miR-517b-3p promotes the progression of portal vein tumor thrombus via activating Wnt/β-catenin signaling pathway in hepatocellular carcinoma

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Abstract
Aims This study was aimed to investigate the expression patterns and prognostic value of microRNA-517b-3p (miR-517b-3p) in hepatocellular carcinoma (HCC) patients with portal vein tumor thrombus (PVTT).

Methods The expression of miR-517b-3p in PVTT tissues and cells was estimated using qRT-PCR. Through Kaplan–Meier survival analysis, Cox regression assay and ROC analysis, the significance of miR-517b-3p was explored. In addition, cell experiments were performed to examine the functional role of miR-517b-3p during progression of PVTT. Moreover, the biological process and biological pathway analyses were conducted through GSEA and FunRich. Besides, the protein–protein interaction (PPI) network of the DEGs was established through cBioPortal website.

Results Compared with the controls, the miR-517b-3p was upregulated in both PVTT tissues and cells. The upregulated miR-517b-3p, which served as a potential diagnostic biomarker to distinguish PVTT from PT and controls, was associated with poor overall survival and acted as an independent prognostic factor. The cell proliferation, migration and invasion were proved to be enhanced by overexpression of miR-517b-3p. Furthermore, Wnt/β-catenin signaling was suppressed by miR-517b-3p knockdown and might be involved in the progression of PVTT.

Conclusion miR-517b-3p may promote PVTT cell proliferation, migration and invasion via activation of Wnt/β-catenin signaling pathway. Meanwhile, miR-517b-3p has overexpression in PVTT samples, and serves as a candidate diagnostic and prognostic biomarker in HCC patients with PVTT.

Keywords Bioinformatic analysis · Hepatocellular carcinoma · miR-517b-3p · Prognosis · Portal vein tumor thrombus

Abbreviations
miR-517b-3p MicroRNA-517b-3p
HCC Hepatocellular carcinoma
PVTT Portal vein tumor thrombus
GSEA Gene set enrichment analysis
PT Paired parenchyma tumor
TCGA The Cancer Genome Altas database
NES Normalized Enrichment Score
AFP Alpha fetal protein
APC Adenomatous polyposis coli protein
GSK-3β Glycogen synthase kinase-3β
CK1 Casein kinase 1
CCND1 Cyclin D1
MMP-7 Matrix metalloproteinase-7
COX-2 Cyclooxygenase-2

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Introduction

In recent years, hepatocellular carcinoma (HCC) has become one of the common malignancies worldwide with increased rates of morbidity and mortality [1]. Most HCC cases are identified as advanced disease by the initial diagnosis, leading to poor outcomes of the cancer patients [2]. HCC is characterized by its tendency to invade the vascular in liver. Vascular invasion has been described as a risk factor for mortality and recurrence of HCC after surgical resection [3]. Portal vein tumor thrombosis (PVTT), which represents a common form of vascular invasion, can be detected in 40–90.2% of advanced HCC cases. At the same time, it has been reported to serve as a predictor of poor prognosis of HCC [4]. The median survival of HCC patients without PVTT was 24.4 months, while this value was only 2.7 months in patients with PVTT [5]. Therefore, it is important to improve the prognosis for HCC patients presenting PVTT. However, the mechanism of tumor thrombosis induced by malignant tumors remains unclear [6].

Aberrant expression levels of MicroRNAs (miRNAs) have been detected in various human malignancies, which are closely correlated with cell proliferation, differentiation, migration, invasion and apoptosis [7]. It is generally considered that PVTT is a special type of metastasis of HCC [8, 9]. Thus, the altered miRNAs can participate in the development of PVTT by regulating the genes involved in tumor metastasis [10]. In addition, the miRNAs aberrant expression has received increasing attention on their diagnostic and prognostic value for diverse kinds of human cancers including HCC [11]. Therefore, investigating the clinical significance of miRNAs involved in the PVTT progression is critical for the treatment of HCC. Overexpression of miR-517b has ever been identified in PVTT tissues compared with paired parenchyma tumor (PT) tissues in HCC patients [12]. MiR-517b-3p, which belongs to miR-517 family in C19MC cluster, is regarded as one of the largest human miRNA gene clusters and includes 54 miRNA members [13]. Thus, our study was aimed to further confirm the expression patterns of miR-517b-3p in PVTT, and explore its potential function and prognostic significance for cancer patients.

Methods and materials

Patients and tissue specimen collection

In this study, a total of 124 HCC patients presenting PVTT had been recruited, and they were pathologically diagnosed at the First Affiliated Hospital of Xiamen University and 900 Hospital of the Joint Logistics Team from 2008 to 2012. No preoperative therapy had been carried out for the patients. The liver PVTT tissues, corresponding PT tissues and adjacent normal tissues were collected from the patients undergoing resection operation, immediately frozen in liquid nitrogen and then stored in − 80 °C for RNA extraction. In addition, the clinicopathological characteristics, including age, sex, tumor size, tumor number, cirrhosis, alpha fetal protein (AFP) and PVTT stage, were obtained from the electronic medical records (see these clinical information in Table 1). Beyond that, a 5 year follow-up survey was conducted for each patient and the survival information was recorded for the subsequent survival analysis.

Cell line and transfection

PVTT-derived cell line CSQT-2 was established at The Fuzong Clinical Medical College of Fujian Medical University according to the method reported previously [14]. Apart from that, human HCC cell line Huh7 and the normal liver cell line THLE-2 were purchased from American Type Culture Collection (ATCC). Furthermore, all the cells were
cultured in DMEM (Gibco, USA) supplemented with 10% of the fetal bovine serum (FBS) (Invitrogen, USA) in a 5% CO₂ incubator at 37 °C. In addition, the cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA) with the vector as follows: miR-517b-3p mimic, miR-517b-3p inhibitor, or their non-specific control miRNAs (mimic-NC, inhibitor-NC). Total of the vectors were synthesized at Ribobio (Ribobio, Guangzhou, China).

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

As per the manufacturer’s instructions, the total RNA in the tissue samples was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). In order to evaluate the concentration and purity of RNA, the ratio of OD A260/A280 was estimated. Only the RNA with ratio of 1.8–2.1 was applied in the subsequent analysis. Single stranded cDNA was synthesized from the RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoorde, Brussel, Belgium), and then adopted as the template of qRT-PCR. The expression levels of miR-517b-3p were estimated by qRT-PCR, which was conducted using SYBR green I Master Mix kit (Invitrogen) and 7300 Real-Time PCR System (Applied Biosystems). In addition, U6 was used as an internal control gene in the reactions. The primers used in the reactions were as follows: miR-517b-3p, forward: 5′-TGACCTCTT AGATGGAAGCAGC-3′, reverse: 5′-TGCATTAACACTCT AAAGGATG-3′; U6, forward: 5′-CTCGCTTCGGCAGCA CATATCT-3′, reverse: 5′-ACGCTTACGAATTTGCG TGTC-3′. The final relative expression of miR-517b-3p was calculated using 2^−ΔΔCt and normalized to U6.

**MTT assay**

MTT assay was used to evaluate the effect of miR-517b-3p on cell proliferation. Besides, CSQT-2 cells were seeded into a 96-well plate (1 × 10^5 cells/well) and incubated at 37 °C with 5% CO₂. After transfection with miR-517b-3p mimic, miR-517b-3p inhibitor, mimic-NC or inhibitor-NC, cells were continually cultured for 3 days. Afterwards, the cell number was examined using MTT assay every 24 h. In addition, 20 μL MTT solution (Sigma Aldrich, 0.5 mg/mL) was added into each well and incubated for another 2 h. Then, 150 μL DMSO (Sigma Aldrich) was added into the wells for 10 min. After that, the absorbance at 490 nm was measured to evaluate the cell viability in each well. All the experiments were performed in triplicate.

**Transwell assay**

Based on Transwell analysis, the migration and invasion abilities of CSQT-2 cells were examined. Cells were collected after transfection, washed twice with PBS, and then seeded in the upper chamber of a transwell with the cell density of 5 × 10^3 cells/mL. The membrane used was coated without matrigel for migration analysis and with matrigel for invasion analysis. Apart from that, the upper chamber was filled with serum free medium, while the lower chamber included medium supplemented with 10% FBS. Afterwards, the wells were incubated in a 5% CO₂ incubator at 37 °C for next 12–24 h. Then the migrated and invaded cells were fixed and dyed. The cell number was counted under a microscope.

**Analysis of miR-517b-3p associated expression genes in HCC**

The potential function of miR-517b in HCC was analyzed by screening miR-517b co-expressed genes with Linkedomics database (http://www.linkedomics.org/login.php). Besides, the KEGG pathway enrichment analysis of miR-517b co-expressed genes was enriched by Gene set enrichment analysis (GSEA) [Rank Criteria is P value, the minimum number of genes (size) is 3, and Simulation is 500]. Furthermore, KEGG analysis was used to predict the signaling pathways in which target genes could participate in.

**Analysis of mRNAs associated with miR-517b-3p expression in HCC and functional enrichment analysis**

LinkedOmics was used to discover, compare, and understand the association’s analysis within and across omics data associated with miR-517b expression of HCC [20]. Beyond that, mRNAs related to miR-517b-3p expression were searched using databases such as “Linkedomics”, “MiRPathDB”, “TargetScan” and “MiRDIP”. The overlapping mRNAs related to miR-517b-3p expression in different datasets was identified by Venny 2.1.0 software (http://bioinfogp.cnb.csic.es/tools/venny/index.html). In addition, the cBioPortal (http://www.cbioportal.org) was used to analyze the network of the hub genes and their co-expression genes [15]. Information about the alteration of the hub genes was available in cBioPortal OncoPrint, and the prognostic information (overall survival) of the 12 alteration genes was available in the HCC datasets (TCGA, Provisional) of the cBioPortal [16]. FunRich is the software used mainly for gene functional classification that provides a comprehensive set of functional annotation for researchers to understand the biological characteristics [17]. At the same time, biological process analysis and biological pathway enrichment analyses of the DEGs were performed through FunRich.
Western-blot

Western-blot was used to analyze the protein levels in the transfected cells. The proteins in cells treated with LiCl (an activator of Wnt/β-catenin pathway) were also evaluated by Western-blot. Briefly speaking, cells lysis was performed using radioimmunoprecipitation assay buffer. Then the concentrations of protein in the cell lysates were estimated with bicinchoninic acid (BCA) protein assay reagent (Pierce, IL). Moreover, the proteins were firstly separated using 10% SDS polyacrylamide gels and then transferred to the polyvinylidene fluoride (PVDF) membranes. Furthermore, the membranes were incubated with the first antibodies [anti-β-catenin, anti-Axin, anti-adenomatous polyposis coli protein (APC), anti-glycogen synthase kinase-3β (GSK-3β), anti-casein kinase 1 (CK1), anti-Cyclin D1 (CCND1), anti-c-Myc (MYC), anti-matrix metalloproteinase-7 (MMP-7), anti-cyclooxygenase-2 (COX-2) and anti-E-cadherin], and then with secondary antibodies coupled with horseradish peroxidase, which could be evaluated by chemiluminescence (Millipore, Billerica, MA).

Statistical analysis

On the one hand, the differences between two groups were analyzed by Student’s t test. On the other hand, the association of miR-517b-3p expression with clinicopathological features of cancer patients was assessed by Chi-square test. The diagnostic significance of miR-517b-3p was evaluated using receiver operating characteristic (ROC) analysis. In addition, the survival curves were plotted for the patients using Kaplan–Meier and the difference between the curves was compared with log-rank test. Apart from that, the prognostic value of miR-517b-3p for HCC patients with PVTT was confirmed by multiple Cox regression analysis. All these statistical analyses were performed using SPSS version 21.0 software (SPSS Inc., Chicago, IL). It was considered that the result with P < 0.05 was statistically significant.

Results

Expression of miR-517b-3p in the HCC tissues

The expression level of miR-517b was significantly increased in HCC tissues of the TCGA database in comparison to the normal adjacent liver tissues (P < 0.001, Fig. 1A). The overall cancer survival was predicted using Kaplan–Meier-plotter online database, and the results were presented through a Kaplan–Meier survival curve. It could be observed that patients with high miR-517b expression had significantly lower 5 year overall survival than those with low miR-517b expression (log-rank P < 0.001, Fig. 1B). According to the qRT-PCR, the expression of miR-517b-3p was found to be significantly upregulated in PVTT tissues in comparison to both the PT nodules and normal tissues (all P < 0.001, Fig. 1C). Moreover, the similar results were obtained in the cell lines. Namely, the higher miR-517b-3p expression was observed in CSQT-2 cells than that in Huh-7 and THLE-2 cells (all P < 0.001, Fig. 1D).

Association of miR-517b-3p expression with clinicopathological features of HCC patients

In order to examine the role of miR-517b-3p during tumor progression of HCC patients with PVTT, the relationship between miR-517b-3p expression and clinicopathological characteristics of the patients was analyzed through Chi-square test. Afterwards, the miR-517b-3p expression was classified into low and high expression groups using the mean expression level (1.095) as a cutoff value. According to the analysis results in Table 1, the revealed that miR-517b-3p expression was correlated with PVTT stage (P = 0.008). However, no significant association was found between miR-517b-3p expression and other parameters, such as age, gender, tumor size, tumor number, cirrhosis, and AFP (all P > 0.05).

Clinical significance of miR-517b-3p in diagnosis and prognosis

As shown in Fig. 1E, the areas under the ROC curve (AUC) values for PVTT and PT were all analyzed. The AUC for PT was 0.818 in comparison to the normal controls, indicating that the miR-517b-3p expression could act as a diagnostic biomarker for HCC patients. Similarly, the miR-517b-3p expression had high diagnostic accuracy and served as a high sensitive and specific diagnostic biomarker to distinguish PVTT from normal controls with an AUC of 0.928, and the sensitivity and specificity were 90.3% and 82.3% respectively at the cutoff value of 0.525. Moreover, the AUC for PTVV compared with PV was 0.723 (cutoff value = 1.070, sensitivity = 67.7%, specificity = 75.8%), suggesting that the miR-517b-3p expression might be a potential diagnostic marker for PVTT.

According to the results of survival analysis in Fig. 1F, patients with high miR-517b-3p expression had significantly shorter survival time than those with low miR-517b-3p expression (log-rank P = 0.03). Furthermore, Cox regression analysis was performed to evaluate the parameters that were independently correlated with overall survival. Based on the data in Table 2, we concluded that miR-517b-3p (HR 3.137, 95%CI 1.570–6.269, P = 0.001) and PVTT stage (HR 1.976, 95%CI 1.067–3.661, P = 0.03) were two independent prognostic factors for HCC patients with PVTT.
Fig. 1 A Analysis of miR-517b expression levels in HCC tissues (n = 375) and normal adjacent liver tissues (n = 50) from TCGA database. B Kaplan–Meier-plotter online database was used to predict the overall cancer survival. HCC Patients with high miR-517b expression had shorter survival time than those with low miR-517b expression (log-rank $P < 0.001$). C MiR-517b-3p expression was increased in PVTT tissues compared with PV tissues and normal controls ($**P < 0.001$). D Expression of miR-517b-3p was upregulated in CSQT-2 cells compared with Huh7 and THLE-2 cells ($***P < 0.001$). E ROC analysis for PVTT and PT. The AUC for PVTT-PT was 0.723, and the sensitivity and specificity were respectively 67.7% and 75.8% at cutoff value of 1.070. The AUC for PVTT-NC was 0.928, and the sensitivity and specificity were respectively 90.3% and 82.3% at cutoff value of 0.525. The AUC for PT-NC was 0.818, and the sensitivity and specificity were respectively 75.8% and 75.8% at cutoff value of 0.440. F The upregulated expression of miR-517b-3p predicted poor overall survival of HCC patients with PVTT (log-rank $P = 0.03$).
Effects of miR-517b-3p on cell proliferation, migration and invasion

In this study, the biological function of miR-517b-3p in PVTT cells was also focused. Through the use of miR-517b-3p mimic and inhibitor, the expression of miR-517b-3p was regulated in CSQT-2 cells. Besides, the remarkably increased miR-517b-3p expression was detected in miR-517b-3p mimic group and decreased miR-517b-3p expression was exhibited in miR-517b-3p inhibitor group in comparison to the corresponding controls (all \(P<0.001\), Fig. 2A). Although cell proliferation in miR-517b-3p mimic group was enhanced, it was suppressed in miR-517b-3p inhibitor group (\(P<0.05\), Fig. 2B). The migration and invasion assay revealed that the overexpression of miR-517b-3p could promote cell migration and invasion, while the knockdown of miR-517b-3p decreased the migration and invasion of CSQT-2 cells (all \(P<0.05\), Fig. 2C–E).

Enrichment analysis of miR-517b gene co-expression in LIHC

We investigated 5617 genes associated with miR-517b expression (\(P<0.05\)) and displayed a gene heat map (spearman correlation analysis) (Fig. 3A) that was positively (Fig. 3B) and negatively (Fig. 3C) correlated with miR-517b expression in 371 samples from the linkedomics database of LIHC. Moreover, the differential expression genes (DEGs) regulated by miR-517b-3p, especially at the post-transcriptional levels, were predicted by online LinkedOMics database. KEGG analysis was used to predict the signaling pathways in which miR-517b co-expression genes could participate (Fig. 3D). In order to explore oncogenic signaling related to miR-517b, we performed GSEA on the LinkedOMics dataset to determine whether miR-517b co-expression gene sets were enriched in different phenotypes. In fact, the DNA replication (NES = 1.716, \(P<0.001\)) (Fig. 4E), CELL CYCLE (NES = 1.681, \(P<0.001\)) (Fig. 3F), T cell receptor signaling pathway (NES = 1.508, \(P=0.002\)) (Fig. 4G), p53 signaling pathway (NES = 1.479, \(P=0.004\)) (Fig. 3H), Pancreatic cancer (NES = 1.434, \(P=0.006\)) (Fig. 3I) and Prostate cancer (NES = 1.292, \(P=0.042\)) (Fig. 3J) were enriched in the miR-517b high expression group, all of which were closely associated with the specific cancer-related gene sets.

Analysis of mRNAs associated with miR-517b-3p expression in HCC

Then, we searched for mRNAs associated with miR-517b through the “Linkedomics”, “MiRPathDB”, “TargetScan” and “MiRDIP” databases, respectively. The overlapping mRNAs in the four databases were analyzed by Venny plot. Finally 12 overlapping mRNAs were obtained (CCNK, EIF4G3, MCHR2, OLFM3, POU4F1, PTK2B, RYBP, SEMA3A, SLITRK1, TNIP3, USP1, WNT4) and the result is shown in Fig. 4A. The network of 12 hub genes was constructed using cBioPortal online platform. As displayed in Fig. 4B, the network below contains 62 nodes, including 12 query genes and the 50 most frequently altered neighbor genes.

Functional enrichment analysis of 12 hub genes

In order to further investigate the biological functions of the 12 query genes, the biological process analysis and biological pathway analysis in FunRich were performed. To be specific, the biological process of the 12 query genes was mainly enriched in the Protein metabolism; Cell communication; Signal transduction; Regulation of nucleobase, nucleoside, nucleotide and nucleic acid; metabolism; Regulation of cell cycle; Apoptosis; Regulation of gene expression, epigenetic; DNA repair (Fig. 4C). Meanwhile, the biological pathway functional enrichment terms of 12 query genes were mainly correlated with the mTOR signaling pathway, Class I PI3K signaling events, Wnt signaling

| Table 2 | Cox regression analysis for miR-517b-3p expression in HCC patients |
| Variables | Univariate analysis | Multivariate analysis |
| | HR | 95%CI | \(P\) value | HR | 95%CI | \(P\) value |
| MiR-517b-3p | 2.377 | 1.271–4.445 | 0.007 | 3.137 | 1.570–6.269 | 0.001 |
| Age | 1.194 | 0.688–2.072 | 0.529 | 1.172 | 0.668–2.058 | 0.580 |
| Gender | 1.124 | 0.612–2.063 | 0.706 | 1.163 | 0.627–2.157 | 0.631 |
| Tumor size | 1.031 | 0.583–1.821 | 0.918 | 1.089 | 0.610–1.943 | 0.773 |
| Tumor number | 1.463 | 0.852–2.513 | 0.168 | 1.124 | 0.619–2.041 | 0.701 |
| Cirrhosis | 1.094 | 0.549–2.180 | 0.799 | 1.028 | 0.510–2.074 | 0.938 |
| AFP | 1.007 | 0.584–1.736 | 0.980 | 1.162 | 0.643–2.100 | 0.620 |
| PVTT stage | 1.969 | 1.094–3.545 | 0.024 | 1.976 | 1.067–3.661 | 0.030 |

\(AFP\) alpha fetal protein, \(PVTT\) portal vein tumor thrombus
network, canonical Wnt signaling pathway, FGF signaling pathway, p53 pathway, ATR signaling pathway, FGF signaling pathway, and signal regulatory protein (SIRP) family interactions. (Fig. 4D).

**Hub alteration analyzed in RNA-sequencing data from the cancer genome atlas and Kaplan–Meier plots of cBioPortal comparing OS in cases with/without alterations**

We investigated the genetic alteration of 12 query genes with a cohort of 360 cases of HCC available in the cBioPortal.
database (TCGA, Provisional). As shown by the result, 169 (47%) cases of HCC in this cohort exhibited genes alteration. The percentage of individual Genetic Alteration is as follows: CCNK 8%, EIF4G3 6%, MCHR2 4%, OLFM3 2.5%, POU4F1 5%, PTK2B 8%, RYBP 6%, SEMA3A 7%, SLITR1 6%, TNIP3 4%, USP1 9%, and WNT4 4% (Fig. 4E). After analysis by Kaplan–Meier plot and log-rank test, the alterations in Query genes were associated with poorer OS in HCC patients of cBioPortal ($P = 0.0154$, Fig. 4F).

**Significantly enriched biological pathway of miR-517b in BCLC C stage HCC**

Furthermore, we crossed the “being significantly correlated with miR-517b expression” with “being significantly associated with overall survival prognosis” by Venny map, and found that 5 Hub genes of miR-517b (SLITRK1, MCHR2, OLFM3, POU4F1, and PTK2B) remained (Fig. 5A). In order to further investigate the biological function of miR-517b, we used FunRich software to perform functional enrichment analysis on five hub genes that might involve the biological pathway. In addition, five hub genes were particularly enriched in 10 pathways, including Wnt signaling network, N-cadherin signaling events, Betal integrin cell surface interactions, and Canonical Wnt signaling pathway (Fig. 4E). Among the above biological pathways, we found that multiple biological pathways were associated with Wnt/β-catenin signaling. Therefore, we hypothesized that the overexpression of miR-517b-3p may promote the progression of portal vein thrombosis by activating Wnt/β-catenin signaling pathway in hepatocellular carcinoma.

**Downregulation of miR-517b-3p suppressed Wnt/β-catenin signaling**

In the cells with decreased miR-517b-3p expression by miR-517b-3p inhibitor, the proteins involved in the Wnt/β-catenin signaling were estimated using Western blot. As shown in Fig. 5C, the concentration of cytoplasm β-catenin (Aliases CTNNB1) was dramatically downregulated in
Fig. 4  

A. The Venny plot reveals that 12 overlapping miRNAs in “Linkedomics”, “MiRPathDB”, “TargetScan”, and “miRDIP” interact with miR-517b. Different colour areas represented different datasets.  

B. The network of 12 hub genes was constructed using cBioPortal online platform.  

C. The biological process of the 12 query genes.  

D. The biological pathway functional enrichment terms of 12 query genes.  

E. The alteration and Expression Heatmap of 12 hub genes in 360 HCC cases from the cBioPortal database. According to the result, 169 (47%) cases of HCC in this cohort exhibited genes alteration.  

F. Kaplan–Meier plots of cBioPortal comparing OS in cases with/without 12 Query genes alterations ($P = 0.0154$).
the miR-517b-3p silencing cells ($P < 0.05$), and a significant decrease in the protein expression of nucleus β-catenin was also observed by the knockdown of miR-517b-3p ($P < 0.05$), indicating that the downregulation of miR-517b-3p in CSQT-2 cells led to the activation of Wnt/β-catenin signaling. In order to confirm this conclusion, the effects of miR-517b-3p on the expression of APC-degradation complex were also investigated. As shown in Fig. 5D, the members of the degradation complex, including Axin, APC, GSK-3β and CK1, were all upregulated by the downregulation of miR-517b-3p ($*P < 0.05$).
downregulation of miR-517b-3p (all \( P < 0.05 \)). Moreover, the decreased Cyclin D1, c-Myc, MMP-7 and COX2 and increased E-cadherin, which represent the downstream targets of the Wnt/β-catenin signaling pathway, were detected in the miR-517b-3p silencing cells (all \( P < 0.05 \), Fig. 5E). All these results indicated that Wnt/β-catenin signaling was inhibited by the knockdown of miR-517b-3p.

**Effects of Wnt/β-catenin signaling on cell proliferation, migration and invasion**

In order to further confirm whether miR-517b-3p exerted its tumor promoting effects according to Wnt/β-catenin signaling, we used LiCl to activate Wnt/β-catenin signaling in the miR-517b-3p silencing cells, and estimated the cell proliferation, migration and invasion abilities. Besides, β-catenin protein levels were checked using western-blot, and the suppressed Wnt/β-catenin signaling induced by miR-517b-3p knockdown was abrogated by LiCl, as evidenced by the elevated expression of β-catenin (\( P < 0.05 \), Fig. 5F). Furthermore, the decreased cell proliferation, migration and invasion result from the downregulation of miR-517b-3p were all increased in the cells treated with LiCl (all \( P < 0.05 \), Fig. 5G, H).

**Discussion**

In recent decades, miRNAs have received increasing attentions for their valuable clinical significance for different types of human cancer [7, 18]. Many members of miRNAs have been identified to act as biomarkers for HCC prognosis [19]. MiR-517b-3p belongs to miR-517 family in C19MC cluster, which is reported to be one of the largest human miRNA gene clusters and includes 54 miRNA members [13]. MiR-517 family consists of three subtypes: miR-517a, miR-517b-3p and miR-517c [20]. At the same time, the sequence of miR-517b-3p has been reported to be similar to the data of miR-517a, indicating that they may have parallel function in various biological processes [21]. Abnormal expression of miR-517a has been observed in various human malignancies, and its differential expression is closely correlated with the tumor progression and prognosis. A study indicated that elevated expression of miR-517a significantly promotes the growth, migration and invasion of glioma cells in vitro and in vivo. Therefore, miR-517a may be a promising therapeutic candidate for glioma [22]. The increased expression of miR-517a has also been reported in melanoma. Silencing the expression of microrna-517a can up-regulate the expression of CDKN1C, inactivate JNK signaling pathway, and induce oxidative stress injury of melanoma cells [23]. In HCC, miR-517a has been described as an oncogene with its significant elevated expression in tumor tissues [24]. The upregulated miR-517b-3p expression is presented in PVTT tissues compared with the PT tissues in HCC patients [12]. However, the association of miR-517b-3p with progression and prognosis of PVTT is rarely reported.

In the present study, it was found that the expression of miR-517b-3p measured by qRT-PCR was upregulated in PVTT tissues compared with PT tissues and normal controls. In agreement with the data in tissues, miR-517b-3p expression was also increased in PVTT cells compared with HCC and normal cells. In addition, the expression of miR-517b-3p in PT tissues was significantly higher than that in the normal tissues. According the ROC analysis, we concluded that the miR-517b-3p expression could be an effective diagnostic biomarker to distinguish PVTT from PV and normal controls. As indicated by the survival analysis results, the upregulated expression of miR-517b-3p was correlated with poor overall survival of HCC patients with PVTT. Furthermore, multivariate Cox analysis revealed that miR-517b-3p expression was an independent prognostic factor for the cancer patients. It is noteworthy that the overexpression of miR-517b-3p was proved to be correlated with the advanced PVTT stage. Therefore, these data above suggested that miR-517b-3p served as an oncogene of HCC and might be involved in PVTT development in HCC patients.

In order to further understand the potential molecular mechanisms underlying the role of miR-517b-3p in HCC, we applied bioinformatic analyses to explore oncogenic signaling related to miR-517b. According to the GSEA results, the miR-517b expression was positively correlated with DNA replication, cell cycle, T cell receptor signaling pathway, p53 signaling pathway, pancreatic cancer and prostate cancer, all of which were closely associated with specific cancer-related gene sets. For further investigating the biological function of miR-517b, we used FunRich software to perform functional enrichment analysis on hub genes that might involve the Biological pathway. Therefore, we hypothesized that the overexpression of miR-517b-3p might promote the progression of portal vein thrombosis by activating Wnt/β-catenin signaling pathway in hepatocellular carcinoma.

In order to further investigate the functional role of miR-517b-3p in progression of PVTT, the effects of miR-517b-3p expression on cell proliferation, migration and invasion of CSQT-2 were analyzed in this study. Through the use of miR-517b-3p mimic and inhibitor, the expression of miR-517b-3p expression was regulated in CSQT-2 cells. According to the analysis results, the cell proliferation, migration and invasion were significantly enhanced in the cells with overexpression of miR-517b-3p, while were suppressed in the miR-517b-3p silencing cells. Thus, we concluded that miR-517b-3p expression could promote the progression of PVTT. It was considered that the over activity of Wnt/β-catenin signaling pathway was closely correlated with tumor initiation and progression of various human
cancers, including HCC [25, 26]. In the present study, by evaluating the related protein concentration of this pathway, we observed that the Wnt/β-catenin signaling pathway was activated by miR-517b-3p. Furthermore, the Wnt/β-catenin signaling was activated by LiCl, and its effects on biological behaviors of PVTT cells were assessed. As revealed by the analysis results, the downregulated cell proliferation, migration and invasion induced by miR-517b-3p knockdown were recovered by activation of Wnt/β-catenin. Thus, we considered that miR-517b-3p might promote tumor progression of PVTT by activating Wnt/β-catenin signaling.

In conclusion, the overexpression of miR-517b-3p may promote PVTT cell proliferation, migration and invasion by activating Wnt/β-catenin signaling pathway. Combining with the bioinformatic analyses above, this study concluded that overexpression of miR-517b-3p in PVTT tissues compared with normal tissues could serve as a candidate predictor for poor prognosis in HCC patients.

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Author contributions The experiments were conceived and planned by R-SK, Z-HL and F-XZ. R-SK and K-ZH drafted and critically revised the manuscript and was responsible for management of the project. L-ZL, YJ and D-SB collected patient clinical data. R-SK, D-SB, H-XW and L-ZL carried out experiments and data analysis and prepared the manuscript. R-SK, J-RY and K-ZH performed experiments, data statistics and submission. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have declared no conflict of interest.

Ethical approval and consent to participate The studies involving patient materials were in agreement with written informed consent for participation and approved by the Ethics Committee of the The First Affiliated Hospital of Xiamen University (Ethics No. XMYY-2020KY060), China.

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