

# Enhanced plasma miR-142-5p promotes the progression of intrahepatic cholangiocarcinoma via targeting PTEN

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**Abstract.** The aim of the present study was to evaluate the expression and specific role of microRNA (miR)-142-5p in the progression of intrahepatic cholangiocarcinoma (ICC). Reverse transcription-quantitative polymerase chain reaction was performed to evaluate miR-142-5p expression in patients with ICC and healthy control subjects. The results revealed that plasma miR-142-5p was significantly increased in patients with ICC compared with the control group. Furthermore, miR-142-5p was also increased in ICC tissues compared with adjacent non-neoplastic tissues. Compared with patients with Ta-T1 stage ICC, miR-142-5p was significantly elevated in patients with ICC  $\geq$ T2 stage. Patients with ICC at G3 stage had much higher plasma miR-142-5p levels compared with those at G1/2 stage. Receiver operating characteristic analysis indicated that miR-142-5p could be used as a biomarker to differentiate patients with ICC from healthy controls. Kaplan-Meier analysis demonstrated that plasma miR-142-5p was negatively correlated with survival in patients with ICC. A dual luciferase reporter assay indicated that miR-142-5p significantly suppressed the relative luciferase activity of pmirGLO-PTEN-3' untranslated region compared with the control group. In summary, the results of the present study provide novel data indicating that plasma miR-142-5p is significantly upregulated in patients with ICC. miR-142-5p may therefore have potential as a biomarker for screening patients with ICC from healthy controls.

## Introduction

Intrahepatic cholangiocarcinoma (ICC) is one of the most common hepatic malignancies worldwide (1), the incidence

and mortality of which has increased in recent years (2). Previous studies have suggested that multiple signaling pathways are involved in the progression of ICC (3,4); however, the specific mechanisms underlying ICC etiology remain unknown. As cystoscopy is invasive and expensive, there is a need to identify potential diagnostic biomarkers for ICC in order to improve the early detection of ICC (5,6).

MicroRNAs (miRs) are small non-coding RNAs ~22 nucleotides in length that are associated with multiple biologic processes including cell proliferation, differentiation and apoptosis (7). Abnormal expression of miRNAs has been widely identified in different diseases (8,9). For instance, miR-590-3p inhibits epithelial-mesenchymal transition in ICC via suppressing Smad interacting protein 1 expression (9). Additionally, several differentially expressed miRNAs have been reported as potential diagnostic biomarkers for patients with ICC; for instance, high miR-146a expression in the plasma and tumor tissues is reportedly associated with prolonged overall survival in surgical patients with ICC (7).

Abnormal miR-142-5p expression has been widely reported in different tumors (10-12). miR-142-5p acts as a tumor suppressor via targeting phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$  in non-small cell lung cancer (10). High miR-142-5p expression is also associated with the biological aggressiveness of colorectal cancer (11) and has been reported as a potential predictive biomarker for recurrence risk in patients with gastric cancer (12). The focus of the present study was miR-142-5p and its specific role in the progression of ICC. The aim was to evaluate the expression of miR-142-5p in ICC tissues and elucidate the potential underlying mechanism.

## Materials and methods

**Cell culture.** Two hundred and ninety-three cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and streptomycin in 25-cm<sup>2</sup> culture flasks at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Patients and specimens.** Human clinical samples were obtained from 100 patients with ICC between December 2016

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and November 2017 at The First People's Hospital of Tongxiang (Tongxiang, China). Corresponding adjacent, non-neoplastic tissues from the macroscopic tumor margin were isolated and used as controls. ICC diagnosis was based on criteria outlined by the World Health Organization (13) and tumor differentiation was based on the classification proposed by Edmondson and Steiner (13). The clinical classification of tumors was performed according to the 7th edition of the tumor-node-metastasis classification system of the International Union Against Cancer (14). Patient characteristics are presented in Table I.

Patients were excluded from the current study if they exhibited: Failure of important organs, including the heart, lungs, kidneys and brain, intolerance to surgery, distant organ metastasis, lymph node involvement beyond the hepato-duodenal ligament, hilar or caval lymph nodes, preoperative chemotherapy or radiotherapy and preoperative liver treatment (arterial chemoembolization, radiofrequency ablation or percutaneous ethanol injection). All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Whole blood samples were prospectively collected from patients with ICC and healthy controls without urologic malignancies. Whole blood (5-8 ml) was collected in EDTA tubes and samples were centrifuged twice at 3,000 x g at 4°C for 15 min. The plasma was then stored at -80°C. All research protocols were approved by The First People's Hospital of Tongxiang and written informed consent was obtained from all participants.

**Plasma RNA isolation.** Total RNA was isolated from whole blood samples using RNAVzol LS or tissue samples using or RNAVzol (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China) according to the manufacturer's protocol. The quality, quantity and integrity of RNA were monitored using a NanoDrop spectrophotometer (ND-1000; Nanodrop; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** A total of 1 µg RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcription enzyme (Applied Biosystems; Thermo Fisher Scientific, Inc.) with specific primers. The temperature protocol used for RT was as follows: 72°C for 10 min; 42°C for 60 min, 72°C for 5 min and 95°C for 2 min. qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in an iCycler iQ real-time PCR detection system. The PCR amplifications were performed in a 10 µl reaction system containing 5 µl SYBR Green Supermix, 0.4 µl forward primer, 0.4 µl reverse primer, 2.2 µl double distilled H<sub>2</sub>O and 2 µl template cDNA. Thermocycling conditions were as follows: 95°C for 10 min followed by 50 cycles of 95°C for 10 sec, 55°C for 10 sec, 72°C for 5 sec, 99°C for 1 sec, 59°C for 15 sec and 95°C for 1 sec, followed by cooling to 40°C. Relative mRNA expression was normalized to U6 using the 2<sup>-ΔΔC<sub>q</sub></sup> method (15). Primer sequences were as follows: miR-142-5p-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTA G-3'; U6-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACAAAATG-3'; miR-142-5p, forward 5'-CATAAAGTAGAAAGCACTACT-3'; U6, forward 5'-GCGCGTCGTGAAGCGTTC-3'; and universal

Table I. Clinicopathological features of patients with ICC and healthy controls.

Variables	Patients with ICC	Healthy controls
Sex ratio (male/female)	75/25	39/11
Age		
≥60 years	64	35
<60 years	36	15
Stage		
Ta	33	-
T1	27	-
T2	17	-
T3	13	-
T4	10	-
Grade		
1	32	-
2	38	-
3	30	-

ICC, intrahepatic cholangiocarcinoma.

reverse primer, 5'-GTGCAGGGTCCGAGGT-3'. The criteria for dividing low and high expression of miR-142-5p were determined as follows: Relative mRNA expression was normalized to U6 using the 2<sup>-ΔΔC<sub>q</sub></sup> method (15). Patients with below average 2<sup>-ΔΔC<sub>q</sub></sup> were classed as low miR-142-5p expression, while those with above average 2<sup>-ΔΔC<sub>q</sub></sup> were classed as high miR-142-5p expression.

**Transient transfection.** A total of 6x10<sup>5</sup> 293 cells were seeded in 6-well plates with 2 ml DMEM supplemented with serum and antibiotics as above. miR-142-5p mimics, inhibitors, or miR negative controls (miR-NC; Shanghai GenePharma Co., Ltd., Shanghai, China) were mixed with HiperFect transfection reagent (Qiagen GmbH, Hilden, Germany) and incubated at room temperature for 10 min. This mixture was then added to cultured 293 cells for 48 h. The interval between transfection and subsequent experimentation was 48 h.

**MiRNA target prediction and dual-luciferase reporter assay.** TargetScan (<https://www.targetscan.org>) was used to predict potential target genes of miR-142-5p. The 3'-untranslated region (3'UTR) of phosphate and tensin homolog (PTEN) was cloned into the pmirGLO plasmid. After 293 cells were cultured for 24 h at 37°C in DMEM medium, miR-142-5p or scramble were cotransfected with blank pmirGLO or pmirGLO-PTEN-3'UTR using Vigofect (Vigorous Biotechnology Beijing Co., Ltd.) according to the manufacturer's protocol. Luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System (E1910; Promega Corp., Madison, WI, USA).

**Statistical analysis.** Data are presented as the mean ± standard error of the mean. Two-tailed unpaired Student's t-tests were used to compare two groups. Multiple group comparisons were made using one-way analysis of variance followed by

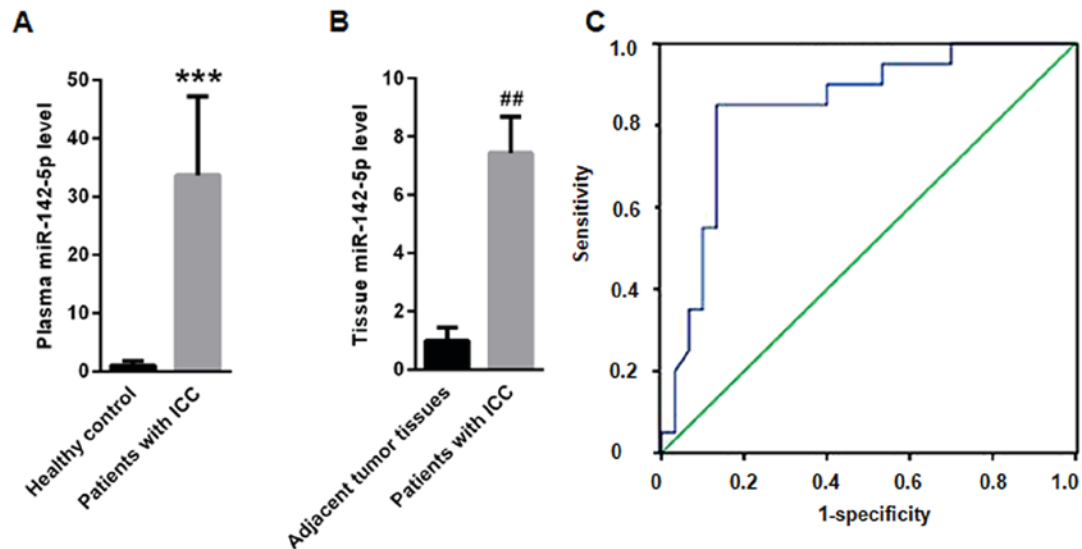


Figure 1. miR-142-5p expression in plasma and tissue samples. (A) Plasma miR-142-5p in healthy controls and patients with ICC. (B) miR-142-5p expression in tumor and adjacent non-neoplastic tissues. (C) Receiver operating characteristic analysis indicated that plasma miR-142-5p could screen patients with ICC from healthy controls. \*\*\*P<0.001 vs. control and ##P<0.01 vs. adjacent non-neoplastic tissues. miR, microRNA; ICC, intrahepatic cholangiocarcinoma.

Table II. Multivariate Cox regression analysis for miR-142-5p in patients with intrahepatic cholangiocarcinoma.

Parameter	Hazard ratio	95% confidence interval	P-value
miR-142-5p	3.508	1.783-6.968	<0.001
Sex	0.843	0.532-1.467	0.486
Age	1.513	0.879-1.987	0.114
Tumor size	1.021	0.621-1.831	0.876
Lymph node metastasis	1.032	0.653-1.902	0.821
Clinical stage	0.926	0.602-1.623	0.821
Histological type	1.024	0.597-1.821	0.921
Differentiation	1.067	0.672-1.906	0.798

miR, microRNA.

Tukey's multiple comparison test. Receiver operating characteristic curve (ROC) analysis was used to assess the efficacy of miR-142-5p as a biomarker. Kaplan-Meier survival analysis was also performed and survival differences were assessed using a log-rank test. Cox regression assay was performed to evaluate the prognostic value of miR-142-5p in patients with ICC. SPSS (version 20.0, SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Plasma and tissue miR-142-5p levels are upregulated in patients with ICC.* Compared with healthy controls ( $1 \pm 0.87$ ), plasma miR-142-5p was significantly upregulated in patients with ICC ( $33.7 \pm 13.5$ ; Fig. 1A). Furthermore, miR-142-5p expression was significantly higher in tumor tissues ( $7.45 \pm 1.23$ ) compared with adjacent non-neoplastic tissues ( $1 \pm 0.45$ ; Fig. 1B). ROC analysis indicated that plasma miR-142-5p could

be used to screen patients with ICC from healthy controls, with an area under the curve of 0.844, (95% confidence interval: 0.730-0.959; Fig. 1C). Furthermore, the prognostic value of miR-142-5p was assessed using Cox analysis. The results revealed that miR-142-5p overexpression was an independent prognostic factor for patients with ICC (hazard ratio 3.508, 95% confidence interval 1.783-6.968; Table II).

*miR-142-5p expression is positively correlated with ICC metastasis and invasion.* Compared with patients with Ta-T1 stage ICC, ( $1 \pm 0.68$ ), plasma miR-142-5p was significantly elevated in patients with ICC with  $\geq T2$  staging ( $21.5 \pm 5.93$ ; Fig. 2A). Furthermore, patients with ICC at G3 stage had significantly higher plasma miR-142-5p levels ( $17.8 \pm 4.56$ ) compared with those at G1/2 stage ( $1 \pm 0.67$ ; Fig. 2B).

*Plasma miR-142-5p is negatively correlated with survival in ICC cancer patients.* The results of Kaplan-Meier analysis revealed that patients with high plasma miR-142-5p had a

Table III. Clinicopathological features of patients with intrahepatic cholangiocarcinoma and healthy control subjects.

Variables	N	miR-142-5p			Overall survival, months		
		Low	High	P-value	Mean	95% CI	P-value
Sex							
Male	75	37	38	0.985	44.67	39.05-51.94	0.435
Female	25	13	12		38.25	32.56-43.15	
Age							
≥60	64	34	30	0.936	40.87	36.87-47.35	0.098
<60	36	18	18		42.86	31.45-50.87	
Stage							
Ta	33	15	18	0.016	52.56	46.87-57.67	0.012
T1	27	13	14		46.53	39.75-53.12	
T2	17	9	8		36.54	30.57-43.22	
T3	13	6	7		29.35	27.01-34.52	
T4	10	4	6		20.91	5.78-32.87	
Grade							
1	32	17	15	0.032	46.08	40.35-50.76	0.009
2	38	19	19		36.45	30.23-43.12	
3	30	16	14		21.34	6.56-35.96	

CI, confidence interval.

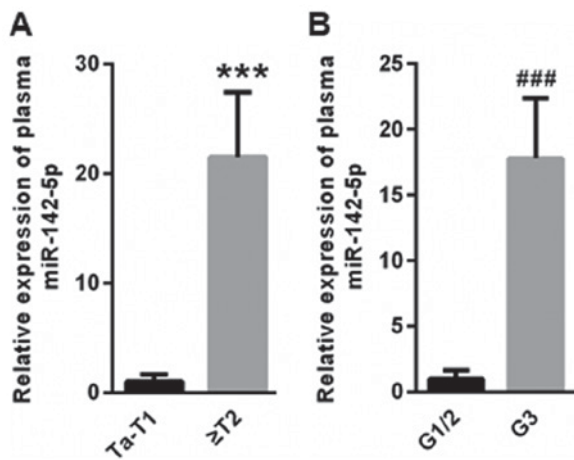


Figure 2. miR-142-5p is positively correlated with ICC metastasis and invasion. (A) Plasma miR-142-5p expression in patients with Ta-T1 and ≥T2 stage ICC. (B) Plasma miR-142-5p expression in patients with TG1/2 and G3 stage ICC. \*\*\*P<0.001 vs. Ta-T1 and ###P<0.001 vs. G1/2. miR, microRNA; ICC, intrahepatic cholangiocarcinoma.

poorer survival rate compared with those with low plasma miR-142-5p, with 5-year overall survival rates of 27.86 and 51.46%, respectively (Fig. 3). In addition, to analyze whether clinical factors, including sex, age, tumor diameter and tumor differentiation affect ICC prognosis, Kaplan-Meier survival curves were plotted and compared using a log-rank test (Table III). Log-rank analysis demonstrated that increased miR-142-5p was significantly correlated with tumor differentiation and malignancy (Table III).

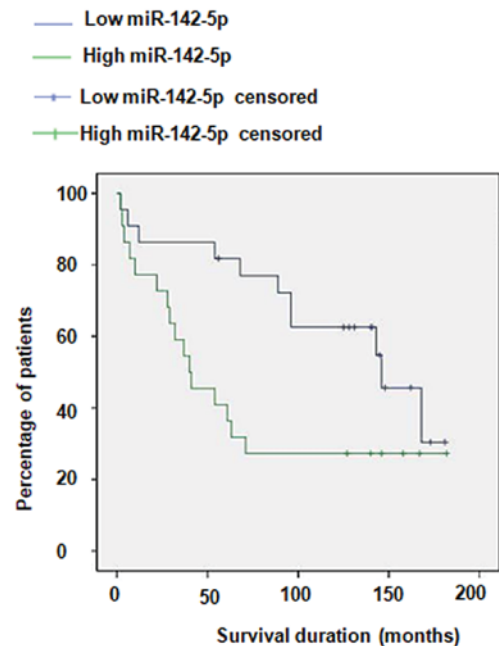


Figure 3. Kaplan-Meier analysis for plasma miR-142-5p expression and the survival of patients with intrahepatic cholangiocarcinoma. miR, microRNA.

*PTEN is a target gene of miR-142-5p.* Based on the results of TargetScan analysis, a conserved binding site of miR-142-5p in the 3'UTR of PTEN was identified (Fig. 4A). Dual luciferase reporter assay results indicated that miR-142-5p significantly suppressed the relative luciferase activity of pmirGLO-PTEN-3'UTR compared with the control (Fig. 4B).



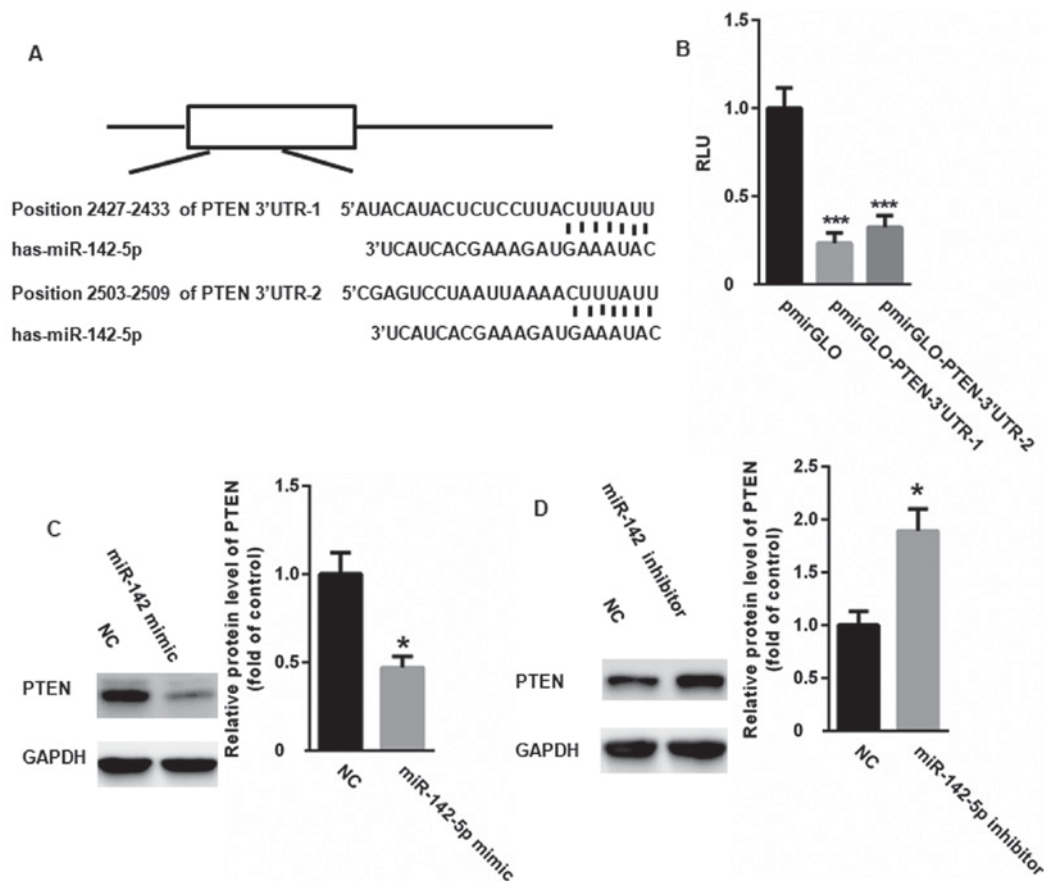


Figure 4. PTEN is a target gene of miR-142-5p. (A) A conserved binding site of miR-142-5p in the 3'UTR of PTEN was identified using TargetScan. (B) A dual luciferase reporter assay was performed to confirm the TargetScan prediction. Western blotting was performed to measure PTEN expression in cells transfected with (C) miR-142-5p mimics and (D) inhibitors. \* $P < 0.05$  vs. miR-NC. \*\*\* $P < 0.001$  vs. pmirGLO. RLU, relative luciferase unit; PTEN, phosphatase and tensin homolog; miR, microRNA; 3'UTR, 3'-untranslated region.

Western blotting also revealed that miR-142-5p overexpression downregulated PTEN (Fig. 4C), while miR-142-5p knock-down resulted in PTEN upregulation (Fig. 4D). These results confirm PTEN as a target gene of miR-142-5p.

## Discussion

ICC is the second most common intrahepatic primary tumor after hepatocellular carcinoma and ICC is highly invasive by nature and frequently metastasizes (16). It has been reported that chromosomal anomalies, genetic polymorphisms and genetic or epigenetic alterations may contribute to the tumorigenesis and progression of ICC (17,18). It is important to detect ICC early in order to improve treatment outcomes. Increasing evidence has suggested that miRNAs may be used as potential diagnostic biomarkers for ICC and may also serve as therapeutic targets (19,20).

miRNAs are able to stably exist in bodily fluids, including serum, plasma, saliva, urine and tears (20,21). Furthermore, miRNAs can be easily detected in small amounts and are resistant to degradation (20,22). These characteristics make miRNAs attractive as potential biomarkers (20). In the present study it was determined that data showed that plasma miR-142-5p was significantly increased in patients with ICC compared with healthy controls. Furthermore, miR-142-5p levels were increased in ICC tumor tissues compared with

adjacent non-neoplastic tissues. Further analysis revealed a positive correlation between miR-142-5p and clinical outcome. Compared with patients with ICC at Ta-T1 stage, miR-142-5p was significantly upregulated in patients with ICC at  $\geq T2$  stage. Additionally, patients with ICC at G3 stage had higher plasma miR-142-5p levels compared with those at G1/2 stage. These data indicate that miR-142-5p expression is positively correlated with therapy and outcome. ROC analysis indicated that miR-142-5p could be used to differentiate patients with ICC from healthy controls. Additionally, Kaplan-Meier analysis revealed that plasma miR-142-5p is negatively correlated with survival in patients with ICC. These data indicate that plasma miR-142-5p may be useful for the early detection of cancer and individualized therapies. The main focus of the present study was PTEN, which is an important tumor suppressor in the development of ICC (23). Mutation and genomic loss of PTEN have been widely reported in a number of cancers (24,25). It has been also demonstrated that liver-specific deletion of PTEN in a mouse model results in the development of ICC (26,27). As an important tumor suppressor, PTEN mainly acts to dephosphorylate phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P<sub>3</sub>], which potently activates 3-phosphoinositide-dependent kinase (PDK) and protein kinase B (AKT) (28). However, PTEN loss leads to excessive recruitment of PtdIns(3,4,5)P<sub>3</sub> at the plasma membrane, thereby activating a subset of proteins, including the AKT family and PDK1 (28). AKT signaling

induces cell survival, cell proliferation, angiogenesis and cellular metabolism via phosphorylating downstream signaling proteins (29,30). The results of the present study revealed that PTEN was a target gene of miR-142-5p. PTEN is widely acknowledged as a tumor suppressor that is mutated in multiple tumors (31-33). In the progression of ICC cancer, PTEN could negatively regulate the AKT/PKB signaling pathway, thereby increasing cancer cell growth and survival (34,35). The results of the present study indicate that miR-142-5p may suppress PTEN expression, thereby resulting in the malignant proliferation and increased viability of cancer cells.

In summary, this study presents novel data indicating that plasma miR-142-5p is significantly upregulated in patients with ICC. Further analysis demonstrated that plasma miR-142-5p could be used to screen patients with ICC from healthy controls by targeting PTEN. However, only limited samples were included in the current study. Thus, in further study, it may be necessary to include more patients to validate the application of miR-142-5p as a potential biomarker.

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### Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

### Authors' contributions

GW performed the experiments and analyzed the data. YY, XH and LJ performed RT-qPCR experiments. DJ designed all experiments, analyzed the data and gave final approval of the version of the manuscript to be published. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The research protocols were approved by the First People's Hospital of Tongxiang (Tongxiang, China) and written informed consent was obtained from the participants.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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