

Down-regulated miR-28-5p in human hepatocellular carcinoma correlated with tumor proliferation and migration by targeting insulin-like growth factor-1 (IGF-1)

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Abstract Hepatocellular carcinoma (HCC) is a rapidly progressing, incurable cancer that frequently spreads to portal vein and lung. New insights are needed to identify therapeutic targets to prevent or retard HCC metastatic progression. Because microRNAs (miRNA) often act as tumor regulators, we investigated their role in preclinical models of HCC. Here we found miR-28-5p is a liver-relevant anti-proliferative miRNA whose expression, functions, and mechanisms were analyzed in human hepatoma cells, HepG2 and Huh7. Interestingly, when evaluating the specific targets of miR-28-5p, we found that ectopic miR-28-5p expression down-regulates insulin-like growth factor 1 (IGF1) protein and that the expression of miR-28-5p correlates negatively with IGF1 protein in HCC cells. Luciferase report in HCC cells expressing miR-28-5p suggests that miR-28-5p reduces luciferase activity by targeting the 3'-UTR of IGF1 mRNA. Additionally, we show that the selective inhibition of either the PI3K/AKT pathway prior to miR-28-5p stimulation prevents the expression of previously described tumor suppressor miRNAs that are family and cluster specific. Together, our results defined miR-28-5p as a critical regulator of IGF1 mRNA translation function, down-regulated miR-28-5p in HCC was associated with tumor growth through PI3K/AKT pathway by targeting IGF1. miR-28-5p-IGF1-PI3K/AKT pathway may play an important role in the development of HCC.

Keywords Hepatocellular carcinoma · miRNA-28-5p · IGF1 · PI3K/AKT

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide and is one of the most human malignant tumors, especially in Asian countries [1, 2]. Nowadays, drug intervention with antineoplastic and laparoscopic surgery is widely utilized to treat HCC patients [3]. But their 5-year survival rate remains low because many patients are diagnosed at the late stage of HCC and due to a high incidence of recurrence [4]. An improved understanding of the pathogenesis of HCC development would facilitate the development of more effective outcomes for the diagnosis and treatment of HCC at earlier stages. Therefore, new therapeutic approaches and prognostic markers are needed. In last decade, new players in tumor biology were identified: microRNAs (miRNAs) [5–7]. miRNA are small, endogenous, non-coding, which are 22–30 nucleotides in length and modulate the expression of various target genes at the post-transcriptional and translational levels [8, 9]. Aberrant expression of miRNA is common in various human malignancies and modulates cancer-associated genomic regions or fragile sites [10].

As for the relationship between miRNA and HCC, several studies have demonstrated that the aberrant expression of specific miRNA can be detected in HCC cells and tissues. However, little is known about the mechanisms of miRNA-related cell proliferation and development. The insulin growth factor (IGF) pathway is activated in several malignancies including HCC [11, 12]. In addition, signaling through IGF1 is essential for normal development and

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growth [13]. Through downstream activation of both PI3K/AKT and MAPK/ERK kinase, IGF1 controls cell proliferation and survival, respectively [14, 15]. Several ongoing clinical trials have focused on strategies for directed targeting of IGF signaling but have had mixed results [16]. Despite these results, IGF signaling continues to be the focus of investigation particularly for the development of targeted therapeutics in selected subgroups of tumor patients [17]. For example, in breast cancer, miR-148a and miR-152 target both IGF1 and insulin receptor substrate (IRS-1) leading to a reduction in both tumor proliferation and angiogenesis [18]. MiR-145 also functions as a potent tumor suppressor in both hepatocellular and colon carcinoma [19, 20]. Nowadays, investigators have identified a few potential targets for miR-28 family, including the cancer-associated gene CCND1, HOXB3, and NM23-H1 [21]. However, the mechanistic role for miR-28-5p as either an oncogene or tumor suppressor particularly in HCC remains largely unknown.

Herein, we demonstrate that down-regulated miR-28-5p expression is observed in the metastatic hepatic carcinoma. The high-expression of miR-28-5p is associated with better prognostic features in HCC. Furthermore, we investigated the role of PI3K/AKT pathway in HCC and found that miR-28-5p inhibits the activation of the PI3K/AKT pathway by suppressing IGF1. Thus, we elucidated the role of miR-28-5p as a regulator in IGF1 expression and PI3K/AKT pathway activity in human HCC, which could be a potential therapeutic target for cancer therapy.

Materials and methods

Clinical samples

Forty six HCC samples were collected from patients including 30 males and 16 females, who underwent the resection of their primary HCC in the Department of Hepatobiliary Surgery, at the Shanghai Changzheng Hospital of Second Military Medical University during January 2008 to December 2011, with a median follow-up time of 38 months. The clinicopathological data are shown in Table 1. Written Informed Consent was obtained from all patients, samples were used after obtaining informed consent. Patients did not receive preoperative chemotherapy or embolization.

Cell culture

Human HCC cell lines HepG2 and Huh7 were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS; GIBCO-BRL) and Penicillin Streptomycin (1×) antibiotics

(GIBCO). Cells were kept in a humidified incubator that was maintained at 37 °C and supplied with 5 % CO₂ and 95 % air.

Prediction of miRNAs targeting IGF1

miRNA target predicting algorithms miRDB (<http://mirdb.org/miRDB/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdcberlin.de/>) were used to predict miRNAs targeting IGF1 and the binding regions.

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), using the standard method. cDNA synthesis was performed with 1 µg of total RNA, using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed on the ABI 7500 cycler (Applied Biosystems, CA, USA), using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Hsa-RNU6B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous controls for miRNAs and mRNAs expression, respectively.

Plasmids constructs and luciferase reporter assay

3'-UTR of IGF1 and a mutation sequence were amplified by PCR using the primers that included a Bgl II restriction site on the 5' and 3' strands. The PCR products were inserted into the Bgl II sites of the pGL3-control vector (Promega, Madison, WI, USA) and identified by DNA sequencing. The wild-type plasmid was created containing the 3'-UTR of IGF1 with complementary sequence of miR-28-5p (pGL3-IGF1-3'-UTR wild) and a mutant plasmid with the mutation sequence without complementary sequence of miR-28-5p (pGL3-IGF1-3'-UTR mut). For the luciferase reporter assay, the HCC cells were seeded on 24-well plates and co-transfected using Lipofectamine 2000 (Invitrogen) with 100 ng per well of the resulting luciferase UTR-report vector, 2 ng per well of pRLCMV vector (internal control, Promega), and 20 ng per well of miR-28-5p precursor molecules or control precursor (Applied Biosystems) following the manufacturer's instructions. After 24 h, the cells were lysed and the relative luciferase activity was assessed with the Dual-Luciferase Assay Reporter System (Promega).

Transfection and immunocytochemical assay

miR-28-5p mimics/inhibitors, obtained from Lifetechnologies (Grand Island, NY, USA), were transfected into HepG2 and Huh7 cells with the FuGENE 6 transfection

Table 1 Correlation between the clinicopathologic characteristics and miR-28-5p expression in HCC patients

Characteristics	No. of patients <i>n</i> = 46	No. of expression		<i>p</i>
		miR-28-5p ^{Low}	miR-28-5p ^{High}	
Age (years)				
<55	24(52.17)	14(58.33)	10(41.67)	0.257
≥55	22(47.83)	10(45.45)	12(54.55)	
Gender				
Male	30(65.22)	17(56.67)	13(43.33)	0.311
Female	16(34.78)	7(43.75)	9(56.25)	
AFP level (ng/ml)				
<400	19(41.3)	8(42.11)	11(57.89)	0.539
≥400	27(58.7)	16(59.26)	11(40.74)	
HBV virus				
Negative	14(30.43)	5(35.71)	9(64.29)	0.074
Positive	32(69.57)	19(59.38)	13(40.62)	
Tumor size(cm)				
<5	12(26.09)	6(50)	6(50)	0.842
≥5	34(73.91)	18(52.94)	16(47.06)	
TNM stage				
I + II	36(78.26)	16(44.44)	20(55.56)	<0.001*
III + IV	10(21.74)	8(80)	2(20)	
Tumor foci				
1	37(80.43)	18(48.65)	19(51.35)	0.061
>1	9(19.57)	6(66.67)	3(33.33)	
Cirrhosis				
Negative	21(45.65)	10(47.62)	11(52.38)	0.117
Positive	25(54.35)	14(56)	11(44)	
Edmondson–Steiner grading				
I + II	31(67.39)	12(38.71)	19(61.29)	0.006*
III + IV	15(32.61)	12(80)	3(20)	
Venous infiltration				
Negative	37(80.43)	16(43.24)	21(56.76)	0.003*
Positive	9(19.57)	8(88.89)	1(11.11)	

* Statistically significant

HCC hepatocellular carcinoma; *AFP* serum alpha-fetoprotein; *TNM* tumor-node-metastasis

reagent (Promega, Madison, USA) at a final concentration of 50/100 nM. siRictor and siRaptor duplexes were synthesized by GenePharma (Invitrogen, Carlsbad, CA, USA), and a non-silencing siRNA was used as a negative control. The transfection protocol for siRNA was the same as that for miR-28-5p mimics. Briefly, cells in wells of 96-well plates were grown to 80–90 % confluence. Cells were incubated with small RNA complexes for 8 h before the medium was changed.

After transfection HepG2 and Huh7 cells cultured in 24-well plates were washed with PBS and fixed with 4 % paraformaldehyde for 15 min. 0.5 % Triton-100 was used for permeabilization. The cells were then blocked in 2 % goat serum (diluted in PBS). After blocking, the cells were incubated with the anti-myosin primary antibody at 37 °C

for 1–2 h, and then the fluorescent secondary antibodies at 37 °C for 1 h. The nuclei were stained with DAPI (Lifetechnologies) for 10 min. Images were captured using fluorescence microscope (Olympus, Tokyo, Japan).

Cell proliferation assay

Cell viability was assayed by a colorimetric procedure, using the Cell Counting Kit-8 (Lifetechnologies, NY, USA) according to the manufacturer's protocol. The absorbance at 460 nm was determined with a microplate reader. To detect the exact proliferation rates of HepG2 and Huh7 cells, an EDU (5-ethynyl-20-deoxyuridine) incorporation assay was executed with the Cell-LightTM EdU In Vitro Imaging Kit (Lifetechnologies, NY, USA)

according to the producer's instructions. Briefly, cells at 70–80 % confluence were treated with 50 nM EDU in ECM medium and incubated for 2 h before fixation in 4 % paraformaldehyde. After EDU staining, cell nuclei were stained with Hoechst 33342 and observed with an inverted fluorescent microscope (Olympus, Tokyo, Japan).

Wound healing assay

Briefly, HepG2 and Huh7 cells were first transfected with miR-28-5p mimics, inhibitors, or negative control (NC) RNAs in a 6-well plate and were allowed to grow overnight until confluent. Next, IGF1 was added to each well, except for the control one, at a final concentration of 100 ng/ml as a 24-h pre-treatment, and IGF1 treatment continued until the end of the test. At the 0 h time point, the cell monolayer in each well was scraped with a sterile 200 μ l pipette tip 3 times to form parallel lines, followed by 1 wash with ECM. The same wound areas were examined and photographed with a Nikon Eclipse TS100 Microscope (Nikon, Japan) at the 0 h and 24 h post-injury time points. The areas of the cells that migrated into the wound fields were measured with Adobe Photoshop software.

In vitro tube network formation assay

For tube network formation assay, we co-cultured human umbilical vein endothelial cells (HUVECs) with the transfection HCC cells; corresponding to this, one group HUVEC cells cultured alone of 96-well plates was pre-coated with 50 μ l of Matrigel (BD Biosciences, Bedford, MA, USA) and allowed to polymerize for 30 min at 37 °C. Then cells were seeded on Matrigel-coated wells at a density of 2×10^4 cells per well in ECM medium containing 1 % FBS at 37 °C. Cells started to form tubes at 4 h. Tube formation was optimal after 6 h. The tube images were taken at 6–8 h with a digital camera attached to an inverted phase-contrast microscope. Total tube length in each well was measured and calculated using image software (IPP).

Cell cycle analysis

HCC cells in wells of 6-well plate were transfected with miR-28-5p mimics, inhibitors, or negative control (NC) for 48 h. Then, the cells were harvested for flow cytometry analysis. Briefly, cells were fixed in 80 % ethanol, suspended in PBS, and incubated with RNase A (0.5 μ g/ μ l) for 30 min at 37 °C. Then the cells were stained with propidium iodide on ice for 1 h and subsequently measured with FACScan Flow Cytometry (Becton–Dickinson, San Jose, USA). The percentage of cells in the G1, S, and G2

phases were analyzed using the Cell cycle analysis Software.

Migration assay

HCC cells migration were determined using a modified two-chamber migration assay with a pore size of 8 μ m (Transwell chamber, Corning, NY, USA). For migration assay, 1×10^5 cells were seeded in serum-free medium in the upper chamber. After 12 h incubation at 37 °C, cells in the upper chamber were carefully removed with a cotton swab and the cells that had traversed the membrane were fixed in methanol, stained with Giemsa, and photographed in five independent $\times 100$ fields for each well.

Western blot

HCC cells in a 6-well plate were scraped in RIPA lysis buffer that was supplemented with 1 mM PMSF. Proteins (20 μ g) were separated on 10 or 12 % (for RhoB protein assay) SDS–polyacrylamide gels and were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Lifetechnologies, NY, USA). After a blocking incubation with 5 % milk-TBST, the membranes were incubated overnight in primary antibodies at dilutions, followed by 1 h incubations in a secondary antibody that was conjugated to horseradish peroxidase (1:10,000 dilution). After incubations in an enhanced chemiluminescence reagent (Lifetechnologies, NY, USA), the images were captured on the image reader LAS-4000 system (Olympus, Tokyo, Japan).

Statistical analysis

All experiments were performed at least three times. Data were presented as the mean \pm SEM. Analyses were conducted with SPSS 19.0 software and GraphPad Prism, using the unpaired Student's *t* test for comparisons between two groups or one-way ANOVA for multiple comparisons. *p* values <0.05 were considered statistically significant.

Results

Clinical significance of miR-28-5p expression in HCC patients

Forty six samples of HCC tissues were determined by qRT-PCR for miR-28-5p expression. We determined 0.68 (mean level of miR-28-5p) as a cutoff value for the expression level of miR-28-5p. The expression of miR-28-5p was considered as either low (<0.68 , $n = 24$) or high (≥ 0.68 ,

$n = 22$). As shown in Table 1, the low-expression of miR-28-5p was prominently associated with venous infiltration ($p = 0.003$), low edmondson and steiner grading ($p = 0.006$), and TNM tumor stage ($p < 0.001$). Thus, our results indicate that low-expression of miR-28-5p is correlated with malignant clinicopathologic characteristics in HCC patients.

miR-28-5p suppress metastasis and recurrence in HCC patients

We tested the expression of miR-28-5p by qRT-PCR and normalized against an endogenous control (hsa-U6 level) in 38 pairs of randomly selected tumor tissues and matched adjacent non-tumor tissues from HCC patients who received liver resection. The expression level of miR-28-5p in HCC tissues was significantly lower than that in matched adjacent non-tumor liver tissues ($p < 0.05$, Fig. 1a). HCC patients cases that showed intrahepatic spreading, venous infiltration or tumor invasion into bile ducts were considered as aggressive HCC cases. As compared with non-aggressive HCC tissues, miR-28-5p levels were prominently down-regulated in aggressive HCC tissues ($p < 0.05$, Fig. 1b). Furthermore, miR-28-5p levels were obviously decreased in tumor tissues arising from patients with intrahepatic tumor recurrence or

extrahepatic metastasis as compared with tumor tissues arising from patients without tumor recurrence ($p < 0.05$). Thus, the expression of different levels of miR-28-5p level was correlated with metastasis and recurrence of HCC patients.

IGF1 is the direct target of miR-28-5p in HCC cells

We searched the sequence in the TargetScan and found that the 3'-UTR of the IGF1 gene contains one separate miR-28-5p-binding seed sequences that are conserved through evolution. To demonstrate the direct interaction between miR-28-5p and IGF1 mRNA, we then constructed the luciferase reporter system containing the binding site (IGF1-3'-UTR-wt) or mutated site (IGF1-3'-UTR-mut) to downstream of the pRLCMV luciferase reporter gene. This vector was co-transfected into 293T cells with miR-28-5p mimic or negative controls. Luciferase activity in miR-28-5p group was decreased markedly compared with negative controls by 37.6 % (Fig. 1d–f). MiR-28-5p mimic did not affect the luciferase activity in the pGL3-IGF-1-mut vector (Fig. 1e). When blocking the expression of miR-28-5p with miR-28-5p inhibitor, we get increased luciferase intensity in 293T cells (Fig. 1f). The pGL3-IGF1-mut vector co-transfected with miR-28-5p inhibitor did not show changes in luciferase activity in 293T cells. These results support

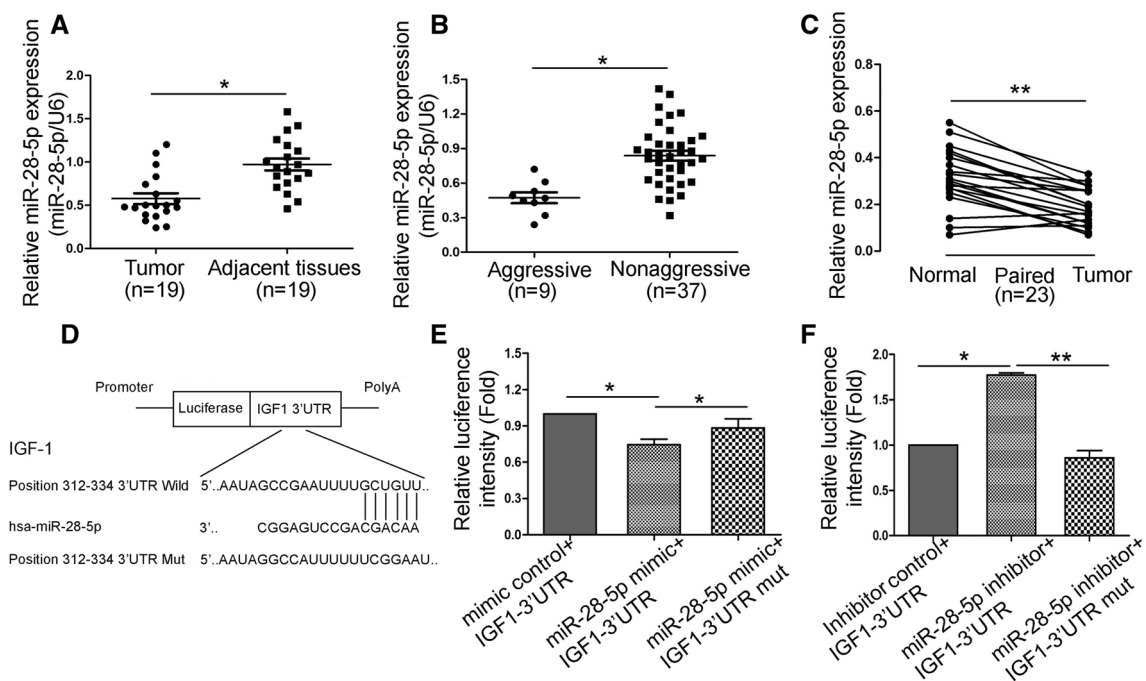


Fig. 1 The expression levels of miR-28-5p in HCC tissues. **a** Comparing differences in the expression levels of miR-28-5p between HCC and matched non-tumor tissues; **b** aggressive and non-aggressive tumor tissues; **c** miR-28-5p was down-regulated significantly in HCC tissues compared to paired tissues; **d** Predict IGF1 was potential

targets for miR-28-5p and its validation. miR-28-5p directly down-regulates IGF1 expression. Luciferase reporter assay was performed to detect the effect of miR-28-5p (**e**) and anti-sense miR-28-5p (**f**) on the luciferase intensity controlled by 3'UTR of IGF1. Values are depicted as Mean \pm SEM; * $p < 0.05$, ** $p < 0.01$ by *t* test

the bioinformatics prediction indicating the 3'-UTR of IGF1 mRNA as a target for miR-28-5p.

miR-28-5p induces early apoptosis and inhibits the proliferation in HCC cells

Considering the important roles of miR-28-5p in HCC tumorigenesis, HepG2 and Huh7 cell lines were transfected with miR-28-5p mimics, inhibitor, or Control group. As shown in Fig. 2A and D, HCC cells overexpressing miR-28-5p mimics grew significantly less ($p < 0.005$, respectively) than which cells transfected with other groups. Therefore, the in vitro results suggest that miR-28-5p significantly suppresses proliferation in HCC cells. Because of poly-(adenosine diphosphate-ribose), polymerase 1 (PARP1) plays a central role in apoptosis determining the cell fate, which induce apoptosis, through apoptosis inducing factor (AIF) activation, as well as necrosis, we detected PARP1 protein as a reference, to test whether miR-28-5p had an effect on cell apoptosis. Results shown cells transfected with miR-28-5p expressed 3.4 and 2.6 times more cleaved-PARP1 form than which cells transfected with control in the HepG2 and Huh7 cell lines, respectively (Fig. 2b, c). Then we examined the cell cycle distribution by FACS after transfection. Compared with the control, cells transfected with miR-28-5p had a significantly higher percentage of cells in G1 phase and a

significantly lower percentage of cells in S phase, suggesting that miR-28-5p causes G1 arrest ($p < 0.05$) (Fig. 2e and F). These data suggest that miR-28-5p has a tumor suppressive role in HCC.

MiR-28-5p inhibits tube network formation and migratory ability of HCC cells

We co-cultured a human HCC cell line with a high-metastatic potential (HepG2) and HUVECs to simulate the interaction of HCC cells in vitro, which evaluate the effect of HCC cells after transfection on tube network formation. In this co-culturing system, the HUVECs proliferate, migrate, and form tubule-like sprouts that mimic the development of microvessels in vivo. The HUVECs were plated onto the inserts, and HCC cells were plated onto the chambers at a HepG2: HUVEC cell ratio of 4:1; the HUVECs cultured alone served as control group. The HUVECs exposed to HepG2 cells after transfect miR-28-5p group also formed less branches in the tube formation assay (Fig. 3a, $p < 0.05$). Further, HepG2 cells promoted the migratory ability of HUVECs, as shown by migration assays (Fig. 3b, $p = 0.004$) and wound healing assay (Fig. 3c, d, $p = 0.037$). These results suggest that miR-28-5p may inhibit the angiogenic and migratory activity of HCC cells.

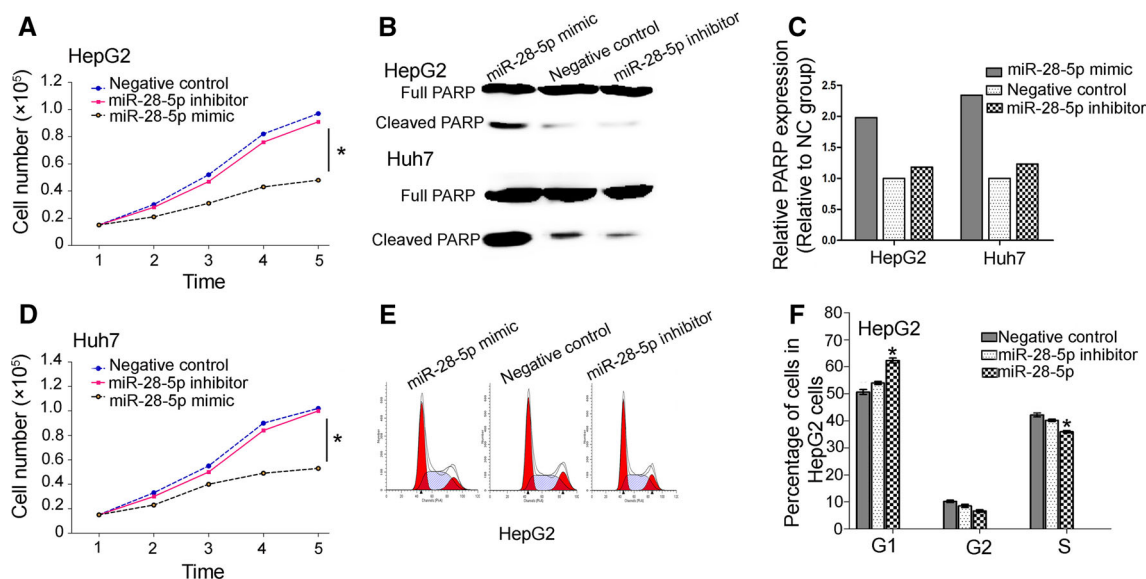
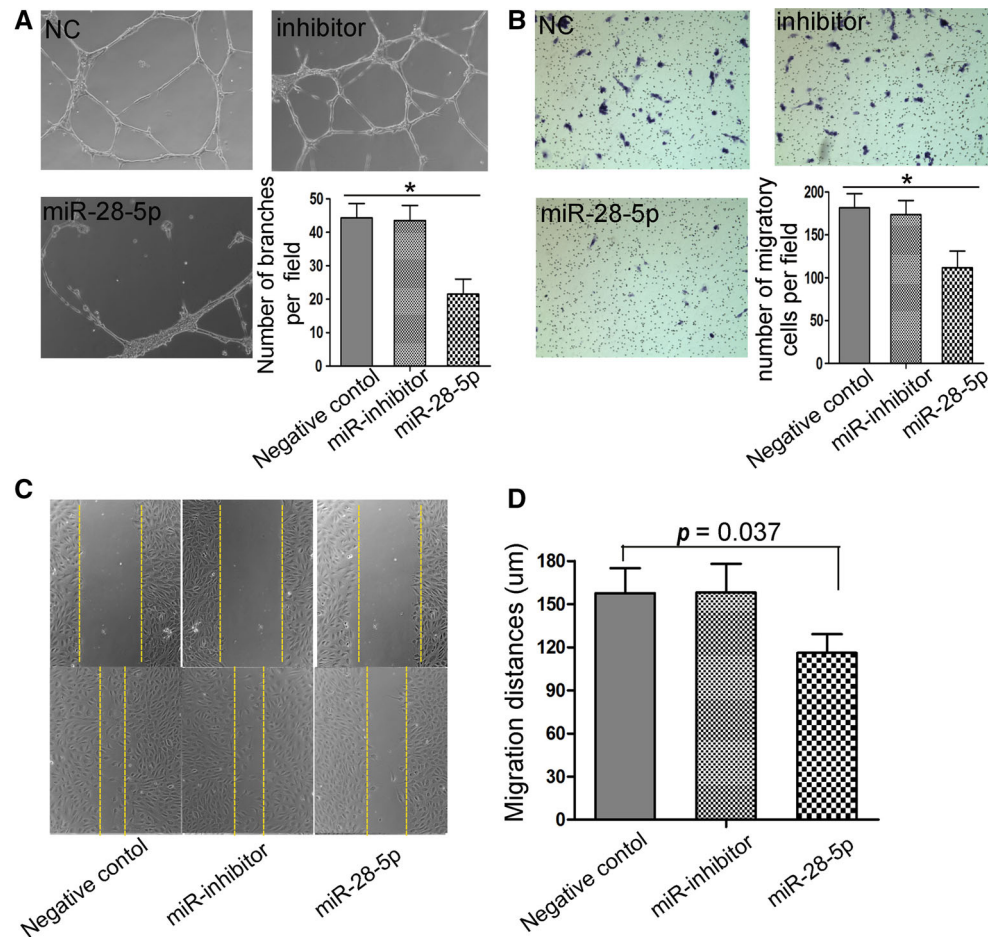


Fig. 2 Cell proliferation were detected in HCC cells transfected with miR-28-5p. HepG2 (a) and Huh7 (c) cells were transfected with miR-28-5p mimics, negative control, and miR-28-5p inhibitor for 5 days as described in the methods section before measurement of the conversion of cell proliferation assay; **b** Immunoblotting with anti-PARP 48 h after transfection of HepG2 and Huh7 cell lines with

negative control, miR-28-5p mimics, or miR-28-5p inhibitor. (C) miR-28-5p can increased PARP1 cleavage form. (E, F) Fluorescent-activated cell sorting analysis 48 h post-transfection with negative control, miR-28-5p, or miR-28-5p inhibitor. Representative experiment was performed in duplicate; mean standard deviation ($*p < 0.05$, Student *t* test), Mean \pm SD ($n = 3$)

Fig. 3 Effect of miR-28-5p on tube formation and migration of HepG2 cells. **a** Images of tube formation assay. **b** Images of two-chamber migration assay. **(c and d)** Representative images of wound healing assay in HepG2 cells with miR-28-5p, miR-28-5p inhibitor or negative control group. Values are depicted as Mean \pm SEM, control versus miR-28-5p, $p = 0.037$, $*p < 0.05$



miR-28-5p inhibits PI3K/AKT pathway activity expression by suppressing IGF1 in HCC

We evaluated the correlation between IGF1 and miR-28-5p expression in our HCC samples and HCC cells. Spearman correlation analysis indicated that miR-28-5p was inversely correlated with IGF1 expression in HCC samples ($r = -0.837$, $p < 0.001$, Fig. 4a). Then the expression of IGF1 levels in miR-28-5p mimics cells was significantly lower than those in miR-28-5p inhibitor cells ($p < 0.05$, Fig. 4b). HepG2 and Huh7 cells that were transfected with miR-28-5p mimics or negative control were subjected to Western blot for IGF1, p-PI3K, p-AKT, and Bcl2 protein expression. As assessed by immunoblotting assay, up-regulation of miR-28-5p obviously suppressed the expression of IGF1 protein and led to down-regulation of PI3K/AKT pathway activity in both HepG2 and Huh7 cells (Fig. 4c, d). Taken together, these data indicate that miR-28-5p directly targets IGF1 expression that subsequently regulates PI3K/AKT progression in HCC.

Discussion

HCC is particularly problematic in China, where the incidence of HCC is much higher than that in other Asian countries. HCC is a highly angiogenic malignancy, which is characterized by a high propensity for vascular invasion [22]. Increasing studies have reported that miRNAs regulated hepatocarcinogenesis-related gene expression, indicating a new insight in the initiation and progression of HCC [23]. In this study, miR-28-5p was identified as a potential biomarker of HCC with high predictive value. We initially detected the expression level of miR-28-5p in 46 pairs of HCC tissues and adjacent non-tumor tissues. Quantification of the data indicated that miR-28-5p expression in tumor tissues was significantly down-regulated as compared with that in adjacent non-tumor tissues. Moreover, our results showed that elevated miR-28-5p expression conferred a significant lower recurrence rate for HCC patients. Clinical data analysis found that miR-28-5p was expressed at significantly lower levels in HCC patients with venous infiltration, low Edmondson-Steiner grading,

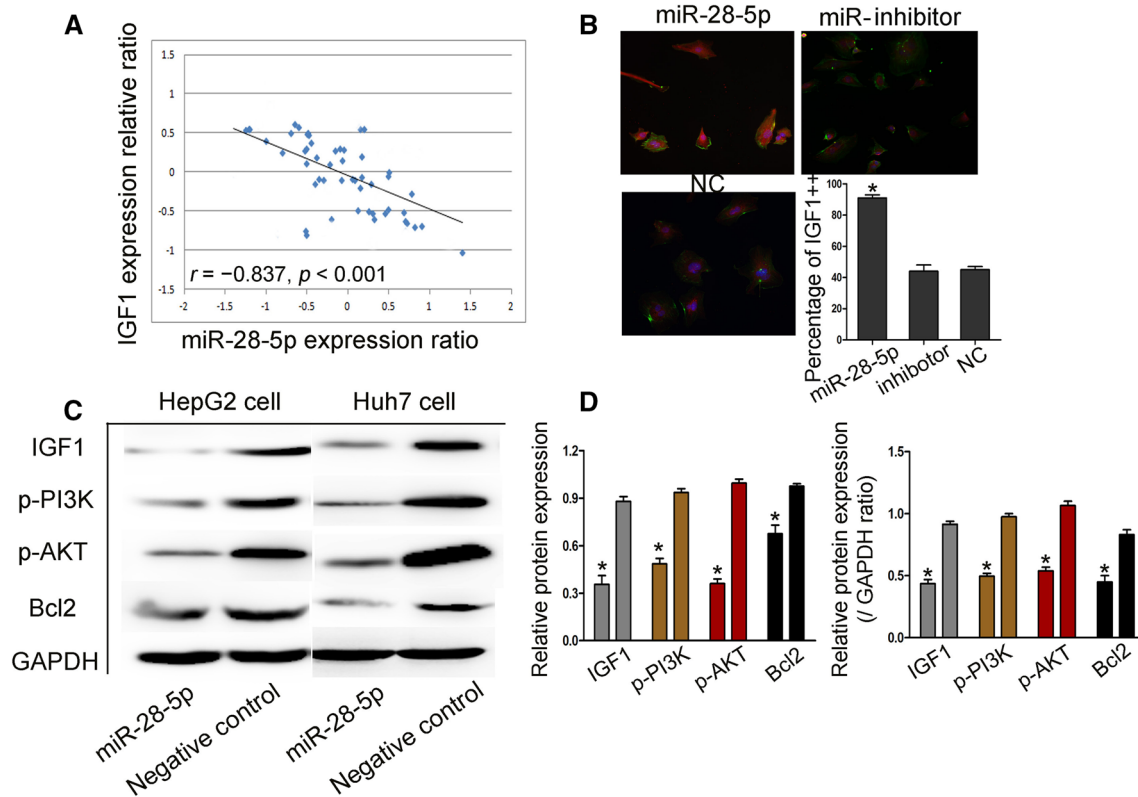


Fig. 4 miR-28-5p inhibits PI3K/AKT pathway activity expression by suppressing IGF1 in HCC. **a** IGF1 expression was negatively correlated with miR-28-5p expression in HCC (linear correlation analysis, $r = -0.837$, $p < 0.001$). **b** Immunocytochemistry assay to compare the activity of IGF1 in miR-28-5p overexpressing and inhibitor, control cells. **c, d** HepG2 and Huh7 cells that were

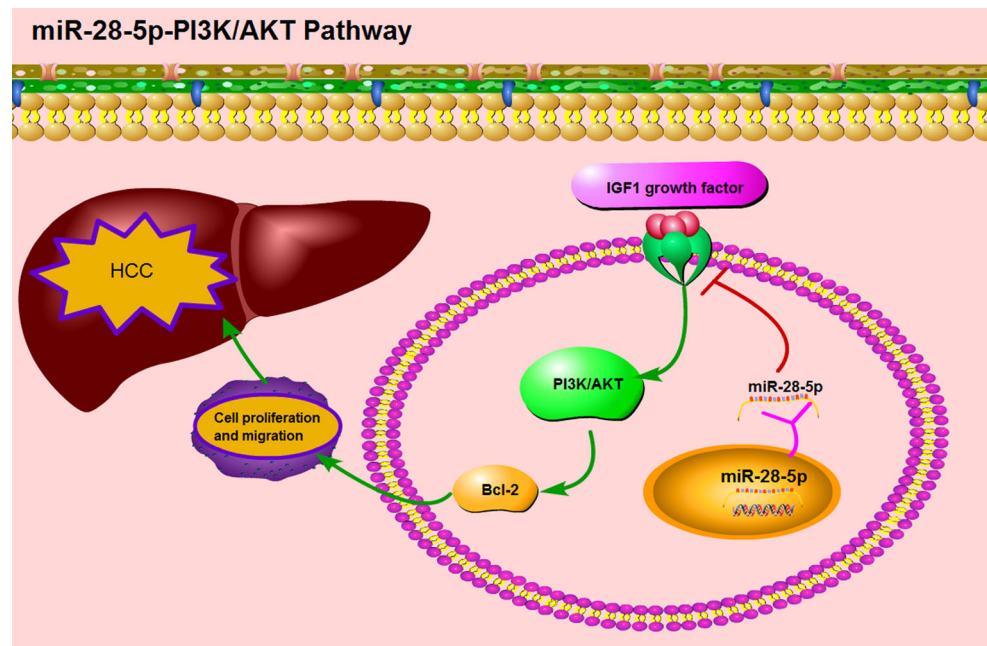
transfected with miR-28-5p inhibitors (anti-miR-28-5p) and miR-28-5p mimics, respectively, were subjected to Western blot for IGF1, p-PI3K, p-AKT, and Bcl2. Representative WB showed that up-regulation of miR-28-5p obviously depressed protein expression of IGF1, p-PI3K, p-AKT, and Bcl2 expression in HCC cells. ($n = 3$ repeats with similar results. $*p < 0.05$)

and low grade TNM tumor stage, suggesting that low-expression of miR-28-5p is obviously correlated with bad prognostic features in HCC. It has also been reported that miR-28-5p was down-regulated in colorectal cancer samples compared with normal colon samples, overexpression of miR-28-5p reduced colorectal cancer cell proliferation, migration, and invasion in vitro [21]. Altogether, these results suggest that miR-28-5p is critical for prognosis determination in tumor patients. Moreover, elevated miR-28-5p expression was observed in HCC cell lines, especially in the highly metastatic HCC cell line HepG2 and Huh7. Our results indicate that miR-28-5p might be critical for the regulation of tumor growth, autophagy, and metastasis in HCC patients. Certainly, cell autophagy may need us for further study.

Insulin-like growth factor 1 (IGF1) is a growth factor controlling cell proliferation, differentiation, and apoptosis. Abnormal expression of IGF1 promoted metastatic activity of tumor cells [24]. IGF1 signaling is a mediator of both PI3K/AKT and ERK/P38 signaling and has been demonstrated to enhance wide variety of tumorigenesis [15, 24,

25]. The emerging importance of miRNAs in the regulation of proteins associated with proliferation, survival, and PI3K/AKT pathway underscores the importance of uncovering mechanisms governing the expression of these small RNAs as a means to better understand the complicated processes of tumorigenesis and cancer cell progression [26, 27]. Determining the roles of endocrine molecules, growth factors, and signaling pathways in the regulation of miRNA expression is essential. Here we demonstrate for the first time a role for IGF1-PI3K/AKT pathway signaling in the regulation of miRNAs in the HCC cell line. Recently, IGF1 in breast cancer cells has been found to be a direct target of let-7 and contribute to MAPK and EMT mediated by miR-let7 [28]. In our study, the inverse correlation between miR-28-5p and IGF1 expression was observed in HCC tissues. Furthermore, we investigated the regulatory effect of miR-28-5p on IGF1, p-PI3K, p-AKT, and Bcl2 in vitro. Our data showed that down-regulation of miR-28-5p increased the expression level of IGF1 and promoted PI3K/AKT in two different HCC cell lines, HepG2, and Huh7. Herein, we validated

Fig. 5 Abridged general view for the interplay among miR-28-5p-PI3K/AKT pathway in HCC. miR-28-5p as a tumor suppressor by targeting IGF1, which decreased the tumorigenesis of HCC cells in vitro through the modulation of the PI3K/AKT pathway. Overexpression of miR-28-5p, which suppresses the expression of IGF1, activates the PI3K/AKT-Bcl2 pathway by increasing the phosphorylation of p-PI3K, p-AKT, and Bcl2 expression, which inhibits cell proliferation and induced apoptosis in HCC



IGF1 as a direct functional target of miR-28-5p in HCC, adding information to previously reported cell types [29]. Notably, the regulatory effect of reduced miR-28-5p expression on IGF1, p-PI3K, and p-AKT was inverted by miR-28-5p mimics in Huh7 cells. Thus, miR-28-5p may suppress PI3K/AKT by inhibiting IGF1 expression in HCC.

The potential relevance of the IGF1 and IGF1R pathway in tumor has long been noted, with many opportunities for therapeutic targeting [30]. Clinical evidence certainly supports this strategy, although a recently published Phase II clinical trial in metastatic HNSCC patients using an IGF-1R monoclonal antibody as monotherapy has proven to be disappointing [31, 32]. Given the complexity of this pathway, with multiple ligand and receptor interactions in epithelial cancer cells, the interplay between the stroma with the malignant epithelial tissues, along with the interactions between the IGF and IGF1Rs, it would have been indeed quite surprising if a single molecular therapy would have been therapeutically effective, delivered as the sole modality. Even so, this current work provides additional biological insights into yet another mechanism by which this complex pathway can be activated in wide variety of tumor [33].

As shown in Fig. 5, the interplay among miR-28-5p, IGF1, and the PI3K/AKT pathway was shown. Prospective studies should be performed to address clinical correlations and systematic experiments should be conducted to identify all potential targets that can explain the distinct biological effects.

Conclusions

In summary, the role of IGF1 with small RNAs or other part of mechanism involved in carcinogenesis has been partly demonstrated in the study. Our studies have suggested IGF1 as a novel target of miR-28-5p. Overexpression of miR-28-5p, which suppresses the expression of IGF1, activates the PI3K/AKT-Bcl2 pathway by increasing the phosphorylation of p-PI3K, p-AKT and Bcl2 expression, which inhibits cell proliferation and induced apoptosis in HCC. We also place special emphasis on the overexpression of miR-28-5p as means of identifies patients at concrete risk and be a novel therapeutic molecular target for HCC.

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Compliance with Ethical Standards

Conflict of interest No conflicts of interest, financial or otherwise, are declared by the author(s).

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