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SP1-regulated LINC01638 promotes proliferation and inhibits apoptosis in non-small cell lung cancer

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Abstract. – OBJECTIVE: To detect the expressions of long intergenic non-protein coding ribonucleic acid 1638 (LINC01638) in non-small cell lung cancer (NSCLC) tissues and cells, and to explore the biological function of LINC01638 and the underlying mechanism of its high expression.

PATIENTS AND METHODS: The relative expression levels of LINC01638 in NSCLC tissues and cells were determined via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The interference sequences of LINC01638 were designed, and the interference efficiency was measured using qRT-PCR. The influences of the interference in LINC01638 expression on the proliferation ability, the cycle distribution and apoptosis of NSCLC cells were detected via cell counting kit (CCK)-8 assay and flow cytometry. The changes in the expressions of the molecular markers in the downstream Phosphatase and Tensin Homolog deleted on chromosome ten (PTEN)/protein kinase B (AKT) signaling pathway of LINC01638 were evaluated via Western blotting. Moreover, the upstream transcription factors of LINC01638 were predicted based on bioinformatics, and the expression of LINC01638 was detected via qRT-PCR after interfering in the expression of specificity protein 1 (SP1).

RESULTS: According to the qRT-PCR results, the expression of LINC01638 was up-regulated in the NSCLC tissues and cells. After interference in LINC01638 expression, the cell proliferation ability was weakened according to the CCK-8 assay results. The flow cytometry results revealed that the cell cycle was arrested in G0/G1 phase, while the apoptosis rate raised. It was found in the Western blotting that the expressions of the molecular markers in the PTEN/AKT signaling pathway were altered. Additionally, the bioinformatics prediction results revealed that the transcription factor SP1 stimulated LINC01638 expression and that it was lowered after interfering in the expression of SP1.

CONCLUSIONS: The expression of LINC01638 is upregulated in NSCLC tissues and cells, and the highly expressed LINC01638 is modulated by the transcription factor SP1 and promotes the proliferation but represses the apoptosis of NSCLC cells via the PTEN/AKT signaling pathway.

Key Words:
Non-small cell lung cancer, LINC01638, SP1, Proliferation, Apoptosis.

Introduction

Lung cancer is one of the leading causes of cancer-related deaths, and its incidence rate is rising[1]. In addition to small cell lung cancer, non-small cell lung cancer (NSCLC) originates from the epithelium among all lung cancers, and it represents about 85% of the total[2]. The treatment of lung cancer and the long-term efficacy depend on the type and stage of tumors and the overall health of patients. The common treatments include surgery, chemotherapy, and radiotherapy, and NSCLC is less sensitive to chemotherapy than the small cell lung cancer[3]. Although many treatment approaches have been widely applied, the long-term survival rate is far from satisfactory in lung cancer patients[4]. As immunotherapy and molecular targeted therapy emerge, it is needed to further raise the understanding of the molecular biological process in the occurrence of lung cancer and to seek the potential marker and novel target treatment.

Long non-coding ribonucleic acids (lncRNAs) are defined as endogenous cellular RNAs with over 200 nt in length, and they can exert many molecular biological functions, such as regulation

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of alternative splicing, chromatin remodeling, and RNA metabolism[5,6]. According to some literature reports, lncRNAs, as crucial regulators, are involved in the development and progression of multiple tumors, including NSCLC. Xue et al[7] reported that lncRNA AFAP1-AS1 binds to LSD1 to inhibit the expression of HBP1, thereby accelerating the formation of the malignant phenotype in NSCLC. Bian et al[8] found that the highly expressed LINC01288 promotes the proliferation of NSCLC cells by stabilizing IL-6 mRNA. However, there are few reports on the expression of long intergenic non-protein coding ribonucleic acid 1683 (LINC01638) and its biological function in NSCLC, and it was showed by the research group that LINC01638 was highly expressed in NSCLC tissues and cells to promote the proliferation but also to inhibit the apoptosis of NSCLC cells.

According to related literature reports, the aberrant expression of lncRNAs is modulated by the upstream transcription factors. The transcription factor KLF5 drives the expression of lncRNA RP1 in breast cancer and further represses the transcription of P27, thereby promoting the proliferation and metastasis of breast cancer cells[9]. It was observed by Ling et al[10] that the transcription factor FOX01 modulates the expression of LINC01197 to inhibit the proliferation of pancreatic cancer cells. In the present study, the bioinformatics prediction results manifested that the promoter region of LINC01638 probably bound to the transcription factor specificity protein 1 (SP1), and the *in vitro* experiment results verified that SP1 participated in regulating the increase in LINC01638 expression to promote the proliferation but repress the apoptosis of NSCLC cells via the Phosphatase and Tensin Homolog deleted on chromosome ten (PTEN)/protein kinase B (AKT) signaling pathway. The experimental findings of the research group provide a theoretical basis and molecular target for the clinical reversal of NSCLC malignant phenotype.

Patients and Methods

Tissue Specimens

A total of 60 NSCLC patients admitted to and treated in the Yankuang New Mile General Hospital were enrolled in the present study, and their lung cancer tissues and the corresponding paracancerous tissues (3 cm away from the cancer tissues) were sampled. After the tissue specimens were surgically removed, they were preserved in liquid nitrogen at -180°C. Meanwhile, the clinicopathological features of the patients were collected. All the patients received no chemotherapy or radiotherapy preoperatively. Moreover, all the samples were collected for the study after having informed subjects. The informed consent was signed by all patients. The Ethics Committee of the hospital approved this investigation.

Cell Culture

The human NSCLC cell strains A549, SPCA-1, PC-9, and H-226 and the human bronchial epithelioid cells 16HBE (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured with the F12K or 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone, South Logan, UT, USA) in the incubator containing 5% CO_2 at 37°C and saturated humidity.

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

NSCLC tissues or cells were added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), from which the total RNA was extracted using the total RNA kit. Then, the purity of the RNAs extracted was measured using an ultraviolet spectrophotometer. After quantification, the total RNAs were reversely transcribed into complementary deoxyribonucleic acids (cDNAs). The cDNAs were taken for qRT-PCR using ABI 7500 fluorescence PCR instrument (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed under the following conditions: pre-denaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 1 min for a total of 40 cycles. The relative expression level was determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are as follows: linc01638, forward, 5′-CCAATCTACTAATGCTAATACCTG-3′, reverse, 5′-CTGCATTCTGACT TTCAGTAAGG-3′; GAPDH, forward, 5′-AGGTCCACCACTGACACGTT-3′, reverse, 5′-GCCTCAAGATCATCAGCAAT-3′; si-linc01638#1: 5’-CGACAGUAGUCGUGTTCAATT-3’; si-linc 01638 #2: 5’-ACUGCAAGACTGCAUGAUATT-3’, si-linc01638#3: 5’-AACCGGUACCGGCU GAUATT-3’; si-SP1: 5’-GATAATATCTGGATTAAUA-3’.

Cell Counting Kit (CCK)-8 Assay

After transfection for 48 h, the cells were taken from both the experiment group and the control

group and seeded into 96-well plates at 100 μL/well (containing about 1×10^4 cells), with 6 parallel wells set in each group. After culture in the incubator for 0, 24, 48, 72, and 96 h, the cells in each well were added with 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) and incubated for other 4 h. Finally, the optical density (OD) of each well was determined at the wavelength of 450 nm, which represented the relative proliferation level of the cells. The experiment was performed in triplicate.

Cell Cycle

After being cultured for 48 h, the cells in the experiment and the control groups were harvested, re-suspended in pre-cooled 75% ethanol, and fixed at -20°C overnight. Then, the content of the cellular DNA was measured via propidium iodide (PI) staining and flow cytometry, and the percentage of the cells in each cell cycle (G0/G1 phase, S phase, and G2/M phase) was calculated using the software.

Cell Apoptosis

After culturing for 48 h, 1×10^5 cells transferred were collected from the experiment group and control group and centrifuged at 1,000 rpm for 5 min. With the supernatant discarded, the resulting cells were re-suspended in 500 μL of binding buffer. Subsequently, they were evenly mixed with 5 μL of annexin V-FITC and then with 5 μL of PI, and the resulting cells were incubated at room temperature in the dark for 5 min. Finally, the cell apoptosis was evaluated using the flow cytometer (BD FACSCalibur, Detroit, MI, USA), and the apoptosis rate was calculated.

Western Blotting

The cells in the experiment group and control group were cultured for 48 h, digested using trypsin, and collected. Then, they were treated with the protein lysate, and the proteins were sampled. The concentration of the protein samples was measured using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA), and the loading volume per well was adjusted according to the protein quantification results. Subsequently, the protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel using the SDS-PAGE apparatus (Bio-Rad, Hercules, CA, USA), and after electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by the wet transfer method, shaken, and blocked in 5% skim milk powder for 2 h. After the membranes were washed with Tris-Buffered Saline with Tween-20 (TBST), the proteins were incubated with the PTEN, AKT, p-AKT, and β-actin primary antibodies in the refrigerator at 4°C overnight. After the membranes were washed using TBST, the proteins were incubated with the secondary antibodies for 1 h, added with the luminescence solution for chemiluminescence development, and photographed using the JS-300 gel image analyzer.

Statistical Analysis

All the experimental data were analyzed using the Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA). The measurement data were in line with the normal distribution and expressed as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for analyzing the measurement data. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ indicated that the difference was statistically significant.

Results

The Expression of LINC01638 Was Upregulated in NSCLC Tissues and Cells

The expression levels of LINC01638 in the cancer tissues and paracancerous tissues of 60 NSCLC patients were determined via qRT-PCR, and it was found that, compared with that in paracancerous tissues, the expression level of LINC01638 was up-regulated in 47 cases (78.3%) (Figure 1A). Then, the relative expression level of LINC01638 in NSCLC was measured via qRT-PCR, and the results revealed that the expression of LINC01638 was raised in NSCLC cells (A549, SPCA-1, PC-9, and H-226) (Figure 1B). A549 and H-226 cells were taken for subsequent experiments, and the interference efficiency of small interfering (si)-LINC01638 was measured via qRT-PCR (Figures 1C and 1D).

Influences of LINC01638 on the Biological Functions of NSCLC Cells

The influence of LINC01638 on the proliferation of NSCLC cells was first evaluated

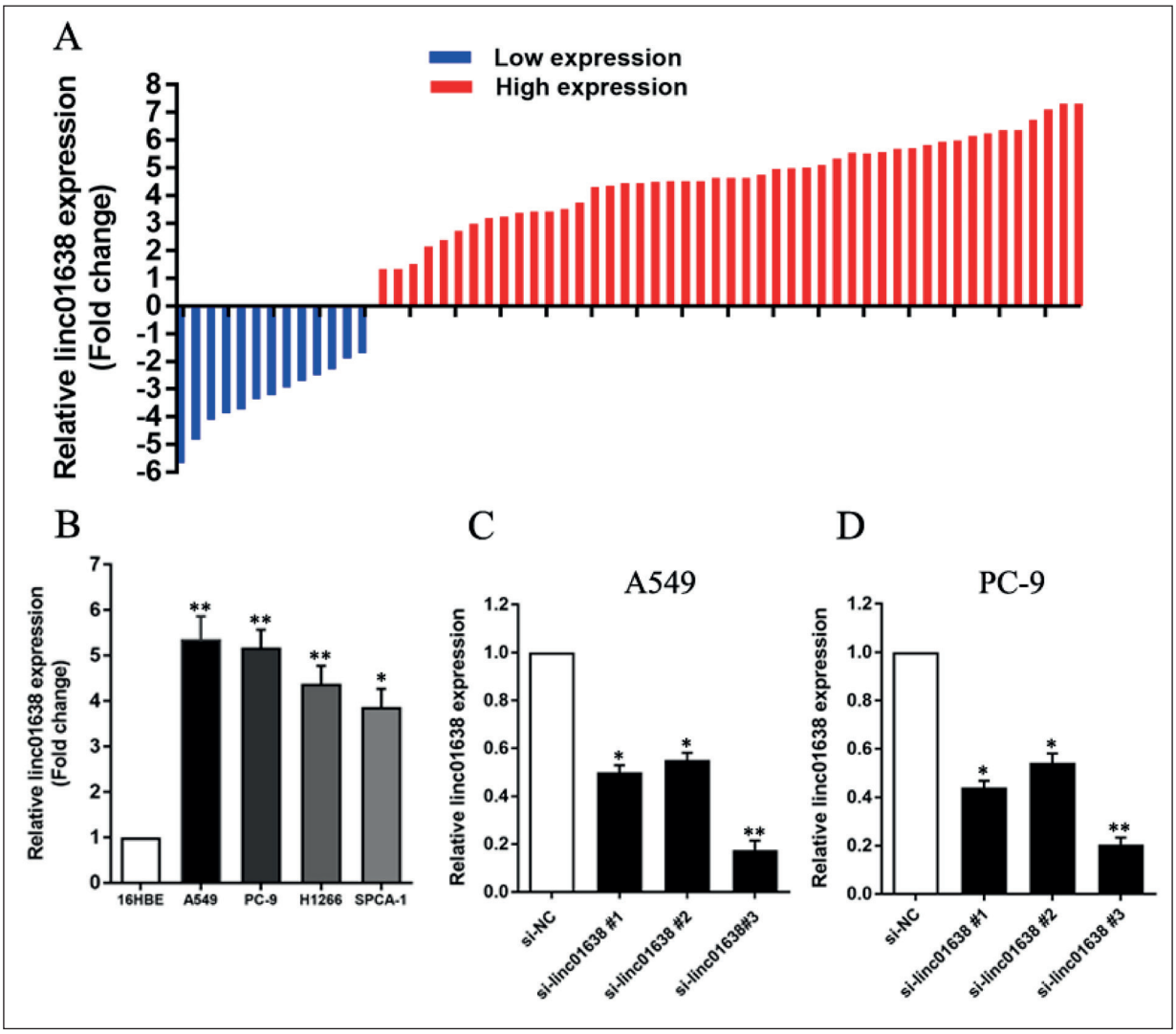

Figure 1. LINC01638 expression is up-regulated in NSCLC tissues and cells. **A,** The relative expression levels of LINC01638 in NSCLC tissues and the paracancerous tissues of 60 NSCLC patients are measured via qRT-PCR, and the results showed that there are 47 cases with up-regulated LINC01638 expression. **B,** With 16HBE cells as the reference, the relative expression levels of LINC01638 in 4 types of NSCLC cells are determined via qRT-PCR, and it is found that LINC01638 expression is up-regulated in NSCLC cells. **C-D,** The interference efficiency of si-LINC01638 determined via qRT-PCR. (**p<0.01, and *p<0.05).

using the CCK-8 assay, and it was found that interfering in the expression of LINC01638 inhibited the proliferation of NSCLC cells (Figures 2A and 2B). Then, the impacts of LINC01638 on the cycle distribution and apoptosis rate of the cells in the experiment group and the control group (si-LINC01638 and si-NC) were observed via flow cytometry, and it was discovered that after interfering in LINC01638 expression, the cycle of NSCLC cells was arrested in G0/G1 phase (Figures 2C and 2D), and their apoptosis rate was elevated (Figures 2E and 2F).

SP1 Promoted the Transcription of LINC01638 to Regulate the PTEN/AKT Signaling Pathway

To explore the downstream regulatory molecules of LINC01638, the proteins were extracted from the cells in both experiment group and control group, and it was found that there were changes in the expression of the downstream signaling pathway (Figures 3A and 3B). The upstream potential transcription regulator of LINC01638 was predicted according to bioinformatics (https://www.genecards.org/), and it was disclosed that interference in SP1 expression re-

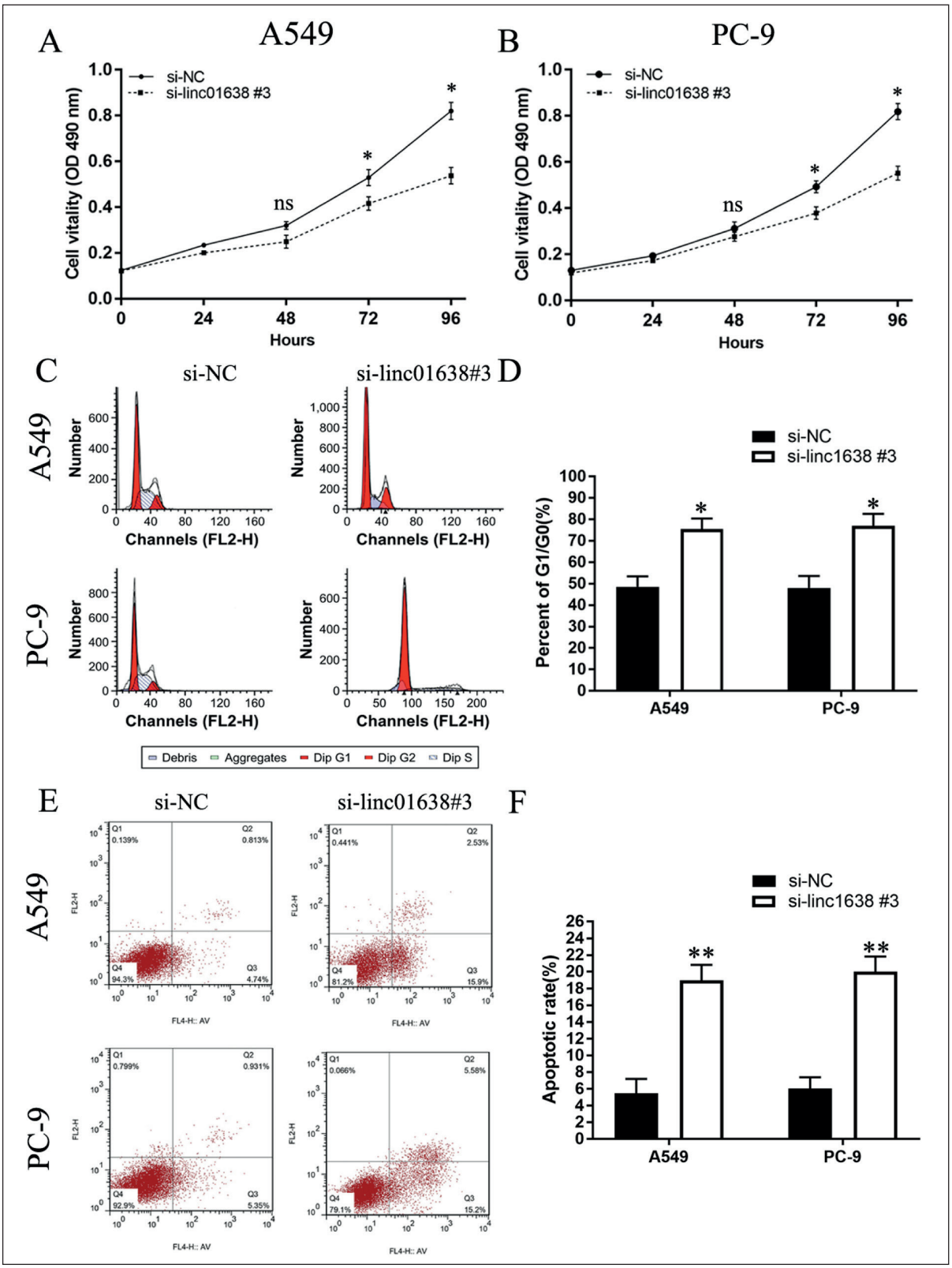

Figure 2. Influences of LINC01638 on the biological functions of NSCLC cells. **A-B,** Impact of LINC01638 on the proliferation activity of NSCLC cells detected via CCK8 assay. **C-D,** Influence of si-LINC01638 on NSCLC cell cycle distribution evaluated via flow cytometry. **E-F,** Impact of si-LINC01638 on the apoptosis rate of NSCLC cells evaluated via flow cytometry. (**p<0.01, and *p<0.05).

pressed the transcription of LINC01638 (Figures 3C and 3D). Furthermore, the Western blotting results manifested that the expressions of the molecular markers in the PTEN/AKT signaling pathway were changed after interference in the SP1 expression (Figures 3E and 3F).

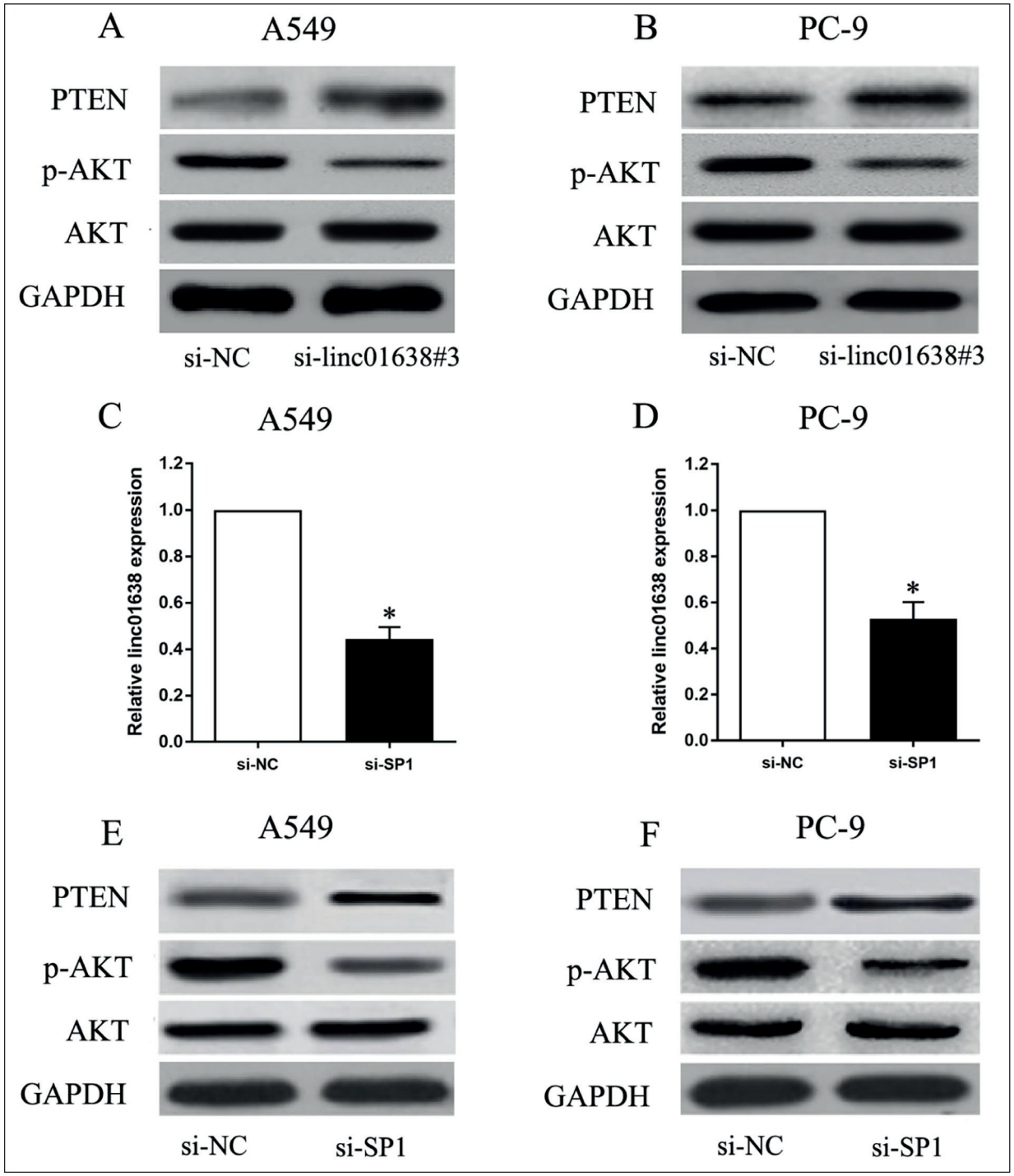

Figure 3. SP1 modulates the PTEN/AKT signaling pathway by stimulating the transcription of LINC01638 **A-B,** According to the Western blotting results, the expressions of the molecular markers in the downstream PTEN/AKT signaling pathway are changed after interfering in the expression of LINC01638. **C-D,** The changes in LINC01638 expression detected via qRT-PCR after interfering in SP1 expression. **E-F,** According to the Western blotting results, after interfering in SP1 expression, the expressions of the molecular markers of the downstream PTEN/AKT signaling pathway were altered. (**p<0.01, and *p<0.05).

Discussion

Most of NSCLC patients have not been definitely diagnosed until middle-advanced stage, with the overall 5-year survival rate <15%[11,12]. Therefore, it is urgent to find novel strategies for the early diagnosis and monitoring of NSCLC. With the development of scientific technologies, the non-coding RNAs have been found by researchers to play an important role in gene regulation. LncRNAs, as a major class of non-coding RNAs, are involved in the growth, invasion, and metastasis of tumors. Moreover, several lncRNAs have been found to affect the development and progression of NSCLC by some complex mechanisms[13,14].

LINC01638 is a lncRNA that can stimulate tumors and acts as a crucial player in the development and progression of normal tumor cells. Currently, it has been found that LINC01638 is abnormally highly expressed in several human malignancies, such as breast cancer[15]. According to literature reports, interfering in the expression of LINC01638 can suppress the proliferation of colorectal cancer cells via action on RUNX2[16]. Highly expressed LINC01638 can regulate the Notch1 signaling pathway to promote the proliferation, migration, and metastasis of prostate cancer cells[17]. It was firstly found by the research group that the expression of LINC01638 was up-regulated in NSCLC and that it promoted the proliferation but inhibited the apoptosis of tumors.

As a sequence-specific DNA-binding protein, the transcription factor SP1 is widely present in the cell nuclei in tissues, and its aberrant expression is closely associated with the development, progression, and prognosis of tumors. SP1 mainly regulates the transcription activity and expressions of angiogenesis factors, oncogenes, tumor suppressor genes, and cell cycle regulatory molecules to promote tumor growth and metastasis, but inhibits the apoptosis of tumor cells[18,19]. Furthermore, SP1 can promote the transcription of lncRNAs to stimulate the development and progression of tumors according to the latest literature reporting that SP1 drives the transcription of LINC01234 in NSCLC and that the highly expressed LINC01234 regulates OTUB1 to promote the formation of NSCLC malignant phenotype[20].

Conclusions

In summary, the transcription factor SP1 stimulated the transcription of LINC01638 and the malignant proliferation of NSCLC cells via the PTEN/AKT signaling pathway. The findings of the research group provide a theoretical basis and molecular target for the clinical reversal of NSCLC malignant phenotype.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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