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# Effects of Linc00460 on cell migration and invasion through regulating epithelial-mesenchymal transition (EMT) in non-small cell lung cancer

Q.-Y. YUE[1], Y. ZHANG[2]

[1]Department of Respiratory Medicine, The First People's Hospital of Yunnan Province, Kunming, China
[2]Department of Respiratory Medicine, People's Hospital of Yuxi City, Yuxi, China

*Cheng hai Wang and Jun Luo contributed equally to this work*

**Abstract. – OBJECTIVE: To detect the relative expression of long intergenic non-protein coding RNA 460 (linc00460) in non-small cell lung cancer (NSCLC) tissues and cells, to investigate the effects of linc00460 on proliferation, migration and invasion of NSCLC and to preliminarily explore its potential molecular action mechanisms.**

**PATIENTS AND METHODS: The relative expression of cancer tissues from 52 patients with NSCLC compared with that of para-carcinoma normal tissues was detected through Real-time quantitative polymerase chain reaction (qRT-PCR). qRT-PCR was also used to test the relative expressions of linc00460 in 4 strains of NSCLC cells. Effects of knockdown of linc00460 expression on the cell proliferation ability were detected by cell counting kit-8 (CCK-8) assay through transfection of small interfering linc00460 (si-linc00460) interference sequence in A549 cells and PC-9 cells. Transwell assay was used to detect the effects of lic00460 on the migration and invasion abilities of NSCLC cells. Western blot was applied to observe the changes of epithelial-mesenchymal transition (EMT)-related proteins. The expression changes of EMT-related proteins were detected by immunofluorescence assay.**

**RESULTS: qRT-PCR results showed that there were 40 cases (76.9%) with up-regulated linc00460 expressions in tissues from 52 patients with NSCLC. The linc00460 expressions in 5 strains of NSCLC cells were higher than those of human bronchial epithelial (16HBE) cells. Interference in linc00460 expression could inhibit the proliferation, invasion and migration of NSCLC cells. Results of Western blot and immunofluorescence found that interfering with the linc00460 expression could affect the expression changes of EMT-related proteins.**

**CONCLUSIONS: The relative expression of linc00460 is upregulated in NSCLC tissues. Cells and linc00460 can promote the tumor invasion and migration through affecting EMT.**

Key Words
NSCLC, linc00460, Proliferation, Invasion and migration, EMT.

## Introduction

At present, lung cancer still has the highest incidence and mortality rates among malignant tumors worldwide. The deaths caused by lung cancer rank first in cancer-related deaths[1]. Lung cancer mainly includes two types, namely non-small cell lung cancer (NSCLC) and small cell lung cancer, the former of which accounts for about 85% in lung cancer[2]. So far, the commonly used treatment methods of NSCLC include surgical resection, radiotherapy and chemotherapy, molecular targeted therapy and immunotherapy developed in recent years. Although the treatment for NSCLC continues to be improved, survival and prognosis of most patients are still not ideal because patients used to be in moderate or late stage when they come to receive treatment; thus, the 5-year survival rate is less than 15%[3,4]. The main factors affecting the prognosis of NSCLC are metastasis and recurrence[5]. Therefore, the research on the invasion and metastasis mechanism of NSCLC has become a hot topic of current study worldwide.

With the completion of human genome project, it has been found that, in addition to about 20000 protein encoding genes (accounting for only about 2% of the genome) in the genome, there also contains a large number of non-coding ribonucleic acids (ncRNAs)[6]. Long non-coding RNA (lncRNA) is a kind of ncRNA with transcript of more than 200 nt in length, which can affect the behavior changes of biolo-

*Corresponding Author:* Yi Zhang, MD; e-mail: 979119632@qq.com

gical cytology through participation in various kinds of biological processes such as epigenetic modification, transcriptional regulation, protein translation and protein degradation[7]. More researches[8,9] report that lncRNA can play roles similar to oncogenes or tumor suppressor genes in participating in the occurrence and development of tumor. However, the expression and biological function of linc00460 in NSCLC tissues and cells have not been reported yet. Linc00460 is located in chromosome 13q33.2 region with a length of 935 bp. At first, Cao et al[10] discovered the upregulation of the linc00460 expression in squamous-cell carcinoma of the head and neck, and the high expression of linc00460 was associated with poor prognosis. Liang et al[11] reported that the expression of linc00460 is high in esophageal cancer tissues and cells and it is closely related with tumor node metastasis (TNM) staging, and lymph node metastasis. The interference in its expression can promote the apoptosis of esophageal cancer cells. In nasopharyngeal carcinoma, linc00460 may promote the occurrence and development of nasopharyngeal carcinoma by adsorbing microRNA (miRNA) 149-5P and upregulating the expression of interleukin-6 (IL-6)[12]. Through *in vitro* experiments, it was found first in this report that the linc00460 has a high expression in NSCLC tissues and cells and interfering with linc00460 expression can affect the invasion and migration of NSCLC via regulating EMT. The result of our work provides new drug targets and monitoring indicators for the clinical treatment of NSCLC.

## Patients and Methods

### Tissue samples

A total of 52 pairs of non-small cell lung cancer tissue samples and corresponding samples of para-carcinoma normal tissues were taken from People's Hospital of Yuxi City, and all non-small cell lung cancer tissues had been diagnosed by pathological examination. At the same time, the type of tissue differentiation was determined. The consent was obtained from patients or the authorized clients before sampling. The investigation was approved and supported by the Ethics Committee of the People's Hospital of Yuxi City. The tissue samples were stored in liquid nitrogen immediately until they were treated according to the requirements of the study.

### Cell strains

Normal human bronchial epithelial cell 16HBE, NSCLC cell strains A549, H226, H1915, secretory pathway $Ca^{(2+)}$ -ATPase pump type 1 (SPCA-1) and PC-9 were all from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in an incubator with 5% $CO_2$ at 37°C in Roswell Park Memorial Institute (RPMI)-1640 or Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA). Then, the mixture of 1% penicillin and streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin) was added to prevent the growth of mixed bacterium.

### RNA extraction and reverse transcription

The total RNA in tissues and cells was extracted according to the experimental procedures provided by the TRIzol kit; the OD260/280 (OD: optical density) value was detected by ultraviolet visible spectrophotometer (Alpha1106) and the concentration of RNA was determined. The synthesis of complementary DNA (cDNA) was achieved based on the experimental steps offered by PrimeScript™ RT Master Mix Kit (Perfect Real-time) and the reaction system (20 μL) was reversely transcribed; qRT-PCR system was prepared in accordance with the instructions of HiScript® II One Step qRT-PCR SYBR® Green Kit. The ABI7500RTC-PCR was used to conduct the Real-time PCR with one-step method, and the target gene was calculated through the $2^{-\Delta\Delta Ct}$ method.

### Synthesis of small interfering RNA (siRNA) and primers

Linc00460 interference sequence (Invitrogen, Carlsbad, CA, USA): 1# AAGTCAAGAATCTGCCTGAACCAGT 2# CCGTCTCCTTACATGAAAGTGAAGA, 3# GCCAACTTCAAGCCATTCATTGTTA. Primers were designed by the ligo7 and Primer 5.0 software (Sangon, Shanghai, China): GAPDH F-GGGAGCCAAAAGGGTCAT, GAPDH R-GAGTCCTTCCACGATACCAA. Linc00460 F-ACG TGCAGACATCTACAACCT, linc00460 R-TACTTCCAACACCCGCAT.

### Cell counting kit-8 (CCK-8) proliferation assay

The transfected NSCLC cells in each group were collected and inoculated on 96-well plates with a cell density of $3\times10^3$ in each well. After re-

gular cultures for 24, 48, 72 and 96 h, CCK-8 reagent with a final concentration of 10% (Beyotime Biotechnology, Shanghai, China) was added at the above mentioned time points. Cells were sheltered from light for 1 h. OD values at wavelength of 450 nm were detected by a microplate reader.

### Scratch test

Cells from the experimental group at logarithmic phase and those of control group were selected and inoculated on 6-well plates with $5\times10^3$ in each well. They were cultured in the serum-free medium and 2 scratches were made at the bottom of incubator. The observation and analysis were performed after 24 h and 48 h, respectively.

### Transwell experiment

The NSCLC cells of experimental group and control group were collected after transfection and were resuspended with serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle Medium (DMEM). Next, they were added to the upper chamber of the transwell chamber (or added to the pre-paved Matrigel matrix) with a cell density of $5\times10^4$ in each well. In the lower chamber, 700 mL complete medium were added and cultured in the incubator for 24 h (48 h). Then, they were removed from the upper chamber to be observed via an optical microscope (200×) after treatment with 4% paraformaldehyde and staining with 0.01% crystal violet.

### Western blot experiment

After collection of cells, the total protein solution was obtained by adding a proper amount of RIPA to perform the lysis. The crosslinking of the solution was conducted with sodium-dodecyl sulphate (SDS) loading buffer in 100°C water bath. Protein samples were treated with polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. The skimmed milk was used to block nonspecific binding. The membrane was incubated with the primary antibodies and secondary antibodies successively. Chemiluminescence method was applied to develop an image so as to detect the changes of EMT-related proteins such as E-cadherin, N-cadherin, vimentin and SNAL after the linc00460 expression interfered.

### Immunofluorescence assay

Cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.02% Triton X-100 (phosphate-buffered saline, PBS) at room temperature for 20 min, added with primary antibodies on each glass and kept in the wet box. Finally, they were incubated overnight at 4°C; dilute fluorophore-conjugated secondary antibodies were added drop by drop, and kept in the wet box for incubation at 20-37°C for 1 h. 4,6-diamino-2-phenylindole (DAPI) were added drop by drop to be incubated for 5 min and protected from light. The cores of specimens were stained and the specimens were washed for 4 times in phosphate-buffered saline with tween-20 (PBST) for 5 min to remove unnecessary DAPI. Specimens were mounted by liquid blocking buffer with anti-fluorescence quencher. Their images were observed and acquired under the fluorescence microscope.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were presented as x±s, and *t*-test was used for comparison among groups. $p<0.05$ suggested that the difference was statistically significant.

## Results

### Detection of linc00460 expression

The expressions of linc00460 were detected through the collection from tumor tissues and para-carcinoma tissue of 52 patients with NSCLC by using qRT-PCR assay. The results showed that compared with the cancer tissues, there were a total of 40 cases with upregulated expressions of linc00460 (Figure 1A). qRT-PCR was used to detect the linc00460 expressions of 5 strains of human bronchial epithelial cells 16HBE in NSCLC cells, and the results revealed that all the expressions were increased in 5 strains of NSCLC cells (Figure 1B). The si-linc00460 and the control sequence were transfected transiently to NSCLC cells by using lipofectamine 2000 (lip2000); RNA cells were collected after 48 h and qRT-PCR experiment was adopted to detect efficiency of interference (Figure 1C and D).

### Effect of linc00460 on proliferation

Cells of the experimental group and control group were inoculated in 96-well plates with 3000 cells in each well where CCK8 solution was added. The observation time points to measure the OD values were set to be 0 h, 24 h, 48 h, 72 h and 96 h; the growth curve was drawn accordingly.

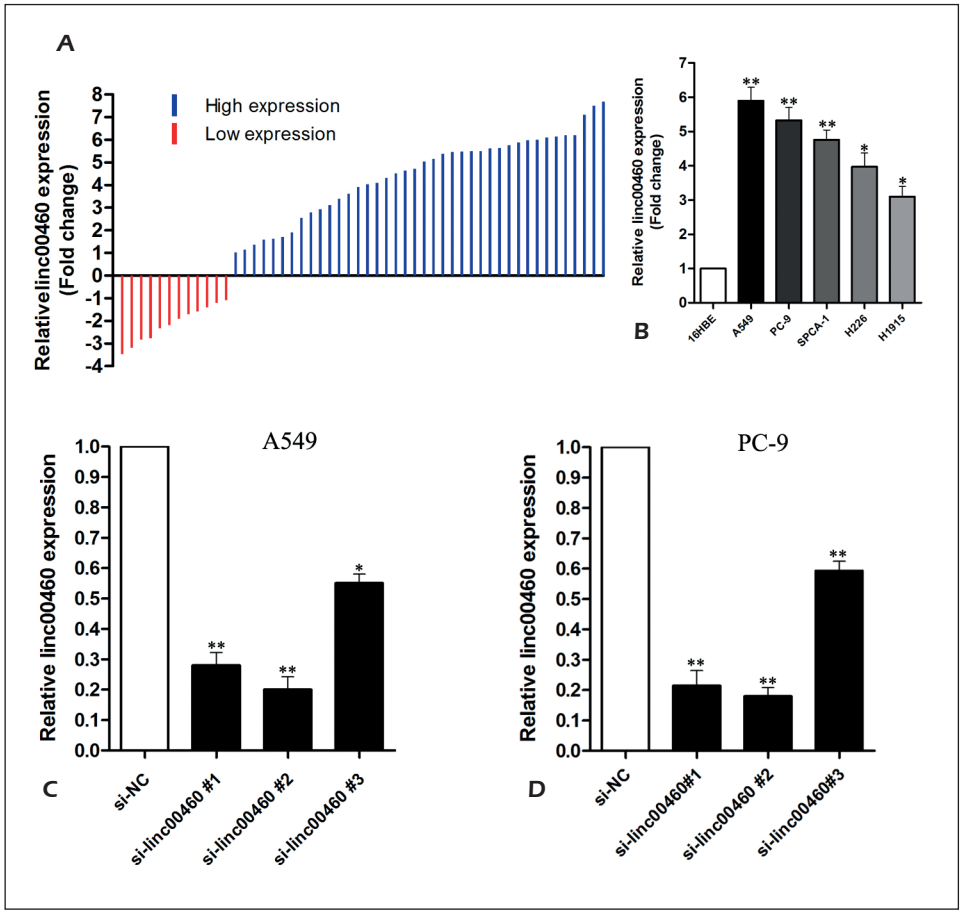


**Figure 1.** The expression of linc00460 in NSCLC. **A**, qRT-PCR assay showed that in 52 cases of NSCLC tissues and adjacent tissues, 40 cases had an upregulated expression of linc00460 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference. **B**, The results of qRT-PCR reports that linc00460 was upregulated in 5 cell strains compared with 16HBE. **C-D**, Si-linc00460 and si-NC were transiently transfected into A549 and PC-9 cells; the transfection efficiency was detected by qRT-PCR assay after 48 h (**$p$<0.01, *$p$<0.05).

The results suggested that the interference with linc00460 expression could inhibit the proliferation of NSCLC cells (Figure 2A, B).

### *Effects of linc00460 on migration and invasion*

Cells of the experimental group and control group were seeded in 6-well plates. The scratch test was applied to detect the effects of linc00460 on cell migration. The results showed that interference in linc0046 expression could inhibit NSCLC cell migration (Figure 2C); after the cells were treated with the same method, transwell results suggested that the interference with linc00460 expression might inhibit the migration and invasion of NSCLC cells (Figure 3A,B).

### *Influence of Linc00460 on EMT*

EMT occurs in the early stage of epithelial cell tumor metastasis, which is one of the important mechanisms for malignant tumor cells to obtain migration and invasion abilities. It plays important roles in the occurrence and development of cancer[13]. In recent years, it has been reported that lncRNA is an important regulatory factor involved in tumor invasion and metastasis through regulating EMT[14]. It was found via Western blot that the expression of EMT-related molecular markers changed after interference with linc00460 expression in NSCLC cells (Figure 4A, B). After the cells were treated with the same method, the expression of EMT-related molecular markers was also changed through immunofluorescence assay (Figure 4C, D).

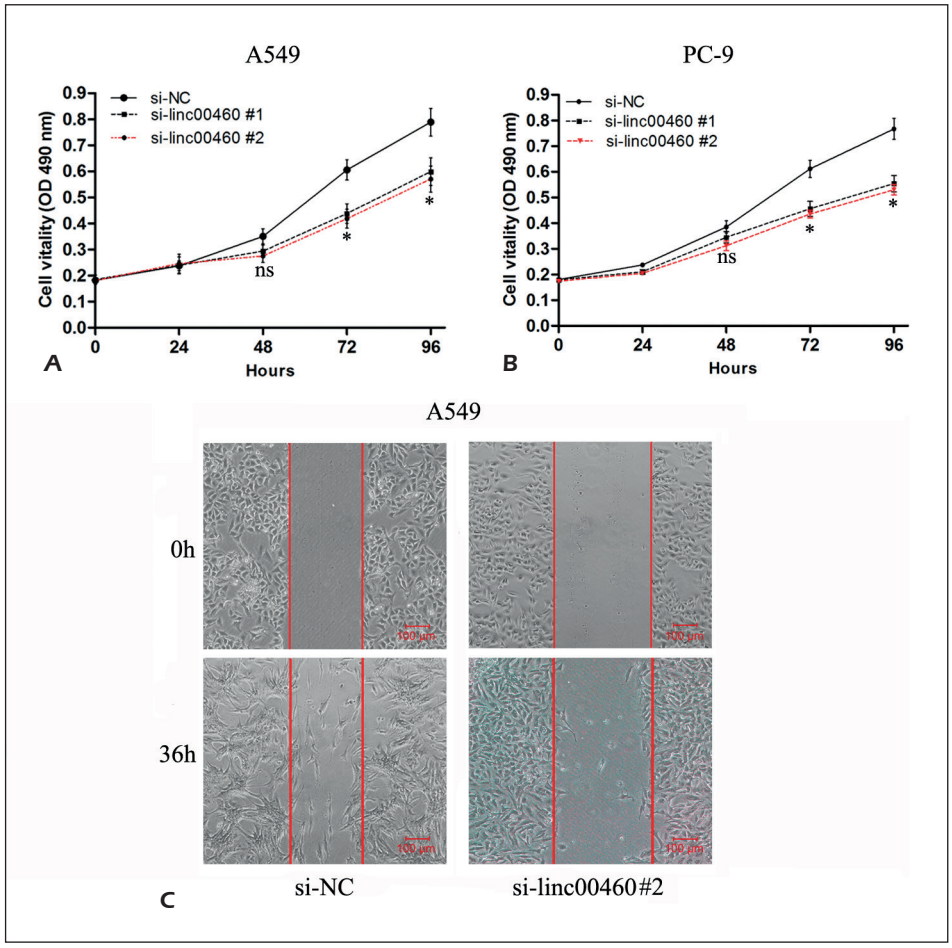


**Figure 2.** Linc00460 promotes NSCLC cell proliferation and migration. **A-B**, Si-linc00460 and control sequence were transiently transfected into NSCLC cells. The CCK8 found that knockdown of linc00460 expression can inhibit the proliferation of NSCLC cells. **C**, Si-linc00460 and control sequence were transiently transfected into NSCLC cells. The scratch test revealed that the low expression of linc00460 may inhibit the migration of NSCLC cells (**$p$<0.01, *$p$<0.05).

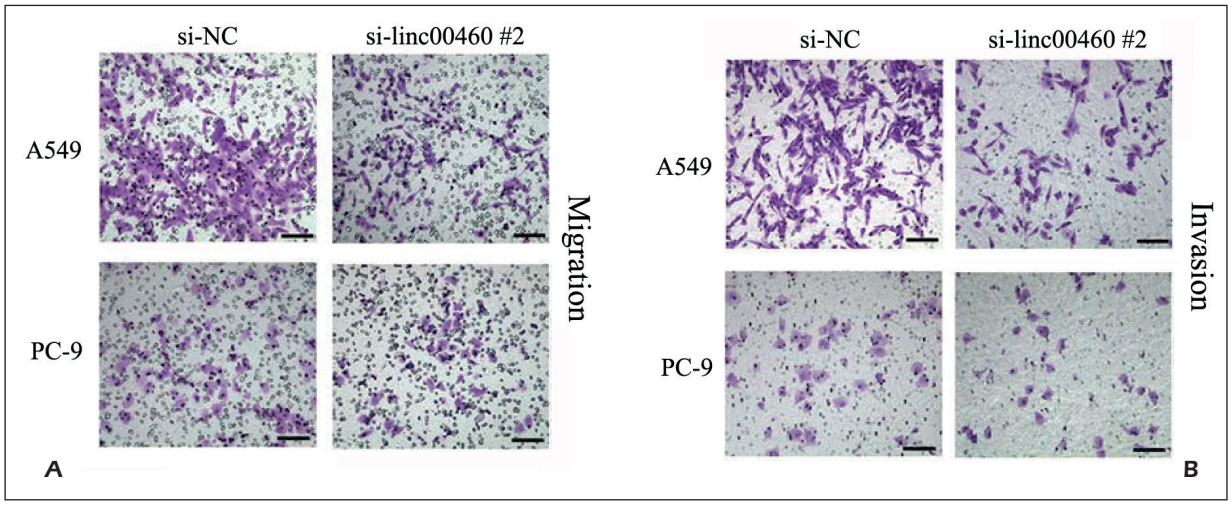


**Figure 3.** Linc00460 promotes the migration and invasion of NSCLC cells. **A**, The interference of linc00460 expression can inhibit the migration of NSCLC cells (without matrigel) via transwell assay. **B**, 48 h after same treatment to NSCLC cells, transwell assay suggested that the interference of lins00460 expression may inhibit the invasion ability of NSCLC cells (with matrigel).

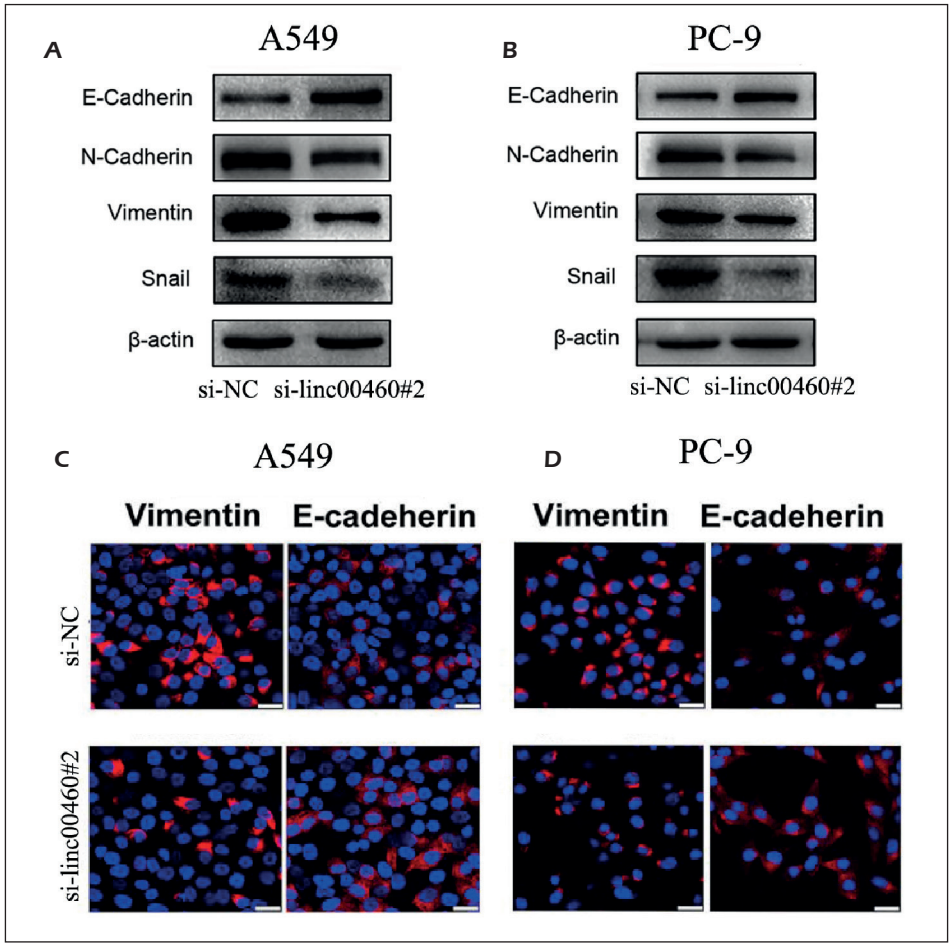


**Figure 4.** Linc00460 regulates EMT. **A-B**, Si-linc00460 and si-NC cells are transfected into NSCLC cells. The total protein of the cells is collected. Through Western blot assay, it was found that the expression of molecular markers in the EMT pathway changed. **C-D**, 48 h after the same treatment to treat NSCLC cells, the expression of EMT markers was detected by immunofluorescence.

## Discussion

NSCLC mainly includes adenocarcinoma and squamous cell carcinoma, which account for about 1/6 of deaths of patients caused by cancer worldwide[1]. Although a lot of progress has been made in clinical and experimental oncology, the overall prognosis of NSCLC patients is still poor. Therefore, understanding the detailed pathogenesis of NSCLC is essential for diagnosis, prevention and treatment. More evidence suggests that lncRNA is involved in the pathogenesis of NSCLC. As an important regulator to control signal pathway related to the occurrence and development of NSCLC and some key genes, the abnormal expression of lncRNA is closely associated with tumor differentiation, drug resistance, invasion and metastasis, and other aspects. It is reported in the literature[15] that lncRNA small nucleolar RNA host gene 20 (SNHG20) promotes the proliferation, invasion, and metastasis of NSCLC cells by silencing the expression of P21. Linc00673 can regulate NSCLC cell proliferation by adsorbing miRNA 150-5P[16]. Ma et al[17] found that knocking down the expression of lncRNA transient receptor potential channels melastatin2-AS (TRPM2-AS) can increase the sensitivity of NSCLC cells to cisplatin by activating the p53-p66 Src homologous-collagen (p53-p66shc) pathway. In this work, we first found that linc00460 was highly expressed in NSCLC tissues and cells; knocking down its expression could inhibit tumor cell proliferation, invasion and metastasis. Epithelial-mesenchymal transition (EMT) refers to the biological process of the transdifferentiation of epithelial cells into mesenchymal cells under

specific physiological or pathological conditions. In the process of EMT, epithelial cells lose the polarity of cells and the epithelial phenotype such as the connection with basement membrane. They acquire the mesenchymal phenotype with higher migration and invasion ability. The main features of EMT include that, the protein expressions of cell adhesion molecules, such as E-cadherin, are reduced; the cell keratin is transformed into cytoskeleton, which mainly refers to the vimentin (VIM). The morphology has the characteristics of mesenchyme[18]. It is reported in the literature that lncRNA can participate in tumor invasion and metastasis through mediating EMT biological processes. For example, Li et al[19] found in breast cancer that the overexpression of lncRNA anti-differentiation ncRNA (ANCR) may partially inhibit EMT induced by transforming growth factor-β (TGF-β), thereby inhibiting the invasion and metastasis of lncRNA cells. Similarly, FEZF1 antisense RNA1 (FEZF1-AS1) can inhibit the epigenetic expression of E-cadherin so as to promote EMT[20]. This work first found that linc00460 can regulate the invasion and metastasis NSCLC cells by regulating EMT.

## Conclusions

This study will help to improve the understanding of the pathogenesis of NSCLC, thereby providing a reliable theoretical basis for diagnosis and treatment of NSCLC. How linc00460 regulates EMT will be deeply discussed in the follow-up study.

### Conflict of Interest

The authors declared no conflict of interest.

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