European Review for Medical and Pharmacological Sciences 2020; 24: 1108-1115

Hsa_circ_0011946 promotes the migration and invasion of hepatocellular carcinoma by inducing EMT process

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Abstract. – OBJECTIVE: Recently, the vital role of circular RNAs is discovered in many diseases including tumor progression and metastasis. Hepatocellular carcinoma (HCC) is one of the most ordinary malignant tumors. The purpose of our study is to detect the potential function of hsa_circ_0011946 in HCC to offer new biomarkers and targets.

PATIENTS AND METHODS: The level of hsa_circ_0011946 in HCC tissues and cell lines was monitored by Real Time Quantitative Polymerase Chain Reaction (RT-qPCR). Pearson's Chi-square test was used to determine the association between hsa_circ_0011946 expression and several clinicopathological factors. Then, hsa_circ_0011946 was knocked down in HCC cells to uncover its function in metastasis of HCC. Cell migrated and invaded ability was measured through transwell assay, Matrigel assay and wound healing assay. Western blot assay was performed to analyze the effect of hsa_circ_0011946 on the epithelial-to-mesenchymal transition (EMT) process.

RESULTS: In this research, the expression level of hsa_circ_0011946 was significantly increased in HCC tissues compared to that in adjacent samples. The expression of hsa_circ_0011946 was also increased in HCC cell lines. The hsa_circ_0011946 expression was associated with lymphatic metastasis in HCC patients. Knockdown of hsa_circ_0011946 led to the inhibition of cell migration and invasion in HCC. In addition, results of further experiments revealed that the EMT-related proteins were regulated *via* the knockdown of hsa_circ_0011946 in HCC.

CONCLUSIONS: The hsa_circ_0011946 could enhance cell migration and invasion of HCC by inducing the EMT process.

Key Words:
Circular RNA, Hsa_circ_0011946, Hepatocellular carcinoma, Epithelial-to-mesenchymal transition.

Introduction

Liver cancer is one of the most common reasons for cancer-related deaths globally, especially in Asia. Hepatocellular carcinoma (HCC) is the major subtype of liver cancer which arises from the liver cells, accounting for approximately 90% of all liver cancer cases[1]. HCC is the fourth most frequent malignancy and the third leading cause of mortality in China where hepatitis B virus (HBV) is particularly prevalent among people[2,3]. The morbidity and mortality of HCC in China contribute to more than a half of the whole world's burden[4]. As the development of HCC, there have been almost 600,000 cases of death despite therapeutic advances in HCC prevention and interventions, with 5-year overall survival rate less than 20%[5,6]. Therefore, it's crucial to understand the molecular basis underlying the metastasis of HCC.

Circular RNAs (circRNAs) are tissue-specific, ubiquitously expressed which are recently discovered as a large class of noncoding RNAs. Due to the resistance to exonucleolytic degradation, circRNAs are more stable than linear RNA[7]. Recently, it has been reported that numerous circRNAs play an important role in tumorigenesis. For example, circRNA_0000285 is overexpressed in patients with radioresistant nasopharyngeal carcinoma which may serve as a prognostic biomarker for nasopharyngeal carcinoma[8]. Circ-ITCH suppresses cells proliferation and induces cells apoptosis in epithelial ovarian cancer which is associated with a prolonged overall survival[9]. Overexpression of circRNA BARD1 inhibits the progression of breast cancer through the miR-3942/BARD1 axis[10]. CircRNA 100146 functions as an oncogene and enhances cell proliferation and cell in non-small cell lung cancer through binding to miR-615-5pand

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miR-361-3p directly[11]. However, the function of circular RNAs in HCC and the potential molecular mechanism have not yet been studied so far. To uncover the role of hsa_circ_0011946 in HCC, its expression was detected in HCC tissues and the related mechanism was studied.

In our work, hsa_circ_0011946 was remarkably upregulated in HCC tissues and cell lines. Moreover, hsa_circ_0011946 promoted migration and invasion of HCC *in vitro*. Our further experiments also showed that hsa_circ_0011946 induced epithelial-to-mesenchymal transition (EMT) process of HCC.

Patients and Methods

Tissue Samples

A total of 55 HCC tissues and para-cancer tissues were obtained at The First Affiliated Hospital of China Medical University. Clinical data of the patients were collected. The clinicopathological characters were analyzed by two pathologists. After surgical resection, all the tissue samples were snap-frozen in liquid nitrogen immediately. This investigation was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Human HCC cell lines (Bel-7402 and HepG2) and a normal liver epithelial cell line (L02) were purchased from the Shanghai Cell Biochemical Institute (Shanghai, China). The culture medium consisted of Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA), and 1% penicillin/streptomycin. Besides, cells were cultured in an incubator containing 5% CO_2 at 37°C.

After HCC cells were cultured for 24 h on 6-well plates, cells were transfected with cDNA oligonucleotides specifically targeting hsa_circ_0011946 (shRNA) and negative control (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was monitored by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR).

RNA Extraction and RT-qPCR

TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) was utilized to separate the total mRNA from tissues and cells. The synthesis of complementary deoxyribose nucleic acids (cDNAs) was conducted through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China), the total RNA was reverse-transcribed to. The primer sequences used for RT-qPCR were as follows: hsa_circ_0011946, forward: 3'-GCTGGTGTTCCTTGACTGGA-5'; hsa_circ_0011946, reverse: 3'-CACTGTAGCAAACCAGCATTTCT-5'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-GCACCGTCAAGCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGCCAGTGGA-3′. The conditions were as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Wound Healing Assay

After transfection, HCC cells were seeded in 6-well plates and incubated in RPMI-1640 medium overnight. Then, cells were scratched with a plastic tip and cultured in serum-free RPMI-1640. Each assay was repeated in triplicate independently. The relate distance was viewed under a light microscope (Olympus Corp., Tokyo, Japan) at 48 h.

Transwell Assay

After transfection, 1×10^5 cells in 200 μL serum-free RPMI-1640 were replanted in the top chamber (Corning, Corning, NY, USA). RPMI-1640 and FBS were added to the lower chamber. Next, they were cultured overnight in an incubator supplemented with 5% CO_2 at 37°C. The top surface of chambers was were treated by methanol for 30 min after wiped by a cotton swab. Then, they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

Matrigel Assay

After transfection, 1×10^5 cells in 200 μL serum-free RPMI-1640 were replanted in top chamber (Corning, Inc., Corning, NY, USA) with 50 μg Matrigel (BD, Bedford, MA, USA). RPMI-1640 and FBS were added to the lower chamber. Then, they were cultured overnight in an incubator supplemented with 5% CO_2 at 37°C. The top surface of chambers was treated by methanol for 30 min after wiped by a cotton swab. Subsequently, they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

Western Blot Analysis

Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after replaced to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-Vimentin, as well as goat anti-rabbit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of protein expression.

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA). Graph PAD 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) helped to present these consequences. Pearson's Chi-square test and independent-sample *t*-test were used when appropriate. Quantitative data were presented as mean ± SD (standard deviation). Moreover, $p<0.05$ was considered a statistically significant difference.

Results

Hsa_circ_0011946 Expression Level was Higher in HCC Tissues and Cells

The hsa_circ_0011946 expression was detected *via* RT-qPCR in 55 HCC patients' tissue samples and matched adjacent samples. Results showed that hsa_circ_0011946 was significantly higher in tumor tissue samples than that in adjacent tissues (Figure 1A). The hsa_circ_0011946 expression in human HCC cell lines and L02 was also detected. The hsa_circ_0011946 expression level was higher in HCC cells than that in L02 (Figure 1B).

The Association Between Hsa_circ_0011946 Expression and Pathological Characters of HCC Patients

The clinical data of HCC patients was analyzed. HCC patients were divided into two groups, low and high hsa_circ_0011946 groups. HCC patients in the high hsa_circ_0011946 expression group present lymphatic metastasis ($p<0.05$), while no differences between two groups were seen in age, gender, TNM stage, and tumor size (Table I). Results suggested that hsa_circ_0011946 might function in the metastasis of HCC.

Hsa_Circ_0011946 Knockdown Inhibited Cell Migration and Invasion in Bel-7402 HCC Cells

Hsa_circ_0011946 was knocked down in Bel-7402 cells and transfection efficiency was detected by RT-qPCR (Figure 2A). To explore how hsa_circ_0011946 affected cell migration of HCC cells, wound healing assay, and transwell assay were performed. As shown in Figure 2B, the wound closure of HCC cells was reduced after hsa_circ_0011946 was knocked down. As shown in Figure 2C, the number of migrated cells was remarkably decreased after hsa_circ_0011946 was knocked down in HCC cells. To explore how hsa_circ_0011946 affected cell invasion of HCC cells, Matrigel assay was performed. As shown in

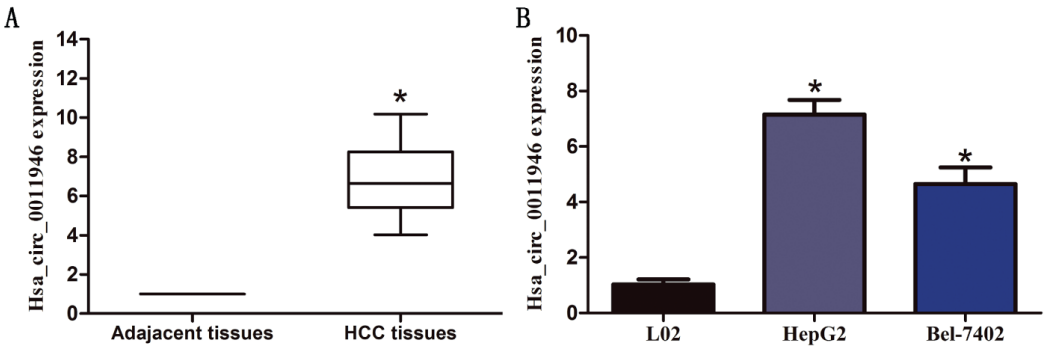

Figure 1. Expression levels of hsa_circ_0011946 were increased in HCC tissues and cell lines. **A,** Hsa_circ_0011946 expression was significantly increased in the HCC tissues compared with adjacent tissues. **B,** Expression levels of hsa_circ_0011946 relative to GAPDH were determined in the human HCC cell lines and L02 by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

Table I. Correlation between hsa_circ_0011946 expression and clinicopathological characteristics in HCC patients.

Characteristics	Patients	Expression of hsa_circ_0011946		*p*-value
		Low group	High group	
Total	55	27	28	
Age (years)				0.504
≤50	26	14	12	
>50	29	13	16	
Gender				0.688
Male	27	14	13	
Female	28	13	15	
TNM stage				0.498
I-II	28	15	13	
III-IV	27	12	15	
Lymphatic metastasis				0.022
No	25	17	8	
Yes	30	12	20	

$p<0.05$ is considered as statistically significant.

Figure 2D, after hsa_circ_0011946 was knocked down, the number of invaded cells was remarkably decreased.

Hsa_circ_0011946 Knockdown Inhibited Cell Migration and Invasion in HepG2 HCC Cells

To further confirm the function of hsa_circ_0011946 in HCC, hsa_circ_0011946 was also knocked down in HepG2 cells and transfection efficiency was detected by RT-qPCR (Figure 3A). As shown in Figure 3B, the wound closure of HCC cells was reduced after hsa_circ_0011946 was knocked down. As shown in Figure 3C, the number of migrated cells was remarkably decreased after hsa_circ_0011946 was knocked down in HCC cells. As shown in Figure 3D, after hsa_circ_0011946 was knocked down, the number of invaded cells was remarkably decreased.

Hsa_circ_0011946 Knockdown Inhibited EMT Process in HCC

Previous studies have reported that the key role of the EMT process in cancer metastasis. In our report, the effect of hsa_circ_0011946 on EMT process was further studied. The EMT-related proteins included E-cadherin, N-cadherin, and Vimentin. Previous studies identified that E-cadherin was downregulated, and N-cadherin and Vimentin were upregulated when the EMT process was induced. RT-qPCR assay showed that the expression of E-cadherin in Bel-7402 cells was higher in hsa_circ_0011946 shRNA group than in control group, while the expression of N-cadherin and Vimentin in Bel-7402 cells was lower in hsa_circ_0011946 shRNA group than in NC group (Figure 4A). Western blot assay showed that the protein level of E-cadherin in Bel-7402 cells was upregulated in hsa_circ_0011946 shRNA group compared to that in NC group, while the expression of N-cadherin and Vimentin in Bel-7402 cells was downregulated in hsa_circ_0011946 shRNA group compared to that in NC group (Figure 4B). The similar effect of hsa_circ_0011946 knockdown on the EMT process was also identified in HepG2 cells (Figures 4C and 4D).

Discussion

Hsa_circ_0011946 is a novel circRNA which has been reported to be upregulated in breast cancer. Furthermore, the low-expression of hsa_circ_0011946 inhibits cell migration and cell invasion in breast cancer through targeting RFC3[12]. Increasing evidence identified the vital role of circular RNAs in HCC. Has a_circ_0005986 functions as a tumor suppressor in HCC through serving as a miR-129-5p sponge which may be a novel biomarker for HCC[13]. By regulating miR-1324/FZD5/Wnt/β-catenin signaling, circ_0067934 facilitates tumor growth and cell migration in hepatocellular carcinoma[14]. Through downregulating the expression of RhoA and circRNA_000839, miR-200b inhibits cell invasion and cell migration in HCC[15]. Hsa_circ_0103809 enhances cell proliferation and inhibits cell apoptosis in HCC by targeting miR-490-5p/SOX2 pathway[16]. In

this study, hsa_circ_0011946 was upregulated in HCC samples and cell lines. More importantly, high hsa_circ_0011946 expression was associated with lymphatic metastasis, which suggested that hsa_circ_0011946 might affect tumor metastasis of HCC.

To further determine whether hsa_circ_0011946 participated in the regulation of HCC metastasis, hsa_circ_0011946 was knocked down in HCC cells. Wound healing assay and transwell assay were conducted in these treated cells. Results showed that hsa_circ_0011946 knockdown repressed cell migrated ability of HCC cells. Moreover, Matrigel assay was also conducted and results showed that hsa_circ_0011946 knockdown repressed cell invaded ability of HCC cells. Above data indicated that hsa_circ_0011946 promoted cell migration and invasion of HCC.

Epithelial-to-mesenchymal transition (EMT) is a crucial biological process involved in a mul-

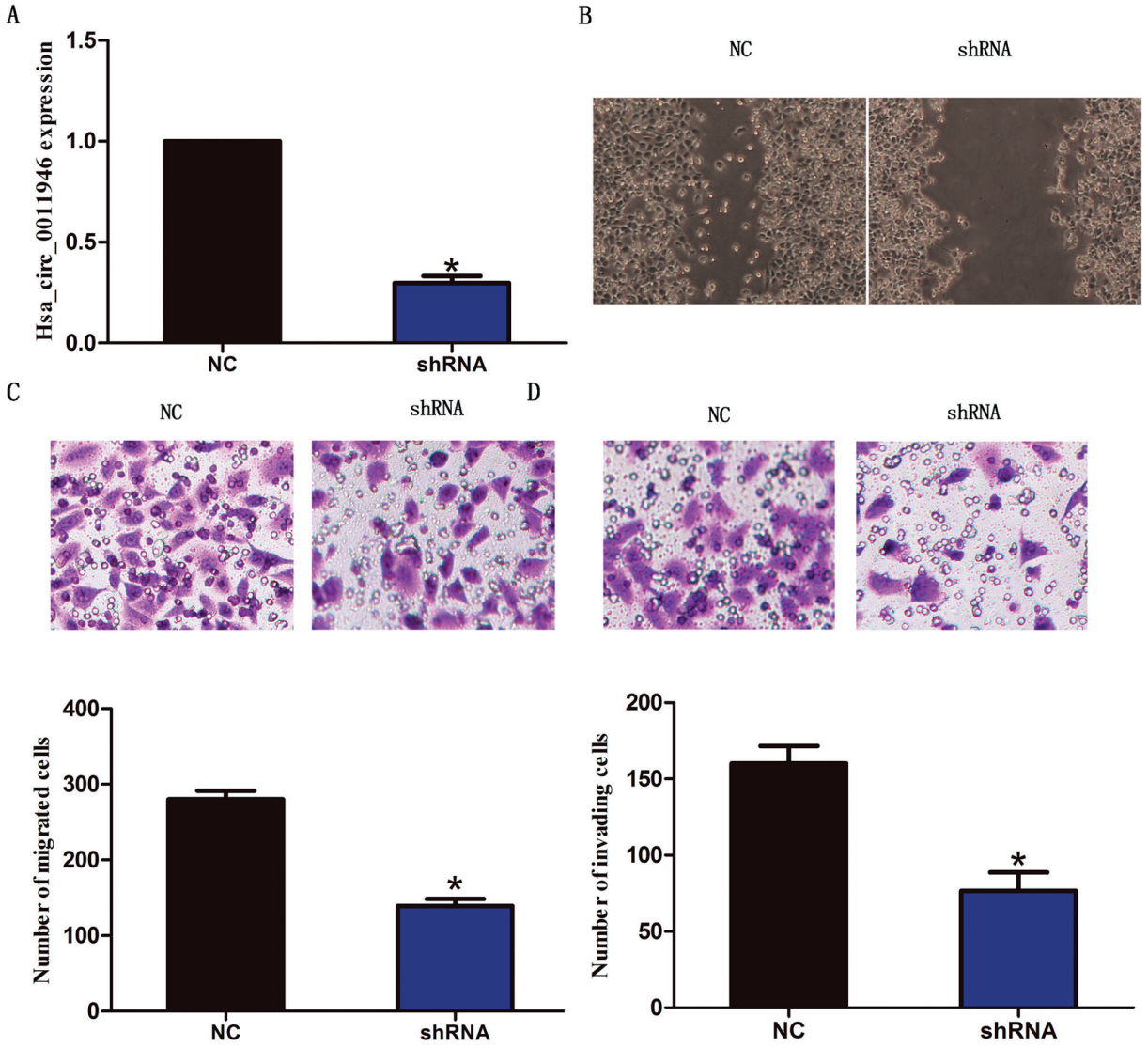

Figure 2. Knockdown of hsa_circ_0011946 inhibited Bel-7402 HCC cell migration and invasion. **A,** Hsa_circ_0011946 expression in Bel-7402 HCC cells transfected with hsa_circ_0011946 shRNA and control were detected by RT-qPCR. **B,** Wound healing assay showed that the migrated length of cells in hsa_circ_0011946 group was significantly decreased compared with control group in HCC cells (magnification: 40×). **C,** Transwell assay showed that knockdown of hsa_circ_0011946 markedly repressed cell migration in HCC cells (magnification: 40×). **D,** Transwell assay showed that knockdown of hsa_circ_0011946 significantly repressed cell invasion in HCC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$.

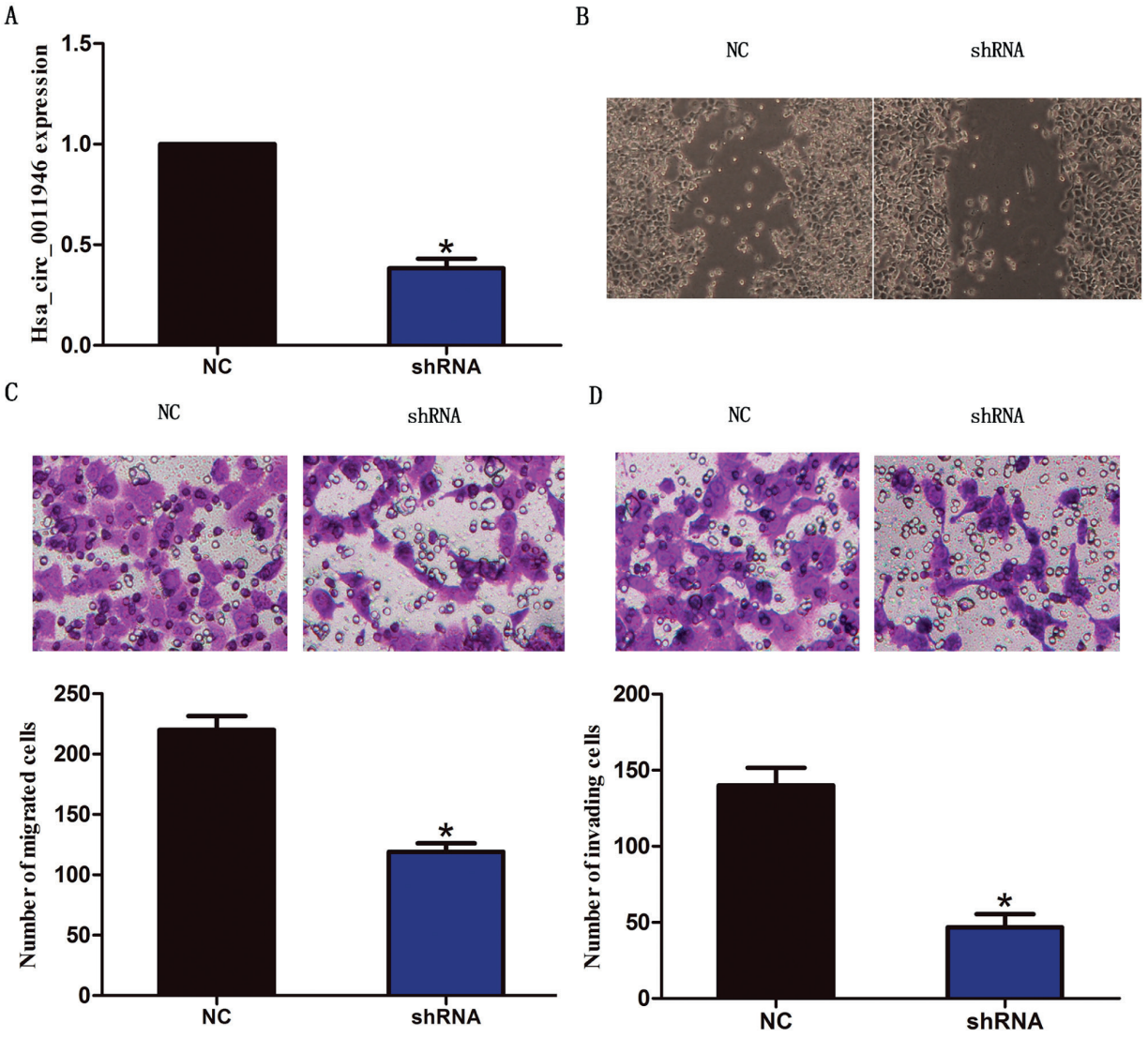

Figure 3. Knockdown of hsa_circ_0011946 inhibited HepG2 HCC cell migration and invasion. **A,** Hsa_circ_0011946 expression in HepG2 HCC cells transfected with hsa_circ_0011946 shRNA and control were detected by RT-qPCR. **B,** Wound healing assay showed that the migrated length of cells in hsa_circ_0011946 group was significantly decreased compared with control group in HCC cells (magnification: 40×). **C,** Transwell assay showed that knockdown of hsa_circ_0011946 significantly repressed cell migration in HCC cells (magnification: 40×). **D,** Transwell assay showed that knockdown of hsa_circ_0011946 markedly repressed cell invasion in HCC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

titude of developmental and pathological events. EMT is characterized by the progressive loss of cell-to-cell contacts resulting in filopodia formation and mesenchymal gene expression which enables cell migration and invasion. In this way, through activation of ZEB1 and interaction with miR-139-5p, lncRNA HCP5 enhances epithelial-mesenchymal transition in colorectal cancer[17]. EMT is associated with poor tumor differentiation in pancreatic ductal adenocarcinoma which can be increased by gemcitabine[18]. Circ MTO1 inhibits tumor metastasis in bladder cancer through serving as a miR-221 sponge and restraining EMT[19]. Moreover, circ-10720 participates in the Twist1-mediated regulation of EMT and promotes the progression of HCC[20]. In this research, the potential interaction between the EMT process and hsa_circ_0011946 was firstly studied. E-cadherin, N-cadherin, and Vimentin are vital proteins in the EMT process. Through detecting the expres-

sion of those proteins in HCC cells, we found that those proteins could be regulated by knockdown of hsa_circ_0011946. All these results indicated that hsa_circ_0011946 could induce EMT process of HCC.

Conclusions

We showed that hsa_circ_0011946 was remarkably upregulated in HCC tissues and was associated with lymphatic metastasis of HCC patients. Hsa_circ_0011946 facilitated cell migration and invasion of HCC by inducing the EMT process. These findings suggest that hsa_circ_0011946 may serve as a prospective therapeutic target for HCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

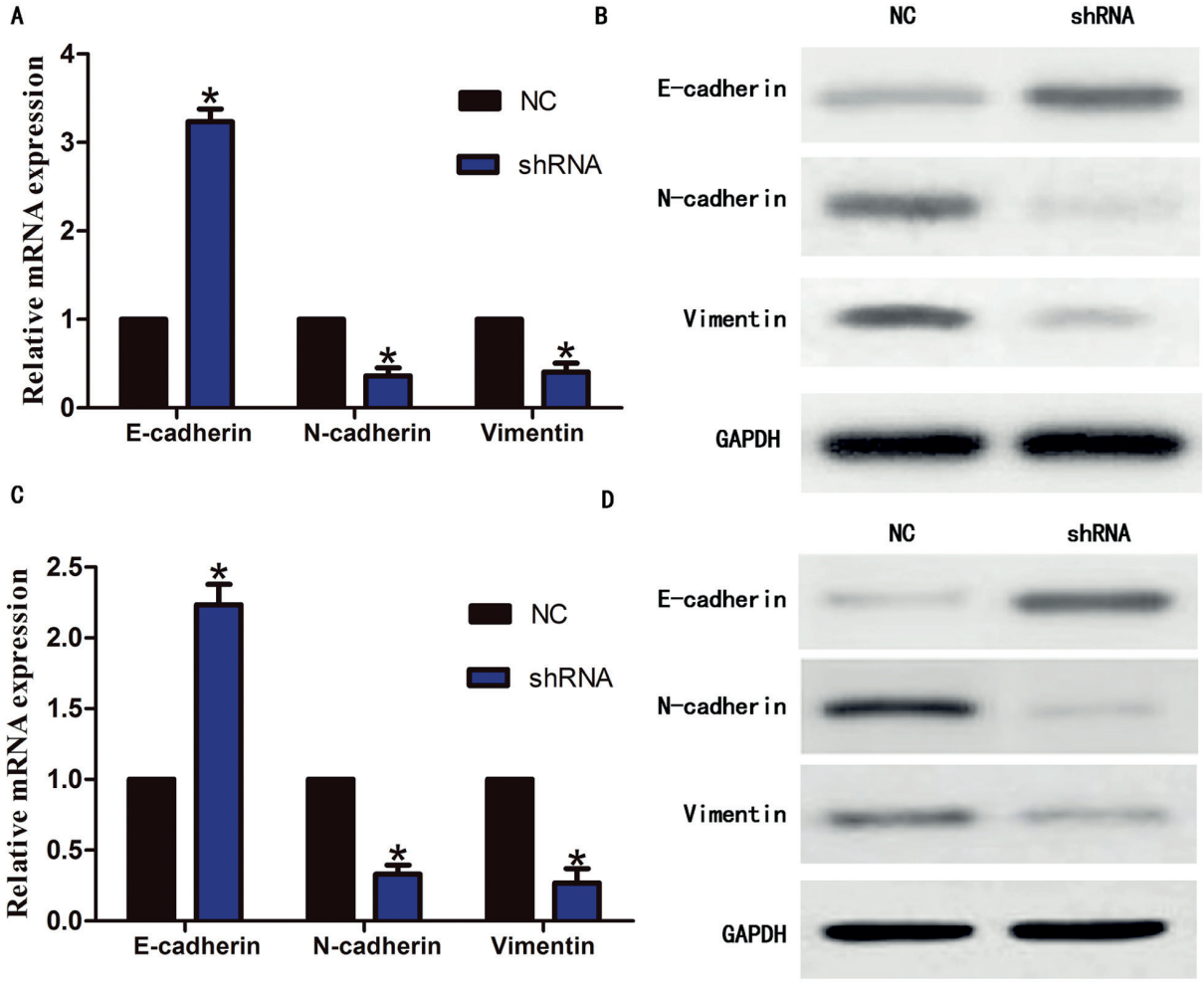

Figure 4. Hsa_circ_0011946 knockdown inhibited EMT process of HCC. **A,** RT-qPCR assay was used to detect the mRNA expression of E-cadherin, N-cadherin and Vimentin of Bel-7402 cells hsa_circ_0011946 in hsa_circ_0011946 shRNA group and NC group. **B,** Western blot assay was used to detect the protein level of E-cadherin, N-cadherin, and Vimentin of Bel-7402 cells in hsa_circ_0011946 shRNA group and NC group. **C,** RT-qPCR assay was used to detect the mRNA expression of E-cadherin, N-cadherin, and Vimentin of HepG2 cells in hsa_circ_0011946 shRNA group and NC group. **D,** Western blot assay was used to detect the protein level of E-cadherin, N-cadherin, and Vimentin of HepG2 cells in hsa_circ_0011946 shRNA group and NC group. Data are presented as the mean ± standard error of the mean. *p<0.05.

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