European Review for Medical and Pharmacological Sciences 2017; 21: 5135-5142

Up-regulation of LINC00346 inhibits proliferation of non-small cell lung cancer cells through mediating JAK-STAT3 signaling pathway

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Abstract. – OBJECTIVE: To investigate the expression levels of LINCRNA00346 in tissues and cells in patients with non-small cell lung cancer (NSCLC) and its biological function.

PATIENTS AND METHODS: The relative expression levels of LINC 00346 in 70 cases of tissues and cells in NSCLC patients were detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). The specific interference sequences of LINC 00346 were designed and the transfection efficiency after 48 h was detected. The effect of LINC 00346 on the proliferation capacity of NSCLC cells was studied via cell counting kit-8 (CCK-8) assay. After the interference in LINC 00346 expression in NSCLC cells, the changes in cell cycle and apoptosis were detected via flow cytometry. The nude-mouse transplanted tumor model was established to study the effect of LINC 00346 on in vivo tumorigenic ability of tumor cells. After the interference in LINC 00346 expression, the changes in expressions of molecular markers in the downstream signaling pathway were detected via Western blotting.

RESULTS: The LINC 00346 expressions were relatively high in 50 cases of tissues and 5 kinds of tumor cells in NSCLC patients. After the interference in LINC 00346 expression, the apoptosis of tumor cells was promoted, the cell proliferation was inhibited, and the cell cycle was arrested in G1-G0 phase. The animal experiment revealed that the interference in LINC 00346 expression could inhibit the *in vivo* tumorigenic ability of tumor cells. Western blotting showed that LINC 00346 could exert its function through partially regulating the Janus kinase/signal transducer and activator of transcription 3 (JAK-STAT3) signaling pathway.

CONCLUSIONS: The expressions of LINC 00346 were relatively high in NSCLC tissues and cells. LINC 00346 promotes the proliferation and inhibits the apoptosis of NSCLC cells through regulating the JAK-STAT3 signaling pathway.

Key Words:
NSCLC, LINC 00346, Proliferation, JAK-STAT3 signaling pathway.

Introduction

Lung cancer is a kind of malignant tumor originated from the bronchial mucosa or gland, as well as one of the malignant tumors with the fastest increase in morbidity and mortality rates, and the greatest threat to the health and life of people. According to statistics, the numbers of incident and death cases of lung cancer rank first in the tumor around the world[1]. Lung cancer includes non-small cell lung cancer (NSCLC) and small cell lung cancer, in which NSCLC accounts for about 85%[2]. The early symptoms of NSCLC are not typical, or there is even no discomfort, so the study on NSCLC pathogenesis and early diagnosis is of great importance in its treatment and prognosis.

Long noncoding RNA (lncRNA) is a kind of RNA molecule with the transcript of more than 200 nt in length, and it does not have the protein-encoding capacity, but regulates the gene expression level at epigenetic, transcription and post-transcription levels[3,4]. Studies have confirmed that lncRNA is widely involved in cell proliferation, differentiation and apoptosis, and plays an important role in pathological processes of a variety of diseases[5]; in particular, it is closely re-

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lated to the incidence, metastasis, prognosis and treatment of tumors[6,7].

LincRNA 00346 (LINC 00346) belongs to the intergenic lncRNA, located on the chromosome 13q34 with a total length of 6322bp. Zhang et al[8], found its up-regulated expression of LINC 00346 in hepatocellular carcinoma (HCC) for the first time, and the high-expression LINC 00346 was negatively correlated with the survival of HCC patients. After that, scholars reported that the LINC 00346 expression is upregulated in breast cancer and can be used as a biomarker for prognosis estimation[9]. Ye et al[10] found that LINC 00346 plays a similar role to oncogene in bladder cancer to promote the formation of malignant phenotype of tumors. However, the expression and biological function of LINC 00346 in NSCLC tissues and cells have not been reported yet.

In this study, the relatively high expression of LINC 00346 in NSCLC tissues and cells was confirmed for the first time *via in vitro* and *in vivo* experiments. The interference in LINCRNA 00346 expression could inhibit the proliferation and promote the apoptosis of tumor cells, and the biological effect exerted by LINC 00346 through partially regulating the Janus kinase/signal transducer and activator of transcription 3 (JAK-STAT3) signaling pathway was preliminarily investigated.

Patients and Methods

Tissue Specimens and Cells

A total of 70 cases of tumor tissues and para-carcinoma tissues (>5 cm away from the border of tumor tissues) were collected from surgical patients in Thoracic Surgery in Dongying People's Hospital of Shandong Province from January 2014 to December 2016. Patients were pathologically diagnosed with NSCLC without receiving chemotherapy, radiotherapy and targeted therapy before operation; the specimens were removed and stored in liquid nitrogen. This study was approved by Ethics Committee of Dongying People's Hospital of Shandong Province and patients agreed and signed the informed consent.

Five kinds of NSCLC cell lines (A549, SPC-A-1, PC-9, H157 and SK-MES-1) and one strain of human bronchial epithelial cell 16HBE were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 1640 or Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS). 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) were added to the medium to prevent the growth of bacteria. Cells were cultured in an incubator containing 50 ml/L CO_2 under the constant temperature at 37°C. The cell culture medium was replaced every other day, and cells were digested with 2 g/L trypsin when about 80-90% cells were fused, followed by passage.

siRNA and Primer Synthesis

LINC00346 interference sequences were designed by Invitrogen (Carlsbad, CA, USA): shRNA-1:5'-CCGGAAGCACAGTGGTCTAAAAGTACTCGAGTACTTTTAGACCACTGTGCTTTTTTTG-3', shRNA-2: 5'-CCGGCTGTAGAAGGTTGAAGGGAA. Primer 5.0 software was used to design primers: GAPDH F: 5'-ACTAGGCGCTCACTGTTCTCT-3', R: 5'-GTTGACTCCGACCTTCACCT-3'. LINC 00346 F: 5'-AGCTTGAATGGCGTTGGAACCTATAG-3, LINC 00346R: 5'-ATAGTCCCTTCCTCGAATCCTAGT-3' (Realgene, Nanjing, China).

Detection of LINC 00346 Expression Via Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from tissues and cells according to the instructions of TRIzol® LS reagent (Invitrogen, Carlsbad, CA, USA), and the RNA extracted was dissolved in diethylpyrocarbonate (DEPC) water. Complementary DNA (cDNA) was synthesized using the reverse transcription kit (Thermo Scientific, Waltham, MA, USA), followed by PCR amplification using the SYBR® Premix Ex TaqTM II (TaKaRa, Dalian, China). The 20 μL reaction system consisted of 10 μL SYBR® Premix Ex TaqTM II (2×), 3.0 μL template, 1.0 μL forward and 1.0 μL reverse primer (10 μM), 5.0 μL ddH_2O. PCR conditions were: pre-denaturation at 95°C for 30 s, 95°C for 10 s, 60°C for 30 s, a total of 35 cycles. After amplification, Ct value was obtained and the expression level of LINC 00346 was calculated using $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit (CCK)-8 Assay

Short hairpin lncRNA (sh-LINC-00346) and control sequences were transfected into NSCLC cells. Cell suspensions were collected and evenly inoculated into 96-well plates with 3×10^3 cells per well. Six repeated wells were set up for the expe-

rimental group and control group. The cells were incubated in an incubator at 37°C and observed at 0 h, 24 h, 48 h, 72 h and 96 h. Then, the medium was removed, and the plate was washed twice with phosphate buffered saline (PBS) and added with 110 mL CCK-8 mixture (the ratio of CCK-8 to serum-free medium was 1:10) each well. The optical density (OD) value at the wavelength of 450 nm was measured using the microplate reader. Finally, the growth curve was drawn.

Flow Cytometry

Sh-LINC00346 and control sequences were transfected into NSCLC cells. After 48 h, cells were digested with trypsin without EDTA enzyme, washed with the pre-cooled PBS at 4°C and precipitated once; then, they were fixed with 70% alcohol in a refrigerator at 4°C overnight. After that, cells were washed with phosphate buffered saline (PBS) again and precipitated once; the cell staining solution propidium iodide (PI) was added, followed by full re-suspension and detection of cell cycle *via* flow cytometry. According to the above method, the cell suspension in experimental group and control group was obtained; next, the cell suspension was added with Annexin V-APC for staining in a dark place at room temperature for 10-15 min, and the apoptosis rate was detected using the flow cytometer.

Nude-mouse Transplanted Tumor Model

A549 cells were selected as the model cells and nude male mice aged 4-5 weeks old were used as the model animals. sh-LINC 00346 and control sequences were transfected into A549 cells. After 24 h, the cells in experimental group and control group were collected and counted. The cells were injected into the armpit of nude mice, observed and recorded every three days, including the state and weight of nude mice and the transplanted tumor size. After 18 d, the nude mice were executed, and the transplanted tumor was removed, photographed and weighed. Half of the transplanted tumor was used for qRT-PCR, while the other half was used for hematoxylin eosin (HE) staining and immunohistochemical assay.

Western Blotting

The cell lysis buffer was added into cells in experimental group and control group, and the protein was extracted and quantified using the Bradford method. Equivalent proteins were taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the isolated protein was transferred onto the polyvinylidene fluoride (PVDF) membrane; then, the membrane was sealed using 5% skim milk for 2 h, and rabbit anti-human antibody JAK1, pSTAT5 and STAT3 (1:1000) (Cell Signaling Technology, Danvers, MA, USA) were added for incubation at 4°C overnight. The membrane was washed with Tris-Buffered Saline Tween (TBST) for 3 times (5 min/time). Next, the secondary antibody (1:2000) was added for incubation at 37°C for 2 h, and the membrane was washed with TBST for 3 times (10 min/times). Finally, the luminescence liquid (Thermo Pierce ECL, Rockford, IL, USA) was added, followed by tableting and development, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference.

Statistical Analysis

Statistical Product and Service Solution 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical treatment of all data. Measurement data were presented as mean ± standard deviation ($\bar{x}\pm s$), and the *t*-test was used for intergroup comparison. $p<0.05$ suggested that the difference was statistically significant.

Results

High LINC 00346 Expressions in NSCLC Tissues and Cells

The results of qRT-PCR showed that the LINC 00346 expressions were upregulated in 50 out of 70 tissues of NSCLC patients (Fold change >1) (Figure 1A); the LINC 00346 expressions in 5 strains of NSCLC cells were upregulated compared with that in human bronchial epithelial cell 16HBE (Figure 1B). Two cell lines, A549 and PC-9, with the highest upregulation fold were selected for subsequent experiments. sh-LINC 00346 and control sequences were transfected into A549 and PC-9 using Lip2000. RNA was collected after 48 h, and the transfection efficiency was detected *via* qRT-PCR (Figure 1C-D).

Research on Biological Function of LINC 00346 via In vitro Experiment

sh-LINC 00346 was transfected into NSCLC cells, and the changes in cell proliferation capacity were detected *via* CCK-8 assay. The results showed that the interference in LINC 00346 expression could significantly inhibit the proliferation capacity of NSCLC cells (Figure 2A-B). A549 and PC-9 were treated using the same

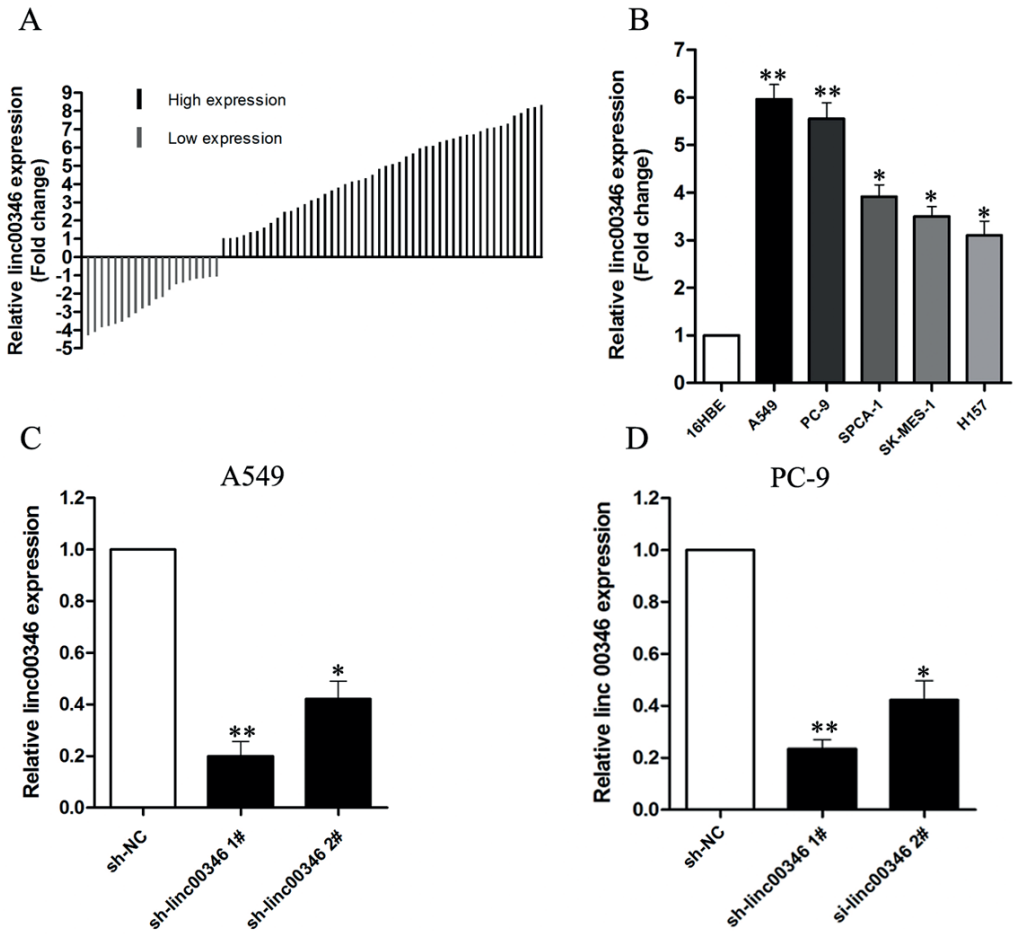

Figure 1. High LINC 00346 expressions in NSCLC tissues and cells. ***(A)*** The expressions of LINC 00346 in 70 cases of tissues in NSCLC patients are detected *via* qRT-PCR. The results show that the expressions of LINC 00346 are upregulated in 50 cases compared with those in para-carcinoma tissues; (B) qRT-PCR is used to detect the relative expression level of LINC 00346 in NSCLC cells. The results show that the LINC 00346 expressions are up-regulated in 5 strains of NSCLC cells compared with those in 16HBE; ***(C-D)*** sh-LINC 00346 and sh-NC are transfected into A549 and PC-9 cells; after 48 h, qRT-PCR is used to detect the transfection efficiency ($^{**}p<0.01$ and $^{*}p<0.05$).

methods and conditions, and the cells were collected after 48 h. The flow cytometry showed that the apoptotic rate of NSCLC cells was increased (Figure 2C-D), and the cell cycle was arrested in G1-G0 phase (Figure 2E-F).

Research on Biological Function of LINC 00346 via In Vivo Experiment

In order to study the effect of LINC 00346 on the *in vivo* tumorigenic ability of NSCLC cells, A549 cells were selected as the model cells and nude male mice aged 4-5 weeks old were used as the model animals. sh-LINC 00346 and control sequences were transfected into A549 cells, cells were collected and injected into the armpit of nude mice, and the transplanted tumor volume was measured every three days (Figure 3A). After 18 d, the nude mice were executed and the transplanted tumor was removed (Figure 3B); the weight of transplanted tumor formed by cells transfected with sh-LINC 00346 sequence was lower than that in control group (Figure 3C). The results of qRT-PCR revealed that the expression of LINC 00346 in transplanted tumor transfected with sh-LINC 00346 was down-regulated compared with that in control group (Figure 3D).

LINC 00346 Regulated the JAK-STAT3 Signaling Pathway

In order to demonstrate the successful establishment of transplanted tumor model, HE

staining was performed for the transplanted tumor (Figure 4A). The results of KI67 staining showed that the proliferation rate of transplanted tumor formed by cells transfected with sh-LINC 00346 was significantly decreased compared with that in control group (Figure 4A). According to literature report, JAK-STAT3 signaling pathway is involved in tumor proliferation and drug resistance[11]. It was found in Western blotting that after interference in LINC 00346 expression in NSCLC cells, the expressions of molecular markers of JAK-STAT3 signaling pathway, JAK1, STAT3 and STAT5, were changed (Figure 4B-C).

Discussion

Noncoding RNA refers to the RNA that does not encode the protein, including rRNA,

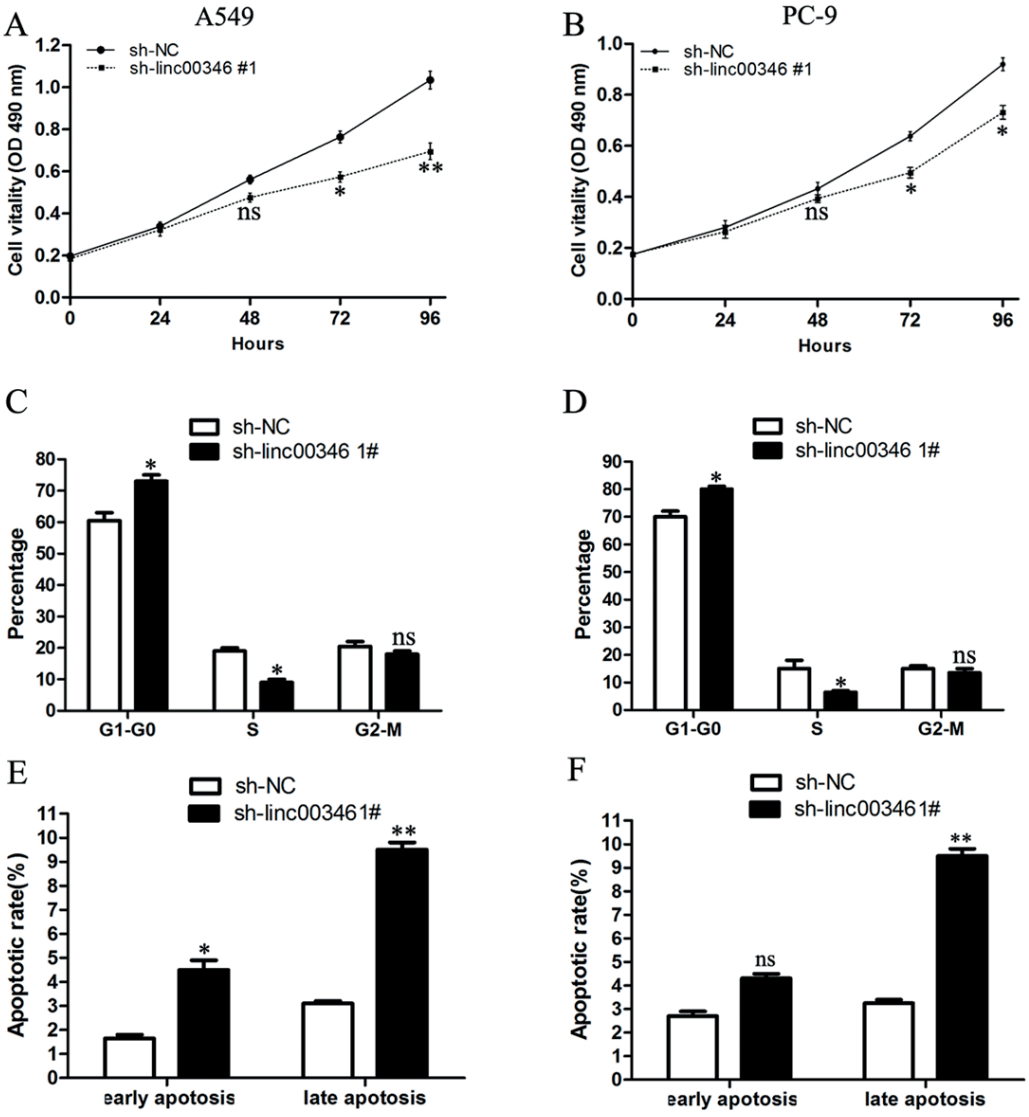

Figure 2. LINC 00346 promotes the proliferation and inhibits the apoptosis of NSCLC cells. (A-B) sh-LINC 00346 and sh-NC are transfected into NSCLC cells. The results of CCK-8 assay show that the interference in LINC 00346 expression can significantly inhibit the proliferation capacity of tumor cells; (C-D) flow cytometry shows that the interference in LINC 00346 expression can arrest the cell cycle in G1-G0 phase; (E-F) flow cytometry shows that the interference in LINC 00346 expression can promote the apoptosis of NSCLC cells (**p<0.01 and *p<0.05).

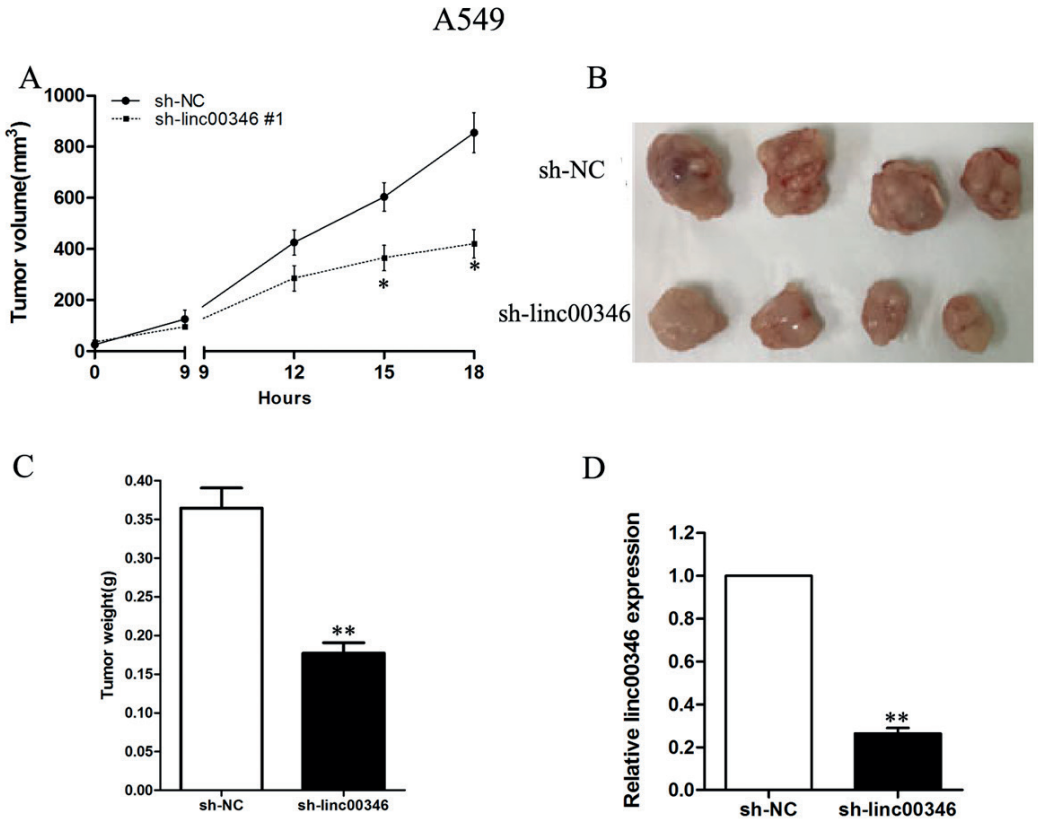

Figure 3. LINC 00346 promotes the *in vivo* tumorigenic ability of NSCLC cells. ***(A)*** The transplanted tumor volume is measured every three days and recorded to obtain the growth curve; ***(B)*** after 18 d, nude mice are executed, and the transplanted tumor is removed and photographed; ***(C)*** the weight of transplanted tumor formed by cells transfected with sh-LINC 00346 is obviously decreased compared with that in control group; ***(D)*** the relative expression level of LINC 00346 in transplanted tumor is detected *via* qRT-PCR. The results show that the expression of LINC 00346 in experimental group is downregulated compared with that in control group ($^{**}p<0.01$ and $^{*}p<0.05$).

tRNA, snRNA, snoRNA, microRNA, lncRNA, and circRNA[12]. Since lncRNA does not encode the protein, it was initially thought to be the "noise" of genomic transcription without the biological function[13]. However, with the continuous development of RNA sequencing, chip and other molecular biology technologies and bioinformatics, more and more evidence has confirmed that lncRNA has complex biological functions[3]. Scholars[14-17] studied and found that PVT1, HOTAIR, ANRIL, MALANT1, MEG3 and GAS5 are expressed abnormally in lung cancer, which is closely related to the occurrence, diagnosis, treatment and prognosis of lung cancer.

Although the LINC 00346 expression is reported to be upregulated in HCC and breast cancer, its biological function is not studied in detail. Besides, LINC 00346 and NSCLC have not been reported yet. In this study, the LINC 00346 expressions in NSCLC tissues and cells were relatively high; *in vivo* and *in vitro* experiments showed that the interference in LINC 00346 could inhibit the proliferation and promote the apoptosis of tumor cells, and inhibit the *in vivo* tumorigenic ability of tumor cells.

JAK-STAT signaling pathway is an important signal transduction pathway, activated by a variety of cytokines, growth factors and receptors[18], which is involved in cell proliferation, differentiation, apoptosis, angiogenesis and immune regulation process. Moreover, it plays an important role in the occurrence and development of tumor and its abnormal activation can lead to the cell clonal proliferation and tumor formation[19]. In recent studies, lncRNA, as an important regulator, is involved in the activation or inhibition of JAK-STAT signaling pathway[20]. In this paper, LINC 00346 could also promote the occurrence and development

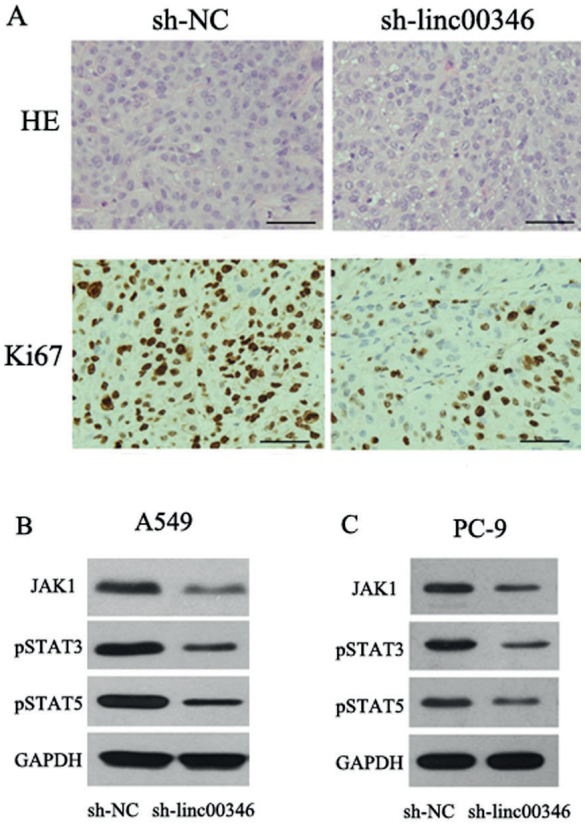

Figure 4. LINC 00346 regulates the JAK-STAT3 signaling pathway. ***(A)*** HE staining for transplanted tumor; The results of KI67 staining show that the proliferation capacity of tumor cells in experimental group is decreased compared with that in control group; ***(B-C)*** detection of changes in molecular marker expressions in JAK-STAT3 signaling pathway *via* Western blotting.

of NSCLC through regulating JAK-STAT pathway.

Conclusions

We showed for the first time that LINC 00346 could promote the occurrence and development of NSCLC through partially regulating JAK-STAT pathway. Our research data provide an important theoretical basis for the clinical reversal of NSCLC malignant phenotype, and the development of new drugs for LINC 00346 can provide important targets for the clinical treatment of NSCLC.

Conflict of interest

The authors declare no conflicts of interest.

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