Investigating the roles of microRNAs associated with cancer stem cells, drug resistance, metastasis and recurrence in hepatocellular carcinoma: A systematic review, network analysis and experimental verification

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Abstract. Cancer stem cells (CSCs) are responsible for drug therapy resistance, recurrence and metastasis in hepatocellular carcinoma (HCC). Certain microRNAs (miRNAs/miRs) are involved in various pathological cancer pathways via their binding interactions with target mRNAs. The objectives of the present study were to explore the potential microRNAs associated with HCC-CSCs, and to investigate their roles in recurrence, metastasis and drug resistance. Initially, a registered systematic review (CRD42024508526; International Prospective Register of Systematic Reviews) identified a group of miRNAs associated with HCC prognosis, and the Coremine Medical data mining tool identified another group of potential miRNAs associated with HCC-CSCs, recurrence, metastasis and drug resistance. Secondly, potential miRNAs that were associated with HCC prognosis, CSCs, recurrence, metastasis and drug resistance were detected by comparing the two groups of miRNAs. Subsequently, the expression levels of a potential miRNA in HCC tissues and its prognostic predictive power were evaluated using the Encyclopedia of RNA Interactomes and Kaplan-Meier plotter online tools. In addition, the expression levels of the target miRNA in Huh7-CSCs and Huh7 cells were measured using reverse transcription-quantitative PCR (RT-qPCR). Finally, the potential biological processes of the miRNA target genes were examined through bioinformatics analysis using the Enrichr database. Briefly, hsa-miR-17-5p was predicted to be associated with stemness, metastasis, recurrence and drug resistance in HCC. Bioinformatics analysis demonstrated that miR-17-5p expression was higher in HCC tissues compared with that in para-tumor tissues and that patients with low miR-17-5p expression demonstrated higher overall survival rates (months). The RT-qPCR results indicated that miR-17-5p expression in Huh7-CSCs was significantly higher compared with that in Huh7 cells. Further bioinformatics analysis suggested that miR-17-5p maintains stemness by targeting hypoxia-inducible factor-1α (HIF1A) and Myc. Additionally, the target genes of miR-17-5p were revealed to be involved in cell fate and metabolic reprogramming pathways. In conclusion, miR-17-5p may be a potential miRNA associated with CSCs, metastasis, recurrence and drug resistance in HCC via cell fate and metabolic reprogramming pathways. miR-17-5p exhibited higher expression in HCC-CSCs compared with that in HCC cell lines, and may target HIF1A and Myc to maintain HCC-CSCs stemness.

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Introduction

Primary liver cancer (PLC) is the sixth most common malignant tumor worldwide (1), and primarily includes hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma. In China, PLC had the second highest mortality rate among all cancers in 2020 (2). HCC is the most prevalent

type of PLC, accounting for ~90% of PLC cases (3). The most common causes of HCC include viral hepatitis, metabolic disorders, alcohol consumption and smoking (4).

Surgical resection is the standard treatment for patients with early stage PLC. Recurrence occurs in ~50% of patients who undergo surgical resection within 2 years and the 5-year survival rate ranges between 50 and 75% (5). Patients with HCC frequently lack noticeable clinical symptoms, resulting in delayed medical attention, and missed opportunities for surgical resection or liver transplantation. Systematic therapies, including immunotherapy, targeted therapy, local radiotherapy and chemotherapy, are often used in patients with intermediate-to advanced-stage disease (6,7). However, drug resistance is the primary cause of treatment failure and can lead to tumor recurrence or metastasis (8). Once recurrence and metastasis occur, the prognosis of patients with HCC decreases. Therefore, high recurrence and metastasis rates due to therapeutic resistance remain major clinical issues.

Cancer stem cells (CSCs) are a rare subpopulation of tumor cells capable of self-renewal and differentiation. CSCs are resistant to chemotherapy and radiotherapy, and are associated with tumorigenesis, recurrence and metastasis (9-11). CSCs exhibit characteristics that are distinct from those of other cancer cells and several surface markers have been identified in previous studies, including CD44, CD133, epithelial cell adhesion molecule (EpCAM) and hepatic leukemia factor (12-15).

MicroRNAs (miRNAs/miRs) are short endogenous non-coding RNAs that regulate gene expression by binding to target mRNAs (16). Previous studies have reported that miRNAs serve key roles in tumor cell proliferation, migration and invasion (17,18). However, potential miRNAs associated with HCC-CSCs and their roles in tumor therapy resistance, recurrence and metastasis remain to be elucidated.

The present study aimed to identify potential miRNAs associated with HCC-CSCs through a systematic review, data mining and bioinformatics analysis. Subsequently, the expression of candidate miRNAs and their biological functions were explored in HCC-CSCs.

Materials and methods

Systematic review. The present study was reported as per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses protocol (19) and registered in the International Prospective Register of Systematic Reviews (CRD42024508526).

Search strategy. All of the research articles used in the present study were gathered from two publicly available literature databases, PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Web of Science (https://www.webofscience.com/wos/), with a search range from the date of establishment of the database to January 15, 2024. A search strategy was collaboratively developed and independently executed by two authors based on the Population, Intervention, Comparison, Outcome and Study type (PICOS) (20). The formulated PICOS framework was as follows: i) The population was composed of patients with HCC; ii) the intervention was the altered expression levels of miRNAs in patients; iii) the comparison was between groups, one with high miRNA expression and the other with low miRNA expression; iv) the outcome was the difference in survival prognosis between patients with HCC with low and high miRNA expression; and v) the study method was comparative research. The key words and search details are shown in Table SI.

Eligibility criteria. Research articles were included based on the following criteria: i) Demonstrated significantly different miRNA expression levels in patients with HCC ($P<0.05$); ii) compared the prognoses of high and low miRNA expression groups; and iii) described a clear follow-up time for patients. Studies were excluded based on the following criteria: i) Did not use human subjects; ii) did not perform a comparative analysis; iii) did not include HCC; iv) did not have available data that could be extracted; or v) were not original research projects.

Study selection and data extraction. After excluding review articles and eliminating duplicate items, the literature search results were imported into Rayyan (https://www.rayyan.ai/), an online tool for systematic literature reviews. Independent screening of titles and abstracts was conducted using inclusion and exclusion criteria. The miRNAs mentioned in the target articles were extracted and arranged in tables that included the first author, year, country, number of patients, follow-up period, type of evidence, miRNA and expression.

Data mining analysis. Coremine Medical (http://www.coremine.com/medical/), an online data-mining tool, was used to explore potential miRNAs associated with CSCs, metastasis, recurrence and drug resistance in HCC. Key words including 'liver carcinoma', 'neoplasm recurrence', 'neoplasm metastasis', 'drug resistance' and 'neoplastic stem cells' were chosen as the search strategy, and the associated genes were downloaded and screened for miRNAs with a significant difference ($P<0.05$), based on the analysis role of the web tool.

Bioinformatics analysis. The ONCO.IO website (https://onco.io/) was used to explore miRNA-target gene interactions and their biological processes in HCC. A regulatory network diagram was created to illustrate these interactions. The Enrichr database (https://maayanlab.cloud/Enrichr/) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Box plots on the Encyclopedia of RNA Interactomes (ENCORI) website (https://rnasysu.com/encori/) indicated the differential expression levels of miRNAs between cancerous and paracancerous tissue samples in liver HCC (LIHC). Kaplan-Meier survival curves (https://www.kmplot.com) were used to assess the effects of different miRNA expression levels on the survival of patients with LIHC.

Cell culture and CSC culture. The Huh7 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and confirmed by short tandem repeat profiling without discrepancies. The cells were cultured in DMEM (cat. no. 11965118; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS [cat. no. E01010; Eva (Suzhou) Biopharmaceutical Technology Co., Ltd.] and 1%

penicillin-streptomycin (cat. no. C100C5; Suzhou Xinsaimei Biotechnology Co., Ltd.). Cell culture was performed at 37˚C in a 5% CO_2 incubator. HCC-CSCs were developed from Huh7 cells using the protocol as described previously (21). The Huh7 cell line was cultured under ultra-low attachment conditions with DMEM supplemented with 1% penicillin-streptomycin, 0.5% N2 supplement (cat. no. 17502-048; Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF; cat. no. HY-P7004; MedChemExpress) and 20 ng/ml epidermal growth factor (EGF; cat. no. HY-P7109; MedChemExpress) at 37˚C in a 5% CO_2 incubator for 5-7 days.

Tumor sphere assays and sphere formation efficiency (SFE). A total of 2,000 cells in the logarithmic growth phase were selected, seeded into 24-well ultra-low adsorption culture plates and cultured in DMEM supplemented with 1% penicillin-streptomycin, 0.5% N2 supplement, 20 ng/ml bFGF, and 20 ng/ml EGF at 37˚C in a 5% CO_2 cell culture incubator for 6 days. Spherical cells were defined as those with diameters ≥100 μm. Imaging was performed using a Motic AE31E inverted microscope (Motic Industrial Group Co., Ltd.). SFE was calculated on day 6 using the following formula: SFE=(number of spheres counted/number of seeded cells) x100.

miRNA extraction and reverse transcription-quantitative PCR (RT-qPCR). miRNAs were extracted and purified from Huh7 and Huh7-CSCs using the miRcute miRNA Isolation Kit (cat. no. DP501; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. The concentration and purity of the miRNAs were measured using a NanoDrop 2000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). The RT-qPCR detection of miRNA was performed using the poly(A) tailing-based method. RT was performed using the miRNA 1st Strand cDNA Synthesis Kit (Tailing A; cat. no. MR201-01; Vazyme Biotech Co., Ltd.). The reverse transcription for miRNA was carried out at 37˚C for 60 min, followed by an enzyme inactivation step at 85˚C for 5 sec. qPCR was performed using a Tap Pro Universal SYBR™ qPCR Master Mix kit (cat. no. Q712-02; Vazyme Biotech Co., Ltd.). The qPCR protocol consisted of an initial denaturation step (95˚C for 30 sec), followed by 40 amplification cycles (denaturation at 95˚C for 30 sec and annealing/extension at 60˚C for 30 sec), and concluded with a melt curve analysis step (95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec). U6 was used as the endogenous control and the $2^{-\Delta\Delta Cq}$ method was used to analyze expression levels (22). The primer sequences of the detected genes were as follows: Hsa-miR-17-5p-q forward (F), 5'-GCCGCAAAGTGCTTACAGTGC-3' and the reverse (R) primer for miR-17-5p was the Universal reverse Q primer from the miRNA 1st Strand cDNA Synthesis Kit; U6 F, 5'-CTCGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCA CGAATTTGCGT-3'.

Statistical analysis. Statistical analyses were performed using the Coremine, ONCO.IO, Enrichr, ENCORI and Kaplan-Meier plotter databases. The ENCORI pan-cancer dataset (version 2024), comprising 10,546 samples from The Cancer Genome Atlas (TCGA), was analyzed. The best-performing percentile was automatically selected as the cutoff. The miRNA expression levels were quantified as reads per million (RPM) and transformed using $\log_2$ (RPM + 0.01) to normalize the data distribution. The RT-qPCR data are presented as the mean ± Standard Error of the Mean and each experiment was performed in triplicate unless otherwise noted. Differences in the RT-qPCR results between the two groups were analyzed using unpaired Student's t-test. Unless specified otherwise, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Study characteristics. The initial literature search retrieved 7,535 research articles, with 2,710 from PubMed and 4,825 from Web of Science. After further screening, 2,396 duplicates were removed, and the titles and abstracts of the remaining 5,139 articles were reviewed. A total of 5,121 articles failed to meet the inclusion criteria, resulting in 18 articles selected for full reading and review. Nine additional articles were excluded because they did not meet the inclusion criteria and the remaining nine were included in the study (23-31). The screening process is illustrated in Fig. 1. These studies focused on nine different miRNAs collected from the serum and tissues of patients with HCC in China and South Korea. All nine miRNAs were reported to have notable prognostic capability for patients with HCC. The details of the nine studies are shown in Table I encompassing 1,318 patients with HCC collectively.

miR-17-5p: Potential miRNA associated with CSCs, drug resistance, recurrence and metastasis in HCC. A total of 34 miRNAs, including miR-7-3HG, miR-100, miR-25, miR-26B, miR-200A, miR-184, miR-190A, miR-21,miR-186, miR-19A, miR-17, miR-196B, miR-22, Let-7d, miR-145, miR-183, miR-34A,miR-17HG, miR-185, miR-107, Let-7c, miR-203A, miR-137, miR-96, miR-191, miR-98, miR-4435-2HG, Let-7b, miR-31, miR-93, miR-146A, miR-15B, miR-221, miR-192, associated with HCC, neoplasm recurrence, neoplasm metastasis, drug resistance and CSCs were identified in the Coremine database. The intersecting miRNA, miR-17-5p, was selected by comparing the nine miRNAs from the included studies with the 34 miRNAs identified in the Coremine database (Fig. 2A). The ENCORI tool demonstrated that miR-17-5p was expressed at significantly higher levels in 370 cancerous tissues compared with that in 50 para-tumor tissues (fold change, 2.08; Fig. 2B). Survival analysis of miR-17-5p, which included 163 patients who were followed-up for 48 months, was performed using the Kaplan-Meier plotter online tool. The results of this analysis revealed a significant association between miR-17-5p expression and overall survival (months) in patients with HCC (Fig. 2C).

The interaction network of the target genes regulated by miR-17-5p is shown in Fig. 2D. These 12 genes were directly identified through functional analysis (pathogenic processes) using the ONCO.IO platform, including two stemness-associated genes, seven drug resistance-associated genes and six metastasis-associated genes. The combined list yielded 12 unique genes. Huh7-CSCs were successfully developed from Huh7 cells. Spherical cells with a diameter ≥100 μm were observed after 6 days of culturing in ultra-low attachment conditions and the SFE of Huh7-CSCs was calculated

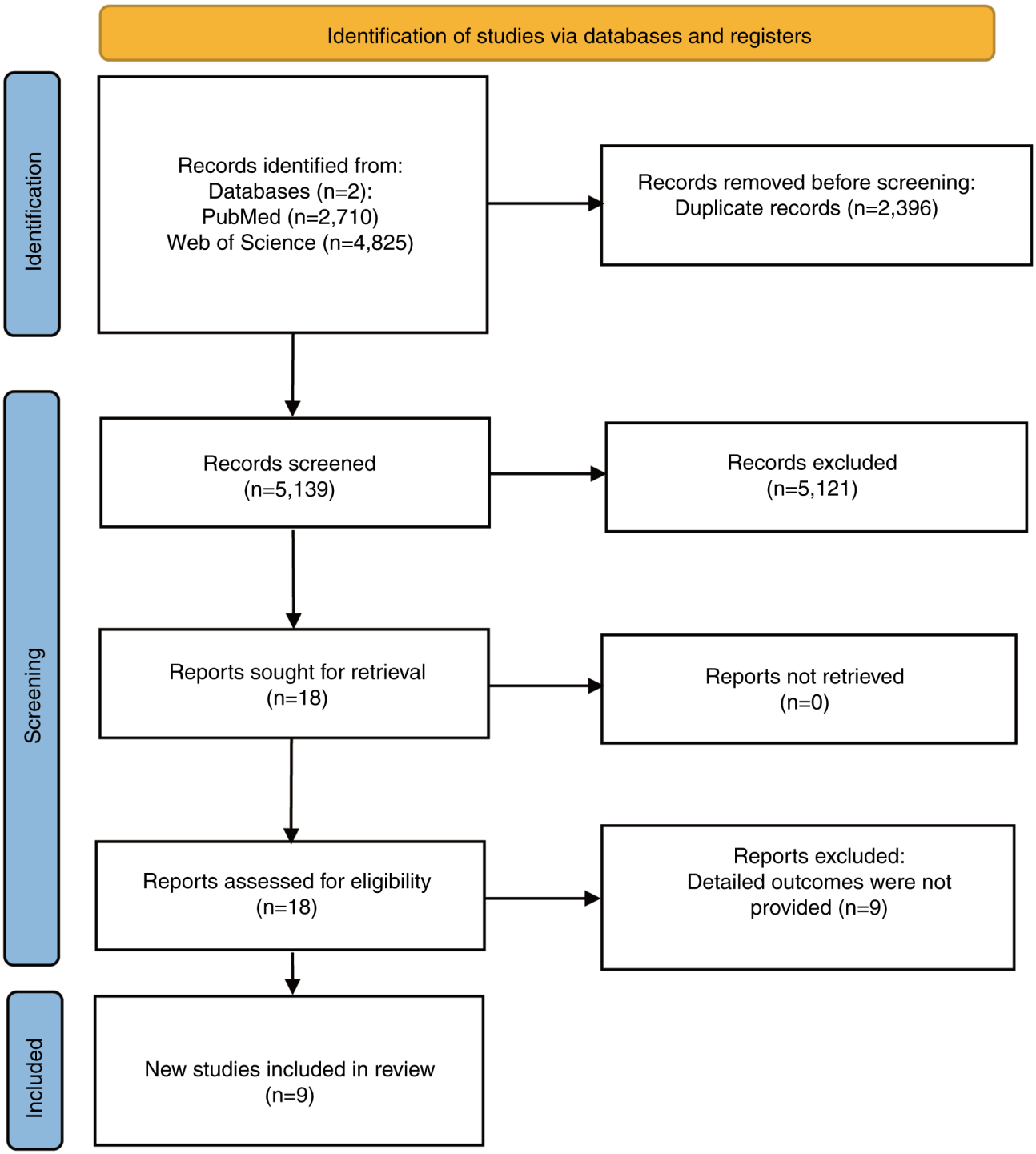

Figure 1. Flow diagram depicting the systematic review article selection process.

to be 4.7% (Fig. 3A). Subsequently, the expression levels of miR-17-5p in Huh7-CSCs were revealed to be significantly higher compared with those in Huh7 cells (Fig. 3B). The primary biological processes regulated by miR-17-5p and its target genes, including proliferation, invasion, migration, metastasis, stemness and drug resistance, were explored using the ONCO.IO database (Table II).

The Enrichr tool was used to explore the potential pathways of the 12 miR-17-5p target genes involved in metastasis, stemness and drug resistance. These 20 pathways are listed in Table III after excluding those associated with other diseases, including acute myeloid leukemia and thyroid cancer. These 20 pathways are primarily involved in the regulation of metabolism, cell fate, hepatitis viruses and the immune checkpoint system. Certain pathways, including central carbon metabolism and proteoglycans are closely associated with cell stemness.

Discussion

Previous studies have identified ~2,000 miRNAs in humans that regulate >60% of protein-coding genes and

Table I. Extracted data of the nine articles selected for the systematic review.

First author, year	Country	No. of patients (male, female, unknown)	Follow-up period	Type of evidence (95% CI)	miRNA	Expression	(Refs.)
Xie *et al*, 2018	China	149 (117, 30, 2)	150 months	OS: HR, 0.072 (0.033-0.159); P<0.001 PFS: HR, 0.194 (0.118-0.317); P<0.001	miR-33a	Downregulated (HCC tissues)	(23)
Zhuang *et al*, 2015	China	182 (155, 27)	656±393 days	OS: HR, 2.793 (1.550-5.033); P=0.001	miR-128-2	Upregulated (HCC serum)	(24)
Sun *et al*, 2015	China	60 (40, 20)	>24 months	DFS: HR, 2.681 (1.306-5.504); P=0.007	miR-9	Upregulated (HCC tissues)	(25)
Chen *et al*, 2012	China	66 (56, 10)	100 months	OS: HR, 0.332 (0.139-0.793); P=0.013 RFS: HR, 0.202 (0.064-0.638); P=0.006	miR-203	Downregulated (HCC tissues)	(26)
Zhou *et al*, 2016	China	38 (30, 8)	25 months	DFS: RR, 3.273 (1.107-9.679); P=0.032	miR-375	Downregulated (HCC tissues)	(27)
Luo *et al*, 2019	China	148 (100, 48)	24 months	OS: HR, 2.226 (1.235-4.012); P=0.008 RFS: 2.662 (1.618-4.38); P<0.001	miR-200c	Downregulated (HCC tissues)	(28)
Ha *et al*, 2019	South Korea	289 (238, 51)	151.4 months	IHRFS: HR, 1.89 (1.16-3.07); P=0.010 DMFS: HR, 2.14 (1.05-4.36); P=0.036 RFS: HR, 2.17 (1.34-3.52); P=0.002	miR-122	Downregulated (HCC tissues)	(29)
Zhu *et al*, 2012	China	266 (225, 41)	81 months	OS: HR, 1.0 (0.7-1.4); P=0.901 TTR: HR, 0.5 (0.3-0.8); P=0.003	miR-29a-5p	Upregulated (HCC tissues)	(30)
Chen *et al*, 2012	China	120 (102, 18)	46 months	OS: RR, 4.96 (1.78-13.82); P=0.002 DFS: RR, 1.79 (1.14-2.98); P=0.042	miR-17-5p	Upregulated (HCC tissues)	(31)

OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; DFS, disease-free survival; IHRFS, intrahepatic recurrence-free survival; DMFS, distant metastasis-free survival; TTR, time to tumor recurrence; HR, hazard ratio; RR, risk ratio; HCC, hepatocellular carcinoma; miR, microRNA; 95% CI, 95% confidence interval.

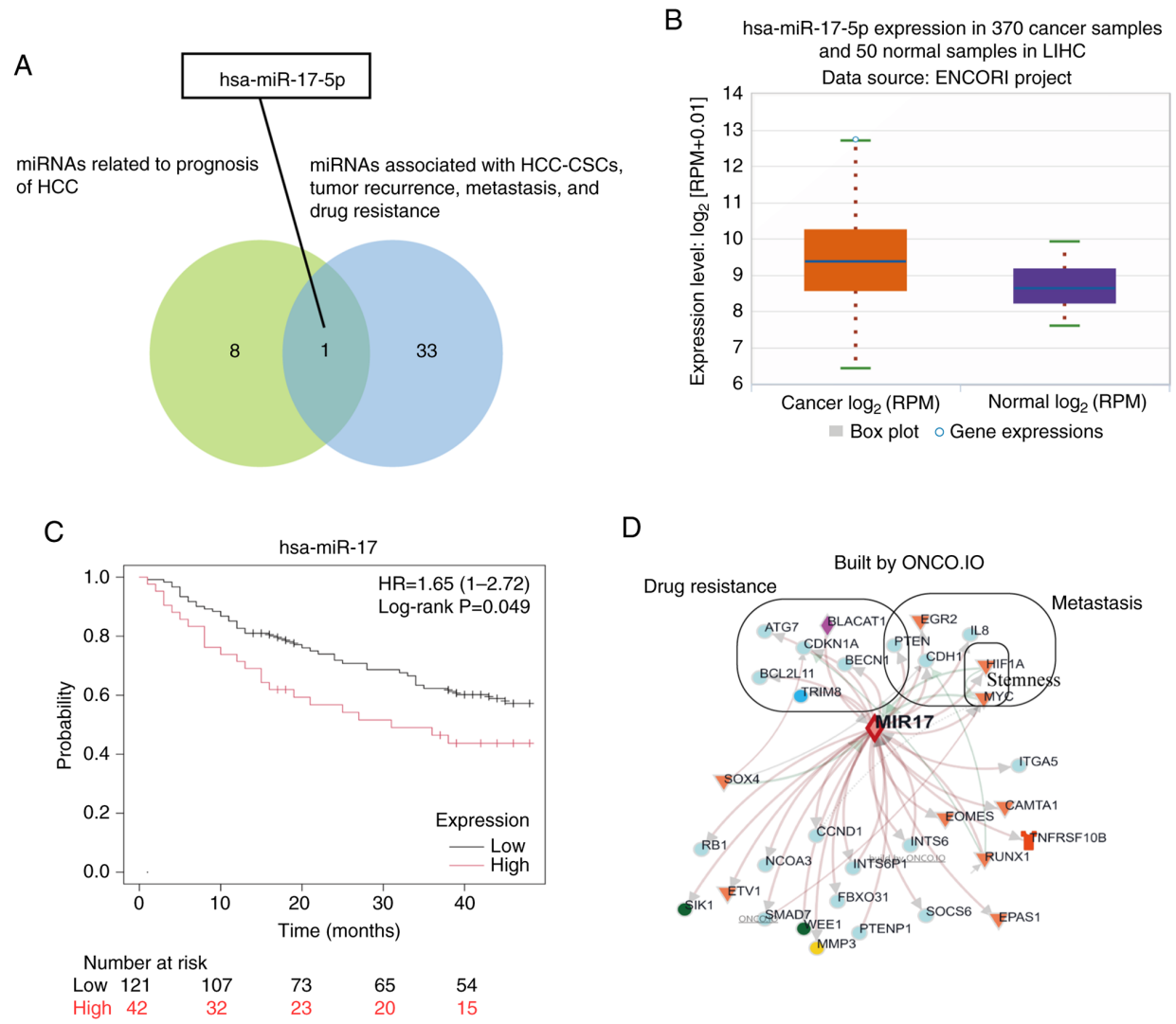

Figure 2. miR-17-5p is a miRNA potentially associated with CSCs, metastasis, recurrence and drug resistance in HCC. (A) Venn diagram illustrating how the potential miRNA (miR-17-5p) was selected by exploring the intersection between the nine miRNAs associated with the prognosis of HCC, and the 34 miRNAs associated with HCC-CSCs, metastasis, tumor recurrence and drug resistance. (B) Box plot of miR-17-5p expression in LIHC. (C) Survival analysis of miR-17-5p in LIHC. (D) Interaction network of targeted genes of miR-17-5p, with the 12 genes involved in metastasis, stemness and drug resistance specially marked. miR/miRNA, microRNA; CSC, cancer stem cell; HCC, hepatocellular carcinoma; LIHC, liver hepatocellular carcinoma; RPM, reads per million.

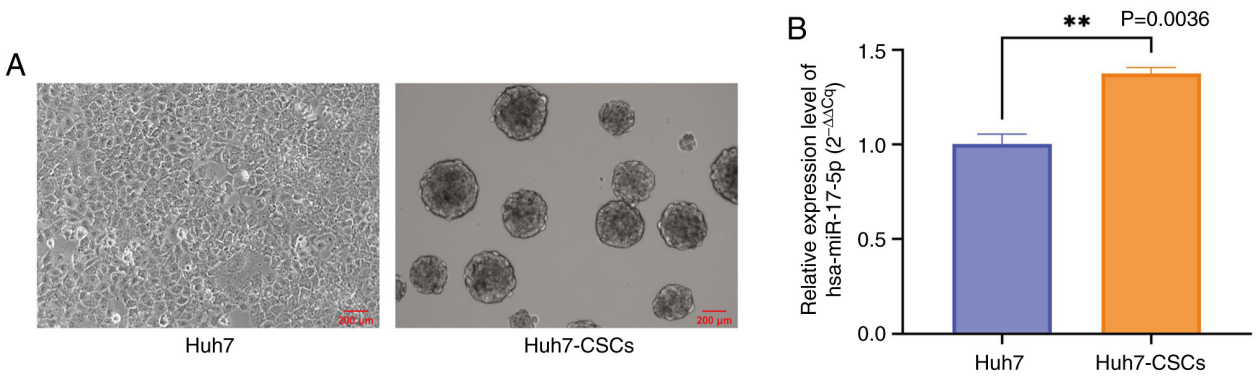

Figure 3. Morphology of HCC-CSCs and expression levels of miR-17-5p. (A) Huh7 cell line and Huh7-CSCs (magnification, x10). (B) Expression levels of miR-17-5p in Huh7 and Huh7-CSCs. **P<0.005. CSC, cancer stem cell; HCC, hepatocellular carcinoma; miR, microRNA.

perform post-transcriptional gene regulation by binding to target genes (32,33). Systematic reviews have been used as a high-evidence tool to detect potential miRNAs with diagnostic and/or prognostic potential in the study and treatment of cancer. For example, a meta-analysis of nine studies (involving 1,624 study participants, of which 957 were

Table II. Role of microRNA-17-5p targeted genes in biological processes of cancer.

Process	No. of genes	Targeted genes
Proliferation	17	RBL2, RB1, NCOA3, SOCS6, UBE2C, ETV1, SIK1, CDKN1A, RND3, Myc, SMAD7, STAT3, Sox4, CCND1, RELA, BLACAT1 and CDH1
Invasion	14	PTEN, EGR2, ITGA5, ITGB1, ETV1, IL8, PTENP1, CD274, Myc, Sox4, STAT3, HIF1A, BLACAT1 and RELA
Migration	10	RB1, PTEN, ETV1, SIK1, PTENP1, Myc, INTS6, INTS6P1, Sox4 and RUNX1
Metastasis	6	PTEN, EGR2, IL8, HIF1A, Myc and CDH1
Stemness	2	HIF1A and Myc
Drug resistance	7	BLACAT1, ATG7, CDKN1A, PTEN, BCL2L11, BECN1 and TRIM8

Table III. The 20 pathways of microRNA-17-5p-targeted genes associated with metastasis, stemness and drug resistance in HCC.

Term	P-value	Genes
Pathways in cancer	2.75×10^{-7}	CDKN1A, BCL2L11, CDH1, Myc, PTEN and HIF1A
Autophagy	9.99×10^{-7}	BECN1, PTEN, HIF1A and ATG7
Central carbon metabolism in cancer	8.83×10^{-6}	Myc, PTEN and HIF1A
miRNAs in cancer	2.54×10^{-5}	CDKN1A, BCL2L11, Myc and PTEN
PI3K-AKT signaling pathway	4.27×10^{-5}	CDKN1A, BCL2L11, Myc and PTEN
FOXO signaling pathway	5.79×10^{-5}	CDKN1A, BCL2L11 and PTEN
Cellular senescence	9.73×10^{-5}	CDKN1A, Myc and PTEN
Hepatitis B	1.09×10^{-4}	EGR2, CDKN1A and Myc
Hepatocellular carcinoma	1.21×10^{-4}	CDKN1A, Myc and PTEN
Proteoglycans in cancer	2.18×10^{-4}	CDKN1A, Myc and HIF1A
Mitophagy	7.35×10^{-4}	BECN1 and HIF1A
p53 signaling pathway	8.47×10^{-4}	CDKN1A and PTEN
ErbB signaling pathway	1.15×10^{-3}	CDKN1A and Myc
PD-L1 expression and PD-1 checkpoint pathway in cancer	1.26×10^{-3}	PTEN and HIF1A
HIF-1 signaling pathway	1.87×10^{-3}	CDKN1A and HIF1A
Cell cycle	2.42×10^{-3}	CDKN1A and Myc
Apelin signaling pathway	2.94×10^{-3}	BECN1 and CDH1
Apoptosis	3.15×10^{-3}	BECN1 and BCL2L11
Hepatitis C	3.84×10^{-3}	CDKN1A and Myc
JAK-STAT signaling pathway	4.08×10^{-3}	CDKN1A and Myc

patients with cervical cancer and 667 were healthy controls) revealed a marked upregulation of miR-21 expression in cervical cancer (34). In the present study, nine miRNAs were found to be associated with the prognosis of patients with HCC in a systematic review. In HCC tumor tissues, the expression levels of miR-33a, miR-203, miR-375 and miR-200c were markedly downregulated, whereas those of miR-9, miR-29a-5p and miR-17-5p were markedly upregulated. Due to the post-transcriptional regulation feature of miRNA, several miRNAs are abnormally expressed during the development of HCC and can affect patient prognosis. These miRNAs bind to multiple target genes and are involved in numerous biological processes in HCC. For example, miR-375, which is downregulated in HCC tissues, inhibits tumor angiogenesis by targeting platelet-derived growth factor C. Additionally, miR-375 has been shown to reduce sorafenib resistance by regulating astrocyte elevated gene-1and sirtuin (SIRT)5 (35,36). By contrast, miR-9 is not only upregulated in HCC tissues but also exhibits higher expression in patients with HCC with early vascular invasion. Mechanistically, miR-9 promotes HCC cell invasion and migration by targeting SIRT1, FOXO1 and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 2 (37). Data mining was combined with a systematic review to further understand the potential impact of these miRNAs on stemness, tumor metastasis, recurrence and drug resistance in HCC. miR-17-5p was specifically identified as a potential miRNA associated with these processes. A previous study reported that miR-17-5p expression is strongly associated with the number of tumor

nodules, pathological grading of HCC and venous infiltration (31). Other studies have demonstrated that miR-17-5p induces hepatocarcinogenesis, and promotes the proliferation and migration of HCC cells by targeting Smad3, Runt-related transcription factor 3 and PTEN (38-40). However, the role of miR-17-5p in HCC-CSCs remains to be elucidated.

CSCs are gradually gaining popularity in research because of their ability to drive tumor metastasis and recurrence (41-43). However, the mechanisms underlying stemness maintenance remain unclear. CSCs are a subpopulation of cancer cells that remain dormant and may contribute to drug resistance and the risk of recurrence (44,45). By contrast, most HCC cells exhibit rapid proliferation and are easily eliminated by DNA damage therapy. A previous study reported that miR-17-5p expression is often higher in cancerous tissues compared with that in paracancerous tissues (46). Notably, the expression levels of miR-17-5p were significantly higher in HCC-CSCs compared with those in HCC cells in the present study. This finding suggests that miR-17-5p expression in HCC-CSCs may differ from that in the majority of HCC cells and the high expression level of miR-17-5p could be a unique biological feature that maintains the stemness of HCC-CSCs.

To reveal the role of miR-17-5p in regulating stemness, the target genes of miR-17-5p and their biological processes were predicted using bioinformatics. Notably, miR-17-5p may be involved in regulating stemness by targeting hypoxia-inducible factor-1α (HIF1A) and Myc. Hypoxia is an essential feature of the tumor microenvironment in the majority of solid tumors, which contributes to tumor metabolism reprogramming and leads to the failure of antitumor therapy (47). According to a previous study, knocking down HIF-1α expression can inhibit the expression of stemness-related genes (Oct3/4, Nanog, BMI-1 and Notch1), as well as suppress self-renewal, migration and chemoresistance in liver cancer cells under hypoxic conditions (48). Myc is one of the most common oncogenes in human carcinogenesis, and acts as a bridge between stem and tumor cells. The Myc family consists of c-Myc, n-Myc and l-Myc, among which c-Myc serves a key role in HCC pathogenesis. A previous study showed that miR-17-5p regulates the expression of c-Myc to influence HCC development (49). The inactivation of Myc enables tumor cells to de-differentiate into normal liver cell lineages. However, upon its reactivation, these cells rapidly regain their malignant characteristics. These tumor cells with stem cell properties are CSCs. It has been demonstrated that in Myc-induced HCC, the inactivation and reactivation of Myc can engage certain cells with stem cell properties (50).

Myc and HIF1A, two stemness-maintaining genes, are closely associated with central carbon metabolism (CCM) and proteoglycans in tumors according to KEGG pathway analysis. CCM primarily involves glycolysis and tricarboxylic acid cycle. CCM reprogramming occurs in various tumors and serves as the primary source of cellular energy (51). The present study revealed that CCM is closely associated with CSCs. Under aerobic conditions, CSCs exhibit dual metabolic modes, utilizing glycolysis and oxidative phosphorylation (OXPHOS) as energy sources that collectively drive tumor progression, recurrence and drug resistance (52). Glycolysis is a key factor in maintaining the stemness of HCC-CSCs. The upregulation of glycolysis-associated genes in HCC-CSCs facilitates the expansion of CSC populations, augments drug resistance and accelerates tumor progression (53). For example, hepatitis B virus X induces Bcl-2/adenovirus E1B 19 kDa-interacting protein 3-like-dependent mitophagy, thereby upregulating glycolytic metabolism. This metabolic shift modulates CSCs stemness through elevated expression of cancer stemness-associated genes (such as ATP-binding cassette subfamily G member 2, octamer-binding transcription factor 4 and B-cell-specific Moloney murine leukemia virus integration site 1), ultimately promoting tumor growth (54). Furthermore, potassium calcium-activated channel subfamily N member 4 potentiates glycolysis in HCC-CSCs, upregulates stemness-associated transcription factors, expands the HCC-CSCs population, and confers resistance to radiotherapy and chemotherapy (55). OXPHOS serves as an alternative energy source for CSCs. Emerging evidence has demonstrated that HCC-CSCs exhibit more robust OXPHOS than HCC cell (56). Furthermore, the enhanced OXPHOS levels in HCC-CSCs can promote stemness maintenance, thereby increasing tumor metastasis. For example, organic cation/carnitine transporter 2 activates peroxisome proliferator-activated receptor-γ coactivator 1α signaling to potentiate OXPHOS, elevating EpCAM/CD24 expression, augmenting CSC sphere-forming capacity, and ultimately driving HCC cell proliferation, migration and invasion (57).

Proteoglycans, macromolecules composed of core proteins and glycosaminoglycan chains, have also been implicated in CSCs (58). These molecules contribute to the maintenance of the CSC phenotype and facilitate tumor progression. Glypican-3, a heparan sulfate proteoglycan subtype, modulates c-Myc activity to regulate the migratory, invasive and CSC-forming capabilities of HCC cells within hypoxic tumor microenvironments (59). Hyaluronic acid (HA) belongs to the glycosaminoglycan family and CD44 is an HA receptor and a surface marker of HCC-CSCs. TGF-β affects the expression levels of pluripotent transcription factors by regulating CD44 subtypes and targeting the MAPK signaling pathway, mediates the formation ability of HCC-CSCs, promotes epithelial-mesenchymal transition and increases the invasion and migration of HCC cells (60). KEGG pathway analysis indicated that miR-17-5p targeted genes associated with stemness, metastasis and drug resistance mainly influenced cell fate and metabolic reprogramming pathways to maintain CSC survival in harsh microenvironments. Therefore, miR-17-5p may represent a potential target that serves an essential role in HCC-CSCs.

The present study had some limitations. First, the nine studies selected in the systematic review only included participants of Chinese and Korean ethnicities, which may have led to bias and missed other potential miRNAs. However, inclusion of only studies with Chinese and Korean ethnicities was due to the current evidence base being limited and the reliability of the present study findings was further validated using pan-ethnic databases, such as TCGA. Second, a single HCC cell line (Huh7) was used in the present study and only the expression levels of miR-17-5p were explored in Huh7 and Huh7-CSCs, suggesting a potential role for miR-17-5p in HCC-CSCs. In

future studies, the role of miR-17-5p will be explored in a larger population and multiple cell lines. Direct investigations of miR-17-5p function in drug resistance, metastasis and recurrence of HCC-CSCs will be conducted, including miRNA mimic/inhibitor experiments, sphere formation and drug resistance assays. In summary, miR-17-5p is a miRNA potentially associated with CSCs, drug resistance, metastasis and recurrence of HCC through cell fate and metabolic reprogramming pathways. miR-17-5p exhibited higher expression in Huh7-CSCs compared with that in Huh7 cells, and may target HIF1A and Myc to maintain the stemness of HCC-CSCs. However, further experimental studies are required to validate these hypotheses.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YJ and XZ confirm the authenticity of all the raw data. YJ, QP and XZ conducted the systematic review and network analysis. YKC, XZ and JW designed the study. MP, PZD, DZ, XW, YKC and JW supervised the experiments and discussion. YJ and XZ wrote and edited the manuscript. YJ, XW and QP performed the cytological experiments. XZ, DZ and MP performed the statistical analyses and interpreted the data. DZ, XW and PZD were involved in reviewing and editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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