

Promotion of proliferation and metastasis of hepatocellular carcinoma by LncRNA00673 based on the targeted-regulation of notch signaling pathway

H. CHEN¹, J.-Z. LIU¹, G.-J. HU¹, L.-L. SHI², T. LAN¹

¹Department of Hepatobiliary and Pancreatic Surgery, The People's Hospital of Cangzhou, Cangzhou, Hebei Province, China

²Department of Obstetrics and Gynecology, The People's Hospital of Cangzhou, Cangzhou, Hebei Province, China

Abstract. – OBJECTIVE: To investigate the relative expression of long non-coding RNA 00673 (lncRNA 00673) in hepatocellular carcinoma (HCC) and HCC cells and study its regulation on the malignant phenotype of HCC cells

PATIENTS AND METHODS: Samples of HCC and adjacent tissues from January 2013 to December 2015 were collected. The expression level of lncRNA00673 in HCC tissues and cells was detected by quantitative Real-time polymerase chain reaction (qRT-PCR) assays. lncRNA00673 specific interference sequences were transiently transfected into HCC cells and the effect of HCC cells on the biological behavior of HCC cells was examined by *in vitro* experiments ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), flow cytometry, transwell, etc.). A tumor model of nude mice with HCC was established for the study of tumor growth condition of tumor-bearing mice after the interference with lncRNA00673 expression. Changes in expression levels of molecular markers on Notch signaling pathway after the interference with lncRNA00673 were detected by Western blot.

RESULTS: lncRNA00673 was highly expressed in HCC tissues and cells. MTT results showed that interfering with lncRNA00673 inhibited cell proliferation. Flow cytometry results showed that HCC cell cycle was retarded in G1-G0 phase, thus promoting apoptosis after the interference with lncRNA00673. Western blot results showed that expression levels of molecular markers on Notch signaling pathway were changed after the interference with lncRNA00673.

CONCLUSIONS: lncRNA00673 is highly expressed in HCC tissues and cells, and can promote the proliferation and metastasis of HCC by the regulation on Notch signaling pathway. lncRNA00673 may be a potential target for the treatment of HCC.

Key Words:

HCC, lncRNA 00673, Notch signaling pathway, Proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors in the world, ranking third in the malignant tumors of China¹. According to cell types, it is divided into hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), in which the proportion of HCC is as high as 90%². Although there are diverse treatments at present, the overall curative effect is poor due to its high malignancy, rapid progress, needs for complex treatments and other characteristics³. In recent years, the research on molecular biology of HCC has become a new field with rapid development, and some HCC targeting drugs (such as sorafenib) have been clinically applied with good curative effects⁴. The basic reason because this field becomes a hot issue is that relevant researches are involved in in-depth studies of the occurrence and development mechanisms of HCC from the molecular level, in which characteristic molecules are regarded as anti-tumor targets, thus opening up a new way for clinical treatment of HCC. According to studies of human genomes, only about 2% of the genes can be transcribed into biologically functional RNAs, and most of them are ncRNAs without protein-coding function, which are, therefore, considered as “dark matters” in a genome⁵. lncRNA is a class of ncRNA

with the length of over 200 nt. Recent studies⁶⁻⁹ have found that lncRNA plays important roles in cell life activities, such as chromatin modification, gene transcription activation and inhibition, cell differentiation and other links. Abnormal expressions of lncRNA are closely related to the occurrence and development of tumors¹⁰. In non-small cell lung cancer (NSCLC), the highly expressed lncRNA PVT1 (plasmacytoma variant translocation 1 gene) promotes the development of non-small cell lung cancer by binding the apparent complex polycomb repressive complex 2 (PRC2) to inhibit the transcription of target gene large tumor suppressor kinases 2 (LATS2)¹¹. Huang et al¹² found that lncRNA ANRIL (antisense non-coding RNA in the INK4 locus) inhibited the apoptosis of HCC cells by inhibiting Kruppel-like factor 2 (KLF2) gene expression, but the biological effects of lncRNA00673 in HCC have not been reported. lncRNA00673 is located in chromosome 17q25.1 region and its overall length is 2275bp. It has been reported in literature that lncRNA00673 abnormal expression plays an important role in the changing process of tumor biological behavior. In NSCLC tissues and cells, the highly expressed lncRNA00673 binds the apparent complex lysine-specific histone demethylase 1 (LSD1) to mediate the demethylation of neurocalcin delta (NCALD) genes at the promoter region, thus promoting the proliferation of NSCLC¹³. Besides, the highly expressed lncRNA00673 also promotes the metastasis of NSCLC by binding the PRC2 protein to inhibit the transcription of homeobox protein A5 (HOXA5)¹⁴. In tongue squamous cell carcinoma, the highly expressed lncRNA00673 can promote the invasion and metastasis of tumor cells¹⁵. In this study, we found for the first time that lncRNA00673 was highly expressed in HCC tissues and cells, and interfering with lncRNA00673 could inhibit the proliferation and metastasis of HCC cells by Notch signaling pathway.

Patients and Methods

Culture of Tissue Specimens and Cells

The patients untreated with chemotherapy, radiotherapy and molecular targeted therapy were selected as the study subjects. This study was approved by the Ethics Committee of the People's Hospital of Cangzhou. Signed written informed consents were obtained from all participants before the study. HCC and its adjacent tissue specimens excised from 55 patients by surgery in

our hospital were collected. Those patients were pathologically diagnosed as HCC, and the specimens excised from the patients after surgery were quickly put into liquid nitrogen at -180°C for preservation. HCC cell lines HepG2, Hep3B, MHCC-97H, and normal liver epithelial cell line L02 were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in Dulbecco modified eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL double anti-penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Then, the cells were conventionally cultured in an incubator with 5% CO₂ at the constant temperature of 37°C. The medium was replaced with a fresh one every 2 days. When the cell fusion degree reached 80-90%, cell passage would begin, and at that time, the well-functioning cells were selected for experiments.

Synthesis of siRNA and qRT-PCR Primers Interfering with lncRNA00673

The effective interference sequence of lncRNA00673 and the control sequence si-NC are shown in Annex 1. The upstream and downstream primer sequences of Real-time fluorescence quantitative PCR are shown in Annex 1. The above sequences are designed and synthesized by Invitrogen, (Carlsbad, CA, USA). The effective interference sequence of lncRNA00673 was si-NC (Table I). The control sequence was si-NC: 5'-CCTATCTGGTCAACACGTATT-3'. The upstream and downstream primer sequences of Real-time fluorescence quantitative PCR were shown in Table I. The above sequences were synthesized by Invitrogen (Carlsbad, CA, USA).

Detection of the Expression of lncRNA00673 by Using Real-time Fluorescence Quantitative PCR

Total RNA was extracted from breast carcinoma and corresponding adjacent tissues by Trizol kits and RNA concentration was measured by ultraviolet spectrophotometer. cRNA was synthesized according to the described procedures of PrimeScript™ RT Master Mix (Perfect Real-time) kits for further Real-time fluorescence quantitative PCR. The reaction system of qRT-PCR Mix (20 μL): 10 μL SYBR qPCR Mix, 0.8 μL (10 μmol/L) upstream and downstream primers, respectively, 2 μL cDNA products and 0.4 μL 50×ROX reference dye. Reaction condi-

Table I. Primers for qRT-PCR experiments.

linc00673	Forward	TACCACACCCCTTTCTTGCCC
	Reverse	ACACTGGCCTCTTTACACGG
GAPDH	Forward	GGGAGCCAAAAGGGTCAT
	Reverse	GAGTCCTTCCACGATACCAA
linc00673	si-1#	GAGAAAUAGUCUGUGUUGCCCUGAA
	si-2#	CAGCCGGAUACAGAGUGAAUAGUUA
	si-3#	UGUGCCUUUGUACUCAGCAAUUCUU

tions: after 1 min of pre-denaturation at 95°C, the cells were pre-denatured at 95°C for 30 s and at 60°C for 40 s, and the cycle were repeated for 40 times. Three parallel wells were designed in the experiment, and all samples were repeatedly tested for three times. The relative expression level of target genes was expressed by $-\Delta C_t$ and $2^{-\Delta\Delta C_t}$ using the relative quantification method, respectively.

Detection of the Proliferation of HCC Cells by MTT Assay

The cell lines were transfected into a 96-well plate at a cell density of 3×10^3 /well and transfected by siRNA. 20 μ L MTT solution (1.55 g/L) was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h, and after the incubation at 37°C for 4 h, 150 μ L dimethyl sulfoxide (DMSO) was added to each well. The absorbance values were read and the cell growth curve was drawn.

Detection of the Changes in Cycles and Apoptosis by flow Cytometry

Concentrations of HCC cells in the logarithmic growth phase were adjusted to 3×10^5 /mL, and they were inoculated into a 6-well plate at 2 mL/well. After 48 h transfection, the mice were divided into the experiment group and the control group. The cells in the two groups were collected after another 48 h-culture. Then double staining was performed for cells with Annexin V/PI (propidium iodide) and they were kept in a dark place for 10 min. The apoptosis rates were measured by flow cytometry. For the other group, cells were collected by the same method and under the same conditions. These cells were resuspended with 75% precooled ethanol and were fixed at -20°C overnight. The cells were divided into G1/G0 phase, S phase and G2/M phase, respectively, when the intracellular DNA contents were detected by flow cytometry and PI staining. The percentage of each phase could be calculated by special software (Version X; TreeStar, Ashland, OR, USA).

Detection of Changes in Abilities of Metastasis and Invasion by Transwell Experiments

Interference sequences and the control sequences were transiently transfected into the model cells. After these transfected cells grew up to 80-90%, pancreatin was used to digest cells. The cells were resuspended in a serum-free medium to form a single cell suspension. The density was calculated and adjusted to 3×10^5 /mL. The above 200 μ L cell suspension was added into the upper transwell chamber (50 mg/L BD Matrigel at 1: 8 dilution was used to coat the upper chamber surface of the membrane at the bottom of transwell chamber). At the same time, the lower chamber was added with 600 μ L 1640 medium containing 20% FBS and then incubated for 24 hours under conventional conditions. In each group, 3 to 5 samples were examined, fixed by formaldehyde and finally received crystal violet staining.

Detection of the Changes in the Expression Level of Notch Signaling Pathway by Western Blot

Cells of the experiment group and control group were collected and the cell lysate was added for the extraction of total protein. Then, the total protein was quantified by Bradford protein assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed and the proteins extracted by electrophoresis were transfected to polyvinylidene fluoride (PVDF) membranes and kept on a confined space with 5% skimmed milk. 1:1000 rabbit anti-human Notch1 (Abcam, Cambridge, MA, USA) and Notch3 antibodies (Abcam, Cambridge, MA, USA) were added, respectively, and these cells were kept overnight at 4°C. The membranes were washed with tris-buffered saline tween-20 (TBST) buffer solution for 3 times for 10 min each time. Next, horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit antibodies (1:2000 diluent) (Abcam, Cambridge, MA, USA) was added and incubated at 37°C for 1 h. The membranes were

fist washed with TBST buffer solution for 3 times for 10 min each time; then, they were washed with tris-buffered saline (TBS) for 10 min. The color developing was detected from luminous compressed tablets, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded as the internal reference.

Nude Mice Tumorigenesis Experiment

4-5-week-old male BALB/c mice were selected as research subjects and HepG2 cells were cultured. The cells were transfected with sh-LncRNA00673 or were not transfected to form a blank control group, respectively. 1×10^7 cells of the experiment group and the control group were suspended in 0.1 mL serum-free DMEM (Gibco, Grand Island, NY, USA), and subcutaneous injection was performed to establish a tumor formation model of nude mice for the observation of the tumor formation in mice *in vitro*.

Immunohistochemical Method

Immunohistochemical method was used to detect the expression level of Ki67 in the specimens. Appearing light brown or brown area was regarded as a positive result, and a standardization analysis was conducted for the detection of results by using imaging mass spectrometry (IMS) cell im-

age analysis system and medical image analysis software (Version X; Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

All experiments were repeated for 3 times and were analyzed by using Statistical Product and Service Solutions 15.0 (SPSS Inc. Chicago, IL, USA). Data were expressed as mean \pm standard deviation, and tested by using the *t*-test. $p < 0.05$ represented that the difference was statistically significant.

Results

Detection of Expression Levels of LncRNA00673 in HCC Tissues and Cells

To detect expression levels of LncRNA00673 in HCC and its adjacent tissues, relative expression levels of LncRNA00673 in 55 pairs of HCC and adjacent tissues were detected by qRT-PCR assays. The results showed that compared with that in adjacent tissues, LncRNA00673 expression was significantly up-regulated in 72.7% (40 of 55, fold ≥ 1.0) HCC tissues (Figure 1A). Then, qRT-PCR assays showed that the expression level of LncRNA00673 in three HCC cell lines (HepG2,

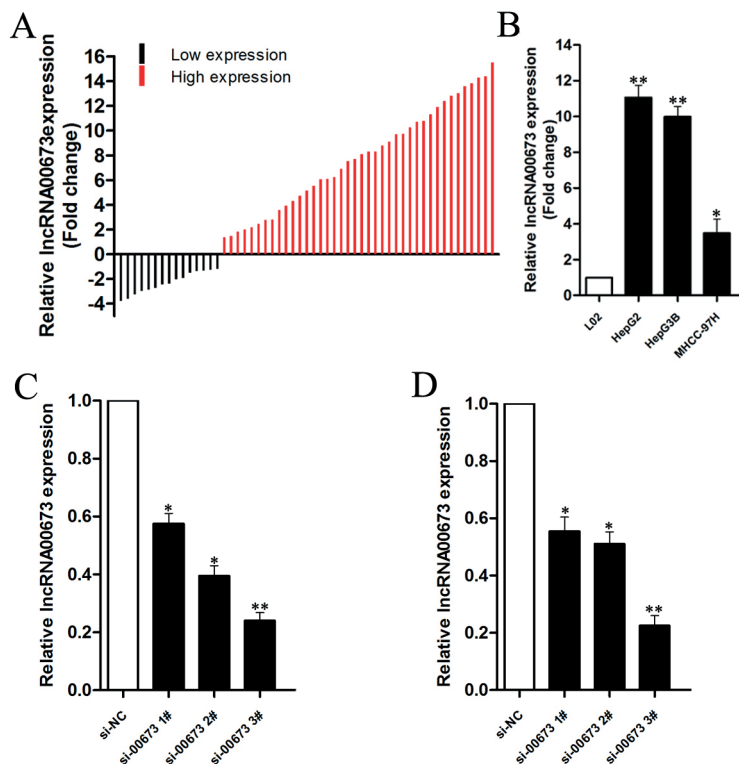


Figure 1. The expression level of LncRNA00673 in HCC tissues and cells. **A**, Relative expression levels of 55 pairs of HCC and adjacent tissues are detected by qRT-PCR assays, in which the expression levels of 40 cases are up-regulated and those of 15 cases are down-regulated. **B**, qRT-PCR assays are used to detect the relative expression levels of HCC cells compared with normal liver cells. **C-D**. Specific interference sequences of LncRNA00673 are designed and synthesized, which transiently transfected into HCC cells. qRT-PCR assays are used to detect interference efficiency. (** indicates $p < 0.01$, and * indicates $p < 0.05$).

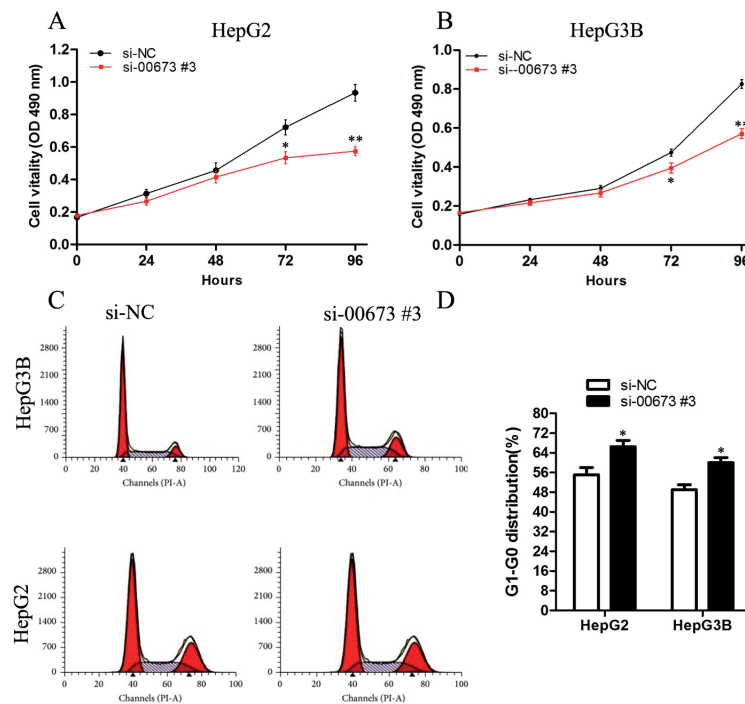


Figure 2. Effects of lncRNA00673 on proliferation ability of HCC cells. *A-B*, After the interference with the expression of lncRNA00673 in HCC cells, cell proliferation activity is decreased. *C-D*, After the interference with the expression of lncRNA00673 in HCC cells, cell cycle progression is detected to be retarded in G1-G0 phase by flow cytometry. (** indicates $p < 0.01$, and * indicates $p < 0.05$).

Hep3B, MHCC-97H) was significantly up-regulated compared with that in the normal liver cell line (L02) (Figure 1B). We selected two cell lines with the most obvious up-regulation signs HepG2 and Hep3B as model cells. In order to study biological functions of lncRNA00673, specific interference sequences of lncRNA00673 were designed and synthesized. The interference sequences and control sequences of target genes were transiently transfected into HCC cell lines by liposome lip2000. 48 hours later, we detected the interference rate of lncRNA00673 and selected 3 sequences for subsequent experiments (Figure 1C,D).

Research on Biological Functions of lncRNA00673 Based on *in vitro* Experiments

First, we investigated effects of lncRNA00673 on the proliferation of HCC cells by MTT assays. Results of MTT assays showed that the proliferation activity of cells in the group transfected with lncRNA00673 was significantly inhibited compared with the control group (Figure 2A,-B). To study whether promoting the proliferation of HCC cells influenced processes of cell cycles, in-

terference sequences of lncRNA00673 were transiently transfected into HepG2 and Hep3B cells. Flow cytometry (FCM) results showed that compared with the control group, cell cycle progressions of HepG2 and Hep3B cells of transfected lncRNA00673 interference sequences were retarded in G1-G0 phase (Figure 2C, D). Next, we investigated the effects of lncRNA00673 on the apoptosis of HCC cells. These cells were treated with the same method and changes in apoptosis were detected by FCM. Results showed that apoptosis rates of HepG2 and Hep3B cells were significantly increased after interference with lncRNA00673 compared with the control group (Figure 3A-B). To investigate the effect of lncRNA00673 on HCC cells' capacities of metastasis and invasion, we conducted transwell experiments. The results showed that HCC cells' capacities of metastasis and invasion were inhibited after the interference with lncRNA00673 (Figure 3C, D).

Research on the Effect of lncRNA00673 on Tumor Formation Capacity of HCC Based on *in vivo* Experiments

In order to investigate the role of lncRNA00673 *in vivo*, we selected the HepG2 cell line with high

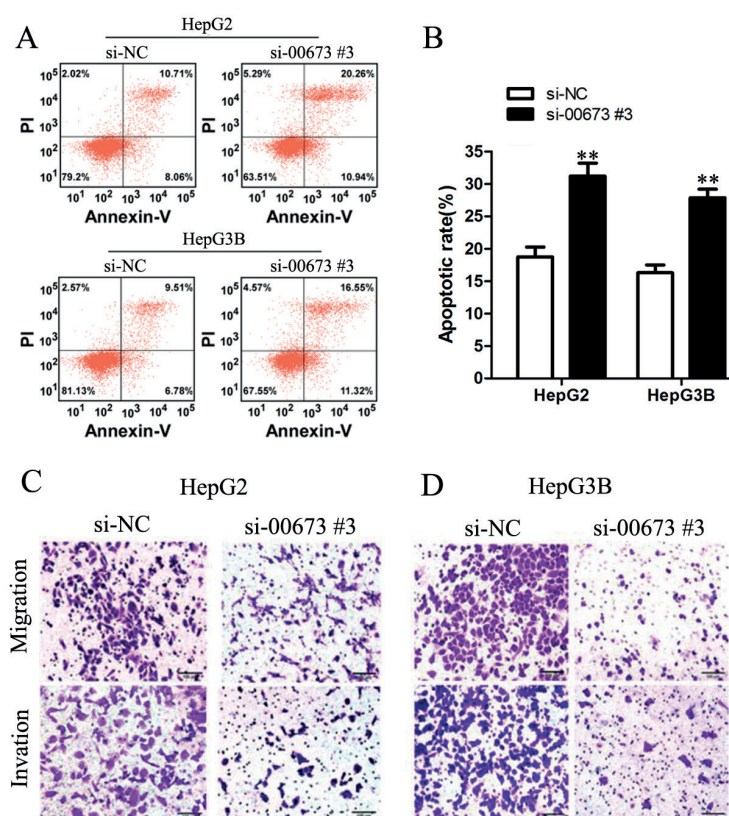


Figure 3. Effects of lncRNA00673 on biological functions of HCC cells *A-B*, After the interference with the expression of lncRNA00673 in HCC cells, the apoptosis rate is detected to be increased by flow cytometry. *C-D*, After the interference with the expression of lncRNA00673 in HCC cells, abilities of metastasis and invasion of cells are detected to be decreased by transwell experiments. (** indicates $p < 0.01$, and * indicates $p < 0.05$).

transfection efficiency. HepG2 cells were transfected with sh-lncRNA00673 or control sequences, and then injected into the armpits of nude mice to establish a transplanted tumor model. 18 days after the subcutaneous injection, the nude mice were killed and the specimens of subcutaneous tumor tissues were taken. Then, we took pictures and weight of them. As shown in Figure 4A, the volume of transplanted tumor derived from transfected sh-lncRNA00673 cells was significantly reduced compared with the control group, and the weight of the tumor in the experiment group was significantly lower than that in the control group (Figure 4C). We extracted total RNA from part of tumor tissues and the relative expression levels of lncRNA00673 in tumor tissues were detected by qRT-PCR assays. The results showed that the expression level of lncRNA00673 in the transplanted tumor from transfected sh-lncRNA00673 cells was significantly lower than that in the control group (Figure 4D). Then, we fixed the transplanted tumor and the immunohistochemistry

was conducted for slices. Afterwards, hematoxylin and eosin (HE) staining and proliferative cell-associated nuclear antigen (Ki67) staining were performed. HE staining results showed that a transplanted tumor model of nude mice was successfully constructed, and Ki67 staining results showed that the positive rate of Ki67 in transplanted tumors from transfected sh-lncRNA00673 cells was significantly lower than that in the control group (Figure 4B), which indicated that the tumorigenic ability of the cells in the experiment group was inhibited. Results of *in vivo* experiments above confirmed that decreased lncRNA00673 could significantly inhibit tumor formation ability of HCC cells.

Proliferation Promotion of HCC Cells Achieved by lncRNA00673 Based on the Regulation of Notch Signaling Pathway

Notch signaling pathway consists of a group of cell membrane ligands, such as Delta/Jagged, receptors and downstream molecules, which is

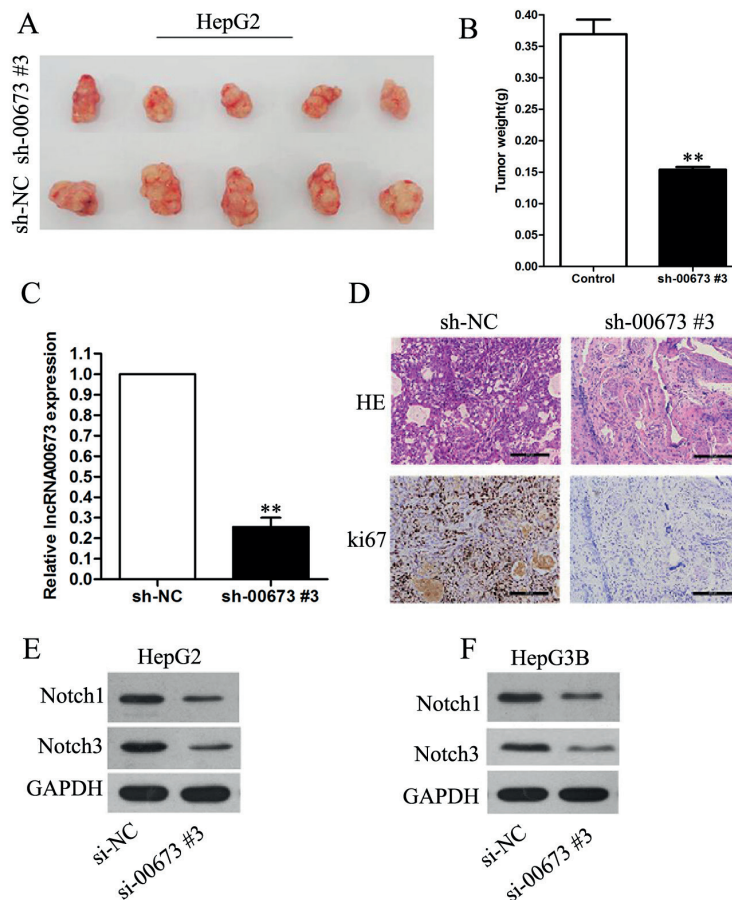


Figure 4. *In vivo* research on the effects of the interference with lncRNA00673 on tumor formation ability of tumor cells **A**, Empty vectors and sh-00673 are transfected into HepG2 cells and injected into male nude mice (n=6), respectively. 18 days after the transfection, we kill nude mice, remove their tumors and take pictures. **B**, We slice up tumor tissues and perform HE staining and Ki67 immunohistochemistry for them. **C**, We weigh the weight of transplanted tumors. **D**, qRT-PCR assays are used to detect the relative expression level of lncRNA00673 in transplanted tumors. **E-F**, After the interference with the expression of lncRNA00673 in HCC cell, changes in molecular markers on Notch signaling pathway are detected by Western blot assays (** indicates $p < 0.01$, and * indicates $p < 0.05$).

the intersection of many important signaling pathways. The abnormal activation or closure of this signaling pathway is closely related to tumor formation¹⁶⁻¹⁸. It has been reported in literature that lncRNA can participate in tumorigenesis by the regulation on Notch signaling pathway; for example, lncRNA HOTAIR promotes the proliferation and metastasis of cervical cancer by regulating Notch pathway¹⁹. However, the regulation relationship between lncRNA00673 and Notch signaling pathway has not been reported. Through Western blot assays, we found that, after the interference with lncRNA00673 in HCC cells, expression levels of molecular markers on the Notch signaling pathway (Notch1 and Notch3) were changed (Figure 4E).

Discussion

HCC is one of the most common malignant tumors in the world and it is also a major disease that threatens human life and social development. It is the second leading cause of global cancer-related death. The incidence rate of HCC is high in China and about 50% of the world new and death cases occur in China, but the current research on the signal transduction pathway of HCC is still not clear. In recent years, with the rapid development of high-throughput sequencing technology, it has been found that lncRNA abnormal expression plays an important role in the process of tumor formation and progression²⁰. Besides, a number of researches²¹ have confirmed that lncRNA plays an important role in the field of tumor, mainly

in tumor occurrence, chemotherapy resistance, metastasis and recurrence and other aspects. At present, abnormally expressed lncRNA found in tumor tissues can be involved in all systems in the whole body, and the distribution is relatively more extensive. Studies have shown that these upregulated lncRNAs in tumor tissues often act as “oncogenes”, such as the highly expressed MALAT-1 (metastasis-associated lung adenocarcinoma transcript-1) in lung cancer, breast cancer, colorectal cancer and other cancers²²⁻²⁴. However, the down-regulated lncRNAs act as “tumor suppressor genes”, such as lowly expressed MEG3 (maternally expressed gene 3) in lung cancer and liver cancer^{25,26}. Some lncRNAs play different roles in different tumors; for example, H19 has a carcinogenic effect in liver cancer, bladder cancer and breast cancer, while it has anti-cancer effect in colorectal cancer²⁷⁻²⁹. Therefore, lncRNAs, which have different expression levels or are specifically expressed in certain tumors, are expected to become new molecular markers for tumor diagnosis and treatment in the future. It was found for the first time in our study that lncRNA00673 was highly expressed in HCC tissues and cells and acted as “oncogenes” to promote the proliferation, invasion and metastasis of HCC cells. Notch signaling pathway is an evolutionally highly conserved signaling pathway that plays an important role in mediating cell differentiation, cell proliferation and apoptosis, embryonic development, tumorigenesis and other physiological and pathological processes by the regulation of downstream genes in, involving almost all organs and tissues³⁰⁻³². The Notch signaling pathway cannot only act on intrahepatic angiogenesis, but also promote the development of liver cancer through a variety of mechanisms. It has been reported in literature that the activation of Notch signals can promote HCC cell proliferation, regulate cell cycle and inhibit apoptosis and epithelial mesenchymal transition (EMT), thus inducing the tumorigenesis of cells^{33,34}. Recent studies have shown that lncRNA-mediated changes in Notch signaling pathways are associated with the development of tumors. For the first time, we found that lncRNA00673 could promote the proliferation and metastasis of HCC by regulating Notch signaling pathway. The regulation relationship between lncRNA00673 and Notch signaling pathway provides new directions and opportunities for the development of more targeted drugs for HCC treatment, and provides a new strategy for the prevention and treatment of HCC.

Conclusions

lncRNA00673 is highly expressed in HCC tissues and cells, and can promote the proliferation and metastasis of HCC by the regulation on Notch signaling pathway. lncRNA00673 may be a potential target for the treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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