MicroRNA-373 exerts anti-tumor functions in human liver cancer by targeting Rab22a

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Abstract. Liver cancer is a one of the most frequent types of tumor worldwide. It has long been recognized that microRNAs are important participants in the progression of various types of cancer. The present study explored the role of microRNA-373 (miR-373) in liver cancer development. Reverse transcription-quantitative polymerase chain reaction was performed to evaluate the transcription level of miR-373 in 96 liver cancer tissues and adjacent normal liver tissues. The association of miR-373 with clinicopathological characteristics was analyzed using the χ^2 test. Kaplan-Meier univariate analysis and multivariate hazard analysis were performed to identify the clinical potential of miR-373 in the prognosis of liver cancer patients. Transfection of miR-373 mimics into Hep3B and HepG2 liver cancer cell lines was conducted to reveal the underlying mechanism in regulating liver cancer progression. The functional assays included proliferation, migration, invasion and luciferase assays. The findings of the present study demonstrated that miR-373 transcription level was markedly downregulated in liver cancer tissues compared with the adjacent normal tissues and was associated with the clinical prognosis of liver cancer patients. Overexpressing miR-373 mimics in liver cancer cell lines decreased cell proliferation and invasion, suggesting that miR-373 exerts anti-tumor effects in liver cancer. In addition, data from the present study demonstrated the direct effect of miR373 on inhibiting the expression and signaling of Ras-related protein Rab22a, a well-known oncoprotein. Taken together, the results from the present study suggested that miR-373 suppresses liver cancer progression and may serve as a promising prognosis prediction biomarker.

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Introduction

Liver cancer is a one of the most common types of cancer with high morbidity and mortality rates, particularly in China (1,2). Therapeutic options for liver cancer are stage-dependent (3). Liver cancer treatment is challenging since more than two-thirds of patients are diagnosed at advanced stages with local or distant metastasis and poor liver function caused by underlying cirrhosis (4,5). Considerable progress in understanding the molecular biology of liver cancer has been made in recent years (6). However, few effective treatments are available for advanced liver cancer. Therefore, efficient biomarkers are essential for improving the diagnosis and treatment of liver cancer.

MicroRNAs (miRNAs) are small non-coding RNAs approximately 19-23 nucleotides in length and are associated with cell proliferation, development and apoptosis (7). Generally, miRNAs can serve oncogene or tumor suppressor roles by repressing translation or inducing degradation of target messenger RNAs. For example, miR-744 expression is positively associated with poor survival of pancreatic cancer patients (8). miR-126 can inhibit non-small-cell lung carcinoma progression *in vitro* and *in vivo* by targeting the epidermal growth factor-like domain 7 (9). miR-29b and miR-195 suppress liver cancer angiogenesis, metastasis and invasion (10,11), whereas miR-10b initiates cancer cell invasion and metastasis in breast cancer (12). These data suggest that miRNAs are potential markers for targeted therapies in cancer.

miR-373 is transcribed from the location on chromosome 19q13.4 (13). Previous studies have indicated the potential prognostic role of miR-373 in cancers, including gliomas (14), bladder (15), and breast cancer (16). However, the association between miR-373 expression and liver cancer progression remains to be elucidated. The present study focused on the role of miR-373 in liver cancer development. The prognostic value of miR-373 in liver cancer and its influence in cancer cell growth and metastasis were investigated. The results indicated that miR-373 may serve as a novel anti-cancer target for liver cancer treatment.

Materials and methods

Patients and tissue samples. A total of 96 human liver cancer specimens and the matched adjacent normal tissue samples were obtained from the Seventh People's Hospital (Shanghai, China) (Table I). The diagnosis of all patients (enrolled between January 2002 and January 2012) was based on histological examination. All patients underwent surgical resections. Additionally, follow-up data were available for all patients. Tumor stages were determined according to 2007 World Health Organization (WHO) classification (17). All patients included in the present study signed written informed consents, and collection of patients' samples was authorized by the Ethical Committee for Clinical Research of the Seventh People's Hospital (Shanghai, China).

Cell culture and transfection. Human liver cancer cells (Hep3B and HepG2) were obtained from the Cell Bank of the Chinese Academy of Sciences (Beijing, China) and maintained with RPMI-1640 containing 10% fetal bovine serum (BSA; both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 1% streptomycin and penicillin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated in a humidified atmosphere of 5% CO_2 at 37˚C. The miR-373 mimics and negative control (NC) used in the present study were produced by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequence of miR-373 mimics was 5'-GAAGUGCUUCGAUUUUGGGGUGU-3'. The NC sequence was 5'-UGGGCGUAUAGACGUGUUACAC-3'. Both miRNAs were used at a final concentration of 50 nM. The transfections were carried out using Lipofectamine® 2000 in accordance with the manufacturer's protocols (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested after 48 h for further experiments.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from tissues (cell density, 10^6 cells/ml) using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.). The absorbance at 260 and 280 nm was used to validate RNA purity and concentration using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Total miRNA was synthesized using miRNA First Strand cDNA Synthesis (Tailing Reaction) kit supplied by Sangon Biotech Co., Ltd. (Shanghai, China) following the manufacturer's protocols (18). RT-qPCR was performed with SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China) to detect the expression of mature miRNAs. The qPCR reactions were performed with an initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, and 60˚C for 35 sec using the ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The $2^{-\Delta\Delta Cq}$ method was applied to calculate the transcription level of miR-373 with U6 small nuclear RNA as the normalization reference gene (19). Primers used were: miR-373 forward, 5'-GTCGTATCCAGTGCAGGGTCCGAGGT-3'; U6 forward, 5'-CGAATTTGCGTGTCATCCT-3'. The universal PCR reverse primer was provided by the cDNA Synthesis kit. The experiments were repeated three times.

Cell proliferation assay. Hep3B and HepG2 cells were reseeded on 96-well plates at $3x10^3$ cells/well following transfection with miRNA mimics for 24 h. The effect of miR-373 on the proliferation of liver cancer cells was evaluated by MTT assay at a daily interval for 4 days; 20 μl of 5 mg/ml MTT was added to each well and incubated for 4 h at each time point. The medium was discarded, and the precipitated formazan was dissolved in 150 μl DMSO. Absorbance was measured at 450 nm using a 96-well plate reader (Thermo Fisher Scientific, Inc.).

Transwell invasion assay. Invasion assays were performed using Transwell chambers (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (20). Cells ($1x10^5$) were re-suspended and added in the upper chamber in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). Then, 20% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber. The membranes were precoated with Matrigel for 1 h at 37˚C. Following invasion across the Matrigel-coated membranes for 48 h, invasive cells were observed by 0.5% crystal violet staining for 10 min at room temperature.

Plasmids, transfection, and luciferase activity assays. The Ras-related protein Rab22a plasmid was purchased from Addgene (Cambridge, MA, USA) and subcloned into pGL3 luciferase vector, the mutant of Rab22a was constructed by site-specific mutagenesis strategy as described by others (21).

Hep3B cells were seeded into a 24-well plate at the density of $1x10^4$ cells/well. Cells were transfected with miR-373 mimics and Rab22a plasmids using Lipofectamine® 2000 in accordance with the manufacturer's protocols (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, a blank vector was introduced as the control of miR-373, which was also co-transfected with either Rab22a-WT (wild-type) or Rab22a-mutant plasmids containing firefly luciferase. The value of relative luciferase activity was evaluated using a dual luciferase assay kit (Promega Corporation, Madison, WI, USA) (22). For each well, luciferase activity was normalized to *Renilla* luciferase activity.

Western blot analysis. Immunoblotting was conducted to test protein levels as described elsewhere (23). Briefly, cultured cells were lysed using RIPA buffer supplemented with protease inhibitor cocktails (Roche Diagnostics, Indianapolis, IN, USA). Following the determination of the extracted protein concentration by a BCA kit, 20 μg of total proteins was subjected to 12% SDS-PAGE and transferred onto nitrocellulose membranes. Following blocking with 5% BSA (Sigma-Aldrich; Merck KGaA) in tris-buffered saline/Tween (0.05%) buffer for 1 h at room temperature, the membranes were incubated with primary antibodies (dilution, 1:1,000) at 4˚C overnight. The antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), including Rab22a (cat. no. sc-390726), E-cadherin (cat. no. sc-71009), and β-actin (cat. no. sc-517582). Corresponding horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (cat. no. sc-2039; Santa Cruz Biotechnology, Inc.; 1:10,000 dilution) were added and incubated for another 1 h at room temperature. The immunoreactivity was detected using the Pierce enhanced electrochemiluminescence western blotting substrate (Thermo Fisher Scientific, Inc.) and X-ray film. Data

Table I. Correlations between miR-373 and clinicopathological factors.

Clinicopathological factor	Number	Percentage	miR-373		P-value[a]
			Low	High	
Age (years)					
<50	58	60.4	33	25	0.384
≥50	38	39.6	25	13	
Sex					
Female	26	27.1	18	8	0.282
Male	70	72.9	40	30	
AFP (U/ml)					
<400	34	35.4	19	15	0.501
≥400	62	64.6	39	23	
Tumor number					
Single	67	69.8	33	34	0.001[b]
Multiple	29	30.2	25	4	
Tumor size (cm)					
≤5	46	47.9	19	27	<0.001[b]
>5	50	52.1	39	11	
Tumor location					
Unilateral	85	88.5	51	34	0.816
Bilateral	11	11.5	7	4	
Histopathological grade					
Well/moderate	46	47.9	23	23	0.045[b]
Poor/undifferentiated	50	52.1	35	15	
Surgical margin (mm)					
≤10	50	52.1	27	23	0.180
>10	46	47.9	31	15	
TNM stage					
I/II	47	49.0	20	27	<0.001[b]
III/IV	49	51.0	38	11	

[a]Analyzed by χ^2 test; [b]statistically significant. miR, microRNA; AFP, α-fetoprotein; TNM, TNM Classification of Malignant Tumors.

were analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as the loading control. Each experiment was performed three times.

Target prediction. TargetScan tool (version 7.1; www.targetscan.org) was used to investigate the potential targets of miR-373. Rab22a gene was one of the predicted targets and was selected for further analysis. Subsequently, the predicted miRNAs targeting Rab22a were investigated using TargetScan (version 7.1) and various miRNAs were identified, including miR-373-3p.

Statistical analysis. Statistical analyses were performed by SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation from ≥3 separate experiments. Difference among two groups was compared by Student's t-test (two-tailed). Comparison between multiple groups was performed by one-way analysis of variance followed by least significant difference post hoc test. The prognosis of patients was evaluated by the Kaplan-Meier method and log-rank test, and multivariate analysis was conducted to test potential clinical variables using Cox regression test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-373 transcription level is downregulated in liver cancer tumors. RT-qPCR was performed to determine the miR-373 expression in liver cancer and adjacent normal tissues. miR-373 transcription level was significantly downregulated in 96 liver cancer tissues compared with the adjacent tissues (Fig. 1A; $P<0.05$). In addition, the expression of miR-373 was negatively associated with cancer stages (Fig. 1B).

Correlation between miR-373 expression and clinicopathological factors in liver cancer patients. As shown in Table I, all 96 liver cancer patients were grouped in high- or low-miR-373 groups based on the median value of miR-373 level in tumor tissues. miR-373 level was significantly associated with

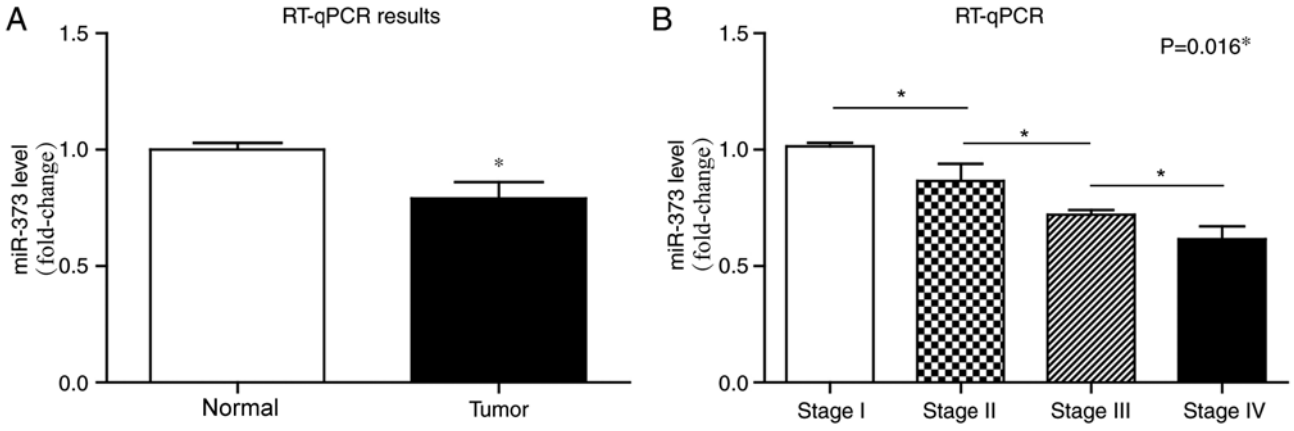

Figure 1. miR-373 is downregulated in liver cancer tissues. (A) miR-373 was downregulated in liver cancer tissues compared with adjacent normal tissues, *P<0.05. (B) Reverse transcription-quantitative polymerase chain reaction analysis of miR-373 in all 4 TNM stages of liver cancer.

Table II. Correlation between clinical factors and survival rates.

Clinical factor	5-year survival rate (%)	P-value[a]
Age (years)		
<50	49.5	0.738
≥50	48.7	
Sex		
Female	40.9	0.100
Male	52.2	
AFP (U/ml)		
<400	36.6	0.964
≥400	56.5	
Tumor number		
Single	56.2	0.014[b]
Multiple	30.9	
Tumor size (cm)		
≤5	63.4	0.098
>5	29.8	
Tumor location		
Unilateral	48.8	0.255
Bilateral	59.7	
Histopathological grade		
Well/moderate	56.0	0.210
Poor/undifferentiated	42.2	
Surgical margin (mm)		
≤10	68.3	<0.001[b]
>10	20.9	
TNM stage		
I/II	64.2	<0.001[b]
III/IV	31.8	
miR-373 level		
Low	31.0	<0.001[b]
High	75.1	

[a]Analyzed by log-rank test; [b]statistically significant. AFP, α-fetoprotein; TNM, Classification of Malignant Tumors; miR, microRNA.

Table III. Cox multivariate analysis.

Clinical factor	HR	95% CI	P-value[a]
Tumor number			
Single	1		
Multiple	0.63	0.26-1.51	0.301
Surgical margin (mm)			
≤10	1		
>10	3.1	1.52-6.43	0.002[b]
TNM stage			
I/II	1		
III/IV	3.2	1.24-8.22	0.016[b]
miR-373 level			
Low	1		
High	0.43	0.17-0.94	0.048[b]

[a]Calculated by Cox-regression model; [b]statistically significant. HR, hazard ratio; CI, confidence interval; miR, microRNA.

tumor number (P=0.001), tumor size (P<0.001), histopathological grade (P=0.045) and TNM stage (P<0.001), but was not correlated to age (P=0.384), sex (P=0.282), α-fetoprotein (AFP; P=0.501), tumor location (P=0.816) or surgical margin (P=0.180).

Prognostic value of miR-373 for liver cancer patients. Survival analysis was performed to test whether clinicopathological factors and miR-373 expression level were associated with liver cancer prognosis. As shown in Fig. 2 and Table II, according to a univariate analysis, tumor number (P=0.014), surgical margin (P<0.001), TNM stage (P<0.001) and miR-373 level (P<0.001) were all significant prognostic factors. Additionally, Cox multivariate analysis indicated that surgical margin [hazard risk (HR)=3.1, 95% confidence interval (CI)=1.52-6.43, P=0.002], TNM stage (HR=3.2, 95% CI=1.24-8.22, P=0.016) and miR-373 level (HR=0.43, 95% CI=0.17-0.94, P=0.048) were independent prognostic indicators for liver cancer (Table III).

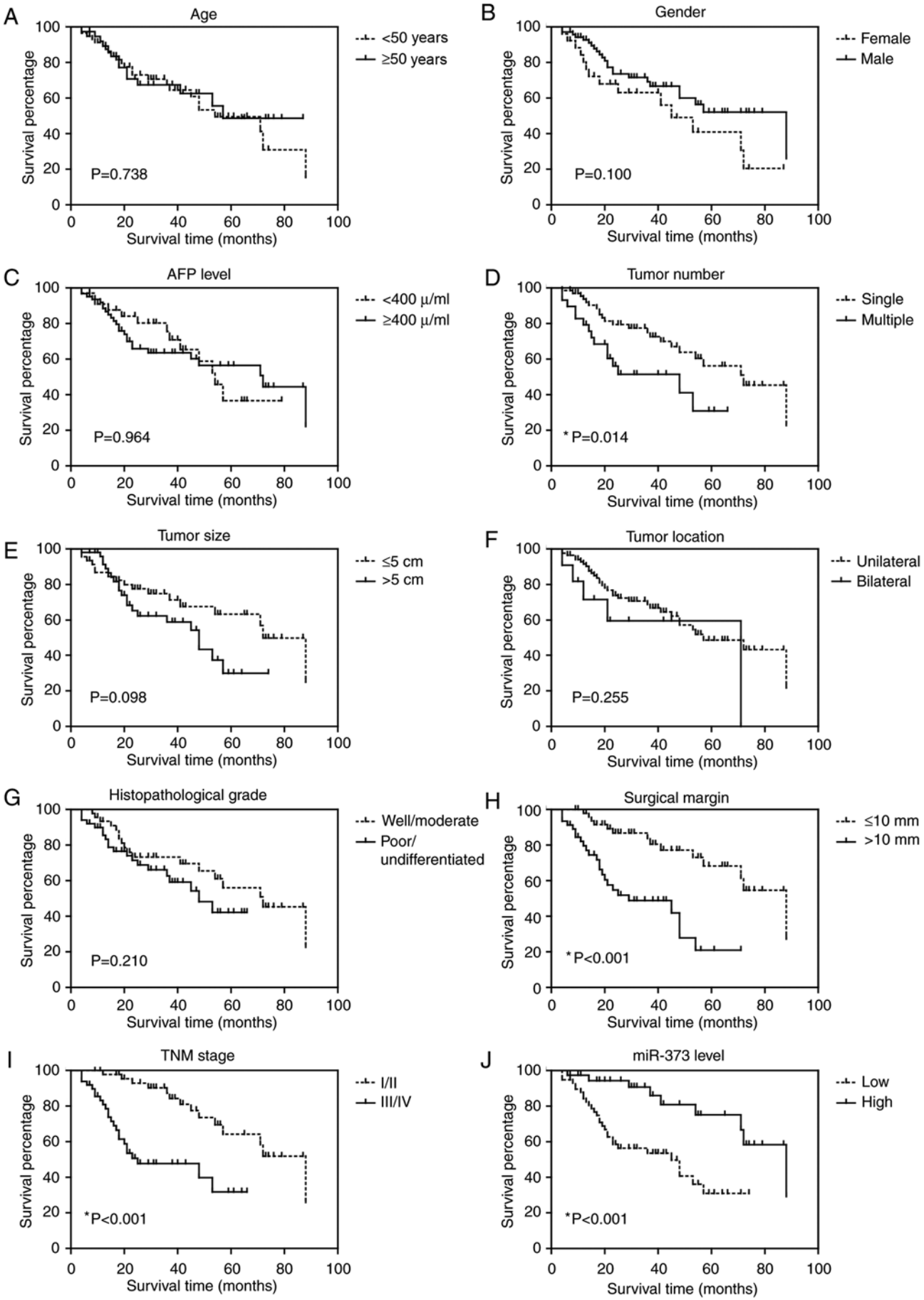

Figure 2. Overall survival curve of liver cancer patients. Kaplan-Meier analysis of the overall survival in 96 liver cancer patients in relation to (A) age, (B) sex, (C) AFP level, (D) tumor number, (E) tumor size, (F) tumor location, (G) histopathological grade, (H) surgical margin, (I) TNM stage and (J) miR-373 level. AFP, α-fetoprotein; miR, microRNA.

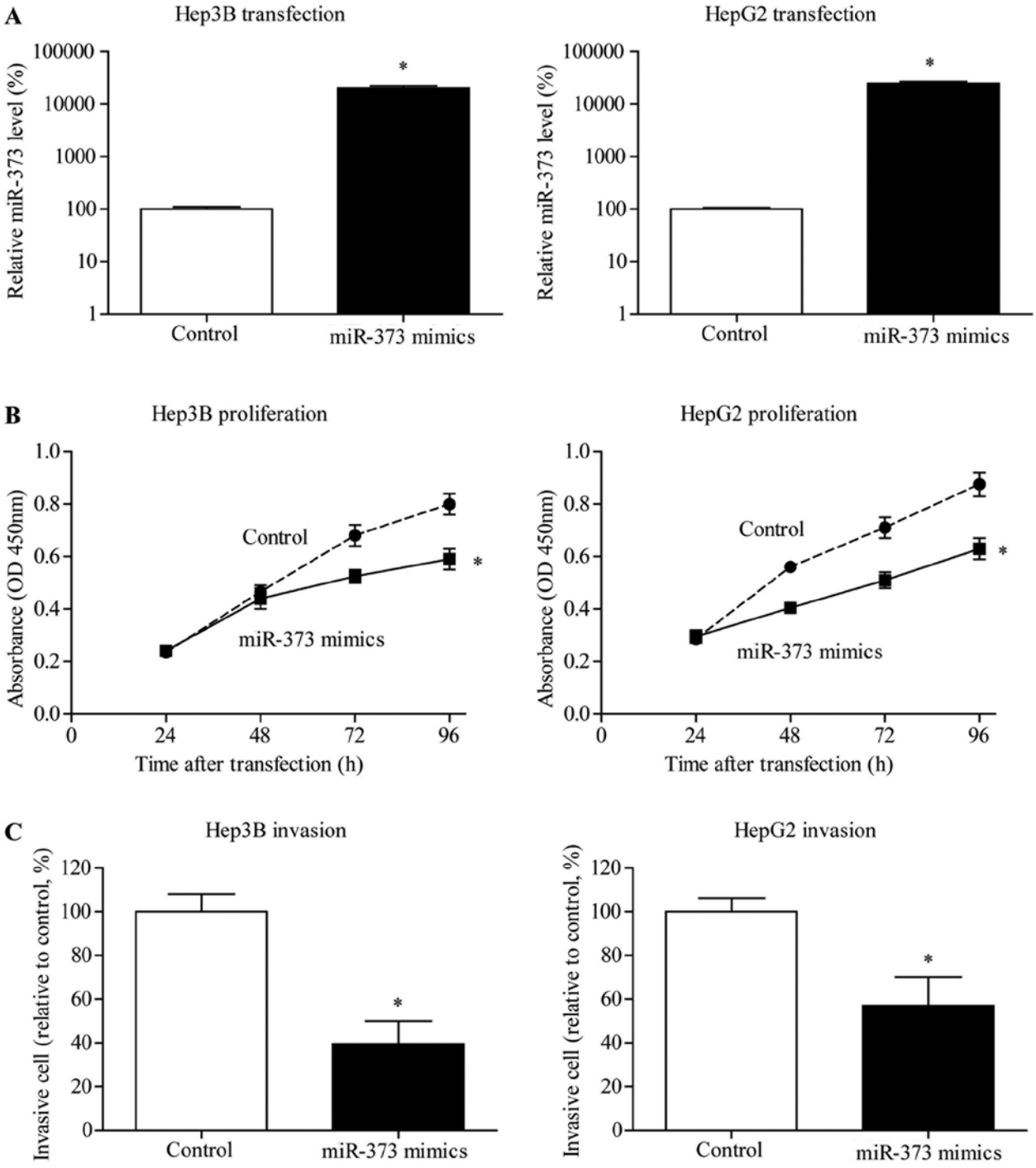

Figure 3. Effect of miR-373 on the proliferation and invasive potential of liver cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-373 expression in Hep3B and HepG2 cells following transfection with mimics. *P<0.05. (B) MTT assays of Hep3B and HepG2 cells following transfection with miR-373 mimics or negative control. *P<0.05. (C) Transwell invasion assays of Hep3B and HepG2 cells with miR-373 mimics or negative control transfection. *P<0.05. miR, microRNA.

miR-373 inhibits the viability of liver cancer cells. To further explore possible the effect of miR-373 on the proliferation of liver cancer, miR-373 transiently transfected cells were established (Fig. 3A). MTT results indicated that proliferation of Hep3B and HepG2 cells in the miR-373 transfection group was notably inhibited compared with NC groups (Fig. 3B).

miR-373 inhibits the invasive capacity of liver cancer cells. To investigate whether miR-373 upregulation served critical roles in the metastasis of liver cancer cells, miR-373 mimic was transfected in Hep3B and HepG2 cell lines. Fig. 3C demonstrates a substantial reduction in the invasion of liver cancer cells following the upregulation of miR-373 expression level.

miR-373 targets Rab22a and elevates E-cadherin level. The present study next explored the underlying mechanism of miR-373 in inhibiting tumor progression in liver cancer. By

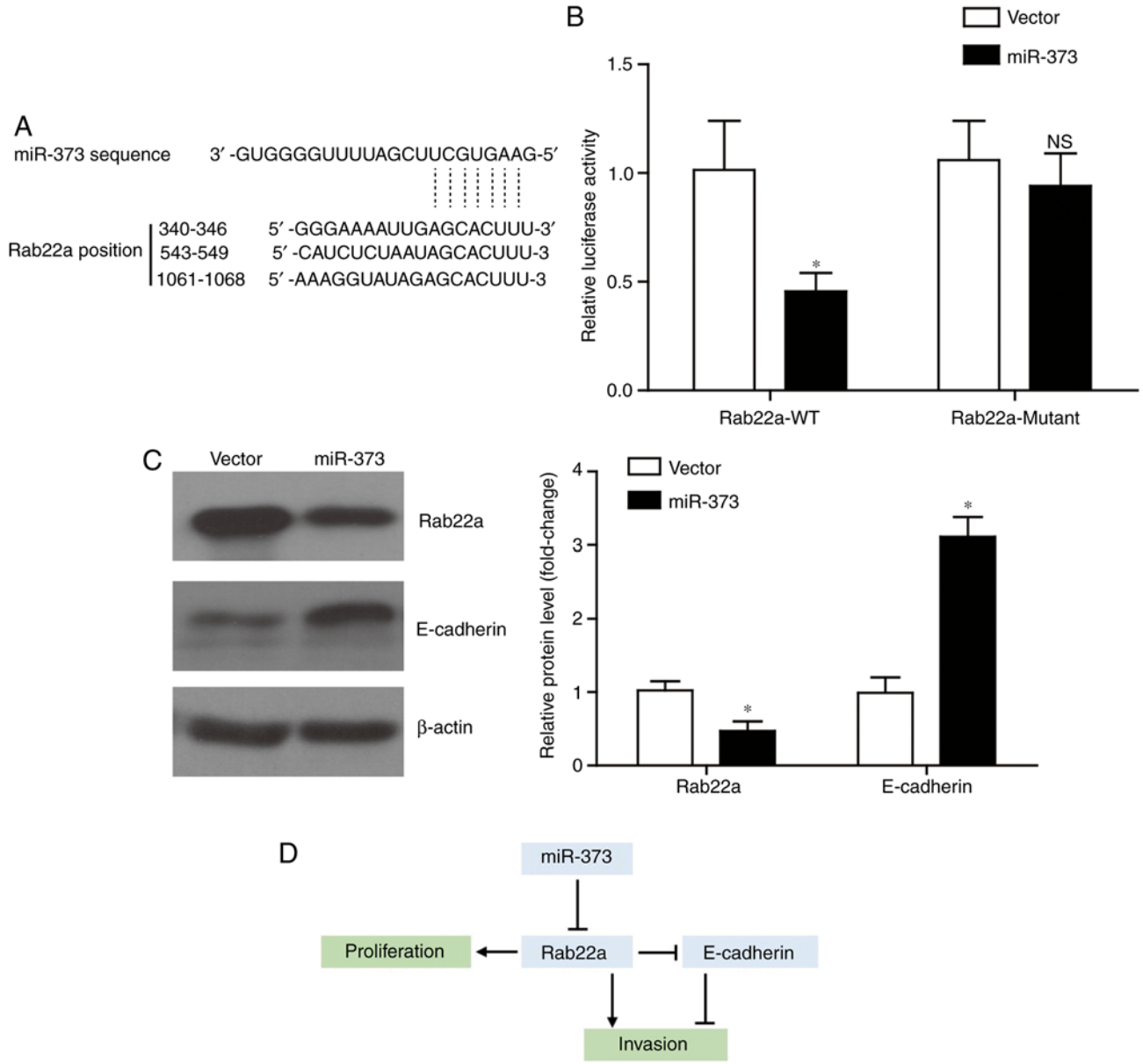

Figure 4. miR-373 directly suppresses Rab22a signaling. (A) The putative miR-373 binding site with Rab22a was predicted by TargetScan (www.targetscan.org). (B) The luciferase activity of wild type (WT) Rab22a was inhibited by miR-373; however, miR-373 demonstrated no significant effect on the luciferase activity of the mutant Rab22a (which mutated all 3 binding sites shown in (A). *P<0.05; NS, no significance. (C) Western blot analysis demonstrated the effect of miR-373 on inhibiting the expression of Rab22a, while increasing the protein level of E-cadherin. *P<0.05 (D) A schematic model demonstrated the hypothetical mechanism of miR-373 on suppressing liver cancer progression.

using the TargetScan tool (www.targetscan.org), it was identified that Rab22a possessed three potential binding sites with miR-373 (Fig. 4A), which is consistent with a recent study (24). Furthermore, it was verified by using luciferase assays that miR373 can directly regulate the transcription of Rab22a (Fig. 4B). By contrast, following the mutation of the binding sites on Rab22a, miR-373 co-transfection exhibited little effect on its luciferase activity. Western blot analysis results demonstrated that miR-373 overexpression can inhibit the expression of the oncoprotein Rab22a, while increasing the protein level of E-cadherin (Fig. 4C). Considering the regulatory role of Rab22a on E-cadherin expression (25), an Rab22a-dependent role for miR-373 on inhibiting liver cancer proliferation and invasion was hypothesized (Fig. 4D).

Discussion

A previous study demonstrated that miRNAs serve non-negligible roles in the progression of various tumors, including liver cancer (26). Previous studies have reported that miR-373 serves as either an oncogene or antioncogene in several cancer types with variable expression. For instance, an upregulated level of miR-373 attenuates TGF-β-induced metastasis of breast cancer cells *in vivo*, indicating the tumor suppressor activity of miR-373 (27). Another study demonstrated that a low level of miR-373 is associated with poorer survival rates in hilar cholangiocarcinoma (28). Conversely, it has been noted that miR-373 is upregulated in human cervical cancer tissues and its overexpression promotes the tumorigenicity of cervical cancer cells by targeting the *YOD1* gene (29). Consistently,

miR-373 can enhance tumor metastasis of breast cancer by directly suppressing thioredoxin-interacting protein (30).

The current study established the clinicopathological role of miR-373 in liver cancer patients. RT-qPCR was performed to evaluate the endogenous transcription of miR-373 and indicated that it was significantly decreased in liver cancer tissues compared with the adjacent normal tissues. Further investigation demonstrated that low miR-373 levels were associated with multiple tumor number, larger tumor size, poorly differentiated histopathological and advanced TNM stages. Kaplan-Meier survival analysis demonstrated that high expression levels of miR-373 in the tissues of patients was a predictor of improved prognosis while low miR-373 expression levels suggested a worse prognosis. Cox multivariate analysis also supported the hypothesis that miR-373 expression level was an independent prognostic factor for the survival rates of liver cancer patients. In addition, transient high expression of miR-373 in Hep3B and HepG2 cells attenuated the proliferation of liver cancer. miR-373 exerted an inhibitory effect in the invasion of liver cancer cells, confirming the antioncogenic role of miR-373 in liver cancer.

Previous studies have revealed specific targets of miR-373 in other tumor types. For example, miR-373 targets the transforming growth factor-β type II receptor and reduces its protein expression, therefore suppressing breast cancer migration and invasion (8,27). miR-373 also demonstrates an inhibitory effect on the expression of estrogen receptor in breast cancer, which subsequently regulates the downstream matrix metalloproteinases signaling and suppresses tumor progression (31). Therefore, the targets and mechanisms could be distinctive in liver cancer due to the histospecificity of miRNAs. According to the data of the present study, the tumor inhibiting effect of miR-373 in liver cancer was exerted, at least partly, by suppressing the Rab22a signaling pathway, which is consistent with its functions in ovarian cancer (24). However, Wu *et al* (32) reported that miR-373 is upregulated in human liver cancer tissues and promotes tumor progression by targeting the protein phosphatase 6 catalytic subunit. It is reasonable to hypothesize that specific miRNAs may serve multiple roles even in the same tumor type. For example, miR-708-5p has been shown to exert different roles in lung cancer by targeting distinct downstream targets (33,34).

Another possible explanation for the differences between Wu *et al* (32) and the present study may be due to the hepatitis effect. The majority of the Chinese liver cancer patients were the result of hepatitis, and the expression of miR-373 has been reported to exhibit significant crosstalk with hepatitis B and C (35-37). However, neither the present study nor that of Wu *et al* retrieved the hepatitis information of enrolled patients. Similarly, whether the patients were treated with anti-hepatitis drugs or anti-tumor drugs prior to specimen collection may also affect the results. Additionally, the results from the present study and those of Wu *et al* were drawn from different medical centers, therefore it may also reflect bias due to limited patient resources in specific regions. Besides, Wu *et al* used pri-miR-373-expressing pcDNA3 vector to overexpress miR-373 in HepG2 cells, while the present study directly transfected cells with miR-373 mimics, which may also be a reason for significant differences. Although a previous study reported that co-expression of miR371, miR372 and miR373 clusters can promote liver cancer progression (38), the authors did not test the individual roles of each miRNA. Therefore, further *in vivo* studies are necessary to verify the exact and multifaceted roles of miR-373 in liver cancer. In summary, the present study revealed that downregulated miR-373 predicted poor prognosis in liver cancer patients. In addition, miR-373 inhibited the proliferation and invasion of liver cancer cells *in vitro*. The outcomes represent a potential drug target for liver cancer therapy.

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Availability of data and materials

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

Authors' contributions

WX designed the present study. YY, LZ, YS and JZ performed the *in vitro* experiments and interpreted the data. GW, JN and SZ analyzed the clinical datasets and performed western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients included in the present study signed written informed consents and collection of patients' samples was authorized by the Ethical Committee for Clinical Research of the Seventh People's Hospital (approval no. v1.0-2015-04-23).

Patient consent for publication

All patients included in this study signed written informed consent.

Competing interests

The authors declare that they have no competing interests.

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