

Expression and Effects of Long Non-Coding RNA, LINC01124, in Non-Small Cell Lung Cancer

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Objective: To investigate the expression and evaluate the clinical significance of long non-coding RNA, LINC01124, in non-small cell lung cancer (NSCLC) and to study its influence in this tumor.

Methods: Hundred specimens of NSCLC tissues and normal lung tissues after surgery were collected. The qRT-PCR for LINC01124 expression was performed on cancerous and normal lung tissues. The correlations between the expression of LINC01124 and pathological characteristics were analyzed. PcDNA-LINC01124 was transfected to upregulate LINC01124 expression in NSCLC cells, and the transfection efficiency was evaluated by the qRT-PCR. CCK8 assay, wound-healing assay, and the Transwell assay were performed to evaluate the effect of ectopic LINC01124 expression on proliferation, migration, and invasive of NSCLC cells.

Results: The expression level of LINC01124 was downregulated in tumor tissues when compared with the paired normal lung tissues ($P < 0.05$). The expression of LINC01124 was associated with patients' age and distant metastasis ($P < 0.05$). Enforced expression of LINC01124 significantly inhibited the proliferation, migration, and invasive ability of NSCLC cells.

Conclusion: The expression of LINC01124 was decreased in patients with NSCLC of older age and with those having distant metastasis. LINC01124 may inhibit cell proliferation, migration, and invasive ability.

Keywords: non-small cell lung cancer, NSCLC, long non-coding RNA, lncRNA, LINC01124

Introduction

Lung cancer is one of the most fatal malignant tumors, with the number of new cases worldwide exceeding 1.8 million per year.¹ In 2015, there were approximately 4,292,000 new cases of cancer in China and 2.8 million associated deaths, among which lung cancer was one of the leading causes.² Many pathological types of lung cancer exist, but approximately 85% of lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC), which has a 5-year survival rate of only 10%.³ Although there has been significant progress made in recent years in improving diagnoses and lung cancer treatments, the prognoses remain unoptimistic. Therefore, further research into the molecular mechanisms behind the development and progression of NSCLC is imperative.^{4,5}

The human genome contains approximately 20,000 protein-coding genes, which account for only 2% of the total genes. More than 90% of transcripts are non-coding RNAs. Long non-coding RNAs (lncRNAs) are a type of non-coding RNA, greater than 200 nucleotides in length.^{6,7} They have no open reading frame, and cannot be translated into protein. Increasing evidence suggests that lncRNAs play indispensable roles in the proliferation, growth, and apoptosis of tumor cells.⁸ The

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Table 1 Sequences of Primers for qRT-PCR

Name	Sequences (5' to 3')
The PCR primers for GAPDH	
Forward	CCACATCGCTCAGACACCAT
Reverse	ACCAGGCGCCCAATACG
The PCR primers for LINC01124	
Forward	AGACTTGTTCTGCCACCTTTG
Reverse	GCCCGCCTCTTCTCCTT

lncRNAs participate in the development and progression of tumors by interfering with processes related to tumorigenesis.⁹ Studies have confirmed that abnormal lncRNA expression is associated with a variety of cancers, including lung cancer, although the specific mechanisms by which lncRNAs influence tumorigenesis remain unclear. The lncRNAs play a dual role in lung cancer, acting as both oncogene and tumor suppressor gene.^{10,11} Therefore, in the vast network of cancer-related processes, it is particularly important to explore the role of lncRNAs in tumor formation and metastasis.

Long intergenic non-protein coding RNA 1124 (LINC01124) is located on chromosome 2q31.1, and contains one exon. In this study, we investigated the expression of LINC01124 in cancer tissue and adjacent normal tissue samples from 100 patients with NSCLC, and analyzed correlations between expression and clinical features of NSCLC. By adopting this approach, we explored whether LINC01124 had the potential to be a new biomarker for the prediction and diagnosis of metastatic NSCLC. Finally, we investigated the effects of LINC01124 on the proliferation, migration, and invasion of lung cancer cells using several in vitro assays.

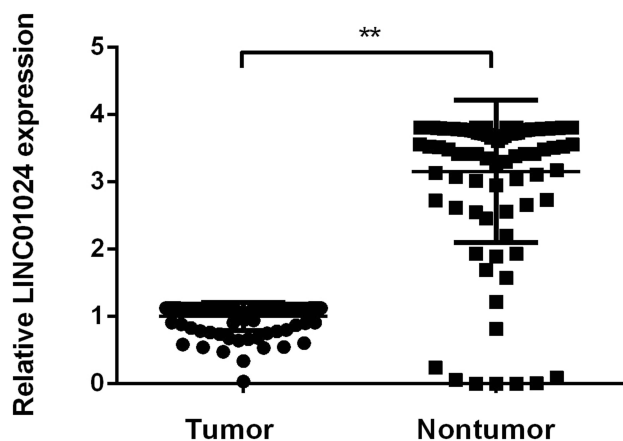


Figure 1 The LINC01124 expression levels in lung cancer tissue and normal lung tissues. ** $P < 0.01$.

Methods

Patients and Tissue Samples

Hundred NSCLC tissues and matched adjacent non-tumor tissues were collected from patients in Shengjing Hospital between 2016 and 2017. All patients recruited in this study were not subjected to preoperative radiotherapy or

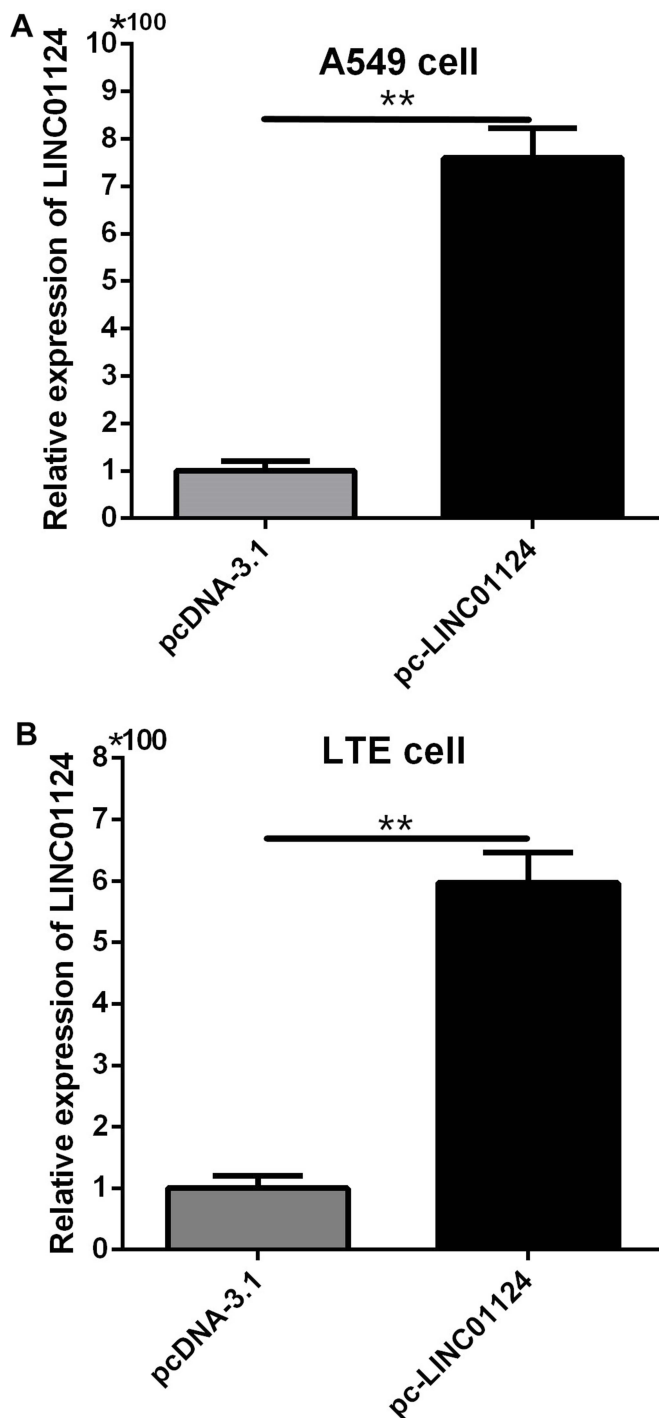


Figure 2 Plasmids and pc-linc01124 were, respectively, transfected into A549 (A) and LTE cells (B) to obtain LINC01124 overexpression. ** $P < 0.01$.

chemotherapy. This research was approved by the Ethics Committee of Shengjing Hospital, and human samples were obtained with written informed consent from all patients. All tissue samples were excised and stored in liquid nitrogen immediately until total RNA extraction.

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from frozen tissue samples or cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. For reverse transcription, 500 ng total RNA was reverse transcribed in a final volume of 20 μ L using the PrimerScript RT reagent Kit (TaKaRa, Beijing, China) according to the manufacturer protocol. The qRT-PCR was performed using the SYBR Premix Ex Taq II (TaKaRa, Beijing, China) on 7900HT Fast Real-Time PCR system according to the manufacturer's instructions. The primers are shown in Table 1. Ct-values were calculated using SDS 2.4 software. GAPDH was used as an internal control. The Ct-value for each sample was calculated with the $\Delta\Delta$ Ct-method, and the results were expressed as $2^{-\Delta\Delta$ Ct to analyze

the fold change (tumor vs normal): $\Delta\Delta$ Ct=(Ct_{target gene}-Ct_{tactin})_{normal}-(Ct_{target gene}-Ct_{tactin})_{tumor}.

Cell Culture

A549 cell line and LTE cell line were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). Cells were incubated at 37.5°C with 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum (10% FBS, GIBCO), 100 U/mL penicillin, and 100 mg/mL streptomycin.

Overexpression Vector Transfection

We used a Linc01124-overexpression vector (pcDNA3.1-linc01124) (Nanjing GenScript Biotech Corp, Nanjing, China) to induce overexpression of linc01124. The A549 and LTE cells were transfected by pcDNA3.1-linc01124 for 48 hrs and the overexpression efficiency was detected by qPCR.

Cell Proliferation Assay

Cell proliferation was determined using the cell counting kit-8 (CCK-8) assay (Dojindo, Japan, Tokyo), according to the manufacturer's instructions. After transfection, A549

Table 2 Correlation Between Clinicopathological Features and LINC01024 Expression in NSCLC Patients

	High Expression	Low Expression	Total Numbers	χ^2	P Value
Gender				1.442	0.23
Male	21	27	48		
Female	29	23	52		
Differentiation				2.679	0.102
Poorly/Medium differentiation	26	41	67		
High differentiation	17	13	30		
Smoking				0.364	0.546
No	26	29	55		
Yes	24	21	45		
Age				10.256	0.001
\leq 60	34	18	52		
>60	16	32	48		
Tumor size				0.04	0.841
\leq 3 cm	25	26	51		
>3 cm	25	24	49		
Lymph node metastasis				0.041	0.84
No	29	28	57		
Yes	21	22	43		
Distant metastasis				5.15	0.023
No	46	21	87		
Yes	4	9	13		

cells and LTE cells were seeded into 96-well plates (2×10^4 cells/well), respectively, and incubated in RPMI 1640 at 37°C and 5% CO₂ atmosphere. At 24, 48, and 72 hrs, CCK-8 (10 µL/well) solution was added to measure cell viability. After 1.5 hrs, the absorbance of each well was measured at 450 nm. Each experiment was repeated at least three times independently.

Wound-Healing Assay

Wound-healing assay was performed to detect the migration ability and compare the difference among the two experimental groups (pcDNA3.1-linc01124 and blank pcDNA3.1) of A549 and LTE cells, respectively. 2×10^5 cells/well were seeded in 6-well plates. When cells were completely adherent, a sterile 20 µL tip was used to scratch a straight line through the cell layer in each well. Cell migration was determined by detecting the average distance the growing cells had migrated into the wound surface under microscopy at the designated time periods. Assays were repeated three times for each clone.

Transwell Assay

The Transwell assay was performed to detect the migration and invasive abilities. A549 and LTE cells were grown in RPMI 1640 medium containing 10% FBS and transfected with pcDNA3.1-linc01124 and the blank pcDNA3.1. After 24 hrs, the cells were harvested by trypsinization and washed once with phosphate-buffered saline.

To measure cell migration, Transwell chambers (8 µm; Corning Incorporated, Corning, NY, USA) were placed into the wells of 24-well culture plates. Matrigel transwell chambers were used to measure cell invasion.

A total of 1×10^5 cells were cultured in 100 µL of serum-free RPMI 1640 medium added into the upper chamber of the Transwells. In the lower chamber, 600 µL of RPMI 1640 containing 20% FBS was added. After 24 hrs of incubation at 37°C with 5% CO₂, the migrated cells were stained with DAPI (Beyotime, China, Shanghai). The number of invaded cells was counted and the average value was determined through three different random fields.

Results

Detection of LINC01124 Expression by qRT-PCR

The qRT-PCR was used to detect the expression levels of LINC01124 in cancer tissue and adjacent normal tissue from NSCLC samples. Results showed that the LINC01124

expression levels in lung cancer tissue were significantly lower than normal tissue ($t = -19.969$, $P < 0.01$) (Figure 1).

The recombinant plasmid pcDNA3.1-LINC01124 and blank-plasmid pcDNA3.1 were transfected into both lung cancer A549 and LTE cells, and LINC01124 expression was detected. Each experiment was repeated at least three times. At 48 hrs after transfection, transfection efficiency was determined using qRT-PCR. As depicted in Figure 2 (A and B), the transfected pcDNA3.1-LINC01124 significantly increased LINC01124 expression in both A549 and LTE cells. We used pcDNA3.1-LINC01124 to perform further analysis.

Correlation Between LINC01124 Expression and Clinical Features of NSCLC

Using the median value of LINC01124 expression levels in cancer tissue as a cutoff, our 100 patient cohort was divided into a high expression group ($n = 50$) and a low expression

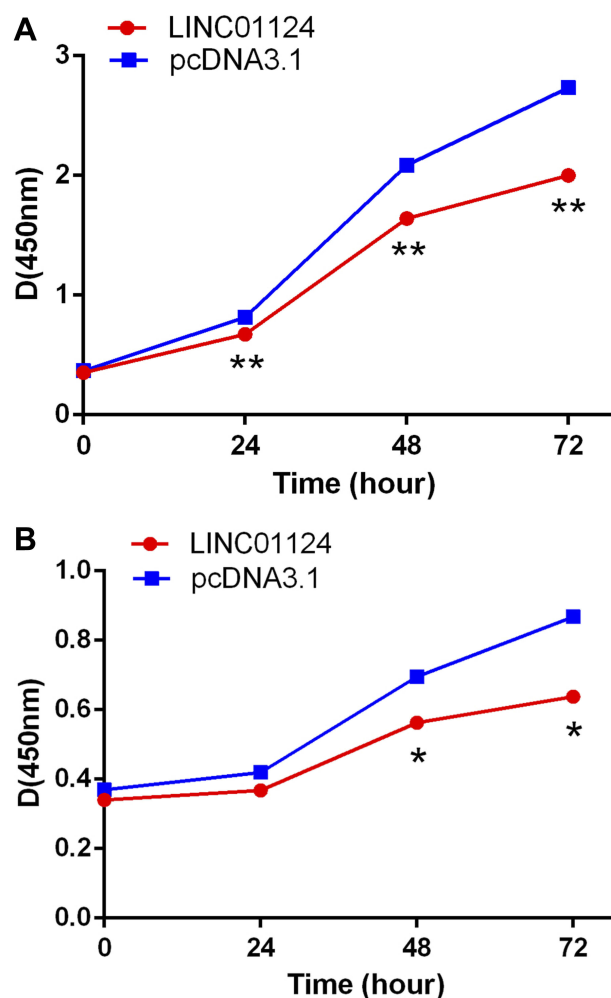


Figure 3 The proliferation ability of LINC01124-overexpressing cells and blank cells (A) A549 cells (B) LTE cells. * $P < 0.05$, ** $P < 0.01$.

group (n=50). Correlations between LINC01124 expression levels and clinical features were analyzed using a chi-squared test. We found that LINC01124 expression levels showed significant correlations with age and distant metastasis ($P < 0.05$); older patient age correlated with lower LINC01124 expression levels, and patients with distant metastasis had further lower LINC01124 expression levels. There were no significant correlations between LINC01124 expression and sex, smoking status, lymph node metastasis, tumor size, or tumor differentiation ($P > 0.05$) (Table 2).

The Effects of LINC01124 Expression on Cellular Proliferation

A CCK-8 assay revealed that LINC01124 expression in A549 and LTE cells was increased following transfection of pcDNA3.1-LINC01124. When compared with the control group (blank plasmid), the proliferation of LINC01124-

overexpressing A549 (Figure 3A) and LTE (Figure 3B) cells was significantly inhibited.

The Effects of LINC01124 Expression on Cellular Migration

To investigate the influence of LINC01124 on migration in NSCLC cell lines, we performed a wound-healing assay. The number of cells in the scratched area in the pcDNA3.1-LINC01124 group was significantly less compared with the control group (blank plasmid) at the designated time periods (24 hrs, 48 hrs). The assay showed that overexpression of LINC01124 leads to prolonged scratch wound closure in A549 and LTE cells as compared with control cells (Figure 4A, B).

Transwell cell invasion assay indicated that the ability of LINC01124-overexpressed A549 cells and LTE cells to

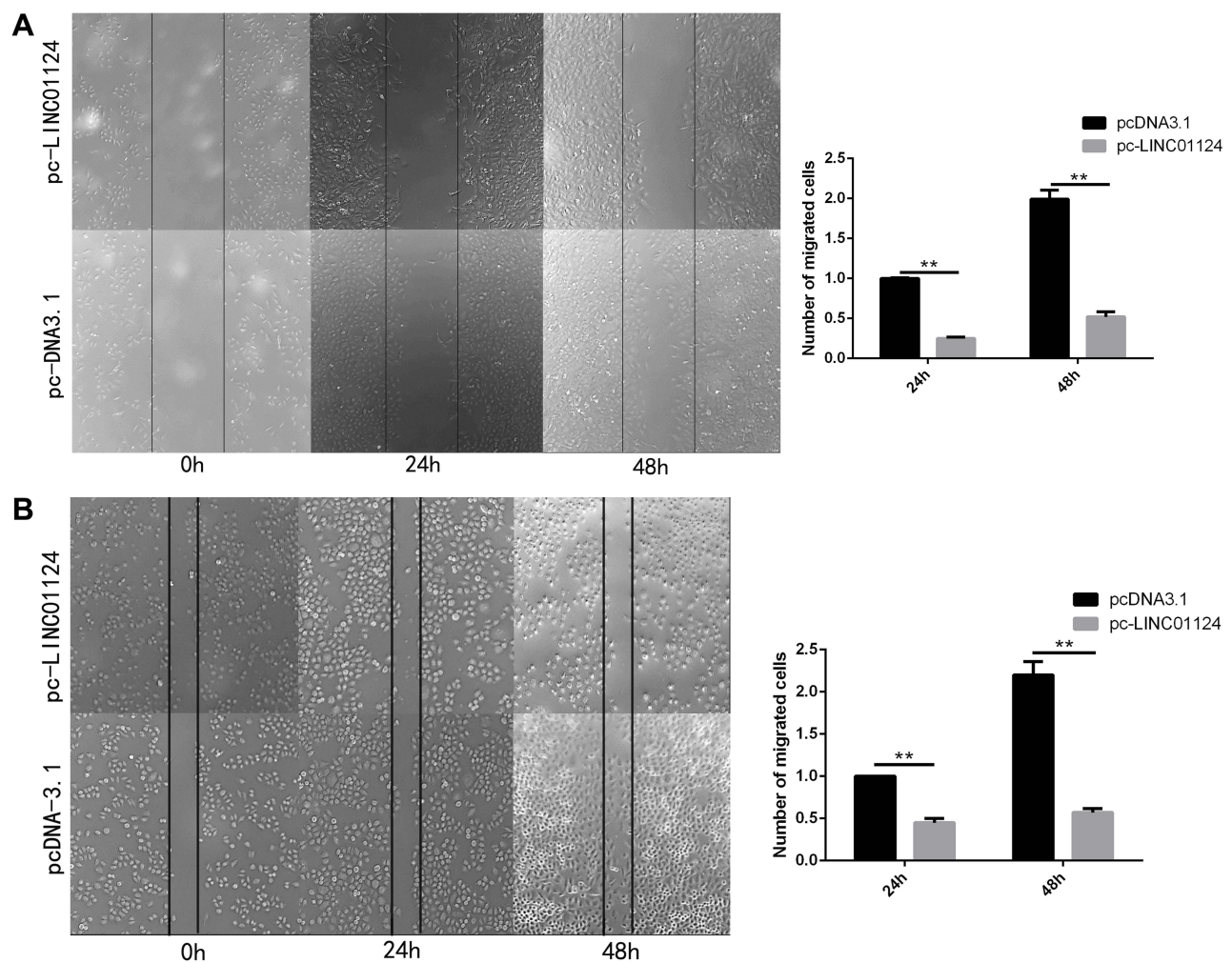


Figure 4 The results of wound-healing assay in LINC01124-overexpressing cells and blank cells. (A) A549 cells (B) LTE cells. ** $P < 0.01$.

migrate in the transwell chamber significantly decreased compared with empty vector controls (Figure 5A, B).

The Effects of LINC01124 Expression on Cellular Invasion

We next performed a Transwell assay with Matrigel, which demonstrated that A549 and LTE cell invasion is inhibited after LINC01124 overexpression as compared with empty vector controls (Figure 5A, B).

Discussion

NSCLC is one of the leading causes of cancer-related deaths worldwide.^{12,13} Due to the limitations in early diagnosis of NSCLC, its 5-year survival rate is still less than 20%.¹⁴ The lncRNAs were once considered to be “transcriptional noise”; however, accumulating evidence suggests that lncRNAs have important biological functions.^{15–17} Studies have

shown that lncRNAs play critical roles in both physiological and pathological cellular processes,^{18,19} are involved in the development of various diseases including tumors,²⁰ and are associated with lymphatic and distant metastasis, impacting on patient prognosis.²¹

A number of NSCLC-related lncRNAs have been discovered.^{22–24} For example, in 2013, Liu et al found that the lncRNA HOTAIR showed significantly elevated expression in NSCLC, and its high expression levels were closely related to lymph node metastases and patient prognoses.²⁵ Zhang et al, in their meta-analysis, found that high expression levels of the lncRNA MALAT1 were associated with lung cancer prognosis.^{26,27} In our study, we found that the lncRNA LINC01124, located on chromosome 2q31.1, may be involved in NSCLC. First, in examining NSCLC tissues, we found that LINC01124 expression levels were significantly lower than those in adjacent normal tissues. Second,

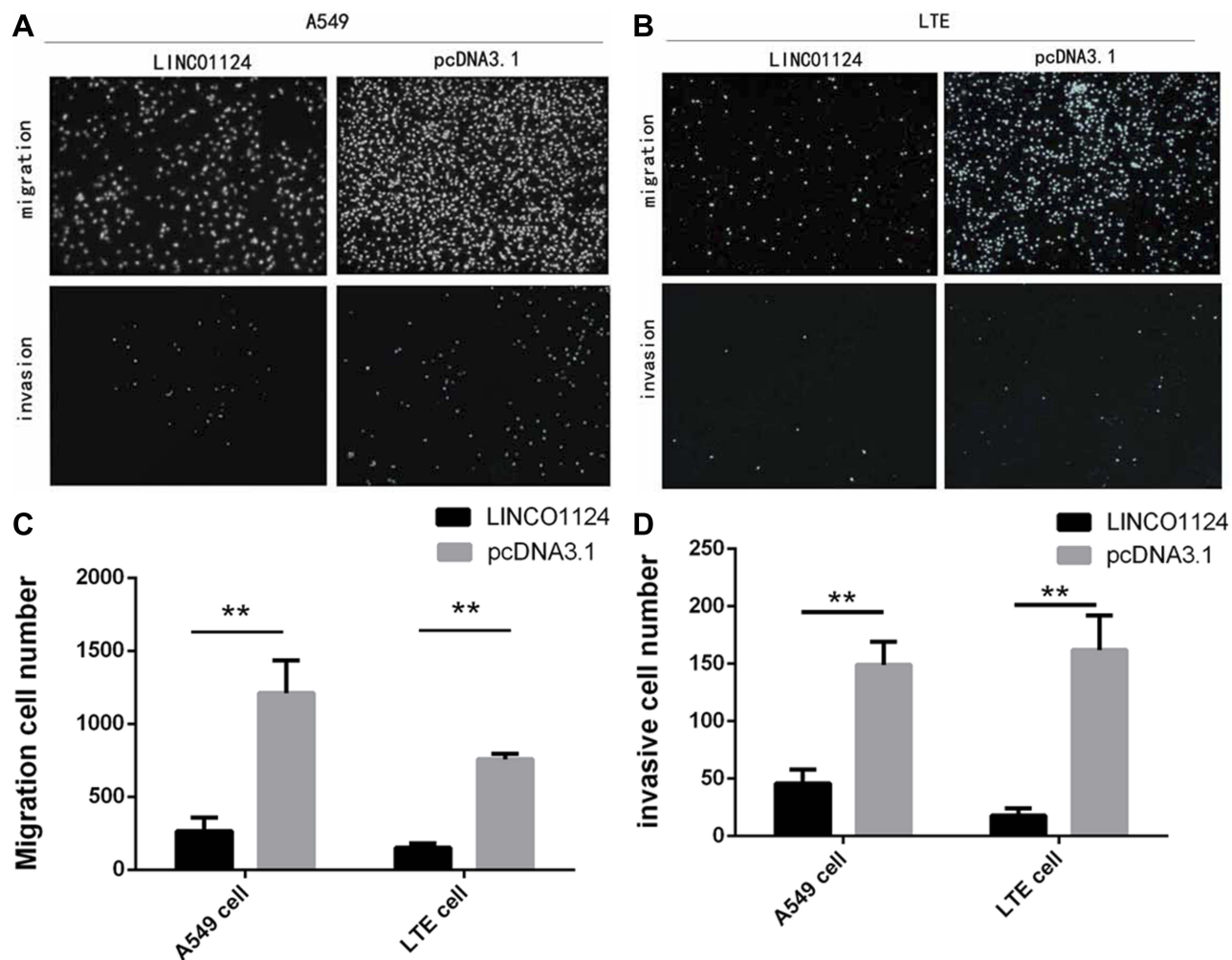


Figure 5 The result of Transwell assay in LINC01124-overexpressing cells and blank cells. (A) A549 cells. (B) LTE cells. (C) Number of cells showing migration. (D) Number of cells showing invasion. **P<0.01.

by increasing LINC01124 expression levels in NSCLC cells in vitro, we significantly inhibited the proliferation, migration, and invasion of these cells, demonstrating that LINC01124 suppresses key tumorigenic properties of NSCLC cells.

We first detected the expression of LINC01124 in the lung cancer tissue samples and adjacent normal tissue samples from 100 patients with NSCLC. We found that the expression of LINC01124 was significantly lower in NSCLC tissues than in normal adjacent tissues. We then analyzed the clinical features of 100 patients and found a significant correlation between LINC01124 levels and patient age and distant metastasis.

We further studied the effect of LINC01124 on in vitro cultured NSCLC cells. We transfected the recombinant plasmid pcDNA3.1-LINC01124 into A549 and LTE cells. The proliferation, migration, and invasion of LINC01124-overexpressed A549 and LTE cells significantly decreased, thereby suggesting that LINC01124 suppressed the proliferation, migration, and invasive abilities of NSCLC cells.

Relatively few studies on LINC01124 have been published, and its function in NSCLC and other cancers remains unclear. Our results suggested that LINC01124 had inhibitory effects in NSCLC, although we lacked in vivo data to support our observations; therefore, in vivo studies are warranted in the future to delineate the precise role of this molecule in lung cancer etiology.

In conclusion, our study demonstrated the important role of LINC01124 in the development of NSCLC and regulation of biological functions of cancer cells. It is expected to be a new biomarker and molecular target for the diagnosis and treatment of NSCLC.

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Disclosure

The authors report no conflicts of interest in this work.

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