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Linc00703 suppresses non-small cell lung cancer progression by modulating CyclinD1/CDK4 expression

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Abstract. – OBJECTIVE: The aim of this study was to detect the expression of linc00703 in non-small cell lung cancer (NSCLC), and to explore the biological function and potential molecular mechanism of linc00703 in NSCLC using *in vitro* experiments.

PATIENTS AND METHODS: The carcinoma tissues and para-carcinoma tissues were collected from 32 patients diagnosed with NSCLC, from which the RNA was extracted. The relative expression of linc00703 in NSCLC tissues was detected *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The NSCLC cells and normal human bronchial epithelial cells were selected, in which the relative expression of linc00703 was determined *via* qRT-PCR. Next, the linc00703 overexpression plasmids were designed and synthesized, and then transiently transfected into NSCLC cells. After 48 h, the overexpression efficiency was detected. Finally, the changes in cell proliferation, apoptosis, cycle distribution and expressions of downstream molecular markers were determined using cell counting kit-8 (CCK8) assay, colony formation assay, flow cytometry and Western blotting, respectively, after overexpression of linc00703 in NSCLC cells.

RESULTS: The results of qRT-PCR revealed that the expression of linc00703 was down-regulated by 5.14 times on average in 29 out of 32 cases of NSCLC tissues, and it was also down-regulated in NSCLC cells. Besides, it was found through CCK-8 assay, colony formation assay and flow cytometry that after overexpression of linc00703 in NSCLC cells, the cell proliferation was inhibited, the apoptosis was enhanced, and the cell cycle was arrested in G1/G0 phase. Furthermore, the results of Western blotting showed that after overexpression of linc00703, the protein expressions of cyclinD1 and cyclin-dependent kinase 4 (CDK4) declined, while those of cyclinE1 and CDK2 did not change.

CONCLUSIONS: The expression of linc00703 is down-regulated in NSCLC, and it suppresses the occurrence and development of NSCLC *via* mediating the expression of cyclinD1/CDK4.

Key Words:
NSCLC, Linc00703, Biological function, CyclinD1, CDK4.

Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers. With the development of molecular biology of lung cancer in recent years, new drugs have emerged, but the 5-year survival rate of patients with advanced lung cancer remains only about 15%[1,2]. Therefore, exploring new treatment approaches of NSCLC, especially the molecular mechanism of its occurrence and development, and searching for effective therapeutic targets are the research hotspots currently.

Long non-coding ribonucleic acids (lncRNAs) are a class of RNAs with more than 200 nucleotides in length, and they have no protein-coding function, so lncRNAs are considered as the ''dark matter'' of gene transcription[3,4]. Recently, it has been found that the abnormal expression of lncRNAs is strictly related to human diseases, and plays an important role in regulating the occurrence, proliferation, invasion, metastasis, recurrence and drug resistance of tumors. Therefore, lncRNAs may serve as new therapeutic targets for tumors[5,6]. It is reported in the literature that lncRNA TTN-AS1 regulates the expression of ROCK2 *via* adsorbing miR-139-5P, thereby regulating the occurrence

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and development of ovarian cancer[7]. Liu et al[8] found that the expression of lncRNA HTTIP is up-regulated in colorectal cancer, and highly expressed HTTIP promotes the proliferation and metastasis of colorectal cancer.

Linc00703, located on chromosome 10p15.1, is 2493 bp in length, which, as a tumor suppressor, can suppress the occurrence and development of gastric cancer *via* regulating the miR-181a/KLF6 axis according to a literature[9]. However, there have been no reports about the expression and biological function of linc00703 in NSCLC. In this study, it was found for the first time that the expression of linc00703 was down-regulated in NSCLC, and the results of *in vitro* experiments revealed that the overexpression of linc00703 inhibited NSCLC cell proliferation, arrested the cell cycle in G1/G0 phase and promoted apoptosis. The research results will provide a potential molecular target for the treatment of NSCLC.

Patients and Methods

Tissue Specimens

A total of 32 NSCLC patients admitted to the Third Hospital of Jilin University from January 2016 to December 2017 were collected, and the specimens were surgically resected. Inclusion criteria: 1) patients pathologically diagnosed with NSCLC, 2) Chinese Han people, 3) those without mental disease, autoimmune disease and other cancers, and 4) those with complete clinical data and pathological tissue specimens. Patients were selected based on the guideline proposed by the Union for International Cancer Control (UICC). The specimens were first placed in a liquid nitrogen container for 10 min, then taken out and stored at -80°C for subsequent treatment. This investigation was approved by the Medical Ethics Committee of The Third Hospital of Jilin University.

Cells and Cell Culture

NSCLC cells (A549, H226, PC-9 and H358) and normal human bronchial epithelial cells (BEAS-2B) were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). BEAS-2B cells were cultured in the Bronchial Epithelial Cell Growth Medium (BEGM) (Lonza, Basel, Switzerland). A549, H226, SPCA1 and H358 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (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). All cells were cultured in an incubator with 5% CO_2 at 37°C.

Extraction of RNA

The cells in each well were lysed with 1 mL of TRIzol (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, and centrifuged at 12,000 rpm and 4°C for 5 min. Then, the supernatant was transferred to a new centrifuge tube, added with chloroform (^{1}/5 volume of lysate) and shaken vigorously till thorough emulsification, followed by centrifugation at 12,000 rpm and 4°C for 15 min. After that, the supernatant was aspirated into a new centrifuge tube, added with an equal volume of isopropanol, turned upside down for 15 times and let to stand for 10 min. After centrifugation at 12,000 rpm and 4°C for 15 min, the supernatant was discarded, 1 mL of 75% ethanol was added, and the mixture was centrifuged at 12,000 rpm and 14°C for 5 min. At last, the precipitate obtained was dissolved with 20 μL of deionized water to obtain the RNA.

Reverse Transcription (RT)

The RT system consisted of 1 μL of RNA, 2 μL of 5 × PrimeScript RT Master (TaKaRa, Otsu, Shiga, Japan) and 7 μL of deionized water, and the RT conditions were as follows: reaction at 37°C for 15 min and 85°C for 5 s, and storage at 4°C.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total qRT-PCR system (25 μL) consisted of 12.5 μL of SYBR premix Ex Taq II, 1 μL of forward primer, 1 μL of reverse primer, 2 μL of real-time quantitative PCR products and 8.5 μL of deionized water. The PCR conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 15 s, annealing at 60°C for 30 s, a total of 30 cycles (TaKaRa, Otsu, Shiga, Japan). After the reaction, the specimens were stored at 4°C. The gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed below: Linc00703 F: 5′-TGGCCTG-CGAATTCTAGCAG-3′; R: 5′-TGTCGG GTAG-ACAACAGCAT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F 5′-CCCTTCATT-GACCTCAACTACA-3′; R: 5′-ATGACAAGCTT CC CGTTCTC-3′

Cell Transfection

NSCLC cells in the logarithmic growth phase were inoculated into a culture plate, added with 4 μg of pcDNA-linc00703 and control plasmids (Genechem, Shanghai, China), mixed and incubated with Lip2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min. After 12 h, the medium was replaced. After transfection for 48 h, the cells were collected for subsequent experiments.

Detection of Cell Proliferative Activity Via Cell Counting Kit-8 (CCK8) Assay

The cells in a 96-well plate were incubated with 100 μL of mixed medium (90 μL of combined medium + 10 μL of CCK-8 buffer) (Biosharp, Hefei, China) in each well at 37°C for 2 h. The absorbance (A) was measured at 450 nm using a microplate reader (SpectraMax 190), indicating the cell proliferative activity, with five replicates in each group.

Colony Formation Assay

The cells in experimental group and control group were inoculated into a 6-well plate (1000 cells/well), and cultured for about 10 d until there were visible cell colonies. After the cells were washed twice with phosphate-buffered saline (PBS), they were fixed with 4% paraformaldehyde for 30 min, stained with 1% crystal violet dye for 10 min, air dried and photographed. Finally, cell colonies in each group were counted to reflect the colony formation ability, with three replicates in each group.

Detection of Cell Cycle Via Flow Cytometry

The cells in each group were fixed with 70% cold ethanol at 4°C overnight, and stained with propidium iodide (PI) (0.05 g/L) and RNase (2 g/L) at room temperature for 30 min. Then, the cell cycle was detected and analyzed *via* flow cytometry, and the percentage of cells in different cell cycles was calculated using Cell Lab Quanta SC software (Beckman Coulter, Miami, FL, USA).

Detection of Apoptosis Via Flow Cytometry

After transfection for 48 h, the cells in each group were digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin using the Annexin V-FITC (fluorescein isothiocyanate) method (BD, Biosciences, Franklin Lakes, NJ, USA). Then, 5×10^4 cells were taken, resuspended with binding buffer and transferred to the flow tube. Afterwards, 5 μL of Annexin V-FITC was added and mixed evenly, followed by incubation at room temperature in the dark for 15 min. At 5 min before detection, the cells were stained with 5 μL of Propidium Iodide (PI), and the apoptosis was detected *via* flow cytometry. The assay was repeated for 3 times, and the average was taken.

Western Blotting

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer in a 6-well plate on ice for more than 30 min, and the protein lysate was collected. Then, 50 μg of proteins were added into 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoretic separation, transferred onto a membrane at 100 V for 90 min, sealed at room temperature for 1 h, and incubated with primary antibodies (cyclinD1, CDK4, cyclinE1, CDK2, 1:3000) (Univ-Bio, Shanghai, China) at 4°C overnight. After the membrane was washed, the proteins were incubated again with secondary antibodies (Univ-Bio, Shanghai, China) at 37°C for 1 h, and the membrane was washed again, followed by image development. Finally, the gray value of cyclinD1, CDK4 and GAPDH protein bands in each group was analyzed using Scion Image software, and the ratio of gray values of cyclinD1 and CDK4 to that of GAPDH was calculated in each group.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation (`χ±s). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ was considered to be statistically significant.

Results

Expression of Linc00703 Was Down-Regulated In NSCLC

The tissue specimens were collected from 32 patients pathologically diagnosed with NSCLC, and the relative expression of linc00703 in NSCLC tissues was detected *via* qRT-PCR. It was

found that compared with that in para-carcinoma tissues, the expression of linc00703 was down-regulated by 5.14 times on average in 29 cases of NSCLC tissues (Figure 1A, 1B). Besides, the relative expression of linc00703 in NSCLC cells was detected *via* qRT-PCR, and the down-regulated expression was also observed (Figure 1C). Then, the possible biological function of linc00703 in NSCLC was detected, the linc00703 overexpression plasmids were constructed, and the overexpression efficiency was determined using qRT-PCR (Figure 1D).

Effect of Linc00703 on Proliferation of NSCLC Cells

The pcDNA-linc00703 and control plasmids were transiently transfected into NSCLC cells, and the changes in the proliferation ability of NSCLC cells were determined using CCK-8 assay. It was confirmed that the cell proliferation was inhibited in experimental group compared with that in control group (Figure 2A, 2B). After the same treatment, the results of colony formation assay revealed that the cell proliferation was suppressed in experimental group, consistent with those in CCK8 assay (Figure 2C, 2D).

Effects of Linc00703 on NSCLC Cell Cycle and Apoptosis

The results of flow cytometry manifested that after overexpression of linc00703 in NSCLC cells, the cell cycle was arrested in G1/G0 phase (Figure 3A, 3B), and the apoptosis rate rose in experimental group compared with that in control group (Figure 3C, 3D).

Linc00703 Regulated CyclinD1/Cyclin-Dependent Kinase 4 (CDK4) Expression

To study the potential molecular mechanism of biological function of linc00703 in NSCLC, the

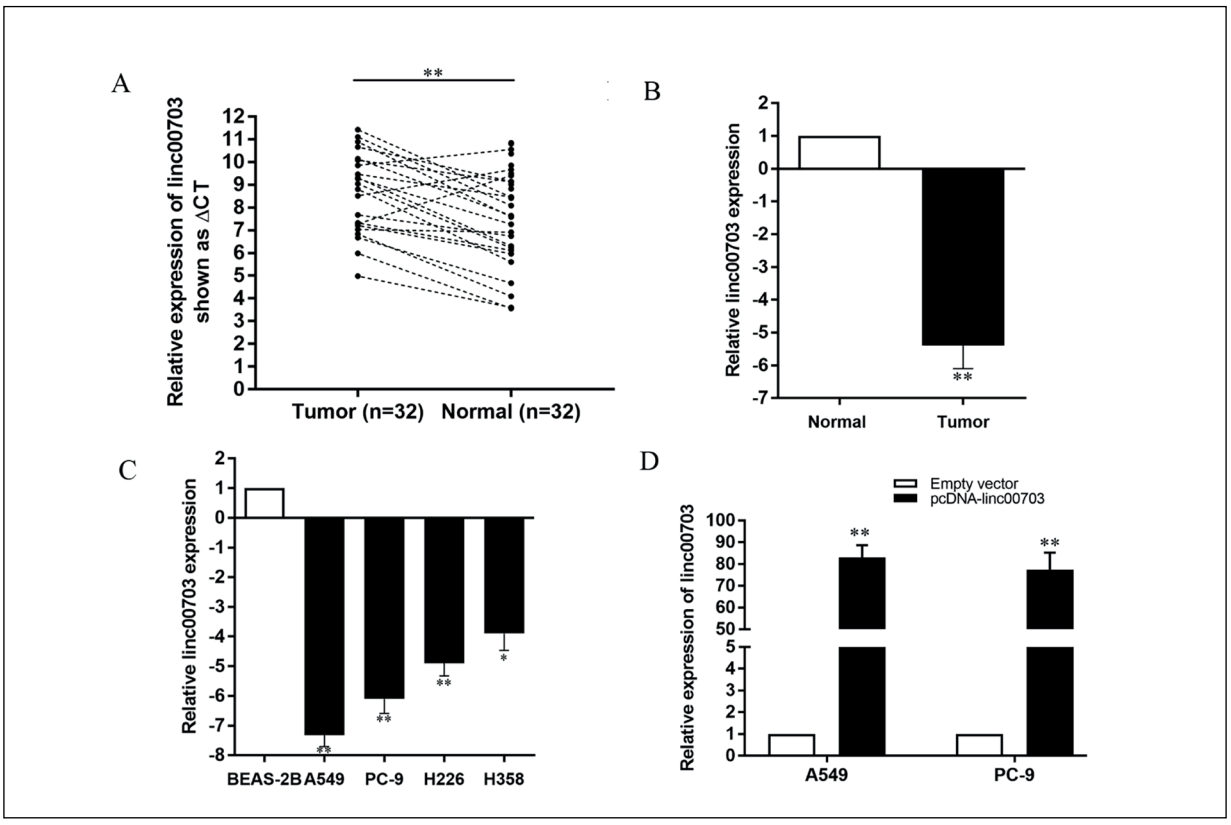

Figure 1. Expression of linc00703 was down-regulated in NSCLC. **A,** The expression of linc00703 in 32 cases of NSCLC tissues was detected via qRT-PCR. The results showed that the expression of linc00703 was down-regulated in 29 cases of NSCLC tissues. **B,** The expression of linc00703 was down-regulated by 5.14 times on average. **C,** The expression of linc00703 in NSCLC cells was detected via qRT-PCR. **D,** The overexpression efficiency was determined using qRT-PCR.

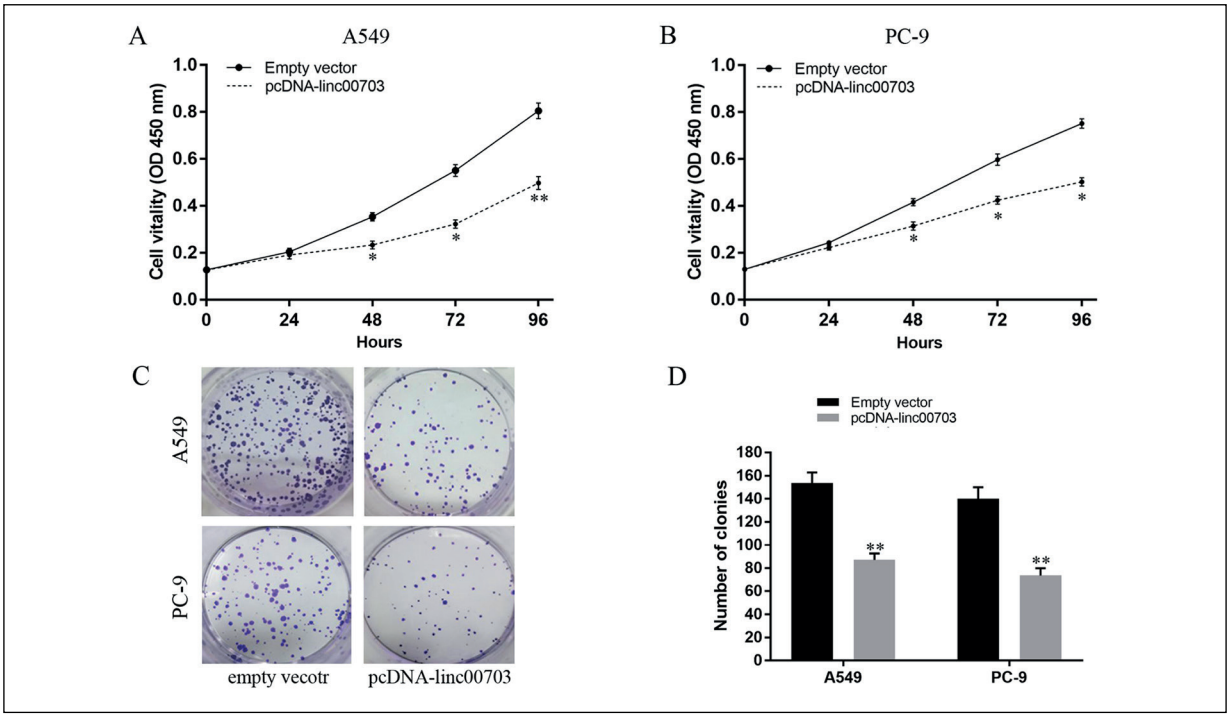

Figure 2. Effect of linc00703 on proliferation of NSCLC cells. **A, B,** The effect of linc00703 on the proliferation of NSCLC cells was determined using CCK-8 assay. It was found that the cell proliferation was weakened after the overexpression of linc00703. **C, D,** The results of colony formation assay revealed that the cell proliferation was suppressed in experimental group compared with that in control group (magnification: 20×).

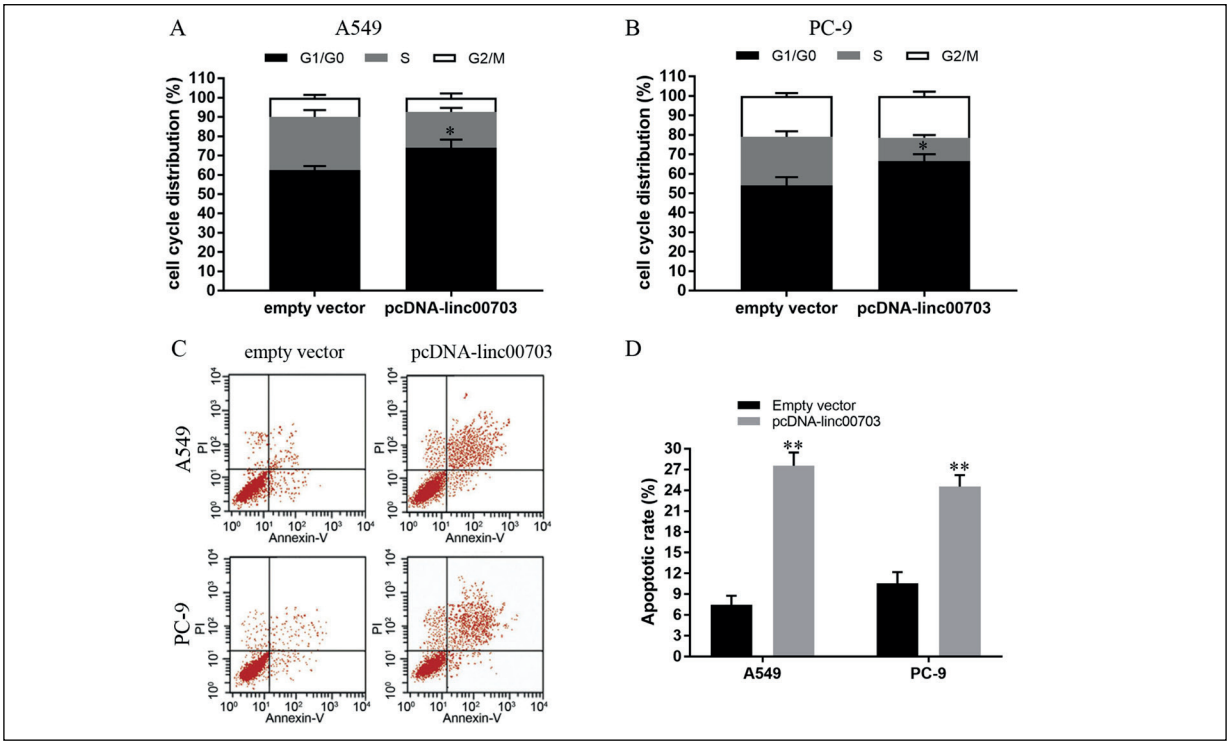

Figure 3. Effects of linc00703 on NSCLC cell cycle and apoptosis. **A, B,** The results of flow cytometry manifested that after overexpression of linc00703, the cell cycle was arrested in G1/G0 phase. **C, D,** The results of flow cytometry manifested that after overexpression of linc00703, the apoptosis rate of NSCLC cells rose.

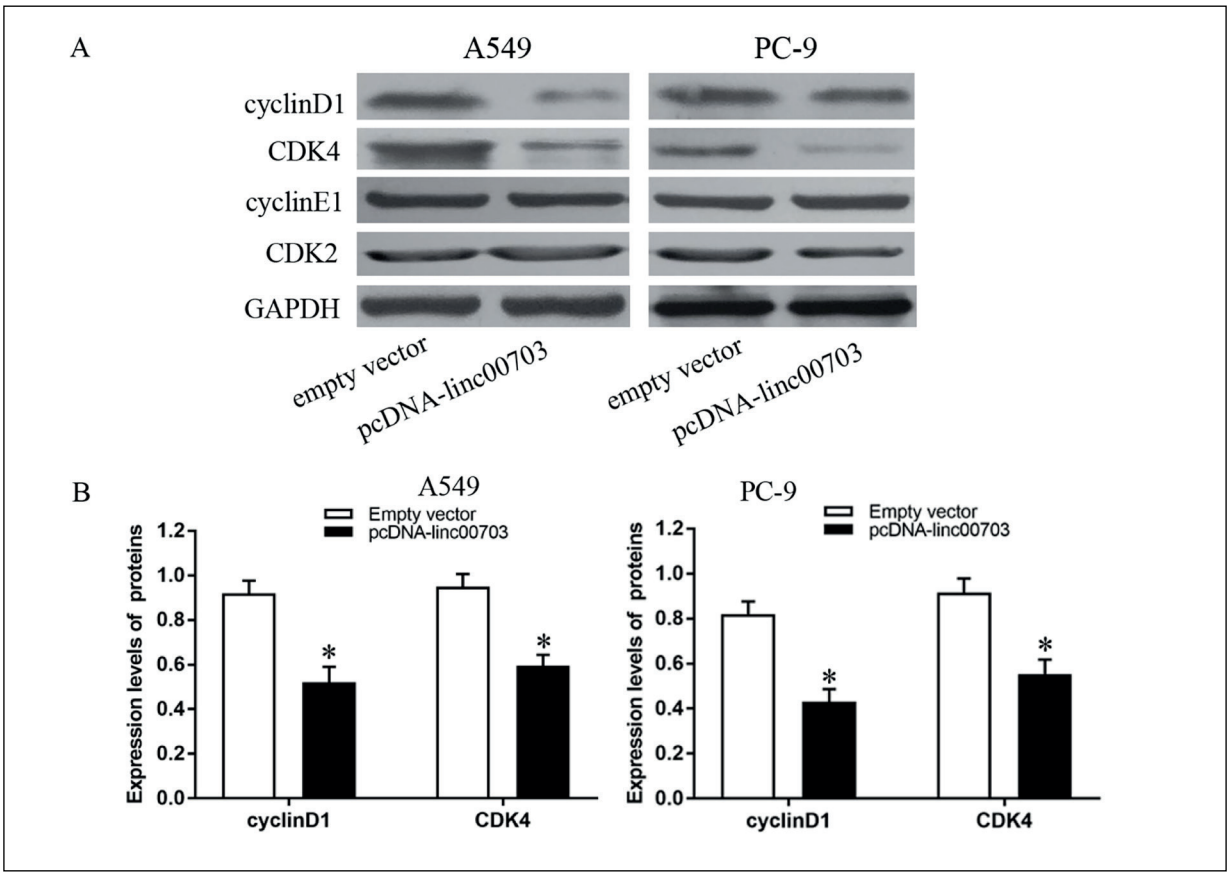

Figure 4. Linc00703 regulated cyclinD1/CDK4 expression. **A, B,** The results of Western blotting showed that after overexpression of linc00703 in NSCLC cells, the protein expressions of cyclinD1 and CDK4 declined, while those of cyclinE1 and CDK2 did not change.

changes in expressions of proteins related to cell proliferation, cycle and apoptosis were detected using Western blotting. As shown in Figure 4A and 4B, after overexpression of linc00703, the protein expressions of cyclinD1 and CDK4 declined, while those of cyclinE1 and CDK2 had no changes.

Discussion

Due to the lack of high-efficient early biomarkers, many NSCLC patients have been in the late stage when diagnosed, missing the best opportunity for treatment. In recent years, the survival rate of NSCLC patients has been significantly improved by specific molecular targeted therapy. However, the 5-year survival rate of a large number of patients is still lower due to the specificity of tumors and individuals. Therefore, it is important to find the molecular markers for early diagnosis and treatment of NSCLC[10].

LncRNAs can regulate cell proliferation, growth and apoptosis, and their abnormal expressions are also closely related to tumorigenesis. LncRNAs are involved in the occurrence and development of NSCLC. It is also reported that lncRNA H19 can regulate the resistance of NSCLC cells to cisplatin, and it can serve as a prognostic biomarker[11]. Moreover, lncRNA RMRP can promote the expressions of KRAS and SOX9 in NSCLC cells *via* inhibiting the expression of miR-206, thereby facilitating the malignant proliferation of NSCLC[12]. LncRNA SNHG7 regulates the expression of FAIM2, thus promoting the spread and metastasis of NSCLC[13]. However, there have been no reports about the expression and function of linc00703 in NSCLC. In this study, the expression of linc00703 in NSCLC was explored for the first time. It was found that linc00703 had a down-regulated expression and played a similar role to a cancer suppressor gene.

The occurrence of tumor is closely related to the cell cycle disorder. The cell cycle is a strict and orderly biological process under the action of a series of positive and negative regulators[14,15]. As important positive regulators promoting the cell cycle progression, cyclinD1 and CDK4/6 form the cyclinD1-CDK4/6 complex, which initiates DNA replication in S phase[16,17]. The expressions of cyclinD1 and CDK4 are up-regulated in a variety of tumors including NSCLC, leading to the out-of-control cell cycle, as well as infinite proliferation and division, and resulting in malignant tumors[18,19]. As important regulators, lncRNAs can be involved in the occurrence and development of tumors through regulating the expressions of cell cycle-related proteins cyclinD1 and CDK4[20]. Fu et al[21] found that lncRNA NR2F2-AS1 can positively regulate CDK4, thereby facilitating the proliferation of prostate cancer cells. In this study, it was found in the *in vitro* experiments that the expressions of cyclinD1 and CDK4 declined after overexpression of linc00703.

Conclusions

Summarily, lowly expressed linc00703 can regulate the expression of cyclinD1/CDK4 in NSCLC, thereby regulating the malignant proliferation of NSCLC cells. Linc00703 may serve as a new target for diagnosis and treatment of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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