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LINC01503 promotes cell proliferation, invasion and EMT process in cholangio-carcinoma

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Abstract. – OBJECTIVE: This work aimed to analyze the relative expression level of long intergenic non-protein coding ribonucleic acid 1503 (LINC01503) in cholangiocarcinoma tissues and cells, and to explore the effects of LINC01503 on cell proliferation, migration and invasion.

PATIENTS AND METHODS: Logarithmic growth phase cholangiocarcinoma cells were selected and transfected with Lipofectamine 2000 (si-LINC01503, si-NC). The expression of LINC01503 was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The proliferation of cells was observed by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect cell apoptosis. Transwell assay was used to observe the cell migration and invasion ability.

RESULTS: The expression of LINC01503 was significantly increased in cholangiocarcinoma tissues compared with adjacent tissues ($p<0.05$), and the up-regulated expression of LINC01503 was associated with lymph node metastasis. Compared with normal bile duct cells (HIBEC), cholangiocarcinoma cells (RBE, QBC939) have higher expression of LINC01503, and si-LINC01503 transfection can effectively reduce the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of cholangiocarcinoma cells.

CONCLUSIONS: LINC01503 is highly expressed in cholangiocarcinoma and can effectively promote the proliferation, migration, invasion and EMT process of cancer cells, and LINC01503 is expected to be a potential biomarker for cholangiocarcinoma.

Key Words:
LINC01503, Cholangiocarcinoma, EMT.

Introduction

Cholangiocarcinoma originates from biliary epithelial cells and is the 2nd most common hepatobiliary malignant tumor. In recent years, although the incidence rate has been found to be different between regions and genders, it has been increasing year by year in the global scale[1,2]. As the incidence of cholangiocarcinoma is concealed and there is no effective diagnosis and treatment, the patients are often found in the middle and late stages of the disease and the prognosis is poor. Therefore, it is particularly important to explain the molecular mechanism of the occurrence and development of cholangiocarcinoma.

The pathogenesis of cholangiocarcinoma is accompanied by the deregulation of multiple genes, and the abnormal expression of long non-coding RNA (lncRNA) has been found in many studies[3] in recent years. As a large class of transcripts that do not have the function of encoding proteins, lncRNA has long been considered as a by-product of the transcription process and lacks biological regulation. However, more and more evidence indicated that these molecules are involved in transcriptional splicing and post-transcriptional modification. Gene network regulation and other processes play important roles and participate in mediating the occurrence and development of a variety of tumors[4-7]. There is evidence that long intergenic non-protein coding ribonucleic acid 1503 (LINC01503) is up-regulated in breast cancer, gastric cancer, lung cancer and liver cancer and is closely related to various clinical features, but the correlation with cholangiocarcinoma has not been reported[8-11]. Therefore, this study ex-

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plored the expression of LINC01503 in cholangiocarcinoma, and further observed its effect on proliferation, apoptosis and invasion of cholangiocarcinoma by intervention expression, to provide new ideas for the prognosis and therapeutic target of cholangiocarcinoma.

Patients and Methods

Specimen Processing and Clinical Information Collection

The specimen was stored in liquid nitrogen for 30 min and then transferred to a -80°C refrigerator for storage. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Jiamusi University, and all patients signed informed consent.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Detection

The expression level of LINC01503 was extracted from tissues and cells by TRIzol (Invitrogen, Carlsbad, CA, USA), and the expression level of RNA was detected by UV spectrophotometer. Reverse transcription synthesis complementary deoxyribonucleic acid (cDNA) was carried out according to the protocol (Roche, Basel, Switzerland). The reaction system was established according to the requirements of SYBR Green (Applied Biosystems, Foster City, CA, USA), and cDNA amplification was performed by qRT-PCR. GAPDH was used as an internal reference gene to detect the expression of LINC01503 in tissues and cells. Among them, LINC01503 primer (GenePharma, Shanghai, China) sequence sense strand: 5'--3', antisense strand: 5'--3'; GAPDH primer sequence sense strand: 5'-GGGAGCCAAAAGGGTCAT-3', antisense strand: 5'- GAGTCCTTCCACGATACCAA-3'.

Cell Culture and Transfection

RBE and QBC939 cells were placed in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and cultured at 37°C and 5% CO_2 at a constant temperature. Penicillin and streptomycin were added to the medium. The logarithmic growth phase cells were selected to be inoculated into a 6-well plate (Corning, Lowell, MA, USA) to a growth density of 50%, and the expression level of LINC01503 was interfered by RNA interference (GenePharma, Shanghai, China). The experimental group was added with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and mixed with si-LINC01503. The same amount of Lipofectamine 2000 and si-NC was added to the control group.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay Test

The proliferative activity of each group of cells was examined. The cells were seeded in 96-well plates (Corning, Lowell, MA, USA), and the cells reached 30% to 50% per well during transfection. At 48, 72, and 96 h after transfection, the plates were removed. MTT (Sigma-Aldrich, St. Louis, MO, USA), 20 μL (5 mg/ml) were added to each well, and cultured for 4 h at 37°C in a 5% CO_2 incubator. The plate was removed and centrifuged for 5 min. Discarded the supernatant, 100 μL of dimethyl sulfoxide (DMSO) was added per well to terminate the reaction and dissolve the purple particles of the Formazan blue. The absorbance at 490 nm was measured by a spectrophotometer. Three wells were set in each group, and the cell growth curve was drawn by the mean value.

Transwell Test

The concentration of trypsin-digested cells was adjusted in a serum-free medium, and 200 μL of cell suspension was dropped into the chamber (Costar, Coppell, TX, USA); the invasion experiment was performed in the chamber layer before dropping the cells into Matrigel gel (BD Biosciences, Frankln Lakes, NJ, USA), and the lower chamber was added with 500 μL 10% FBS complete medium. After 24 h of incubation, the upper chamber cells were wiped off (the Matrigel Matrigel should be wiped off at the same time), then fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) for 15 min, and crystal violet (Hyclone, South Logan, UT, USA) for 15 min. The microscope was observed under high magnification to detect the migration and invasion ability.

Cell Apoptosis

Cells smears were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Then, the cells were incubated with deoxynucleotidyl transferase-mediat-

ed deoxyuridine triphosphate (dUTP)-biotin Nick End Labeling (TUNEL) reaction. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and visualized. The number of TUNEL-positive nuclei was expressed as a percentage of total nuclei.

Western Blot Detection

The total protein of each group was extracted, the protein concentration was determined by bicinchoninic acid (BCA); the protein was denatured and then subjected to dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. 5% skim milk powder solution was blocked at room temperature for 2 h, and primary antibody was added [dilution concentration: E-cadherin (Cell Signaling Technology, Danvers, MA, USA), N-cadherin (Abcam, Cambridge, MA, USA) and Vimentin (Cell Signaling Technology, Danvers, MA, USA), 1:1000; GAPDH 1:15000] overnight at 4°C. The membrane was washed by using Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), the secondary antibody was added (dilution concentration: 1:8000, SouthernBiotech, Birmingham, AL, USA) for 2 h at room temperature. The membrane was washed with TBST solution 3 times. The gray value of the strip was determined by an image analyzer, the measured gray value reflects the expression of the protein.

Statistical Analysis

Data were analyzed using SPSS 17. 0 statistical software (SPSS Inc., Chicago, IL, USA). The *t*-test was used for comparison between groups, and the difference was statistically significant with $p<0.05$.

Results

LINC01503 Was Highly Expressed in Cholangiocarcinoma Tissues and Cells

Compared with qRT-PCR, the relative expression of LINC01503 in cholangiocarcinoma was significantly higher than that in the adjacent normal tissues ($p<0.05$, Figure 1A). Cholangiocarcinoma cells (RBE, QBC939) were relatively higher expressed compared to normal bile duct cells (HIBEC; $p<0.05$, Figure 1B). Furthermore, as shown in Figure 1C, LINC01503 expression in the N1 group was markedly elevated compared with the N0 group, which indicated that the expression level of LINC01503 was significantly correlated with lymph node invasion. The results showed that LINC01503 expression was relatively higher in cholangiocarcinoma tissues and cells than in the adjacent tissues and normal bile ducts, suggesting that LINC01503 was likely to promote the malignant biological processes of proliferation, migration and invasion of cholangiocarcinoma.

LINC01503 can Promote the Proliferation Activity and Inhibit Apoptosis of Cholangiocarcinoma Cells

RBE and QBC939 cells were transfected with si-NC or si-LINC01503 respectively. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) results showed inhibition of LINC01503 expression in cells transfecting with si-LINC01503 as compared with the si-NC group (Figure 2A, 2B). In the MTT assay, the proliferation of RBE and QBC939 cells was observed after transfecting with si-LINC01503 and si-NC. The proliferative ability of cells transfecting with si-LINC01503 was inhibited by about 60.2% as compared with the control group (Figure 2C, 2D). In addition,

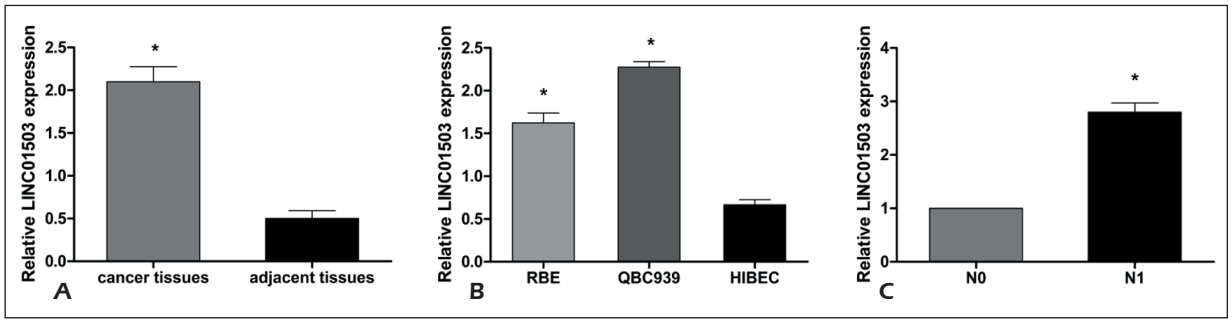

Figure 1. LINC01503 was highly expressed in cholangiocarcinoma tissues and cells. ***A***, The relative expression of LINC01503 in cholangiocarcinoma tissues was detected by qRT-PCR. ***B***, The relative expression of LINC01503 among cholangiocarcinoma cells (RBE, QBC939) and a normal bile duct cell (HIBEC) were detected by qRT-PCR. ***C***, The relative expression of LINC01503 between N1 group and N0 group were detected by qRT-PCR. $p<0.05$.

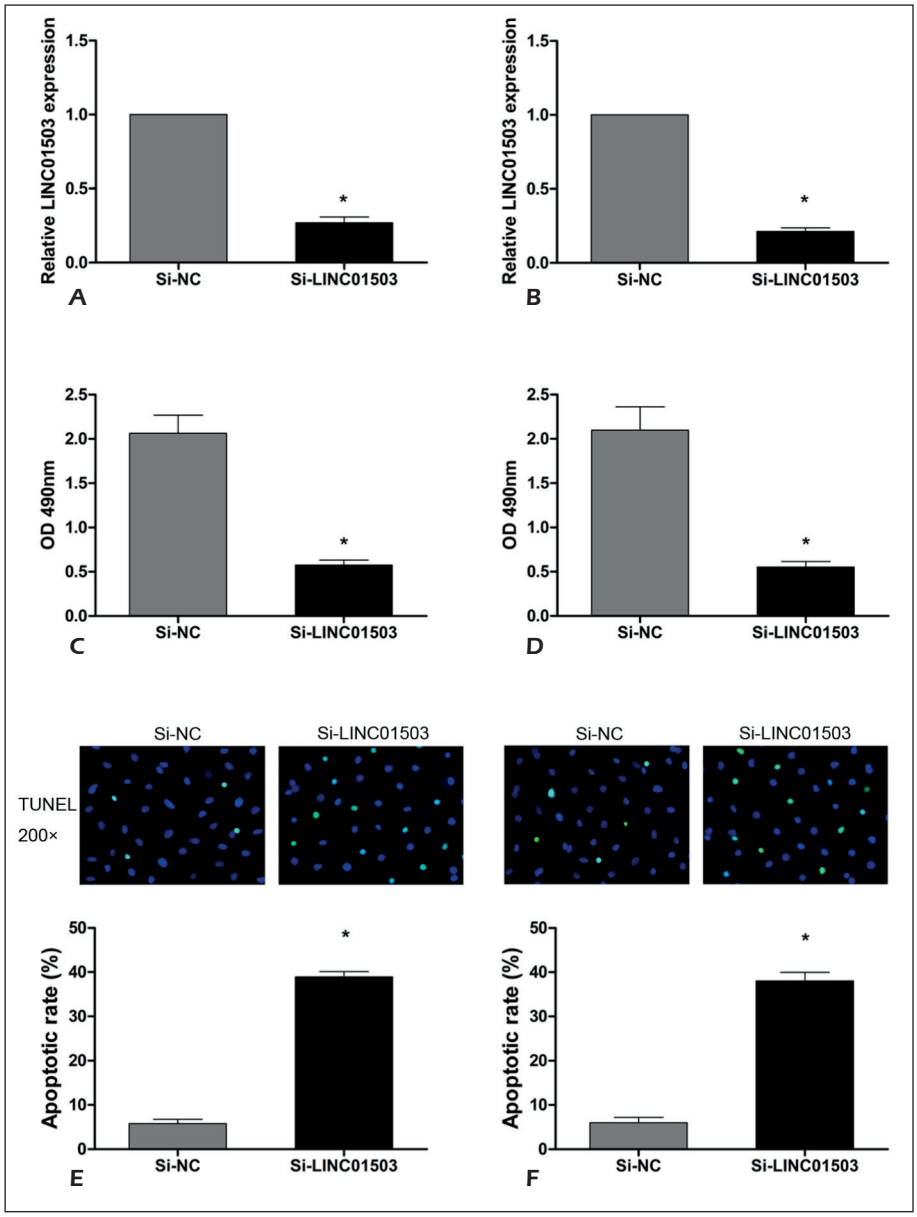

Figure 2. LINC01503 can promote the proliferation activity and inhibit apoptosis of cholangiocarcinoma cells. ***A-B***, RBE and QBC939 cells were transfected with si-NC or si-LINC01503 respectively and confirmed by qRT-PCR. ***C-D***, The proliferation of RBE and QBC939 cells was detcted by MTT assay. ***E-F***, The apoptosis of RBE and QBC939 cells was revealed by TUNEL assay. $p<0.05$.

in the TUNEL assay, the apoptosis of RBE and QBC939 cells was observed after transfecting with si-LINC01503 and si-NC. The apoptosis of cells transfecting with si-LINC01503 was elevated by about 7 times than the control group (Figure 2E, 2F). This finding revealed that LINC01503 can promote the proliferation activity and inhibit apoptosis of cholangiocarcinoma cells.

LINC01503 Promoted Cell Migration and Invasion

To further understand the ability of LINC01503 to migrate and invade cholangiocarcinoma cells, this study examined the effects of LINC01503 on migration and invasion of cholangiocarcinoma by observing the number of cells in the lower chamber of the chamber. The results showed that the migration cells of cholangiocarcinoma were decreased in the si-LINC01503 transfection group. Continued observation showed a decrease in the number of cell invasion in the si-LINC01503 transfection group (Figure 3). Thus, LINC01503 has the effect of promoting cells migration and invasion.

Effect of LINC01503 on the Progression of EMT in Cholangiocarcinoma Cells

Epithelial-mesenchymal transition (EMT) has been identified to participate in tumor cell invasion[12-14]. Here, to evaluate whether LINC01503

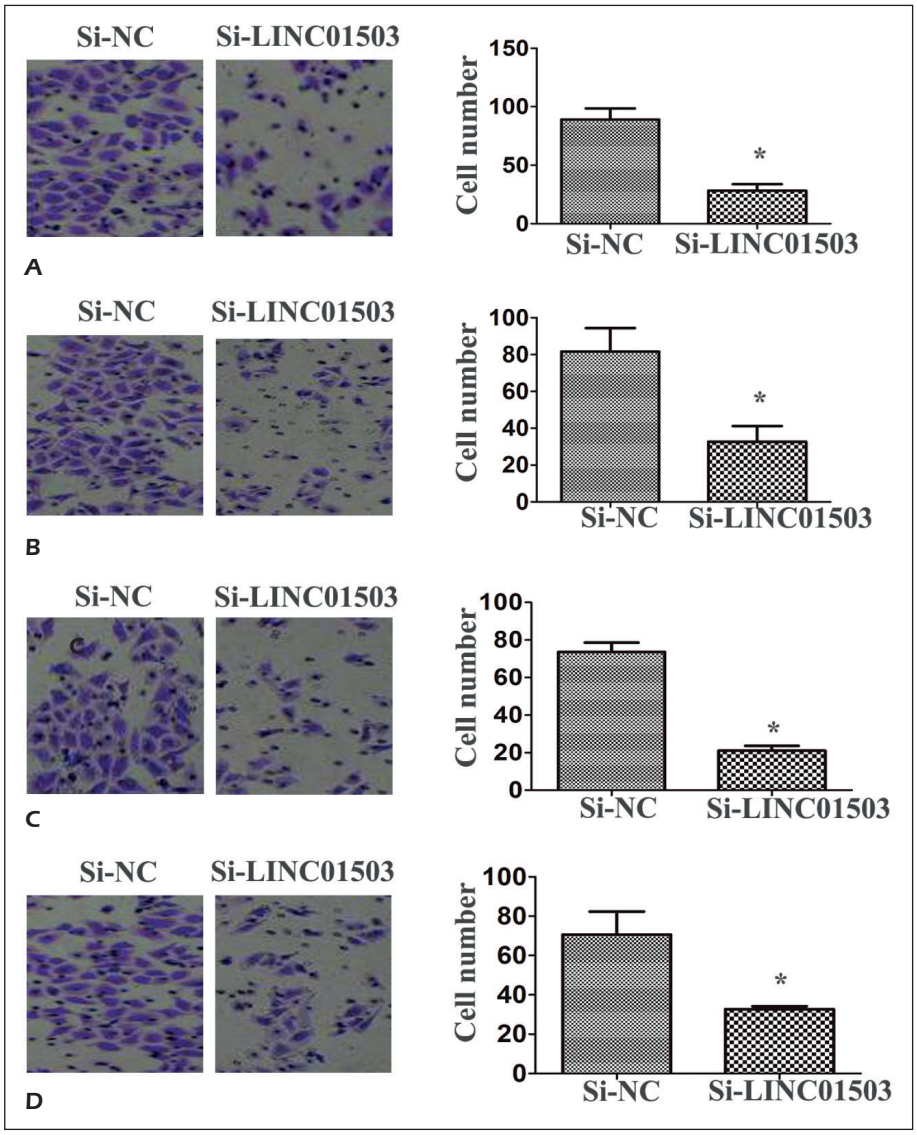

Figure 3. LINC01503 promoted cell migration and invasion. ***A-B***, The effects of LINC01503 on RBE and QBC939 cells migration were detected by transwell assay. ***C-D***, The effects of LINC01503 on RBE and QBC939 cells invasion were found by transwell assay with Matrigel. $p<0.05$.

was able to influence the progression of EMT in cholangiocarcinoma cells, we conducted Western blot assays. Interestingly, the results of Western blot showed that compared with the si-NC group, the expression of E-cadherin protein in the si-LINC01503 group was increased, and the expression levels of N-cadherin and Vimentin protein in the si-LINC01503 group were decreased (Figure 4A, 4B). This finding demonstrated that LINC01503 can enhance the EMT process in cholangiocarcinoma cells to promote cell migration and invasion. Moreover, the mRNA levels of E-cadherin, N-cadherin and Vimentin were detected between cholangiocarcinoma tissues and adjacent tissues. As presented in Figure 4C, 4D, 4E, the relative E-cadherin expression in cancer tissues was lower than that in the adjacent tissues; meanwhile, both the relative N-cadherin and Vimentin expression levels in cancer tissues were higher than that in the adjacent tissues.

Discussion

LncRNA is a linear transcript that is widely distributed in eukaryotic cells over 200 nt in length and cannot encode proteins due to the lack of an open reading frame (ORF). In recent years, a large number of studies have shown that lncRNA plays an important role in different biological links such as transcription, translation, shearing and modification of human genes, and the imbalance of such molecules is closely related to many physiological and pathological processes of the hu-

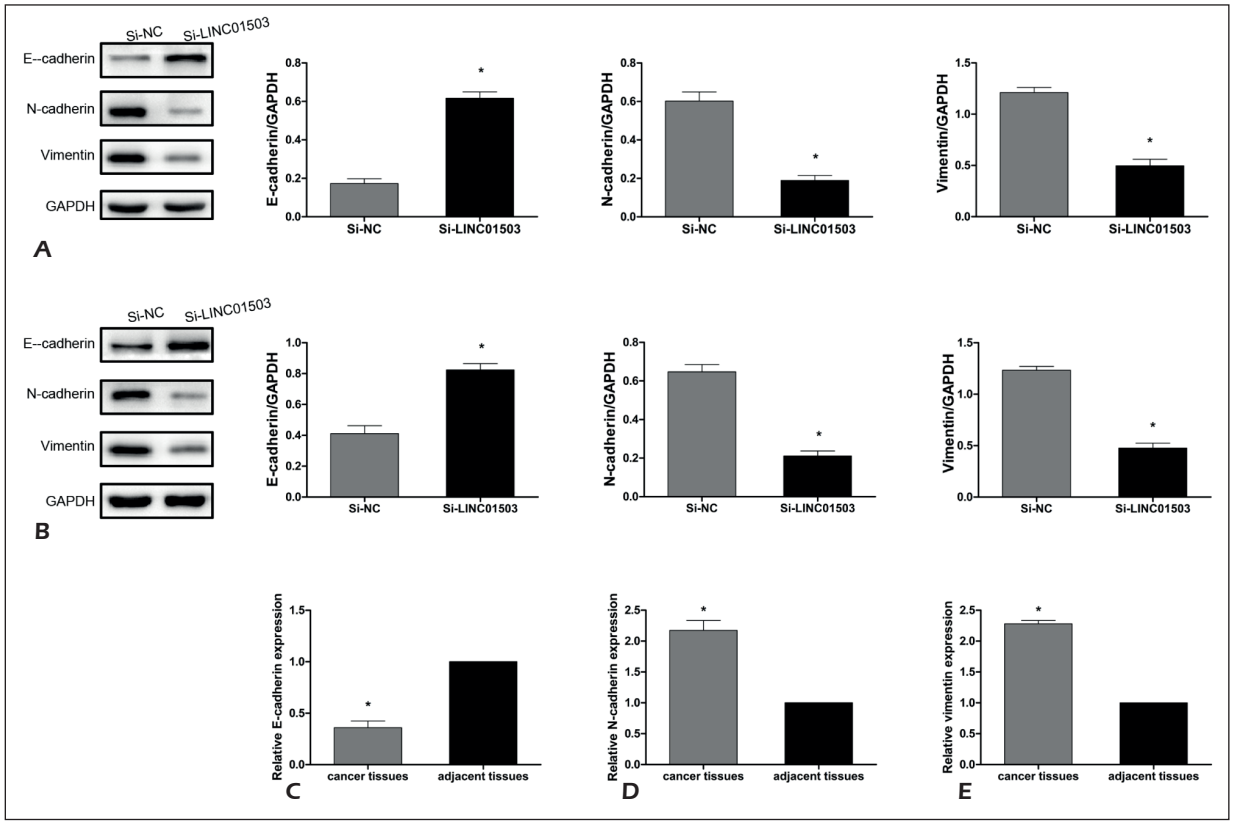

Figure 4. Effect of LINC01503 on the progression of EMT in cholangiocarcinoma cells. ***A-B***, The expression levels of E-cadherin, N-cadherin and Vimentin protein were detected by Western blot assay. ***C-E***, The mRNA levels of E-cadherin, N-cadherin and Vimentin were detected between cholangiocarcinoma tissues and adjacent tissues. $p<0.05$.

man body, especially the development of tumors is most obvious[15]. With the deepening of research, the important role of lncRNA in various tumors has been revealed. For example, overexpressed highly up-regulated in liver cancer (HULC) in liver cancer can promote the proliferation of liver cancer by regulating cyclooxygenase-2 (Cox-2), and can activate tumor epithelial-mesenchymal transformation to affect the prognosis of the patients[16]. CCAT1 can promote the expression of proliferative protein BRD4 in rectal cancer cells and has the potential to judge the prognosis of patients[17]. Up-regulated MALAT1 in breast cancer can inhibit the expression of CD133, thereby regulating the invasion and epithelial-mesenchymal transformation of breast cancer cells[18].

EMT (epithelial-mesenchymal transformation) refers to the process of transforming epithelial cells into mesenchymal cells, which is a physiological phenomenon during embryonic development. Loss of E-cadherin expression and gain of N-cadherin expression have been considered as markers of EMT phenomenon[19].

Our study used qRT-PCR to detect the overexpression of LINC01503 in cholangiocarcinoma tissues and cell lines. Statistical analysis showed that lncRNA was significantly associated with lymph node metastasis of cholangiocarcinoma. In addition, to further clarify that the molecule can promote the progression of the malignant biological behavior of cholangiocarcinoma, MTT, TUNEL and transwell experiments were applied. Therefore, LINC01503 is identified as a potential biomarker and therapeutic target of cholangiocarcinoma. This work also provides an experimental basis for individualized and accurate diagnosis and treatment of cholangiocarcinoma.

The results of this study indicated that LINC01503 plays a significant role in the development of cholangiocarcinoma. The experimental results are consistent with the trend of LINC01503 expression in other tumors, such as colorectal cancer[20] and squamous cell carcinoma[21]. The high expression of LINC01503 has the ability to promote tumor cell proliferation, invasion and EMT, and the up-regulated expression

of LINC01503 in tumor is associated with lymph node invasion, which shows that the molecule is a tumor-promoting factor. This experiment preliminarily revealed that LINC01503 is involved in mediating the proliferation, apoptosis, migration and invasion of cholangiocarcinoma.

Conclusions

We demonstrated that LINC01503 overexpression is closely related to cholangiocarcinoma lymph node invasion and can effectively affect the malignant biological phenotype of cholangiocarcinoma by promoting cell proliferation, apoptosis, migration and invasion, thus providing a theoretical basis for analyzing the mechanism of LINC01503.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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