

## Original Article

# microRNA-96-5p induces the epithelial-mesenchymal transition to promote the metastasis of hepatocellular carcinoma by post-transcriptionally downregulating Talin 1

Hongying Tang, Yi Liu, Wei Cheng, Zili He, Ning Zhou

*Laboratory of Hepatobiliary Molecular Oncology, Department of Hepatopancreatobiliary Surgery, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, Changsha, P. R. China*

Received February 20, 2019; Accepted March 27, 2019; Epub May 1, 2019; Published May 15, 2019

**Abstract:** Numerous microRNAs (miRNAs) have been shown to play an important regulatory role in the progression of hepatocellular carcinoma (HCC). miR-96-5p, a cancer-related microRNA, was previously reported to inhibit cell apoptosis in HCC, but the function and underlying mechanism of miR-96-5p's involvement in HCC metastasis and progression still remain unknown. In this study, we showed that a significant up-regulation of miR-96-5p in HCC tissues and cell lines, and its increased expression, are associated with microvascular invasion and with the TNM stages of HCC patients. Gain-of-function assays revealed that miR-96-5p induced the epithelial-mesenchymal transition (EMT) to promote the migration and invasion of HCC in vitro. The expression of TLN1 (Talin 1) is significantly decreased in HCC tissues and is inversely correlated to miR-96-5p levels. Notably, through a luciferase reporter assay and a Western blot analysis, TLN1 was confirmed to be a direct target gene of miR-96-5p. Furthermore, results of cell functional assays revealed that the over-expression of TLN1 partially reverses the promotive effects of miR-96-5p overexpression on the migration, invasion, and EMT of HCC. Overall, data from the present study demonstrate that miR-96-5p induces EMT to promote the migration and invasion of HCC by post-transcriptionally downregulating TLN1, indicating that the miR-96-5p/TLN1 axis might provide a potential therapeutic target for the treatment of HCC.

**Keywords:** HCC, miR-96-5p, EMT, TLN1, treatment

## Introduction

Hepatocellular carcinoma (HCC) is among the most fatal malignancies and is the third leading cause of cancer-related death throughout the world [1]. The carcinogenesis and development of HCC are the result of complex and multi-step processes associated with epigenetic and genetic changes [2]. There are no obvious symptoms during the early stages of HCC, and HCC patients are commonly diagnosed at the advanced or unresectable stages. Although remarkable improvements have been made in surgical techniques and perioperative management, the prognosis of HCC patients after hepatectomy is still unsatisfactory because of the high rates of recurrence and metastasis [3, 4]. Therefore, it is essential to elucidate the under-

lying molecular mechanisms of HCC metastasis and to develop novel strategies for the early diagnosis and treatment of HCC.

microRNAs (miRNAs) are a class of evolutionarily conserved small noncoding RNAs composed of 15~22 nucleotides that usually bind with the 3'-untranslated regions (3'-UTRs) of target genes to suppress their expression [5]. As post-transcriptional regulators of gene expression, miRNAs have broad effects on innumerable biological processes, including differentiation, metastasis, apoptosis, and the cell cycle [6]. Recently, the aberrant expression of miRNAs has been shown to play an important role in the pathogenesis and tumorigenicity of various human malignancies [7-9]. For example, the up-regulation of miR-1271 promotes

## miR-96-5p induces EMT to promote migration and invasion in HCC

non-small-cell lung cancer cell proliferation and invasion by targeting HOXA5 (homeobox A5) [10]. miR-152 inhibits tumor cell growth by directly targeting RTKN (Rhotekin) in HCC [11]. The overexpression of miR-613 inhibits the proliferation and invasion of renal cell carcinoma cells by targeting FZD7 (Frizzled class receptor 7) [12]. miR-101-3p upregulation suppresses cell invasion and proliferation and enhances chemotherapeutic sensitivity in salivary gland adenoid cystic carcinoma by directly targeting Pim-1 (Pim-1 proto-oncogene, serine/threonine kinase) [13].

miR-96-5p is one member of the miR-183-96-182 cluster, and it is located on human chromosome 7q32.2. Accumulating evidence has suggested that miR-96-5p is upregulated and plays a key role in different types of cancer, including breast [14], colorectal [15], and ovarian cancer [16]. Additionally, a previous study has demonstrated that miR-96-5p inhibits cell apoptosis by targeting the caspase-9 gene in HCC [17]. However, an understanding of the exact role and mechanism of miR-96-5p on HCC progression and metastasis is still needed. Recently, Fang et al. demonstrated that TLN1 (Talin 1) correlates with the malignancy potential of the HCC MHCC-97 L cell [18]. Further, emerging studies have suggested that TLN1 is significantly downregulated in HCC tissues compared with adjacent non-tumor tissues, and low TLN1 expression is associated with HCC progression and a poor prognosis [19]. TLN1 knockdown induces the epithelial-mesenchymal transition and promotes migration and invasion in SK-Hep-1 cells and HepG2 cells [19]. Interestingly, a bioinformatics analysis predicts that miR-96-5p is able to bind TLN1 mRNA 3'-UTR. Thus, we suspected miR-96-5p might have an important role in HCC progression by inhibiting TLN1 expression. In this study, we explored the possibility that miR-96-5p might be involved in HCC cell progression and metastasis, and we partially elucidated the underlying molecular mechanism.

### Materials and methods

#### *Cell lines and cell culture*

The human normal liver cell line (L02) and human liver cancer cell lines (Hep3B, Huh7, and HCCLM3) were obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai,

China. These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 µg/ml streptomycin (Sigma, USA), and 100 U/ml penicillin (Sigma, USA) at 37°C in a humidified chamber containing 5% CO<sub>2</sub>.

#### *Cell transfection*

The miR-96-5p mimic (5'-UUUGGCACUAGCAC-AUUUUUGCU-3') and the corresponding mimic negative control (mimic NC, 5'-CAAUAACCAUGACUUGACCU-3'), as well as the TLN1 expressing vector and the corresponding negative control vector (NC vector) were designed and synthesized by RiboBio Co., Ltd (Guangzhou, China). Huh7 and HCCLM3 cells were transfected with 50 nmol/L miR-96-5p mimic and mimic NC and/or 1 µg TLN1 expressing vector and NC vector by utilizing the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. At 48 h post-transfection, the transfection efficiency was evaluated in each experiment by qRT-PCR or Western blot analysis.

#### *Patients and tissue samples*

This study was approved by the Ethics Board of Hunan Provincial People's Hospital (Changsha, China), and it complied with the Declaration of Helsinki. All patients or the legal guardians of the patients agreed to participate in this study. Informed consent was obtained from each participant. HCC tissue specimens were obtained from 51 patients who underwent a surgical resection of their HCC lesions at the Department of Hepatopancreatobiliary Surgery between January 2007 and December 2010. None of the patients had received prior radiotherapy or chemotherapy. Fresh HCC tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. The pathological diagnosis was based on the World Health Organization criteria, and the tumor differentiation grades were based on the classification proposed by Edmondson and Steiner [20]. Tumor stage was determined in conformity with the TNM classification system proposed by the 2010 International Union Against Cancer [21]. After their surgeries, the patients were monitored until December 31, 2016. Overall survival (OS) was defined as the time between surgery and death or between surgery and the last observation

## miR-96-5p induces EMT to promote migration and invasion in HCC

time. The OS data were censored at the last follow-up for the surviving patients.

### *Reverse transcription-quantitative polymerase chain reaction (qRT-PCR)*

TRIzol solution (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cell lines and frozen tumor specimens according to the manufacturer's protocols. Synthesis of complementary DNA (cDNA) was performed by a TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) or PrimeScript Reverse Transcriptase reagent Kit with gDNA Eraser Kit (Takara, Osaka, Japan). U6 small nuclear 1 (U6) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. qPCR was performed using a 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Hieff™ qPCR SYBR® Green Master Mix (Yisheng Biotechnology Co., Ltd; Shanghai, China), in accordance with the manufacturer's protocols. The primers used for qPCR used are as follows: miR-96-5p (sense) 5'-ACACTCCAGCTGGGTTTGGCACTAGCACATTT-3', miR-96-5p (antisense) 5'-CTCAACTGGTGTCTGTCGTCGGCAATTCAGTGTAGAGCAAAA-3'; U6 (sense) 5'-CTCGCTTCGGCAGCACA-3', U6 (antisense) 5'-AACGGTTCACGAATTTGCGT-3'; TLN1 sequences of forward and reverse primers: 5'-GTGCCCTATCCACTTCC-3' and 5'-TGGCTACCAGTCGTCCAG-3'; GAPDH sequences of forward and reverse primers: 5'-CTGGGCTACACTGAGCACC-3' and 5'-AAGTGTCGTTGAGGGCAATG-3'. The reaction condition was 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 sec and annealing/elongation step at 60°C for 30 sec. The expression levels of miR-96-5p and TLN1 were normalized by U6 and GAPDH to produce a  $2^{-\Delta\Delta C_t}$  value for relative expression [22].

### *Luciferase reporter assay*

The prediction of potential miR-96-5p target genes was performed using the publicly available algorithm TargetScanHuman 7.2 ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)). Human TLN1 3'-untranslated region (UTR), including the complimentary binding region of miR-96-5p, was amplified by PCR and inserted into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to construct the wild type (WT)-TLN1 reporter plasmid. The mutant binding region was generated

using a Quick Change mutagenesis kit (Takara, Japan), and was also cloned into a pmirGLO vector to produce mutant (MUT)-TLN1 reporter plasmid. For the luciferase reporter assay, co-transfection with WT or MUT-TLN1 reporter plasmid and miR-96-5p mimic and mimic NC were introduced into Huh7 and HCCLM3 cells by using the Lipofectamine 2000 transfection reagent. Luciferase activities were measured at 48 h post-transfection by a Dual-Luciferase Assay kit (Promega, Madison, WI, USA), according to the manufacturer's protocols.

### *Cell migration assay*

A wound-healing assay was performed to evaluate cell migration. Briefly, Huh7 and HCCLM3 cells ( $3 \times 10^5$  per/well) transfected with miR-96-5p mimic and mimic NC and/or TLN1 expressing vector and NC vector were seeded into six-well culture plates and cultured with DMEM medium and FBS to form a tight monolayer. Scraped lines were created with 200  $\mu$ l sterile pipette tips, and the cell debris were removed with PBS, and the remaining cells were incubated for 24 h at 37°C with no serum-containing DMEM medium. The migrated distances of the growing edge on the monolayer were observed by a lighted microscope (ECLIPSE TS-100; Nikon Corporation, Tokyo, Japan) under a 400 $\times$  microscope field at 0~12 h after being wounded.

### *Cell invasion assay*

For the cell transwell invasion assay, Matrigel-precoated 6-well and 8- $\mu$ m pore transwell chambers (Costar, Cambridge, MA, USA) were used to evaluate the cell invasion. HCC cells ( $3 \times 10^5$  per/well) were dispensed into the upper chamber with a 200  $\mu$ l no serum-containing DMEM medium, while a 500  $\mu$ l normal serum-containing DMEM medium was added in the under chamber. A cotton swab was used to remove the gel and cells in the upper chamber after 48 h of incubation, and the cells that invaded to the lower membrane of the chamber were fixed using 4% paraformaldehyde (Beyotime Biotechnology, Shanghai, China) for 30 min at 37°C and stained with 0.1% crystal violet dye (Beyotime Biotechnology, Shanghai, China) at 37°C for 20 min. Finally, the invaded cells were counted (at  $\times 200$  magnification) in five microscopic fields using a lighted micro-

# miR-96-5p induces EMT to promote migration and invasion in HCC

scope (ECLIPSE TS100; Nikon Corporation, Tokyo, Japan).

## Western blot

Western blot was performed to evaluate the protein expression. In brief, the total protein from the cell lines was extracted using a RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocols. A BCA Protein Assay Kit (Pierce, IL, USA) was used to quantify the concentration of each sample. The protein was then subjected to 8% SDS-polyacrylamide gel electrophoresis (Beyotime Biotechnology, Shanghai, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After that, the membranes were blocked with 5% non-fat milk for 2 h at 37°C and then incubated with TLN1 antibodies (dilution: 1:500; no. ab137843), GAPDH antibodies (dilution: 1:5000; no. ab181-602), E-cadherin antibodies (dilution: 1:1000; no. ab1416), N-cadherin antibodies (dilution: 1:500; no. ab76011), and vimentin antibodies (dilution: 1:2000; no. ab92547) at 4°C overnight. These antibodies were obtained from Abcam (Cambridge, MA, USA). After washing with a TBS-T buffer for 15 min, the membranes were incubated with appropriate second antibodies (Cambridge, MA, USA) for 1 h at 37°C. Positive bands were conducted using an ECL (enhanced chemiluminescence) system (Pierce, IL, USA). The protein expressions of TLN1, E-cadherin, N-cadherin and vimentin were analyzed by using Image-Pro plus software 6.0 (Media Cybernetics Inc, Silver Springs, MD, USA), comparing the density ratio with GAPDH.

## Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA). Data were presented as the mean  $\pm$  standard deviation (SD). The statistically significant differences between different groups were analyzed by one-way ANOVA followed by post hoc tests or Student's *t*-test, as appropriate. Correlations were determined by Spearman's correlation analysis. The relationship between miR-96-5p expression and the clinicopathologic characteristics of HCC patients was analyzed using a chi-square test. Survival curves were evaluated using a Kaplan-Meier survival analysis. A value of  $P < 0.05$  indicated significant differences.

## Results

*Expression of miR-96-5p was significantly increased in HCC tissues and cell lines*

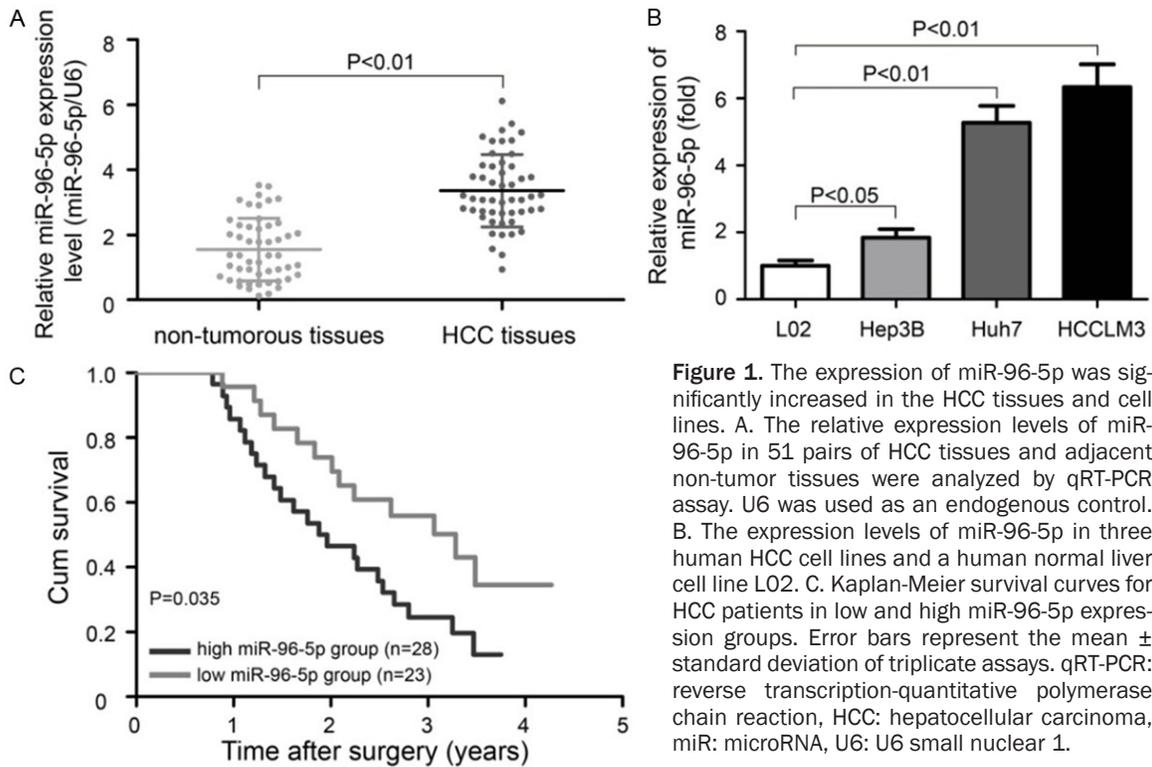
To investigate the role of miR-96-5p in the progression of HCC, we first detected the levels of miR-96-5p by qRT-PCR in the HCC tissues and in the adjacent non-tumorous tissues from 51 patients with HCC. As shown in **Figure 1A**, the expression of miR-96-5p in the HCC tissues was significantly higher than that in the adjacent non-cancerous tissues ( $P < 0.01$ ).

A qRT-PCR assay was further applied to quantify miR-96-5p expression in three HCC cell lines, including Hep3B, Huh7, and HCCLM3, and the human normal liver cell line L02. The results revealed that the expression of miR-96-5p was significantly upregulated in the three HCC cell lines compared with L02 cells (**Figure 1B**,  $P < 0.05$ ), especially in the case of the Huh7 and HCCLM3 cells. According to the relatively high levels of expression of miR-96-5p in the Huh7 and HCCLM3 cells, these cells were selected for further study in vitro.

*Up-regulation of miR-96-5p associated with microvascular invasion, TNM stage and the poor prognosis of patients with HCC*

Next, we investigated the clinicopathological characteristics and prognosis of miR-96-5p in patients with HCC. Based on the median value of the miR-96-5p expression level, all 51 HCC patients were divided into two subgroups: the low miR-96-5p group ( $n = 23$ ) and the high miR-96-5p group ( $n = 28$ ). The association between miR-96-5p expression and the clinicopathological parameters of all HCC patients, including age, gender, alcoholism, cirrhosis, hepatitis B surface antigen (HBsAg) status, tumor size, TNM stage, differentiation and microvascular invasion, were analyzed. As shown in **Table 1**, an increase of miR-96-5p expression was significantly correlated with the parameters of the TNM stages ( $P = 0.004$ ) and microvascular invasion ( $P = 0.015$ ), but not with age, gender, tumor size, differentiation, alcoholism, cirrhosis, or HBsAg status. The Kaplan-Meier survival analysis revealed that patients with high miR-96-5p expression had a shorter overall survival (OS) times than those patients with low miR-96-5p expression (**Figure 1C**,  $P < 0.05$ ). Thus,

## miR-96-5p induces EMT to promote migration and invasion in HCC



**Figure 1.** The expression of miR-96-5p was significantly increased in the HCC tissues and cell lines. A. The relative expression levels of miR-96-5p in 51 pairs of HCC tissues and adjacent non-tumor tissues were analyzed by qRT-PCR assay. U6 was used as an endogenous control. B. The expression levels of miR-96-5p in three human HCC cell lines and a human normal liver cell line L02. C. Kaplan-Meier survival curves for HCC patients in low and high miR-96-5p expression groups. Error bars represent the mean  $\pm$  standard deviation of triplicate assays. qRT-PCR: reverse transcription-quantitative polymerase chain reaction, HCC: hepatocellular carcinoma, miR: microRNA, U6: U6 small nuclear 1.

our findings indicated that the upregulation of miR-96-5p might play a potential role in promoting the malignant progression of HCC.

### *The over-expression of miR-96-5p promoted the migration, invasion and EMT of HCC cells*

To explore the functional role of miR-96-5p in HCC, gain-of-function assays were performed in the Huh7 and HCCLM3 cells. Known from the qRT-PCR assay, the levels of miR-96-5p in Huh7 and HCCLM3 cells were successfully overexpressed by the transfection of the miR-96-5p mimic (**Figure 2A**,  $P < 0.01$ ). Results of both the wound healing and transwell invasion assays revealed that the HCC cells transfected with the miR-96-5p mimic had a higher migratory and more invasive capacity than the cells treated with mimic NC (**Figure 2B** and **2C**,  $P < 0.05$ ). Furthermore, miR-96-5p was studied on HCC metastasis by regulating EMT. The expressions of the EMT markers were detected and the results indicated that the level of E-cadherin, as an epithelial marker, was decreased after miR-96-5p upregulation in Huh7 and HCCLM3 cells, but the levels of mesenchymal markers including N-cadherin and vimentin were increased (**Figure 2D**,  $P < 0.05$ ). Therefore, these results suggest that miR-96-5p induces EMT to promote the migration and invasion of HCC.

### *TLN1 level was inversely correlated with miR-96-5p expression in HCC tissues*

miR-96-5p was predicted to be able to bind TLN1 mRNA 3'-UTR; therefore, our next objective was to determine the levels of TLN1 mRNA present in the 51 HCC samples using a qRT-PCR assay and analyzing the relationship between TLN1 level and miR-96-5p expression. As shown in **Figure 3A**, TLN1 mRNA expression was significantly lower in the HCC tissues than it was in the adjacent non-cancerous tissues ( $P < 0.01$ ). Spearman's correlation analysis showed a significant inverse correlation between TLN1 level and miR-96-5p expression in HCC tissues (**Figure 3B**,  $P < 0.01$ ).

To further test the association between miR-96-5p and TLN1, we quantified expression of endogenous TLN1 in the HCC cells after they were transfected with the miR-96-5p mimic or the mimic NC. The data showed that transfection of the miR-96-5p mimic resulted in a dramatically decreased protein expression of TLN1 in both HCC cell lines (**Figure 3C**,  $P < 0.01$ ).

### *TLN1 was found to be one of the targets of miR-96-5p*

To further investigate the underlying mechanism of how miR-96-5p exerts its functional

## miR-96-5p induces EMT to promote migration and invasion in HCC

**Table 1.** The association of miR-96-5p expression with the clinicopathological features of patients with HCC

Features	Patients	miR-96-5p expression		P value
		Low (n = 23)	High (n = 28)	
Age (years)				
< 60	19	6	13	0.135
≥ 60	32	17	15	
Sex				
Male	38	15	23	0.168
Female	13	8	5	
Tumor size				
< 5 cm	25	10	15	0.473
≥ 5 cm	26	13	13	
TNM stage				
I-II	27	7	20	0.004
III-IV	24	16	8	
Differentiation				
Well/Moderately	30	16	14	0.158
Poorly	21	7	14	
Microvascular invasion				
Absent	35	20	15	0.015
Present	16	3	13	
Alcoholism				
No	23	11	12	0.723
Yes	28	12	16	
Cirrhosis				
Without	17	8	9	0.842
With	34	15	19	
HBsAg status				
Negative	8	2	6	0.269
Positive	43	21	22	

HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen.

effects on HCC cells, identification of the target genes for miR-96-5p was performed through an algorithm from TargetScanHuman 7.2. Among these genes, TLN1, an important tumor suppressor gene in HCC, was chosen for further experiments. As shown in **Figure 4A**, a complimentary binding region for miR-96-5p was found in the 3'-UTR of TLN1 mRNA. To determine whether TLN1 was a direct target gene of miR-96-5p, a luciferase reporter assay was performed. The results showed that an overexpression of miR-96-5p was able to decrease the luciferase activity of the WT-TLN1 reporter plasmid in both HCC cell lines; however, no significant change of the luciferase activity was found with the MUT-TLN1 reporter plas-

mid after modulating the miR-96-5p expression (**Figure 4B** and **4C**,  $P < 0.01$ ). Taken together, these data suggested that TLN1 could be a direct downstream target of miR-96-5p in HCC.

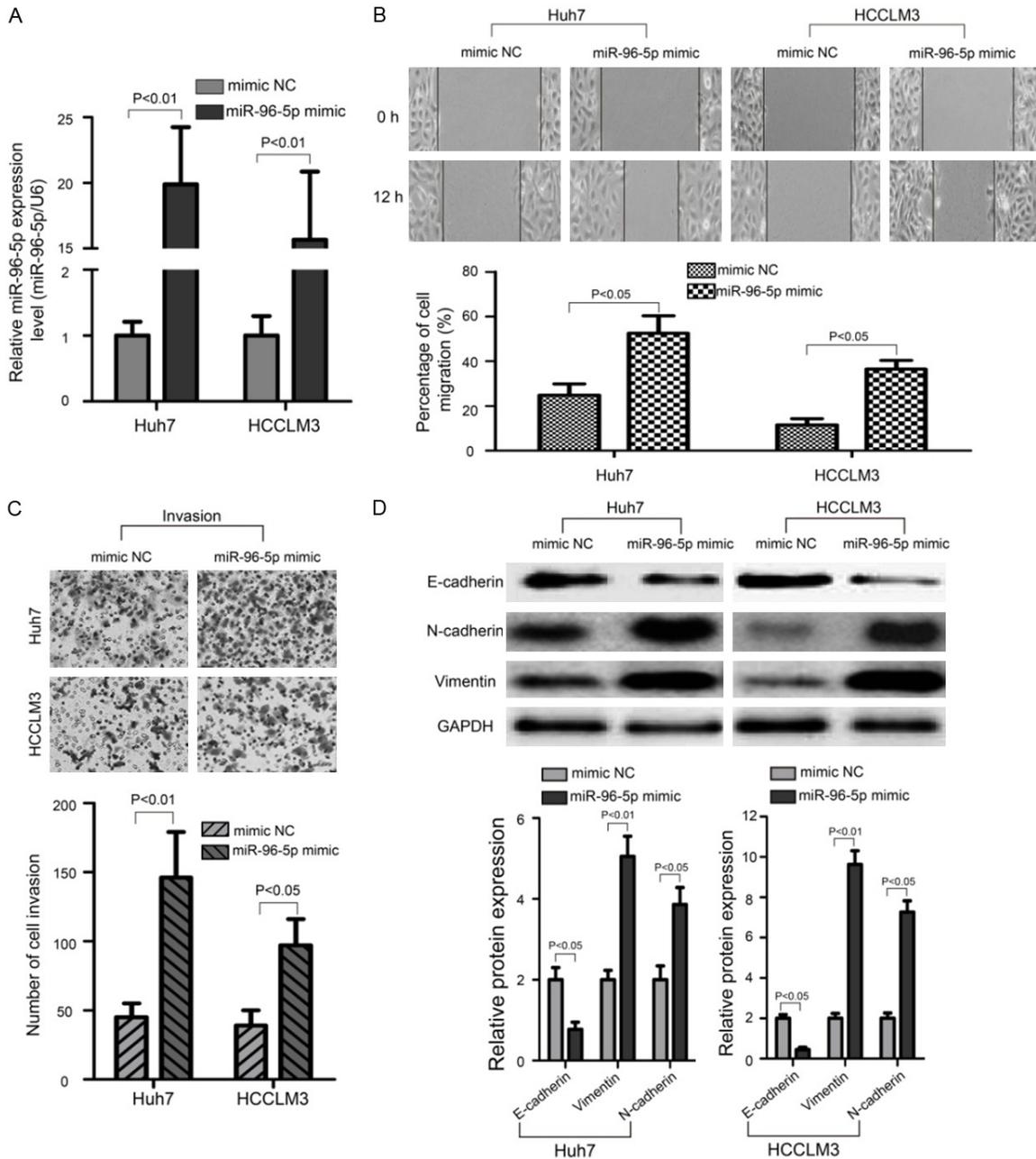
*TLN1 mediated the effects of miR-96-5p on the invasion, migration, and EMT of HCC cells*

Finally, rescue experiments were performed to confirm whether miR-96-5p executed its functional effects by suppressing TLN1 in HCC cells. TLN1 expression was restored by the TLN1 expressing vector after the overexpression of miR-96-5p in Huh7 and HCCLM3 cells (**Figure 5A**,  $P < 0.01$ ). Restoration of TLN1 expression attenuated the miR-96-5p-mediated promotive effects on the migration, invasion, and EMT in both HCC cell lines (**Figure 5B-D**,  $P < 0.05$ ). These findings strongly suggested that TLN1 acts as a functional downstream target of miR-96-5p in HCC cells, indicating miR-96-5p induces EMT to promote the migration and invasion of HCC by the downregulation of TLN1.

### Discussion

Deregulated miRNAs have been shown to be involved in the development and progression of HCC, which function as either tumor suppressor genes or oncogenes [23]. For instance, Wang, et al. showed that miR-300 regulates the EMT and invasion of HCC by targeting the FAK/PI3K/AKT signaling pathway [24]. Luo, et al. found that miR-501-3p suppresses the metastasis and progression of HCC by targeting LIN7A (lin-7 homolog A, crumbs cell polarity complex component) [25]. Yang, et al. reported that miR-1301 inhibits HCC cell migration, invasion, and angiogenesis by decreasing Wnt/ $\beta$ -catenin signaling through targeting BCL9 (B-cell lymphoma 9) [26]. Cao, et al. demonstrated that miR-23b suppressed EMT and metastasis in HCC via targeting PTK2B (protein tyrosine kinase 2 beta) [27]. Although several studies have demonstrated the roles of miRNAs in the metastasis and progression of HCC, the under-

## miR-96-5p induces EMT to promote migration and invasion in HCC



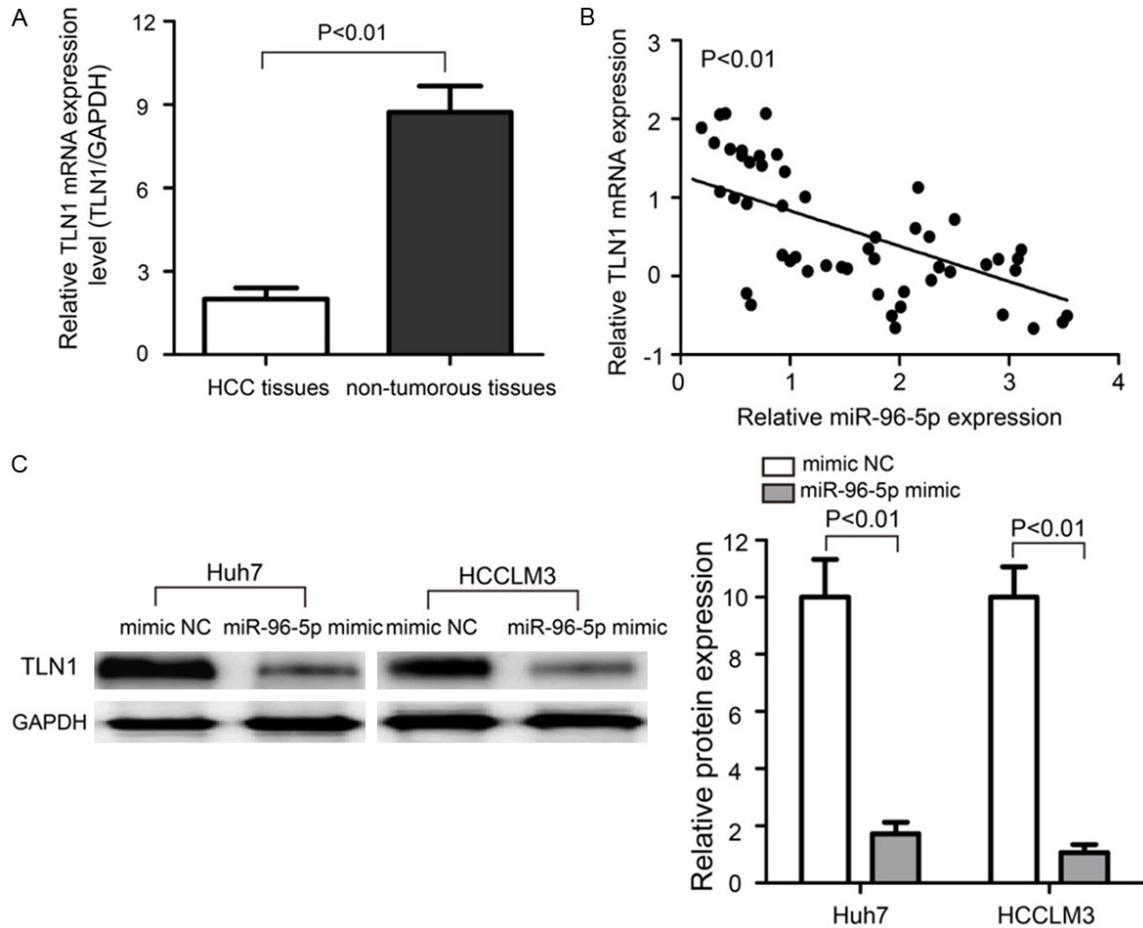
**Figure 2.** The over-expression of miR-96-5p promoted the migration, invasion, and EMT of HCC cells. A. Huh7 and HCCLM3 cells transfected with miR-96-5p mimic and mimic NC were subjected to qRT-PCR for the analysis of the miR-96-5p expression. B. The migratory capacity of the HCC cells was strengthened by miR-96-5p overexpression (at  $\times 400$  magnification). C. Invasion of Huh7 and HCCLM3 cells that were transfected with the miR-96-5p mimic and mimic NC was determined using a transwell invasion assay (at  $\times 200$  magnification). D. miR-96-5p upregulation decreased the expression of E-cadherin and increased the levels of N-cadherin and vimentin in Huh7 and HCCLM3 cells. The experiments were performed in triplicate. mimic NC: corresponding mimic negative control, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

lying molecular mechanisms by which miRNAs regulate HCC metastasis are still unclear.

As a multifunctional miRNA, miR-96-5p plays crucial roles in a variety of physiological and

pathological processes, such as hepatic stellate cell activation [28], autophagy [28], craniofacial and dental development [29], and neuroprotection [30]. As an oncogene, miR-96-5p has been shown to be overexpressed in several

miR-96-5p induces EMT to promote migration and invasion in HCC



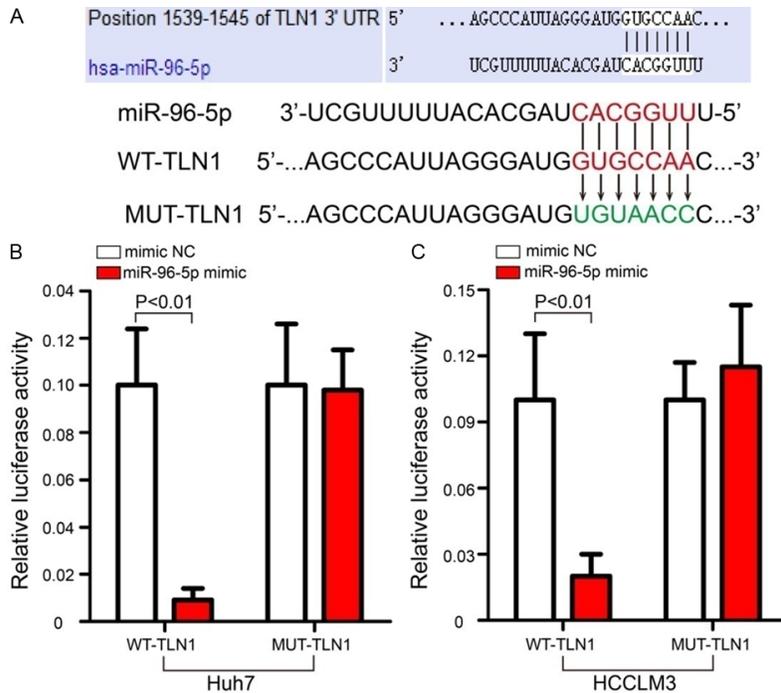
**Figure 3.** TLN1 levels were downregulated in HCC tissues and inversely correlated with miR-96-5p expression. A. The expression levels of TLN1 from HCC tissues and adjacent non-tumor tissues, as determined by a qRT-PCR assay. GAPDH was used as an endogenous control. B. The correlation between the miR-96-5p expression and the TLN1 levels in the HCC samples. C. miR-96-5p overexpression reduced the expression of TLN1 protein in the Huh7 and HCCLM3 cells. Values are expressed as the mean  $\pm$  standard deviation of three independent experiments. TLN1: Talin 1.

types of human cancers, including breast, colorectal, and ovarian cancer, and is closely related to the migration and metastasis of malignant tumors [14-16]. In the present study, our data showed that miR-96-5p was significantly up-regulated in HCC tissues and cell lines. The presence of upregulated miR-96-5p expression in HCC was consistent with the results of previous studies [14-16]. Combined with the clinicopathological features, our results revealed that an increased expression of miR-96-5p in patients with HCC was associated with microvascular invasion, TNM stage, and a poor prognosis. These data strongly suggest that the upregulation of miR-96-5p is correlated with the metastasis and progression of HCC.

The recurrence and metastasis of HCC are the main culprits of the poor prognosis of HCC pa-

tients [31]. miRNAs have been found to play an important role in modulating the invasion and metastasis of HCC cells [24-27]. Following the determination of the clinical significance of miR-96-5p in HCC samples, the biological roles of miR-96-5p were analyzed in HCC cell lines. The results indicated that miR-96-5p plays a key role in the progression of HCC by promoting cell migration and invasion in vitro. EMT is a biological process by which cancer cells lose their epithelial polarity to assume a mesenchymal phenotype, and it is characterized by a decreased expression of the epithelial marker (E-cadherin) and an increased expression of the mesenchymal markers (vimentin and N-cadherin) [32]. EMT has been demonstrated as the critical mechanism for the invasion and metastasis of human cancer cells. Accumulating evidence has demonstrated that miRNAs play a

## miR-96-5p induces EMT to promote migration and invasion in HCC



**Figure 4.** TLN1 was a direct target gene of miR-96-5p. A. The potential miR-96-5p binding site in the 3'-UTR of TLN1 mRNA was computationally predicted by TargetScanHuman 7.2. B. Luciferase reporter assay analysis of the effects of miR-96-5p over-expression on the luciferase activities of wild type (WT) and mutant (MUT) TLN1 reporter plasmids in Huh7 cells. C. Luciferase activities of WT and MUT TLN1 reporter plasmids in HCCLM3 cells co-transfected with miR-96-5p mimic and mimic NC. The experiments were performed in triplicate.

critical role in the EMT of HCC cells [33, 34]. Here, we also found that the over-expression of miR-96-5p decreases E-cadherin expression and increases N-cadherin and vimentin expression. These data suggest that miR-96-5p could promote the migration and invasion of HCC by inducing EMT.

To explore the molecular mechanisms by which miR-96-5p promotes the progression and metastasis of HCC, we identified TLN1 as a direct target gene of miR-96-5p in HCC. TLN1, a large 270 kDa cytoskeletal protein that contains 2541 amino acids, is mainly expressed in the liver, spleen, stomach, kidney, lung, and vascular smooth muscle and plays an essential role in integrin activation [35]. Previous studies have demonstrated that aberrantly expressed TLN1 is associated with oral squamous cell carcinoma and prostate cancer [36, 37]. TLN1 knockdown induces the epithelial-mesenchymal transition and promotes migration and invasion in SK-Hep-1 cells and HepG2 cells [19]. TLN1 has been reported to be a target gene of miR-9 in ovarian carcinoma [38] and

miR-124 in prostate cancer [39]; however, the interaction between miR-96-5p and TLN1 has not been reported in HCC. Here, by using a luciferase reporter assay, we confirmed that the TLN1 gene was a direct target of miR-96-5p. The over-expression of miR-96-5p significantly suppressed the expression levels of the TLN1 protein in HCC cells. Excitingly, the restoration of TLN1 expression attenuated miR-96-5p-mediated promotive effects on migration, invasion, and EMT in both HCC cell lines. These results indicated that TLN1 was a direct and functional target of miR-96-5p in HCC. The limitations of this study are as follows: 1) the clinical samples of HCC were insufficient; 2) the target genes of miR-96-5p were not limited to TLN1 in HCC; 3) the function and mechanism of miR-96-5p in the

nude mouse tumor model remains to be elucidated.

In conclusion, our results demonstrate that miR-96-5p is upregulated in HCC tissue and cell lines, and high miR-96-5p expression is significantly associated with microvascular invasion, TNM stage, and a poor prognosis in patients with HCC. The over-expression of miR-96-5p induces EMT to promote the migration and invasion of HCC by the suppression of TLN1. Understanding the role of the miR-96-5p/TLN1 axis' involvement in HCC metastasis and progression will enable us to use it as a therapeutic target in treating HCC.

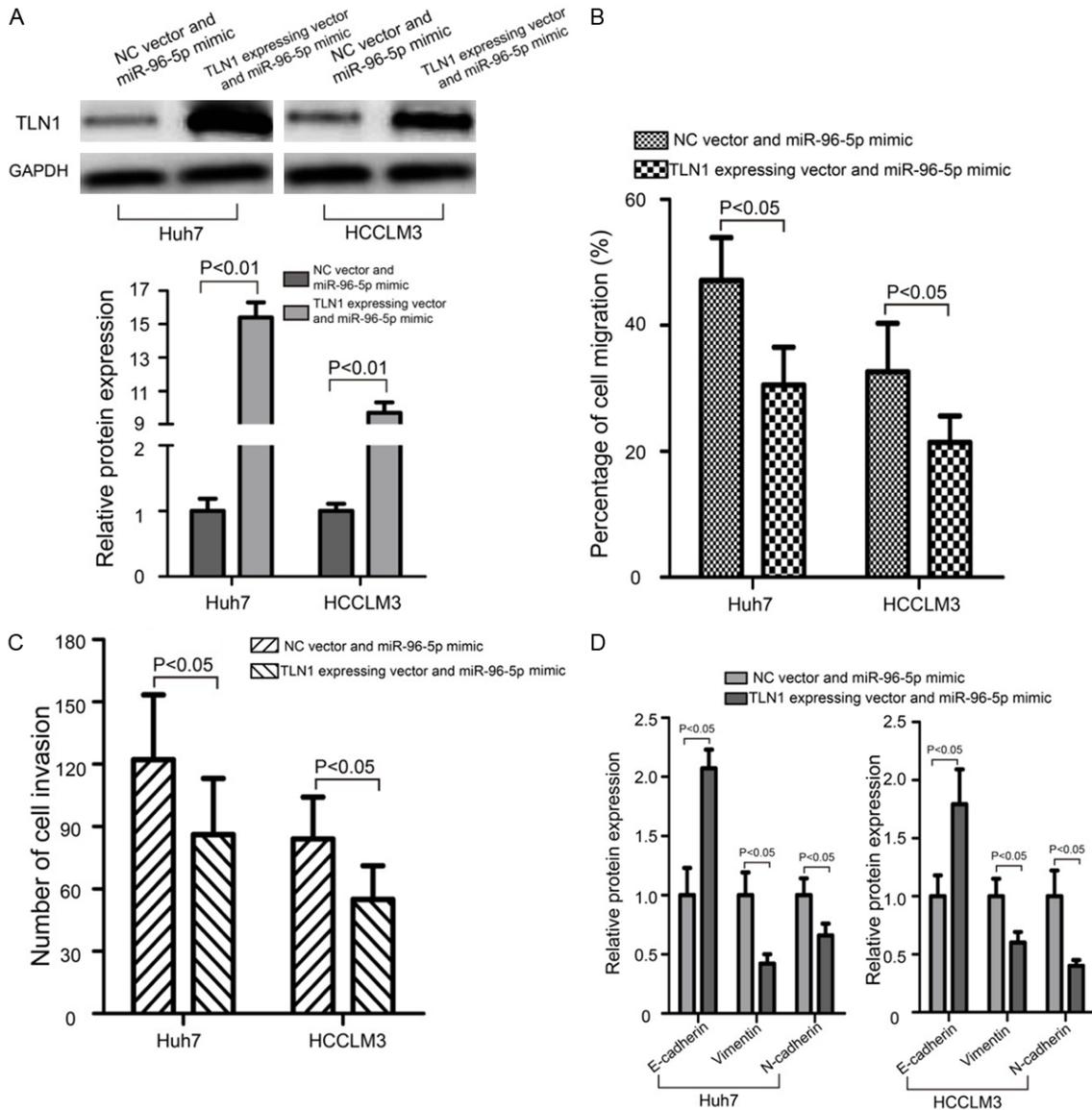
### Acknowledgements

This work was supported by the Hunan Provincial Natural Science Foundation (no. 2017-JJ3176) and the Youth PhD Fund of Hunan Provincial People's Hospital (BSJJ201808).

### Disclosure of conflict of interest

None.

# miR-96-5p induces EMT to promote migration and invasion in HCC



**Figure 5.** TLN1 mediated the effects of miR-96-5p on the invasion, migration, and EMT of HCC cells. A. A Western blot analysis was performed to measure the TLN1 protein levels in the Huh7 and HCCLM3 cells treated with the TLN1 expressing vector and the miR-96-5p mimic. GAPDH was used as an internal control. B. SMMC-7721 and HepG2 cell migration following transfection with the miR-96-5p mimic with or without TLN1 expressing vector. C. Restoration of TLN1 expression attenuated the miR-96-5p-mediated promotive effects on the invasion in both HCC cell lines. D. Western blot analysis of E-cadherin, N-cadherin and vimentin protein levels in the SMMC-7721 and HepG2 cells transfected with the miR-96-5p mimic with or without the TLN1 expressing plasmid. The experiments were performed in duplicate and repeated three times.

**Address correspondence to:** Dr. Ning Zhou, Laboratory of Hepatobiliary Molecular Oncology, Department of Hepatopancreatobiliary Surgery, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, 61 West Liberation Road, Changsha 410011, P. R. China. Tel: 86-731-83929140; E-mail: zhouning\_198401@126.com

## References

- [1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65: 87-108.
- [2] Nishida N and Goel A. Genetic and epigenetic signatures in human hepatocellular carcinoma: a systematic review. *Curr Genomics* 2011; 12: 130-137.

## miR-96-5p induces EMT to promote migration and invasion in HCC

- [3] Dhanasekaran R, Limaye A and Cabrera R. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics. *Hepat Med* 2012; 4: 19-37.
- [4] Karaman B, Battal B, Sari S and Verim S. Hepatocellular carcinoma review: current treatment, and evidence-based medicine. *World J Gastroenterol* 2014; 20: 18059-18060.
- [5] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215-233.
- [6] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; 116: 281-297.
- [7] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR and Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; 435: 834-838.
- [8] Ruvkun G. Clarifications on miRNA and cancer. *Science* 2006; 311: 36-37.
- [9] Farazi TA, Hoell JI, Morozov P and Tuschl T. MicroRNAs in human cancer. *Adv Exp Med Biol* 2013; 774: 1-20.
- [10] Wang Y, Xu L and Jiang L. miR-1271 promotes non-small-cell lung cancer cell proliferation and invasion via targeting HOXA5. *Biochem Biophys Res Commun* 2015; 458: 714-719.
- [11] Zhou J, Zhang Y, Qi Y, Yu D, Shao Q and Liang J. MicroRNA-152 inhibits tumor cell growth by directly targeting RTKN in hepatocellular carcinoma. *Oncol Rep* 2017; 37: 1227-1234.
- [12] Song H, Nan Y, Wang X, Zhang G, Zong S and Kong X. MicroRNA613 inhibits proliferation and invasion of renal cell carcinoma cells through targeting FZD7. *Mol Med Rep* 2017; 16: 4279-4286.
- [13] Liu XY, Liu ZJ, He H, Zhang C and Wang YL. MicroRNA-101-3p suppresses cell proliferation, invasion and enhances chemotherapeutic sensitivity in salivary gland adenoid cystic carcinoma by targeting Pim-1. *Am J Cancer Res* 2015; 5: 3015-3029.
- [14] Shi Y, Zhao Y, Shao N, Ye R, Lin Y, Zhang N, Li W, Zhang Y and Wang S. Overexpression of microRNA-96-5p inhibits autophagy and apoptosis and enhances the proliferation, migration and invasiveness of human breast cancer cells. *Oncol Lett* 2017; 13: 4402-4412.
- [15] Kara M, Yumrutas O, Ozcan O, Celik OI, Bozgeyik E, Bozgeyik I and Tasdemir S. Differential expressions of cancer-associated genes and their regulatory miRNAs in colorectal carcinoma. *Gene* 2015; 567: 81-86.
- [16] Wang L, Zhu MJ, Ren AM, Wu HF, Han WM, Tan RY and Tu RQ. A ten-microRNA signature identified from a genome-wide microRNA expression profiling in human epithelial ovarian cancer. *PLoS One* 2014; 9: e96472.
- [17] Iwai N, Yasui K, Tomie A, Gen Y, Terasaki K, Kitaichi T, Soda T, Yamada N, Dohi O, Seko Y, Umemura A, Nishikawa T, Yamaguchi K, Moriguchi M, Konishi H, Naito Y and Itoh Y. Oncogenic miR-96-5p inhibits apoptosis by targeting the caspase-9 gene in hepatocellular carcinoma. *Int J Oncol* 2018; 53: 237-245.
- [18] Fang KP, Dai W, Ren YH, Xu YC, Zhang SM and Qian YB. Both Talin-1 and Talin-2 correlate with malignancy potential of the human hepatocellular carcinoma MHCC-97 L cell. *BMC Cancer* 2016; 16: 45.
- [19] Chen P, Lei L, Wang J, Zou X, Zhang D, Deng L and Wu D. Downregulation of Talin1 promotes hepatocellular carcinoma progression through activation of the ERK1/2 pathway. *Cancer Sci* 2017; 108: 1157-1168.
- [20] Zhou L, Rui JA, Zhou WX, Wang SB, Chen SG and Qu Q. Edmondson-Steiner grade: a crucial predictor of recurrence and survival in hepatocellular carcinoma without microvascular invasion. *Pathol Res Pract* 2017; 213: 824-830.
- [21] Baba Y, Hayashi S, Ueno K, Nakajo M, Ueno S, Kubo F, Hamanoue M, Hasegawa S, Tsubouchi H and Komorizono Y. Comparison of survival rates between patients treated with transcatheter arterial chemoembolization and hepatic resection for solitary hepatocellular carcinoma. *Oncol Lett* 2010; 1: 905-911.
- [22] Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3: 1101-1108.
- [23] Chen E, Xu X, Liu R and Liu T. Small but heavy role: microRNAs in hepatocellular carcinoma progression. *Biomed Res Int* 2018; 2018: 6784607.
- [24] Wang R, Yu Z, Chen F, Xu H, Shen S, Chen W, Chen L, Su Q, Zhang L, Bi J, Zeng W, Li W, Huang X and Wang Q. miR-300 regulates the epithelial-mesenchymal transition and invasion of hepatocellular carcinoma by targeting the FAK/PI3K/AKT signaling pathway. *Biomed Pharmacother* 2018; 103: 1632-1642.
- [25] Luo C, Yin D, Zhan H, Borjigin U, Li C, Zhou Z, Hu Z, Wang P, Sun Q, Fan J, Zhou J, Wang X, Zhou S and Huang X. microRNA-501-3p suppresses metastasis and progression of hepatocellular carcinoma through targeting LIN7A. *Cell Death Dis* 2018; 9: 535.
- [26] Yang C, Xu Y, Cheng F, Hu Y, Yang S, Rao J and Wang X. miR-1301 inhibits hepatocellular carcinoma cell migration, invasion, and angiogenesis by decreasing Wnt/beta-catenin signaling through targeting BCL9. *Cell Death Dis* 2017; 8: e2999.
- [27] Cao J, Liu J, Long J, Fu J, Huang L, Li J, Liu C, Zhang X and Yan Y. microRNA-23b suppresses epithelial-mesenchymal transition (EMT) and metastasis in hepatocellular carcinoma via tar-

## miR-96-5p induces EMT to promote migration and invasion in HCC

- getting Pyk2. *Biomed Pharmacother* 2017; 89: 642-650.
- [28] Yu K, Li N, Cheng Q, Zheng J, Zhu M, Bao S, Chen M and Shi G. miR-96-5p prevents hepatic stellate cell activation by inhibiting autophagy via ATG7. *J Mol Med (Berl)* 2018; 96: 65-74.
- [29] Gao S, Moreno M, Eliason S, Cao H, Li X, Yu W, Bidlack FB, Margolis HC, Baldini A and Amendt BA. TBX1 protein interactions and microRNA-96-5p regulation controls cell proliferation during craniofacial and dental development: implications for 22q11.2 deletion syndrome. *Hum Mol Genet* 2015; 24: 2330-2348.
- [30] Kinoshita C, Aoyama K, Matsumura N, Kikuchi-Utsumi K, Watabe M and Nakaki T. Rhythmic oscillations of the microRNA miR-96-5p play a neuroprotective role by indirectly regulating glutathione levels. *Nat Commun* 2014; 5: 3823.
- [31] Zhang Y, Shi ZL, Yang X and Yin ZF. Targeting of circulating hepatocellular carcinoma cells to prevent postoperative recurrence and metastasis. *World J Gastroenterol* 2014; 20: 142-147.
- [32] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; 2: 442-454.
- [33] Yu M, Xue H, Wang Y, Shen Q, Jiang Q, Zhang X, Li K, Jia M, Jia J, Xu J and Tian Y. miR-345 inhibits tumor metastasis and EMT by targeting IRF1-mediated mTOR/STAT3/AKT pathway in hepatocellular carcinoma. *Int J Oncol* 2017; 50: 975-983.
- [34] Dou C, Liu Z, Xu M, Jia Y, Wang Y, Li Q, Yang W, Zheng X, Tu K and Liu Q. miR-187-3p inhibits the metastasis and epithelial-mesenchymal transition of hepatocellular carcinoma by targeting S100A4. *Cancer Lett* 2016; 381: 380-390.
- [35] Xu YF, Ren XY, Li YQ, He QM, Tang XR, Sun Y, Shao JY, Jia WH, Kang TB, Zeng MS, Liu N and Ma J. High expression of Talin-1 is associated with poor prognosis in patients with nasopharyngeal carcinoma. *BMC Cancer* 2015; 15: 332.
- [36] Lai MT, Hua CH, Tsai MH, Wan L, Lin YJ, Chen CM, Chiu IW, Chan C, Tsai FJ and Jinn-Chyuan Sheu J. Talin-1 overexpression defines high risk for aggressive oral squamous cell carcinoma and promotes cancer metastasis. *J Pathol* 2011; 224: 367-376.
- [37] Xu N, Chen HJ, Chen SH, Xue XY, Chen H, Zheng QS, Wei Y, Li XD, Huang JB, Cai H and Sun XL. Upregulation of Talin-1 expression associates with advanced pathological features and predicts lymph node metastases and biochemical recurrence of prostate cancer. *Medicine (Baltimore)* 2016; 95: e4326.
- [38] Tang H, Yao L, Tao X, Yu Y, Chen M, Zhang R and Xu C. miR-9 functions as a tumor suppressor in ovarian serous carcinoma by targeting TLN1. *Int J Mol Med* 2013; 32: 381-388.
- [39] Zhang W, Mao YQ, Wang H, Yin WJ, Zhu SX and Wang WC. MiR-124 suppresses cell motility and adhesion by targeting talin 1 in prostate cancer cells. *Cancer Cell Int* 2015; 15: 49.