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C-MYC-induced upregulation of LINC01503 promotes progression of non-small cell lung cancer

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Abstract. – OBJECTIVE: The purpose of this study was to detect the expression of long intergenic non-protein-coding RNA 1503 (LINC01503) in non-small cell lung cancer (NSCLC), and to further study its biological function, as well as the regulatory relationships of c-MYC with LINC01503 and the extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway in NSCLC.

PATIENTS AND METHODS: Tissue specimens were collected from 36 NSCLC patients, and the relative expression level of LINC01503 in the 36 cases of NSCLC tissue specimens and NSCLC cells was then determined using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Then, the effects of LINC01503 on the proliferation and apoptosis of NSCLC cells were detected in vitro via Cell-Counting Kit (CCK)-8 assay, colony-forming assay and flow cytometry. Besides, the possible LINC01503 promoter-binding transcription factor was predicted using bioinformatics. After interference with c-MYC expression, the changes in the expression of LINC01503 were examined through qRT-PCR. Finally, the changes in the expressions of the molecular markers in the ERK/MAPK signaling pathway after interference with LINC01503 and c-MYC expressions were evaluated using Western blotting.

RESULTS: According to qRT-PCR results, the expression of LINC01503 was upregulated in 30 out of 36 cases of NSCLC tissues. Compared with that in human normal bronchial epithelial cells, the expression of LINC01503 was elevated in NSCLC cells. As shown by the CCK-8 assay and colony-forming assay, the proliferation ability of NSCLC cells was weakened after interference with LINC01503 expression, and the flow cytometry results revealed the apoptosis rate of NSCLC cells was raised after interference with LINC01503 expression. Moreover, the bioinformatics prediction showed that c-MYC might be the LINC01503 promoter-binding transcription factor. Additionally, it was found through the qRT-PCR that the expression of LINC01503 declined after interference with c-MYC expression. Finally, based on Western blotting results, the expressions of phosphorylated ERK1/2 (p-ERK1/2) and p-MAPK/ERK kinase (MEK), the molecular markers in the ERK/MAPK signaling pathway, were inhibited after interference with c-MYC and LINC01503 expressions.

CONCLUSIONS: The transcription factor c-MYC promotes the expression of LINC01503 in NSCLC and activates the ERK/MAPK signaling pathway to drive the development and progression of NSCLC.

Key Words:
NSCLC, c-MYC, LINC01503, ERK/MAPK signaling pathway, Biological function.

Introduction

Lung cancer is now one of the most common malignancies worldwide, with the morbidity and fatality rates ranking first among all malignant tumors[1]. Non-small cell lung cancer (NSCLC), the most prevalent type of lung cancer, accounts for about 85% of all lung cancers[2]. Despite great advances in the treatment of NSCLC in recent years, most patients have not been diagnosed until the advanced stage due to the atypical early symptoms, and their 5-year survival rate is still low. The development and progression of NSCLC is a complex process involving multiple genes and its specific molecular pathogenesis has not yet been fully elucidated[3].

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Long non-coding ribonucleic acids (lncRNAs) are mainly distributed in the cytoplasm or nucleus[4], and they have attracted extensive attention since they can participate in chromatin modification, transcription activation or interference and other regulatory processes. LncRNAs are abnormally expressed in many tumor tissues and may be implicated in the development and progression of tumors[5]. There is a literature report that lncRNA ELFN1-AS1 absorbs microRNA (miR)-183-3p to regulate the expression of GFPT1, thereby facilitating the progression of esophageal cancer[6]. Ding et al[7] discovered that lncRNA PCAT1 plays a role as an oncogene in epithelial ovarian cancer and modifies the cyclin D1/CDK4 signaling pathway to promote the formation of malignant phenotype of epithelial ovarian cancer.

Long intergenic non-protein-coding RNA 1503 (LINC01503) is a lncRNA firstly discovered in esophageal squamous cell carcinoma and located on human chromosome 9q34.11[8]. Its expression is upregulated in colorectal cancer, gastric cancer and bile duct cancer tissues. Thus, the highly expressed LINC01503 can contribute to the development and progression of tumors[9-11]. However, the relationships of LINC01503 with the development and progression of NSCLC remain elusive. Therefore, the changes in LINC01503 expression in NSCLC tissues were firstly observed, and the clinical significance in the present study was explored.

Patients and Methods

Tissue Specimens

A total of 36 pairs of NSCLC tissues and the normal paracancerous tissues surgically removed in Ganzhou People's Hospital from January 2016 to December 2018 were collected from the patients. Inclusion criteria: 1) patients who were firstly diagnosed with NSCLC, 2) those receiving no anti-tumor treatment before surgery, and 3) those definitely diagnosed post-operatively by histopathological examination. Exclusion criteria: 1) patients with malignant tumors at other sites, 2) those who received any anti-tumor treatment before surgery, or 3) those with incomplete clinicopathological data or follow-up data. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). The present study was approved by the Medical Ethics Committee of Ganzhou People's Hospital, and all the patients provided the informed consent.

Cell Culture

NSCLC cells and human bronchial epithelial cells (BEAS-2B) were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The NSCLC cells were first inoculated into the Roswell Park Memorial Institute-1640 (RPMI-1640) medium or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA) and cultured in a 5% CO_2 incubator at 37°C. Upon reaching about 80% confluence, the cells were trypsinized and sub-cultured at 1:4. The well-growing third-generation cells in the logarithmic phase were used for subsequent experiments.

Cell Transfection

The third-generation NSCLC cells growing well in the logarithmic phase were taken, seeded into 6-well plates, randomly assigned into small interfering RNA (si)-LINC01503 group and si-negative control (si-NC) group and cultured in the 5% CO_2 incubator at 37°C. Upon reaching about 50% confluence, the cells were transfected with 5 μL of siRNAs and 5 μL of transfection reagent according to the instructions of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). At 12 h after transfection, the cells were further cultured in a fresh 10% FBS-containing complete medium for 36 h. At 48 h after transfection, the two groups of cells were collected, and the transfection efficiency was determined using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The siRNA (si-LINC01503) and its negative sequence (si-NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Extraction of Total RNAs and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

NSCLC tissues and cells were added with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and lysed to extract the total RNAs. Then, RNAs were air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China), and the eligible

RNAs were used for subsequent experiments. With 50 μg of total RNAs as the template, complementary deoxyribonucleic acids (cDNAs) were synthesized using the Super Master Mix synthesis kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Next, qRT-PCR was conducted in the 7300 real-time PCR system (ABI, Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s according to the instructions of SYBR premix Ex Taq (TaKaRa Biotechnology Co., Ltd., Dalian, China). Finally, the relative expression level of LINC01503 was determined using $2^{-\Delta\Delta Ct}$, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primers are as follows: LINC01503 F 5'-TGTACCTACGTGTCAGGGAGG-3', R 5'-GGGGAGGGTGTATTCAGAG AG-3'. GAPDH F 5'-GTCTCCTCTGACTTCAACAGCG-3', R 5'-ACCACCCTGTTGC TGT AGCCAA-3'. c-MYC F 5'-GGCTCCTGGCAAAAGGTCA-3', R 5'-CTGCGTAGTTGTGCTGATGT-3'.

Cell Counting Kit (CCK)-8 Assay

A total of 5×10^3 cells were first inoculated into 96-well plates and treated by the designated experimental method. The original medium was discarded at the end of the cell treatment. Then, each well of cells was added with 100 μL of fresh medium and 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) and incubated in the incubator at 37°C for another 2 h. After incubation, the optical density of each sample in the 96-well plates was determined using a multifunctional microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Colony-Forming Assay

First, each well of 6-well plates was added with 10 mL of medium and 1,000 cells. After the cells were cultured under standard conditions for 10-14 d, the formation of colonies was observed. When macroscopically visible colonies were formed, the culture was terminated and the medium was removed. Subsequently, the resulting cells were washed by phosphate-buffered saline (PBS) for 3 times, added with 4% formaldehyde at 1 mL/well and fixed for 15 min. With the fixation solution discarded, the cells were stained with 1 mL of crystal violet dye for 15 min. After the dye was washed away slowly using running water, the cells were photographed and counted.

Detection of Cell Apoptosis by Flow Cytometry

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was performed as follows: At 48 h after transfection, the two groups of cells were rinsed by PBS twice and digested using ethylenediaminetetraacetic acid (EDTA)-free trypsin (Gibco, Rockville, MD, USA). Then, the density of cells was adjusted using PBS to 5×10^4 cells/mL, and the cells were re-suspended in 100 μL of 1× binding buffer and incubated with 5 μL of Annexin V-FITC (Beyotime Biotechnology, Shanghai, China) at room temperature in the dark for 5 min. Within 1 h, the cells were detected using a flow cytometer (BD, Franklin Lakes, NJ, USA).

Western Blotting

Protein lysis buffer was first added into the cells, and the supernatant was aspirated, added with loading buffer, and denatured at 99°C for 7 min. Then, protein concentration was measured by the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China) according to the instructions of the kit. Subsequently, 10% separation and stacking gels were prepared. The loading volume was calculated based on the protein concentration determined. Afterwards, 20 μg of proteins were subjected to electrophoresis with the controlled voltage and time of 80 V for 30 min and 120 V for 1 h and transferred onto polyvinylidene difluoride membranes (PVDF; Roche, Basel, Switzerland) by the wet transfer method. Subsequently, the resulting proteins were incubated with the primary antibodies against phosphorylated extracellular signal regulated kinase (p-ERK)/p-mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) at 4°C overnight, washed using Tris-Buffered Saline with Tween-20 (TBST), incubated with the secondary antibodies at room temperature for 1 h, and rinsed with TBST, followed by enhanced chemiluminescence (ECL) image development.

Statistical Analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used in this study. Measurement data were expressed as mean ± standard deviation. The results were compared using independent-samples *t*-test, and $p<05$ suggested that the differences were statistically significant.

Results

Up-Regulation of LINC01503 Expression

The tissue specimens were collected from 36 patients definitely diagnosed with NSCLC *via* pathological examination, and the expression level of LINC01503 in the tissue specimens was then determined using qRT-PCR. According to the results, the expression of LINC01503 was raised in 30 cases of NSCLC tissues compared with that in the paracancerous tissues (Figure 1A, 1B). Subsequently, the expression level of LINC01503 in NSCLC cells was measured using qRT-PCR, and it was found that the expression of LINC01503 was upregulated in NSCLC cells (Figure 1C). Besides, the specific interference sequence of LINC01503 was designed and transiently transfected into cells to study the biological function of LINC01503 in NSCLC cells. After 48 h, the interference efficiency was measured using qRT-PCR (Figure 1D).

LINC01503 Promoted the Proliferation and Inhibited the Apoptosis of NSCLC Cells

The effect of LINC01503 on the proliferation ability of NSCLC cells was first detected *via* CCK-8 assay, and the results showed that the proliferation of NSCLC cells with the knockdown of LINC01503 was inhibited (Figure 2A, 2B). Then, it was found through the colony-forming assay that the proliferation ability of cells in si-LINC01503 group was poorer than that in si-NC group (Figure 2C, 2D). Moreover, the effect of LINC01503 on the apoptosis rate of NSCLC cells was detected *via* flow cytometry, and the results revealed that the apoptosis rate of NSCLC cells was elevated after interference with LINC01503 expression (Figure 2E, 2F).

C-MYC Facilitated the Transcription of LINC01503 and Regulated the ERK/MAPK Signaling Pathway

To further explore the mechanism of the upregulation of LINC01503 expression in NSCLC tissues and cells, the possible LINC01503 promoter-binding factor (http://jaspar.genereg.net/) was predicted *via* bioinformatics. It was discovered that the transcription factor c-MYC might bind to the promoter of LINC01503. Subsequently, the binding relationship was verified: the si-c-MYC interference efficiency was first determined *via* qRT-PCR and Western blotting

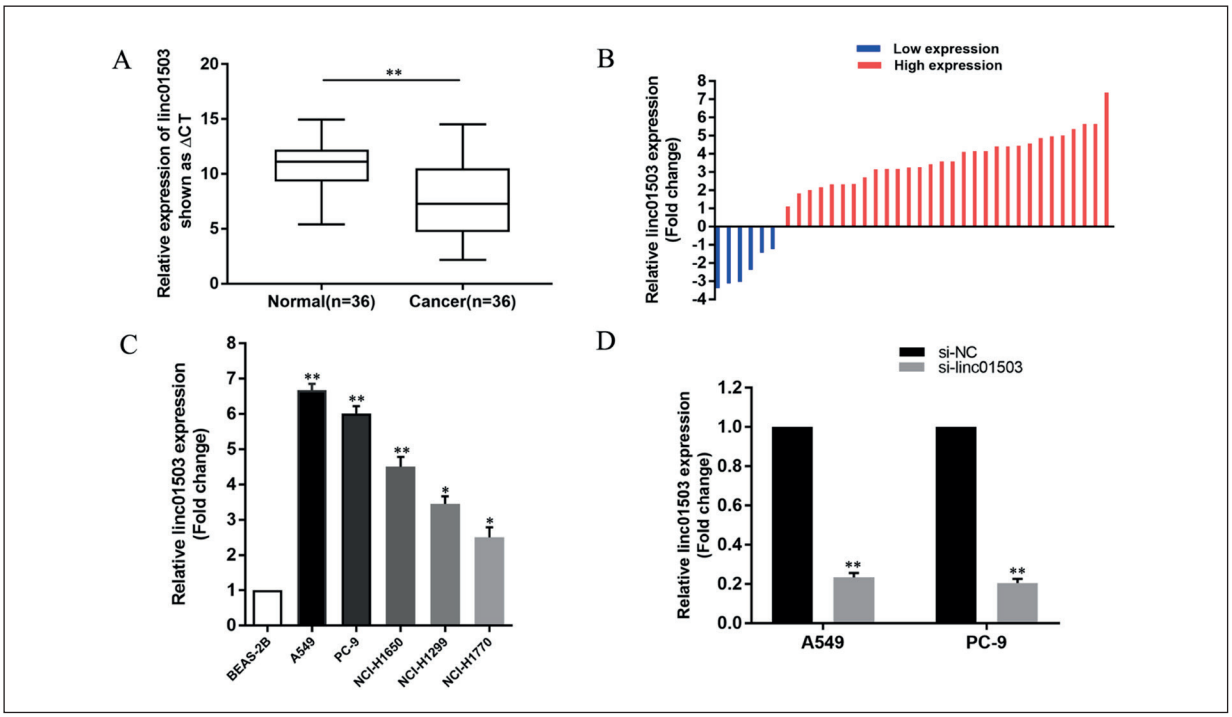

Figure 1. Up-regulation of LINC01503 expression. **A,** Upregulation of LINC01503 expression in 36 cases of NSCLC tissues detected via qRT-PCR. **B,** Based on the qRT-PCR results, the expression of LINC01503 was raised in 30 out of 36 cases of NSCLC tissues. **C,** The qRT-PCR results showed the up-regulation of LINC01503 expression in NSCLC cells. **D,** Interference efficiency of si-LINC01503 determined *via* qRT-PCR.

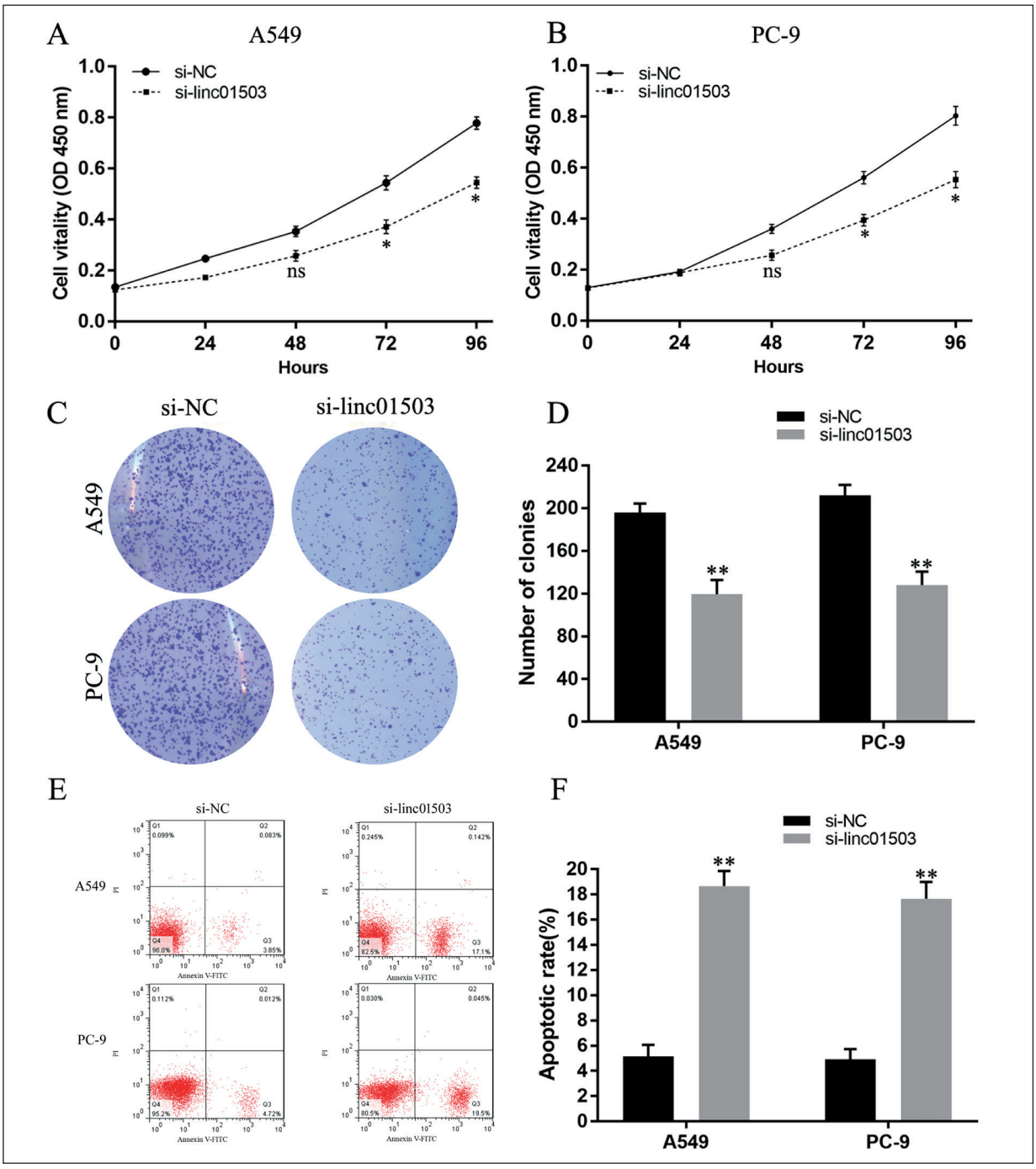

Figure 2. LINC01503 promoted the proliferation of NSCLC cells and repressed their apoptosis. **A-B,** Changes in cell proliferation ability detected via CCK-8 assay after interference with LINC01503 expression in NSCLC cells. **C-D,** According to the colony formation assay results, the proliferation ability of NSCLC cells was weakened after interference with LINC01503 expression in NSCLC cells. (magnification: 40×). **E-F,** Based on the flow cytometry results, the apoptosis rate of cells in si-LINC01503 group was raised compared with that in si-NC group.

(Figure 3A, 3B). Then, it was found through the qRT-PCR that the expression of LINC01503 was downregulated after interference with c-MYC expression (Figure 3C). Finally, the downstream molecular mechanism of LINC01503 in regulating the biological function of NSCLC cells was investigated. Based on the Western blotting results, the expressions of the downstream ERK/

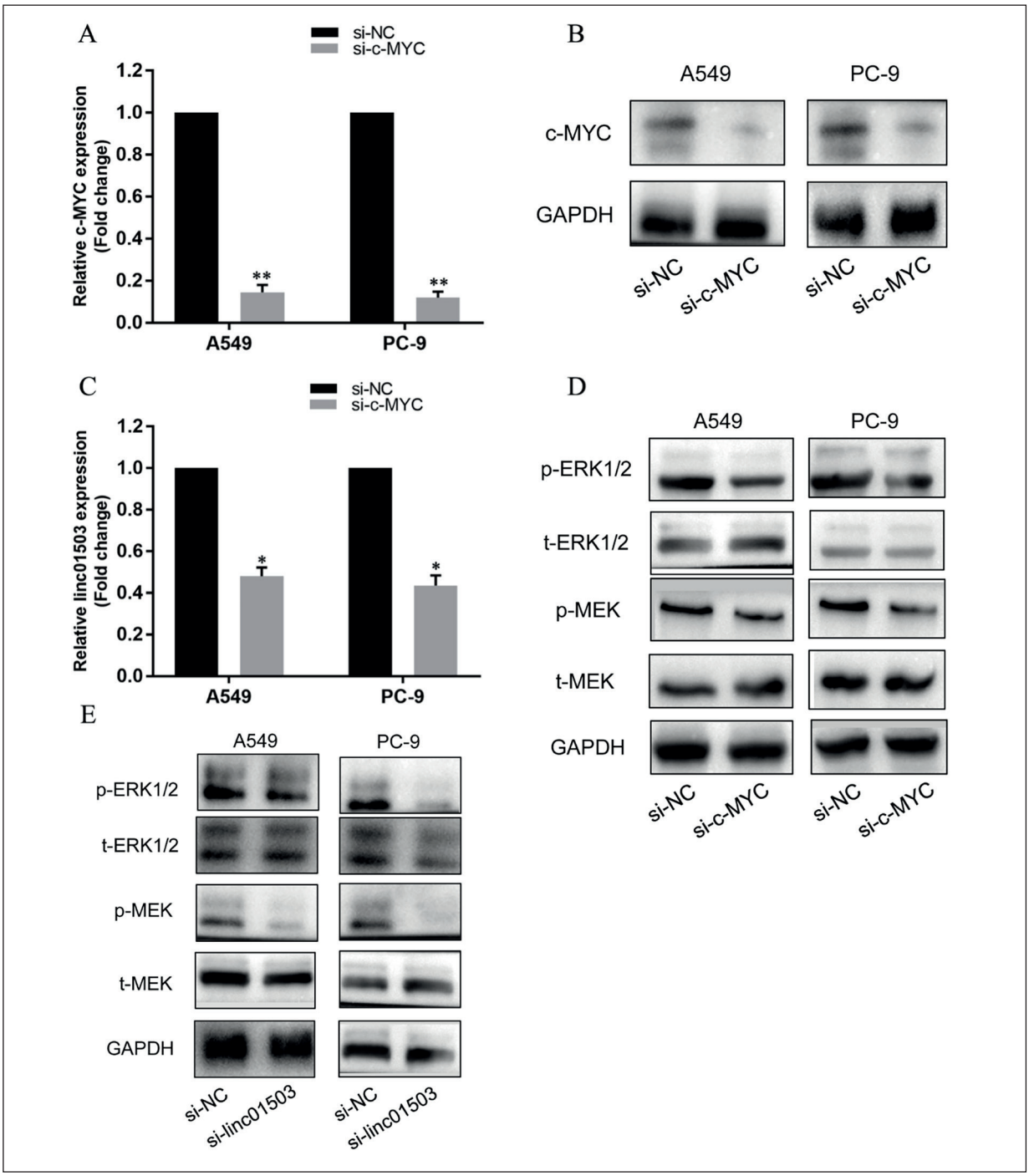

Figure 3. C-MYC contributed to the transcription of LINC01503 and regulated the ERK/MAPK signaling pathway. **A-B,** Si-c-MYC interference efficiency determined via qRT-PCR and Western blotting. **C,** LINC01503 expression detected by qRT-PCR after interference with c-MYC expression. **D-E,** Expressions of the molecular markers in the ERK/MAPK signaling pathway disclosed *via* Western blotting after interference with c-MYC and LINC01503 expressions.

MPAK signaling pathway molecular markers p-ERK1/2 and p-MEK declined after separate interference with c-MYC and LINC01503 expressions (Figure 3D, 3E).

Discussion

The morbidity and fatality rates of lung cancer have been markedly increasing in recent years,

and the disease has become a leading cause of cancer-related deaths, about 85% of which is NSCLC. Besides, over 70% patients are definitely diagnosed with locally advanced or advanced NSCLC[12]. Although molecular targeted drugs and immunotherapy can prolong the survival time of locally advanced or advanced NSCLC patients to a certain extent, their 5-year survival rate remains low. At present, the molecular mechanisms of the development and progression of NSCLC have not yet been fully clarified[13]. Therefore, it is of great significance to further explore the molecular mechanisms of the development and progression of NSCLC and search for novel treatment targets and strategies for improving the prognosis of patients.

LncRNAs are transcribed from RNA polymerases II, and since the fragments of lncRNAs lack open reading frames, they cannot encode proteins and were initially considered as the noises in transcription[14]. They can participate in epigenetic modification and regulation of post-transcriptional gene expression through multiple pathways, thereby playing an important role in the growth and development, as well as cell apoptosis and differentiation. LncRNAs are aberrantly expressed in various malignant tumor tissues and regulate cell proliferation, apoptosis and migration to participate in the development and progression of malignancies[15]. Xie et al[16] found that lncRNA LOX1-AS1 drives the proliferation and migration of NSCLC cells through the targeted regulation of miR-324-3p. According to the results of this study, the relative expression level of LINC01503 in the NSCLC tissues was higher than that in the normal paracancerous tissues, and the interference with LINC01503 repressed the proliferation of NSCLC cells and promoted their apoptosis.

MYC, a regulatory gene in tumorigenesis, has a substantially enhanced expression in many types of tumors and its gene product can regulate the transcription of various protein-encoding genes and non-coding RNAs. The MYC family has 3 members: c-MYC, N-MYC (MYCN) and L-MYC (MYCL). Of them, c-MYC is an important oncogene, and the lncRNA/c-MYC regulatory network has been detected to play a crucial role in the development and metastasis of tumors. c-MYC can promote the expression of lncRNA at the transcription level[17]. Yu et al[18] demonstrated that c-MYC directly binds to the E-box element of lncRNA colon cancer-associated transcript-1 (CCAT1) to mediate CCAT1 expression and activate CCAT1, further affecting the growth of pancreatic cancer cells. At present, the relationship between c-MYC and LINC01503 transcription regulation has not been reported in cancers. Firstly, we predict that c-MYC can bind to the LINC01503 promoter by bioinformatics. It was found that c-MYC could promote the expression of LINC01503 *in vitro*. However, whether the C-Myc is directly integrated into the linc01503 promoter needs to be verified by Chromatin immunoprecipitation (CHIP) experiment.

MAPK/ERK1/2 is an important signaling pathway that modulates numerous cellular functions, including cell proliferation and apoptosis[19,20]. LINC01503 inhibits the ERK/MAPK signaling pathway to restrain the proliferation and metastasis of gastric cancer cells. The *in vitro* assays in the present study verified that the interference with LINC01503 suppressed the expressions of the molecular markers in the ERK/MAPK signaling pathway[21].

Conclusions

Altogether, these findings show that c-MYC facilitates the expression of LINC01503 in NSCLC and activates the ERK/MAPK signaling pathway to drive the proliferation of NSCLC cells and repress their apoptosis, which provides a novel biomarker for the diagnosis and treatment of NSCLC patients and treatment strategies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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