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Down-regulation of LncRNA NR027113 inhibits cell proliferation and metastasis via PTEN/PI3K/AKT signaling pathway in hepatocellular carcinoma

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Abstract. – OBJECTIVE: Hepatocellular Carcinoma (HCC) is a worldwide common and malignant tumor. It is discovered in recent years that long non-coding RNAs (lncRNAs) participate in many biological processes of HCC. However, their specific role in HCC has not been entirely clarified yet. In this research, we aimed to explore biological functions, clinical significance and the underlying molecular mechanisms of lncRNA NR027113 in HCC.

PATIENTS AND METHODS: qRT-PCR was performed to test the expression of NR027113 in HCC tissue samples and HCC cell lines. The association of NR027113 expression with overall survival, disease-free survival and clinicopathological factors was analyzed. MTT assays, Colony formation assay, flow cytometry and transwell invasion assays were performed to determine the effect of NR027113 in the regulation of biological behaviors of HCC cells. Western blot was performed to determine the activation of the PTEN/PI3K/AKT signaling pathway.

RESULTS: In the present study, we proved that is significantly up-regulated in HCC tissues and cell lines. HCC patients with higher NR027113 expression were associated with significantly shorter overall survival and disease-free survival. NR027113 knockdown inhibited the proliferation and metastasis of HCC cells *in vitro*. In addition, NR027113 knock-down was found to inhibit the activity of the PI3K/Akt signaling pathway and restrain the EMT process. Furthermore, we found that PTEN silencing could reverse the inhibitory effect of NR027113 knockdown on Akt phosphorylation and HCC cells function.

CONCLUSIONS: A brand new lncRNA NR027113 was found, which can promote the proliferation, invasion and metastasis of HCC via the PTEN/PI3K/AKT signaling pathway, and may be a potential therapeutic target in the future treatment of HCC.

Key Words:
LncRNA, Hepatocellular carcinoma, PTEN/PI3K/AKT signaling, EMT.

Abbreviations

HCC = Hepatocellular carcinoma; lncRNA = Long non-coding RNA; EMT = Epithelial to mesenchymal transition; PTEN = Phosphatase and Tensin homologue deleted on chromosome 10; PI3K = phosphatidylinositol 3-kinase; Akt = Protein kinase B; PVDF membrane = polyvinylidene difluoride membrane.

Introduction

Hepatocellular carcinoma (HCC) is the world's fifth most common cancer with high annual mortality rate[1]. Although some progress and breakthroughs have been made in the comprehensive treatment of HCC, the five-year survival rate is still less optimistic[2]. The poor prognosis of HCC is largely due to its biological characteristics[3]. Although studies have confirmed that many genes are associated with the poor prognosis of malignant tumors, the specific biological process that regulates HCC remains to be elucidated[4]. Therefore, determining the molecular mechanism of HCC is of great importance to discover new opportunities for preventing HCC patients from poor treatment outcomes. Long non-co-

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ding RNAs (LncRNAs) are a kind of RNA with a length of more than 200 nucleotides and without protein-coding ability[5]. Studies have shown that lncRNAs play an important role in epigenetic regulation, cell differentiation and other life activities[6,7]. Meanwhile, lncRNAs are closely related to the carcinogenesis, progression and prognosis of various tumors[8-10], such as AFAP1-AS1 in breast cancer[11], PVT1 in gastric cancer[12] and HULC in HCC[13]. Therefore, lncRNAs are now identified as a promising target for tumor therapy. Previous studies have shown that a lot of lncRNAs regulate the biological process of HCC[14,15]. However, it is unclear whether other lncRNAs are involved in the progression of HCC. The present study confirmed that NR027113, as a new lncRNA, is highly expressed in HCC tissues, and promotes the proliferation, invasion and metastasis of HCC through the PTEN/PI3K/AKT signaling pathway.

Patients and Methods

Patients and Samples

A total of 134 paired HCC tissue samples and adjacent normal tissues were obtained from Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. The diagnosis of HCC was based on pathology or dynamic contrast-enhanced imaging (computed tomography scan or magnetic resonance imaging) according to the diagnostic criteria of the European Association for the Study of the Liver (EASL, 2012). None of the patients received chemotherapy or radiotherapy before the operation. This study was approved by the Ethics Committee of the hospital and written informed consent for operative treatment of all the patients was obtained. The clinicopathological features of HCC patients are summarized in Table I. Meanwhile, multivariate analysis revealed independent prognostic importance of NR027113 expression in Table II.

Cell Culture

Human HCC cell lines (Bel-7402, SK-HEP-1, PLC/PRF/5, MHCC97H, and SMMC-7721) and normal hepatic cell lines LO2 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All the cells were grown routinely in 1640 or DMEM containing10% fetal bovine serum (Gibco, Grand Island, NY, USA). All cells were used for experiments at least 3 passages after thawing.

Table I. The relationship between Lnc NR027113 and clinicopathological factors of patients with HCC.

	LncNR027113 expression level		
Clinical characteristic	High expression n=78 (59%)	Low expression n=56 (41%)	*p*-value
Age (year)			
<50	40	20	0.075
≥50	38	36	
Gender			
Male	43	38	0.144
Female	35	18	
AFP (ng/ml)			
<20	34	19	0.206
≥20	44	39	
Tumor number			
Solitary	42	26	0.393
Multiple	36	30	
Tumor size (mm)			
<50	45	21	0.027*
≥50	33	35	
TNM stage			
I, II stage	32	10	<0.012*
III, IV stage	46	46	

Values represent the mean±SEM obtained with 6 animals in each group. $p<0.05$ compared with those of the water control, vehicle control and Difflam® solution (Dunnett's test). Histological healing scores determined as described in the Materials and methods section.

Table II. Multivariate analyses of HCC patients with Cox regression analysis.

Variable	Category	*p*-value
Age (year)	<50	
	≥50	1.205
Gender	Male	
	Female	0.564
AFP (ng/ml)	<20	0.371
	≥20	
Tumor number	Solitary	0.205
	Multiple	
Tumor size (mm)	<50	0.167
	≥50	
TNM stage	I, II stage	0.098
	III, IV stage	
NR027113	Low	
	High	0.016*

Quantitative Real-Time PCR

TRIzol reagent (TaKaRa, Shanghai, China) was used to extract RNA from fresh HCC tissues and HCC cell lines. The RNAs were then reversely transcribed into cDNA using PrimeScript RT reagent Kit (TaKaRa, Shanghai, China). Quantitative Real-Time PCR detection was performed with SYBR Green qPCR Master Mix (TaKaRa, Shanghai, China). The PCR primers used in this study are as follows: GAPDHForward 5'-GAAGGTGAAGGTCGGAGTCAACG-3', Reverse 5'-TGCCATGGGTGGAATCATATTGG-3', NR027113, Forward 5'-ACCAATCACATAGCCCTGCC-3', Reverse 5'-TCAGAGCTGCAGATGTGGTC-3', Short Hairpin RNAs (shRNAs) which targeted NR027113 were purchased from Gene Pharma (Shanghai, China).

Cell Transfection

NR027113 short hairpin RNAs (shRNA) target (Sh- #1 and Sh- #2) or negative control (Sh-NC) were packaged into 293T cell, and the viruses were collected by centrifugation. The targeted HCC cell lines were then infected with the lentivirus, and then screened with 1 mg/ml puromycin for 2 weeks to get a stable cell line.

MTT Cell Viability Assays

The cells were inoculated in 96-well plate with the density of 1000 cells in each well with 5 sub-wells for each kind of cells, and cultured for 3-5 days. Then, 5 mg/ml MTT (methyl thiazolyl tetrazolium colorimetric method) was added into each well following by 4 hours of continued incubation. Next, the supernatant was discarded and 150 ul DMSO was added into each well. The absorbance value of each well was measured at 490 nm.

Cell Apoptosis

After transfection with shRNAs, stable cell lines were got. The cells were cultured for 2 days, then, the cells were trypsinized and apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson, Franklin Lakes, NJ, USA). Then, cells were pelleted and washed with cold PBS suspended. The SMMC-7721 and SK-HEP-1 cells were then treated with Annexin V-propidium iodide (PI) in the dark for 20 min at room temperature. The cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA).

Clone Formation Assay

The cells were inoculated in 6-well culture plate, with 500 cells in each well. Then the cells were incubated routinely in the incubator. After 2 weeks culture, the cells were fixed with 5% paraformaldehyde, stained by crystal violet, rinsed by PBS twice, and counted as well as observed microscopically.

Migration and Invasion Assay

100 μl culture medium without serum was used to dissolve 1×10^5 cells, and seeded into the upper chamber of transwell cell culture plate (24 wells with 8 mm diameter; Corning, Corning, NY, USA). The bottom chamber was filled with 600 ul culture medium containing 20% fetal bovine serum. After 1-2 days, the cells were fixed by 5% paraformaldehyde and stained by crystal violet. The bottom of the chamber was wiped off, and the cell amount was counted microscopically. For invasion assay, Matrigel (R&D Systems, Minneapolis, MN, USA) was used to coat the porous membrane of the upper chamber.

Wound-Healing Assay

The cells were cultured in 6-well plate. When the cells grew to 90% area of the plate, scratching was done with 200 ul gene gun, fresh culture medium with without fresh (5%) fetal bovine serum was added and a photograph was taken at 0 and 48 hours, respectively.

Western Blotting

The protein concentration was detected by BCA method using cell lysis buffer (p0013 RIPA lysis, Beyotime, Shanghai, China). Then the cell lysis solution was boiled and ran the gel with 8% SDS-PAGE electrophoresis. The proteins were transferred to PVDF membranes (Millipore Sigma-Aldrich, Burlington, MA, USA). Then, the

PVDF membranes were incubated with primary antibodies at 4°C overnight: PI3K, p-PI3K, AKT, p-AKT, PTEN (Cell Signaling Technology, Danvers, MA, USA, 1:1000), and GAPDH (Proteintech, Wuhan, China, 1:500). Then, the membranes were incubated with secondary antibody Santa Cruz Biotechnology (Santa Cruz Biotechnology, Philadelphia, Santa Cruz, CA, USA, 1:10000) for 1 h at room temperature.

Statistics Analysis

Statistical analysis was performed with SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). The data was expressed by the mean value plus or minus standard deviation. Cumulative survival was calculated by the Kaplan-Meier analysis and the log-rank test. Quantitative data and categorical data were analyzed by the Student's *t*-test and Fisher's exact test, respectively. $p<0.05$ was considered statistically significant.

Results

Up-regulation of lncRNA NR027113 in HCC Tissues Confers a Poor Prognosis in HCC Patients

To determine the role of LncRNA NR027113 in HCC, 134 paired of HCC tissues and adjacent normal tissues were detected by quantitative Real-Time PCR. Results showed that NR027113 was highly expressed in HCC tissues compared with adjacent tissues ($p<0.001$, Figure 1A). The expression level of NR027113 in 5 kinds of human HCC cell lines was further detected, and normal human hepatic cell line LO2 was taken as a control. SMMC-7721 and SK-HEP-1 had a significantly higher expression of NR027113 (Figure 1B). Next, the relationship between NR027113 expression and the patients' survival time was studied. Kaplan-Meier survival curve analysis and disease-free survival curve confirmed that HCC patients with high NR027113 expression

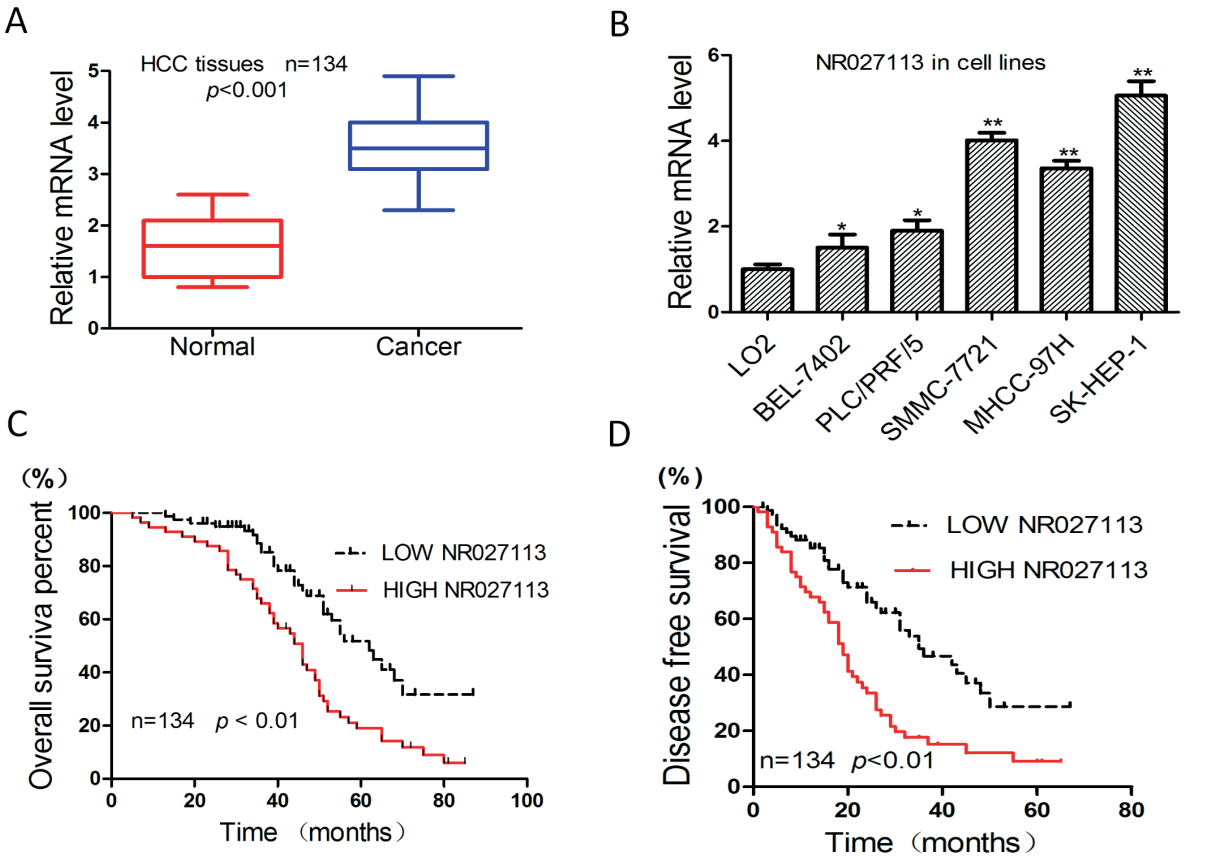

Figure 1. LncRNA NR027113 is highly expressed in human HCC tissues and cell lines. ***A,*** NR027113 expression in HCC tissues and adjacent normal tissues. ***B,*** The expression of NR027113 in HCC cell lines. ***C,*** Kaplan-Meier survival curve analysis of NR027113 in collected samples. ***D,*** Disease free survival curve analysis of NR027113 in collected samples.

were associated with significantly shorter overall survival (p=0.001, Figure 1C and D). The above data showed that the the abnormal expression of NR027113 may be closely related to the progression of HCC. Thus, the NR027113 expression level in all HCC samples was classified into two (low or high) groups. Clinicopathological features tests revealed that the NR027113 expression levels were significant in Tumor size (p=0.027) and TNM stage (p<0.012, Table I). Meanwhile, multivariate analysis revealed NR027113 could serve as an independent prognostic biomarker in HCC patients (p=0.016, Table II).

Knockdown of NR027113 Inhibited HCC Cell Proliferation

To investigate the biological effect of NR027113 on HCC cells, we first checked the expression level of NR027113 knocked down (Figure 2A). Then, we conducted a MTT cell viability assay, cell apoptosis assay, and clone formation assay in cell lines stably knocked down NR027113. The results showed that after NR027113 knocked down, cell proliferation was inhibited compared with the control group (Figure 2B). Flow cytometry analysis revealed that knocking down NR027113 in SMMC-7721 and SK-HEP-1 cells significantly increased the cell apoptosis rate compared with control groups (Figure 2C), indicating the important role of NR027113 in the apoptosis regulation of HCC cells. The number of clone formation also significantly decreased in the NR027113 knockdown group compared with the control group (Figure 2D). The above results suggested that NR027113 is associated with the proliferation ability of HCC cells.

Knockdown of NR027113 Attenuates Cell Migration and Invasion In vitro

The effect of NR027113 on the migration and invasion ability of HCC cells was then studied. Wound healing assays showed that silencing NR027113 inhibited the migration ability of SMMC-7721 and SK-HEP-1 cells (Figure 3A). Furthermore, transwell assay results showed that the migration and invasion ability were significantly attenuated after NR027113 knockdown compared with the control group (Figure 3B and C).

NR027113 Regulates Tumor Progression Through PTEN/PI3K/Akt Signaling Pathway

Evidence suggests that cell cycle and cell proliferation are affected by the PI3K/Akt signaling pathway. To study the specific mechanism of NR027113 in promoting HCC proliferation and invasion, we assessed the activation status of the PI3K/Akt pathway under conditions of NR027113 knockdown. After the knockdown of NR027113, the phosphorylation of PI3K and Akt were significantly decreased, while the total protein expression of the PI3K and Akt remained unchanged (Figure 4A). Moreover, PTEN, the upstream of the PI3K/Akt signaling pathway, was up-regulated after the inhibition of NR027113 (Figure 4A). To evaluate whether NR027113 is involved in the EMT process in HCC and promote invasion and metastasis ability of HCC cells the levels of EMT-related proteins were detected in SMMC-7721 and SK-HEP-1 cells after knockdown NR027113. The results showed that silencing NR027113 increased the expression levels of E-cadherin and decreased the expression levels of other proteins (Figure 4B). The above results suggested that NR027113 regulated tumor progression through the PTEN/PI3K/Akt signaling pathway, meanwhile activated EMT-related proteins.

PTEN Silencing could Reverse the Inhibitory Effect of NR027113 Knock-Down on Akt Phosphorylation and HCC Cells Function

To further explore the role of NR027113 in the regulation of the PTEN/PI3K/Akt pathway, we knocked down the expression of PTEN under conditions of NR027113 knockdown. Knockdown of PTEN significantly restored the phosphorylation of Akt initially down-regulated by NR027113 knockdown (Figure 5A). Furthermore, PTEN knockdown could also restore the ability of proliferation (Figure 5B), migration (Figure 5C) and invasion (Figure 5D) of HCC cells inhibited by the NR027113 knockdown. The above results suggested that NR027113 regulates tumor progression through the PTEN/PI3K/Akt signaling pathway.

Discussion

LncRNAs play an important role in the tumorigenesis and progression of various tumors and are considered as new biomarkers to predict patient prognosis[16-18]. In our study, we focused on a novel HCC related lncRNA NR027113 and the role of NR027113 in HCC was first investigated. We showed that NR027113 was significantly up-regulated in HCC tissues and HCC cell lines.

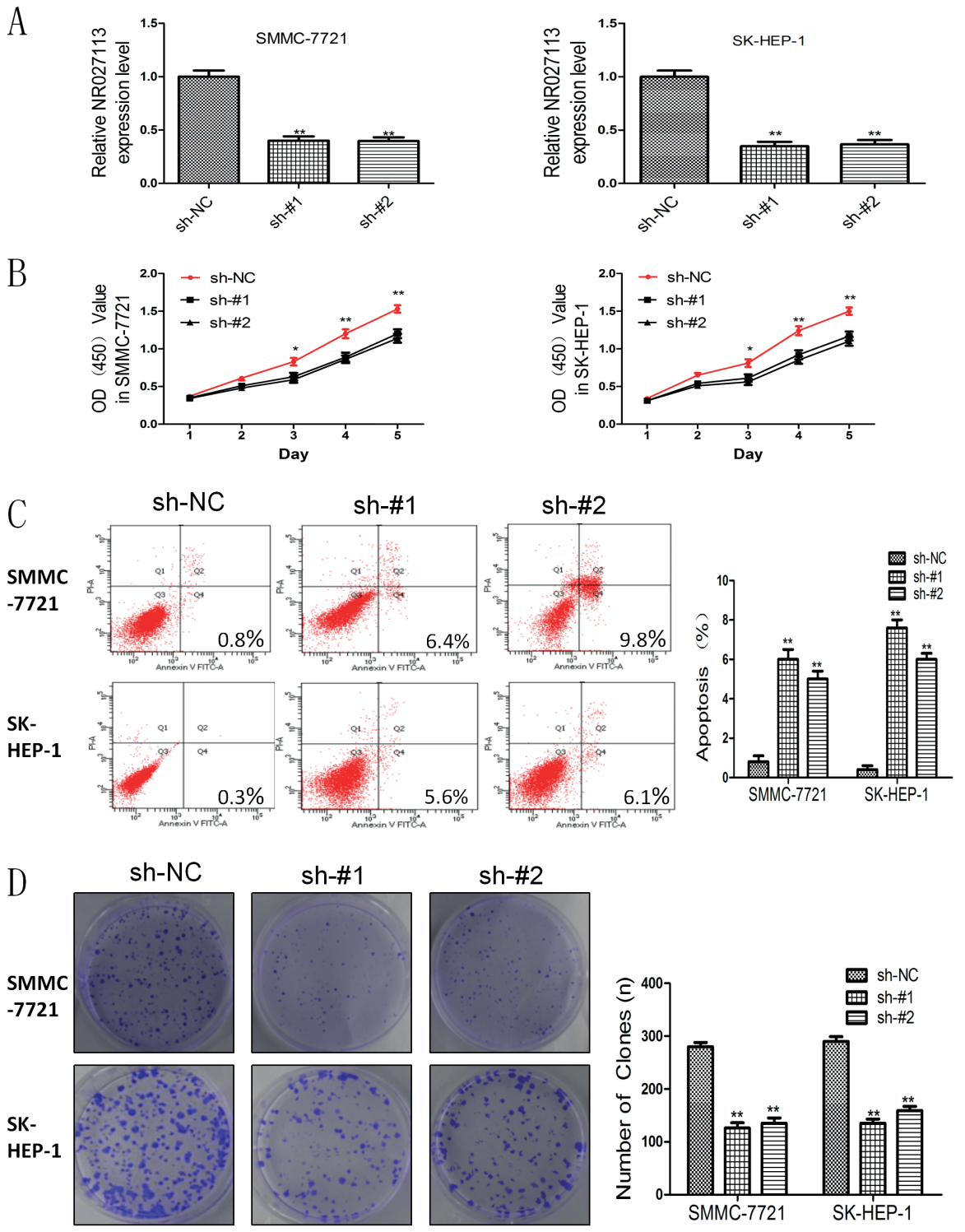

Figure 2. Stable knockdown of NR027113 attenuated the proliferation of HCC cells. ***A,*** NR027113 stable knockdown cell lines was successfully constructed via sh-#1 and 2 of NR027113. ***B,*** Downregulation of NR027113 inhibited HCC cell viability as shown by MTT cell viability assays. ***C,*** NR027113 knockdown promoted cell apoptosis in SMMC-7721 and SK-HEP-1. ***D,*** NR027113 knockdown inhibited cell colony formation in SMMC-7721 and SK-HEP-1.

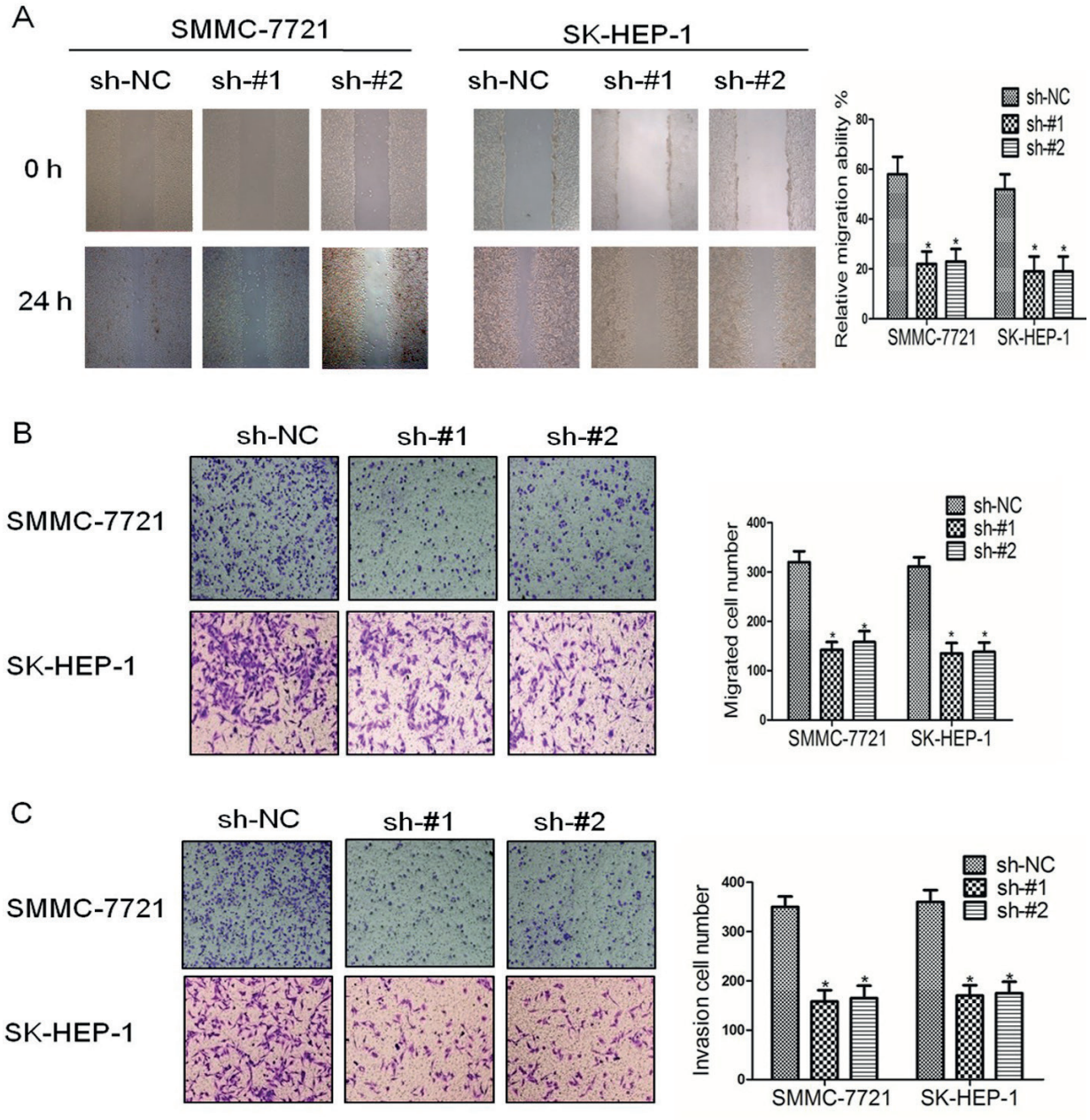

Figure 3. Knockdown of NR027113 inhibited cell migration and invasion *in vitro*. ***A,*** Wound healing assays showed that knockdown NR027113 inhibited the migration ability of SMMC-7721 and SK-HEP-1. ***B,*** NR027113 knockdown inhibited cell migration in SMMC-7721 and SK-HEP-1. ***C,*** NR027113 knockdown inhibited cell invasion in SMMC-7721 and SK-HEP-1.

The previous study[19] has found that lncRNAS in HCC were associated with the late TNM stage, which was also demonstrated in our study. Tumor size and TNM stage were associated with NR027113 expression level as clinicopathological features. Multivariate analysis proved that NR027113 could become an independent indicator for HCC survival. All results supported that NR027113 expression could serve as an oncogene in HCC.

After the clinical significance of NR027113 in HCC, we tried a variety of cell function tests. After the knock-down of NR027113, the ability of proliferation, migration and invasion of HCC cells were significantly decreased. The above results demonstrated that NR027113 has a role in promoting tumorigenesis and development in HCC.

Next, the oncogenetic mechanism of NR027113 was further investigated. At present, more and more cancer-associated lncRNAs are being found and recognized[20,21]. Many of these lncRNAs have been shown to regulate protein or histone modification in cancer cells, thereby affecting the epigenetic regulation of transcription[22] and participating in various signaling pathways[23,24]. Our results showed that NR027113 plays its oncogenetic role in HCC through the PTEN/PI3K/AKT signaling pathway. Accumulating evidence suggests that tumor cell cycle, invasion and metastasis are affected by the PI3K/Akt signaling pathway[25,26]. In this study, we showed that NR027113 was necessary for the activation status of the PI3K/Akt pathway. And this effect was probably through up-regulated PTEN expression in HCC cells. Furthermore, EMT (epithelial-mesenchymal transition) is a vital mechanism involved in tumor invasion and metastasis abilities[27,28]. Increasing evidence proved that lncRNAs could promote tumor metastasis through the EMT process[29,30]. We found that EMT-associated protein E-cadherin was an over-regulated expression, while other markers, especially N-cadherin, vimentin, slug were down-expressed after knock-down NR027113. Meanwhile, PTEN silencing could reverse the inhibitory effect of NR027113 knockdown on Akt phosphorylation and HCC cells function. Thus our results enriched the understanding of the oncogenic effect of lncRNAs in HCC.

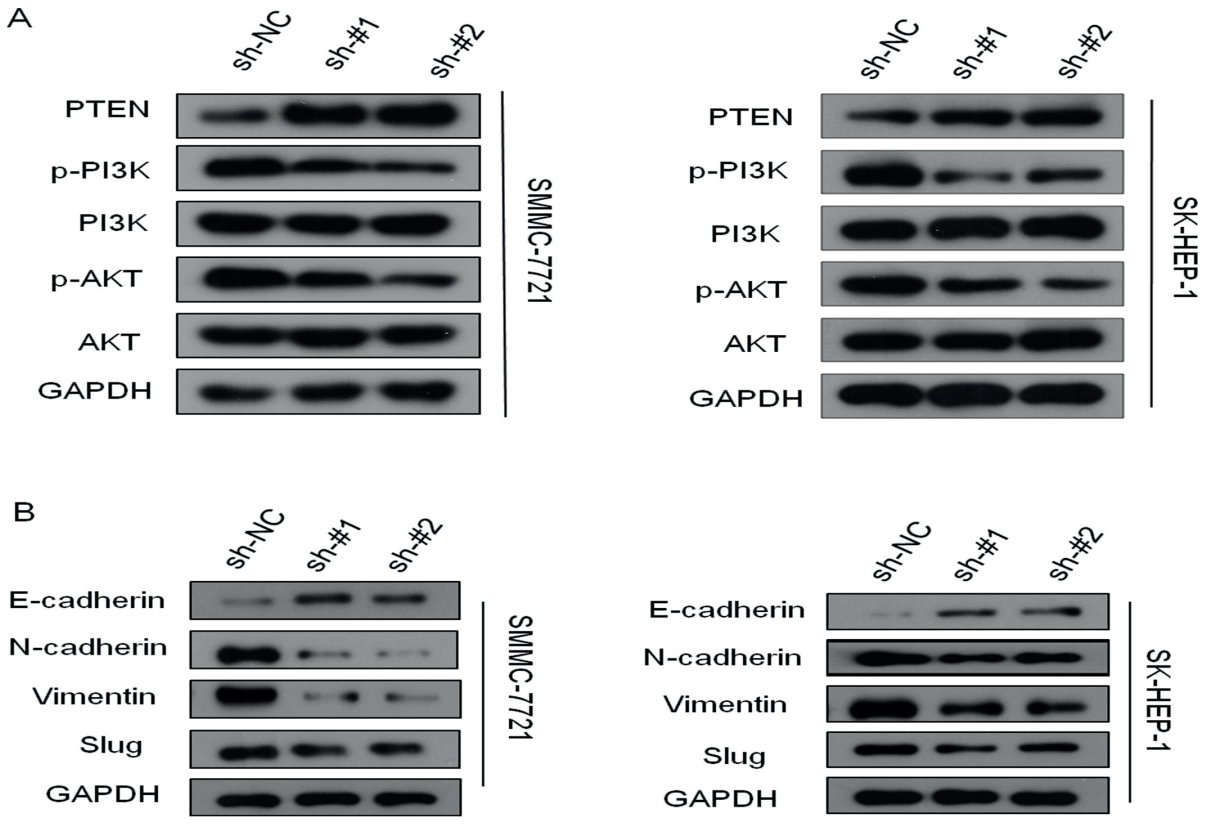

Figure 4. NR027113 regulates tumor progression through PTEN/PI3K/Akt signaling pathway and EMT proteins. ***A,*** Western blotting detection of protein expression of PTEN/PI3K/Akt pathway in SMMC-7721 and SK-HEP-1 stably knockdown of NR027113. ***B,*** EMT-related proteins expression after knockdown NR027113.

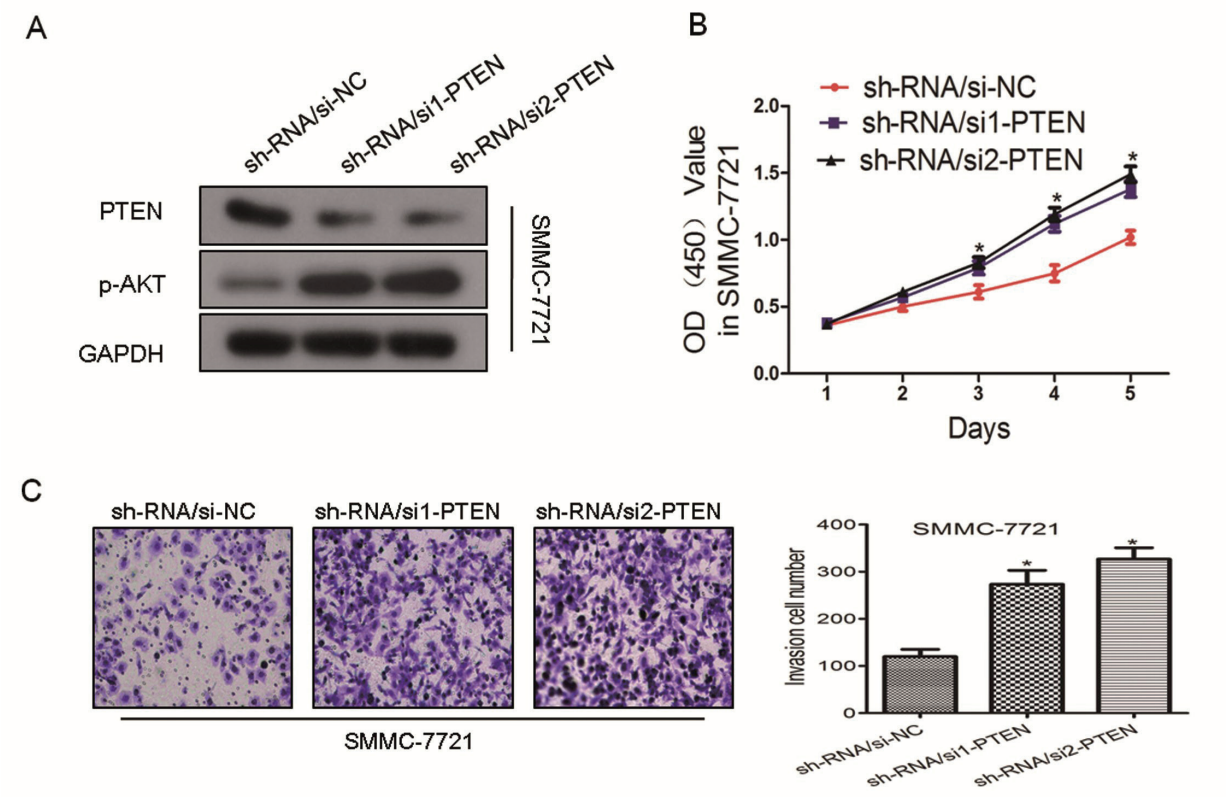

Figure 5. PTEN silencing could reversed the inhibitory effect of NR027113 knockdown on Akt phosphorylation and HCC cells function. ***A,*** Transient knockdown of PTEN enhanced the expression of p-AKT initially down-regulated by NR027113 knockdown. ***B,*** Transient knockdown of PTEN reversed cell viability by MTT assays when NR027113 knockdown. ***C,*** Transient knockdown of PTEN dramatically reversed the migration ability of SMMC-7721 detected by migra-tion assay.

Conclusions

We showed that NR027113 was significantly up-regulated in HCC tissues and was associated with poor prognosis of HCC. In addition, our results suggested that NR027113 is necessary for HCC cells proliferation, migration and invasion. This effect was probably through the TEN/PI3K/AKT signaling pathway and EMT process. Other possible pathway and mechanisms need to be further investigated in the future. This finding will improve understanding of the mechanism involved in cancer progression and provide novel targets for the molecular treatment of HCC.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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