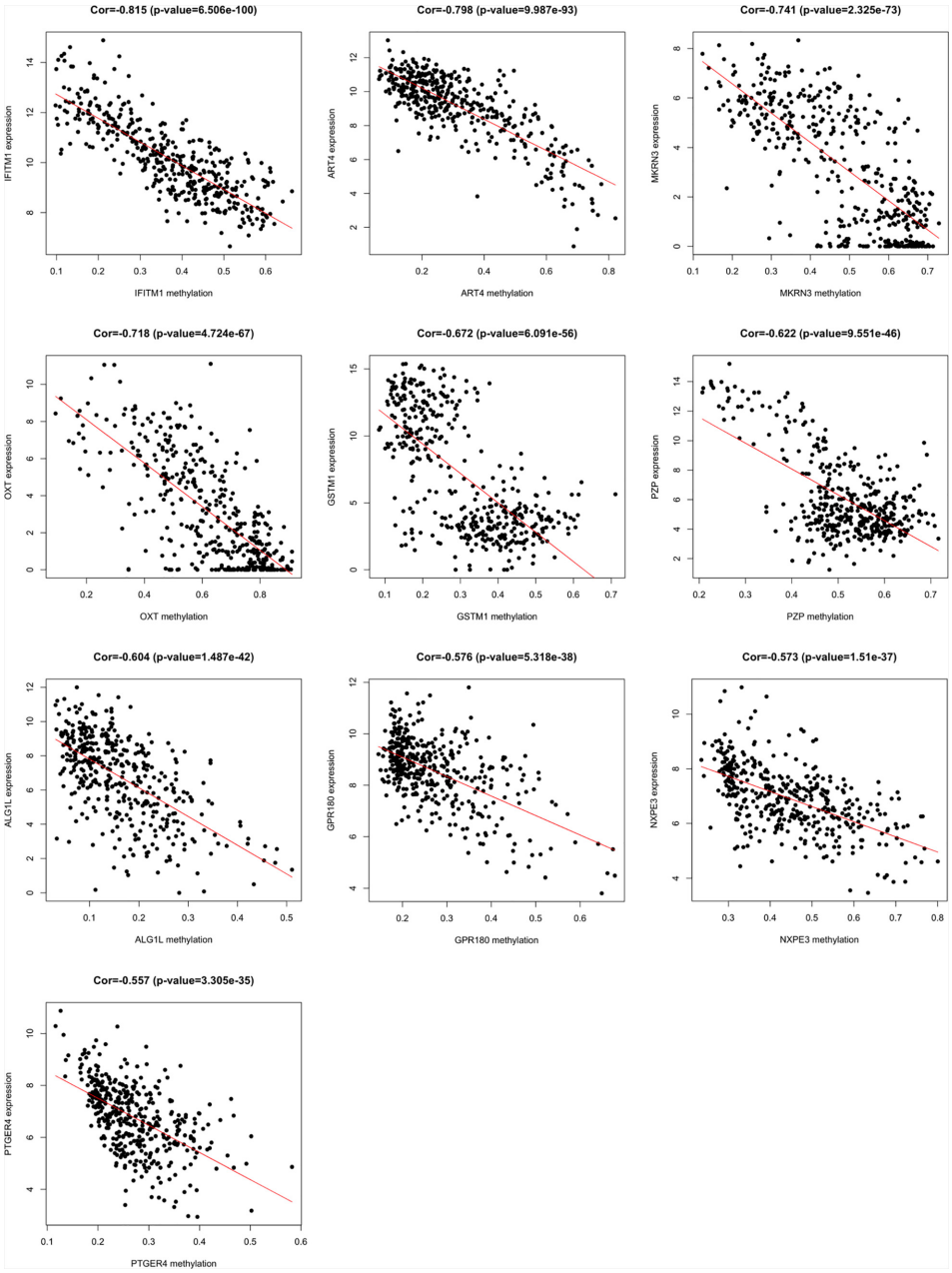

FIGURE 3 | Methylation-regulated differentially expressed genes (MeDEGs) with the top ten correlation coefficients. Spearman's correlation analysis for methylation (horizontal axis) and expression (vertical axis) of MeDEGs. Spearman's correlation coefficient and P-values are shown in each plot.

to transcription dysregulation. For instance, Kruppel-like factor 4 (KLF4) has been identified as a transcription factor that can suppress the expression of Ring1- and YY1-binding protein and inhibit HCC tumorigenesis (18). Moreover, KLF4 expression was epigenetically inhibited by CpG-island hypermethylation (19). Therefore, it was speculated that methylation can indirectly control gene expression by regulating transcription factors. The present study also identified tumor-associated calcium signal transducer 2 (TACSTD2) as a MeDEG, which has been reported to be down-regulated in primary HCC tissue. However, no research has demonstrated that TACSTD2 is regulated by methylation in HCC, which will be the subject of future studies. KEGG pathway analysis further defined the role of MeDEGs in HCC. It is noteworthy that "Pathways in cancer" was the most enriched pathway in which Ras-related C3 botulinum toxin substrate 2 (RAC2), guanine nucleotide-binding protein subunit beta-4 (GNB4), prostaglandin E2 receptor EP4 subtype (PTGER4), G1/S-specific cyclin-E2

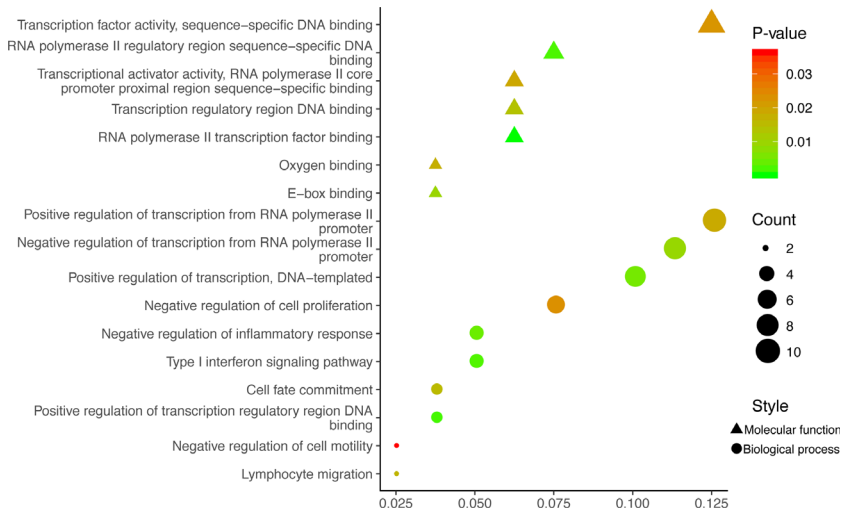

FIGURE 4 | MeDEG gene ontology enrichment analysis. Molecular function and biological process terms for MeDEGs are shown as "triangles" and "circles", respectively. "Count" represents the number of genes. MeDEGs, methylation-regulated differentially expressed genes.

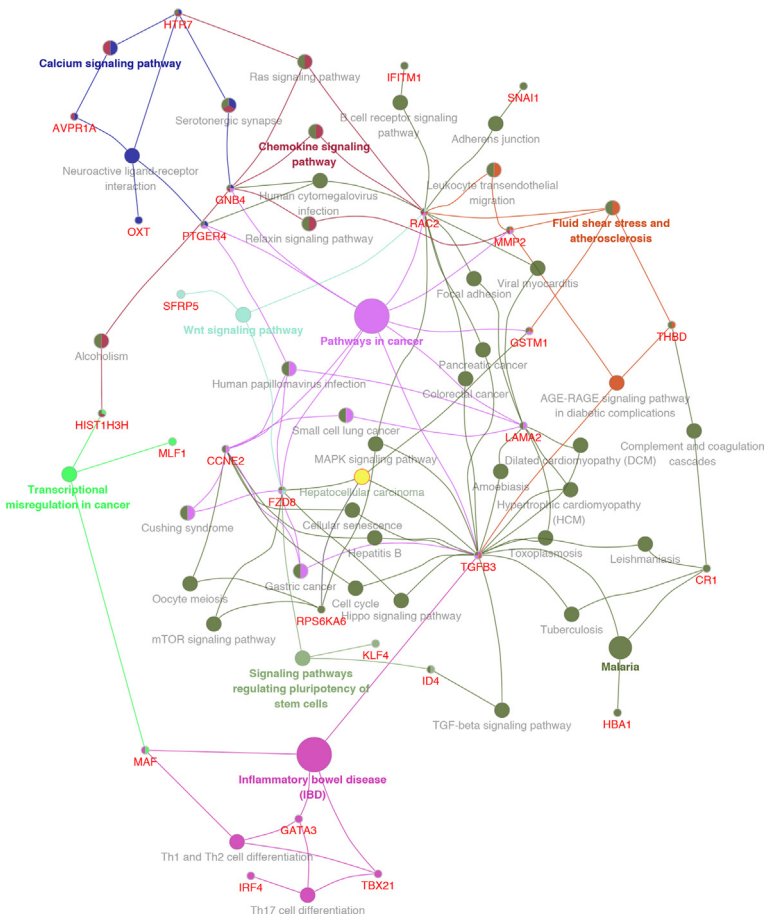

FIGURE 5 | KEGG pathway enrichment network diagram for MeDEGs. MeDEGs and enrichment pathways constitute a regulatory network. The larger the pathway circle, the more genes were enriched.

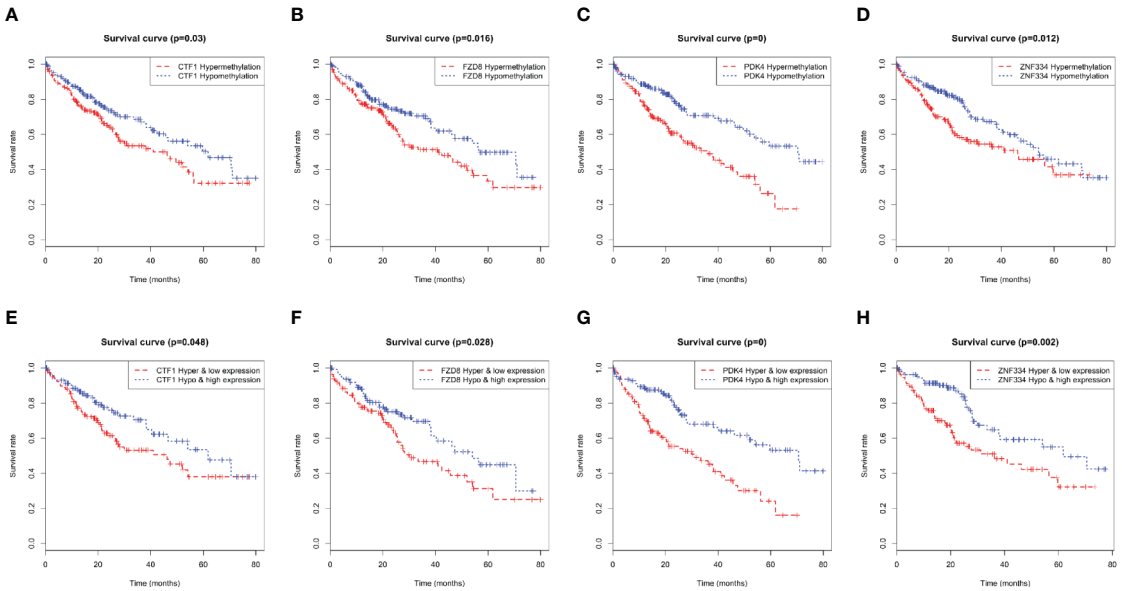

FIGURE 6 | Kaplan-Meier curves for methylation expression of MeDEGs are associated with overall survival. **(A)** CTF1, **(B)** FZD8, **(C)** PDK4 and **(D)** ZNF334 were ranked by the median of methylation and then scored for each patient in accordance with high- or low-level methylation value. **(E)** CTF1, **(F)** FZD8, **(G)** PDK4 and **(H)** ZNF334 were ranked by the median of methylation and expression and then scored for each patient in accordance with high- or low-level methylation value and high or low-level expression value. The horizontal axis represents the overall survival time and the vertical axis represents survival function.

(CCNE2), Frizzled-8 (FZD8), transforming growth factor beta-3 (TGFB3), laminin subunit alpha-2 (LAMA2), glutathione S-transferase Mu 1 (GSTM1), and matrix metalloproteinase-2 (MMP2) are involved. Though RAC2 (20), GNB4 (21), PTGER4 (22, 23), CCNE2 (24), FZD8 (25), TGFB3 (26), LAMA2 (27), and GSTM1 (28, 29) have been reported to be regulated by methylation in multiple cancers, very little is known about the regulatory mechanisms by which methylation is involved in HCC. Moreover, the term "Hepatocellular carcinoma" is enriched with FZD8, ribosomal protein S6 kinase alpha-6 (PRSAKA6), TGFB3 and GSTM1. FZD8 has been demonstrated to be an important cell membrane receptor that mediates the Wnt signaling pathway in HCC (30, 31). Feng et al. (32) have indicated that TGFB3 can function as a modulator to promote the metastatic phenotype of non-metastatic HCC cells induced by TGFB1. GSTM1 polymorphisms have been identified as biomarkers of HCC development and risk in different regions (33–37).

In addition, methylation status of cardiotrophin-1 (CTF1), FZD8, pyruvate dehydrogenase kinase 4 (PDK4), and ZNF334 was associated with overall survival. Similar results were obtained by performing conjoint analysis of methylation,

TABLE 1 | Association between methylation of prognosis-related MeDEGs and clinical features.

Variable	CTF1 methylation		*P*-value	FZD8 methylation		*P*-value	PDK4 methylation		*P*-value	ZNF334 methylation		*P*-value
Age at diagnosis (yr)	Low	High		Low	High		Low	High		Low	High	
<60	89	80	0.366	100	69	0.001**	79	90	0.310	82	87	0.708
≥60	87	97		76	108		97	87		94	90	
Gender												
female	46	65	0.043*	49	62	0.180	42	69	0.003**	55	56	0.971
male	130	112		127	115		134	108		121	121	
T stage												
T1	97	78	0.038*	92	83	0.368	100	75	0.009**	91	84	0.489
T2-4	79	99		84	94		76	102		85	93	
N stage												
N0	173	170	0.202	171	172	0.993	174	169	0.055	170	173	0.741
N1	3	7		5	5		2	8		6	4	
Pathologic stage												
I + II	138	124	0.094	134	128	0.485	140	122	0.031*	133	129	0.564
III + IV	38	53		42	49		36	55		43	48	

*$P < 0.05$, **$P < 0.01$.

TABLE 2 | Cox regression analyses of association between prognosis-related MeDEGs and clinicopathological characteristics.

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Age at diagnosis (≥60 *vs* <60)	0.843 (0.582-1.220)	0.365		
Gender (female *vs* male)	0.756 (0.516-1.108)	0.151		
T stage (T2-4 *vs* T1)	2.776 (1.881-3.709)	<0.001**	1.761 (1.283-2.417)	0.003**
N stage (N1 *vs* N0)	1.104 (0.750-1.651)	0.186		
Pathologic stage (III + IV *vs* I + II)	2.122 (1.319-3.471)	<0.001**	1.391 (0.967-2.002)	0.076
CTF1 methylation (high *vs* low)	1.557 (1.055-2.106)	0.022*	1.487 (1.023-2.177)	0.032*
FZD8 methylation (high *vs* low)	1.463 (1.002-2.042)	0.048*	1.245 (0.806-1.838)	0.309
PDK4 methylation (high *vs* low)	1.505 (1.065-2.128)	0.021*	1.506 (1.083-2.313)	0.018*
ZNF334 methylation (high *vs* low)	1.491(1.006-2.102)	0.041*	1.391 (0.967-2.002)	0.076

*$P < 0.05$, **$P < 0.01$.

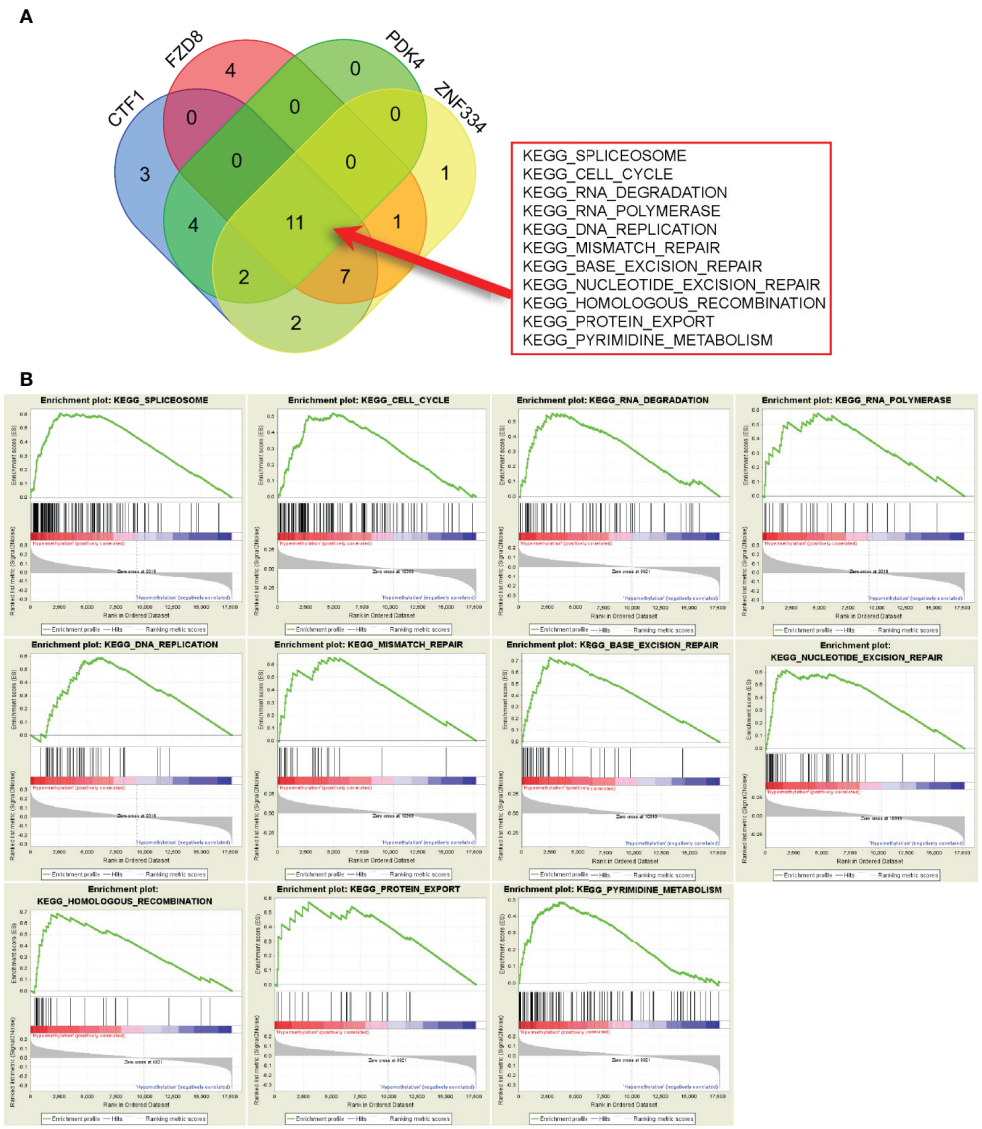

FIGURE 7 | Gene set enrichment analysis (GSEA) of prognosis-related MeDEGs. **(A)** Intersection of pathways enriched by hypermethylation of CTF1, FZD8, PDK4, and ZNF334 is shown in the diagram. **(B)** GSEA of PDK4 is shown as example.

expression, and prognosis. CTF1 is a mitogenic cytokine of the interleukin 6 family, which is a hepatocyte survival factor that is up-regulated during liver regeneration in animal models (38). Bustos et al. (39) have indicated that CTF1 prevented colon cancer cell proliferation in the liver depending on T and NK cells. However, the function and regulatory mechanism of CTF1 in HCC remains controversial. A recent study has indicated that prognosis-related PDK4 is down-regulated in HCC tissues, while PDK4 knockdown promotes HCC cell proliferation, migration, and invasion (40). Moreover, arsenic-induced silencing of PDK4 in hepatic cells is mediated by histone H3 lysine 9 methylation in the promoter (41). ZNF334 lymphocyte expression can be regulated by tumor necrosis factor α. However, little is known about ZNF334 in tumors (42). The present study first indicated that methylation of PDK4 and CTF1 is a potential independent biomarker for prognosis prediction. More studies are needed to verify this hypothesis.

GSEA clarified the mechanisms by which prognosis-related MeDEGs drive tumorigenesis. A total of 11 pathways that involved prognosis-related MeDEGs and the "spliceosome" pathway were the most significantly enriched. The spliceosome consists of five ribonucleoprotein subunits and protein cofactors and has been demonstrated as a critical and complicated mechanism in mRNA synthesis regulation of eukaryotic cells (43). Krogh et al. (44) have indicated that ribose methylation interrupts snRNA interactions and affects the splicing process in a T cell leukemia model. According to the GSEA results, almost every step of gene transcription and translation is enriched by methylation of prognosis-related MeDEGs.

The present study has some limitations. Firstly, the present research is mainly based on bioinformatic analysis of TCGA database and verification of identified genes and pathways is insufficient. Secondly, it is generally known that microsatellite-instability (MSI) is associated with aberrant methylation in HCC. Illumina Human Methylation 450K BeadChip data analyzed in the present research did not supply any MSI information. Thus, it is difficult to reveal the relationship between MSI, methylation and prognosis and more validation experiments are needed in the future.

In conclusion, MeDEGs were identified by analyzing the expression profiles and methylation data of HCC samples from TCGA database. GO and KEGG pathways analyses verified the MeDEG mechanisms. Furthermore, four prognosis-related MeDEGs and methylation status of PDK4 and CTF1 were identified as potential biomarkers for survival prediction and treatment.

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

H-MY conducted the study. YL, BM, and PJ applied the experiments on TCGA project. YL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Germline Variants and Genetic Interactions of Several EMT Regulatory Genes Increase the Risk of HBV-Related Hepatocellular Carcinoma

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Epithelial-mesenchymal transition (EMT) plays an important role in the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). We hypothesized that germline variants in the major EMT regulatory genes (*SNAIL1*, *ZEB1*, *ZEB2*, *TWIST1*) may influence the development of HBV-related HCC. We included 421 cases of HBsAg-positive patients with HCC, 1371 cases of HBsAg-positive subjects without HCC [patients with chronic hepatitis B (CHB) or liver cirrhosis (LC)] and 618 cases of healthy controls in the case-control study. Genotype, allele, and haplotype associations in the major EMT regulatory genes were tested. Environment-gene and gene-gene interactions were analysed using the non-parametric model-free multifactor dimensionality reduction (MDR) method. The *SNAIL1*rs4647958T>C was associated with a significantly increased risk of both HCC (CT+CC *vs*. TT: *OR*=1.559; 95% confidence interval [*CI*], 1.073-2.264; *P*=0.020) and CHB+LC (CT+CC *vs*. TT: *OR*=1.509; 95% *CI*, 1.145-1.988; *P*=0.003). Carriers of the *TWIST1*rs2285681G>C (genotypes CT+CC) had an increased risk of HCC (CG+CC *vs*. GG: *OR*=1.407; 95% *CI*, 1.065-1.858; *P*=0.016). The *ZEB2*rs3806475T>C was associated with significantly increased risk of both HCC ($P_{recessive}$ =0.001) and CHB+LC ($P_{recessive}$<0.001). The CG haplotype of the rs4647958/rs1543442 haplotype block was associated with significant differences between healthy subjects and HCC patients (*P*=0.0347). Meanwhile, the CT haplotype of the rs2285681/rs2285682 haplotype block was associated with significant differences between CHB+LC

and HCC patients (*P*=0.0123). In MDR analysis, the combination of *TWIST1*rs2285681, *ZEB2*rs3806475, *SNAIL1*rs4647958 exhibited the most significant association with CHB+LC and Health control in the three-locus model. Our results suggest significant single-gene associations and environment-gene/gene-gene interactions of EMT-related genes with HBV-related HCC.

Keywords: hepatocellular carcinoma, epithelial-mesenchymal transition, polymorphisms, interaction, multifactor dimensionality reduction

HIGHLIGHTS

1. The functional *SNAIL1* exon variant rs4647958T>C, the *ZEB2* promoter exon variant rs3806475T>C and the *TWIST1* promoter exon variant rs2285681G>C are associated with increased risk of HBV-related HCC.
2. The CG haplotype of the rs4647958/rs1543442 haplotype block was associated with significant differences between healthy control subjects and HCC patients. Additionally, the CT haplotype of the rs2285681/rs2285682 haplotype block was associated with significant differences between CHB+LC and HCC patients.
3. *TWIST1* rs2285681 and *SNAIL1* rs4647958 showed a significant environment-gene interaction for the development of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC), a common malignant tumour of the digestive system, is the second leading cause of cancer-related death in China. HCC is characterized by high malignant potential, concealed pathogenesis, rapid progress, poor prognosis and a high mortality rate. It is typically diagnosed during the middle and late disease stages, when surgery is no longer a viable option (1). Therefore, it is important to identify genetic loci that may be valuable predictors for the development of both HCC and chronic hepatitis B virus (HBV) infection in order to evaluate the risk of HCC in patients with HBV infection.

In recent years, the significance of epithelial-mesenchymal transition (EMT) in tumours has been extensively studied. There are many complex factors that may influence the process of tumour metastasis; however, the specific underlying mechanisms are not yet clear. A great many studies have revealed that EMT plays an important role in tumour invasion and metastasis. To date, three well-established transcriptional regulatory groups have been identified as important factors in regulating the expression of EMT molecular markers (2). Studies have shown that several EMT regulators are involved in the process of tumour metastasis and that the phenotypic changes associated with EMT play a key role in the development of invasive phenotypes in colon cancer, thyroid cancer and breast cancer (3). In addition, increasing evidence demonstrates that EMT is involved in promoting other aspects of tumour progression (4–6). A more comprehensive understanding of the role of EMT in regulating the growth and metastasis of tumours is critical for improving the diagnosis and treatment of these tumours.

Previous work has demonstrated that *SNAIL* and *TWIST* are the major regulators of EMT, which subsequently induces HCC (7). Overexpression of *SNAIL* and *TWIST* is associated with greater tumour volume, increased recurrence, and shorter disease-free and overall survival in HCC patients (7). In addition, *SNAIL* and *TWIST* expression is associated with decreased E-cadherin expression in HCC. *In vitro* experiments have confirmed that overexpression of *SNAIL* or *TWIST* promotes invasion and increases the interstitial phenotype of tumour cells. Overexpression of *SNAIL* or *TWIST* in Huh7 cells suppresses E-cadherin expression and induces EMT (3, 8, 9). In addition, previous studies have demonstrated that EMT leads to increased chemotherapeutic resistance in poorly differentiated HCC cell lines (4–6). Wu et al. constructed gemcitabine-resistant HCC cell lines and found that these cells develop an EMT-related phenotype (10). Furthermore, real-time PCR has been used to demonstrate the downregulation of E-cadherin expression and increased expression of *TWIST*1, further confirming the development of EMT (11).

Genetic variations in EMT-related regulatory genes may affect the process of EMT and thus influence the development of HCC or chronic HBV infection. However, there has been no published research on the association of these variants with HCC or chronic HBV infection risk. Moreover, although several genetic variants associated with these liver diseases have been revealed by GWAS, little research has been done on the link between these genes and disease progression. Therefore, it is of great value to identify which genetic loci of EMT-related genes are related with the development of HCC. Thus, we assessed whether Genetic variations in EMT-related regulatory genes are associated with the progress of HCC and chronic HBV infection.

A common analysis method for genotype data is to perform a single gene locus or haplotype analysis on a single gene, that is, to detect the association between each locus or gene and disease separately. However, when we want to explain the genetic changes in complex diseases, the usefulness of this analysis is

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; CHB, chronic hepatitis B; LC, liver cirrhosis; EMT, epithelial-mesenchymal transition; SNP, single-nucleotide polymorphism; LD, linkage disequilibrium; MDR, multifactor dimensionality reduction; MAF, minor allele frequency; mean ± SD, mean ± standard deviation; M(QR), median(quartile interval); OR, odds ratio; CI, confidence interval; EDTA, ethylenediamine-tetra-acetic acid; 3'-UTR, 3'-untranslatedregion.

limited (12). Because the risk of a particular disease may be explained by genetic mutations at other loci, discovering gene-to-gene interactions is more conducive to a comprehensive understanding of the factors that affect disease risk (13). In this study, we investigated possible genetic interactions between EMT-related genes (*SNAIL1, ZEB1, ZEB2* and *TWIST1*) in HBV-related HCC in the Han population and their relevance as potential biomarkers for HBV and HCC. This approach may help develop new therapy or individualized treatments for HBV-related HCC and chronic HBV infection.

MATERIALS AND METHODS

Study Subjects

Case-control studies were conducted to investigate HBV-related HCC and chronic HBV infection in northern China. To evaluate HBV-associated mutations and their correlation with HCC risk, 421 HBsAg-positive patients with HCC, 1371 HBsAg-positive patients without HCC [691 cases of chronic hepatitis B (CHB) and 680 cases of liver cirrhosis (LC)] and 618 controls without HBV infection were enrolled. All subjects are independent of each other and are ethnically Han Chinese. All participants were recruited between January 2010 and March 2014 from the First, Second and Fourth Hospitals of Hebei Medical University and the Fifth Hospital of Shijiazhuang City. Each subject provided demographic characteristics as well as a one-time 2 mL blood sample. All subjects signed a written informed consent forms to study initiation. This study was approved by the institutional review board of Hebei Medical University (Ethics Committee of Hebei Medical University: No. 2017053).

Healthy individuals were defined as (i) HBsAg, antibodies against HBc (anti-HBc) and other HBV biomarkers were free; (ii) blood routine and biochemical indexes were normal; (iii) without a history of hepatitis B vaccination; (iv) without endocrine, cardiovascular, renal or other liver diseases. CHB patients were defined as (i) serum HBsAg was positive; (ii) HBeAg was positive; (iii) anti-HBe was negative; (iv)serum HBV-DNA >2000 IU/mL lasting for >6 months; (v) the value of alanine aminotransferase (ALT) was persistent or repeated rising; (vi) liver histology showed hepatitis. LC patients were defined by clinical manifestations of portal hypertension (e.g., varicose oesophageal or gastric fundus, ascites and splenomegaly) and imaging results of ultrasonography, computed tomography, and magnetic resonance imaging (14, 15). HBV-related HCC patients were defined as pathologic diagnosis and/or blood alpha-fetoprotein (AFP) >400 ng/mL, at the same time combined with imaging examination results (16, 17). Patients were excluded from this study if they with alcoholic liver disease, positive laboratory tests for HCV (identified by the presence of anti-HCV and/or HCV-RNA) and HIV or suspected autoimmune diseases with an antinuclear antibody titre greater than 1:160.

The personal information of the research subjects was obtained through questionnaires, which included the subjects' gender, age, smoking status, and drinking status. The definition of smoking and drinking here is: an individual who smokes every day and has smoked for more than 1 year is defined as a smoker, and an individual who drinks once or more a week for more than 6 months is defined as a drinker. We collected about 2 mL of anticoagulated venous blood by ethylenediamine tetra-acetic acid (EDTA) from each subject. Each subject signed an informed consent form. The study protocol adhered to the ethical guidelines set forth by the 1975 Declaration of Helsinki and was approved by the Hebei Medical University ethics committee.

Polymorphisms Selection and Genotyping

According to the dbSNP database (http://www.ncbi.nlm.nih.gov/), we selected 6 EMT gene loci located in the promoter, regulator coding region and 3'-UTR. All putative functional single-nucleotide polymorphisms (SNPs) of the genes encoding the aforementioned EMT regulators (*SNAIL1* rs4647958T>C, *SNAIL1* rs1543442G>A, *ZEB1* rs7349C>T, *ZEB2* rs3806475T>C, *TWIST1* rs2285681G>C and *TWIST1* rs2285682T>G) with a minor allele frequency greater than 5% in the Chinese population were selected. The location information in gene region for the selected SNPs was shown in **Table 1**. A Genomic DNA Purification Kit purchased from Promega was used for genomic DNA extraction and time of flight mass spectrometry technology from SOLARBIO Technology Co., Ltd. was used for all sample SNP genotyping. Primers for the five SNP alleles were designed by the Bio Miao Biological Company with the aid of MassARRAY® Assay Design 4.0 Software (Sequenom Inc., San Diego, CA, USA). SNPs were genotyped using TaqMan-based PCR. Basic information for the selected SNPs was shown in **Supplementary Table 1**.

Statistical Analysis

SPSS version 18.0 (SPSS Inc., Chicago, IL, USA), Haploview 4.2 software (Copyright (c) 2003-2006 Broad Institute of MIT and Harvard, United States) and MDR 3.0.2 software (https://sourceforge.net/projects/mdr/) were used to perform statistical analyses. Categorical variables were described using frequencies, while continuous data with abnormal distribution were described using the median and interquartile range. The comparisons of continuous data sets were done using Kruskal-Wallis *H* test and evaluation of differences in categorical variables between groups was done using Pearson chi-square test. The bonfferny method was used for pairwise comparisons between groups when there was a significant difference in the overall distribution of each factor in the three groups. Calculation of odds ratios (*OR*) and 95% confidence intervals (95% *CI*) was done using unconditional logistic regression. Analysis of correlations between genetic variants and HCC stages was done by Spearman's rank correlation. Linkage disequilibrium (LD) and haplotype block analyses were used to investigate the LD of EMT SNPs using Haploview 4.2 software. Multifactor dimensionality reduction (MDR) method as a nonparametric alternative was used to analyse the environment-gene and gene-gene interactions. The MDR analyses were performed by MDR 3.0.2 software. This extensive search for genetic interactions was done for HCC. Up to four loci interactions were tested using 10-fold crossvalidation in a search considering all possible SNP combinations. The SNP combination with maximum cross-validation consistency (CVC)

TABLE 1 | Associations between the SNPs in candidate EMT regulators and risk of chronic HBV infection in the discovery set.

SNP	Location in Gene Region	HCC *n* = 421	CHB+LC *n* =1371	Health Control *n* = 618	MAF			P^a	P^b	P^c
					HCC	CHB+LC	Health Control			
rs4647958T>C	*SNAIL1*, exon	338/78/2	1106/246/8	530/70/5	0.098	0.096	0.066	0.009	0.728	0.001
rs1543442G>A	*SNAIL1*, 3'-UTR	168/194/57	544/621/196	226/293/95	0.368	0.372	0.393	0.715	0.694	0.421
rs7349C>T	*ZEB1*, 3'-UTR	269/135/14	848/452/54	409/173/29	0.195	0.207	0.189	0.485	0.920	0.068
rs3806475T>C	*ZEB2*, promoter	120/216/84	305/769/287	133/237/72	0.457	0.493	0.431	<0.001	0.020	<0.001
rs2285681G>C	*TWIST1*, promoter	209/173/33	704/541/95	343/223/48	0.288	0.273	0.260	0.043	0.516	0.180
rs2285682T>G	*TWIST1*, promoter	331/80/8	1026/297/35	481/119/11	0.115	0.135	0.115	0.800	0.247	0.228

[a]*HCC vs health control;* [b]*HCC vs CHB+LC;* [c]*CHB+LC vs health control.*

P value of association test from the best-fitted genetic model calculated by the unconditional logistic regression, adjusted for age, gender, smoked and drink, which owned the smallest Akaikein formation criterion value.

was considered to be the best model (*see* METHODS in the **Supplements**). All hypothesis tests were based on two-sided. When *P* values were less than 0.05, it is considered statistically significant.

RESULTS

Subject Characteristics

Baseline characteristics of the 1371 HbsAg-positive patients without HCC (CHB+LC), 421 HBsAg-positive patients with HCC and 618 healthy control subjects were shown in **Supplementary Table 2**. The age, gender, and tobacco and alcohol use distributions were significantly different among all studied groups ($P<0.05$). Smoking and drinking were significantly lower in healthy patients versus in HBsAg-positive patients with and without HCC. The proportion of males was higher in the HBsAg-positive patients versus the healthy subjects, while patients older than 45 years old were more frequent in the HBsAg-positive patients with HCC. We adjusted for these factors in the multivariate logistic regression models.

Genotypes of EMT Regulators and Their Association With Hepatocellular Carcinoma and Chronic HBV Infection Risk

The genotype distributions of the six EMT regulators and their associations with HCC and CHB+LC are presented in **Tables 1** and **2**. Based on the best genetic model (defined as the model with the smallest AIC value), the *SNAIL1* exon variant rs4647958T>C was significantly associated with an increased risk of both HCC ($P_{dominant}$ =0.020) and CHB+LC ($P_{dominant}$ =0.003). The *ZEB2* promoter variant rs3806475T>C was significantly associated with an increased risk of both HCC ($P_{recessive}$ =0.001) and CHB+LC ($P_{recessive}$<0.001). Further, the *TWIST1* promoter variant rs2285681G>C was significantly associated with an increased risk of HCC ($P_{dominant}$ =0.016). However, no significant association was

TABLE 2 | Associations between the SNPs in EMT regulators and diseases risk under different genetic models.

SNP	Gene	Additive model		Dominant model		Recessive model	
		OR	*P* value	*OR*	*P* value	*OR*	*P* value
HCC vs. Health							
rs4647958T>C	*SNAIL1*	0.343(0.068-1.728)	0.194	1.559(1.073-2.264)	0.020	0.316(0.063-1.593)	0.163
rs1543442G>A	*SNAIL1*	0.841(0.552-1.282)	0.420	0940(0.711-1.245)	0.668	0.853(0.578-1.259)	0.424
rs7349C>T	*ZEB1*	0.733(0.360-1.492)	0.391	1.063(0.797-1.418)	0.679	0.707(0.350-1.430)	0.335
rs3806475T>C	*ZEB2*	1.327(0.853-2.065)	0.210	0.701(0.512-0.961)	0.027	1.918(1.313-2.803)	0.001
rs2285681G>C	*TWIST1*	1.201(0.712-2.025)	0.492	1.407(1.065-1.858)	0.016	1.023(0.617-1.697)	0.928
rs2285682T>G	*TWIST1*	1.263(0.451-3.537)	0.657	1.112(0.793-1.559)	0.538	1.240(0.444-3.464)	0.682
HCC vs. CHB+LC							
rs4647958T>C	*SNAIL1*	0.899(0.162-4.982)	0.903	1.122(0.831-1.516)	0.452	0.881(0.159-4.872)	0.884
rs1543442G>A	*SNAIL1*	0.969(0.673-1.394)	0.864	1.065(0.839-1.352)	0.605	0.923(0.658-1.295)	0.642
rs7349C>T	*ZEB1*	0.895(0.470-1.707)	0.737	0.958(0.751-1.223)	0.732	0.906(0.478-1.718)	0.763
rs3806475T>C	*ZEB2*	0.750(0.531-1.059)	0.102	0.692(0.530-0.904)	0.007	0.984(0.736-1.316)	0.916
rs2285681G>C	*TWIST1*	1.091(0.694-1.714)	0.706	1.145(0.904-1.450)	0.262	1.024(0.661-1.587)	0.916
rs2285682T>G	*TWIST1*	0.667(0.297-1.499)	0.327	0.792(0.598-1.051)	0.106	0.699(0.312-1.567)	0.385
CHB+LC vs. Health							
rs4647958T>C	*SNAIL1*	0.448(0.166-1.209)	0.113	1.509(1.145-1.988)	0.003	0.416(0.154-1.122)	0.083
rs1543442G>A	*SNAIL1*	0.860(0.642-1.152)	0.311	0.876(0.719-1.069)	0.193	0.921(0.705-1.204)	0.548
rs7349C>T	*ZEB1*	0.885(0.553-1.418)	0.612	1.211(0.988-1.484)	0.065	0.820(0.515-1.307)	0.404
rs3806475T>C	*ZEB2*	1.745(1.253-2.430)	0.001	0.975(0.772-1.230)	0.829	1.988(1.503-2.630)	<0.001
rs2285681G>C	*TWIST1*	0.958(0.659-1.393)	0.824	1.157(0.953-1.404)	0.141	0.889(0.617-1.280)	0.528
rs2285682T>G	*TWIST1*	1.469(0.736-2.931)	0.274	1.213(0.961-1.530)	0.104	1.415(0.710-2.818)	0.324

OR, odds ratio; adjusted in a logistic regression model that included age, gender, smoking and drinking.

observed between any of the other loci and the risk of HbsAg-positive HBV with or without HCC. Therefore, we further analysed the *SNAIL1* rs4647958T>C, *ZEB2* rs3806475T>C and *TWIST1* rs2285681G>C SNPs. As shown in **Table 2**, the rs4647958T>C SNP was associated with a significantly increased risk of both HCC (CT+CC *vs.* TT: *OR*=1.559; 95% confidence interval [*CI*], 1.073-2.264; P = 0.020) and CHB+LC (CT+CC *vs.* TT: *OR*=1.509; 95% confidence interval [*CI*], 1.145-1.988; P = 0.003) under the dominant model. Carriers of the *TWIST1* rs2285681G>C genotypes (CT+CC) had an increased risk of HCC (CG+CC *vs.* GG: *OR*=1.407; 95% confidence interval [*CI*], 1.065-1.858; P = 0.016) under the dominant model.

The stratification analysis showed that the rs4647958 genotype-associated risk of HCC development was more pronounced in non-smoking individuals (*OR*, 2.053; 95% *CI*, 1.372-3.072) versus those who did smoke (*OR*, 0.878; 95% *CI*, 0.461-1.673; Breslow-day test, P = 0.027) under the dominant model (see **Figure 1A**). Meanwhile, the rs3806475 genotype-associated risk of HCC development was more pronounced in non-drinking individuals (*OR*, 2.410; 95% *CI*, 1.577-3.683) versus those who did drink (*OR*, 1.117; 95% *CI*, 0.621-2.009; Breslow-day test, P = 0.036) under the recessive model (see **Figure 1B**). Last, the rs3806475 genotype-associated risk of CHB+LC development was more pronounced in non-drinking individuals (*OR*, 2.425; 95% *CI*, 1.732-3.395) compared with those who did drink (*OR*, 1.276; 95% *CI*, 0.770-2.114; Breslow-day test, P = 0.037) under the recessive model (see **Figure 1C**). None of the other SNPs observed were associated with any significant differences in disease characteristics.

SNAIL1, *ZEB2* and *TWIST1* Genotypes and Their Correlation With HbsAg-Positive HBV With and Without HCC Progression

EMT has been widely studied in the metastatic process of epithelial malignancies (18). We therefore analysed the correlation between SNPs and HCC clinical stages as shown in **Supplementary Table 3**. We found that the rs4647958 *SNAIL1* genotypes were correlated with HCC progression with a lower correlation-coefficient in non-smoking patients (r_s= 0.087, P<0.001). Additional correlations were identified in the following patient groups: age less than 45 years (rs4647958: r_s= 0.113, P=0.001), female (rs4647958: r_s= 0.079, P=0.026; rs2285681: r_s= 0.074, P=0.038) and non-drinking (rs4647958: r_s= 0.074, P=0.002; rs3806475: r_s= 0.054, P=0.025).

LD and Haplotype Block Analysis

Haplotype block LD mapping demonstrated that the rs2285681 and rs2285682 SNPs are in tight LD in a 0-kb sequence, while the rs4647958 and rs1543442 SNPs are in tight LD in a 4-kb sequence (**Supplementary Figure 1**). As shown in **Table 3**, the CG haplotype of the rs4647958/rs1543442 haplotype block is associated with significant differences between healthy control subjects and HCC patients (P=0.0347). Meanwhile, the CT haplotype of the rs2285681/rs2285682 haplotype block is associated with significant differences between CHB+LC and HCC patients (P=0.0123). However, no significant correlations were identified between other observed SNPs.

MDR Models of Environment-Gene and Gene-Gene Interactions

We searched for possible genetic interactions of the four genes studied in the context of HCC. We evaluated up to three-locus interactions with 6 polymorphic sites and 3 environmental factors (gender, tobacco smoking and alcohol drinking). For HCC and health subjects as comparative groups, gender in one-locus models was the best, while the balanced accuracy (BA) for testing the dataset was 60.55% and the CVC was 10/10. For HCC and CHB+LC as comparative groups, the combination drinking, smoking in the two-locus model was the best, while the BA was 57.48% and the CVC was 9/10. For CHB+LC and health subjects as comparative groups, the combination *TWIST1*rs2285681, *ZEB2*rs3806475, *SNAIL1*rs4647958 of the three-locus model was the best model with a BA of 56.99% and CVC of 9/10. **Table 4** summarizes the MDR results for the one- to three-locus models. **Figures 2**–**4** show the detailed distribution of high- and low-risk genotypes in the best three-locus model for the HCC and CHB+LC. These results were all significant, with empirical p-values of <0.001 in 10000 permutation tests.

DISCUSSION

We hypothesized that EMT genes play an important role in HCC and chronic HBV infection, and that environment-gene and gene-gene interactions are important. We found significant genetic associations for single EMT genes with HCC and chronic HBV infection, as well as environment-gene and gene-gene interactions. The MDR results indicated that interactions of environment-gene and gene-gene contribute significantly to HCC and chronic HBV infection, even when individual EMT genes do not.

In this study, we found that EMT-related genes were important in HCC and chronic HBV infection. Our findings demonstrate that the *SNAIL1* rs4647958T>C, *ZEB2* rs3806475T>C and *TWIST1* rs2285681G>C SNPs are associated with increased susceptibility to both HCC and chronic HBV infection. In addition, interactions among potentially related polymorphic sites were associated with the development of HCC through the MDR method. MDR is a suitable method to analyse environment-gene and gene-gene interactions by reducing multi-locus genotypes into high-risk and low-risk groups in case-control studies (19). This method marks the genotype in each cell as high or low risk based on whether the ratio of case to control cell is greater than or less than the threshold (20). Furthermore, the MDR method is further extended to model-based MDR, generalized MDR and surviving MDR, etc., to apply to different situations (21–23). Our study found *SNAIL1* rs4647958 showed a significant environment-gene interaction for chronic HBV infection with or without HCC in the MDR results. *TWIST1* rs2285681 showed a significant environment-gene interaction for the development of HCC.

For HCC and health subjects as comparative groups, the one-locus model was found to be optimal for the prediction of HCC in terms of BA (60.55%). However, the BA values between the two- and three-locus combination models in HCC didn't have any meaningful difference. For HCC and CHB+LC as

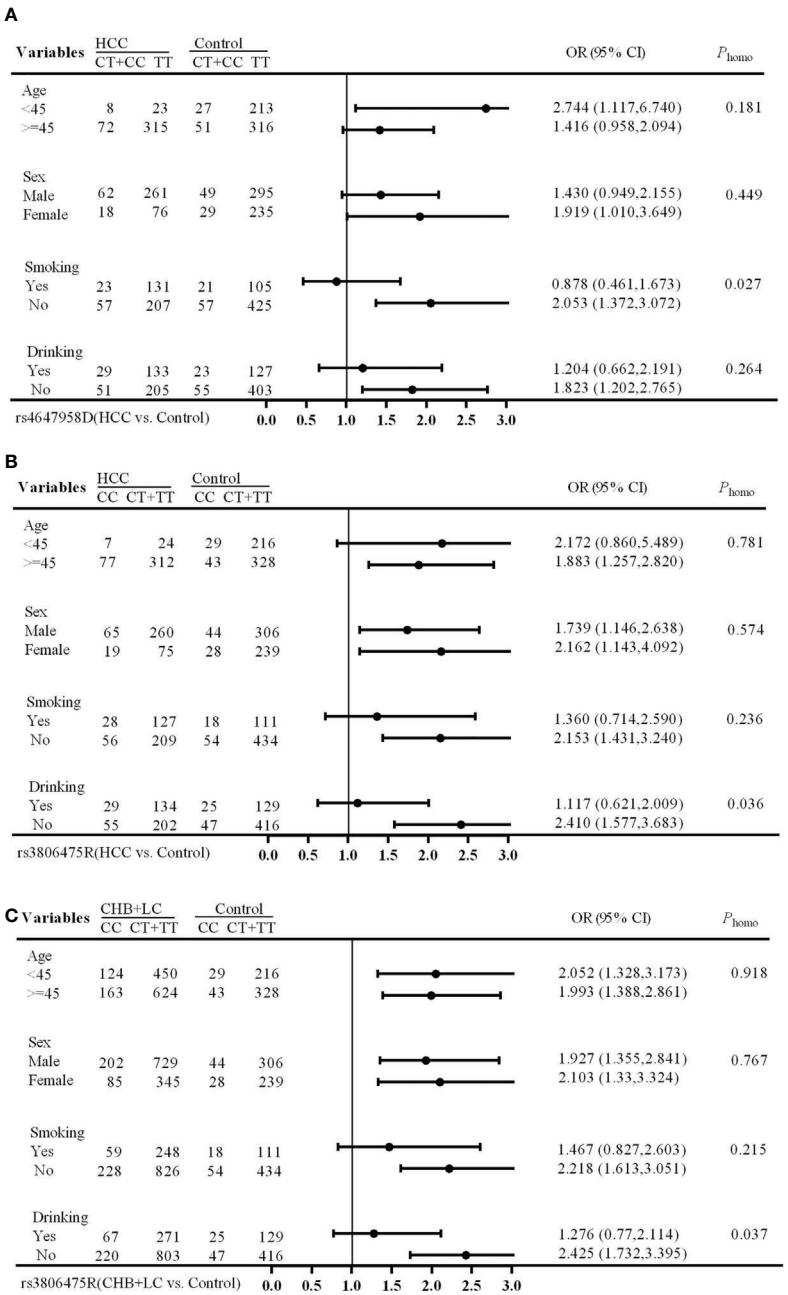

FIGURE 1 | Stratification analysis of associations between EMT regulatory genes and HBV-related HCC risk. **(A)** HCC *vs* Health Control (rs4647958D); **(B)** CHB+LC *vs*. Health Control (rs3806475R); **(C)**, HCC *vs* Health Control (rs3806475R). CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Phomo from the homogeneity test in each stratum was tested by the Breslow-Day Test.

comparative groups, the two-locus model was found to be the best in terms of BA (57.48%). Similarly, the BA values between the one- and three-locus combination models didn't have any meaningful difference. For CHB+LC and health subjects as comparative groups, the four-locus combination model (*TWIST1* rs2285681, *ZEB2* rs3806475 and *SNAIL1* rs4647958) was the best for BA (56.99%) and the CVC was 9/10.

In the three-locus combination models for HCC and CHB+LC, *SNAIL1* rs4647958 was common to both liver diseases, while the other two factors differed. Comparatively, while we compared HCC and CHB+LC, CHB+LC and health subjects, *TWIST1* rs2285681 appeared in both three-locus combination models at the same time. Our finding of the EMT-related gene interaction seemed to support the clinical observation that

TABLE 3 | Haplotype analysis between HCC and patients with CHB + LC by Haploview.

	Haplotype	Freq.	Case, Control Ratio Counts	Case, Control Frequencies	Chi Square value	*P* value
Comparison between health control and HCC						
Block 1	GT	0.729	598.4: 243.6, 912.9: 319.1	0.711, 0.741	2.327	0.1271
	CT	0.157	147.6: 694.4, 178.8: 1053.2	0.175, 0.145	3.431	0.0640
	CG	0.114	96.0: 746.0, 140.3: 1091.7	0.114, 0.114	<0.001	0.9906
Block 2	TG	0.536	450.8: 389.2, 660.6: 573.4	0.537, 0.535	0.003	0.9535
	TA	0.382	307.7: 532.3, 485.5: 748.5	0.366, 0.393	1.562	0.2114
	CG	0.082	81.5: 758.5, 87.9: 1146.1	0.097, 0.071	4.458	0.0347
Comparison between CHB+LC and HCC						
Block 1	GT	0.722	598.4: 243.6, 1985.4: 750.6	0.711, 0.726	0.723	0.3952
	CT	0.149	147.6: 694.4, 383.7: 2352.3	0.175, 0.140	6.266	0.0123
	CG	0.128	95.9: 746.1, 363.4: 2372.6	0.114, 0.133	2.066	0.1506
Comparisons between health control and CHB+LC						
Block 1	GT	0.730	1985.7: 750.3, 912.8: 321.2	0.726, 0.740	0.842	0.3589
	CT	0.142	383.4: 2352.6, 178.7: 1055.3	0.140, 0.145	0.156	0.6931
	CG	0.127	363.4: 2372.6, 141.2: 1092.8	0.133, 0.114	2.579	0.1083

Block 1, rs2285681 and rs2285682; Block 2, rs4647958 and rs1543442.

SNAIL1 rs4647958 and *TWIST1* rs2285681 had an impact on patients with HCC and chronic HBV.

In this study, we found significant associations between germline variants of six EMT regulators and the development of chronic HBV infection and HCC revealed that the *SNAIL1* exon variant rs4647958T>C and the *ZEB2* promoter exon variant rs3806475T>C are significantly associated with the risk of developing both diseases. Additionally, the *TWIST1* promoter exon variant rs2285681G>C is associated with an increased risk of HBV-related HCC. Furthermore, the *SNAIL1* rs4647958T>C genotype is associated with decreased probability of HBV-related HCC metastasis at diagnosis among smokers.

The stratified analysis showed that *SNAIL1* genotypes (rs4647958) are associated with the development of a more aggressive form of HCC in non-smokers, *ZEB2* genotypes (rs3806475) are associated with increased risk of HCC development in non-drinkers, and *TWIST1* genotypes (rs3806475) are associated with increased risk of CHB and LC. Meanwhile, the *SNAIL1* SNPs (rs4647958) are correlated with HCC stages in smokers, though not significantly. *SNAIL1* is an important factor involved in inducing and promoting EMT. *SNAIL1* is also involved in the pathogenesis of hepatitis B virus mutations in HCC patients. Our findings are remarkably consistent with previously published studies. Chen et al. (24) found that *SNAIL* is negatively correlated with E-cadherin expression and positively correlated with *MMP-2* expression in HCC tissues. Further, these changes in E-cadherin and MMP-2 expression help to promote HCC invasion. Woo et al. (25) used immunohistochemistry to study HCC and found that *SNAIL* expression is correlated with low E-cadherin expression and poor differentiation in hepatocellular carcinoma. The occurrence and development of HCC are related to many signal pathways, and the expression of *SNAIL* can play a role in the process of HCC by affecting these signal pathways. Kim et al. (26) found that Notch1 and ROS synergistically upregulate the expression of *SNAIL* protein in hepatoma carcinoma cells through the PI3K/Akt signalling pathway, thereby increasing cancer cell invasion. Cheng et al. (27) demonstrated that increased expression of *SNAIL1* can promote liver tumour initiation, progression, and metastasis. High *SNAIL1* expression was also reported in liver tissues,

TABLE 4 | MDR models of analyse the environment-gene and gene-gene interactions.

Comparative group	Model	Training Balanced Accuracy (%)	Testing Balanced Accuracy (%)	Cross Validation Consistency	Chi Square value	*p*-value
HCC *vs* Health	Gender	60.55	60.55	10/10	47.1755	<0.0001
	Gender, *ZEB1* rs7349	61.69	60.32	8/10	56.0426	<0.0001
	Gender, *SNAIL1* rs4647958, *ZEB1* rs7349	62.76	58.77	5/10	67.1358	<0.0001
HCC *vs* CHB+LC	Smoking	57.63	56.09	7/10	36.9906	<0.0001
	Drinking, Smoking	58.34	57.48	9/10	38.4753	<0.0001
	Drinking, Smoking, *TWIST1* rs2285681	58.99	55.13	6/10	43.0362	<0.0001
CHB+LC *vs* Health	Gender	56.00	54.91	8/10	25.4365	<0.0001
	Gender, *ZEB1* rs7349	57.51	55.74	6/10	38.7054	<0.0001
	TWIST1 rs2285681, *ZEB2* rs3806475, *SNAIL1* rs4647958	58.67	56.99	9/10	50.6300	<0.0001

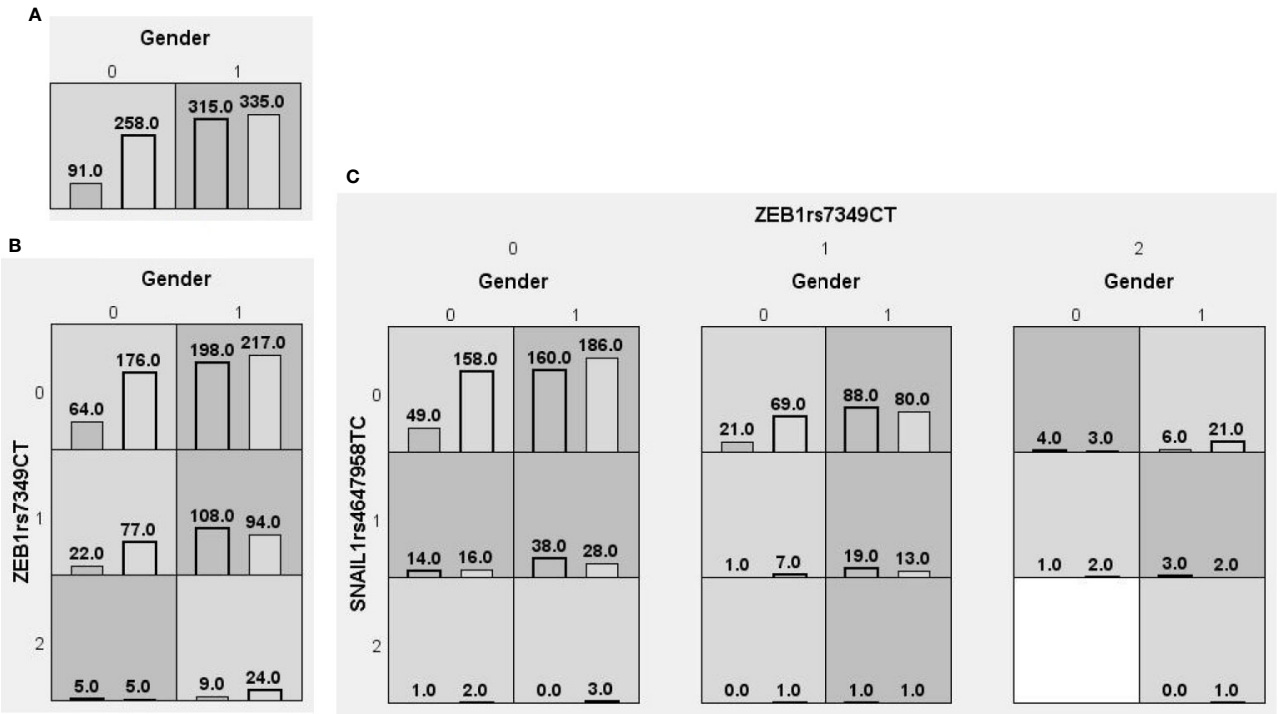

FIGURE 2 | Distributions of high-risk and low-risk genotypes between HCC and health control. **(A)** Single-locus model; **(B)** Two-locus model; **(C)** Three-locus model. Dark gray and light gray boxes presented the high-risk and low-risk SNP combinations, respectively. Left bars inside each box represented major depressive disorder while the right bars represented control. The heights of the bars are proportionate to the sum of samples in each group. The patterns of high-risk and low-risk cells differ across each of the different multi-locus SNP dimensions.

suggesting that it also contributes to HCC pathogenesis (28). These pieces of evidence all indicate that the *SNAIL* variant (rs4647958) is functional and contributes to increased risk of HCC and chronic HBV infection.

The *TWIST1* protein (also known as *Twist*) can regulate the expression of many specific genes and participates in many different biological processes required for normal growth and development (29). However, *TWIST* also plays an oncogenic role in tumour cells. Yang et al. (30) showed that *TWIST* plays a key role in the vascular invasion and lung metastasis of cancer cells. During the process of tumour metastasis, primary tumour cells undergo EMT and then metastasize to distant organs *via* the circulatory system. *TWIST* stimulates tumour metastasis by promoting the occurrence of EMT in tumour cells. In addition, *TWIST* can inhibit apoptosis and senescence pathways and immortalize cells (31).

The *ZEB2* protein plays an important role as a transcription factor in the *TGF* signalling pathway. This signalling pathway is essential during early foetal development (32). *ZEB1* and *ZEB2* can bind to the CACCT (G) sequence in the promoter of the E-cadherin gene, causing epithelial cells to lose their epithelial-like characteristics and transform into mesenchymal cells, thus leading to EMT (33). Gene mutations can result in the production of non-functional *ZEB2* proteins or can completely inactivate the gene. The absence of *ZEB*2 proteins influences the biological processes of many organs. *ZEB2* mutations are the underlying cause of irregular development of the neural crest (34). Our study is the first to demonstrate that mutations in the *ZEB2* gene are related to HCC.

As a case-control hospital-based study, some limitations in our study are inevitable. For example, selection and information biases are unavoidable. However, our identification of associations between gene variants and HBV-related HCC risk are unlikely to be solely due to chance, as these findings were confirmed by the results of functional assays.

Longo et al. (35) concluded that the liver microenvironment of HCC patients is more immunosuppressed, accompanied by an increase in the number of regulatory T cells (Tregs), tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC), which is associated with tumor progression and poor prognosis. Here we described and analysed the single-gene associations and environment-gene/gene-gene interactions of EMT-related genes with HBV-related HCC. We found that EMT genes play a role in HBV-related HCC and genetic factors at multi-levels, from alleles and genotypes to haplotypes and environment-gene/gene-gene interactions. Our study suggests that these SNPs are not only candidate predictors for HCC and chronic HBV infection risk but may also be a genetic determinant for the development of HCC in the chronic HBV infection population. Future studies should be

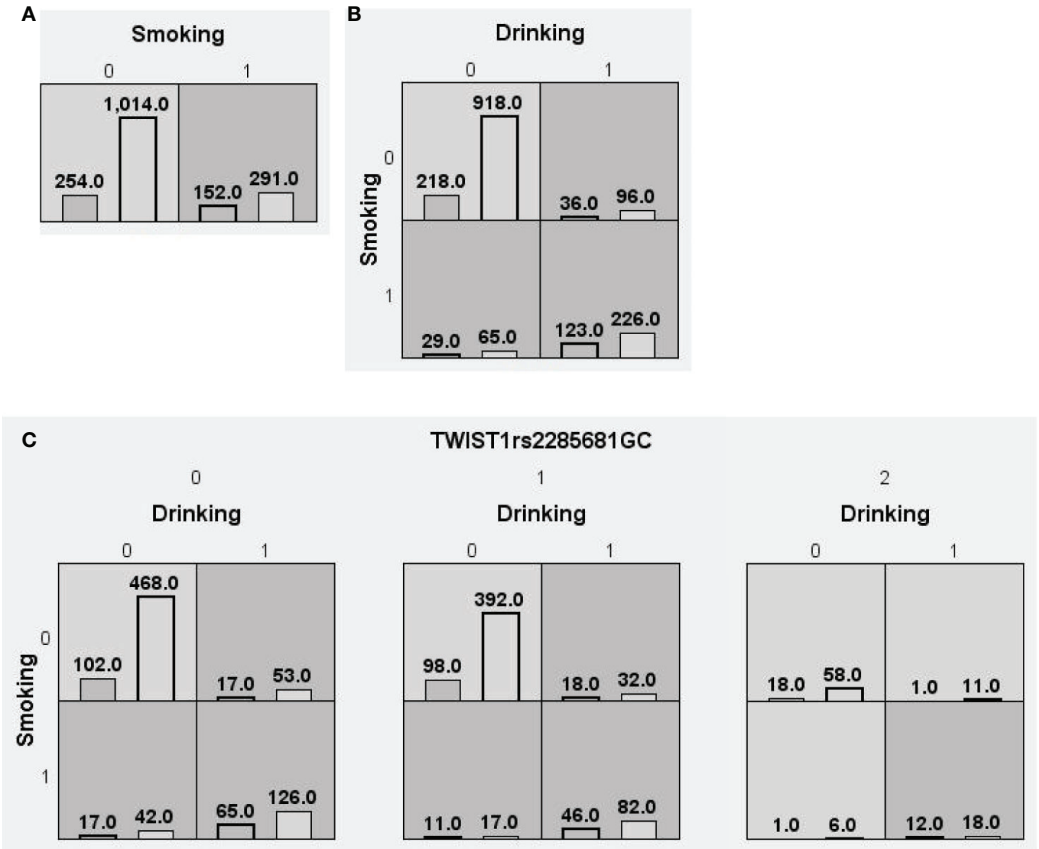

FIGURE 3 | Distributions of high-risk and low-risk genotypes in between HCC and CHB+LC patients. **(A)** Single-locus model; **(B)** Two-locus model; **(C)** Three-locus model. Dark gray and light gray boxes presented the high-risk and low-risk SNP combinations, respectively. Left bars inside each box represented major depressive disorder while the right bars represented control. The heights of the bars are proportionate to the sum of samples in each group. The patterns of high-risk and low-risk cells differ across each of the different multi-locus SNP dimensions.

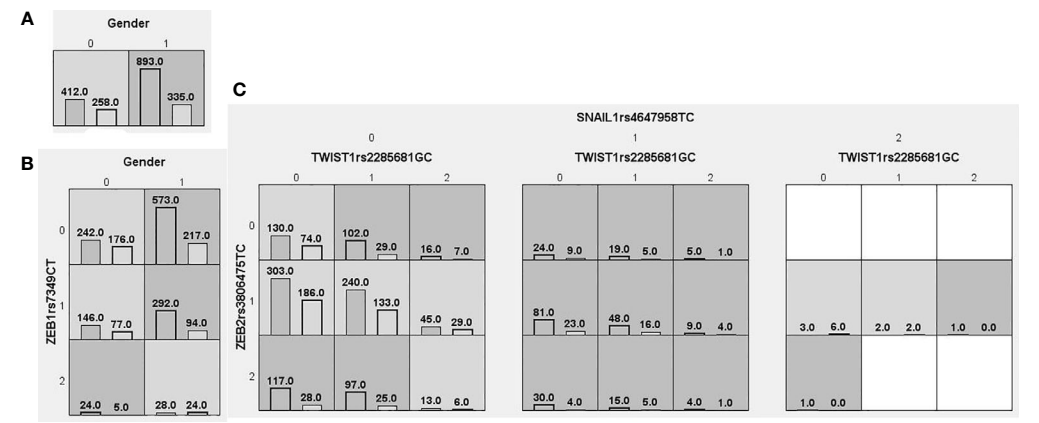

FIGURE 4 | Distributions of high-risk and low-risk genotypes in between CHB+LC patients and health control. **(A)** Single-locus model; **(B)** Two-locus model; **(C)** Three-locus model. Dark gray and light gray boxes presented the high-risk and low-risk SNP combinations, respectively. Left bars inside each box represented major depressive disorder while the right bars represented control. The heights of the bars are proportionate to the sum of samples in each group. The patterns of high-risk and low-risk cells differ across each of the different multi-locus SNP dimensions.

performed in a larger population encompassing multiple ethnic groups in order to confirm our findings.

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**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Hebei Medical University. 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: W-XL, LY, XG, and D-WL. Data curation: L-NY, NM, X-LZ, and L-MT. Data analysis: XG, W-XL, LY, and D-WL. Funding acquisition: XG and D-WL. Investigation: LY, H-MY, L-NY, NM, X-LZ, L-MT, and XG. Methodology: XG, D-WL, and L-MT. Project administration: D-WL, XG, and LY. Resources: XG and D-WL. Supervision: XG, D-WL, W-XL, and LY. Writing the original draft: XG and D-WL. Reviewing and editing: W-XL, LY, H-MY, L-NY, NM, X-LZ, L-MT, XG, and D-WL. 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.2021.564477/full#supplementary-material

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HYPOTHESIS AND THEORY
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Consensus of Minimally Invasive and Multidisciplinary Comprehensive Treatment for Hepatocellular Carcinoma – 2020 Guangzhou Recommendations

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In China, the majority of patients with hepatocellular carcinoma (HCC) result from long-term infection of hepatitis B. Pathologically, HCC is characterized by rich blood supply, multicentric origins, early vascular invasion and intrahepatic metastasis. Therefore, HCC is not a local disease but a systemic disease at the beginning of its occurrence. For this reason, a comprehensive treatment strategy should be adopted in the management of HCC, including local treatments (such as surgical resection, radiofrequency ablation, microwave ablation, chemical ablation and cryoablation, etc.), organ-level treatments [such as transcatheter arterial infusion of chemotherapy and transcatheter arterial chemoembolization (TACE)], and systemic treatments (such as immunotherapy, antiviral therapy and molecular targeted therapy, etc.). This consensus sets forth the minimally-invasive and multidisciplinary comprehensive guideline of HCC, focusing on the following

eight aspects (1) using hepaticarteriography, CT hepatic arteriography (CTHA), CT arterial portography (CTAP), lipiodol CT (Lp-CT), TACE-CT to find the intrahepatic lesion and make precise staging (2) TACE combined with ablation or ablation as the first choice of treatment for early stage or small HCC, while other therapies are considered only when ablation is not applicable (3) infiltrating HCC should be regarded as an independent subtype of HCC (4) minimally-invasive comprehensive treatment could be adopted in treating metastatic lymph nodes (5) multi-level subdivision of M-staging should be used for individualized treatment and predicting prognosis (6) HCC with severe hepatic decompensation is the only candidate criterion for liver transplantation (7) bio-immunotherapy, traditional Chinese medicine therapy, antiviral therapy, and psychosocial and psychopharmacological interventions should be advocated through the whole course of HCC treatment (8) implementation of multicenter randomized controlled trials of minimally-invasive therapy versus surgery for early and intermediate stage HCC is recommended.

Keywords: hepatocellular carcinoma, minimally-invasive therapy, multidisciplinary comprehensive treatment, consensus, Guangzhou recommendations

INTRODUCTION

Primary liver cancer is the fourth most common malignant tumor and the third leading cause of cancer death in China, which seriously threatens the lives and health of Chinese people. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancer (1, 2). In China, chronic hepatitis B virus (HBV) infection is the main cause of HCC. Approximately 85% of HCC cases are associated with HBV infection, and only approximately 10% are associated with hepatitis C virus (HCV) infection. Conversely, approximately 70% of HCC cases in European countries, North America, and Japan are associated with alcohol and HCV infection (1, 3). China has a large population of patients with liver cancer. There is not yet a worldwide consensus on the treatment strategy for liver cancer, and there is significant divergence in the guidelines for liver cancer treatment in different countries. The epidemiological characteristics, pathogenesis, biological behaviors, staging, diagnosis, treatment, and prognosis of HCC in China are significantly different from those in Europe, North America, and Japan. Therefore, the HCC guidelines developed by the Liver Disease Associations of the European countries, North America, and Japan are not fully applicable to HCC diagnosis and treatment in China. Even the "guidelines or expert consensus" representing the opinions of professionals in different disciplines or different societies are controversial in China. In China, the vast majority of HCC cases result from long-term HBV infection and cirrhosis development. Pathologically, HCC is characterized by a rich blood supply and multicenter origins, with early invasion of small branches of the portal vein and intrahepatic metastasis. Therefore, HCC is not only a local organ disease but also a systemic disease from the beginning of its occurrence (4). In recent years, with the continuous advancement of minimally invasive interventional treatment techniques for HCC guided by imaging and studies of related large-scale randomized clinical trials, the efficacy of minimally invasive interventional therapy has been enhanced. In the meantime, the benefits of a multidisciplinary comprehensive treatment regimen for HCC have also been widely recognized in the clinic. In 2015, Minimally Invasive Therapy in Oncology of Chinese Anti-Cancer association published an article in the "National Medical Journal of China" (also called "Zhonghua Yi Xue Za Zhi") to preliminarily describe the strategy for minimally invasive, multidisciplinary and comprehensive diagnosis and treatment of HCC (5). On the basis of the preliminary strategy, this consensus further summarizes previous achievements and experience in HCC treatment and highlights the following trends in HCC treatment: 1. More accurate diagnosis and staging; 2. Interventional and minimally invasive treatment, biological immunotherapy, Chinese herbal medicine, psychosocial and psychopharmacological interventions, and humanistic care, which constitute the basic framework for a modern HCC treatment approach; 3. Further explanation of the "constructive treatment concept and strategy" for tumors consistently advocated by the authors, i.e., while effectively inactivating the tumor, the physiological functions, immune function, and quality of life of the patients are optimally preserved. When choosing treatment strategies and methods, minimally invasive interventional therapy combined with multidisciplinary comprehensive treatment is preferred, and extensive wound damage should be avoided or reduced as much as possible. This consensus integrates the clinical diagnosis and treatment strategies of HCC in China and aims to reflect the individualized, rational, and humanistic features of a constructive treatment regimen for HCC.

DIAGNOSIS

HCC diagnosis comprises two major aspects: clinical diagnosis and pathological diagnosis. Clinical diagnosis primarily depends on determination of cirrhosis history caused by chronic hepatitis

(HBV and/or HCV) infection and/or other causes, serological diagnosis, and imaging diagnosis.

Serological Diagnosis

More than 60% of HCC patients in China show serum alpha-fetoprotein (AFP) levels >400 ng/ml. Therefore, AFP is of significant importance for surveillance and diagnosis of HCC in China (6). For patients with AFP≥400 μg/L for more than 1 month, ≥200 μg/L for 2 months, or a gradually increased and stabilized AFP level but without pregnancy, gonadal embryoma, or active liver disease, HCC should be highly suspected. However, notably, when the AFP level is normal or below the diagnostic criteria, HCC cannot be completely excluded. Approximately 30% of patients with HCC have AFP levels below 20 ng/mL, and 10% to 42% of AFP abnormalities are caused by pregnancy, gonadal embryoma, active hepatitis, the active inflammatory stage of cirrhosis, or metastatic liver tumors (7). Therefore, AFP cannot be used as the only indicator for HCC surveillance and diagnosis. At present, many studies have found that des-γ-carboxy prothrombin (DCP) [also known as protein induced by vitamin K absence or antagonist (PIVKA) II] and AFP-L3/AFP assessments can improve the sensitivity and specificity of early liver cancer diagnosis. DCP>40 mAU/mL or AFP-L3/AFP>15% suggests the possibility of liver cancer (8). Application of the methylation spectrum of circulating tumor DNA (ctDNA) in the diagnosis of tumors, which is one of the "liquid biopsy" markers, is a hotspot in the field of cancer research using circulating tumor nucleic acids. Xu et al. (9) examined the methylation level of specific loci on ctDNA using a few milliliters of blood for early diagnosis of HCC and achieved a diagnostic sensitivity of 84.8% and a specificity of 93.1%.

Imaging Diagnosis

Imaging plays a crucial role in HCC diagnosis. Currently, the imaging examination methods used for HCC diagnosis primarily include ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), digital subtraction angiography (DSA), and positron emission tomography (PET)/CT. Dynamic enhanced CT and/or MRI are the main diagnostic tools for HCC. CT arterial portography (CTAP)/CT hepatic arteriography (CTHA) combined with lipiodol CT (Lp-CT) can improve the sensitivity and specificity of HCC diagnosis (especially for lesions with a diameter <1 cm). However, since CTAP, CTHA, and Lp-CT are invasive diagnostic procedures, they are used as secondary imaging-based diagnostic methods for HCC. Ultrasound examination is easily available and convenient to perform for initial HCC screening, but the results tend to be affected by the skill of the operators, equipment, liver texture, patient's body built, obstacles from bone and air. In addition, cholangiocarcinoma tends to contribute to false positive findings in contrast-enhanced ultrasound. PET/CT is beneficial in small population for evaluating the extension of HCC. Therefore, ultrasonography and PET/CT are not included in the present HCC diagnostic criteria. Moreover, diagnosis of specific liver cancer types should be emphasized, such as infiltrative and small HCC.

As the primary imaging-based diagnostic method, the characteristic manifestations indicative of HCC diagnosis observed with dynamic contrast-enhanced CT and/or MRI are arterial phase enhancement and washout during the portal venous phase or delayed phase (10, 11). Statistically, the specificity of dynamic enhanced CT and/or MRI for diagnosis of lesions 1-2 cm in diameter with typical nodule manifestations is 96.6%, and the sensitivity is 62%. For nodules with a diameter > 2 cm, the sensitivity reaches 96% (12, 13). Therefore, for nodules with a diameter >1 cm, when dynamic enhanced CT and/or MRI demonstrate arterial phase enhancement and washout during the portal venous phase or delayed phase, HCC should be highly suspected. However, attention should be paid to the specific manifestations of infiltrative HCC. Infiltrative HCC is defined as an HCC lesion with unclear borders diffusely distributed in multiple hepatic segments, occupying an entire hepatic lobe or the entire liver. Infiltrative HCC accounts for 7% to 13% of all HCC cases. It is more commonly seen in patients with HBV infection, frequently associated with portal vein tumor thrombosis, and has a poor prognosis. Infiltrative HCC is often accompanied by cirrhosis and produces nodules similar to cirrhosis, which are difficult to detect with CT and MRI. MRI diagnosis is more meaningful than CT and is based on an uneven, slightly lower signal on T1 WI, a slightly higher signal on T2 WI, limited diffusion, mostly uneven or miliary enhancement in the arterial phase, and no washout in the portal venous phase, which, in contrast, demonstrates continuous enhancement (14, 15). Due to the poor sensitivity of dynamic enhanced CT/MRI in the diagnosis of HCC lesions smaller than 1 cm, the current European and American diagnostic criteria only apply to HCC larger than 1 cm, and the diagnostic value of liver-specific contrast agents is not emphasized in those guidelines. Although liver-specific contrast agents can improve the diagnostic rate of MRI for HCC smaller than 1 cm, the sensitivity is still low (approximately 46%) (16). When HCC is highly suspected in clinical practice, and CT and MRI cannot detect a lesion with a typical imaging manifestations, work-up with combined application of secondary imaging diagnostic methods should be done.

As a secondary imaging diagnostic method, CTAP reveals a HCC on the basis of portal venous blood supply, which is displayed as a filling defect on the background of highly enhanced normal liver tissue. On CTHA, HCC appear as enhanced nodules at arterial phase, which should be distinguished from arterial-portal shunt and abnormal perfusion. CTHA combined with CTAP can reduce the false positive rate and significantly improve diagnostic sensitivity (from up to 80% to 95%) and specificity for HCC (especially lesions with a diameter ≤1 cm) (17). Some studies have found that CTHA/CTAP can detect 32.8% of lesions that are not detected with dynamic enhanced CT, especially in patients with an HBV (-) status, multiple nodules, and intrahepatic recurrence or metastasis after treatment (18, 19). CTHA/CTAP can accurately assess the extent of the lesion and identify disseminated intrahepatic foci. In the meantime, CTHA/CTAP is more sensitive to intrahepatic hemodynamic changes, which is beneficial for assessment of the infiltration status of intrahepatic blood vessels (even tiny blood vessels) (20, 21). Therefore, CTHA/CTAP provides more precise

pretreatment tumor staging and facilitates selection of the optimal treatment choice. Clinically, approximately 15% of small HCC lesions or foci cannot be detected by CTHA/CTAP and require combined examination *via* Lp-CT (22–26). The Lp-CT is performed by injecting 3 to 4 mL of lipiodol through the hepatic artery, followed by liver CT 2 weeks later, and HCC is manifested as intrahepatic lipiodol deposition foci. Lipiodol deposits in HCC are generally dense and uniform, and lipiodol deposits in some necrotic HCC are incomplete and distributed in the periphery or center of the lesion. Lp-CT is of significant importance for detection of HCC with a low degree of differentiation, micro-HCC, small lesions with multicenter origins and intrahepatic micrometastases, and lipiodol also has certain therapeutic effects (22, 27, 28). As early as in 2003, Wu and co-editors monographed a book in Chinese "Minimally Invasive and Multidisciplinary Comprehensive Treatment of Hepatocellular Carcinoma" detailed the diagnosis and treatment of micro-HCC (29). Micro-HCC is defined as follows: 1. Lesion diameter ≤ 0.5 cm; 2. Clinically elevated AFP level (AFP ≥ 400 μg/L for more than 1 month or ≥ 200 μg/L for 2 months); 3. Lesions that were detected by CTAP/CTHA or/and present as spotted lipiodol deposits in Lp-CT; 4. AFP decreased or returned to normal level after Lp-CT or transcatheter arterial embolization (TAE). In view of the invasiveness and high cost of CTHA/CTAP and Lp-CT, these examinations are recommended as secondary diagnostic tools or combined with transarterial chemoembolization (TACE) or TAE to facilitate accurate diagnosis and staging and to simultaneously achieve therapeutic goals.

Pathological Diagnosis

Pathological diagnosis of HCC is recommended when imaging examination shows noncirrhotic liver nodules, uncertain or atypical imaging findings in a cirrhotic liver, or when serology and imaging diagnoses are contradictory. Studies have found that the positive biopsy rate of lesions smaller than 2 cm is only approximately 60% (30), and therefore, negative biopsy results do not completely rule out HCC diagnosis, and still requires further diagnostic work-up or clinical follow-up. **Figure 1** shows the HCC diagnosis process.

TREATMENTS

HCC occurrence and development are complicated dynamic processes involving multiple factors that are conjoined in multiple steps and include different modifications and gene mutations in many molecular pathways. HCC usually originates from chronic hepatitis, proceeding to cirrhosis and then to HCC. In China, the majority of liver tumors have a rich blood supply, and are accompanied by a background of hepatitis or cirrhosis, and exhibit multicenter occurrence, portal vein tumor thrombus and intrahepatic dissemination even at the its early presentation (4). Therefore, single local treatment cannot achieve a curative effect, and a comprehensive treatment regimen should be adopted that includes local treatment (surgery and ablation) combined with organ level treatment (TACE and perfusion chemotherapy) and systemic treatment (psychotherapy, antiviral treatment, molecular targeting, biological immune therapy, and Chinese traditional medicine treatment).

This consensus emphasizes that minimally invasive treatment should be considered first for early-stage HCC when tumor ablation is safe. If minimally invasive treatment is not applicable for the patient after detailed evaluation, the more traumatic surgical treatment can be considered. The various current treatments and comprehensive treatment principles are described below.

Local Ablation

Local ablation is minimally invasive, safe, effective, and can be performed repeatedly. Ablation methods include physical ablation (such as radiofrequency ablation (RFA), microwave ablation (MWA), cryoablation, high intensity focused ultrasound (HIFU) ablation, and irreversible electroporation (IRE) ablation) and chemical ablation [such as percutaneous ethanol injection (PEI) or percutaneous acetic acid injection (PAI)]. For single HCC lesion with a diameter ≤ 2 cm, local ablation treatment can achieve long-term efficacy similar to or superior to surgical treatment, with the advantages of less liver function damage, fewer complications, faster recovery, and shorter hospitalization and therefore should be considered the top option (31–33). For a single HCC lesion with a diameter of 3 to 5 cm or 2 to 3 lesions with diameters < 3 cm, the therapeutic effect can be improved by combining TACE treatment with a suitable ablation technique (34). For tumors with a diameter of more than 5 cm or more than three tumor lesions, local ablation cannot inactivate all of the tumor tissue; small satellite lesions are easily missed, and the local recurrence rate is high. Therefore, TACE combined with ablation is significantly superior to ablation alone under these circumstances. A potential drawback is that such combination requires two consecutive treatment sessions, which poses an increased risk for multifocal tumor recurrence or focal progression through repeated manipulation (35). For a tumor to be considered inside the ablation safety zone, the lesion must not be close to or contiguous to the gallbladder, the hilum of the liver, stomach, intestines, or the heart. Ablation of tumors in these dangerous areas may require comprehensive ablation methods, including the water insulation technique (hydrodissection), combined with chemical ablation and particle (^{125}I seed) implantation.

RFA and MWA are the most widely used local ablation techniques, with well documented therapeutic effects. When the lesion is larger than 2 cm, RFA is superior to PEI. For tumors adjacent to a large blood vessel or for a large tumor, the effect of MWA may be better due to its shorter ablation procedure time, larger ablation zone, and lower heat sink effect compared with RFA. However, the current RFA and WMA comparison studies show no significant difference in local efficacy and occurrence of complications between the two methods (36–38). Cryospheres formed by cryoablation can be easily observed at imaging (especially CT and MRI) studies, which is convenient for controlling the ablation zone to avoid damaging surrounding normal structures or tissues. However, the incidence of complications, such as hemorrhage, in cryoablation is significantly higher than that in RFA, and thus, cryoablation is not widely used in liver cancer treatment (39, 40). HIFU, by combining non-touch, conformal, and real-time treatment, has advantages for the treatment of multiple tumors and/or liver cancer in some specific locations. However, HIFU treatment takes a longer time, and it is

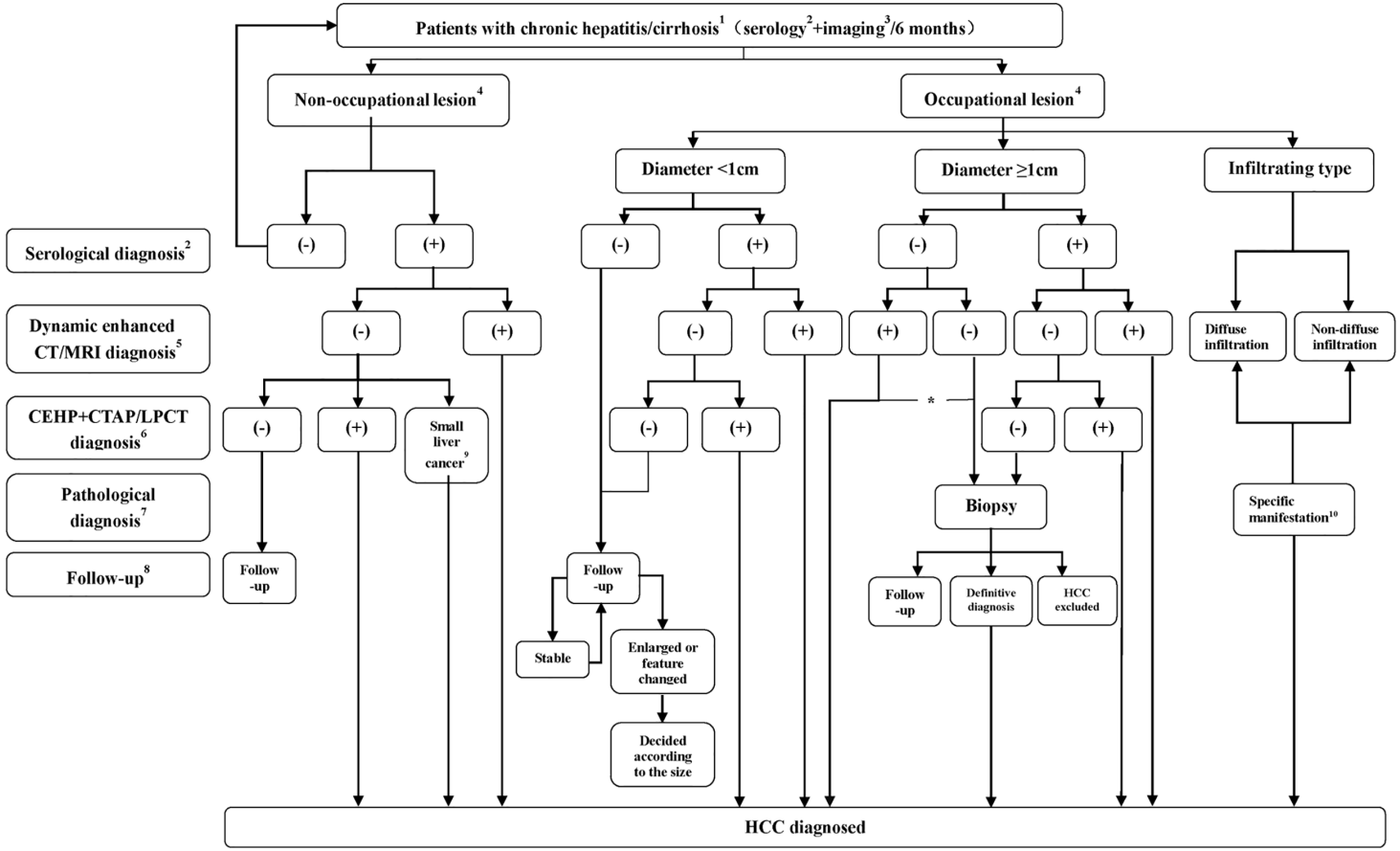

FIGURE 1 | Flowchart of HCC diagnosis. [1] The flowchart is mainly applicable to the diagnosis of HCC patients with HBV and/or HCV infection or a liver cirrhosis background related to various causes. For intrahepatic space-occupying lesion patients without hepatitis or a cirrhosis history, diagnosis is usually made by using biopsy; [2] or serological diagnosis including AFP, AFP-L3, or DCP detection. Serological diagnosis (**+**) refers to AFP ≥ 400 μg/L for more than 1 month or ≥ 200 μg/L for 2 months, DCP > 40 mAU/mL or AFP- L3/AFP>15%; [3] including ultrasound or preliminary CT/MRI examination, with ultrasound more commonly performed; [4] No nodule refers to no nodule detected in the liver during imaging follow-up, and intrahepatic nodule refers to a nodular lesion detected in the liver during imaging follow-up; [5] Significant enhancement during the arterial phase and washout during the portal venous phase or delayed phase; [6] Nodule enhancement in CTHA, filling defect in CTAP and lipiodol deposits in the nodules 2 to 3 weeks after lipiodol injection revealed by Lp-CT; [7] Biopsy (-) does not rule out the possibility of HCC, which still requires close follow-up; [8] Serological plus imaging monitoring is performed every 3 months during the first 2 years, and the patient is considered stable if there is no significant change and is then followed up at 6 months intervals; [9] Micro-HCC is defined as follows: 1. Lesion diameter ≤ 0.5 cm; 2. Clinically elevated AFP (AFP ≥ 400 μg/L for more than 1 month or ≥ 200 μg/L for 2 months); 3. Lesions that were detected by CTAP/CTHA or/and present as spotted lipiodol deposits in Lp-CT or TACE; 4. AFP decreased or returned to a normal level after Lp-CT or TACE; [10] Refers to nondiffuse infiltration and diffuse distribution of lesions with an unclear boundary in the liver. T1 WI shows an uneven, slightly low signal, and T2 WI shows a slightly high signal, limited diffusion, and uneven or miliary enhancement or slight enhancement in the arterial phase and no washout in the portal venous phase, which instead would demonstrate continuous enhancement; *For nodules with a diameter of more than 1 cm with typical imaging manifestations, when serological diagnosis is negative, the lesions should be distinguished from primary HCC and other rare pathological types. If the patients choose nonsurgical treatment, a needle biopsy is recommended.

relatively difficult to locate the tumor. Color Doppler ultrasound monitoring can be performed in real-time and is convenient; however, due to the interference of the ribs and gastrointestinal gases, the efficacy of this treatment is dependent on the operator's experience and skill. Furthermore, the high echo shadow generated during ablation affects observation of the treatment efficacy. HIFU treatment has the deficiencies of therapeutic dosimetry and unclear effects on normal tissues (nonablation areas) after therapeutic ultrasound. Clinically, there is a lack of studies comparing HIFU with other ablation treatments, and thus, the efficacy of HIFU is difficult to evaluate (41–43). As a new nonthermal ablation technique, IRE employs high voltage to irreversibly damage cell membranes and induce apoptosis. IRE treatment has the advantages of a short procedure time, precise ablation zone, no influence of a heat sink effect, and no damage to large blood vessels or bile ducts. Therefore, IRE provides a new option for HCC treatment at certain locations (such as lesions adjacent to large blood vessels and bile ducts and the subcapsular area). However, its effectiveness and safety still await validation in larger cohorts of patients (44–46).

Surgical Treatment

Surgical treatment is one of the main treatments for HCC and includes liver resection and liver transplantation. For patients in good general condition with a sufficient liver function and remnant liver (15-min retention rate of indocyanine green test <14%), and no severe disease involving the heart, lung, kidney, or other important organs, hepatectomy can be performed. The indications for liver transplantation in patients with HCC are controversial. In China, the incidence of HCC is high, donor livers are not easily available, the cost of transplantation is high, and long-term use of immunosuppressive drugs after liver transplantation is necessary, which leads to inevitable postoperative recurrence. This situation is incompatible with the rapid development and progress in tumor immunology research in the past 10 years. Therefore, liver transplantation is not the first choice or routine treatment for HCC, especially for the treatment of early-stage HCC without cirrhosis decompensation and liver failure.

Hepatic Artery Interventional Treatments

Trans-arterial interventional treatments for HCC primarily include TAE, TACE, hepatic arterial infusion chemotherapy (HAIC) and transcatheter arterial radioembolization (TARE). Lp-TACE [also known as conventional TACE (cTACE)] has the widest clinical applications.

For patients with permitting liver function, lipiodol-TACE (Lp-TACE) should be the first treatment choice and can accurately stage and detect subcentimeter lesions and achieve organ level treatment. Lp-TACE as the first-line treatment for patients with intermediate stage HCC (2 to 3 lesions with diameters >3 cm or >3 lesions without portal vein tumor thrombus or extrahepatic metastasis) can effectively block the arterial blood supply to the liver tumor and continuously release a high concentration of chemotherapeutic drugs, which result in ischemic necrosis, shrinkage of the tumor, and control of tumor growth while having little effect on normal liver tissue. In Lp-TACE, ultra liquid lipiodol is fully mixed with chemotherapeutic drugs to form an emulsion, which is injected into the tumor blood supplying artery *via* microcatheter superselection. Commonly used chemotherapeutic drugs are anthracyclines and platinum, and combined administration is better than single drug use (47, 48). Enhanced embolization refers to the combination of gelatin sponge embolization with (after) Lp-TACE treatment, which can increase the efficacy of Lp-TACE. Drug-eluting bead (DEB)-TACE employs bead, which can carry a sufficient drug amount and slowly release chemotherapeutic drugs to achieve and maintain a lethal dose in the tumor tissue for several days to several weeks, while the drug concentration in systemic blood circulation is very low. After DEB-TACE treatment, the rate of tumor necrosis is high, and the adverse effects of systemic chemotherapy are mild. Previous study showed that the occurrence of locoregional complications and global hepatic damage after DEB-TACE, such as bile duct injury, intrahepatic biloma, and liver function impairment (presented as high baseline prothrombin value), was significantly higher than that with Lp-TACE alone and was more obvious in patients with severe cirrhosis (49). Moreover, the antitumor effect and overall survival between the two treatments are not significantly different (50–53). It is therefore suggested that Lp-TACE may be more appropriate than DEB-TACE in patients with less advanced cirrhosis.

Lp-TACE can provide diagnostic information and choice of treatment with the following advantages: 1. Induction of necrosis and tumor shrinkage to achieve a downstaging effect and obtain opportunities for surgery or ablation; 2. Detection of missed lesions on other imaging modalities, especially inconspicuous foci scattered small lesions; 3. Reduction of the blood supply inside and around the tumor, thereby reducing the impact of a heat sink effect; 4. Deposited lipiodol is has a positioning effect, thereby improving treatment accuracy (54, 55). For patients with early- and intermediate-stage HCC with good liver function, TACE treatment is recommended first (56). However, TACE alone cannot lead to complete tumor necrosis (the complete necrosis rate is only approximately 20%) and has difficulty destroying peripheral tumors surrounding the tumor lesion. Repeated TACE treatments impair liver function, and therefore, locally enhanced treatments, such as local ablation, surgery, and biotherapy, are necessary to eliminate residual tumors. A repeated contrast-enhanced CT or MRI of the liver at 3 to 4 weeks after the initial TACE treatment is recommended. Subsequent combined ablation treatment can achieve a complete tumor necrosis rate of more than 90%. Repeated TACE treatments are not recommended because they can cause liver function impairment and aggravate cirrhosis. For tumors smaller than 5 cm, it is recommended to perform one TACE followed by combined ablation therapy; for tumors larger than 5 cm, subsequent combined ablation treatment can be performed after two to three TACE treatments (57, 58). On the basis of the follow-up evaluation, another TACE treatment, possibly in combination with other treatments, can be done if needed.

TARE is a trans-arterial interventional treatment in which ^{90}Y or ^{131}I microspheres or similar reagents are intra-arterially injected for continuous brachytherapy of cancer cells. The main indications of TARE include the following: 1. For the treatment of patients with a large tumor or multifocal or diffuse disease, which are not suitable for TACE; 2. For patients with portal vein tumor thrombus; 3. Disease progression after TACE or sorafenib treatment. Since arterial-venous or arterial-portal shunt

formation are common in patients with cirrhosis, the treatment efficacy of TARE is influenced. Therefore, its application still awaits further clinical confirmation (59, 60). In China, most liver cancer is accompanied by cirrhosis, and thus, the effects of TARE on cirrhosis also need further long-term observation.

Furthermore, when TACE cannot be performed due to liver dysfunction of the patients, CTHA, CTAP, or Lp-CT can still be performed for detection of subcentimeter lesions.

Molecular Targeted Therapy

Sorafenib and Lenvatinib, approved by the China Food and Drug Administration, are first-line treatment options for advanced HCCs (61). The STORM study suggested that adjuvant sorafenib after surgery to be ineffective (62). Retrospective studies have found that combined administration of sorafenib and TACE in the treatment of advanced liver cancer is superior to sorafenib alone (63–65); however, randomized controlled studies indicate that in European, American and Asian populations sorafenib administration on top of TACE does not improve treatment efficacy (66). The efficacy of sorafenib in adjuvant therapy after surgery, local ablation for early-stage patients, or in combination with TACE for intermediate-stage patients still awaits validation in larger cohorts of patients or prospective clinical studies.

Radiation Therapy

Radiation therapy consists of external radiotherapy involving external irradiation of tumors and internal radiotherapy in which the radionuclide is directly implanted into the tumor or lumen invaded by the tumor. In the past, due to less developed radiotherapy equipment, radiotherapy was prone to cause radiation-induced liver disease and aggravate liver dysfunction. Moreover, most liver cancer patients with cirrhosis tolerated the treatment poorly, and thus, the application of radiotherapy in HCC was limited. Since the mid-1990s, modern, precision radiotherapy techniques (e.g., three-dimensional conformal radiotherapy, intensity-modulated conformal radiotherapy, and stereotactic radiotherapy) have developed rapidly. Radiation therapy now is mature, and have been widely used in clinical practice (67). Radioactive particle implantation dose distribution is continuously optimized, achieving satisfactory results in liver cancer treatment, especially in the treatment of portal vein tumor thrombus and hilar lymph nodes (68).

Biological Immunotherapy

Immunotherapy for HCC primarily includes immunomodulators [interferon α, thymosin α1 (thymalfasin), etc.], immune checkpoint blockers [cytotoxic T lymphocyte-associated antigen (CTLA)-4 blocker, and programmed cell death protein 1 (PD-1) and ligand (PD-L1) blocker], tumor vaccines (dendritic cell vaccines) and cellular immunotherapy [cytokine-induced killer (CIK)]. Biotherapy can improve antitumor efficacy and enhance immunity. Phase I/II clinical trials to assess immunotherapy have found that dendritic cell treatment is safe and effective for advanced HCC (69). Studies have shown that reduction of HCC tumor volume combined with dendritic cell-CIK biotherapy can postpone the time to tumor recurrence and benefit patient survival (70, 71). Comprehensive application of CIK in cancer therapy, especially in those with clinical evaluation of complete remission and radical removal of tumor load, is considered an effective method to prevent tumor recurrence (72).

Traditional Chinese Medicine

Many traditional Chinese medicine drugs and treatments are beneficial for reconstructing the immunity of patients, improving quality of life, reducing adverse reactions to radiotherapy and chemotherapy, and putting off tumor progression. In addition to decoction medicine, over the years, many proprietary Chinese medicines have been approved for HCC treatment, and each has their own characteristics and certain effects and has demonstrated good compliance, safety, and tolerance in patients (73). Traditional Chinese medicine advocates treatment of both the symptoms and cause of the disease. Removal of visible tumors treats the cancer-related symptoms but not the underline disease. Improving the constitutional environment and enhancing and improving immune function treat the causes and are important factors to prevent recurrence.

Antiviral Treatment

According to the "Expert consensus of antiviral treatment of HBV/HCV-related hepatocellular carcinoma" (74), antiviral treatment for HBV/HCV-related HCC patients can reduce the recurrence and mortality of HCC, decrease HBV/HCV reactivation, control disease progression, improve liver function, and reduce the occurrence of end-stage liver disease.

Other Treatments

Symptomatic supportive treatments primarily include analgesics, liver protection, cholagogues, improvement of nutritional status, correction of anemia and hypoproteinemia, control of ascites or pleural effusion, and prevention and treatment of gastrointestinal hemorrhage. These symptomatic supportive treatments can alleviate patient suffering, improve quality of life, ensure smooth progression of anticancer treatments, and even improve treatment efficacy or provide opportunity for further treatment.

Comprehensive Treatment Principles and Multidisciplinary Comprehensive Treatment

The proposed treatment algorithm of HCC is shown in **Figure 2**.

EIGHT HIGHLIGHTS OF THE CONSENSUS

Hepatic Angiography, CTHA, CTAP, Lp-CT, and TACE-CT Are Useful in Detection of Focal Liver Lesions and Accurate Staging

Imaging examinations play an important role in HCC diagnosis, and typical imaging characteristics indicating HCC are arterial phase enhancement and washout during the portal venous phase and delayed phase on CT or MRI, which have been included in the guidelines in different countries worldwide (75–78). However, the sensitivity of imaging examinations is limited, especially in the diagnosis of small HCC lesions. Moreover, HCC has multicenter origins and often exhibits early invasion of the small branches of the portal vein, intrahepatic metastasis,

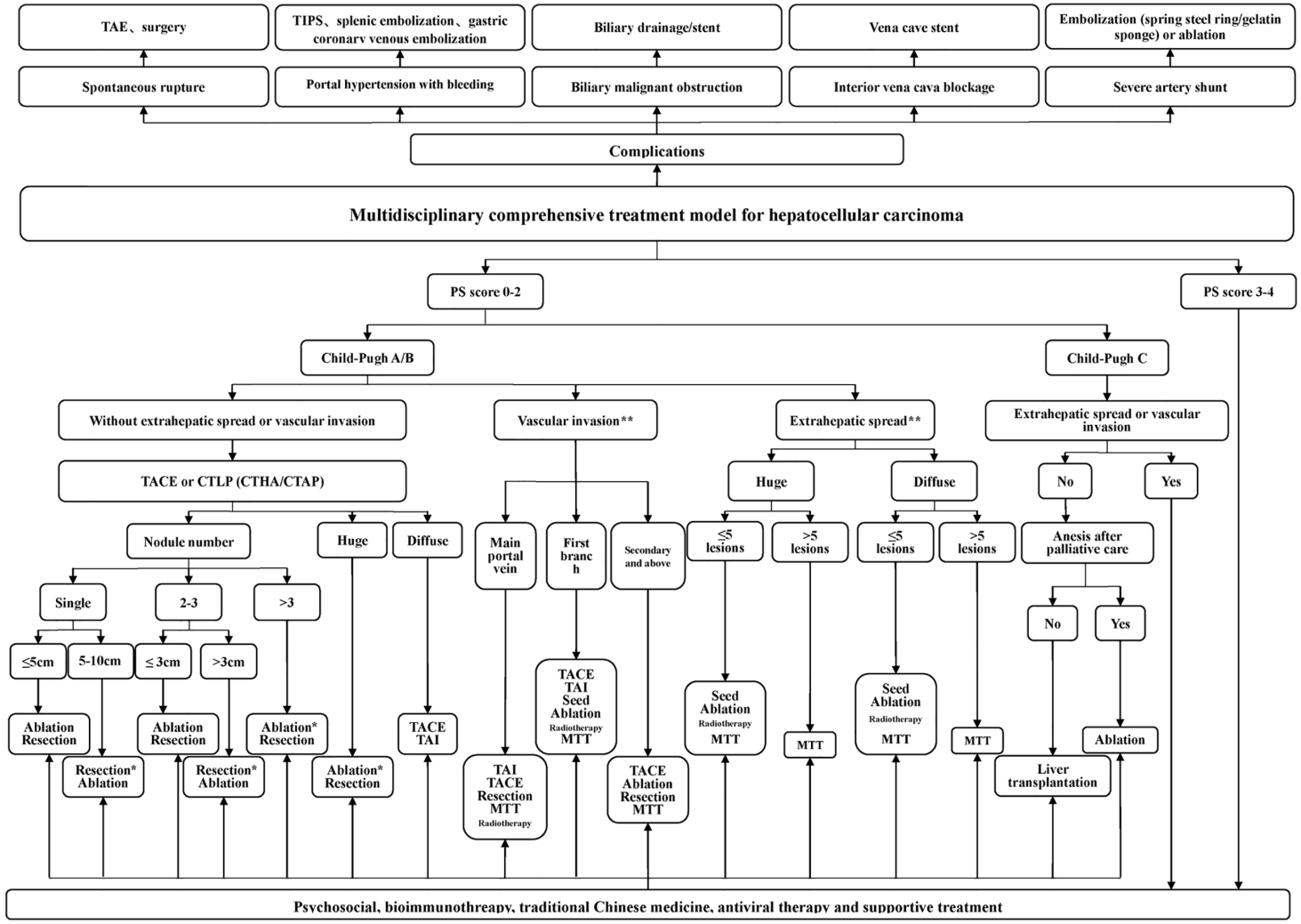

FIGURE 2 | Multidisciplinary, comprehensive treatment model. TIPS: transjugular intrahepatic portosystemic shunt; PS (performance status) score: systemic condition test score; Massive type: maximum tumor diameter > 10 cm; Diffuse type: extensive and diffusely distributed intrahepatic cancer lesions with unclear borders, often accompanied by cirrhosis; Ablation: RFA, MWA, and PEI, among others, but primarily RFA and MWA; *Efficacy comparison of surgery and TACE combined with ablation awaits confirmation in further randomized multicenter clinical studies. **Treatment of portal vein invasion and extrahepatic metastasis should be based on control of intrahepatic lesions, which mostly requires multidisciplinary, comprehensive treatment. 1. Tumors in the safe area are preferably ablated. When a tumor is in a dangerous area and is not suitable for ablation, surgery is considered. 2. Ablation treatment can be repeated. For a tumor that is difficult to ablate in one treatment, the residual portion can be ablated again after follow-up examinations.

and high incidence of recurrence after liver resection. Therefore, early detection of small lesions in the liver and accurate evaluation of focal liver lesions and intrahepatic metastasis are important for disease treatment and prognosis assessment. Hepatic angiography, CTHA, CTAP, Lp-CT, and TACE-CT are useful in detection of focal liver lesions, accurate staging and planning of a suitable treatment regimen (**Figures 3** and **4**). TACE-CT can detect approximately 15% of lesions (most of which are < 5 mm or even < 3 mm) that are unrecognizable in a conventional CT scan (22–26).

TACE/Ablation as the First Choice for Treatment of Early-Stage HCC

The 2019 National Comprehensive Cancer Network (NCCN) guidelines recommend surgical resection or topical treatment for early-stage liver cancer (75). The third edition of the 2019 Japanese Liver Disease Association recommends surgical resection for patients with a single tumor and grade A or B liver function and further recommends RFA as an alternative regimen for patients with tumors smaller than 3 cm (77). The 2018 edition of the European Association of Liver Research guidelines recommend RFA for very early-stage liver cancer (BCLC stage 0) and surgical resection, liver transplantation and ablation treatment for early-stage liver cancer (BCLC stage A) (76). The 2019 edition of China specifications for diagnosis and treatment of primary liver cancer lists surgical resection and ablation as treatment options for early-stage liver cancer (78). Therefore, currently, whether surgical resection or ablation is the preferred treatment for early-stage liver cancer is still controversial.

At present, some clinical studies compared RFA and surgical resection in the treatment of a single small HCC (31, 32, 37, 79–83), and the controversial results remained. Peng et al. (37) enrolled 145 patients with early-stage HCC, of whom 71 underwent RFA and 74 underwent surgical resection. They found that RFA was superior in efficacy and safety to surgical resection, especially when the tumor lesions were more than 3 cm away from the Glisson's capsule. Liu et al. (79) found that among 79 patients with RFA and 79 patients with surgical resection, tumor recurrence and survival were better in the surgical group than in the RFA group and concluded that surgical resection should be considered the preferred treatment method. Chen et al. (80) conducted a prospective, randomized, controlled study of patients with HCC ≤ 5 cm; 71 patients received RFA, and 90 patients underwent surgical resection. There was no survival difference between the two groups of patients, and subgroup analysis based on tumor size (≤ 3 cm and 3 to 5 cm) showed similar results. Therefore, the authors concluded that RFA, which is less invasive, had an efficacy similar to that of surgical resection. Huang et al. (81) compared RFA and surgical treatment for HCC patients with a single lesion <3 cm and found no difference in tumor control and survival between the 121 patients who underwent ablation and the 225 patients who underwent surgical resection, and the quality of life score in the ablation group was significantly better than that in the surgery group. Kang et al. (82) compared 198 patients with early-stage HCC using propensity-matching analysis and reached a similar conclusion that the efficacy of RFA treatment was comparable to that of surgical resection, and patients with RFA had fewer complications, faster recovery, and a significantly shortened hospitalization time. Kutlu et al. (83) used the US National Cancer Institute's "surveillance, epidemiology, and end results (SEER) database" to conduct a large-scale study of 1,894 patients with HCC between 2004 and 2013 and found that there was no significant difference in the efficacy of RFA and surgical resection for tumors < 3 cm. Majumdar et al. (32) systematically reviewed four clinical studies that included 574 patients to compare RFA with

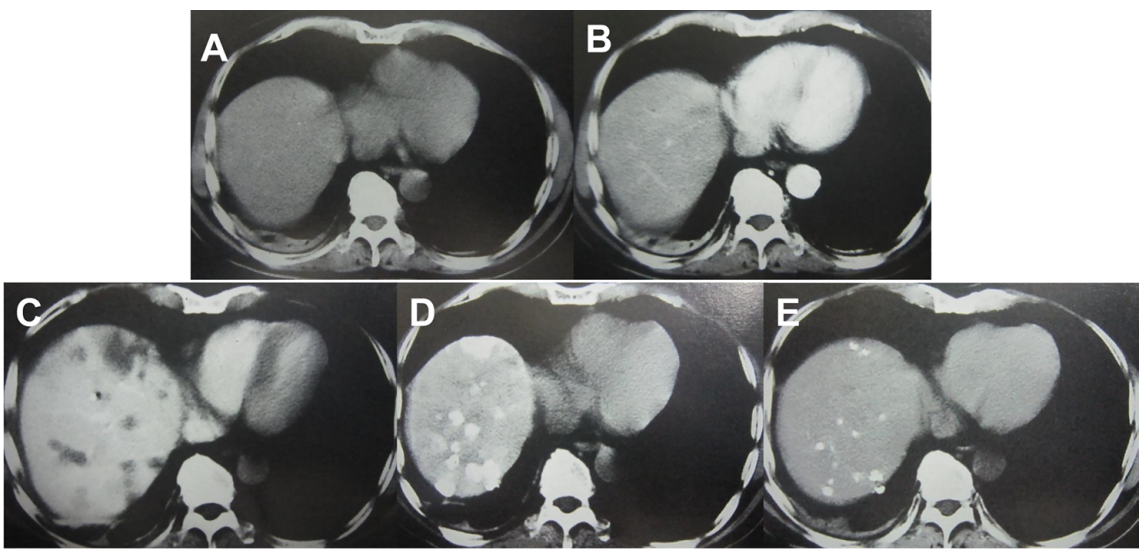

FIGURE 3 | Imaging examination of an HCC patient with a progressively elevated AFP level. **(A)** CT plain scan showed no lesions in the liver; **(B)** A contrast-enhanced CT scan failed to reveal any lesions in the liver; **(C)** CTAP showed intrahepatic low-density lesions; **(D)** CTHA showed intrahepatic high-density lesions; **(E)** Lp-CT showed multiple intrahepatic lipiodol deposits after 3 weeks.

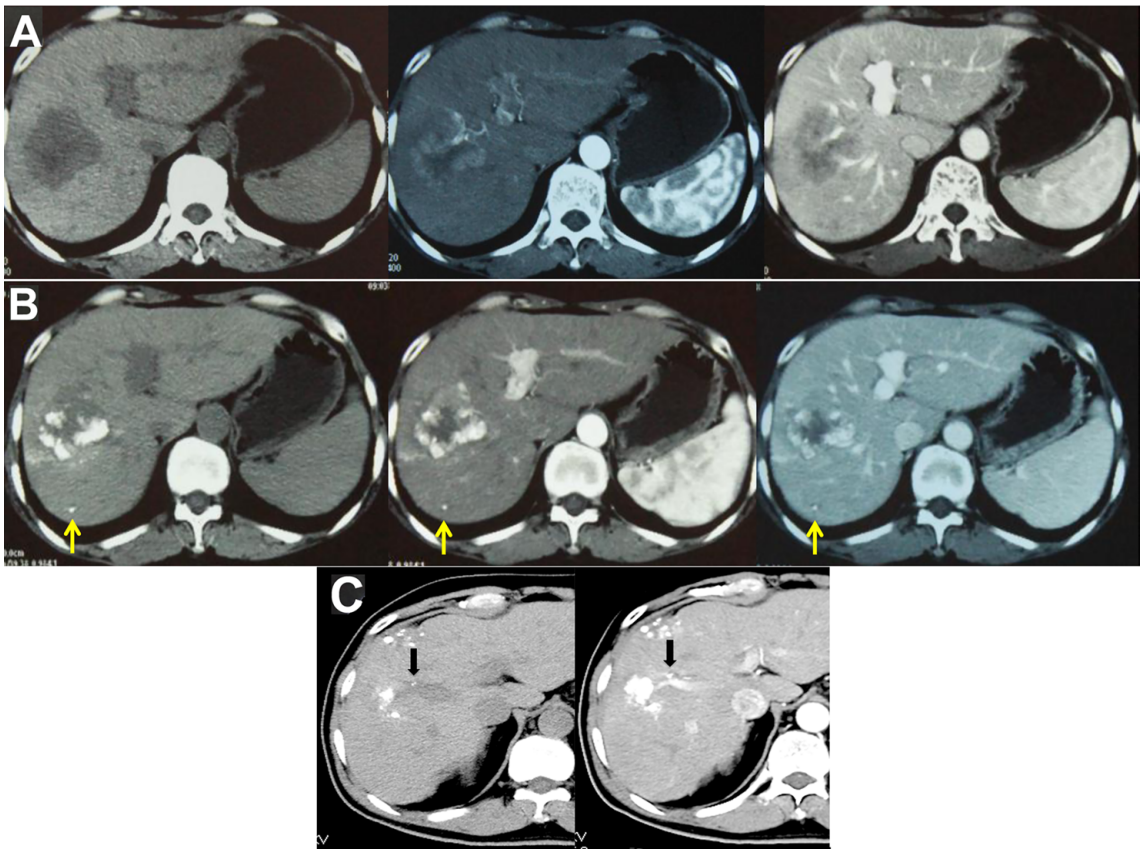

FIGURE 4 | TACE-CT enables detection of focal loci and precise staging. **(A)** CT before treatment showed a 7-cm tumor in the S5 and S6 region of the liver; **(B)** Follow-up CT examination at 4 weeks after TACE showed that the tumor volume at S5 and S6 was reduced, while a small lesion was detected at S6, as indicated by the arrow; **(C)** In another male patient, follow-up CT examination at 3 weeks after TACE showed intravascular lipiodol deposits (indicated by the arrow), suggesting the formation of an intravascular small tumor thrombus.

surgical resection and found that there was no significant difference in survival between the two treatments. In summary, ablation and surgery have similar efficacy in the treatment of early-stage HCC, and ablation is more advantageous from a health economics perspective (84). Therefore, the authors emphasize prioritizing interventional ablation treatment and only suggest surgical resection under circumstances when ablation is not appropriate.

TACE is a holistic treatment at the organ level and is applied at the first step of minimally invasive treatment. Its main role is to reduce tumor blood supply and reduce tumor load. Emulsified lipiodol and chemotherapeutic drugs are deposited in small lesions or foci that are difficult to detect by conventional contrast-enhanced CT scanning, thereby treating small lesions or foci while also revealing them and guiding the next step in minimally invasive treatment. TACE combined with ablation has demonstrated a higher survival rate and better tumor control rate than simple ablation and does not significantly increase the incidence of complications (85–87). Therefore, TACE/ablation has obvious advantages as a primary treatment for early-stage HCC (**Figure 5**).

Infiltrative HCC as an Independent Subtype

Infiltrative HCC is divided into diffuse and nondiffuse types. The imaging manifestation of infiltrative HCC is a lack of a clear boundary between tumor and normal liver tissue. Patients often have a history of cirrhosis. This HCC type accounts for approximately 7% to 13% of total HCC cases (14, 15). Risk factor analysis has shown that infiltrative HCC is more common in patients with HBV infection (88). Moreover, this type of HCC has diffuse distribution characteristics and is more likely to invade the portal vein system. Statistically, portal vein invasion in infiltrative HCC is much more common than in the noninfiltrating type (68% *vs.* 25%, $P < 0.001$) (14), and the prognosis of infiltrative HCC is worse than that of nodular type HCC with an intact capsule (**Figure 6**). Benvegnu et al. (88) reported that the 1-year and 3-year survival rates for HCC patients with an intact capsule were 75.4% and 46.0%, respectively, while those for infiltrative HCC patients were 33.3% and 13.6%, respectively. Kneuertz et al. (14) reported that the 1-year and 3-year survival rates for infiltrative HCC patients were 43% and 29%, respectively, and the median survival time was only 10 months.

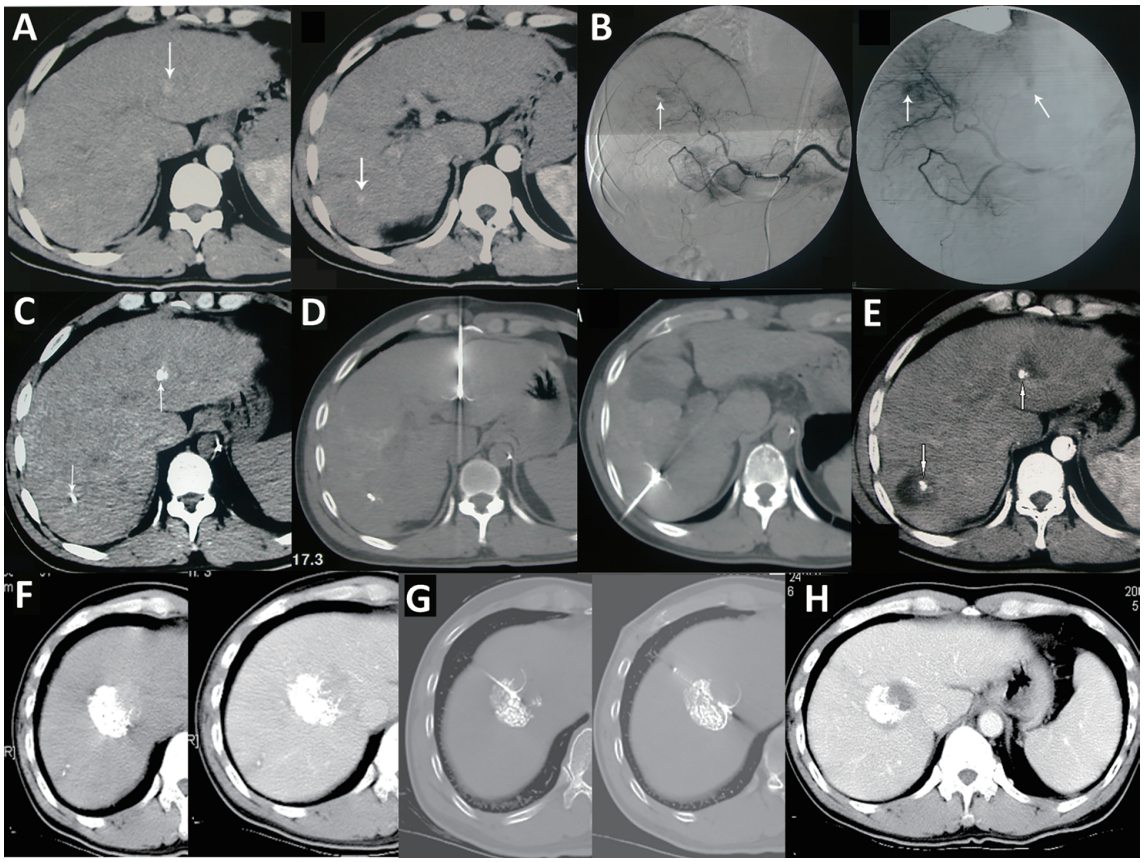

FIGURE 5 | TACE combined with ablation is the preferred treatment for HCC at an ideal location. **(A)** A case of HCC with a progressively increased AFP level. Liver CT shows two lesions in the left lateral segment and right posteroinferior segment of the liver; **(B)** DSA during the TACE treatment showed staining of tumors in the left and right lobe (arrow); **(C)** Lp-CT confirmed two lesions; **(D)** CT-guided radiofrequency ablation of lipiodol deposited lesions; **(E)** One month after combined treatment, AFP decreased to a normal level, and no tumor recurrence was observed five year later in the follow-up CT scan. **(F)** Another case of HCC after TACE treatment combined with radiofrequency ablation treatment **(G)**; **(H)** A 15-year postoperative follow-up examination showed complete inactivation of the lesion.

The treatment options for infiltrative HCC are also limited because the majority of patients are in the advanced stage, and tumor infiltration and growth are typically accompanied by portal vein invasion, which is a contraindication for surgical resection and liver transplantation. Transarterial intravascular treatment is an effective and feasible treatment for infiltrative HCC. Studies conducted by Lyu et al. (89, 90) indicated that HAIC for patients with advanced HCC accompanied by portal vein tumor thrombus was effective. A phase II, single-arm clinical trial showed that 49 patients achieved a good tumor control rate, a considerable survival rate, fewer adverse reactions, and a higher quality of life after treatment. Further comparison with administration of oral sorafenib, a targeted drug, showed that the median progression-free survival time of the HAIC group was longer than that of the sorafenib group [based on the modified response evaluation criteria in solid tumors (mRECIST), 7.4 months *vs.* 3.6 months, P< 0.001] and the median survival time was better than that of the sorafenib group (14.5 months *vs.* 7.0 months, P < 0.001). In the meantime, a study of 147 propensity matched pairs further confirmed the reliability of the results. Multivariate regression analysis also confirmed that HAIC was a favorable factor for tumor control (P < 0.001) and extended survival time (P < 0.001) of patients. For specific protocols for hepatic arterial infusion chemotherapy, refer to the literature (91).

Minimally Invasive and Comprehensive Treatment of Metastatic Lymph Nodes

In advanced patients with extrahepatic metastases *via* molecular targeted therapy (sorafenib), although effective, extends survival time by only 3 months. Furthermore, patients at this stage usually die of intrahepatic lesion progression rather than extrahepatic metastasis. Therefore, multidisciplinary comprehensive therapy consisting of treatment of intrahepatic lesions combined with local treatment of extrahepatic metastases is still advocated (92–97) (**Figure 7**). The principles of treatment are as follows: 1. Protection of lymph nodes with normal function; 2. Inactivation of metastatic lymph nodes; 3. Close observation and follow-up of suspect lymph nodes.

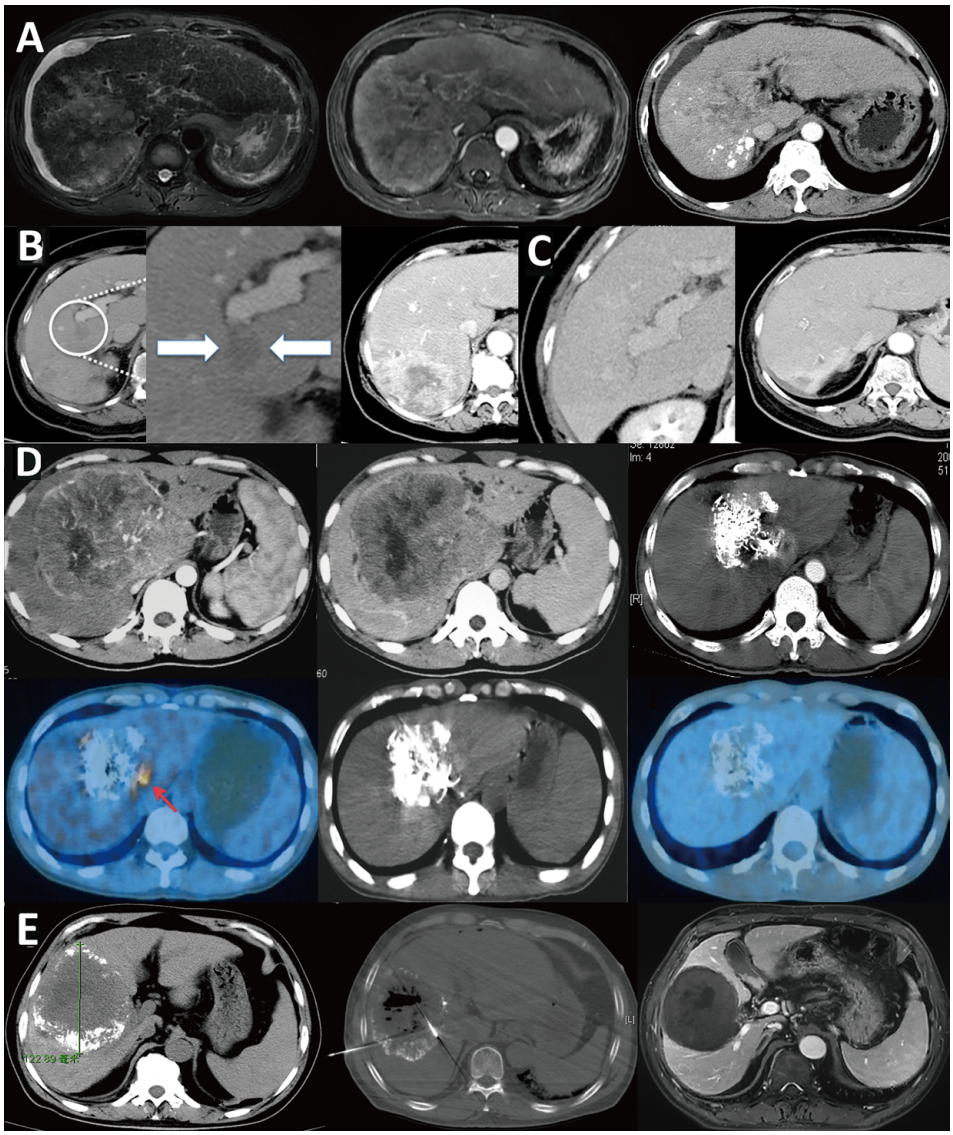

FIGURE 6 | Infiltrative HCC is different from massive HCC and should be classified separately. **(A)** Infiltrative HCC in a patient with hepatitis B cirrhosis. No obvious boundary around the tumor, combined with tumor thrombus in the portal vein and its branches. After TACE treatment, scattered lipiodol was deposited. The treatment effect and prognosis were poor; **(B)** Massive HCC in a patient with a large16-cm liver tumor in the right lobe that has an intact tumor capsule and clear boundary. After three TACE treatments, the tumor was significantly reduced to 8 cm. Follow-up PET/CT examination showed a residual active portion at the edge of the tumor, and another ablation treatment was performed. Follow-up checks showed no tumor activity, and complete remission was achieved. The patient has survived for 16 years and is still alive; **(C)** Another massive liver cancer lesion in the right lobe of a patient. After three TACE treatments combined with microwave ablation, the tumor was completely inactivated; **(D)** Infiltrative HCC patient with an unclear tumor boundary and right portal vein tumor thrombus; **(E)** The tumor and tumor thrombus were significantly reduced after nine courses of HAIC.

Multilevel Subdivision of M Staging Used for Guiding Treatment and Predicting Prognosis

Stage IV HCC should be subdivided to distinguish patients with limited metastasis from patients with a heavy metastatic tumor load. For example, patients with a single metastatic lesion in a single metastatic organ are defined as M1-1, patients with multiple metastatic lesions in a single metastatic organ are defined as M1-m, and so on (**Table 1**). For patients at different stages, limited metastatic lesions can be eliminated by ablation or particle implantation, resulting in longer survival (93, 94, 97) (**Figure 8**), rather than treated by administration of a molecular targeted drug alone as suggested in the guidelines.

HCC Patients With Severe Hepatic Decompensation Are Candidates for Liver Transplantation

In recent years, immunotherapy has become a research hotspot and is the future trend in cancer treatment. The 2018 Nobel Prize

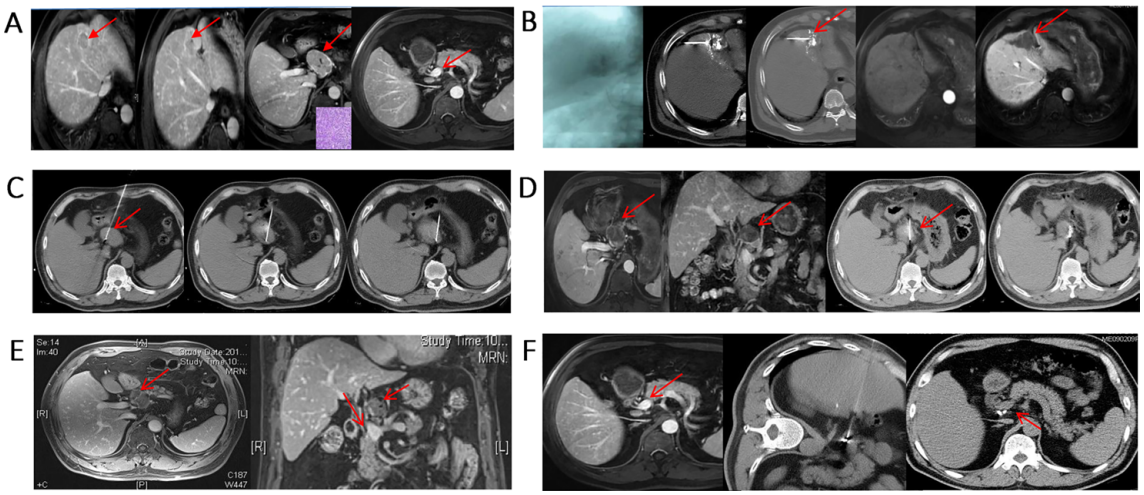

FIGURE 7 | Minimally invasive, comprehensive treatment for metastatic lymph nodes. **(A)** S4 HCC with metastatic lymph nodes in the portal vena cava space that were pathologically confirmed as HCC. The AFP level was 439 ng/mL before treatment; **(B)** Two weeks after TACE treatment, subsequent RFA was performed; **(C)** RFA ablation of hepatic portal metastatic lymph nodes; **(D)** Lymph nodes were basically inactivated, and 125I particles were implanted in the remaining portion; **(E)** Metastatic lymph nodes were completely necrotic, and the AFP level decreased to 14 ng/mL; **(F)** After adjuvant CIK treatment, the metastatic lymph nodes were completely inactivated, and AFP was further reduced to 2 ng/mL. The patient is still alive 10 years after treatment.

TABLE 1 | Multilevel subdivision of M staging.

M staging	Criteria
M1-1	1 organ, 1 metastatic lesion
M1-2	1 organ, 2 metastatic lesions
M1-3	1 organ, 3 metastatic lesions
M1-4	1 organ, 4 metastatic lesions
M1-m	1 organ, multiple metastatic lesions
M2-2	2 organs, 2 metastatic lesions
M3-3	3 organs, 3 metastatic lesions
Mm-m	> 3 organs, ≥ 5 metastatic lesions

M, metastasis; m, multiple.

in Medicine was awarded to two cancer immunotherapy scientists. Tumor immunotherapy is a treatment method for controlling and clearing tumors by reactivating and maintaining the recognition and killing abilities of the immune system toward tumor cells and restoring the normal antitumor immune responses of the body. Some studies suggested that future cancer treatment should be combined with immunotherapy rather than employ a single treatment regimen (98). The CheckMate 040 phase I/II phase clinical trial validated the efficacy and safety of the anti-PD-1 antibody nivolumab in patients with advanced HCC (99). In March 2020, the US Food and Drug Administration (FDA) approved nivolumab plus ipilimumab for HCC previously treated with sorafenib (100). However, liver transplant patients, who suffer from tremendous surgical trauma, need relatively longer recovery time, require many economic and medical resources, and still have issues of recurrence and metastasis. In the meantime, long-term use of immunosuppressive agents is required after surgery, and thus, the valuable opportunity for immunotherapy is missed for many patients, which conflicts with the future direction of cancer treatment.

Promotion of Bioimmunotherapy, Traditional Chinese Medicine Therapy, Antiviral Therapy, and Psychosocial and Psychopharmacological Interventions, Which Should Be Involved in All Stages of Treatment

Liver cancer requires treatment of both the symptoms and the underlying basic causes. Direct treatment of tumors is treatment of the symptoms, and protection of a patient's biological immune functions and establishment of excellent psychosocial support treat the underlying basis. HCC is an inflammation-related cancer, and studies have confirmed that immune remission is associated with tumor and patient outcome (101). Biotherapy can strengthen a patient's immunity and ultimately improve antitumor effects (**Figure 9**). Current immuno-therapeutic strategies are based on two fundamental principles: 1. The ability to evoke current immune responses; 2. The need to stimulate new or different immune responses. Unleashing current immune response relies on reactivity of a pre-existing immunity to cancer which is restricted by micro-environmental factors, such as inhibitory receptors on T cells especially PD-1 and CTLA-4, or alternatively immunosuppressive cytokines such as TGF-β. Checkpoint inhibitors fall within this category. Conversely, antibodies that directly target molecules expressed on HCC, such as alpha- AFP, are within the second category. These strategies can be enhanced by coupling these antibodies to effector cells, such as T cells or even NK cells. The first-line checkpoint inhibitors approved for use in HCC by NCCN (75) are as following: Atezolizumab + bevacizumab is preferred regimens, while Nivolumab is applicable if patient is ineligible for tyrosine kinase inhibitors [TKIs] or other anti-angiogenic agents. As subsequent-line therapy if disease progression, Nivolumab,

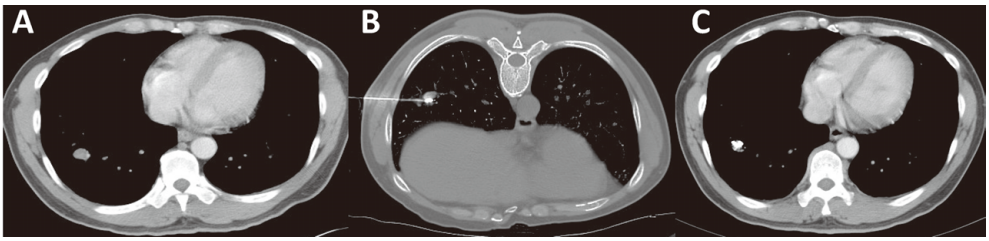

FIGURE 8 | Minimally invasive treatment for HCC with pulmonary oligo-metastasis. **(A)** CT of an HCC patient showing newly developed right lung oligo-metastatic lesions. **(B)** Percutaneous particle implantation was performed. **(C)** A 6-month follow-up CT showed that the metastatic tumor had shrunk and exhibited no activity.

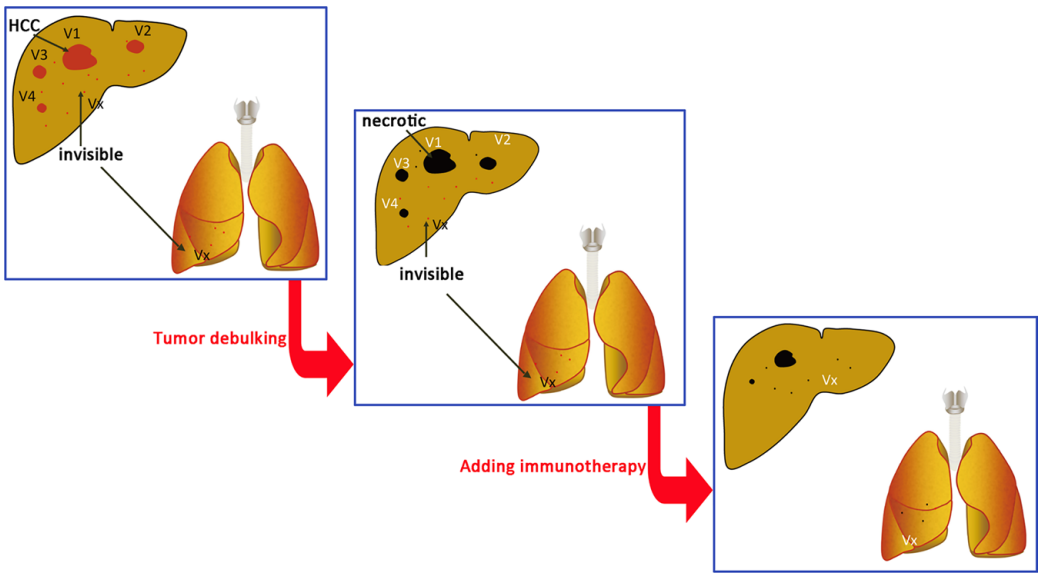

FIGURE 9 | Immune cell treatment performed at the same time or after tumor debulking. V1, V2, V3…Vn are radiographically visible intrahepatic tumors. Vx is a lesion that has not been detected by intrahepatic or extrahepatic imaging examination. The total tumor burden (volume total, VT) = V1 + V2 + V3… + Vn + Vx. The goal of treatment is to reduce the tumor burden to VT = Vx and then use immune cells to treat Vx.

Nivolumab + ipilimumab, Ramucirumab, and Pembrolizumab are optional.

Efficient combination of the various drugs and treatment methods provided by traditional Chinese medicine can enhance the body's immunity, reduce adverse reactions to radiotherapy and chemotherapy, and improve the quality of life of patients. In addition to decoction medicine, the China drug regulatory authorities have approved several modern Chinese medicine preparations for HCC treatment (102), and all have demonstrated unique characteristics and certain effects, with good patient compliance, safety and tolerance, and thus can be used as appropriate. Sustained infection with HBV and/or HCV is an important risk factor for HCC development, progression, and recurrence. Antiviral therapy is very important in liver cancer patients with HBV infection and active replication of the virus. Antiviral therapy can reduce the rate of postoperative recurrence (103, 104). Therefore, antiviral therapy should be involved throughout the liver cancer treatment process. The mental status of patients with liver cancer and their families should be considered, and effective measures should be employed to help them face the disease positively and to reduce depression, fear, and anxiety.

However, the treatment strategy for clinical patients should be made according to their own characteristics, so that patients can get the most benefit from treatment. Bioimmunotherapy, traditional Chinese medicine therapy, antiviral therapy, and psychosocial and psychopharmacological interventions cover a very wide area involving multidisciplinary approach. Therefore, determination of appropriate therapy should include a careful patient/physician discussion. Many clinical trials on combined treatments for HCC are ongoing, including assessing combinations with immune checkpoint inhibitors. Further clinical evidences are required to

explore the reliable treatment schedule, which might allow a more precise selection of treatment in well-defined patients.

Implementation of Multicenter, Randomized, Controlled Studies of Minimally Invasive Treatment and Surgery for Early- and Intermediate-Stage HCC

In choosing treatment methods for early- and intermediate-stage HCC, there are still major differences in different countries and in different disciplines (96), and multicenter, randomized, controlled studies are lacking. For example, in the newly published China Liver Cancer 2019 guidelines, there are three treatment options for early-stage liver cancer: surgery, ablation, and liver transplantation. These three completely different treatment methods confuse both patients and doctors. The aforementioned questions still await verification in additional large-scale multicenter randomized controlled studies. Therefore, conducting multicenter, randomized, controlled clinical studies of minimally invasive and surgical treatments for early- and intermediate-stage HCC is recommended.

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

All authors discussed the recommendations. Q-FC, WL, and PW developed the first outline. All the authors were involved in revising when the first draft had been developed. All the authors refined the contents with feedbacks, and comments. All authors contributed to the article and approved the submitted version.

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Exosomal miR-125b Exerts Anti-Metastatic Properties and Predicts Early Metastasis of Hepatocellular Carcinoma

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Background & Aims: Cancer metastasis is responsible for the majority of cancer-related deaths. Exosomal miRNAs have emerged as promising biomarkers for cancer, serving as signaling molecules that can regulate tumor growth and metastasis. This study examined circulating exosomal miRNAs that could predict hepatocellular carcinoma (HCC) metastasis.

Methods: Exosomal miRNA was measured by quantitative real-time PCR (qRT-PCR) in a large set of patients (n = 284). To investigate the role of exosomal miRNA in HCC, we performed a series of *in vitro* tests, such as exosome labeling, qRT-PCR, reverse transcription PCR, wound healing assay, transwell assay, and Western blot assay.

Results: Exosomal miR-125b was drastically downregulated in HCC patients with metastasis than in those without metastasis. *In vitro*, we observed the uptake of miR-125b by exosome in recipient cells. Exosome-mediated miR-125b significantly inhibited migration and invasion abilities and downregulated the mRNA expressions of MMP-2, MMP-9, and MMP-14 in recipient cells *via* intercellular communication. Further investigation revealed that miR-125b suppressed SMAD2 protein expression in recipient cells by binding to its 3′ untranslated regions. Exosome-mediated miR-125b transfer also disrupted TGF-β1–induced epithelial–mesenchymal transition and TGF-β1/SMAD signaling pathway in recipient cells by leading to a decrease of SMAD2 protein expression. Moreover, exosomal miR-125b was downregulated after metastasis compared with that at baseline in patients with serial measurements before and after metastasis.

Conclusions: The results imply that exosome-mediated miR-125b exerts anti-metastatic properties in HCC. These findings highlight that circulating exosomal miR-125b might represent a reliable biomarker with diagnostic and therapeutic implications for extrahepatic metastasis from HCC.

Keywords: hepatocellular carcinoma, exosome, metastasis, epithelial – mesenchymal – transition, biomarker, miR-125b

INTRODUCTION

Hepatocellular carcinoma (HCC) is a lethal cancer with the third highest mortality in the world. One of the most adverse prognostic events of HCC is vascular invasion contributing to treatment resistance and extrahepatic metastasis. Cancer metastasis refers to the dissemination of malignant cells to distant sites through blood vessels and is responsible for the majority of cancer-related deaths (1). To ensure patient survival and the potential for a cure for patients presenting with metastasis, it is essential to predict or diagnose cancer metastasis in its early stages. Considering that metastasis is spread by various biological signals in the blood, biomarkers for metastasis are expected to be present in the blood. Unfortunately, there is still a lack of valid and reliable biomarkers for early detection of metastasis from HCC.

Exosomes, 30 to 150 nm nanosized extracellular vesicles that are secreted from a wide variety of cells into biological fluids, have received extensive attention because they act as cell-to-cell communication mediators by horizontally transferring their cargos, including nucleic acids, proteins, and lipids (2). MicroRNAs (miRNAs) are short non-coding RNA molecules that regulate gene expressions post-transcriptionally and cellular functions epigenetically by directly binding to 3′ untranslated regions (UTRs) of target mRNAs (3). Given that specific miRNAs are useful in clinical applications for disease (4), exosome-encapsulated miRNAs that overcome tumor heterogeneity are clinically relevant and deserve further investigation (5, 6). Accumulating evidences have shown that tumor-derived exosomes promoted the spread of metastasis by transferring various signals through the blood (7). Moreover, the exosome-mediated transfer of specific miRNAs contributes to behaviors of metastatic capacity *via* paracrine and endocrine signaling (5, 8, 9). Thus, exosomal miRNAs could open an innovative window as promising biomarkers of metastasis in the future. Nevertheless, circulating exosomal miRNAs that can predict extrahepatic metastasis have not been extensively studied in HCC.

The aim of this study was to explore exosomal miRNAs that could predict extrahepatic metastasis in patients with HCC. By analyzing miRNA profiles, we identified the potential role of circulating exosomal miR-125b as a biomarker for early detection of metastasis from HCC. Subsequent studies on mechanisms underlying exosome–target cell interactions indicated that the transfer of miR-125b by exosomes suppressed migration and invasion abilities of recipient cells by attenuating epithelial to mesenchymal transition (EMT) *via* inhibition of TGF-β1/SMAD signaling. Moreover, novel biomarker functions of exosomal miR-125b were confirmed in a large set of patients with HCC.

MATERIALS AND METHODS

miRNA Microarray

To analyze microarray, serum samples were collected from patients with or without HCC. Microarray analysis was performed by GenoCheck (Ansan, Korea). In brief, total RNA was extracted from serum and labeled with alkaline phosphatase. Hybridization was then performed using an Agilent hybridization system on Agilent Mouse miRNA v17.0 array to conduct DNA chip assay. Raw data were analyzed using GeneSpring GX v11.5.1 to evaluate miRNA expressions.

Patient Samples

This study examined serum samples from 239 HCC patients and 45 non-HCC patients at Seoul St. Mary's Hospital, Catholic University of Korea (Seoul, South Korea) between June 2007 and January 2019. Among these, serial measurements for exosomal miRNA were performed for nine HCC patients who had serial samples available before and after metastasis. The diagnosis of HCC was based on histological evidence, α-fetoprotein levels, or typical radiological findings according to the KNCC guideline (10). Metastasis was diagnosed based on pathology, bone scan, computed tomography, or magnetic resonance imaging. Based on tumor extent (11), patients diagnosed as HCC were categorized into the following three groups: 1) "under Milan group," a single tumor < 5 cm or multiple tumors (number ≤ 3, each < 3 cm in diameter) without metastasis; 2) "over Milan group," HCC exceeding Milan criteria but without metastasis; and 3) "metastasis group," HCC exhibiting extrahepatic metastasis. This study was approved by the ethics committee of The Catholic University of Korea. Informed written consent was obtained from all patients (IRB approval number KC17TESI0664).

Abbreviations: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; BCLC, barcelona clinic liver cancer; CHB, chronic hepatitis B; CM, conditioned media; EMT, epithelial to mesenchymal transition; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; miR, micro RNA; MMP, matrix metalloproteinase; mRNA, messenger RNA; NC, negative control; NTA, nanoparticle tracking analysis; PCR, polymerase chain reaction; PVT, portal vein thrombosis; qRT-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SFM, serum-free media; SMAD, small mothers against decapentaplegic; TB, total bilirubin; TEI, total exosome isolation kit; TEM, transmission electron microscopy; TGF-β, transforming growth factor beta; UTR, untranslated region.

Exosome Isolation and Characterization

Exosomes were isolated from sera and cell culture-conditioned media (CM) using an ExoQuick™ (System Biosciences, Palo Alto, CA, USA) and a total exosome isolation kit (TEI; Invitrogen, Carlsbad, CA, USA), respectively. In brief, serum was centrifuged at 3000*g* for 15 min at 4°C to remove cellular debris. Exosomes were then isolated from sera according to the manufacturer's instructions. To isolate exosomes from CM, cells were washed with PBS when reaching 80% confluence and incubated with serum-free media (SFM) for 48 h. CM was collected and then ultrafiltered with Amicon Ultra Centrifugal Filters (Millipore, Bedford, USA). Exosomes were subsequently isolated from CM according to the manufacturer's instructions. For exosome characterization, the exosome pellet was resuspended in PBS. Exosomes were visualized by transmission electron microscopy (TEM). Size distribution and quantification of exosomes were determined using nanoparticle tracking analysis (NTA). Exosomal markers were detected by Western blot assay.

miRNA Transfection Into Cells and Exosomes

HCC cells were transfected with hsa-miR-125b-5p mimic (miR-125b; Genolution Pharmaceuticals, Seoul, Korea) and negative control mimic (miR-NC; Genolution Pharmaceuticals) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The final concentration of miRNA mimics used in this study was 50 nM. Exosomes were also loaded with miRNA mimics based on a previously reported method (12). In brief, Huh7 cell-derived exosomes (Huh7-exo) were loaded with miR-125b (Exo-125b) and miR-NC (Exo-NC) using Lipofectamine 2000 (Invitrogen). These miRNA-loaded exosomes were purified using TEI (Invitrogen) to remove any un-transfection mixture. Transfection or loading efficiency was analyzed by qRT-PCR.

Co-Culture Experiment

Recipient cells (SK-HEP1 and SNU449 cells) were seeded into six-well plates. After reaching 80% confluency, these cells were treated with Exo-125b or Exo-NC suspended in SFM for 24 h.

Transfer of Exosomes and Exosomal miRNA in Cell-to-Cell Communication

To assess the transfer of miRNA by exosomes, Huh7-exo were loaded with Cy3-labeled miR-125 (Genepharma, Shanghai, China) or miR-NC as described above. These miRNA-loaded exosomes were labeled with a PKH67 green fluorescent cell linker for general cell membrane labeling (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Following purification of exosomes using TEI, recipient cells were co-cultured with exosomes for 24 h. Images were then taken with a confocal microscopy (LSM800, Carl Zeiss, Germany).

Cell Migration and Invasion Assay

Wound healing and transwell assays were performed to assess cell migration and invasion abilities. In brief, following transfection in six-well plates, cell monolayers were wounded with a sterile yellow tip. Cells were then washed and replaced with completed media supplemented with 10% FBS. Images were taken 24 and 48 h later using an optical microscopy. Transwell assays were conducted using corning insert and Biocoat matrigel invasion chamber (Corning Inc, Corning, NY, USA) according to the manufacturer's instructions. In brief, cells were trypsinized following transfection and resuspended in SFM. The cell suspension was seeded into each upper chamber after rehydration in SFM for 2 h. The lower chamber was added with 10% FBS-containing media and incubated for 48 h at 37°C in 95% air and 5% CO_2. Migrated and invaded cells were stained with Diff-Quick (Sysmex, Japan) and counted.

Matrix Metalloproteinase (MMP) Expression

The mRNA expression of MMPs was examined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from recipient HCC cells using Qiazol reagent (Qiagen, Germany). cDNA was synthesized using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA,USA). The primer pairs used for the detection of MMP-2, MMP-9, and MMP-14 are shown in **Table S1**. The cDNA was amplified with 10-μM primers using Maxime PCR PreMix Kit (Intron Biotechnology, Seoul, Korea). GAPDH was used as an endogenous control.

EMT Cell Model

To induce EMT, after miRNA-loaded exosomes co-culture, Huh7 cells were treated with 5 ng/ml transforming growth factor beta-1 (TGF-β1; R&D system, Minneapolis, MN, USA) for 48 h. Following incubation, RNA and protein were extracted from cells for further experiments.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and SPSS 20.0 software (IBM, Armonk, NY, USA). Data were presented as mean ± standard error of the mean (SEM) or median. Comparisons between groups were appropriately performed using Student's *t*-test, Mann-Whitney U test, or Wilcoxon signed-rank test. Survival analysis was analyzed with Kaplan-Meier method and log-rank test. Statistical significance was denoted as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Additional Information

Additional experimental methods, including cell culture, Western blot assay, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR), are provided in supporting data.

RESULTS

Circulating miRNAs Screening in HCC Patients

To identify circulating miRNAs for HCC tumorigenicity, miRNA microarray was performed using sera of HCC and non-HCC

cirrhotic patients. A total of 10 miRNAs were selected based on the following criteria: fold change ≥ 1.5 and p value < 0.05 (**Figure S1A**). Among them, two promising miRNAs of our interest and three additional miRNAs found by searching PubMed were analyzed by qRT-PCR. As a result, upregulation of miR-125b and miR-100 and downregulation of miR-3180, miR-130a, and miR-320a were found to be associated with HCC (**Figure S1B**). Of these, considering that miR-125b and miR-100 act as tumor suppressor miRNAs in HCC (13, 14), they were further analyzed in our study. To determine expressions of candidate miRNAs in exosomes, we first attempted to isolate exosomes from patients' sera. Isolated vesicles were characterized by TEM for visual confirmation (**Figure S2A**). NTA was also performed to determine size distribution and concentration (**Figure S2B**). The presence of HSP70 and CD63 commonly used as exosomal markers was detected in vesicles (**Figure S2C**), confirming the successful isolation of exosomes. In serum exosomes of HCC patients, two miRNAs (miR-125b and miR-100) were confirmed by qRT-PCR. Of these, circulating exosomal miR-125b expression was significantly downregulated in the sera of HCC patients with metastasis compared to those without metastasis (p = 0.030; **Figure S2D**). Based on the abovementioned results, we hypothesized that exosomal miR-125b could regulate extrahepatic metastasis from HCC.

Exosomal miR-125b Can Transfer to Recipient Cells

To test the above hypothesis, we performed co-culture experiments using exosomes (**Figure 1A**). We employed cells with high-metastatic potential (SK-HEP-1 and SNU449 cells) as recipient cells and low-metastatic cells (Huh7 cells) as donor cells to investigate the role of exosomal miR-125b in metastasis (15–17). Exosomes were isolated from Huh7 cells and characterized as shown in **Figures 1B, C**. Overexpression of miR-125b in Exo-125b compared with that in Exo-NC was confirmed (**Figure 1D**). As indicated in **Figures 1E, F**, exosomal miR-125b internalization into recipient cells was observed by confocal microscopy and confirmed by qRT-PCR. Altogether, these results indicate that miR-125b can be loaded into Huh7-exo and taken up into recipient cells *via* exosome transfer.

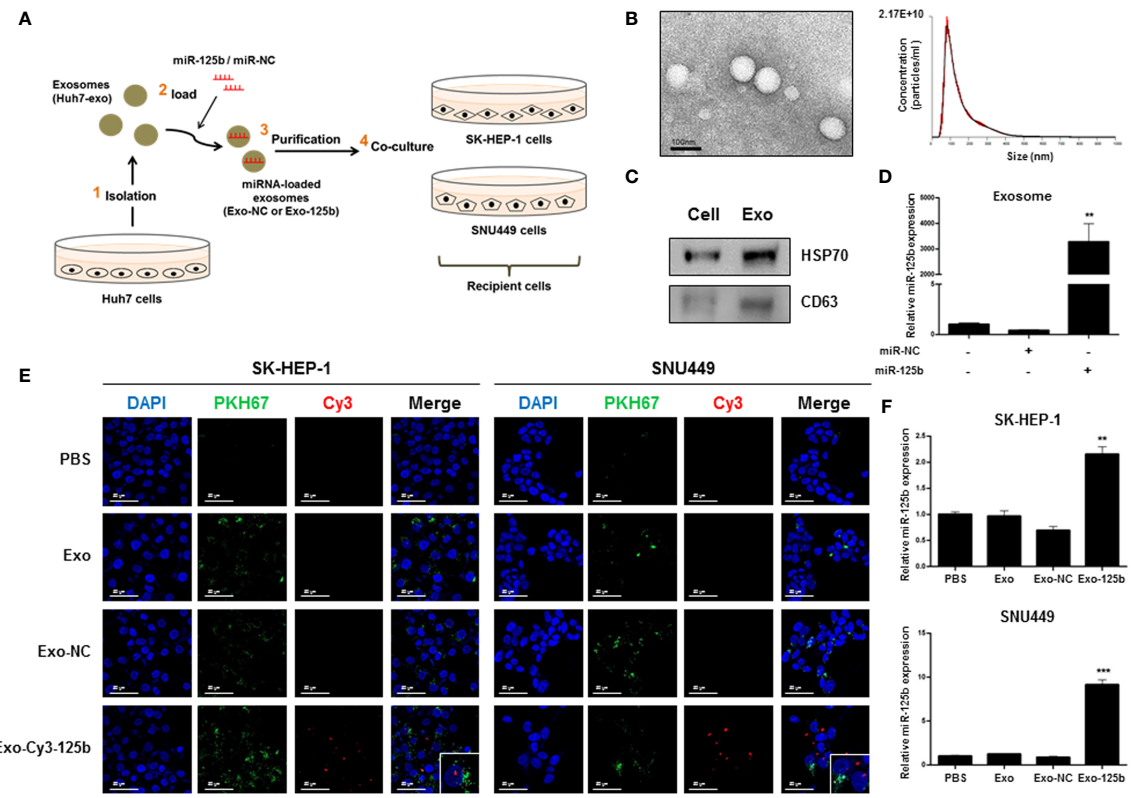

FIGURE 1 | Exosomes mediate the transfer of miR-125b into recipient cells. **(A)** Schematic diagram showing the co-culture experimental procedure. In step 1, exosomes were isolated from Huh7-CM. In step 2, isolated exosomes were loaded with miRNA mimics. In step 3, miRNA-loaded exosomes were purified by TEI. In step 4, purified exosomes were co-cultured with recipient cells. **(B)** Exosomes were characterized by TEM and NTA. Scale bar, 100 nm. **(C)** Detection of exosomal markers in cell lysate and exosomes. **(D)** Upregulation of relative miR-125b expression in Exo-125b compared with that in Exo-NC. **(E)** Confocal microscopy images of recipient cells treated with Exo-Cy3-125b (Cy3-labeled miR-125b-loaded exosomes) or Exo-NC. Original magnification, ×400 or ×800. Scale bar, 50 μm. Red: Cy3-labeled miR-125b; green: exosome; DAPI: nuclei. **(F)** Relative miR-125b expression determined by qRT-PCR after recipient cells were treated with Exo-125b or Exo-NC. Data are presented as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

Exosomal miR-125b Inhibits Migration and Invasion Capacities of Recipient Cells

Cellular miR-125b was reported to inhibit migration and invasion abilities of HCC cells (13). Overexpression of miR-125b significantly impaired wound healing capacity compared with that of miR-NC (**Figures S3A, B**). However, Huh7 cells known to have low-metastatic capacity did not show noticeable difference in wound healing capacity between miR-125b and miR-NC mimics. Next, we examined effects of exosome-mediated miR-125b on recipient cells. Similar to the results observed in cells, wound healing capacity was suppressed in recipient cells treated with Exo-125b (**Figure 2A**). Moreover, transwell assay showed that the number of migration and invasion cells was significantly decreased in recipient cells treated with Exo-125b (**Figure 2B**). MMPs are known to promote cancer migration and invasion (18). Expression of all the MMPs in different type was found to be strongly repressed in recipient cells treated with Exo-125b as compared with those treated with Exo-NC (**Figure 2C**). Taken together, these results suggest that exosomal miR-125b exerts tumor suppressive function by inhibiting metastatic ability of recipient cells *via* cell-to-cell transfer.

SMAD2 Is a Direct Target of Exosomal miR-125b in Recipient Cells

miRNAs are involved in various cellular activities by repressing protein expressions of target genes (3). Thus, to identify target genes of miR-125b for anti-metastatic properties, we used open-source bioinformatics algorithms, including TargetScan, miRWalk, miRDB, TargetRank, and Exiqon. Candidate target genes were screened by Western blot assay (**Figure S4**). Among various candidates, SMAD2 protein expression was most significantly suppressed in Huh7 cells transfected with miR-125b mimic. With functional annotation analysis by Database for Annotation, Visualization, and Integrated Discovery, the target genes of miR-125b were most strongly associated with the TGF-β signaling pathway (p = 0.004; **Table S2**). Two seed regions of miR-125b and SMAD2 were predicted with TargetScan and well matched as shown in **Figure 3A**. This result is consistent with previous reports showing that SMAD2

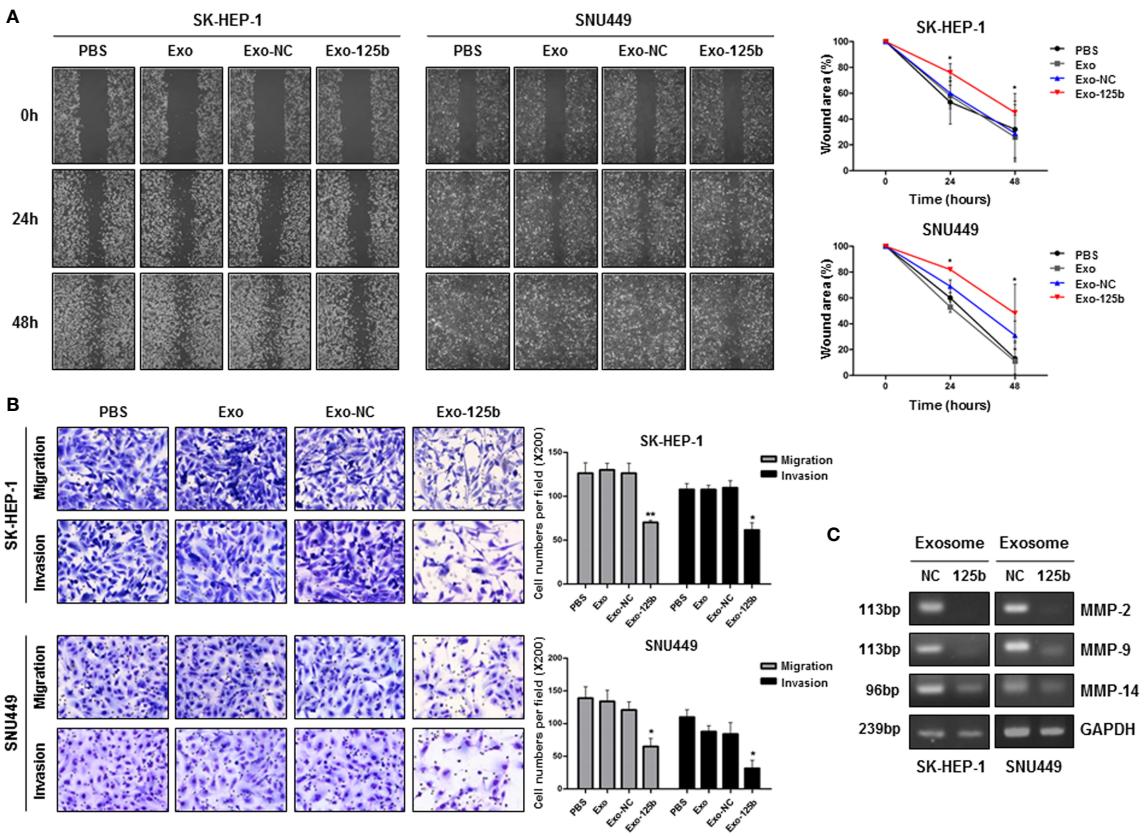

FIGURE 2 | Exosomal miR-125b inhibits migration and invasion abilities of HCC cells. Cell migration and invasion abilities were assessed by wound healing and transwell assays. **(A)** Percent (%) of wound area was determined as the ratio of average wound closure at a given time points (24, 48 hours) relative to the initial wound closure. **(B)** Numbers of migration and invasion cells were counted in indicated groups. **(C)** RT-PCR results displayed reduced mRNA expression levels of MMP-2, MMP-9, and MMP-14 in recipient HCC cells treated with Exo-125b compared to those in cells treated with Exo-NC. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$ *vs.* Exo-NC.

is a target of miR-125b in HCC and that it is a strongly related to cancer metastasis (19, 20). To determine whether miR-125b regulates protein expression of SMAD2 in recipient cells by intercellular communication, recipient cells were overexpressed by treatment with Exo-125b. As a result, SMAD2 protein expression was found to be significantly decreased in recipient cells after treatment with Exo-125b (**Figures 3B, C**). These results indicate that exosomal miR-125b suppresses post-transcriptional SMAD2 protein expression in recipient cells through intercellular communication.

Exosomal miR-125b Attenuates EMT Induced by TGF-β1 and Blocks TGF-β1/SMAD Pathway

TGF-β plays a pivotal role in EMT and SMAD2 is a key regulator of TGF-β signaling pathway (21). Thus, we further explored whether miR-125b could interfere with TGF-β signaling pathway by suppressing SMAD2. When Huh7 cells were treated with TGF-β1, miR-125b expression was significantly downregulated (**Figure 4A**). To elucidate the relevance of EMT and miR-125b, low metastatic Huh7 cells were used as recipient cells to induce EMT. After TGF-β1 treatment, the Exo-125b group only showed changes slightly with a spindle-shaped morphology while the Exo-NC group clearly displaying spindle-shaped cells (**Figure 4B**). As indicated in **Figure 4C**, mRNA expression level of an epithelial marker (E-cadherin) was decreased in response to TGF-β1, whereas expression levels of mesenchymal markers (N-cadherin and Vimentin) were significantly increased. Overexpression of vimentin mRNA by TGF-β1 was significantly reduced after Exo-125b treatment. Next, we examined changes in protein levels of EMT markers in response to TGF-β1. Consistently, E-cadherin was downregulated, whereas N-cadherin was upregulated in Huh7 cells (**Figures 4D, E**). Although either Exo-NC or Exo-125b treatment alone resulted in no significant change in protein expressions of target genes or EMT markers, combined treatment with Exo-125b and TGF-β1 resulted in significant upregulation of E-cadherin protein expression and significant downregulation of N-cadherin, SMAD2, SMAD2/3, and p-SMAD2/3, suggesting a drastic suppression of metastatic potential by transfer of exosomal miR-125b in EMT-promoting cells. Collectively, these findings demonstrate that exosomal miR-125b can block EMT and TGF-β1/SMAD pathway by repressing protein expression of SMAD2.

Exosomal miR-125b Is Downregulated in the Sera of HCC Patients With Metastasis

Based on our *in vitro* results of exosome-mediated miR-125b, we evaluated whether its anti-metastatic properties could serve as a biomarker for early detection of metastasis or therapeutic implications in patients with HCC. For this purpose, we tested exosomal miR-125b expression in sera of 284 patients with available sera samples. As a result, exosomal miR-125b expression was correlated with patient outcomes, showing increasing trends with tumor stage progression. However, these expression levels were significantly decreased with metastasis (**Figure 5A**). Survival analysis was examined based on circulating exosomal miR-125b expression profiles. The low exosomal miR-125b expression group had higher rates of extrahepatic metastasis ($p = 0.025$), as well as trends for worse overall survival ($p = 0.202$) than the high expression group (**Figures 5B, C**). Furthermore, when analyzing patients with serial samples available before and after metastasis, we found that exosomal miR-125b expression was significantly downregulated after metastasis in all patients but one (**Figure 5D**). Overall, these data indicate that exosomal miR-125b is a strong predictor of early extrahepatic metastasis in HCC patients. Clinical characteristics of patients are provided in **Table 1**.

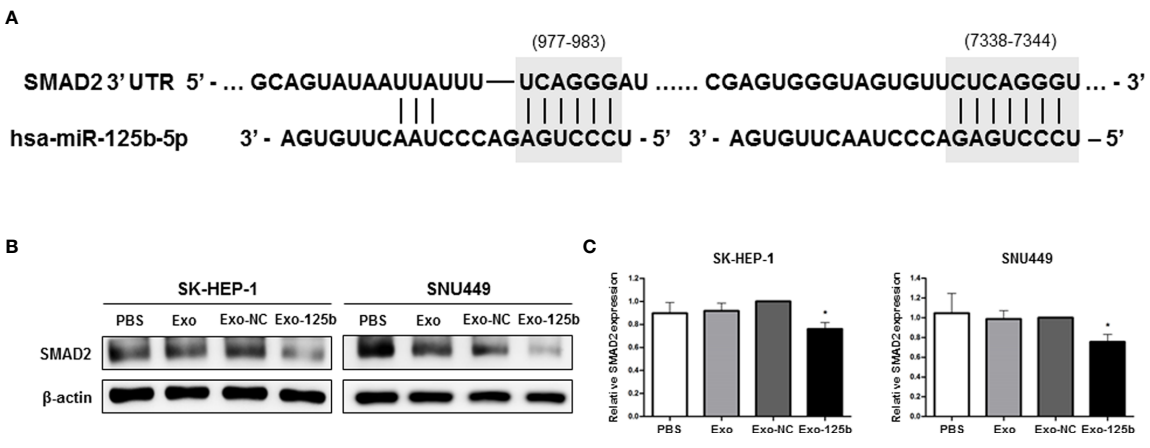

FIGURE 3 | SMAD2 is a direct target of exosomal miR-125b in recipient HCC cells. **(A)** Two potential seed regions (gray) indicate the binding site of miR-125b in SMAD2 3′ UTR. **(B)** Western blot assay showing decreased protein expression of SMAD2 after treatment with Exo-125b compared to that after treatment with Exo-NC. **(C)** Statistical analysis of SMAD2 protein expression in recipient HCC cells. Data are presented as mean ± SEM. *$p < 0.05$ *vs*. Exo-NC.

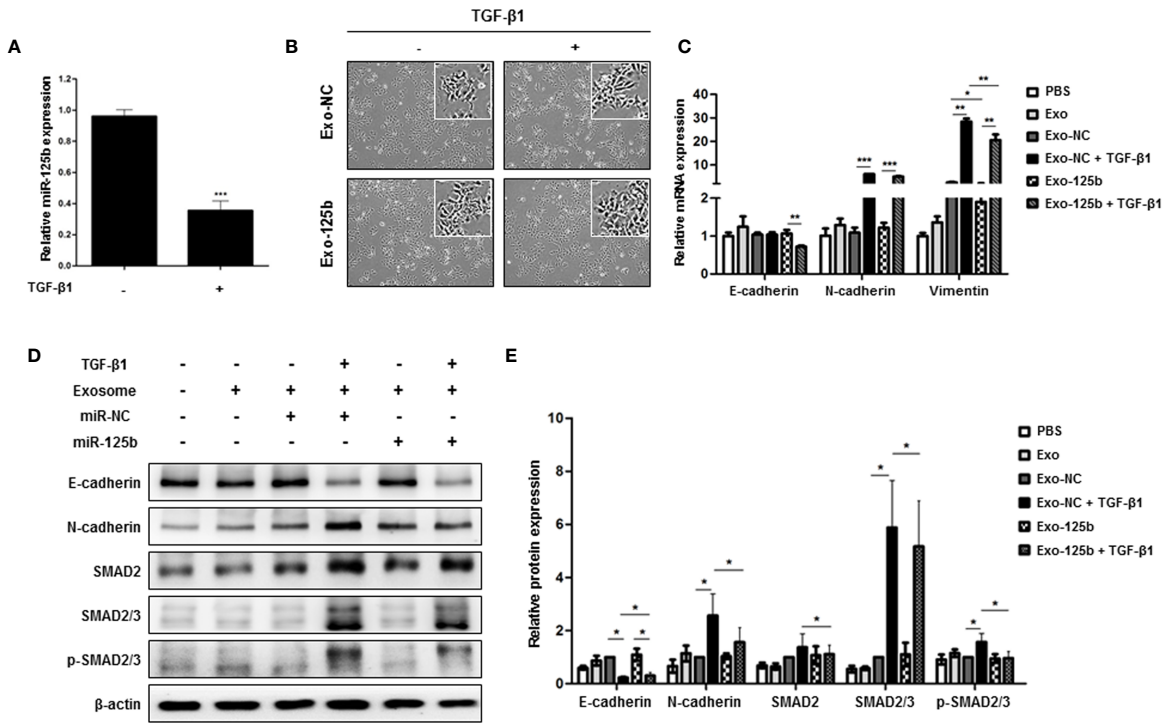

FIGURE 4 | Exosomal miR-125b blocks TGF-β1-induced EMT and TGF-β1/SMAD pathway signaling in recipient HCC cells. Low metastatic Huh7 cells were induced to show EMT by TGF-β1. **(A)** Relative miR-125b expression in Huh7 cells following treatment with TGF-β1. **(B)** Changes of morphology observed by optical microscopy after EMT induction by TGF-β1. **(C)** qRT-PCR results of mRNA expression levels of EMT marker genes in cells. **(D)** Protein expression levels of TGF-β1-EMT pathway genes based on Western blot assay. **(E)** Statistical analysis of protein expression levels in cells. Relative expression levels were normalized β-actin. Data are presented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

DISCUSSION

Metastasis is incurable without early diagnosis tools. Therefore, efficient biomarkers for metastasis that fill gaps of in-depth knowledge should be developed. To this end, we first employed miRNA PCR array to screen serum miRNAs predictive of extrahepatic metastasis. Among several candidate miRNAs, exosomal miR-125b showing the strongest association with metastasis was subjected to more extensive investigations. Based on a series of *in vitro* tests and clinical data, it was found that exosome-mediated miR-125b had significant anti-metastatic properties in HCC. Specifically, the transfer of miR-125b by exosomes inhibited migration and invasion abilities of recipient HCC cells. Exosomal miR-125b also interfered with TGF-β1-induced EMT by suppressing SMAD2 protein expression. Furthermore, a significant downregulation of exosomal miR-125b was detected in a large set of patients with metastasis. More importantly, anti-metastatic effects of exosomal miR-125b were further confirmed by a decrease in its levels at the time of metastasis in patients with serial measurements. These findings indicate the utility of exosomal miR-125b for early diagnosis of extrahepatic metastasis and provide insights into its novel exosome-based therapeutic strategy for inhibiting metastasis in HCC patients.

It is noteworthy that exosomal miRNAs, unlike intracellular miRNAs, modulate cellular processes within recipient cells by indirectly cell-to-cell signaling to distant cells (2). Our observation of cellular internalization and expression of Cy3-labeled miR-125b into recipient cells supports the active role of exosomal miR-125b in cell-to-cell communication (**Figures 1E, F**). It has been reported that miR-125b plays dual roles as an oncogene and a tumor suppressor (13). Oncogenic miR-125b reportedly accelerated cellular proliferation, drug resistance, and migration by controlling target genes in colon, lung, and pancreatic cancers. However, in HCC, miR-125b inhibited these functions by targeting Bcl2, PIGF, LIN28B, and Mcl-1 (13). We found that exosome-mediated delivery of miR-125b effectively mitigated the metastatic potential of recipient HCC cells (**Figures 2A, B**). This finding extends the tumor-suppressive function of cellular miR-125b to the setting of metastasis suppression by exosomal miR-125b and indicates the fundamental role of miR-125b as a key regulator of HCC metastasis. Currently, the role of exosome-mediated signaling in cancer metastasis is highly emerging (7, 22). Tumor cell-derived exosomes can elicit paracrine signaling, whereas exosome-delivered miRNAs mostly target metastasis-related pathways, thereby contributing to the spread of tumors (5, 8, 9). In this regard, exosome-delivered miRNAs, as shown in our results, likely have promising future implications as diagnostic and therapeutic tools for cancer metastasis.

EMT is a key driver that confers metastatic properties on cancer cells by promoting mobility and invasion (23). Among

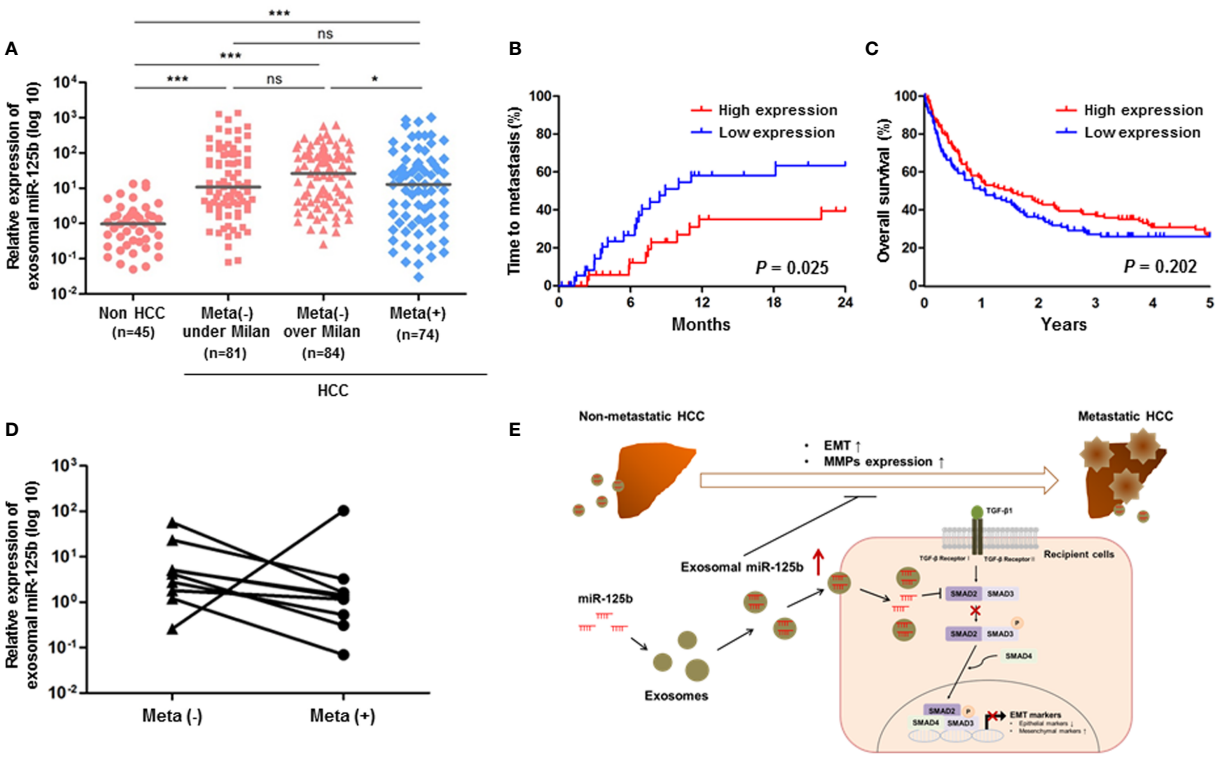

FIGURE 5 | Circulating exosomal miR-125b is a strongly predictive biomarker for extrahepatic metastasis from HCC. **(A)** Relative expression of exosomal miR-125b in sera of HCC patients (n = 239) and non-HCC patients (n = 45). **(B, C)** Kaplan-Meier survival analysis of time to metastasis for meta(-) over Milan patients group and 5 years overall survival. **(D)** Comparison of exosomal miR-125b expression in HCC patients with serial samples available before and after metastasis (n = 9). **(E)** A paradigm of exosomal miR-125b regulating extrahepatic metastasis in HCC. Data are presented as median. Mann-Whitney U test or Wilcoxon signed-rank test was used for data analysis. ns, not significant; *p < 0.05; ***p < 0.001.

EMT-related markers, activated SMAD2 represents a critical molecule that can accelerate cancer metastasis. SMAD-dependent TGF-β signaling pathways are potent inducers of EMT (21). Therefore, targeting SMAD2 represents one of effective strategies against metastasis. In our study, SMAD2 protein expression was significantly downregulated following exosomal miR-125b transfer in highly metastatic recipient cells (**Figures 3B, C**). Furthermore, when low metastatic Huh7 cells were treated with TGF-β1, exosome-delivered miR-125b also drastically abolished TGF-β1-induced EMT in recipient cell (**Figures 4C–E**). To the best of our knowledge, this is the first study to show the crucial role of exosome-mediated transfer of miR-125b in EMT regulation within recipient cells treated with TGF-β1. Altogether, these results indicate that exosome-delivered miR-125b can repress EMT by inhibiting TGF-β1/SMAD signaling. Thus, it has implications for potential anti-metastatic strategy.

MMPs also play essential roles in metastasis by destroying extracellular matrix (18). MMP-2 and MMP-9 mRNA expression levels have been reported to be upregulated in HCC patients with metastasis (24). Although a number of miRNAs have been implicated in the regulation of MMPs (25), the effects of exosome-associated miRNAs on MMP functions in HCC have not been reported. Through exosome-mediated transfer of miR-125b, we found that MMP-2, MMP-9, and MMP-14 mRNA expression levels were markedly decreased in recipient cells (**Figure 2C**). As MMP-2 was also a direct target of miR-125b (26), the results of the present study indicate powerful anti-metastatic functions of exosome-mediated miR-125b by targeting the two major pathways for metastasis including MMP and EMT process.

It could be argued that observed trends of upregulating exosomal miR-125b with tumor stage progression partly contradict a prior study showing that exosomal miR-125b was downregulated in patients with HCC than in non-HCC patients (27). Such discrepancy might be because of the increased total number of exosomes in tumor cells compared with normal cells and the use of different methods between studies (28, 29). Such paradoxical findings indicate the complexity of exosomal miR-125b in clinical evaluation depending on tumor status. The mechanism of exosome packaging and secretion of miRNAs remains incompletely understood. In addition, our study tested only one donor cell-derived exosome and lacked evaluation of exosomes derived from non-hepatocyte liver cells, such as Kupffer cells, fibroblasts, and stellate cells. Given that exosomes can be secreted by various liver cells besides tumor cells (30), the

TABLE 1 | Baseline characteristics of patients with hepatocellular carcinoma.

Characteristics	(n = 239), n (%)
Sex	
Male	185 (77.4)
Female	54 (22.6)
Age (years)	60 ± 11.6
Causes	
Viral	201 (84.1)
Non-viral	38 (15.9)
AST (U/L)	57 (14-799)
ALT (U/L)	54 ± 47.7
TB (mg/dL)	0.9 (0.2-18.2)
AFP level (ng/ml)	101.6 (0.9-448240)
Child-Pugh class	
A	177 (74.1)
B	57 (23.8)
C	5 (2.1)
BCLC stage	
0	28 (11.7)
A	46 (19.3)
B	42 (17.6)
C	121 (50.6)
D	2 (0.8)
Tumor size (cm)	7.6 ± 5.7
Tumor number	
Single	113 (47.3)
Multiple	126 (52.7)
PVT	
Presence	98 (41)
Absence	141(59)
Metastasis	
Presence	74 (31)
Absence	165 (69)

Data are expressed as mean ± standard deviation or median (interquartile range). Data are presented as the n (%) for categorical variable, unless otherwise indicated. AST, aspartate transaminase; ALT, alanine aminotransferase;TB, total bilirubin; AFP, α-fetoprotein; BCLC, barcelona clinic liver cancer; PVT, portal vein thrombosis.

complex exosome circuitry within the tumor microenvironment could be better evaluated in future studies employing human liver 3D geometrical and functional models.

Among multiple candidate exosome biomarkers developed from basic research, only a few can progress to clinical applications largely because of the lack of verification involving sufficient numbers of well-described patient populations. In this regard, out study has strengths including the recruitment of a large number of patients and serial measurements before and after metastasis for biomarker verification, as well as comprehensive description of exosome-mediated cell-to-cell cargo transfer and its molecular regulation involving metastasis.

In conclusion, this study reveals that tumor-derived, exosome-mediated miR-125b possesses anti-metastatic properties by targeting SMAD2, as well as by inhibiting MMPs and TGF-β1/SMAD signaling pathway in EMT *via* intercellular communication (**Figure 5E**). It also serves as a useful predictor of early metastasis in HCC. These findings highlight that circulating exosomal miR-125b has promising non-invasive diagnostic and therapeutic implications for extrahepatic metastasis of HCC.

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**.

ETHICS STATEMENT

This study was approved by the Ethics Committee of The Catholic University of Korea. Informed written consent was obtained from all patients (IRB approval number KC17TESI0664). 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) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Study concept and design: JJ. Acquisition of data: HN, PS, SB, JC, and SY. Analysis and interpretation of data: WH, HK, and JJ. Experiment: HK, NP, JK, and WH. Drafting of the manuscript: HK. Study supervision: JJ. 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.2021.637247/full#supplementary-material

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REVIEW
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The Progress in the Treatment of Hepatocellular Carcinoma With Portal Vein Tumor Thrombus

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Hepatocellular carcinoma (HCC) is one of most prevalent cancer and is a serious healthcare issue worldwide. Portal vein tumor thrombus (PVTT) is a frequent complication and remains as the blockage in the treatment of HCC with high recurrence rate and poor prognosis. There is still no global consensus or standard guideline on the management of HCC with PVTT. In western countries, Sorafenib and Lenvatinib are recommended as the first-line treatment options for HCC patients with PVTT where this condition is now regarded as BCLC Stage C regardless of PVTT types. However, there is growing evidence that supports the close relationship of the extent of PVTT to the prognosis of HCC. Besides the targeted therapy, more aggressive treatment modalities have been proposed and practiced in the clinic which may improve the prognosis of HCC patients with PVTT and prolong the patients' survival time, such as transarterial chemoembolization, radiotherapy, hepatic resection, liver transplantation, and various combination therapies. Herein, we aim to review and summarize the advances in the treatment of HCC with PVTT.

Keywords: hepatocellular carcinoma, portal vein tumor thrombus, transarterial chemoembolization, radiotherapy, liver transplantation, targeted therapy

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INTRODUCTION

Liver cancer is the sixth most commonly diagnosed cancer and the fourth cause of cancer-related deaths worldwide (1). In the last decade, the incidence and mortality of liver cancer keep increasing rapidly (2–4). In 2008, an estimated number of 748,300 new liver cancer cases and 695,900 deaths occurred globally (2). According to global cancer statistics, nearly 841,000 new liver cancer cases and 782,000 deaths were estimated to occur in 2018 (4).

Hepatocellular carcinoma (HCC) is the major histological subtype, accounting for 75% – 85% of cases among the primary liver cancers, while intrahepatic cholangiocarcinoma and other rare types only account for 10% – 15% of cases (4). The symptoms of early HCC are often imperceptible, and about 70% – 80% of patients are already in the advanced stage at the time of diagnosis (5, 6). The overall outcome of HCC still remains unsatisfactory, especially when the HCC is accompanied by the invasion of intrahepatic vessels (the portal vein or hepatic vein branches). It is one of the most

common complications of advanced HCC and has been proven to be closely related with the poor prognosis (7).

Portal vein tumor thrombus (PVTT) is the most frequent form of macrovascular invasion that occurs in 44.0% – 62.2% of HCC patients (8), while the incidence of hepatic vein tumor thrombus (HVTT) (1.4% – 4.9%) (9) or the inferior vena cava/intra-right atrial tumor thrombus (3% – 4%) is rare (10). Llovet et al. (11) analyzed the natural history of HCC patients associated with PVTT and reported that the median survival time (MST) was only 2.7 months without treatment. Giannelli et al. (12) retrospectively analyzed 150 HCC patients and found that the occurrence of PVTT was the most important and reliable negative prognostic factor (P<0.01). Recently, Mahringer-Kunz et al. (13) carried out a retrospective cohort study of 1317 HCC patients. The results showed that 484 patients presented with PVTT and it counted for 36.8% of the cases. The MST of patients with PVTT was 7.2 months, which was significantly shorter than the patients without PVTT (35.7 months, P < 0.001). The study found that the degree of PVTT is not a determined factor, because even the minor PVTT could lead to a very poor prognosis of HCC patients. Taken together, PVTT is an independent risk factor and associated with a dismal prognosis in HCC patients.

At present, there is still no global consensus or standard guidelines on the management of HCC with PVTT. According to the Barcelona Clinic for Liver Cancer (BCLC) staging system and treatment guidelines which are widely used in Europe and America, HCC patients with PVTT are regarded as BCLC Stage C which strongly indicates an advanced stage of the disease (7, 14–17). These guidelines recommend Sorafenib as the standard first-line treatment option but the effect is modest (18). In recent years, Lenvatinib was also approved and recommended as the first-line therapy for HCC (7). In order to improve the prognosis of HCC patients with PVTT, the more aggressive treatment modalities have been proposed in the Asia–Pacific region (6, 19, 20). Besides the small molecular targeted therapy, transarterial chemoembolization (TACE), radiotherapy (RT), hepatic resection, and liver transplantation (LT) have been practiced in the clinical and recognized gradually. Herein, we aim to review and summarize the advances in the diagnosis and treatment of HCC with PVTT.

DIAGNOSIS AND CLASSIFICATION OF PVTT

On the basis of the diagnosis of HCC, we need to distinguish PVTT from Portal vein thrombus (PVT) which usually occurred in cirrhosis patients and is important for the selection of treatment and the prognosis of HCC. Pathological analysis remains the gold standard to diagnose PVTT so far, but the clinical diagnosis mainly relies on computed tomography (CT) scan and magnetic resonance imaging (MRI) (21, 22). Kim et al. (23) retrospectively analyzed the gadoxetic acid–enhanced MR imaging of 366 HCC patients, and found that the characteristic imaging features of PVTT group were the enhancement, vessel expansion, continuity of the tumor, increased T2 signal intensity, and diffusion restriction. Agarwal et al. (24) presented a case report and put forward that ^{18}F-FDG PET/CT scan has good diagnostic performance in differentiating the malignant from benign thrombus. This view was subsequently validated by Wu et al. (25). Recently, by evaluating the radiographic features and clinical characteristics, Sherman et al. (26) found that the alpha-fetoprotein (AFP) >1000 ng/dL, venous expansion, thrombus enhancement, neovascularity, and adjacent to HCC were the characteristics of PVTT. They further proposed a noninvasive diagnostic criterion named the A-VENA criteria. The presence of 3 or more of these criteria could accurately differentiate PVTT from PVT (26).

The prognosis of HCC is not only related to the existence of PVTT, but also closely related to the extent of PVTT (27). Various classification systems for PVTT have been developed in different centers (28–32). Currently, there are two PVTT classification systems which are widely used in clinical practice (**Table 1**, **Figure 1**). The Japanese Vp classification (28, 33) is the first PVTT classification system which comprises five grades based on the extent of PVTT: 1) Vp0 for no PVTT; 2) Vp1 for tumor thrombus involving segmental PV; 3) Vp2 for tumor thrombus involving the second-order branches of PV; 4) Vp3 for tumor thrombus involving the first-order branches of PV; and 5) Vp4 for tumor thrombus involving the main trunk and/or contralateral branch of PV. In the Asia-Pacific, the more applicable classification system is the Chinese Cheng's classification (29, 30). It classifies PVTT macroscopically into four types based on the medical imaging results: 1) Type I, the tumor thrombus invades segmental PV or above. If the postoperative pathological result shows that the tumor thrombus is confined to microvascular, it is classified as Type I_0; 2) Type II, the tumor thrombus invades the right or/and left PV; 3) Type III, the tumor thrombus invades the main PV; and 4) Type IV, the tumor thrombus invades the superior mesenteric vein. Recently, Cao et al. (34) proposed a decision tree algorithm-

TABLE 1 | Classifications of PVTT.

Extent of tumor thrombus	Japanese Vp classification	Chinese Cheng's classification
no PVTT	Vp0	NA
microvascular	NA	Type I_0
segmental PV or above	Vp1	Type I
the second-order branches of PV	Vp2	Type I
the right or left PV	Vp3	Type II
the right and left PV	Vp4	Type II
the main trunk	Vp4	Type III
the superior mesenteric vein	Vp4	Type IV

PVTT, portal vein tumor thrombus; PV, portal vein; NA, not available.

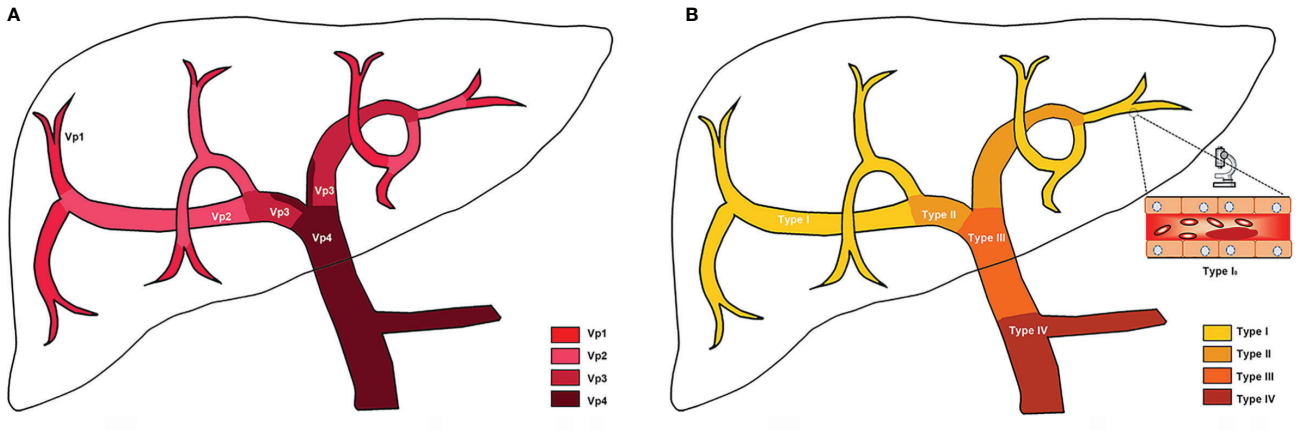

FIGURE 1 | Schematic of PVTT classifications. **(A)** Japanese Vp classification; **(B)** Chinese Cheng's classification. More details of classifications have been listed in **Table 1**.

based classification system by comprehensively considering both the extent of PVTT and HVTT, and generated 13 vascular invasion sub-classes. The classification system enables to personalize the management of HCC patients with vascular invasion, but its performance needs further assessment in more clinical studies.

TREATMENT

Targeted Therapy

Considering the damage to liver function, limited survival benefits and patients' drug intolerance, the traditional cytotoxic chemotherapy is not routinely recommended to HCC patients with PVTT. Targeted therapy remains the main option of systemic therapy for the patients.

Sorafenib, an oral small- molecule multi-kinase inhibitor, is the first approved targeted drug for treatment of HCC patients with PVTT based on two phase III randomized, double-blind, and placebo-controlled trials (18, 35). The MST of patients treated with Sorafenib alone was 10.7 months based on the result of the Sorafenib HCC Assessment Randomized Protocol (SHARP) study. Moreover, the MST was 6.5 months in Asia-Pacific region study, the survival time has only been prolonged for 2 – 3 months compared with placebo (18, 35, 36). In SHARP trial and Asia-Pacific population study, the stable disease (SD) and disease control rate (DCR) were 71% and 43%, 54% and 35.3%, respectively (18, 35). Bruix et al. (37) carried out an exploratory pooled analysis based on the two placebo-controlled in phase III studies. They observed that hepatitis C patients had a greater survival benefit who mainly distributed in the West. Without extrahepatic metastasis and lower neutrophil-to-lymphocyte ratio were also positive prognostic factors. The vascular invasion and high AFP were strong prognostic factors for poor outcome. In summary, sorafenib provides a survival benefit in HCC patients with PVTT but the effect is less than satisfactory.

In addition, the effect of Sorafenib in real-world clinical practice may be worse than the trials due to the selection bias. Jeong et al. (38) investigated the practical effect in 30 HCC patients with PVTT who received sorafenib monotherapy. The MST was 3.1 months and only 3 (10.0%) patients responded partially. SD and DCR were 30.0% and 33.3% respectively and were lower than the data from SHARP and Asia-Pacific trials. The common adverse events of Sorafenib are hand-foot skin reaction and gastrointestinal upset. Hepatic damage occurs occasionally, however it may lead to severe consequences (38, 39). In order to elucidate the safety and efficacy of Sorafenib monotherapy on HCC with PVTT, Kuo et al. (40) enrolled and analyzed 113 patients' clinical data, including 56 (49.5%) Vp3 and 57 (50.5%) Vp4. The incidence rate of hepatic decompensation was 18.2% and 37% for Vp3 patients and Vp4 patients, respectively ($p = 0.028$). Multivariate analysis indicated that Vp4 ($p = 0.041$) and baseline AFP ≥ 200 ng/ml ($p = 0.032$) were the associated factors with hepatic decompensation. Therefore, they suggested that Sorafenib should not be recommended as the first-line treatment for Vp4 patients with higher AFP, which was consistent with the previous viewpoint by the Japan Society of Hepatology (JSH) (41). Additionally, a phase III randomized study (STORM trial) of Sorafenib as adjuvant treatment after resection or ablation for HCC indicated that Sorafenib is not an effective intervention (42). A phase III STAH trial showed that Sorafenib plus TACE tended to prolong overall survival (OS) for HCC patients with PVTT compared with Sorafenib alone, although it is not statistically significant (43).

Lenvatinib is a novel anti-angiogenesis multi-kinase inhibitor which had shown its antitumor activity against advanced HCC on the basis of a randomized phase 3 noninferiority trial (44). Compared to Sorafenib, Lenvatinib was non-inferior in MST (13.6 vs. 12.3 months, HR 0.92, 95% CI 0.79 – 1.06), which had higher objective response rate (24.1% *vs.* 9.2%, OR 3.13, 95% CI 3.59 – 7.01, $p < 0.0001$) and longer progression-free survival (7.4 *vs.* 3.7 months, HR 0.66, 95% CI 0.57 – 0.77, $p < 0.0001$) with acceptable toxicity. The most common adverse events were

hypertension, diarrhea, decreased appetite, and decreased weight. Recently, Lenvatinib had been approved as the first-line treatment for unresectable HCC in the European Union, America, Japan and China currently (44–46). A case of advanced HCC was reported by Takeda et al. (47), In this case, the radiological examination showed clearly portal vein invasion, after 11 months of Lenvatinib monotherapy, the PVTT was undetectable, and vascularization of the main tumor was disappeared. The patient remained alive for more than 5 years after the initiation of Lenvatinib monotherapy. This case showed that Lenvatinib monotherapy might be a considerable therapy. But there were also some toxic effects during the treatment period, such as thrombocytopenia and proteinuria. Whether the curative effect of Lenvatinib was prior to other small molecule inhibitors or not was unclear, needing further investigation and long-term observation.

Beyond Sorafenib and Lenvatinib, there are several targeted drugs that have been studied and applied clinically as the second-line therapy for HCC patients with PVTT (48). Regorafenib is the first drug which demonstrated the efficacy for Sorafenib-intolerant patients, although the MST was only 10.6 months (placebo: 7.8 months, HR = 0.63, p <0.0001) (49). Hypertension and hand–foot skin reaction were the most common grade 3 or 4 adverse events (49). Apatinib, a selective inhibitor of vascular endothelial growth factor receptor (VEGFR)-2 with low price, had shown the safety and survival benefit in HCC patients with PVTT when combined with TACE (50). At present, Hu et al. (51) attempt to perform a multicenter, open-label, randomized controlled trial to assess the efficacy and safety of stereotactic body RT (SBRT) combined with Camrelizumab and Apatinib for HCC patients with PVTT. The efficacy of Cabozantinib in the previously treated patients with advanced HCC was evaluated in a phase 3 randomized trial. The results showed that the MST of Cabozantinib group was longer than placebo group, but with higher rate of high-grade adverse events (52). Ramucirumab, an anti-VEGFR2 monoclonal antibody, has demonstrated clinical benefit for HCC patients with AFP > 400 ng/ml in the recent phase 3 trial (REACH-2) (53). The development of new drugs is advancing and finding the biomarkers to predict responses to immunotherapies is the focus of future research (54).

TACE

TACE is considered as a standard locoregional treatment option and is widely used to treat unresectable HCC by many clinical practice guidelines (7, 19, 55). However, TACE was not administered to HCC patients with PVTT due to the potential risk of liver failure resulting from ischemia after TACE (56). The view is changing gradually with the development of medicine. Lee et al. (57) conducted a prospective controlled study and proposed that PVTT patients may benefit from TACE when the patients' liver function was at good level (Child-Pugh A) and adequate collateral circulation around the occluded PV has been established. Then, more studies about TACE applied in PVTT patients were performed and the results are similar. Chung et al. (58) retrospectively analyzed the survival data of 125 HCC patients with PVTT from 2003 to 2007, which showed improved MST for TACE group compared to supportive care group (5.6 *vs.* 2.2 months, P < 0.001). Another two prospective studies also confirmed that TACE had more survival benefit compared with conservative treatment (7.1 *vs.* 4.1 months, P < 0.001; 8.67 *vs.* 1.4 months, P < 0.001) (59, 60). Thus, for some HCC patients with PVTT, after careful selection, those patients with good liver function and well-establishment collateral circulation might be acquire more benefits from TACE than supportive care.

Research indicates that the extent of PVTT might affect the therapeutic effect of TACE. Silva et al. (61) made a meta-analysis involving 13 trials which comprised 1,933 patients to evaluate the safety and efficacy of TACE in the treatment of HCC with PVTT. Results showed that the MST was 8 (5–15) months, the incidence of liver failure and post-treatment complications were 1% and 18%, respectively. Patients with PVTT in main portal vein trunk had worse survival than with segmental PVTT (p < 0.001), but the modified RECIST criteria response rates were similar. Xiang et al. conducted a multicenter retrospective study in 1,040 patients. The results showed that TACE could significantly improve the OS rate than the other best supportive care for type I-III patients but not type IV (62). In addition, Kim et al. (63) assessed survival data of 331 HCC patients with segmental PVTT who underwent TACE as an initial treatment, and found four risk factors were related to the dismal OS after TACE: a major tumor burden (up-to-11criteria out), extrahepatic spread, Child-Pugh class B, and no response to TACE (stable disease or progressive disease). The study suggested that TACE should not be recommended for patients with 2 – 4 risk factors due to the poor prognosis. Yang et al. (64) retrospectively analyzed the clinical data of 379 HCC patients with PVTT who were treated with TACE as the first-line treatment, and found that patients with positive lipiodol deposition in PVTT was associated with an improved survival. In summary, for carefully evaluated HCC patients with PVTT, TACE could be a safe considerable treatment modality and the degree of lipiodol deposit in PVTT may help to assess the prognosis after TACE.

Though TACE might be an option for HCC patients with PVTT according to above researches, the efficacy of TACE alone is still limited given the MST is less than 10 months. TACE plus other treatments as a new therapeutic strategy, may improve the survival of HCC patients with PVTT. Takano et al. (65) reported a case of HCC patient with PVTT who received curative hepatectomy after TACE and sorafenib, and the disease-free survival (DFS) time was more than 12 months. A meta-analysis of 25 trials involving 2,577 patients showed that 1-year survival rate for the TACE plus RT group was significantly better than that of the TACE alone group (OR 1.36, 95% CI 1.19 – 1.54) (66). Similarly, another meta-analysis of 5 studies involving 973 patients showed that 6-month and 1-year OS rate for the TACE plus sorafenib group were significantly better than that of the TACE alone group (OR 3.47, 95% CI 2.47 – 4.89; OR 3.10, 95% CI 2.22 – 4.33). Chu et al. (67) used propensity score matching analysis to compare the effectiveness of TACE plus RT and TACE plus sorafenib groups in the treatment of HCC patients with PVTT, and found that PFS and OS did not differ significantly between these two combined strategies.

In addition, the effectiveness of TACE is associated with the embolizing agents. TACE with drug-eluting beads has been applied in clinical but its effects need more researches to support (68). Hepatic arterial infusion chemotherapy (HAIC), another locoregional treatment, much like TACE, may be another option for advanced HCC patients which showed a better response and improved prognosis compared to sorafenib in previous studies (69, 70). The conclusion was validated by a retrospective study which showed that the PFS of HCC patients with main PVTT in HAIC group was significantly longer than in sorafenib group (1.9 vs. 6.0 months, p<0.001) (71). By means of meta-analysis, Liu et al. (72) also demonstrated that HAIC is superior to sorafenib in HCC patients with PVTT, especially in type III – IV patients (Cheng's classification). However, the study showed that HAIC was more likely to cause myelosuppression. Of note, the efficacy and safety of HAIC must be evaluated in multicenter randomized controlled trials.

Radiation Therapy

In the past, RT was not regarded as a feasible treatment for HCC patients with PVTT because of the liver's poor tolerance to radiation (73). But this opinion has been changed with the rapid development of precision radiotherapy technology and application of new radioisotope. Several prospective and retrospective studies have applied RT to HCC management and shown that RT could improve the prognosis, especially in patients with PVTT (74–76). The therapeutic method divided into two forms according to different administration pathways: the external beam radiation therapy and selective internal radiation therapy.

External Radiotherapy

Advanced external radiation techniques could deliver a higher radiation dosage to the targeted regions without damage to the adjacent normal liver, including three-dimensional conformal RT (3D-CRT), intensity modulated RT (IMRT), SBRT and proton beam RT. Yu et al. (77) explored the role of external RT in the treatment of HCC patients with PVTT and showed that the objective response rate was 40% to 60% and the MST was 15 to 20 months in responders. The review presented that RT could be an effective local treatment modality. In a prospective study of Kishi et al. (78), preoperative SBRT targeting PVTT in HCC patients showed high pathological response rate and low toxicity. Postoperative RT also could improve survival outcomes for patients with resectable HCC and PVTT. Wei et al. (79) conducted an open-label randomized controlled study to evaluate the efficacy of neoadjuvant 3D-CRT in HCC patients with PVTT after hepatectomy. Results showed that the 1- and 2-years OS rates were significantly better in the neoadjuvant 3D-CRT group than the surgery-alone group (75.2% and 27.4% vs. 43.1% and 9.4%, P<0.001). Another randomized controlled trial showed that postoperative adjuvant IMRT could significantly improve the 1-, 2-, and 3-years OS rates (76.9%, 19.2%, and 11.5% *vs.* 26.9%, 11.5% and 0%, P=0.005) (80).

In clinical practice, several studies indicated that adding RT to combined treatment could improve survival for HCC patients with PVTT. Positive PVTT response to combined treatment was the most significant prognostic factor for PFS (HR 0.33, 95% CI 0.25-0.42, P < 0.001) (81). Li et al. (82) made a network meta-analysis of 15 studies involving 2,359 patients to evaluate the efficacy and safety of different modalities in patients with advanced HCC and PVTT. These modalities included SBRT, HAIC, sorafenib, TACE, SBRT plus TACE, 3D-RT plus HAIC or TACE, and TACE plus sorafenib. Results showed that RT combined with HAIC or TACE produced better survival benefit than other regimens. Im et al. (83) reported a retrospective study about 985 HCC patients with PVTT who received RT and demonstrated that RT with combined treatment is a better approach which had better OS than without combined treatment. Wu et al. (84) also suggested that compared with TACE or RT alone, RT plus TACE is a better choice in treating advanced HCC patients with PVTT. After comparing the MST of patients who received RT-TACE and TACE-RT (13.2 vs.7.4 months, P = 0.020), Li et al. (85) suggested that RT followed by TACE is a better combined therapy strategy for HCC patients with PVTT. Besides treatment methods, radiation dose is another important factor which is still controversial in clinical practice. Im et al. (83) demonstrated that the equivalent RT dose >45 Gy was a significant positive factor for OS. Due to the liver's high sensitivity to radiation, the best radiation dose should be confirmed in further prospective studies.

Internal Radiotherapy

Iodine-125 (^{125}I) seed implantation, a type of brachytherapy, has been widely applied in treating HCC patients with PVTT and the treatment responses are favorable. Clinically, ^{125}I seed implantation is always applied in the combination with TACE or portal vein stent (86, 87). Yuan et al. (87) made a meta-analysis of 8 studies involving 1,098 patients to evaluate the efficacy and safety of ^{125}I seed implantation in HCC patients with PVTT. Results showed that compared with TACE alone, ^{125}I seed implantation plus TACE can significantly improve patients' survival rate (HR 0.27, 95% CI 0.14 – 0.40, p=0.000), reduce patient's mortality risk (HR 0.46, 95% CI 0.37 – 0.54, p=0.000), and did not increase the incidence of adverse event (OR 1.07, 95% CI 0.92 – 1.25, p=0.262). The recommended dose of ^{125}I is more than 110 Gy. Another retrospective study showed that combining endovascular implantation of 125I seed with stent placement, TACE, and sorafenib may provide better OS and PFS than TACE plus sorafenib in HCC patients with PVTT (88).

Transarterial radioembolization (TARE) with yttrium-90 (^{90}Y) is a special treatment which successfully interweaves the microembolic procedure and RT. The available evidence showed that TARE is a safe and effective therapy for HCC patients with PVTT. The response rate ranges from 50% to 75%, and the MST is approximately 10 months (89). Two phase III trials showed that the OS of TARE and sorafenib were not significantly different (90, 91). A meta-analysis involving 17 studies showed that the 6-month and 1-year OS rate were 76% and 47% in TARE group, more than in sorafenib group (54% and 24%) (92). The incidence of grade 3 or 4 adverse events in TARE group was lower than in sorafenib group (9% *vs.* 28%, P = 0.129). Abdominal pain, nausea and fatigue were the frequent adverse events of TARE (92). Thus, the tolerance of TARE may help to

recommend its clinical use. Spreafico et al. (93) found that bilirubin level, extension of PVTT and tumor burden were firmly associated with prognosis of patients with HCC and PVTT treated with TARE, and proposed to build a prognostic stratification to identify suitable candidates. The effectiveness of the prognostic model had been validated by two retrospective single-center study (94, 95), and should be further evaluated in prospective studies.

Compared with external radiotherapy, internal radiotherapy is a more invasive radiotherapy. However, internal radiotherapy has a high dose and continuous release radiation for PVTT and low damage to the nearby normal liver tissues. Especially for patients with malignant stenosis or occlusion of the portal vein, internal radiotherapy plus portal vein stent could not only greatly alleviate the portal hypertension, but also prevent the reinvasion of PVTT into the portal vein (96–98). For HCC patients with PVTT, the selection of external radiotherapy or internal radiotherapy remains unclear. In a retrospective study, Tan et al. (96) showed that internal radiotherapy plus TACE had longer OS than external radiotherapy plus TACE (13.1 vs. 8.0 months, p= 0.021). Internal radiation therapy might be more effective but also more invasive. Most of HCC patients with PVTT are at the end stage, the doctors need to evaluate the condition of specific patients carefully, to choose a better therapy.

Surgical Resection

Liver resection is the main treatment for patients with HCC that may offer the best chance of cure (7). However, the presence of PVTT, regardless of the extent, has been viewed as a contraindication of surgery by BCLC staging system in western countries (15). Therefore, most patients lost the chance for radical operation and the possibility of cure is almost zero. However, with the advances in surgical technologies and improvements in perioperative management, aggressive surgical resection has been proposed and adopted to treat some selected HCC patients with PVTT in several center. Surgical treatment has been considered as a possible choice when the primary tumor and PVTT could be completely resected, without distant metastasis and damage to liver function (5). Hepatectomy and thrombectomy are carried out according to the location and extent of tumor and PVTT. The en bloc resection of PVTT with tumor is considered when the PVTT lies within the liver resection line (Type I – II or Vp1 – Vp3), including segmental hepatectomy and hemihepatectomy. When the PVTT lies beyond the resection line (Type III – IV or Vp4), hepatectomy plus thrombectomy could be considered. Portal vein resection and reconstruction should be performed when the PVTT invading the main portal vein wall (99–101).

Up to now, a number of studies have evaluated the efficacy of surgical treatment on the disease, especially in Asian liver centers. Kokudo et al. (102) published a large retrospective study of 6,474 HCC patients with PVTT in Japan, including 2,093 patients who underwent liver resection and 4,381 patients who received other therapeutic interventions. Results showed that the MST of surgical group was significantly longer than that of non-surgical group (2.87 vs. 1.10 years, P < 0.001) with good liver function (Child-Pugh A). A further subgroup analysis indicated that liver resection could result in survival benefits as long as the PVTT is limited to a first-order branch (Vp1 – Vp3). However, the benefit was not significant in patients whose PVTT affected the main trunk or contralateral branch (Vp4). Similar results were reported by Wang et al. (103). They retrospectively analyzed 1,580 HCC patients with PVTT from four largest tertiary hospitals in China and figured out that the treatment was an independent risk factor of OS. The MST of the surgical group for types I and II patients were 15.9 and 12.5 months respectively, significantly longer than nonsurgical counterparts. What's more, TACE plus RT may provide more survival benefit to types III patients than surgical treatment (8.9 vs. 6.0 months, P=0.063). A similar result is obtained by Chen et al. (104). In a word, HCC patients with PVTT could benefit from surgery but the prognosis is affected by the extent of PVTT.

In order to identify which factors might affect the survival outcome, Huo et al. (99) retrospectively analyzed the clinical data of 487 HCC patients with PVTT who underwent liver reresection. Results showed that the liver function and tumor differentiation were risk factors of short-term and longer-term survival respectively, while AFP was associated with both short-term and longer-term survivals. Zhang et al. (105) developed an EHBH/PVTT scoring system to guide the HCC patients' selections with PVTT (Vp1 – Vp3) who could benefit from negative margin (R0) liver reresection. The score was calculated by using total bilirubin (≥17.1 μmol/L=1), AFP (≥20 μg/L=2), tumor diameter (3-5 cm=1, >5 cm=2), and satellite lesions (Yes=1). Liver resection was recommended for patients when EHBH-PVTT score ≤3. After analyzing a nationwide database of 1,590 HCC patients with PVTT who underwent liver resection, Chen et al. (106) found that the actual 3-year survival rate of patients was 11.7%. The independent prognostic factors of long-term survival included total bilirubin, AFP, types of hepatectomy, extent of PVTT, intraoperative blood loss, tumor diameter, tumor encapsulation, R0 resection, liver cirrhosis, adjuvant TACE, postoperative early recurrence (< 1 year), and recurrence treatments. In addition, postoperative adjuvant TACE could improve the survival of HCC patients with PVTT (107).

The surgical technique may be an important factor which influences the prognosis. "Liver resection first" is the most common major operation performed on HCC patients, PVTT is often removed after hepatectomy in previous studies which concluding that type III/IV PVTT patients were unable to gain a survival advantage through surgery. Ban et al. (108) performed tumor thrombectomy prior to the hepatectomy for 19 Vp4 patients. The 3- and 5-year OS rates in the study were 41.8% and 20.9% respectively, which were significantly higher than in other studies. Peng et al. (100) put forward a concept of "thrombectomy first", which means the PVTT should be removed prior to liver resection when it is located in the main PV, the bifurcation or the contralateral PV. They subsequently shared three types III/IV (Vp4) cases which were treated with "thrombectomy first" method and achieved good long-term survival, the DFS were 13, 9 and 4.6 years respectively (100).

The new surgical technique may improve the management of HCC patients with PVTT, especially for type III/IV PVTT patients. The efficacy of "thrombectomy first" approach should be further validated in multi-center and randomized trials.

Liver Transplantation

Compared to liver resection, LT can not only completely resect the lesion but also restore liver function. As a curative treatment for HCC patients, the indication of LT is expanding. Lots of studies indicated patients beyond the conventional Milan criteria are also suitable for LT, but in most studies, PVTT remains as an absolute contraindication due to the high rate of recurrence and poor prognosis (109–111). In recent years, several centers tried to do LT in HCC patients accompanied by PVTT, and the clinical data have shown that LT can provide survival benefit for selected HCC patients with PVTT. Herein, we reviewed the related literature and crested a summary in **Table 2**.

Xu et al. (124) considered that LT was an efficient treatment but palliative treatment for HCC patients with PVTT. They retrospectively analyzed the survival data of 24 HCC patients with PVTT who received deceased donor LT (DDLT), and compared it with 27 patients who underwent liver resection. The OS rates at 6-month, 1-and 2-year were 66.7%, 29.5% and 23.6% for the LT group, and 33.3%, 22.2% and 14.8% for the resection group (P=0.0335), respectively. But the tumor recurrence rate was as high as 66.7% for the LT group. Zhou et al. (122) compared the therapeutic effects of LT and other therapies on HCC patients with PVTT. Results showed that the 1-, 3-year OS rate in LT group were 30% and 10%, which was better than the conservative treatment (12% and 4%), but inferior to resection combined with adjuvant chemotherapy (70% and 20%). Our previous study showed that pre-transplant AFP level and 18 F-FDG standard uptake value (SUV max) were independent risk factors for HCC recurrence

TABLE 2 | Liver transplantation for HCC patients with PVTT.

Author, Year	Country	Study design	N (Enrollment Period)	Treatment	Downstaging before LT	Classification of PVTT (n)	Survival time	DFS rate (1-,3-,5-year)	OS rate (1-,3-,5-year)
Yang, 2020 (112)	China	Retrospective study	75 (2016-2018)	DDLT	NA	Vp2-3 (47)	NA	44.4%,40.0%, NA	74.1%, 65.4%, NA
						Vp4 (28)	NA	28.6%,21.4%, NA	64.3%, 30.6%, NA
Assalino, 2020 (113)	Switzerland	Retrospective study	30 (2004-2018)	DDLT/ LDLT	Yes	Vp1 (7); Vp2 (12); Vp3 (5); Hepatic vein (6)	NA	63.3%, 56.3%, 56.3%	76.7%, 66.2%, 59.6%
Soin, 2020 (114)	India	Prospective study	46 (2006-2017)	LDLT	Yes	Vp1 (1); Vp2 (12); Vp3 (11); Vp4 (1)	NA	77%, 77%, 51%	82%, 57%, 57%
					No	Vp1 (5); Vp2 (13); Vp3 (3); Vp4 (0)	NA	63%, 48%, 40%	80%, 59%, 48%
Jeng, 2018 (115)	China	Case report	1 (2013)	DDLT	Yes	Type II	DFS is more than 20 months	NA	NA
Levi, 2017 (116)	Italy	Case series	4 (2002-2015)	DDLT	Yes	Vp1 (3); Vp3 (1)	Median DFS: 39.1 (6–76) months	NA	NA
Lee, 2017 (117)	Korea	Retrospective study	11 (2009-2013)	LDLT	Yes	Vp3 (3); Vp4 (1)	Mean DFS: 8.3 (1-20) months	63.6%, 45.5%, 45.5%	72.7%, 63.6%, 63.6%
					No	Vp2 (3); Vp3 (1); Vp4 (3)			
Jeong, 2017 (118)	Korea	Retrospective study	17 (2007-2014)	LDLT	Yes	Vp2 (7); Vp3 (7); Vp4 (1); Hepatic vein (2)	NA	70.6%, 57.8%, NA	87.45%, 60.5%, NA
Choi, 2017 (119)	Korea	Retrospective study	34 (2005-2015)	LDLT	NA	Type I (27)	NA	68.2%, 63.9%, 63.9%	85%, 60.3%, 50.3%
						Type II (7)	NA	28.6%, 14.3%, 14.3%	71.4%, 14.3%, 14.3%
Han, 2016 (120)	Korea	Retrospective study	8 (2011-2012)	LDLT	Yes	Type II, Type III	MST: 33 (22–48) months	87.5%, NA, NA	NA
Ettorre, 2010 (121)	Italy	Case report	1 (2009)	DDLT	Yes	Type II	survival for more than 4 years	NA	NA
Zhou, 2011 (122)	China	Retrospective study	12 (2003-2010)	DDLT	No	Type II (6); Type III (6)	MST: 7 months	NA	30.0%, 10.0%, NA
Wang, 2010 (123)	China	Retrospective study	62 (2001-2007)	DDLT	NA	Type I_0 (12); Type I-III (50)	NA	29.6%, 13.4%, NA	NA
Xu, 2004 (124)	China	Retrospective study	24 (1999-2003)	DDLT	NA	Type II (14); Type III (10)	MST: 8 months	29.5%, NA, NA	23.2%, NA, NA

PVTT, portal vein tumor thrombus; DDLT, deceased donor liver transplantation; LDLT, living donor liver transplantation; MST, median survival time; DFS, disease free survival; OS, overall survival; NA, not available.

fonc.2021.635731. The study also proposed that patients with AFP < 1000 ng/mL and SUV max < 5 might be suitable for LT.

Given the shortage of donor organs, DDLT is still limited in the treatment of HCC patients with PVTT. In recent years, the number of living donor LT (LDLT) is increasing, which provided a therapeutic option for curing HCC patients with PVTT. Choi et al. (119) retrospectively analyzed 34 HCC patients with PVTT who underwent LDLT. The 1-, 3- and 5-year OS and DFS rates for segmental PVTT group were 85%, 60.3%, 50.3% and 68.2%, 63.9%, 63.9%, respectively, which were higher than lobar PVTT group (71.4%, 14.3%, 14.3% and 28.6%, 14.3%, 14.3%, respectively). They proposed that segmental PVTT could benefit from LT, especially when the AFP level less than 100 ng/mL. Similar result was reported by Lee et al. (117). The 5-year OS rates and DFS rates were 63.6% and 45.5% in their study. They proposed that PVTT is not an absolute contraindication for LDLT. LDLT was considered to be a curative treatment option when the PVTT did not extend into the main PV and the multiplication of AFP and protein induced by vitamin K absence/antagonist-II (PIVKA) score is less than 20000. Therefore, LT can improve the survival of HCC patients with PVTT, especially for carefully selected recipients.

Bridging treatment before LT could help HCC patients with PVTT downstage to meet the qualifications for LT, such as TACE, HAIC, TARE, CCRT (125). Chapman et al. (126) reported 17 HCC patients with macrovascular invasion underwent LT after successful downstaging to within the Milan criteria through TACE. The result was satisfied, the 5-year OS rate was up to 93.8%. Levi Sandri et al. (116) reported 4 patients in BCLC stage C received TARE with ^{90}Y before LT. Result showed patients had a complete response for the PVTT and eventually accepted LT, the median DFS was 39.1 months. A similar case reported by Ettorre et al. (121, 125) showed that an HCC patient with PVTT was successfully downstaged through TARE and received LT, then survived for more than four years. Another typical case reported by Jeng et al. (115) showed that an HCC patient with tumor thrombus invading right main PV received DDLT after successful downstaging by multimodal treatments, and the survival time was more than 20 months without tumor recurrence or metastasis. Assalino et al. (113) conducted a multi-center retrospective cohort study and demonstrated that HCC patients could be considered for LT when the vascular invasion achieved radiological complete regression after locoregional therapies and the pretransplant AFP < 10 ng/ml.

Downstaging treatment is also suitable for LDLT. Han et al. (120) reported 8 HCC patients with PVTT who accepted LDLT after successful downstaging of tumor through CCRT and HAIC. The MST was 33 months. Moreover, Jeong et al. (118) reported 17 HCC patients with major vascular invasion who received LDLT after combined treatment modalities. The DFS rates and OS rate at 1- and 3-year were 70.6% and 57.8%, 87.4 and 60.5%, respectively. Recently, Soin et al. (114) shared treatment experience with LDLT in HCC patients with PVTT. Compared to the patients without the downstaging before LDLT, the 1-, 3- and 5-year DFS rates were improved in patients with successful downstaging (77%, 77%, and 51% *vs.* 63%, 48%, and 40%, P=0.35), although without statistical significance. Taken together, these results demonstrate that the downstaging could actually improve survival of HCC patients with PVTT before LT.

All in all, LT could be a promising treatment modality for HCC patients with PVTT. Downstage treatment for these patients is quite important. Combined therapy before LT seems to play an important role in the downstaging strategy for LT candidates. However, the number of related studies is still less. More prospective studies and randomized controlled trials are needed to assess the application value of LT in HCC patients with PVTT. In addition, it is urgently necessary to develop a scoring system to identify suitable candidates for LT.

Other Strategies

Besides, with the development of immunotherapy in the area of cancer therapy, the combination of small molecular targeted therapy and immunotherapy might be a promising direction. Programmed death 1 (PD1) inhibitors have gained great success in some types of cancer treatment. For hepatocellular carcinoma treatment, PD-1 inhibitors showed promising clinical activity in phase 1/2 studies (127, 128). However, the response rates were range of 15-20% in single-agent treatment studies, they did not improve overall survival, either (129, 130). It has been reported that antiVEGF therapies could reduce VEGFmediated immunosuppression within the tumor and its microenvironment (131–133). So, anti-VEGF therapies might also enhance the anti PD-1 or anti PD-L1 efficacy by reversing immunosuppression in tumor (134, 135). Bevacizumab, a monoclonal antibody, which targets VEGF (136), inhibits angiogenesis, and showed response rates of 13 to 14% in single agent phase 2 studies (137–140). Atezolizumab, which targets PDL1 to prevent interaction with receptors PD1 and B71, activate T-cell in immunotherapy. The combination of atezolizumab and bevacizumab showed a promising antitumor ability with acceptable side effect in treatment of untreated unresectable hepatocellular carcinoma. The reported response rate was 36%, and the median progression free survival was 7 months (141). Another global, multicenter, phase 3 randomized trial, IMbrave150 showed us inspiring results. Compare to sorafenib treatment alone, the overall survival at 12 months was 67.2% in combo therapy group, but 54.6% in sorafenib group, median progressionfree survival was 6.8 months (95% CI, 5.7 – 8.3) and 4.3 months (95% CI, 4.0 – 5.6), respectively. Grade 3 or 4 adverse events occurred in 56.5% of 329 patients who received at least one dose of atezolizumab-bevacizumab and in 55.1% of 156 patients who received at least one dose of sorafenib. Serious adverse events occurred more frequently with atezolizumab-bevacizumab (125 patients,38.0%) than with sorafenib (48 patients, 30.8%) (142). Though atezolizumab plus bevacizumab therapy prolong overall survival and PFS in unresectable hepatocellular carcinoma patients, the high rate of serious side effects needs to be on the alert.

CONCLUSION

In conclusion, PVTT remains as the blockage in the treatment of HCC, which contributes in the high recurrence rate and poor prognosis. Besides Sorafenib and Lenvatinib, no other standard treatment regimen is currently available for HCC with PVTT.

For these patients with HCC and PVTT, the surgery, TACE, RT and various combination therapies were effective and safety choices, which could help to prolong the survival time and promote the quality of life. LT may be a curative treatment option for highly selected patients, especially LDLT. In the future, larger scale randomized trials are needed to develop better treatment strategy to manage HCC patients with PVTT.

AUTHOR CONTRIBUTIONS

FL and ML collected related papers and drafted the manuscript. FL drafted the figures. JD participated in the design of the review. SZ was responsible for the supervision of the work. All authors contributed to the article and approved the submitted version.

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Acute Kidney Injury in Adult Patients With Hepatocellular Carcinoma After TACE or Hepatectomy Treatment

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Background: Acute kidney injury (AKI) is one of the most common complications in patients with cancer, yet the specific reasons, mechanisms, and the influence of AKI are not clear in hepatocellular carcinoma (HCC) after treatment. This meta-analysis aimed to find out the risk factors and the impact on mortality of AKI in adult patients with HCC after treatment using available published data.

Methods: We performed a systemic literature search using PubMed, Web of Science, and Embase, encompassing publications up until November 30, 2021 (inclusive), with 17 cohort studies involving 11,865 patients that fulfilled the prespecified criteria for inclusion in the meta-analysis. The number of AKI/non-AKI patients identified by risk factors, the number of AKI/non-AKI-related deaths, the incidence rates, the mortality rates, and the irreversible rates of AKI were derived and analyzed using STATA.

Results: Age, diabetes mellitus (DM), and the number of transarterial chemoembolization (TACE) sessions are risk factors for AKI in patients with HCC after TACE. On the other hand, male gender, age, DM, major resection of the liver, and operation-related transfusion are risk factors for AKI in patients with HCC after hepatectomy. The risk of mortality in those with renal failure due to AKI was up to 4.74 times higher than in those without AKI in a short-term observation period after TACE treatment.

Conclusions: Attention should be paid to the risk of AKI in HCC patients with DM. The occurrence of AKI during TACE treatment is especially dangerous and should be considered a strong red flag, obviously with regard to the extremely high risk of death in a short period. Furthermore, studies are needed to detect more associations of AKI in patients with HCC.

Keywords: AKI, risk, mortality, hepatocellular carcinoma, HCC

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1 INTRODUCTION

As a global health problem, hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer-related death in men and the sixth in women (1). Although liver transplantation (LTx) is the most effective among all the therapeutic options, only about 5% of HCC patients are eligible for this therapy due to the strict indications (2). According to the National

Comprehensive Cancer Network (NCCN) Guidelines, partial hepatic resection is the preferred option in patients without severe liver cirrhosis (excluding patients with Child–Pugh scores in classes B and C); meanwhile, locoregional therapy is the preferred option in patients unsuitable for surgery, which includes ablation, arterially directed therapies, and external beam radiation therapy (EBRT) (3).

Acute kidney injury (AKI) is one of the most common complications in cancer patients (4, 5). It refers to a rapid (hours to days) deterioration of renal function, which results in the failure to excrete waste and to maintain fluid balance, which can be severe as to require renal replacement therapy (RRT) (6). The management of these patients is a significant therapeutic challenge for physicians, and the chance of receiving optimal treatment might be less for those with poor kidney function since mortality from AKI remains high, particularly in critically ill patients (6). Efforts made to prevent AKI progression may contribute to survival and reduce the possibility of progressing to chronic kidney disease (CKD). As a clinical syndrome that results from severe or persistent events that may act as triggers, any diagnostic approach to investigating AKI should take into account the associated epidemiology (6). Although a large cohort study based on a Danish population reported that the risk of developing AKI in 1 year was about 33% in patients with liver cancer (7), there is still a lack of research focused on the association between AKI and HCC after treatment, especially regarding locoregional therapy or hepatectomy—the two major treatment options for HCC patients. The few previous studies that described AKI had limitations of a small study size, collection of data from a single medical center, or discussion of the incidence rates of AKI in newly diagnosed cancer, which present obvious restrictions regarding the generalizability of the findings. More comprehensive analyses are urgently needed to examine their authentic relationship in order to help provide proper management and to improve the clinical outcomes.

This meta-analysis was conducted based on HCC patients receiving locoregional therapy and hepatectomy, aiming to examine the risk factors and the impact on mortality of AKI in these HCC patients using available published data.

2 MATERIALS AND METHODS

Search Strategy

The protocol for this meta-analysis has been registered in the International Prospective Register of Systematic Reviews (PROSPERO no. CRD42020183617). A systematic literature review was performed by two authors (MZX and LC) independently through PubMed, Web of Science, and Embase, employing the search terms "acute kidney injury" OR "acute renal failure" AND "hepatocellular carcinoma" OR "liver cancer" OR "hepatoma" and including publications up until November 30, 2021 (inclusive). The search terms "contrast induced nephropathy" (CIN) AND "hepatocellular carcinoma" were also used as the previous recognition of renal dysfunction in HCC to investigate the incidence of AKI among adult patients with HCC. Each study was evaluated for inclusion or exclusion in this analysis (see below). No language or date restrictions were applied. This meta-analysis was conducted and reported according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; http://www.prisma-statement.org/).

Study Selection Criteria: Risk Factors, Outcomes, and Follow-Up

Firstly, potential eligible studies must meet the Population, Interventions, Comparison and Outcomes (PICO) criteria to fulfill the purpose of this analysis. The inclusion criteria were as follows: HCC patients underwent locoregional therapy or liver resection; the original cohort studies provided data on the AKI events based on adult patients (age, ≥18 years) with HCC; the Child–Pugh score is in Child–Pugh class A or B; the clinical characteristics/prognosis related to AKI could be clearly identified by the number of patients; and the definitions of AKI or RRT were not considered.

The following studies were excluded: those regarding LTx for HCC; comprising patients who had end-stage renal disease or were undergoing RRT; AKI cannot be identified by the number of patients; including other types of hepatobiliary cancers; and case/case series reports including ≤10 patients. Research works from the same hospital were carefully evaluated for exclusion. No restrictions on language or year were applied in the full text.

Data Extraction and Study Quality

To extract the necessary data from each included study, a spreadsheet template (Excel, Microsoft Corporation, Redmond, WA, USA) was established. After a careful review of each included article, the following data were collected: first author, publication year, regions, risk factors of AKI, the number of HCC, AKI, or irreversible renal failure (RF) patients, the number of AKI/non-AKI-related deaths, AKI definitions, and the observation period. Studies that did not base AKI on the number patients required careful calculation to maintain the accuracy. Some original data unpublished online were obtained from authors after communication (8, 9).

Quality assessment for the included studies was conducted using the Newcastle–Ottawa Quality Assessment Scale (NOS), which comprises three aspects (selection, comparability, and outcomes) and eight items (10). This scale enables the researchers to score studies from 0 to 9, whereby those with a score ≥6 were considered of high methodological quality.

Definitions of AKI, Risk Factors, Locoregional Therapy, and Observation Period

Although they have been validated in numerous patients and seem to work similarly (6), there are still over 30 AKI definitions used in the literature (11). RIFLE (Risk of renal failure, Injury to the kidney, Failure of kidney function, Loss of kidney function, and End-stage kidney disease), AKIN (Acute Kidney Injury Network), and KDIGO (Kidney Disease Improving Global Outcomes) are the three widely accepted criteria for the

definition of AKI (12, 13). In this meta-analysis, AKI was accepted in the case of the original study having identified its occurrence regardless of any definition.

According to the NCCN Guidelines, locoregional therapy comprises the following: 1) ablation, including radiofrequency, cryoablation, percutaneous alcohol injection, and microwave ablation; 2) arterially directed therapies, including bland transarterial embolization (TAE), transarterial chemoembolization (TACE), TACE with drug-eluting beads (DEB-TACE), and radioembolization (RE) with yttrium-90 (Y-90) microspheres (3).

All potential risk factors that possibly affect the renal function of patients with HCC after treatment should be identified and screened.

Long-term refers to prognosis being observed after 1 year from AKI, whereas short-term indicates observation being conducted within 3 months from AKI.

Statistical Analysis

STATA statistical software (version 16.0; StataCorp LLC, College Station, TX, USA) was utilized for statistical analysis. In the analysis, random effects models and the DerSimonian–Laird method were applied to analyze dichotomous variables (the number of AKI/non-AKI patients identified by risk factors and the number of AKI/non-AKI-related deaths), continuous variables, and proportion variables (the incidence rates, mortality rates, and the irreversible rates of RF). Double arcsine transformation was applied for the meta-analysis of low proportion variables to ensure normality. The I^2 test was used to assess heterogeneity. Pooled risk ratios (RRs), the weighted mean difference (WMD), and their corresponding 95% confidence intervals (CIs) were used to evaluate the risk factors of developing or the risk of mortality with AKI. The *Z*-test was used to assess the significance of the pooled RRs/WMDs, and a forest plot was drawn to graphically display the results of all statistical analyses. Statistically significant heterogeneity among studies is defined as χ^2-value <0.05 or I^2 test >50%. Subgroup analyses were performed to investigate the original source of significant heterogeneity, and a *Z*-test *p*-value <0.05 was considered a statistically significant difference.

3 RESULTS

Literature Search and Study Characteristics

The flow diagram showing the selection process and the reasons for the exclusion of a systematic review is presented in detail in **Figure 1**. Three databases provided a total of records (PubMed, *n* = 117; Web of Science, *n* = 320; Embase, *n* = 260). After the exclusion of duplicates (262 records), the titles and abstracts of 435 articles were manually screened for eligibility. Then, studies on *in vitro*/animal, machine learning, transplantation, or pediatric/neonatal populations; case reports; conference abstracts; and review articles were excluded. Thereafter, the full texts of the 48 remaining articles were reviewed for eligibility. The remaining studies that included only ≤10 patients, not enough papers using the same treatment method, studies from the same cohort, and national reports (duplicated representative population) were also excluded after careful review.

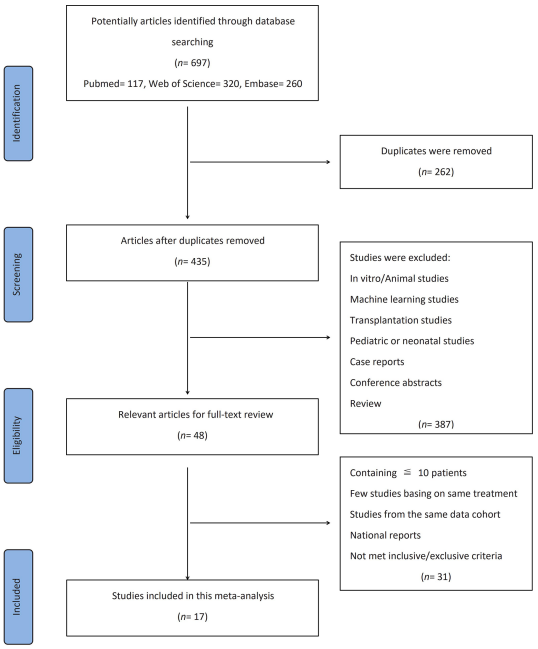

FIGURE 1 | Flow diagram for this meta-analysis.

Finally, 17 cohort studies involving a total of 11,865 patients that fulfilled the prespecified criteria were included in the meta-analysis (**Tables 1**, **2**). Among them, 10 studies were based on TACE, 1 study was based on TACE and TAE (these 11 studies would be analyzed together, hereinafter as "TACE"), and 6 studies were based on liver resection. Nine studies (52.9%) with a score ≥6 were considered of high quality according to the NOS criteria (**Table 3**). Sixteen studies (94.1%) reported the outcomes with clearly defined AKI, 14 studies (82.4%) reported at least one risk factor for developing AKI, while 10 studies (58.8%) reported AKI-related death.

Patient Characteristics

In this study, 11,865 adult patients with HCC between July 1996 and December 2019 were identified as being eligible for analysis. Of these patients, 30.2% (*n* = 3,581) received TACE treatment and 69.8% (*n* = 8,284) received hepatectomy.

For the analysis of HCC-AKI patients receiving TACE, eight studies reported on 2,377 men and 651 women suffering from HCC, four studies recorded the ages of 1,429 patients with HCC, five studies reported on 373 HCC patients with multiple tumor, six studies reported diabetes mellitus (DM) as a comorbidity for 216 patients with HCC, five studies reported on 517 HCC patients with HBsAg(+), three studies reported on 1,362 patients receiving non-steroidal anti-inflammatory drug (NSAID) treatment, four studies provided the number of TACE sessions on 707 patients, and three studies described the amount of contrast on 1,289 patients. In this series analysis, patients with a history of renal insufficiency were excluded.

For the analysis of HCC-AKI after hepatectomy, 6,197 (74.8%) men and 2,087 (25.2%) women with HCC were

TABLE 1 | Characteristics of the studies about transarterial chemoembolization (TACE).

Study	Year	Region	Risk factors for AKI	No. of HCC patients	No. of AKI patients	Death with RF	AKI definitions	Observation period	Irreversible RF (inclusive of death with RF)
Huo et al. (14)	2004	Taiwan	Gender, age, multiple tumor, DM, HBsAg, TACE sessions	140	12	1	KDIGO	11 weeks	4
Huo et al. (15)	2004	Taiwan	Gender, age, multiple tumor, DM, HBsAg, TACE sessions, amount of contrast	235	56	25	KDIGO	Long term	27
Park et al. (16)	2008	Korea	Gender, age, multiple tumor, DM, HBsAg, TACE sessions, amount of contrast, NSAID	236	24	Short-term[a]: 1 Long-term[b]: 19	AKIN	Short-term Long-term	Short-term 6 Long-term 4
Hsu et al. (17)	2009	Taiwan	Gender, multiple tumor, DM, HBsAg	87	11	Short-term: 2 Long-term: 9	KDIGO	Short-term Long-term	Short-term: 4
Cho et al. (11)	2011	South Korea	N/A	91	18	5	Scr >25% within 2–4 days	In-hospital	N/A
Hayakawa et al. (9)	2014	Japan	N/A	115	8	1	Scr >25% within 2–3 days	N/A	N/A
Lee et al. (18)	2017	Taiwan	NSAID	1,132	72	N/A	N/A	N/A	N/A
Zhou et al. (19)	2018	China	Gender, age, DM, amount of contrast, NSAID	818	38	3	KDIGO	1 month	4
Lin et al. (8)	2019	Taiwan	Gender, multiple tumor, DM, HBsAg, TACE sessions	96	17	1	KDIGO	1 month	N/A
Sohn et al. (20)	2020	South Korea	N/A	347	37	N/A	ICA-AKI	Short-term	N/A
Si et al. (21)	2021	China	Gender	284	28	N/A	Scr >25% within 2–3 days	4 days	N/A

HCC, hepatocellular carcinoma; AKI, acute kidney injury; RF, renal failure; DM, diabetes mellitus; TACE, transarterial chemoembolization; NSAID, non-steroidal anti-inflammatory drug; N/A, not applicable; AKIN, Acute Kidney Injury Network; KDIGO, Kidney Disease Improving Global Outcomes; ICA, International Club of Ascites.

[a]*Short-term: the results were observed within 3 months.*

[b]*Long-term: the results were observed after 1 year.*

TABLE 2 | Characteristics of the studies about hepatectomy.

Study	Year	Region	Risk factors for AKI	No. of HCC patients	No. of AKI patients	Death with RF	AKI definitions	Observation period	Irreversible RF (inclusive of death with RF)
Tsai et al. (22)	2014	Taiwan	DM, major resection	5,924	62	N/A	ICD-9-CM 584	N/A	N/A
Lim et al. (23)	2016	France	Gender, age, DM, cirrhosis, major resection, transfusion	457	67	Short-term[a]: 25 Long-term[b]: 46	KDIGO	Short-term Long-term	32
Ishikawa et al. (24)	2017	Japan	Gender, age, DM, cirrhosis, major resection, transfusion	228	27	N/A	AKIN	3 years	N/A
Moon et al. (25)	2017	Korea	Gender, DM, transfusion	1,173	77	N/A	AKIN	1 year	42
Bressan et al. (26)	2018	Canada	Gender, DM, cirrhosis, major resection	80	16	2	AKIN	1 month	N/A
Xu et al. (27)	2018	China	Gender, age, DM, cirrhosis, major resection	422	48	N/A	KDIGO	3 months	N/A

HCC, hepatocellular carcinoma; AKI, acute kidney injury; RF, renal failure; DM, diabetes mellitus; N/A, not applicable; HRS, hepatorenal syndrome; KDIGO, Kidney Disease Improving Global Outcomes; AKIN, Acute Kidney Injury Network.

[a]*Short-term: the results were observed within 3 months.*

[b]*Long-term: the results were observed after 1 year.*

TABLE 3 | Newcastle–Ottawa Scale for assessing the quality of cohort studies.

Study	Representativeness of the exposed cohort	Selection Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration of the outcome of interest being not present at the start of study	Comparability Comparability of cohorts on the basis of the design or analysis	Outcomes Assessment of outcome	Was follow-up long enough for out-comes to occur?	Adequacy of follow-up of cohorts	Score
Huo et al., 2004	★	★	★		★	★	★		6
Huo et al., 2004	★	★	★		★	★	★		6
Park et al., 2008	★	★	★		★	★	★		6
Hsu et al., 2009	★	★	★		★		★		5
Cho et al., (2011)	★	★	★	★	★				6
Hayakawa et al., (2014)	★	★	★	★	★				7
Tsai et al., (2014)	★	★	★		★				4
Lim et al., (2016)	★	★	★	★	★	★	★		7
Lee et al., 2017	★	★	★		★				4
Moon et al., 2017	★	★	★	★	★				5
Ishikawa et al., 2017	★	★	★	★	★	★			6
Zhou et al., 2018	★	★	★		★	★			5
Bressan et al., 2018	★	★	★	★	★		★		6
Xu et al., 2018	★	★	★	★	★				5
Lin et al., 2019	★	★	★	★	★	★			6
Sohn et al., 2020	★	★	★			★	★		5
Si et al., 2021	★	★	★				★		4

The Newcastle-Ottawa Scale quality instrument is scored by awarding a point for each answer that is marked with a star below. Total points are 4 points for Selection, 2 points for Comparability, and 3 points for Outcomes.

recorded in six studies (three studies recorded the ages of 1,107 patients), with 3,236 of them undergoing DM simultaneously. Five studies reported on 1,564 patients who underwent major resection of the liver and 5,547 patients who had minor resection, while 226 patients who needed transfusion due to surgery were recorded in three studies. In this series analysis, patients with a history of end-stage renal disease were excluded.

Analysis of the Risk Factors for AKI in HCC Patients Receiving TACE Treatment

Overall, 249 patients with HCC developed AKI during TACE treatment. The incidence rate of AKI in these HCC patients was about 11.9% (95% CI = 8.3–15.5, $p < 0.001$, $I^2 = 87.9\%$, $\chi^2 p < 0.001$) (see **Figure 2A**). Subgroup analyses were performed according to the number of enrolled HCC patients to examine the original source of significant heterogeneity. We found that there was no significant heterogeneity observed in the <100 patient subgroup ($I^2 = 0$, $\chi^2 p = 0.39$); however, the significant heterogeneity was still high in the >100 patient subgroup ($I^2 = 89.5\%$, $\chi^2 p < 0.001$), which indicated that the significant heterogeneity may have come from the sample size.

Subsequently, we carried out a series meta-analysis to detect the risk factors for AKI. When male gender was taken as a risk factor for AKI, the results showed no significant difference (pooled RR = 1.22, 95% CI = 0.72–2.06, $p = 0.47$) (**Figure 2B**) and significant heterogeneity ($I^2 = 43.3\%$, $\chi^2 p = 0.1$), indicating that male gender is not a risk factor for developing AKI in patients with HCC receiving TACE.

Subsequent analysis indicated that multiple tumors (inclusive of diffuse tumor; pooled RR = 1.25, 95% CI = 0.90–1.72, $p = 0.187$, $I^2 = 0$, $\chi^2 p = 0.701$) (**Figure 2C**), positive HBsAg (pooled RR = 0.72, 95% CI = 0.47–1.11, $p = 0.14$, $I^2 = 34\%$, $\chi^2 p = 0.195$) (**Figure 2E**), the amount of contrast (WMD = −1.71, 95% CI = −5.99 to 2.56, $p = 0.43$, $I^2 = 9.96\%$, $\chi^2 p = 0.33$) (**Figure 2G**), and NSAID use (pooled RR = 0.89, 95% CI = 0.58–1.37, $p = 0.606$, $I^2 = 0$, $\chi^2 p = 0.58$) (**Figure 2H**) were also not risk factors for AKI. Significant heterogeneity was not observed.

On the other hand, when the meta-analysis was conducted taking DM as a risk factor, the results showed that the risk of AKI in patients with DM was 1.69 times higher than in those without DM (pooled RR = 1.69, 95% CI = 1.24–2.3, $p = 0.001$) (**Figure 2D**), but no significant heterogeneity was observed ($I^2 = 0$, $\chi^2 p = 0.692$).

In addition, having more TACE sessions (pooled WMD = 0.63, 95% CI = 0.20–1.06, $p = 0.004$, $I^2 = 0$, $\chi^2 p = 0.499$) (**Figure 2F**) or older age (pooled WMD = 2.03, 95% CI = 0.12–3.94 years, $p = 0.04$, $I^2 = 0$, $\chi^2 p = 0$) (**Figure 2I**) would contribute to developing AKI more easily. These results demonstrated that age, DM, and the number of TACE sessions may act as risk factors for AKI.

3.3 Dangers of AKI in HCC Patients Receiving TACE Treatment

In total, 14 died out of 128 HCC patients with AKI in the short-term observation (within 3 months). The mortality rate of AKI in HCC patients receiving TACE was about 10.0% (95% CI = 4–16, $p < 0.001$, $I^2 = 0.49\%$, $\chi^2 p = 0.42$) (see **Figure 3A**) during this period.

Although there was no difference in the mortality risk with AKI and without AKI (pooled RR = 1.17, 95% CI = 0.95–1.43, $p = 0.13$,

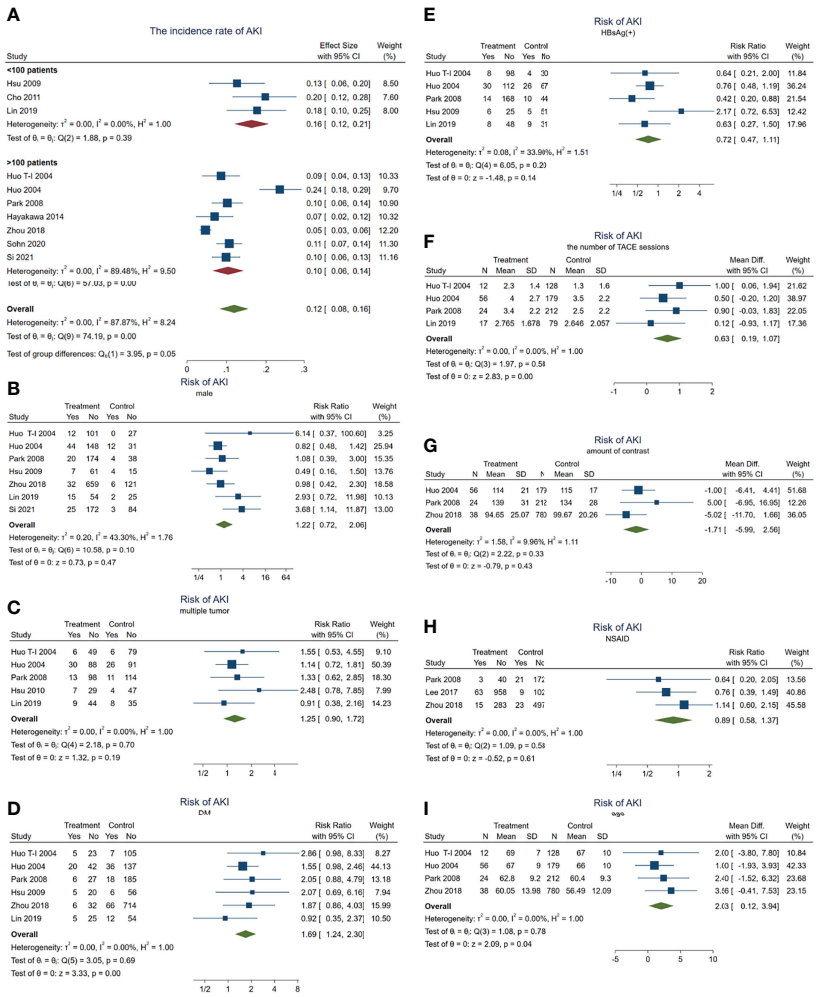

FIGURE 2 | Forest plots of the included studies assessing the risk factors for AKI in patients with HCC who received TACE treatment. The *solid vertical line* indicates no effect. The *horizontal lines* represent the 95% confidence intervals (CIs). **(A)** Incidence rates of AKI in these patients. **(B–I)** Supposing male gender **(B)**, multiple tumor **(C)**, DM **(D)**, HBsAg(+) **(E)**, the number of TACE sessions **(F)**, amount of contrast **(G)**, NSAID use **(H)**, and age **(I)** as risk factors. AKI, acute kidney injury; HCC, hepatocellular carcinoma; TACE, transarterial chemoembolization; DM, diabetes mellitus; NSAID, nonsteroidal anti-inflammatory drug.

I^2= 41.29%, $\chi^2 p$ = 0.16) (**Figure 3D**) after long-term observation (after 1 year), the mortality risk with AKI reached up to 4.74 times higher than in those without AKI in the short-term period (pooled RR = 4.74, 95% CI = 1.44–15.58, p = 0.01, I^2 = 47.38%, $\chi^2 p$ = 0.13) (**Figure 3B**). In addition, 18 HCC patients with AKI progressed to irreversible kidney injury during the short-term period, with the irreversible rate of AKI being about 22% (95% CI = 4–16, p < 0.001, I^2 = 0.49%, $\chi^2 p$ = 0.42) (see **Figure 3C**). These results indicated that TACE-related AKI is not only a dangerous signal related to death but also presents a high possibility of progressing to CKD in these patients within a short period.

Analysis of the Risk Factors for AKI in HCC Patients After Hepatectomy

In total, 235 patients with HCC progressed to AKI after hepatectomy. The incidence rate of AKI in these HCC patients was about 12% (95% CI = 8–16, p = 0.04, I^2 = 87.94%, $\chi^2 p$ < 0.001) (see **Figure 4A**). Subgroup analyses were performed according to the different ethnicities to examine the original source of the significant heterogeneity. We observed no significant heterogeneity in the non-Asian subgroup (I^2 = 20.24%, $\chi^2 p$ = 0.26); however, the significant heterogeneity was still high in the Asian subgroup (I^2 = 83.1%, $\chi^2 p$ < 0.001), indicating that the significant heterogeneity may have come from the ethnicity difference.

Subsequently, a series meta-analysis was also carried out to examine the risk factors for AKI in these patients. Men presented 1.83 times higher risk than women (pooled RR = 1.83, 95% CI = 1.23–2.74, p < 0.001, I^2 = 0, $\chi^2 p$ = 0.8) (**Figure 4B**), having DM was 1.64 times higher than that without DM (pooled RR = 1.64, 95% CI = 1.24–2.16, p < 0.001, I^2 = 9.9%, $\chi^2 p$ = 0.35) (**Figure 4C**), after major resection of the liver showed 2.43 times higher risk

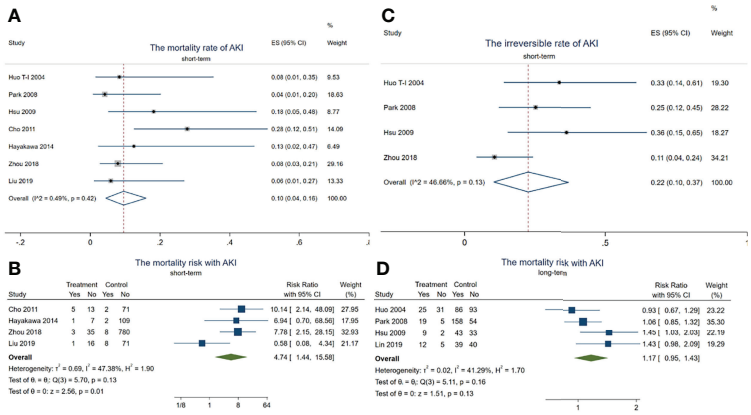

FIGURE 3 | Forest plots of the included studies assessing the risk of acute kidney injury (AKI) in patients with hepatocellular carcinoma (HCC) who received transarterial chemoembolization (TACE) treatment. The *solid vertical line* indicates no effect. The *horizontal lines* represent the 95% confidence intervals (CIs). **(A)** Mortality rates of AKI in these patients. **(B)** Mortality risk with AKI during the short term. **(C)** Irreversible rates of AKI in these patients. **(D)** Mortality risk with AKI after long-term observation.

than after minor resection (pooled RR = 2.43, 95% CI = 1.82–3.23, $p < 0.001$, $I^2 = 16.71\%$, $\chi^2 p = 0.31$) (**Figure 4D**), and having received transfusion during hepatectomy had 2.34 times higher risk than without a need for transfusion (pooled RR = 2.34, 95% CI = 1.71–3.22, $p < 0.001$, $I^2 = 0$, $\chi^2 p = 0.78$) (**Figure 4E**). Finally, older patients would also develop AKI (pooled WMD = 3.81, 95% CI = 1.37–6.26, $p = 0$, $I^2 = 0$, $\chi^2 p = 0.39$) (**Figure 2F**) more frequently.

In a word, male gender, age, DM, major resection, and transfusion may act as risk factors for AKI during hepatectomy for HCC.

Due to the limited data available, the risk of AKI in patients with HCC after hepatectomy was not analyzed.

4 DISCUSSION

The major findings of these published data based on the meta-analyses were as follows: firstly, DM is a risk factor for AKI in patients with HCC either receiving TACE or hepatectomy, which means that close attention should be paid to HCC patients with DM for risk of AKI during these treatments. Secondly, the number of TACE sessions is another risk factor for AKI in patients with HCC receiving TACE treatment. Moreover, male gender, major resection of the liver, and transfusion due to hepatectomy are other risk factors for AKI in HCC patients after hepatectomy. Lastly, the incidence of AKI during TACE treatment is especially dangerous: the risk of mortality with AKI was up to 4.74 times higher than in those without AKI in the short-term period.

A lot of advanced HCC patients require locoregional treatment due to inadequate hepatic reserve, liver-confined disease, being inoperable by performance status, comorbidity, or having uncertain extrahepatic diseases (3). The efficiency and safety of the TACE procedure have been improved for several decades; it is also considered the main treatment option for patients who had four or more HCCs and with liver function assessed as Child–Pugh class A or B (28). Previously recognized as CIN, TACE treatment of patients with HCC is the third leading cause of hospital-acquired AKI, which contributes to prolonged hospital stay and readmission rates (11). The specific reasons, mechanisms, and the influence of AKI in these patients are still unclear. HCC often develops from chronic liver disease that has already progressed to advanced cirrhosis, which may contribute to the development process of AKI. Abnormal systemic hemodynamics, splanchnic arterial vasodilatation, and extrahepatic vasoconstriction are possibly involved in cirrhosis-related AKI (29). The application of iodinated radiocontrast agents potentially is an acute event further exaggerating the already disturbed hemodynamics and/or renal vasoconstriction in advanced cirrhosis, finally leading to renal dysfunction. In addition, nephrotoxic drugs such as iodinated radiocontrast agents, adriamycin, and lipiodol can lead to renal microcirculatory dysfunction, cell apoptosis, or endothelial injury independently (6). On the other hand, renal endothelial cells would be impaired even at quite early exposure to a hyperglycemic milieu, whereas prolonged hyperglycemia would promote the mesenchymal transition and fibrosis of endothelial cells (30), resulting not only in endothelial dysfunction and aggravating kidney fibrosis but also in being vulnerable to nephrotoxicity by radiocontrast agents. Once DM is a comorbidity, the nephrotoxicity of radiocontrast agents will obviously be strengthened due to either transient or persistent hyperglycemic conditions. These theories were further demonstrated in our research, where the incidence of AKI during TACE treatment is not radiocontrast agent dose-dependent, and a small dose is strong enough to cause AKI in HCC-DM patients. The age-related susceptibility of AKI in older individuals has been reported both in TACE and hepatectomy,

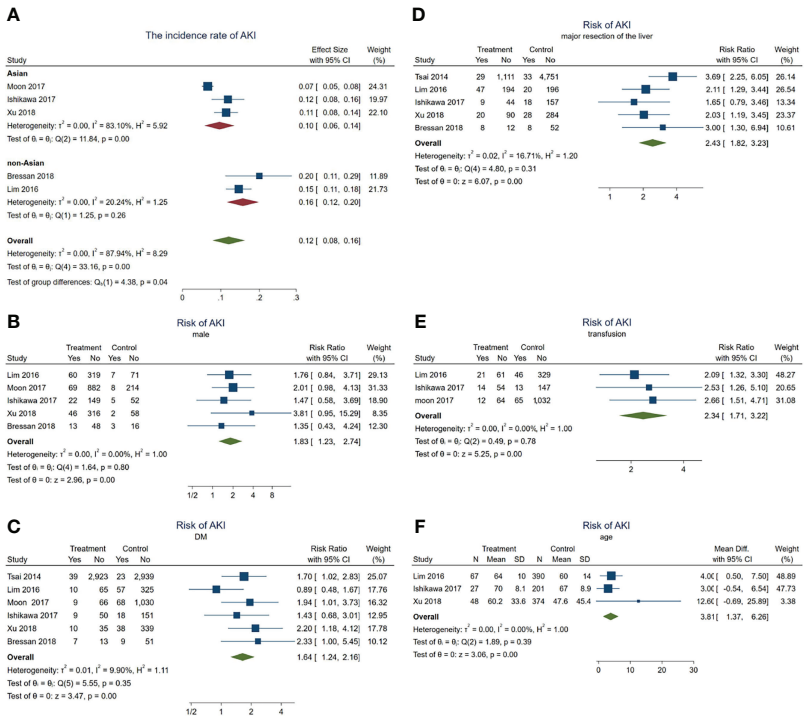

FIGURE 4 | Forest plots of the included studies assessing the risk factors for acute kidney injury (AKI) in patients with hepatocellular carcinoma (HCC) after hepatectomy. The *solid vertical line* indicates no effect. The *horizontal lines* represent the 95% confidence intervals (CIs). **(A)** Incidence rates of AKI in these patients. **(B–F)** Supposing male gender **(B)**, diabetes mellitus (DM) **(C)**, major resection of the liver **(D)**, receiving transfusion **(E)**, and age **(F)** as risk factors.

and the reason may be that older age enhances renal vulnerability as well (9, 26).

The hemodynamic alterations following liver resection are similar to advanced cirrhosis (31). In addition, AKI in patients with HCC ensues from hepatectomy probably more related to transient/prolong renal hypoperfusion or ischemia, while major resection of the liver or preoperative/postoperative transfusion could significantly aggravate this course due to the persistence of extensive blood loss and reduction of oxygen delivery. Firstly, ischemia would induce a significant functional impairment or structural damage of small renal tubular and vascular malfunction (30), which serves as the initiation of systemic inflammatory response activation and leads to renal inflammation injury and microcirculation dysfunction (32). In addition, microvascular damage could obviously affect endothelial cell expansion, apoptosis, or necrosis, in turn leading to microvascular obstruction, further inhibiting post-ischemic reperfusion and delaying kidney regeneration. Furthermore, ischemia would diminish the total surface intrarenal vascular area, along with endothelial–mesenchymal transition, together leading to the loss of important intrinsic physiological defense mechanisms and finally increasing the vulnerability of nephrons to oxygen-free radicals (30, 32). Therefore, even a minor or a laparoscopic liver resection should not be considered a less harmless operation and the prevention of intraoperative hemorrhage should also be paid the same attention, and vice versa.

Different from the hypothesis of ischemia–reperfusion injury, investigation of the association between diabetes-induced endothelial dysfunction and ischemia leading to the vulnerability of the kidney is rare. However, this relationship has been found based on several animal research works: a diabetic mouse model showed a higher vulnerability to ischemia than did non-diabetic controls, and ischemia was even induced quite early (33). On the other hand, non-diabetic rats completely recovered from functional impairment and tissue damage caused by renal ischemia, while diabetic rats failed within about 2 months observation (34). Tumor protein 53 (*TP53*) is the most frequently mutated tumor-suppressor gene in HCC. Inactivating mutations of *TP53* possibly present in 20% of HCCs in western countries, while they present in >50% of HCCs in aflatoxin B1 (AFB1)-exposed regions (35–38). Peng and colleagues demonstrated that P53 played a protective role against AKI in diabetic animal models, either in diabetic mice inducing P53-specific siRNAs or in proximal tubule-specific P53-knockout mice inducing diabetes (33). This may be one explanation for the different incidence rates of AKI consistently observed between the different ethnicities in this study.

Despite ischemia–reperfusion injury or the hemodynamic instability of renal perfusion, transfusion of red blood cells

may be an independent risk factor for postoperative AKI: impaired oxygen unloading of hemoglobin due to 2,3diphosphoglycerate deficiency, less deformability of stored red blood cells leading to the obstruction of smaller capillaries, increase in circulating free iron from stored red blood cell hemolysis, release of procoagulant phospholipids, and the accumulation of pro-inflammatory phospholipids together exaggerate the existing inflammatory response and lead to sepsis-associated AKI (39–41). Hepatorenal syndrome (HRS) describes a reversible AKI in patients with advanced hepatic failure, including advanced cirrhosis. Its varied performance depends on the volume and quality of the remnant liver after hepatectomy (steatosis/cirrhosis). Hepatic microcirculation is already impaired by steatosis or cirrhosis, and the liver presents more mitochondrial dysfunction and is less resistant to ischemia–reperfusion injury. TACE or hepatectomy intervention is a probable acute incident prompting the sudden decrease in the glomerular filtration rate (GFR) and renal perfusion. The potential pathophysiological mechanisms comprise significant splanchnic vasodilation and elevated abdominal pressure accompanied by ascites, causing overactivity of the renin–angiotensin–aldosterone system (RAAS) and sympathetic nervous system (SNS), followed by vasoconstriction/structural damage of the kidney and intravascular hypovolemia, accompanied by necrosis/apoptosis of tubular cells, which would drop off and obstruct the lumen, together causing complete deterioration of the GFR (31–41).

The study has several limitations. Firstly, analyses of the influence and the potential for publication bias could not be effectively performed due to the limited number of original studies (<10) for every meta-analysis. Secondly, statistical heterogeneity was always observed in the meta-analysis. One potential origin of the heterogeneity may be the ethnicity. When a subgroup analysis was performed according to the different ethnicities, the results showed no heterogeneity in the incidence rates of AKI in the non-Asian subgroup, but the statistical heterogeneity existing in the Asian group needs further exploration. As previously mentioned, P53 plays important roles both in HCC and AKI during DM; future studies could probably focus on HCC-AKI in diverse ethnicities. The accurate moment of earlier diagnosis of AKI by any definition is indeed difficult to establish in these patients due to the varied efficacy–efficiency balance of biomarker measurements, which is one of the reasons the International Club of Ascites (ICA) spent several years developing the new expert consensus on the diagnosis and treatment of AKI in patients with liver cirrhosis. This contention may be another source of the heterogeneity. Finally, since the clinical data are from publications and have limitations in terms of availability, not only could further sub-analyses not be performed (TNM stage, duration of hepatectomy, and tumor size, among others), but AKI in patients with HCC receiving other treatments (radiofrequency, microwave ablation, or systemic therapy) could also not be analyzed. Incidentally, further studies are still needed to support the conclusions and demonstrate more associations of AKI in HCC patients.

In conclusion, age, DM, and the number of TACE sessions are risk factors for AKI in patients with HCC receiving TACE, while age, male gender, DM, major resection of the liver, and operation-related transfusion are risk factors for AKI in patients with HCC after hepatectomy. Finally, the occurrence of AKI during TACE treatment is especially dangerous and should be considered a strong red flag, obviously with regard to the extremely high risk of death in a short period. Furthermore, studies are needed to detect more associations of AKI in patients with HCC (especially in patients receiving other treatments).

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

CL had the idea for the study and formulated its design, having had full access to all data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis. ZM and TG contributed to data acquisition and the writing of the report. ZM contributed to critical revisions of the report and to the statistical analysis. All authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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