European Review for Medical and Pharmacological Sciences 2020; 24: 230-237

Effects of lncRNA SNHG20 on proliferation and apoptosis of non-small cell lung cancer cells through Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To investigate the influence of long non-coding ribonucleic acid (lncRNA) small nucleolar host gene 20 (SNHG20) on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells through the Wnt/β-catenin signaling pathway.

PATIENTS AND METHODS: The human NSCLC cells were cultured and lncRNA SNHG20 was inhibited using si-SNHG20 and overexpressed using SNHG20-OE. Then, flow cytometry was used to detect the apoptotic rate. The targets of lncRNA SNHG20 were detected via dual-luciferase reporter gene assay, and the changes in the protein level were detected via Western blotting.

RESULTS: LncRNA SNHG20 was highly expressed in the cancer tissues and serum of patients with NSCLC. LncRNA SNHG20 could promote the proliferation and inhibit the apoptosis of NSCLC cells. LncRNA SNHG20 could bind to micro RNA (miR)-197 in a targeted manner. Besides, nuclear translocation of β-catenin was significantly enhanced after transfection of miR-197. After the down-regulation of miR-197 by small interfering RNA (siRNA), the key molecules TCF and LEF1 of the Wnt/β-catenin pathway were significantly down-regulated.

CONCLUSIONS: LncRNA SNHG20 promotes the proliferation and inhibits the apoptosis of NSCLC cells by targeting miR-197 through the Wnt/β-catenin signaling pathway.

Key Words:
LncRNA SNHG20, Wnt/β-catenin signaling pathway, Non-small cell lung cancer, Proliferation, Apoptosis.

Introduction

Lung cancer is the most common reason for global cancer-related death and non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancers. Patients have been mostly at an advanced stage when diagnosed[1]. In recent years, considerable development has been achieved in the treatment of NSCLC, but its five-year overall survival rate still remains at 11-15%[2]. The main reason for its high mortality is the continuous proliferation and metastatic potential of cancer cells[3,4]. The occurrence of lung cancer is a complicated biological process due to expression disorders of many tumor-related genes. Therefore, it is essential to improve early diagnosis, prevention, and timely treatment to determine the molecular mechanisms of the development and progression of NSCLC.

Previous research on the mechanism of tumorigenesis mainly focused on protein-coding genes. Recently, the transcriptome analysis has revealed that 2% of the non-coding RNAs (ncRNAs), the main part of the human genome, encodes proteins. NcRNAs are classified into small ncRNA (shorter than 200 nucleotides) and long ncRNA (longer than 200 nucleotides), but not translated into proteins[5,6]. Increasing evidence suggests that lncRNAs participate in many biological processes including cell proliferation, growth, cycle progression, and apoptosis[7]. Therefore, lncRNAs are abnormally expressed in various human diseases,

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especially malignant tumors[8,9]. Therefore, it has important significance for understanding the molecular biology of development and progression of tumors to identify tumor-related lncRNAs and study its molecular mechanisms and biological functions.

The Wnt/β-catenin signaling pathway plays an important role in regulating multiple malignant tumors, including lung cancer[10]. When the Wnt ligand binds to the seven-span transmembrane frizzled protein (FZD) receptor and its co-receptor, the low-density lipoprotein receptor-related protein 5/6((LPR5/6)/ROR2/RYK in the Wnt/beta-catenin pathway will be activated to inhibit the compound (compound of Axin-APC-GSK3) from gathering on the plasma membrane, leading to the stabilization of β-catenin and its accumulation in the cytoplasm. Dissociated β-catenin accumulates and transfers to the nucleus and binds to T-cell factor/lymphoid enhancer factor (LEF) in the nucleus to regulate the expression of target genes such as c-myc, cyclin D1, and E-cadherin.

In this research, it was observed that the expression of lncRNA SNHG20 was significantly up-regulated in NSCLC. However, the clinical importance of lncRNA SNHG20 and the molecular mechanism controlling its function are still unidentified. Therefore, the present study aims to compare the expression of lncRNA SNHG20 between NSCLC tissues and adjacent non-tumor tissues, and study its function in malignant progression of NSCLC.

Patients and Methods

Clinical Specimens

The tissue and serum specimens were collected from 116 NSCLC patients admitted in the Oncology Department of our hospital from January 2017 to December 2018. All patients were informed of the study and the project was approved by the Ethics Review Committee of our hospital.

Cell Culture

Three kinds of NSCLC cell lines (A549, NCI-H520, and H1299) and normal human bronchial epithelial cell line (16HBE) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in the Roswell Park Memorial Institute-1640 (RPMI-1640; Hyclone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C and the medium was replaced every other day. Cells were passaged after reaching the confluence of 80-90%.

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

The total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 and 280 nm. Then, the total RNA was synthesized into complementary deoxyribonucleic acid (cDNA) using the PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan), followed by PCR using the specific primers (Table I).

Cell Proliferation Assay

The cell proliferation was detected using the cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The cells were inoculated into a 96-well plate at an initial density of 2×10^4/mL. At 12, 24, 48 or 72 h, the CCK-8 solution was added (10 μL/well) to measure the cell viability. After incubation under 5% CO_2 at 37°C for 2 h, the absorbance of each well was measured at 450 nm.

Apoptosis Assay Via Flow Cytometry

After different treatments, the cells were cultured. The relative number of Annexin V positive and/or PI positive cells was determined *via* flow cytometry. The FlowJo software was used

Table I. Primer sequences.

Gene	Forward primer	Reverse primer
LncRNA SNHG20	5'-CCACTCCTTTAGTCGTTGTGC-3'	5'-GGTCCCTCATTCCTATCCC-3'
MiR-197	5'-CTGGAGAATAACAGAGGGATGC-3'	5'-CCTGGCTCCTCACTTGGC-3'
β-actin	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

for data analysis. The apoptosis was quantified using PE Annexin V apoptosis assay kit II (BD Biosciences, San Jose, CA, USA), and the redistribution of phosphatidylserine on the plasma membrane was measured. The cells were washed twice with cold phosphate-buffer saline (PBS) and resuspended in 1000 mL of binding buffer (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of 1×10^6/mL. Then, 100 µL of the mixture (containing 1×10^5 cells) was transferred into a 5 mL culture tube, added with 5 µL of Annexin V-PE and 5 µL of 7-AAD, and gently vibrated. After incubation in a dark place at room temperature of 25°C for 15 min, 400 µL of binding buffer was added into each tube. The cells were analyzed using the flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ, USA) with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA) to distinguish the living cells, dead cells, early apoptotic cells, and late apoptotic cells. The percentage of early apoptotic cells to late apoptotic cells was compared with that in control group in each experiment. The assay was repeated 3 times.

Western Blotting (WB)

The protein was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) buffer, separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membrane. Then, the membrane was sealed with 5% skim milk at 37°C for 2 h, incubated with the primary antibody at 4°C overnight and incubated again with the horseradish peroxidase-conjugated (HRP) secondary antibody at 37°C for 1 h. Finally, the bands were observed using the enhanced chemiluminescence (ECL) assay kit according to the manufacturer's instructions.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) were used for statistical analysis. All results were expressed as mean ± standard deviation, and the data were analyzed using the two-tailed *t*-test. Comparison between multiple groups was done using the One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested the statistically significant difference.

Results

LncRNA SNHG20 was Highly Expressed in Cancer Tissues and Serum of NSCLC Patients

The expression of lncRNA SNHG20 in the tissues of 116 NSCLC patients was detected *via* qRT-PCR. The results revealed that the expression of lncRNA SNHG20 in cancer tissues was significantly higher than in para-carcinoma tissues ($p<0.001$) (Figure 1A). Besides, the expression of lncRNA SNHG20 in the serum of patients was also detected. Results showed that the expression of lncRNA SNHG20 in NSCLC patients

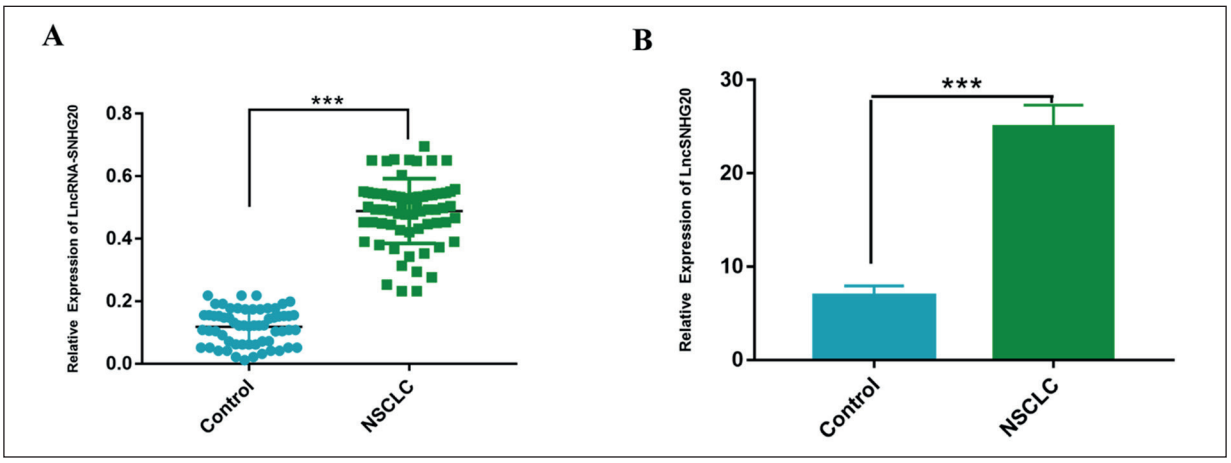

Figure 1. A, Expression of lncRNA SNHG20 in cancer tissues and para-carcinoma tissues of 116 NSCLC patients detected *via* qRT-PCR, **B,** Expression of lncRNA SNHG20 in serum of NSCLC patients detected *via* qRT-PCR.

was increased compared to that in healthy controls (p<0.001) (Figure 1B). The above results indicate that lncRNA SNHG20 may be a potential risk factor for NSCLC.

LncRNA SNHG20 Promoted Proliferation of NSCLC Cells

The NSCLC cells were transfected with Si-SNHG20 and SNHG20-OE. Then, the cell proliferation was detected. It was found that the proliferation ability of cells transfected with Si-SNHG20 was significantly weakened, while that of cells transfected with SNHG20-OE was significantly enhanced (p<0.01), indicating that lncRNA SNHG20 can facilitate cell proliferation (Figure 2).

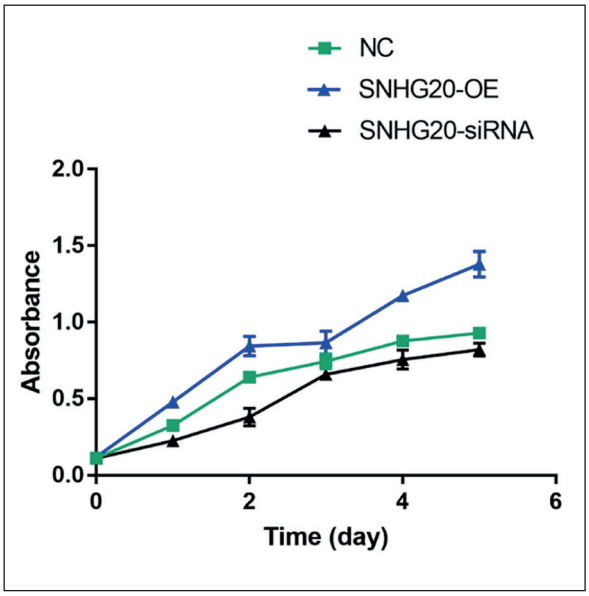

Figure 2. Cell proliferation assay: proliferation ability of cells transfected with Si-SNHG20 is significantly weakened, while that of cells transfected with SNHG20-OE is significantly enhanced (p<0.01).

LncRNA SNHG20 Inhibited Apoptosis

After HUVEC cells were transfected with lncRNA SNHG20, the apoptosis rate was detected via flow cytometry. It was found that the apoptosis rate was (1.21±0.22)% and (21.3±1.34)%, respectively, in lncRNA SNHG20 group and control group, showing a statistically significant difference (p<0.05) (Figure 3A).

Once A549 cells were transfected with SNHG20-OE, the expression of active caspase-3 in each group was detected. The results showed that SNHG20-OE could down-regulate the protein expression of active caspase-3 compared with control group (p<0.01) (Figure 3B).

LncRNA SNHG20 Bound to MiR-197 in a Targeted Manner (Luciferase Reporter Gene Assay)

The targets of lncRNA SNHG20 were predicted using the bioinformatics method. Results showed that lncRNA SNHG20 contained the complementary sites of the 3'-untranslated region (UTR) of miR-197 (Figure 4A) and the binding sites of the mutant-type lncRNA SNHG20. The results of luciferase reporter gene assay showed that the fluorescence intensity markedly declined only after the interaction between wild-type lncRNA SNHG20 and miR-197 (p<0.05) (Figure 4B), demonstrating that lncRNA SNHG20 binds to miR-197 in a targeted manner.

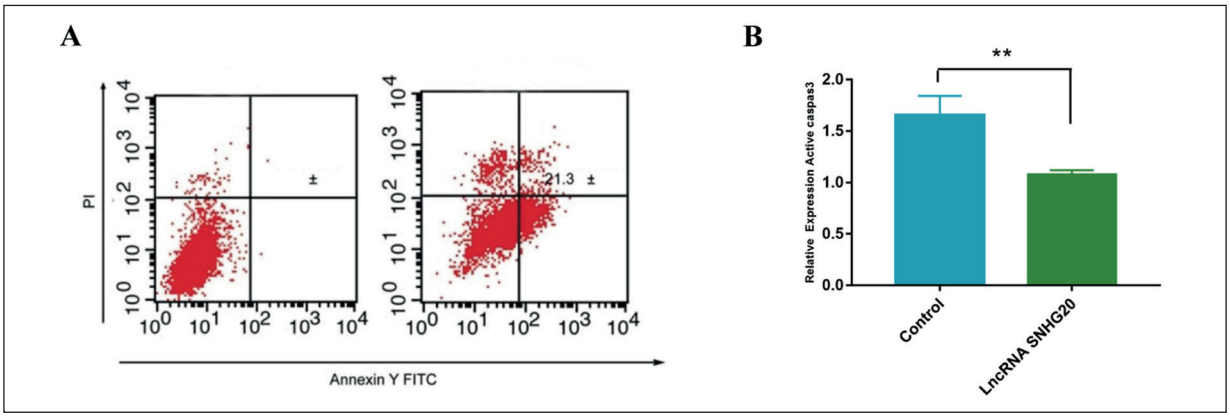

Figure 3. A, Apoptosis rate detected via flow cytometry: the apoptosis rate is (1.21 ± 0.22)% and (21.3 ± 1.34)%, respectively, in lncRNA SNHG20 group and control group, **B,** Expression of active caspase-3 detected *via* WB: SNHG20-OE can down-regulate the protein expression of active caspase-3 (p<0.01).

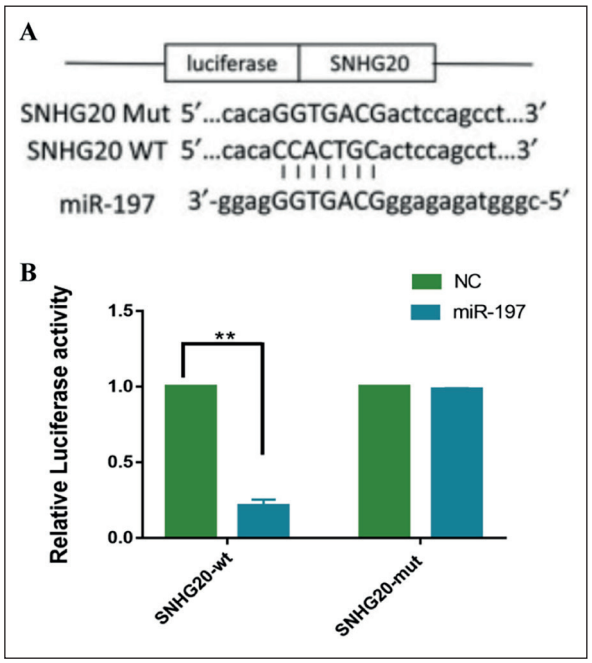

Figure 4. A, LncRNA SNHG20 contains the complementary sites of the 3'-UTR of miR-197, **B,** Fluorescence intensity significantly declines after the interaction between wild-type lncRNA SNHG20 and miR-197.

MiR-197 Regulated Wnt/β-Catenin Pathway in a Targeted Manner

To further clarify the mechanism of lncRNA SNHG20 in regulating proliferation and apoptosis of NSCLC cells through targeting miR-197, miR-197 was overexpressed and the pathway was screened. The bioinformatics analysis revealed that the Wnt/β-catenin pathway was activated after miR-197 was overexpressed (Figure 5A). According to further analysis, the expression levels of Wnt/β-catenin pathway-related molecules were increased (Figure 5B).

Furthermore, miR-197 targeting Wnt/β-catenin pathway was verified and the results of immunofluorescence and Western blot analysis showed that the nuclear translocation of β-catenin was remarkably enhanced after transfection of miR-197 (Figure 6A) ($p<0.05$). After the down-regulation of miR-197 using siRNA, the key molecules of Wnt/β-catenin pathway TCF and LEF1 were remarkably down-regulated (Figure 6B) ($p<0.05$).

Discussion

Malignant tumor cells are characterized by the infinite proliferation, invasion, and apoptosis, so the development of effective therapeutic targets for human tumors is a great challenge. Some studies[11-13] have demonstrated that a large number of lncRNAs are involved in the development of malignant tumors. However, the bioactive mechanism, biological functions, and signaling pathways of most lncRNAs remain unknown. In the present study, it was found that the expression of lncRNA SNHG20 was significantly up-regulated in NSCLC tissues and serum of patients compared to that in para-carcinoma

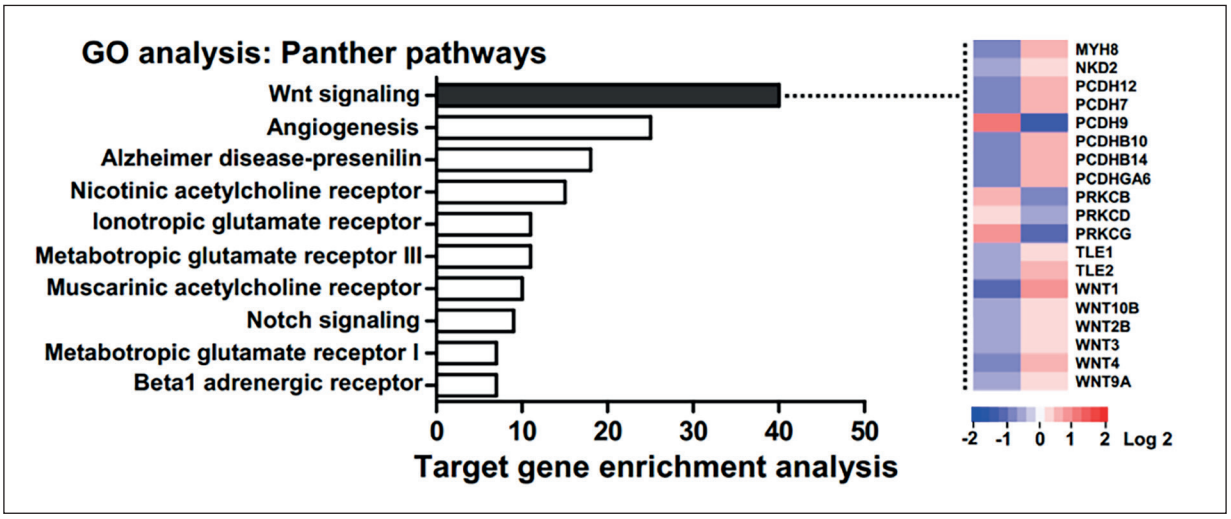

Figure 5. Bioinformatics analysis: after miR-197 is overexpressed, the Wnt/β-catenin pathway is activated and the expression levels of Wnt/β-catenin pathway-related molecules are increased.

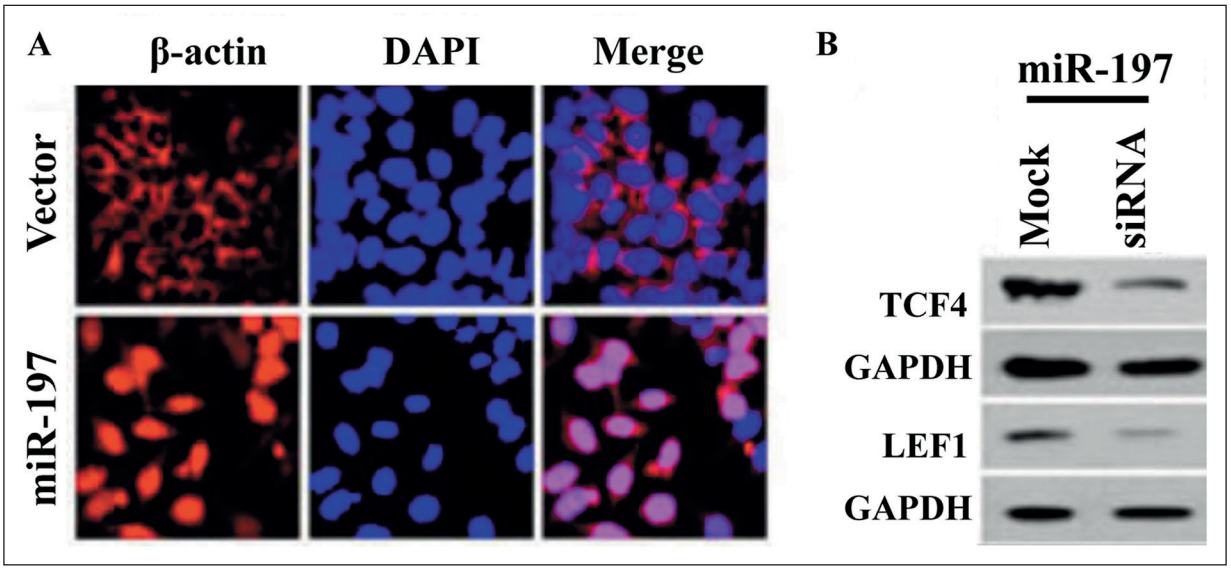

Figure 6. A, Immunofluorescence: nuclear translocation of β-catenin is remarkably enhanced after transfection of miR-197 (magnification: 40×), **B,** Western blot analysis: after the down-regulation of miR-197 using siRNA, the key molecules of Wnt/β-catenin pathway TCF and LEF1 are remarkably down-regulated.

normal tissues. Researches[14,15] showed that the high-expression SNHG20 in NSCLC patients is positively correlated with the poor prognosis. In addition, the up-regulation of SNHG20 can predict the poor prognosis of colorectal cancer and hepatocellular carcinoma. The functional assays in this study showed that the down-regulation of SNHG20 could significantly inhibit proliferation *in vitro* and promote apoptosis. On the contrary, the ectopic expression of SNHG20 could induce malignant tumor cell behaviors. To sum up, SNHG20 may serve as an oncogene and play an important role in the development and progression of NSCLC.

Generally, lncRNAs affect cancer cell behaviors by regulating the expression of target genes. In the present work, the expression of important regulators of cell cycle and growth was explored after knockout of SNHG20 in NSCLC cells, and miR-197 was determined as a new target for SNHG20 in NSCLC cells. LncRNAs can regulate the gene expression at various levels, including chromatin modification, transcriptional, and post-transcriptional processing[16]. To verify the regulatory mechanism of SNHG20, bioinformatics prediction was performed and verified via the dual luciferase reporter gene assay, and it was found that miR-197 could be regulated through SNHG20.

MiRNAs play important roles in tumorigenesis and epithelial-mesenchymal transition (EMT) and they can regulate a variety of gene targets to control the metastasis and progression of different cancers[17]. In the present research, the miRNA microarray database was analyzed and it was found that miR-197 may exert its function in NSCLC through Wnt/β-catenin. Moreover, the effect of miR-197 on EMT was observed in previous studies, but its regulatory mechanism is different[18,19].

The abnormal activation of the Wnt/β-catenin signaling pathway has been confirmed in several cancers. Some studies have found that many lncRNAs target the key molecules of the Wnt/β-catenin pathway to regulate the occurrence and development of tumors. LncRNA CCAL promotes the progression of colorectal cancer by targeting the activator protein 2α, thereby activating the Wnt/β-catenin signaling pathway[20]. Furthermore, HOTAIR inhibits the expression of WIF-1, thereby activating the Wnt/β-catenin signaling pathway[21]. Once the Wnt/β-catenin signal is activated, β-catenin escapes from degradation by the Axin complex and the accumulated β-catenin translocates to the nucleus. Besides, β-catenin can also activate the expression of Snail and Slug, inhibit the expression of E-cadherin, and induce the EMT and tumor invasion and metastasis[22]. Some studies have revealed the association between lncRNA and EMT. Likewise, MALAT-1 induces migration of bladder cancer cells through acti-

vating the Wnt signal, thereby inducing EMT. This association contributes to the activation of Wnt/β-catenin and to the subsequent down-regulation of E-cadherin.

Conclusions

The results of this investigation identified that SNHG20 is up-regulated in NSCLC tissues, which is associated with the poor prognosis of NSCLC patients. SNHG20 may promote the proliferation and migration of NSCLC cells by silencing the expression of miR-197. The better understanding of the mechanism of SNHG20 in the molecular etiology of lung cancer will contribute to the development of lncRNA-based cancer diagnostic and therapeutic drugs. This study provides a new perspective for the role of SNHG20 as a non-coding oncogene in NSCLC, so SNHG20 is a new early diagnostic marker and a target in the treatment of NSCLC. However, other possible mechanisms of SNHG20 in NSCLC cell functions remain to be fully understood.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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