MicroRNA-367 promotes progression of hepatocellular carcinoma through PTEN/PI3K/AKT signaling pathway

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Abstract

The study aimed to investigate the functional roles of microRNA (miR)-367 in progression of hepatocellular carcinoma (HCC), as well as its regulation on PI3K/AKT pathway. The relative expression of miR-367 in HCC tissues and cell line was detected using quantitative real-time polymerase chain reaction (qRT-PCR) method. Chi-square test was applied to analyze the relationship between miR-367 expression and clinical characterizes of HCC patients. The influences of miR-367 expression on cell proliferation, migration and invasion were analyzed using MTT and transwell assays respectively. Western blot assay was performed to for protein analysis. HCC tissues and cell lines exhibited significant up-regulation of miR-367. Moreover, the elevated expression of miR-367 was positively correlated with tumor size ($P=0.005$), metastasis ($P=0.004$) and TNM stage ($P<0.001$). Knockdown of miR-367 expression could inhibit cell proliferation, migration and invasion in vitro. While, enhanced miR-367 expression exhibited opposite effects. Besides, inhibition of miR-367 might enhance PTEN expression, reduce the levels of p-GSK3β and p-AKT. PTEN might be a target of miR-367 in HCC. The inhibition of PTEN could reverse the anti-tumor action caused by the knockdown of miR-367. MiR-367 serves as an oncogene in HCC through activating the PI3K/AKT pathway by targeting PTEN.

**Key words:** Hepatocellular carcinoma; MiR-367; PTEN; PI3K/AKT pathway
Introduction

Hepatocellular carcinoma (HCC) is a prevalent malignant disease, with increasing incidence rate around the world [1]. The cancer is characterized by silent growth at early stages, and rapid metastasis, thus most of the patients present advanced stages when initial diagnosis, leading to poor prognosis [2, 3]. Although there are various available treatments for HCC, the clinical outcomes of the patients still remain dismal [4]. The five-year survival rate of HCC patients is less than 20% [5]. HCC is a heterogeneous disease which is regulated by the interactions of environmental and genetic factors [6]. A variety of risk factors have been confirmed for HCC, including cirrhosis, hepatitis virus infection, alcohol abuse, obesity, liver disease, smoking, and type 2 diabetes [7]. However, the key factors to drive the progression of HCC are still unconfirmed.

Growing evidences have demonstrated that tumor genetics play a key role in development and progression of HCC [8]. The dysregulation of the genes which are involved in cell cycle, growth, motility, apoptosis, may contribute to the progression of HCC [9]. MicroRNAs, a group of endogenous RNAs, have no protein encoding ability, but they can take part in gene regulation through binding to the 3’ untranslated regions (UTR) of their target mRNAs [10, 11]. MiRNAs take part in various biological processes, such as development, differentiation, cell cycle, tumorigenesis [12, 13]. The dysregulation of miRNAs has been frequently observed in cancer cells, revealing their close association with tumorigenesis. The expression profiles of miRNAs are significantly correlated with cancer development and progression, which may be used for cancer monitoring and therapy [14]. To explore the function of miRNAs may provide a new insight into the etiology of cancer.

MicroRNA-367 (MiR-367) is a common member of miRNA family, and its dysregulation has been reported in several human cancers, including non-small cell lung cancer [15], uveal melanoma [16], osteosarcoma [17], renal cell carcinoma [18], etc. In HCC, Wang et al. [19] reported that the expression of miR-367 was up-regulated, and its elevated expression predicted poor prognosis for the patients. However, the molecular mechanisms of miR-367 in HCC had been rarely reported.

In current study, we investigated the expression of miR-367 in HCC, as well as its clinical significance. In addition, the cell experiments were designed to explore the molecular mechanisms underlying the function of miR-367 in HCC.
Materials and methods

Study subjects and tissue collection
A total of 126 HCC patients were recruited from Hunan Provincial Tumor Hospital. The surgical HCC tissues and adjacent normal tissues were collected from each patient. None of the patients had received any preoperative treatments, such as chemotherapy, radiotherapy, or immune treatments. The tissue specimens were immediately frozen in lipid nitrogen, and kept at -80°C. The baseline characteristics of the patients, such as age, gender, smoking, tumor size, lymph node metastasis and TNM stage were collected from their medical records.

The current study was approved by the Ethic Committee of the hospital. The written informed consents were obtained from all the subjects before tissue collection.

Cell line and culture
Human HCC cell line HepG2 (code: SCSP-510) and human immortalized hepatocytes THLE-3 (code: GNHu40) cell lines were cultured in RPMI-1640 medium with supplement of 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were cultured in an incubator with 5% CO₂ at 37°C. Both of the cell lines were bought from the Cell Bank of the Chinese Academy of Science (CBP600232; Shanghai, China).

RNA extraction and quantitative analysis
Total RNA was extracted from HCC tissue and cell specimens using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) following the instruction of the manufacture. Then, the first strand of cDNA was synthesized by the reverse transcription which was carried out by PrimerScript RT reagent kit (Takara, Dalian, China). The relative expression of the target genes was estimated through quantitative real-time polymerase chain reaction (qRT-PCR). The specific primer sequences were as follows: 

- **U6** forward: 5’-CTCGCTTCGGCAGCACA-3’, reverse: 5’-AACGCTTCACGAATTTGCGT-3’;
- **miR-367** forward: 5’-TTCTCCGAACCTTGTGTCACGTTT-3’, reverse: 5’-ACGTGACACGTTCGGAGAATT-3’;
- **GAPDH** forward: 5’-TGCACCACCAACTGCTTAGC-3’, reverse: 5’-GGCATGGACTGTGGTCATGAG-3’;
- **PTEN** forward: 5’-TTCCTTCCTCCAGCAAA-3’, reverse: 5’-GATTCCTCCGGCTGT-3’.
forward, 5'-TGGATTCGACTTAGACTTGACCT-3’, reverse, 5'-GGTGGGTTATGGTCTTCAAAAGG-3'. *U6* acted as internal reference for miRNA, while *GAPDH* was employed as internal control for mRNA. The results were calculated by $2^{-\Delta\Delta Ct}$ method. Each test was repeated in triplicate.

**Cell transfection**

HCC cell line HepG2 at logarithmic phase were harvested from cell transfection. *MiR-367* mimic, *miR-367* inhibitor, si-*PTEN*, over-expression *PTEN*, as well as the corresponding negative controls were constructed by HANBIO company (Shanghai, China; supplementary Table 1). The collected cells were digested using 0.25% trypsin, and seeded into a 6-well plate with the density of $1\times10^5$. Cell transfection was performed by Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacture’s introduction. Then the cell medium was cultured at 37°C with 5% CO₂. 48h later, the cells were collected, and the relative expression level of the target genes were detected using qRT-PCR method to estimate the transfection efficacy.

**Cell proliferation**

The proliferation ability of the cells was estimated using MTT assay. 48h after cell transfection, the cells were seeded to the 96-well plate ($2\times10^4$ cells/well), then the cells were kept in an incubator containing 5% CO₂ at 37°C. Then, 50μL MTT (5mg/mL) was added to cell medium at each detected time point, including culturing for 0h, 24h, 48h and 72h. The cells were incubated with MTT for 4h, and then he absorbance at 490 nm was read with a Microplate Reader (TECAN, Salzburg, Austria).

**Cell motility**

The cell motility was estimated by transwell assay (8.0 μm pore size, Costar, Shanghai, China). The lower chamber contained 500μL RPMI-1640 medium with 10% FBS, while 200μL serum-free RPMI-1640 medium was added to the upper chamber. For cell invasion, the upper chamber was coated with Matrigel (Corning Glass Works, Corning, N.Y., USA). 200μL cell suspension solution ($5\times10^4$/mL) was seeded to the upper chamber, and then the chamber was cultured at 7°C with 5% CO₂. 48h later, the lower chamber was stained by crystal violet, and the cells were numbered using an inverted microscope (IX31; Olympus Corporation, Tokyo, Japan). Five random files were selected.
Each test was repeated three times.

**Western blot**

In our study, western blot assay was performed for protein analysis. The procedures were carried out according to the standards. In brief, the protein samples were isolated from the cultured cells using RIPA Lysis and Extraction Buffer (Thermo Scientific, Waltham, MA, USA), and quantified by BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Next, the same volume of protein specimens were loaded to 10% SDS-PAGE. The proteins were transfected to a polyvinylidene fluoride membrane (PVDF) (0.45 µm pore size; EMD Millipore, Billerica, MA, USA) under 10mA for 40min. Then, the membranes were blocked with 5% skim milk powder at room temperature for 2 h. Subsequently, the membranes were included with the primary specific antibodies for overnight at 4°C, including anti-PTEN antibody (dilution, 1:1,000; cat. No. ab32199l, Abcam), anti-p-AKT antibody (dilution, 1:1,000; cat. No. ab38449, Abcam), AKT antibody (dilution, 1: 2,000; cat. No. ab28422, Abcam), GSK3β antibody (dilution, 1:1,000; cat. No. ab93926, Abcam), p-GSK3β antibody (dilution, 1:1,000; cat. No. ab75745, Abcam). GAPDH was employed as internal control, and the primary GAPDH antibody was bought from Sigma-Aldrich (Germany, dilution, 1:500; cat. No. SAB4300645-100UG). Then, the membranes were incubated with the secondary anti-rabbit IgG antibody (dilution, 1:2,000; cat. No. ab6709; Abcam) for 2 h at room temperature. The results were analyzed by the Chemi Genius gel imaging system.

**Luciferase reporter assay**

The bioinformatic analysis demonstrated that *miR-367* could bind to the 3’UTR of *PTEN* gene. Thus, luciferase reporter assay was performed to confirm whether *PTEN* was a potential target of *miR-367* in HCC. The 3’UTR of *PTEN* containing the binding site of *miR-367* (PTEN-wt), as well as the mutated region without the binding site of *miR-367* (PTEN-mt) was amplified through PCR method. Then the amplified fragments were inserted into firefly luciferase reporter vector pGL3. Subsequently, the HepG2 cells were co-transfected by PTEN-wt or PTEN-mt and *miR-367* mimic or mimic NC. 48h later, the luciferase activity of the cells was measured using Dual-Luciferase Reporter Assay System (Promega Corporation).
**Statistical analysis**

All the data calculation was finished using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The continuous data were shown as mean ± standard deviation (SD), and their comparisons between two groups were performed by student’s t test. Chi-square test was used to estimate the association of miR-367 expression with clinical characteristics of HCC patients. All the tests were two-tailed, and *P* values less than 0.05 predicted the statistical significance of the results.

**Results**

**Baseline characteristics of the study subjects**

In our study, 126 patients who were pathologically diagnosed with HCC were included. There were 76 males (60.32%) and 50 females (39.58%), and their average age was 58.76 ± 12.78 years. 68 (53.97%) patients had smoking history, and 67 patients (53.17%) exhibited tumor size more than 3 cm. Lymph node metastasis was observed in 48 patients (38.10%). According to Tumor Node Metastasis (TNM) staging system, 69 patients were confirmed with stages I-II, while 57 (45.24%) cases were diagnosed with stages III-IV. The baseline characteristics of the patients were summarized in Table 1.

**Up-regulation of miR-367 in HCC tissues and cells**

The expression patterns of miR-367 were detected in HCC tissues and cells. As shown in Figure 1, the levels of miR-367 were significantly higher in HCC tissues (Figure 1A) and cell lines (Figure 1B) than that in the non-cancerous specimens (*P*<0.001).

**Relationship between miR-367 expression and clinical characteristics of HCC patients**

The included patients were divided into high expression group (n=58) and low expression group (n=68) based on their mean expression levels of miR-367 in HCC tissue samples. Chi-square test was performed to estimate the association of miR-367 with clinical characteristics of HCC patients. The results demonstrated that the expression of miR-367 showed positive association with tumor size (*P*=0.005), lymph node metastasis (*P*=0.004), and TNM stage (*P*<0.001). Meanwhile, the expression of miR-367 had no significant association with patients’ age (*P*=0.734), gender (*P*=0.069), or
smoking history ($P=0.333$) (Table 1).

**Effects of cell transfection on expression of miR-367 in HepG2 cells**

To estimate the functional roles of miR-367 in HCC, the miR-367 mimic and miR-367 inhibitor were designed in our study. QRT-PCR method was used to detect the expression of miR-367 in the transfected cells. The results indicated that compared to the controls, the transfection of miR-367 mimic could obviously enhance the expression of miR-367 in HepG2 cells ($P<0.01$), while miR-367 inhibitor transfection might reduce miR-367 expression ($P<0.001$) (Figure 2).

**MiR-367 could promote HCC cell proliferation, migration and invasion**

The biological behaviors of the transfected cells were detected. MTT assay demonstrated that the transfection of miR-367 mimic could enhance the proliferation ability of HepG2 cells ($P<0.01$), while the inhibition of miR-367 obviously suppressed cell proliferation ($P<0.01$) (Figure 3A). Transwell assay demonstrated that the migration and invasion abilities of the cells transfected by miR-367 mimic were significantly enhanced, while the transfection of miR-367 inhibitor could inhibit cell motility ($P<0.01$) (Figure 3B and C).

**MiR-367 targeted PTEN in HCC**

Bioinformatics analysis revealed that miR-367 could bind to 3’UTR (position 420-430) of PTEN (Figure 4A). Luciferase reporter assay demonstrated that the luciferase activity of the cells co-transfected by PTEN-wt and miR-367 mimic was significantly decreased compared to the co-transfection of PTEN-wt and mimic NC ($P<0.01$). However, the co-transfection of PTEN-mt and miR-367 mimic had no significant effects on luciferase activity, compared to the controls ($P>0.05$) (Figure 4B). The results suggested that miR-367 could bind to the 3’ UTR of PTEN gene.

QRT-PCR method was performed to detect the relative expression of PTEN mRNA HCC cells. We found that the expression of PTEN mRNA was lower in HepG2 cells than that in the normal hepatic cells ($P<0.01$) (Figure 5A). Moreover, the knockdown of miR-367 could significantly enhance the expression of PTEN ($P<0.001$) (Figure 5B). However, the transfection of over-expression PTEN vector had no obvious influences on expression of miR-367 ($P>0.05$) (Figure 5C). PTEN might be located at the down-stream of miR-367 in HCC.


MiR-367 activated PI3K/AKT signaling pathway

It had been reported that PTEN might be a negative switch of PI3K/AKT signaling pathway [20]. Thus, we hypothesized that miR-367 might influence PI3K/AKT signaling pathway. Western blot analysis demonstrated that the expression of p-AKT and p-GSK3β was significantly increased after the transfection of miR-367 mimic, while their expression exhibited decreased trend with the inhibition of miR-367 (P<0.05) (Figure 6). Meanwhile, miR-367 expression had no significant effects on expression of AKT and GSK3β (P>0.05 for all). All the data revealed that miR-367 might activate the PI3K/AKT signaling pathway.

MiR-367 activated PI3K/AKT pathway through inhibiting PTEN, thus contributing to malignant progression of HCC

In order to explore the molecular mechanisms underlying the functional roles of miR-367 in progression of HCC, the HCC cells were co-transfected by miR-367 inhibitor and si-PTEN. The cells transfected by miR-367 inhibitor served as internal control. Western blot analysis demonstrated that the expression of p-AKT and p-GSK3β was significantly enhanced after the co-transfection of miR-367 inhibitor and si-PTEN (P<0.01) (Figure 7). MiR-367 might activate PI3K/AKT signaling pathway through suppressing PTEN expression in HCC.

In addition, we also found that the cell proliferation, migration and invasion abilities of the cells co-transfected with miR-367 inhibitor and si-PTEN were significantly enhanced, compared to the controls (P<0.05 for all) (Figure 8). PTEN might reverse the function of miR-367 in HCC. Therefore, miR-367 promoted PI3K/AKT signaling pathway through targeting PTEN, thus contributing to malignant progression of HCC.

Discussion

Accumulating evidences have demonstrated that miRNAs play important roles in cancer through regulating gene expression. The dysregulation of miRNAs may alter multiple cellular signaling pathways, thus contributing to human diseases, like cancer [21]. In tumorigenesis, miRNA could serve as tumor suppressors or oncogenes. In HCC, various miRNAs have been confirmed. For
example, let-7b could promote HCC cell proliferation through activating Wnt/β-catenin signaling pathway, which might be a potential oncogene in HCC [22]. MiR-23c reduced the expression of ERBB2IP, thus suppressing the tumorigenesis of HCC, and its down-regulation predicted poor prognosis for the patients [23]. MiRNAs have the capacity to act as indicators and therapeutic targets for HCC. To explore the functional roles of miRNA in progression of HCC may provide new insight into the pathogenesis of the cancer.

In current study, we investigated the functional roles of miR-367 in HCC. We found that the expression of miR-367 was significantly increased in HCC tissues and cells, compared to non-cancerous specimens. Moreover, the up-regulation of miR-367 was closely correlated with large tumor size, positive lymph node metastasis, and advanced TNM stage. The knockdown of miR-367 might suppress HCC cell proliferation, migration and invasion in vitro. All the data revealed that miR-367 might act as an oncogene in HCC, and its over-expression enhanced malignant biological behaviors of HCC cells, thus contributing to malignant cancer progression. The conclusion was consistent with the previous study. Meng et al. reported that the expression level of miR-367 was positively correlated with malignant proliferation and invasion of HCC cells [24]. Up-regulation of miR-367 predicted aggressive HCC progression, and its knockdown could suppress the malignant behaviors of the cancer cells that miR-367 might be a potential therapeutic target for HCC.

Despite lack of protein encoding ability, miRNAs can bind to the 3'UTR of their targeted mRNAs, thus regulating gene expression. MiRNAs take part in tumorigenesis through regulating the expression of cancer-related genes. In order to explore the molecular mechanisms of miR-367 in progression of HCC, we studied the potential targets of miR-367 in HCC. The bioinformatics analysis demonstrated that miR-367 could bind to the 3'UTR of PTEN gene. The hypothesis was confirmed by the luciferase reporter assay. Moreover, the expression of PTEN was negatively correlated with miR-367 level, and the regulation of PTEN level had no significant effects on expression of miR-367. Thus, we calculated that miR-367 targeted PTEN in HCC, and PTEN was located at the down-stream of miR-367. Meng et al. reported that miR-367 could negatively regulate PTEN in HCC [24]. Their study supported our results. However, Xu et al. suggested that miR-367 might bind to 3'UTR of MDM2, thus contributing to metastasis of HCC [25]. In addition, miR-367 might target Rab23 in gastric cancer [26], and regulate the expression of MTA3 in renal cell carcinoma [18]. In tumorigenesis, miR-367 might target multiple genes, thus taking part in various
cellular processes.

Several published articles have demonstrated that PTEN was a negative switch of PI3K/AKT signaling pathway [20, 27, 28]. Furthermore, we confirmed that miR-367 could target PTEN in HCC. Therefore, we hypothesized that miR-367 might influence PI3K/AKT activity through targeting PTEN. Western blot analysis indicated that the levels of p-AKT and p-GSK3β were positively correlated with miR-367 expression. MiR-367 could activate PI3K/AKT pathway, and its down-regulation might lead to the inactivation of the pathway. Additionally, PTEN hold the ability to reverse the function of miR-367 in HCC. Therefore, we calculated that miR-367 activated PI3K/AKT signaling pathway through suppressing the expression of PTEN, thus contributing to malignant progression of HCC.

Despite of the encouraging results, several limitations in current study should be stated. First, the sample size was relatively small that reduced the statistical power of our results. Second, the animal experiments were not designed to verify our conclusion. In addition, miR-367 might take part in progression of HCC through multiple targets or signaling pathways. In our study, we only proved that miR-367 promoted HCC progression through PTEN/PI3K/AKT axis. Therefore, further researches will be required to verify and improve our results, including the comparison of p-AKT and p-GSK3β expression levels between HCC cells and normal hepatic cells.

In conclusion, the elevated expression of miR-367 predicts aggressive disease progression for HCC patients. MiR-367 activates PI3K/AKT pathway through negatively regulating PTEN, thus contributing to malignant progression of HCC.

Declaration

This study was supported by the Ethics Committee of Hunan Provincial Tumor Hospital and also has been carried out in accordance with the World Medical Association Declaration of Helsinki.

The subjects had been informed the objective. Certainly, written consents were signed by every subject in this study.

Author Contributions

All authors have contributed in the design, analysis, and drafting and approval of the final
manuscript. All authors have approved the final version of the manuscript.

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Figure legends

Figure 1. The expression of miR-367 was obviously increased in HCC tissues (A) and cells (B), compared to the non-cancerous samples. Every experiment is triplicate. ***: P<0.001

Figure 2. The levels of miR-367 in the transfected cells. The transfection of miR-367 mimic could significantly enhance the expression ability of miR-367 in HepG2 cells, while miR-367 inhibitor transfection inhibited the expression of miR-367 in HCC cells. Every experiment is triplicate. ***: P<0.001, **: P<0.01

Figure 3. The transfection of miR-367 mimic could enhance cell proliferation, migration and invasion, while the knockdown of miR-367 inhibit cell proliferation, migration and invasion. Every experiment is triplicate. **: P<0.01

Figure 4. The direct target relationship predicted by StarBase v2.0 between miR-367 and PTEN (A). The luciferase activity of the cells co-transfected by PTEN-wt and miR-367 mimic was significantly decreased compared to the co-transfection of PTEN-wt and mimic NC (B). However, the co-transfection of PTEN-mt and miR-367 mimic had no significant effects on luciferase activity, compared to the controls. Every experiment is triplicate. **: P<0.01

Figure 5. The levels of PTEN mRNA were significantly decreased in HepG2 cells compared to normal hepatic cells (A). The knockdown of miR-367 could enhance PTEN expression in HepG2 cells (B). However, the enforced expression of PTEN in HepG2 cells had no remarkable effects on miR-367 expression (C). Every experiment is triplicate. ***: P<0.001, **: P<0.01

Figure 6. The over-expression of miR-367 could activate PI3K/AKT signaling pathway through promoting the levels of p-AKT and p-GSK3β, meanwhile, the knockdown of miR-367 exerted the opposite effects. The expression of AKT and GSK3β had no significant association with miR-367 level. Every experiment is triplicate. **: P<0.01, *: P<0.05

Figure 7. Protein expressions in transfected HepG2 cells. Compared to miR-367 inhibitor HepG2 cells, the HepG2 cells co-transfected by miR-367 inhibitor and si-PTEN exerted increased expression of p-AKT and p-GSK3β, revealing the activation of PI3K/AKT pathway. The HCC cells transfected by si-PTEN served as control. Every experiment is triplicate. **: P<0.01, *: P<0.05

Figure 8. The cell proliferation (A), migration (B) and invasion (C) abilities of the cells co-transfected with miR-367 inhibitor and si-PTEN were significantly enhanced, compared to the controls. The cells transfected by miR-367 inhibitor served as control. Every experiment is triplicate.
***: P<0.001, **: P<0.01, *: P<0.05
### Table 1. The association of miR-367 expression with clinical characteristics of HCC patients

| Characteristics                  | N (n=126, %) | miR-367 low expression (n=68, %) | miR-367 high expression (n=58, %) | P values |
|----------------------------------|-------------|----------------------------------|----------------------------------|---------|
| Age (years)                      |             |                                  |                                  | 0.734   |
| ≥60                              | 74 (58.73)  | 39 (57.35)                       | 35 (60.34)                       |         |
| <60                              | 52 (42.27)  | 29 (42.65)                       | 23 (39.66)                       |         |
| Gender                           |             |                                  |                                  | 0.069   |
| male                             | 76 (60.32)  | 46 (67.65)                       | 30 (51.72)                       |         |
| female                           | 50 (39.68)  | 22 (32.35)                       | 28 (48.29)                       |         |
| Smoking                          |             |                                  |                                  | 0.333   |
| yes                              | 68 (53.97)  | 34 (50.00)                       | 34 (58.63)                       |         |
| no                               | 58 (46.03)  | 34 (50.00)                       | 24 (41.37)                       |         |
| Tumor size (cm)                  |             |                                  |                                  | 0.005   |
| ≤3                               | 67 (53.17)  | 44 (64.71)                       | 23 (39.66)                       |         |
| >3                               | 59 (46.83)  | 24 (35.29)                       | 35 (60.34)                       |         |
| Lymph node metastasis           |             |                                  |                                  | 0.004   |
| yes                              | 48 (38.10)  | 18 (26.47)                       | 30 (51.72)                       |         |
| no                               | 78 (61.90)  | 50 (73.53)                       | 28 (48.28)                       |         |
| TNM stage                        |             |                                  |                                  | <0.001  |
| I-II                             | 69 (54.76)  | 48 (70.59)                       | 21 (36.21)                       |         |
| III-IV                           | 57 (45.24)  | 20 (29.41)                       | 37 (63.79)                       |         |

Notes: HCC: hepatocellular carcinoma; TNM: tumor node metastasis
Relative expression of miR-367

- miR-367 mimic: **
- mimic NC: -
- miR-367 inhibitor: ***
- Inhibitor NC: -
A

PTEN-WT 3' - UTR

5' ... CCCUUUGCACUUGUGGCAACAGA

hsa-miR-367-5p

3' UCUCAACGUAAUU - CGUUGUCA

PTEN-MT

5' ... CCCUUUGCACUUGUGGCUGUUGUCA

B

Relative luciferase activity

- miR-367 mimic
- mimic NC

PTEN-wt PTEN-mt

**
A

miR-367 inhibitor

miR-367 inhibitor + si-PTEN

OD (490nm)

Time (h)

0.0

0.5

1.0

1.5

0

24

48

72

B

Number of migrate cells

miR-367 inhibitor

miR-367 inhibitor + si-PTEN

100

200

300

C

Number of invasive cells

miR-367 inhibitor

miR-367 inhibitor + si-PTEN

100

150

200

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Supplementary Table 1. Sequences of new constructs.

| Names                  | Sequences                                                                 |
|------------------------|---------------------------------------------------------------------------|
| MiR-367 mimic          | 5'-CCATTACTGTGCTAAATATGCAACTCTGTTGAAATATAATTTGGAATTGC                     |
|                        | ACTTTAGCAATGGTGATGG-3’                                                   |
| MiR-367 mimic NC       | 5'-CGCGATTGTAATCGTGAGCTTTTTGTGATGACATAAATTGTCGGAAAAAT                     |
|                        | CTTCTGTGAGCATATAA-3’                                                    |
| miR-367 inhibitor      | 2'-O-methyl-5'-CCATTACTGTGCTAAATATGCAACTCTGTTGAAATATAATTTGGAATTGC       |
|                        | GGAATTGCACCTTTAGCAATGGTGATGG-3’                                        |
| miR-367 inhibitor NC   | 2'-O-methyl-5'-CGCGATTGTAATCGTGAGCTTTTTGTGATGACATAAATTGTCGGAAAAAT         |
|                        | CGGAAAAATCTTTCTGTGAGCATATAAATAATTTGGAATTGC                               |
| PTEN siRNA             | 5'-AACCCACCACACAGCUAGAAC tt -3’                                         |
|                        | 5'-AAGUUCUAGCUGUGGUGGG tt -3’                                           |
| Control siRNA          | 5'-UUCUCCAAGCGUGUACAGCU tt -3’                                          |
|                        | 5'-ACGUGACACGUUCCGGA A tt -3’                                          |
| Over-expression PTEN   | 5'-CCGGAATTTGCGATGGCCATGGCAACCAAAGG-3’                                  |
|                        | 5'-CCCAAGCTTTCTAGACTTTTGTATGC-3’                                        |