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LncRNA H19 promotes the development of hepatitis B related hepatocellular carcinoma through regulating microRNA-22 *via* EMT pathway

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Abstract. – OBJECTIVE: To explore the relationship between long non-coding RNA (lncRNA) H19 expression and prognosis of hepatitis B-related hepatocellular carcinoma (HBV-related HCC), and its underlying mechanism.

PATIENTS AND METHODS: Expression level of lncRNA H19 in 36 HBV-related HCC tissues and para-cancerous tissues was detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The relationship between lncRNA H19 expression and prognosis of HBV-related HCC was analyzed by Kaplan-Meier method. Serum DNA levels of HBV were detected by fluorescence quantitative polymerase chain reaction (FQ-PCR). For *in vitro* experiments, lncRNA H19 expression in HCC cell line, HBV-related HCC cell line and normal liver cell line was detected by qRT-PCR. After plasmids construction, the effects of lncRNA H19 on cell viability, migration, and invasion were detected by cell counting kit-8 (CCK-8), colony formation and transwell assay, respectively. Finally, protein levels of epithelial-mesenchymal transition (EMT) pathway-related genes were detected by Western blot.

RESULTS: LncRNA H19 was highly expressed in HBV-related HCC tissues. The expression of lncRNA H19 was positively correlated with lymph node metastasis and distant metastasis, whereas negatively correlated with the overall survival of HBV-related HCC patients. Results of *in vitro* experiments showed that lncRNA H19 knockdown significantly downregulated cell proliferation and invasion. However, lncRNA H19 knockdown significantly upregulated apoptosis of HBV-related HCC cells. Western blot results demonstrated that lncRNA H19 remarkably decreased the protein expressions of EMT pathway-related genes, including N-cadherin, Vimentin, β-catenin and MMP-9. In addition, rescue experiments demonstrated that lncRNA H19 remarkably promoted malignant development of HBV-related HCC *via* regulating microRNA-22.

CONCLUSIONS: LncRNA H19 promotes malignant development of HBV-related HCC through regulating microRNA-22 *via* EMT pathway.

Key Words:
HBV, Hepatocellular carcinoma (HCC), LncRNA, MicroRNA, EMT.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. There are about 42,220 new cases and 30,200 deaths in the United States each year[1]. In China, the mortality and morbidity of HCC rank fifth and tenth among all malignancies, respectively[2]. Epidemiological and experimental studies have shown that about 80% of HCC cases are associated with HBV infection and/or HCV infection[3,4]. In addition to viral infection, alcoholic liver disease, smoking, and obesity may also involve in HCC occurrence[5]. The high rate of hepatitis infection contributes to heavy burden on HCC patients and their families[6]. Clinically, more than half of HCC patients experience micro-metastases before radical surgery. Meanwhile, micro-metastases are a direct cause of metastasis and recurrence after HCC surgery[7]. Nowadays, the pathogenesis of HCC has not yet been fully elucidated. Difficulty in early diagnosis and treatment is one of the important reasons for high morbidity and

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mortality of HCC[8]. Therefore, it is urgent to explore the molecular mechanism of HBV-related HCC. With advanced development of molecular biology, HBV-related HCC is considered as the result of long-term interaction between genetic and environmental factors. Alterations[9] in oncogenes and tumor suppressor genes eventually lead to cellular dysfunctions, including proliferation, apoptosis, differentiation, etc. The specific molecular mechanism of HBV-related HCC should be well investigated, thereby providing basis for improving clinical outcomes of affected population. Non-coding RNA has been confirmed to play a crucial role in the occurrence and progression of tumors. Among them, long non-coding RNA (lncRNA) is a kind of non-coding RNA with more than 200 nucleotides in length. LncRNA regulates gene expressions at transcriptional and post-transcriptional level. Functionally, lncRNA is widely involved in the physiological and pathological processes of the body[10]. Huarte[11] found that lncRNAs are differentially expressed in various tumors with tissue specificity. Meanwhile, lncRNAs can promote the proliferation, invasion and metastasis of tumor cells through multiple mechanisms. LncRNA has been found abnormally expressed in various malignancies, such as prostate cancer, colorectal cancer, bladder cancer, and kidney cancer[12-14]. However, the molecular regulation mechanism of lncRNAs in tumors has not been completely understood. In general, lncRNAs involve in chromosome recombination, gene imprinting, epigenetic regulation, nucleoplasm transport, mRNA splicing and translation. Winkle et al[15] have also indicated that biological processes can be regulated by certain lncRNAs, such as proliferation, cell cycle, apoptosis, differentiation and metastasis of tumor cells. Through literature review[16,17], we have found that lncRNA SNHG6 and MINCR can promote HCC. However, lncRNA PVT1 and LNC473 inhibit HCC[18,19]. However, the exact role of lncRNA in HBV-related HCC is poorly understood. The aim of this study was to investigate the role of lncRNA H19 in HBV-related HCC and its possible underlying mechanism.

Patients and Methods

Patients and HCC Samples

A total of 36 HCC tissues and normal adjacent tissues were surgically resected. Enrolled patients were pathologically diagnosed as hepatitis B-related HCC according to TNM stage in the 8th edition of UICC/AJCC. HCC patients did not receive any preoperative anti-tumor treatments. This study was approved by the Ethics Committee of Qingdao Municipal Hospital of Shandong University. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Human HCC cell line (HepG2), HBV-related HCC cell line (HepG2.2.15) and normal liver cell line (LO2) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and maintained in an incubator with 5% CO_2 at 37°C. Culture medium was replaced every 2 days.

Cell Transfection

Corresponding plasmids of lncRNA H19 (si-RNA NC and si-LncRNA H19) were constructed by Gene Pharma (Shanghai, China). Cells were first seeded into 6-well plates. When the confluence was up to 70%, cell transfection was performed according to the manufacturer`s instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, transfected cells were collected for the following experiments.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 2000 cells per well. After culture for 6 h, 24 h, 48 h and 72 h, respectively, 10 μL of cell counting kit-8 solution (CCK-8, Beyotime Biotechnology, Shanghai, China) were added in each well. The absorbance at 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Cells in logarithmic phase were collected and seeded into 6-well plates at a density of 1×10^4/L. A total of 200 cells were added in each well. After culture for 1-2 weeks, the cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for another 30 min. Colony formation was observed and captured under a light microscope (Olympus, Tokyo, Japan).

Transwell Assay

Transfected cells were centrifuged, washed with phosphate-buffered saline (PBS) for three

times, and re-suspended in serum-free medium. The concentration of cells was adjusted to 2×10^5 /mL. For transwell assay, 100 μL cell suspension and 500 μL Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) were added in the upper and lower chamber, respectively. After culture for 48 h, the cells were washed with phosphate-buffered saline (PBS), fixed with ethanol and stained with crystal violet. 6 randomly fields were randomly selected for each sample. Migrating cells were observed and captured using an inverted microscope (Nikon, Tokyo, Japan).

Fluorescence Quantitative Polymerase Chain Reaction (FQ-PCR)

Serum level of HBV DNA in HCC patients was detected using FQ-PCR. Serum samples were detected and analyzed using ABI prism 7300 SDS software (Applied Biosystems, Foster City, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in transfected cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). Subsequently, reverse transcription was performed according to the manufacturer`s instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed in strict accordance with SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). 3 replicates were set in each group. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: lncRNA H19, F: 5'-CGATGCACCTGTACGATCA-3', R: 5'-TCTTTCAACACGCAGGACAG A-3'; microRNA-22, F: 5'-GCAGAGGGAAGAGTTCCCCAGG-3', R: 5'-CCTTGGTCTGGTAGGAGACG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Western blot analysis was used to determine the expression levels of proteins in cells. Briefly, proteins with different molecular weight were separated by gel electrophoresis, and were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies of anti-N-cadherin (Abcam, Cambridge, MA, USA), anti-Vimentin (Cell Signaling Technology, Danvers, MA, USA), anti-β-catenin (Abcam, Cambridge, MA, USA), anti-MMP (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing with Tris-buffered saline and Tween 20 (TBST) (Beyotime, Shanghai, China), the membranes were incubated with horse reddish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 2 h. Western Blot Detection kit and Image J software (NIH) were used to measure the blot signal and density (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Quantitative data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between two groups. Kaplan-Meier method was introduced for evaluating overall survival, and log-rank test was performed for comparing the difference between curves. p-values < 0.05 were considered statistically significant.

Results

LncRNA H19 Was Highly Expressed in HBV-Related HCC Tissues and Cell Lines

LncRNA H19 was overexpressed in HBV-related HCC tissues when compared with para-cancerous tissues (Figure 1A). Besides, the level of HBV DNA in these HCC tissues was significantly higher (Figure 1B). Subsequently, we detected lncRNA H19 in human HCC cell line (HepG2), HBV-related HCC cell line (HepG2.2.15) and normal liver cell line (LO2). Among them, HepG2.2.15 cells expressed the highest level of lncRNA H19 (Figure 1C).

LncRNA H19 Expression was Correlated with Clinical Stage, Lymph Node Metastasis, Distance Metastasis and Overall Survival in HBV-Related HCC Patients

Basic characteristics of enrolled patients were recorded, including age, gender, tumor stage, lymph node metastasis and distant metastasis (Table I). We found that lncRNA H19 expression was positively correlated with lymph node metastasis and distant metastasis, whereas negatively correlated with the overall survival of HBV-related HCC patients. To investigate the correlation between lncRNA H19 expression and prognosis

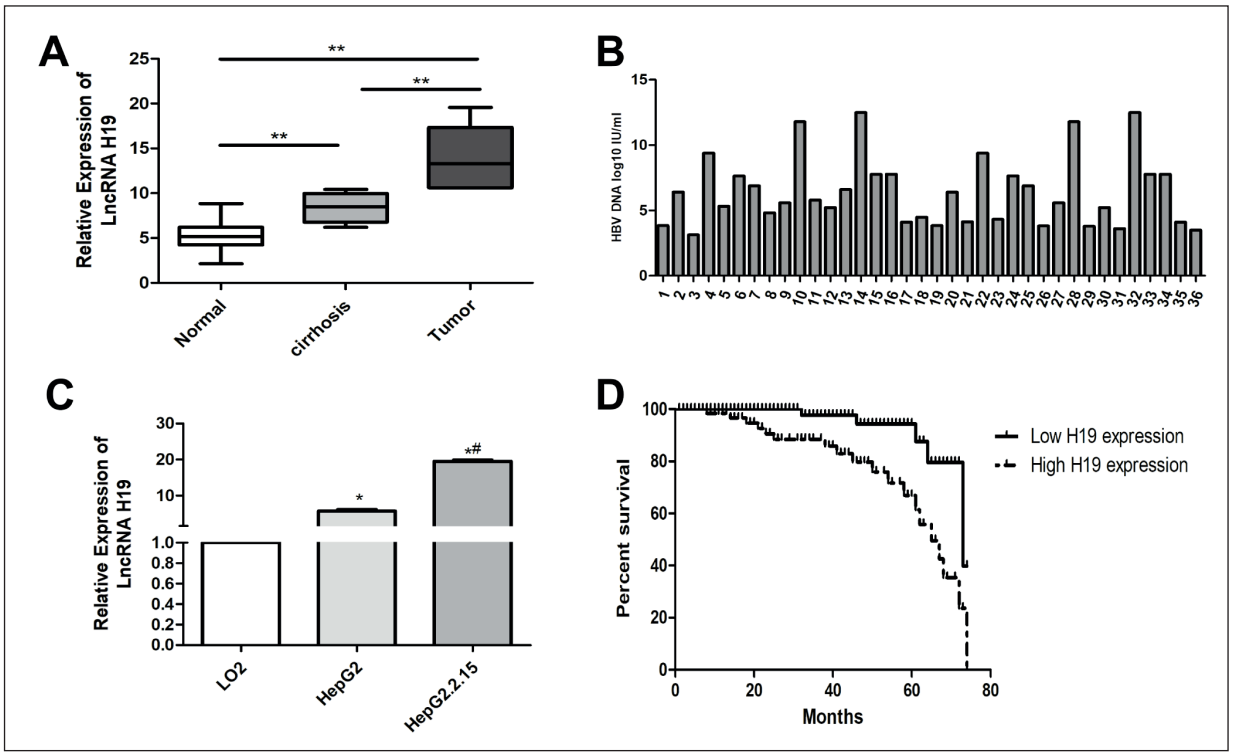

Figure 1. The expression of LncRNA H19 in HBV-related HCC tissues and cell lines. ***A,*** The expression of lncRNA H19 in HCC tissues was significantly increased. $^{**}p<0.01$. ***B,*** HBV DNA levels in serum specimens of HCC patients (n=36). ***C,*** Expression levels of lncRNA H19 in 2 HCC cell lines (HepG2, HepG2.2.15) and 1 normal hepatic cell line (LO2). $^{*}p<0.05$, compared with LO2; $^{\#}p<0.05$, compared with HepG2. ***D,*** Kaplan-Meier survival curves of patients with HCC based on lncRNA H19 expression. Patients in high expression group had a significantly more unfavorable prognosis than those in low expression group.

of HBV-related HCC, follow-up data were collected. Kaplan-Meier results showed that lncRNA H19 overexpression was related to poor prognosis (Figure 1D), indicating that lncRNA H19 might be a novel biomarker for predicting prognosis of HBV-related HCC.

Table I. Relationship between lncRNA H19 expression and clinicopathological characteristics of hepatitis B virus-associated hepatocellular carcinoma.

Parameters	No. of cases, n	LncRNA H19 expression		*p*-value
		Low, n (%)	High, n (%)	
Age (years)				0.733
< 60	14	8 (44.4)	6 (33.3)	
≥ 60	22	10 (55.6)	12 (66.7)	
Gender				0.505
Male	19	11 (61.1)	8 (44.4)	
Female	17	7 (38.9)	10 (55.6)	
T stage				0.044*
T1-T2	19	13 (72.2)	6 (33.3)	
T3-T4	17	5 (27.8)	12 (66.7)	
Lymph node metastasis				0.018*
No	18	13 (72.2)	5 (27.8)	
Yes	18	5 (27.8)	13 (72.2)	
Distance metastasis				0.007*
No	19	14 (77.8)	5 (27.8)	
Yes	17	4 (22.2)	13 (72.2)	

$^{*}p<0.05$.

Knockdown of lncRNA H19 Inhibited Cell Proliferation

To investigate the biological functions of lncRNA H19 in HBV-related HCC cells, we first constructed siRNA NC and si-lncRNA H19. Transfection efficacies were verified by qRT-PCR (Figure 2A). CCK-8 results indicated that knockdown of lncRNA H19 significantly inhibited the proliferation of HBV-related HCC cells (Figure 2B). Similar results were obtained in colony formation assay (Figure 2C, 2D).

Knockdown of lncRNA H19 Inhibited Cell Migration and Invasion

Results of transwell assay demonstrated that knockdown of lncRNA H19 resulted in obvious less penetrating HCC cells than controls, indicating inhibited migratory capacity (Figure 3A). Similarly, invasive ability of HCC cells was also remarkably reduced after transfection of si-lncRNA H19 (Figure 3B).

Knockdown of lncRNA H19 Activated EMT Signaling Pathway

H19 has been proved to promote the progression of diverse types of cancer by promoting proliferation, inhibiting apoptosis and enhancing metastasis. Hence, we speculated that EMT pathway might be involved in HBV-related HCC. Western blot results showed that the protein expressions of key genes in EMT pathway were significantly downregulated after knockdown of lncRNA H19 in HCC cells, including N-cadherin, Vimentin, β-catenin and MMP-9 (Figure 4A). The data illustrated that lncRNA H19 regulated EMT pathway in HCC cells.

MicroRNA-22 Modulated lncRNA H19 Expression in Human Hepatitis B Virus-Associated HCC cells

To explore the specific mechanism of lncRNA H19 in regulating HBV-related HCC, we found that microRNA-22 could be the target gene of

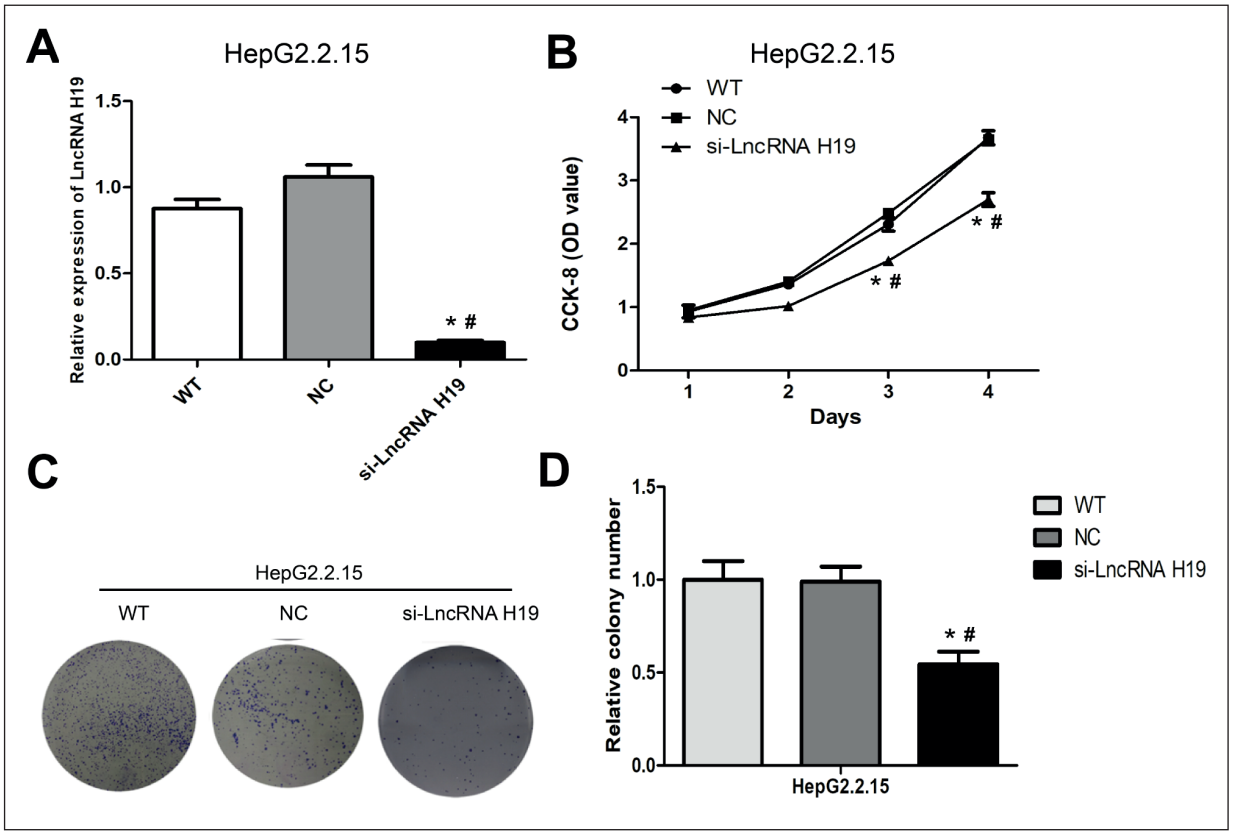

Figure 2. Knockdown of lncRNA H19 inhibited cell proliferation. ***A,*** QRT-PCR was used to verify the efficiency of lncRNA H19 knockdown in HepG2.2.15 cells. ***B,*** Growth curve analysis showed the growth of HepG2.2.15 cells with lncRNA H19 knockdown. ***C, D,*** The efficiencies of cell colony formation in HepG2.2.15 cells with lncRNA H19 knockdown. *$p<0.05$, compared with WT; $^{\#}p<0.05$, compared with NC.

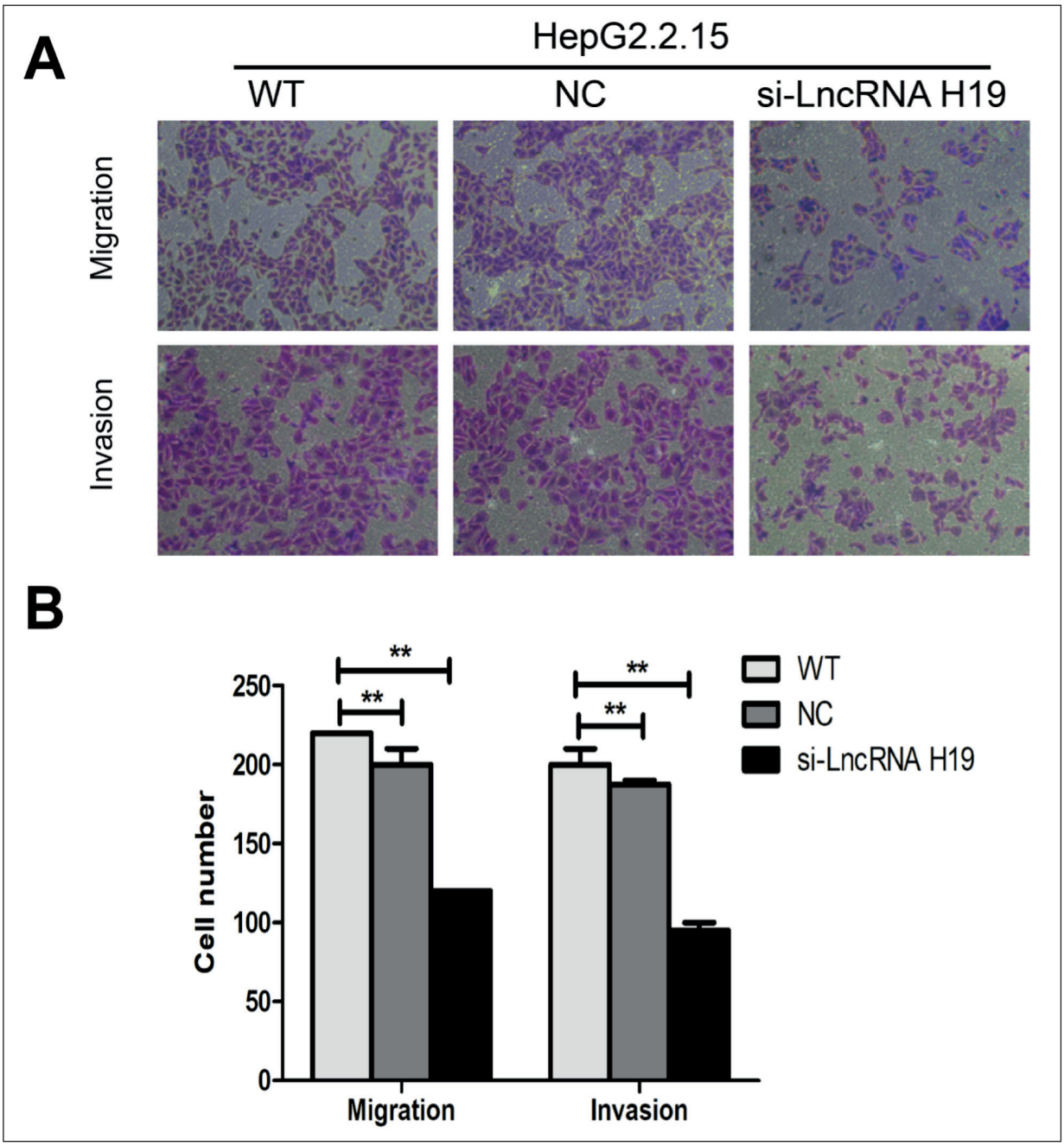

Figure 3. Knockdown of lncRNA H19 inhibited cell migration and invasion. ***A, B,*** HepG2.2.15 cells transfected with si-lncRNA H19 displayed significantly lower migration and invasion capacities. $^{**}p<0.01$.

lncRNA H19 by online prediction. As reported previously, microRNA-22 was downregulated in liver cancer, which could inhibit metastasis[20]. Therefore, we speculated that there might be a regulatory effect of H19 on microRNA-22 as a ceRNA. In this study, results showed that microRNA-22 was significantly downregulated in HCC tissues than that of controls (Figure 4B). Meanwhile, microRNA-22 expression in HCC cells was significantly lower than that of LO2 cells (Figure 4C). Subsequently, we detected the expressions of microRNA-22 and lncRNA H19 in HepG2.2.15 cells. Results showed that microRNA-22 was negatively regulated by lncRNA H19 (Figure 4D and 4E). Rescue experiments suggested that inhibited migration and invasion in-

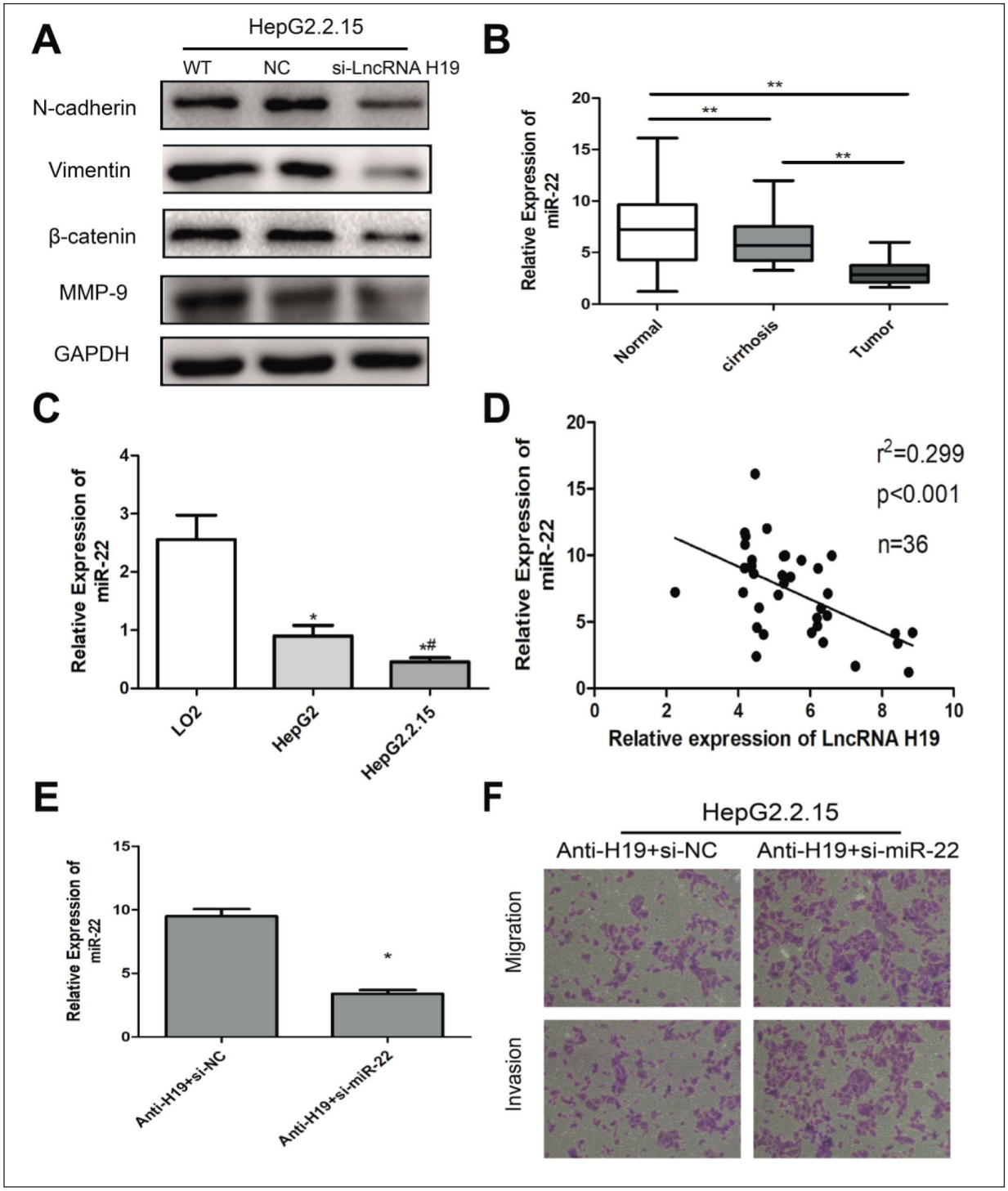

Figure 4. LncRNA H19 regulated microRNA-22 *via* EMT pathway. ***A,*** Knockdown of lncRNA H19 expression significantly decreased the expression of EMT signal pathway, including N-cadherin, Vimentin, β-catenin and microRNA-22. ***B,*** The mRNA expression level of microRNA-22 relative to GAPDH in human HCC tissues and corresponding adjacent tissues was detected by qRT-PCR. $^{**}p<0.01$. ***C,*** The mRNA expression level of microRNA-22 in cell lines was detected by using qRT-PCR. $^{*}p<0.05$, compared with LO2, $^{\#}p<0.05$, compared with HepG2. ***D,*** A negative correlation was found between lncRNA H19 expression and microRNA-22 expression in tumor samples. ***E,*** The expression of microRNA-22 was verified by qRT-PCR in co-transfected cell lines. $^{*}p<0.05$. ***F,*** The roles of lncRNA H19 and microRNA-22 in the regulation of HCC cell migration and invasion were examined by transwell assay.

duced by lncRNA H19 knockdown were reversed by microRNA-22 knockdown (Figure 4F). The above findings further suggested that lncRNA H19 regulated HBV-related HCC *via* microRNA-22.

Discussion

Currently, the high incidence of HCC has become a serious disease burden worldwide. Molecular genetic changes in HCC cells exert important effects on tumor phenotypes. At present, the study of multiple tumor signaling pathways has promoted the development of new therapeutic targets[21]. The early diagnosis rate of HCC patients in China is extremely low. Moreover, most of HCC patients are already in the middle and late stages when first diagnosed[7]. Therefore, early diagnosis and effective treatment are considered important in the treatment of HCC[2]. In China, hepatitis B virus infection is the leading cause of HCC. Therefore, explorations of HBV-related HCC are of great significance. Recent investigations have indicated that some certain lncRNAs can serve as biomarkers in predicting and treating different types of tumors. Our goal is to search for novel lncRNA that is related to HBV-related HCC, eventually developing individual treatment for HBV-related HCC patients. In recent years, it has been revealed various molecular targets are regulated by lncRNAs. Accumulating evidences have shown that lncRNAs exert a crucial role in the biological behavior of HCC, which can provide a new strategy for diagnosis and treatment of HCC in the future[22]. Some certain lncRNAs have been found to be differentially expressed in HCC[23,24]. It is believed that lncRNA can affect cell proliferation, apoptosis, chemotherapy sensitivity and tumor metastasis. Meanwhile, revealing the role and mechanism of lncRNA provide targets for the development of novel anti-hepatoma drugs[2]. In this study, we investigated the clinical characteristics of lncRNA H19 in HBV-related HCC. The expression level of lncRNA H19 in 36 pairs of HBV-related HCC tissues and para-cancerous tissues was first detected. The results showed that overexpressed lncRNA H19 was positively correlated with stage, lymph node metastasis, distant metastasis, and poor prognosis of HBV-related HCC. To further explore the role of lncRNA H19 in HBV-related HCC, we constructed lncRNA H19 knockdown model by transfecting small interfering RNA *in vitro*. The data demonstrated that lncRNA H19 could significantly promote the migration and invasion of HBV-related HCC cells. EMT signaling pathway is an important signaling pathway related to tumorigenesis[25]. A great number of studies[25,26] have shown that EMT is associated with the occurrence and development of epithelial cell malignancy. EMT plays a pivotal role in primary and secondary metastases of breast cancer, colon cancer, lung cancer, prostate cancer, liver cancer, and pancreatic cancer[26]. Therefore, investigating the mechanism of EMT is of great significance for finding of malignant tumor treatment target. In this experiment, results of Western blot showed that knockdown of lncRNA H19 significantly decreased the expressions of key proteins in EMT pathway, such as N-cadherin, Vimentin, β-catenin and MMP-9. This indicated that lncRNA H19 might regulate HBV-related HCC *via* EMT pathway. MicroRNA is a type of endogenous, small-molecule, single-stranded RNA, with 18 to 25 nt in length. Researches have shown that the abnormal expression of microRNAs is closely related to the proliferation, invasion, and metastasis of various malignant tumors[27,28]. Zuo et al[29] have observed that microRNA-22 inhibits gastric cancer growth and metastasis by directly targeting MMP14 and Snail. Palacios et al[30] have found that microRNA-22 promotes the proliferation of chronic lymphocytic leukemia B-cells though activating PI3K/AKT pathway. In our study, our findings indicated that the expression of microRNA-22 was downregulated in HCC tissues and HCC cells. Additionally, our rescue experiments showed that microRNA-22 was regulated by lncRNA H19 in HBV-related HCC.

Conclusions

We pointed out that lncRNA H19 is overexpressed in HBV-related HCC. LncRNA H19 expression is correlated with tumor stage, distant metastasis and poor prognosis of HBV-related HCC. Moreover, lncRNA H19 promotes malignant development of HBV-related HCC through regulating microRNA-22 via EMT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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