

## Original Article

# LINC00961 functions as an anti-oncogene in non-small cell lung carcinoma by regulation of miR-3127

Yong-Gang Liu<sup>1</sup>, Jia Li<sup>2</sup>, Fang Nie<sup>1</sup>, Gao-Wa Jin<sup>3</sup>

<sup>1</sup>Department of Thoracic Oncology, Baotou Cancer Hospital, Baotou 014000, Inner Mongolia, China; <sup>2</sup>Department of Immunization Planning, Baotou Disease Control and Defense Center, Baotou 014000, Inner Mongolia, China;

<sup>3</sup>Department of Oncology, Ordos Central Hospital, Ordos 017000, Inner Mongolia, China

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**Abstract:** Background: This study set out to explore the regulatory relationship between LINC00961/miR-3127 axis and non-small-cell lung carcinoma (NSCLC), so as to provide a new and effective molecular target for targeted therapy of NSCLC. Methods: RNA-seq and miRNA-seq data of NSCLC and normal samples were obtained from The Cancer Genome Atlas (TCGA) database for analyzing LINC00961 and miR-3127 expression. Eighty-six pairs of clinical NSCLC tissues and adjacent normal tissues as well as NSCLC cell lines were obtained. Measurements of LINC00961 and miR-3127 levels were done using real-time-quantitative polymerase chain reaction (RT-qPCR). Furthermore, LINC00961 and miR-3127 in NSCLC cell were regulated respectively. The NSCLC cell proliferation, invasion and migration were determined with MTT assay, Transwell and wound healing assays, respectively. The levels of invasion- and apoptosis-related proteins were detected using western blots, and the connection of LINC00961 and miR-3127 was identified using dual luciferase reporter (DLR) assay. Results: Differential analysis results of TCGA databases identified that LINC00961 was ubiquitously expressed at low levels in NSCLC, while miR-3127 was highly expressed. Similar expression trends of LINC00961 and miR-3127 were observed in clinical NSCLC samples and cell lines. Overexpression of LINC00961 and knockdown of miR-3127 significantly reduced NCI-H1299 cell migration, invasiveness, and multiplication, decreased MMP-2, MMP-9 and Bcl-2 protein levels, and increased E-cadherin, Bax and Caspase-3 protein levels. The DLR assay confirmed that miR-3127 can be targeted by LINC00961. Conclusion: LINC00961 functions as an anti-oncogene in NSCLC by modulating miR-3127.

**Keywords:** LINC00961, miR-3127, NSCLC, invasion, anti-oncogene

## Introduction

As a common subtype of lung cancer, non-small-cell lung carcinoma (NSCLC) accounts for up to 80% of cases [1]. Among newly diagnosed cancer cases in 2020, lung cancer accounts for 11.4% [2]. Most NSCLC patients have adverse prognosis, with a five-year survival rate of <18% [3]. Clinically, the treatments of NSCLC are mainly surgical treatment or surgery combined with chemotherapy, but due to drug resistance of cancer during treatment, the chemotherapy effect is far from satisfactory [4, 5]. Currently, scholars believe that the occurrence of NSCLC is closely related to environmental deterioration, bad living habits, aberrant expression profiles of long non-coding RNAs (lncRNAs) or microRNAs (miRNAs), immune escape and autophagy [6-9]. Moreover, lncRNAs

and miRNAs have been confirmed to regulate each other and jointly participate in the occurrence and development of NSCLC [10]. Therefore, it is necessary to investigate its pathogenesis and discover more biomarkers to guide NSCLC diagnosis and treatment.

The existing studies show that lncRNAs are critical in carcinogenesis and progression such as gastric carcinoma [11], osteosarcoma [12], liver carcinoma [13], colorectal carcinoma [14], and NSCLC [15]. lncRNAs are epigenetic regulatory molecules that can not only modulate the chemical modification of histones and DNA, but also regulate genes of the epigenetic pathway themselves, thus fundamentally affecting the expression of genomes [16]. As competitive endogenous RNAs, lncRNAs and miRNAs can regulate tumor proliferation and metastasis [17].

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LINC00961, located on chromosome 9 with 1546 bp in length, is down-regulated in a variety of tumors such as NSCLC [18] and renal cell carcinoma [19], and can inhibit tumor invasion and metastasis, while miR-3127 can be both an anti-oncogene and an oncogene in tumors, such as glioma [20], lung carcinoma [21] and liver carcinoma [22]. Notably, the regulatory relationship and the function of LINC00961 and miR-3127 in NSCLC remain to be elucidated.

The present study investigated the expression profiles and function of LINC00961 and miR-3127 in NSCLC, together with their effects on NSCLC proliferation and invasion, aiming at clarifying the biologic implications of both in NSCLC and providing targets for clinical treatment of NSCLC.

### Materials and methods

#### *Data acquisition and pre-processing*

From The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>), miRNA-seq and RNA-seq data of 1016 NSCLC (lung squamous cell carcinoma [LUSC] + lung adenocarcinoma [LUAD]) were retrieved. RNA-seq included 515 LUAD samples with 59 paired normal tissue samples, and 501 LUSC samples with 49 paired normal tissue samples. miRNA-seq data included 513 LUAD samples with 46 paired normal tissue samples, and 478 LUSC samples with 45 paired normal tissue samples. Samples without expression of LINC00961 or miR-3127 in the downloaded data were excluded. R software v4.0.3 was employed for statistical processing, and the differences in gene and miRNA expression between the two groups were analyzed using the Wilcoxon rank-sum test, with a significance level set at  $P < 0.05$ .

#### *Tissue samples*

Tissue samples were provided by Baotou Cancer Hospital and Ordos Central Hospital. From September 2017 to November 2018, 86 pairs of surgically resected NSCLC tissues and normal counterparts were stored in liquid nitrogen at  $-196^{\circ}\text{C}$ . All patients were pathologically confirmed as NSCLC. Patients were aged 43-75 years old (mean:  $56.85 \pm 4.61$ ), without chemoradiotherapy or biotherapy before surgery. The Ethical Committee of the Baotou Cancer

Hospital reviewed and ratified this study, and all participants provided the signed informed consent authorizing the use of their tissue specimens for research.

#### *Cell culture*

Human normal lung epithelial cell line BEAS-2B and human NSCLC cell line NCI-H1299, were supplied by Procell Life Science and Technology (Wuhan, China). In an incubator at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ , BEAS-2B was cultivated in the Roswell Park Memorial Institute (RPMI) 1640 medium while NCI-H1299 cells in the Dulbecco's modified eagle medium (DMEM), both containing 10% fetal bovine serum (FBS). The culture medium was changed in 24 h, and subculture was performed once within 48 h.

#### *Cell transfection*

pcDNA 3.1-NC (negative control), pcDNA3.1-LINC00961, inhibitor NC and miR-3127 inhibitor were supplied by Shanghai GenePharma (China). pcDNA3.1 vector was chosen as the supporter of LINC00961 overexpression in which full length sequence (NCBI Reference Sequence: NM\_001348107.3) was cloned into the KpnI and BamHI sites of a pcDNA expression vector (Invitrogen, Carlsbad, CA, USA). When NCI-H1299 cells were observed to be at logarithmic growth stage, they were seeded into 6-well culture plates and transfected with the above vectors with Lipofectamine<sup>TM</sup>2000 Kits (Invitrogen, USA) following the manufacturer's instructions. Serum-free medium was replaced for transfection when the cell confluency reached 50%. According to the transfection, NCI-H1299 cells were divided into pcDNA3.1-NC (transfected with empty pcDNA3.1 vector), pcDNA3.1-LINC00961 (transfected with pcDNA3.1-LINC00961), inhibitor NC (transfected with corresponding negative control) and miR-3127 inhibitor (transfected with miR-3127 inhibitor) groups. miR-3127 inhibitor sequence: 5'-CUUCCAGUCGAGGAUGUUUACA-3'; Corresponding inhibitor NC sequence: 5'-CA-GUACUUUUGUGUAGUACAA-3'.

#### *Quantitative real-time polymerase chain reaction (qRT-PCR) assay*

TRIzol-extracted (Invitrogen, USA) total RNA from NSCLC tissues or cells was subjected to reverse transcription of cDNA by PrimeScript<sup>TM</sup>

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**Table 1.** Sequences of the primers

| Subject gene | Primer sequence   |
|--------------|---|
| LINC00961    | Forward: 5'-CTGTTCTGGATGGGAGCGAA-3'<br>Reverse: 5'-ACAGTCACCACGAACAGCAC-3'      |
| miR-3127     | Forward: 5'-CGGGCTGTGGAATGGTAAGC-3'<br>Reverse: 5'-CTGTCAGCTCCCATTC-3'          |
| U6           | Forward: 5'-CTCGCTTCGGCAGCACATATACT-3'<br>Reverse: 5'-ACGCTTCACGAATTTGCGTGTG-3' |
| GAPDH        | Forward: 5'-AATGGGCGCCGTTAGGAAA-3'<br>Reverse: 5'-TGAAGGGTCATTGATGGCA-3'        |

RT reagent Kit (TaKaRa, Japan), after which qRT-PCR was carried out using SYBR Green reagent (Takara, Japan). Shanghai Sangong Bioengineering was responsible for the design and synthesis of primer sequences presented in **Table 1**. PCR reaction system was: cDNA (1  $\mu$ L), forward and reverse primers (0.5  $\mu$ L each), SYBR Premix Ex Taq (10  $\mu$ L), and ddH<sub>2</sub>O (8  $\mu$ L). Reaction conditions: 95°C/7 min (initial denaturation), followed by 95°C (5 s), 60°C (30 s) and 72°C (3 min) for a total of 40 cycles. LINC00961 and miR-3127 levels relative to GAPDH and U6 respectively were obtained using the formula  $2^{-\Delta\Delta Ct}$ .

### Cell multiplication assay

The multiplication of NCI-H1299 cells ( $1 \times 10^4$  cells/mL) inoculated on a 96-well culture plate was determined by MTT assay. At 0, 24, 48, and 72 h post-incubation, 20  $\mu$ L (5 g/L) MTT reagent (Promega) was added for further culture for 4 h, and the supernatant was removed by pipette. After that, 150  $\mu$ L/well DMSO was added for shaking on a shaker for 10 min. After the blank well was set to zero, a microplate reader was applied to read the absorbance at 570 nm.

### Invasion assay

100  $\mu$ L FBS-free medium (containing  $1 \times 10^4$  cells) was seeded onto the apical chamber pre-coated with Matrigel (Sigma, Japan), while 250  $\mu$ L complete medium +20% FBS was put into the basolateral chamber, for routine culture in an incubator under the condition of 37°C and 5% CO<sub>2</sub>. After 24 h, the chamber was washed twice with PBS and the invaded cells were stained with 0.1% crystal violet after fixation with polymethanol for cell counting under the microscope.

### Wound healing assay

Cells were seeded onto 24-well plates with  $1 \times 10^5$  cells/well. Scratches were made with a sterile small pipette tip when confluence was observed in cells. Then, the cells were washed and incubated for 0, 24 and 48 h to observe the wound healing under the microscope. Cell mobility = area of migrated cells/total area  $\times 100\%$ .

### Western blot

After cell lysis with lysis solution (Beyotime Biotechnology, P0013), the proteins were isolated, and the concentration was identified with the BCA method. Then 30  $\mu$ g/lane of the extracted protein was isolated by SDS-PAGE and then transferred to a PVDF membrane at 300 mA constant current for 90 min. After that, the membrane was processed for 2 h of blocking with 5% nonfat-dried milk as well as overnight cultivation with the following primary antibodies all diluted at 1:1000 and manufactured by Cell Signaling Technology: E-cadherin, MMP-2, MMP-9, Bcl-2, Bax, Caspase-3, and GAPDH. The next day, it was immersed in the HRP-linked anti-rabbit IgG (Cell signaling technology, 1:2000) for 2 h of incubation at indoor temperature. After three PBST rinses (10 min/time), it was developed using the ECL kit. Each sample was repeatedly determined three times. GAPDH level was used as loading control.

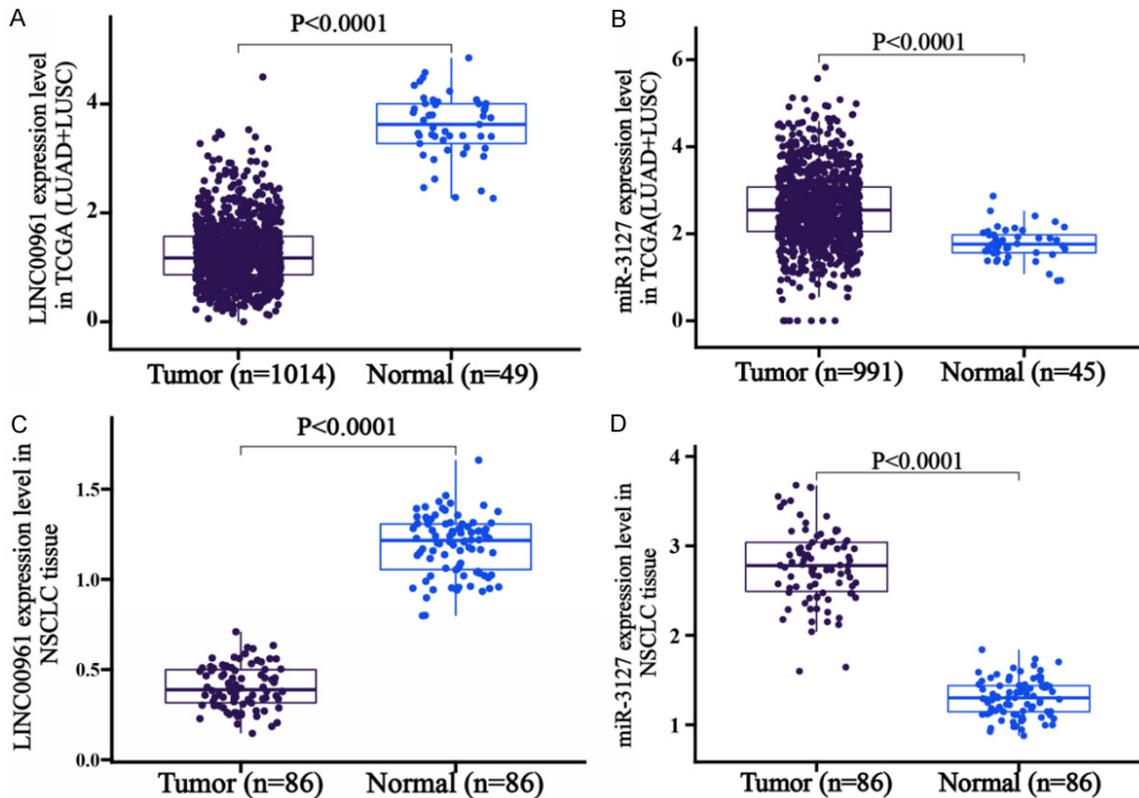
### Dual luciferase reporter (DLR) assay

First, the possible binding loci of LINC00961 and miR-3127 were predicted using bioinformatics software RNA22 Version 2.0. NCI-H1299 cells with 70% confluence were inoculated on 96-well plates, followed by the amplification and subcloning of wild-type or mutant LINC00961 into the pmirGLO vector by QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene; Agilent Technologies, Inc.). With use of Lipofectamine 2000 (Invitrogen), miR-3127 mimic and mimic NC were then co-transfected with either pmirGLO-WT-LINC00961 or pmirGLO-MUT-LINC00961 into cells. Using a DLR system (Promega), the luciferase activity was tested and normalized to the activity of Renilla luciferase.

### Statistical methods

Statistical processing was done by GraphPad Prism 6.0. Measured data were expressed as

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**Figure 1.** Expression levels of LINC00961 and miR-3127 in NSCLC. A: LINC00961 expression level in LUAD+LUSC from TCGA; B: miR-3127 expression level in LUAD+LUSC from TCGA; C: LINC00961 expression level in clinical tissue samples; D: miR-3127 expression level in clinical tissue samples; 2-tailed Student's t-test between two groups;  $P < 0.0001$ .

mean  $\pm$  SD; the differences were analyzed with 2-tailed Student's t-test or one-way ANOVA followed by Tukey's honest significance test, with  $P < 0.05$  as the significance level. Each experiment was conducted in triplicate.

### Results

#### Expression of LINC00961 and miR-3127 in NSCLC

By analyzing LINC00961 and miR-3127 expression in TCGA (LUAD+LUSC), we found that LINC00961 was down-regulated while miR-3127 was up-regulated in NSCLC tissues ( $P < 0.05$ ), **Figure 1A, 1B**. qPCR results confirmed that LINC00961 was lowly expressed in NSCLC tissues, while miR-3127 was highly expressed compared to adjacent normal tissues, **Figure 1C, 1D**.

#### Expression of LINC00961 and miR-3127 in NSCLC cells

By comparing LINC00961 and miR-3127 expression in BEAS-2B and NCI-H1299 cell lines,

we found that LINC00961 was lower while miR-3127 was higher in NCI-H1299 cells compared to BEAS-2B cells ( $P < 0.05$ ), **Figure 2A, 2B**. After transfection of pcDNA 3.1-LINC00961 into NCI-H1299 cells, LINC00961 increased and miR-3127 decreased significantly in pcDNA 3.1-LINC00961 group compared with pcDNA 3.1-NC group ( $P < 0.05$ ). miR-3127 inhibitor did not significantly alter the expression of LINC00961 ( $P > 0.05$ ) but significantly decreased miR-3127 expression as compared with inhibitor NC ( $P < 0.05$ ), **Figure 2C, 2D**.

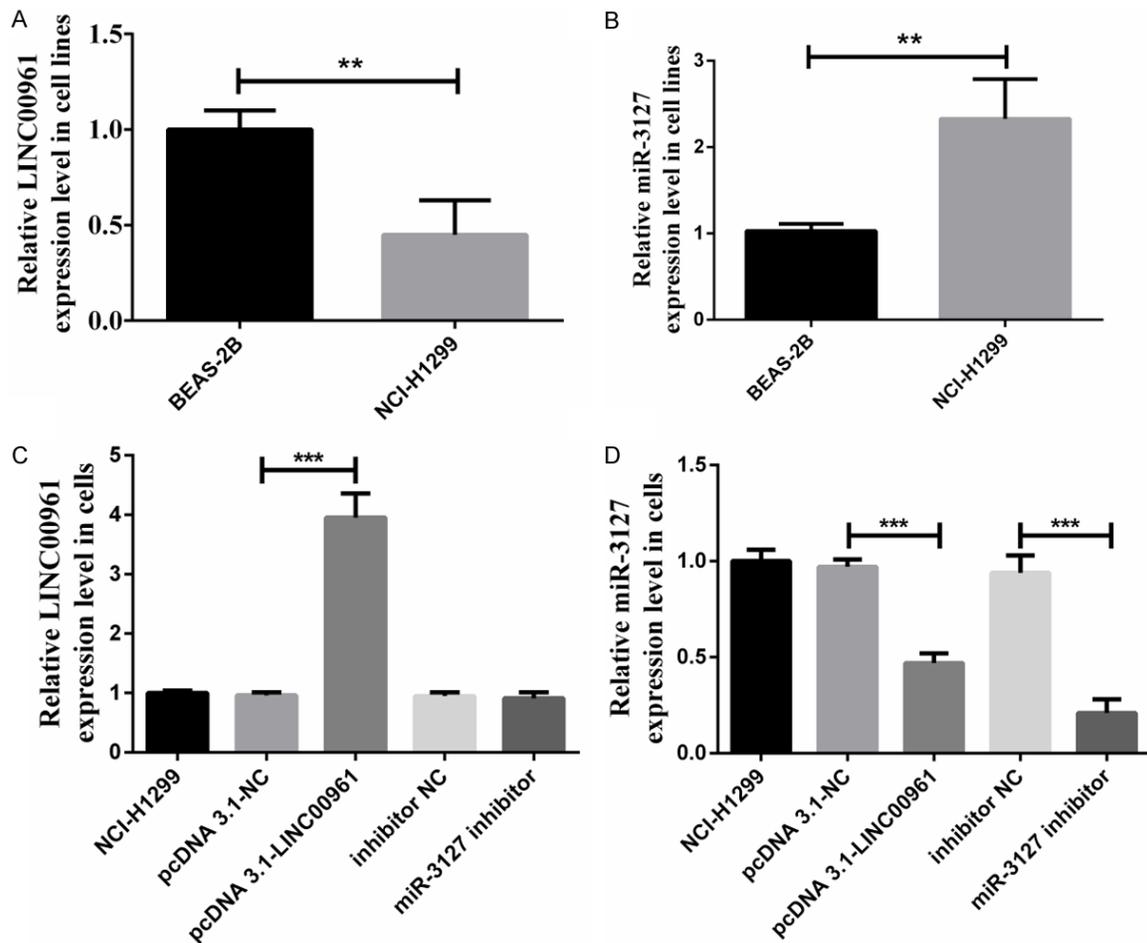
#### Cell proliferation activity of NCI-H1299 cells

MTT assay results showed markedly lowered proliferation activity of NCI-H1299 cells transfected with pcDNA 3.1-LINC00961 and miR-3127 inhibitor at 24 h, 48 h and 72 h compared with the blank control group ( $P < 0.05$ ), **Figure 3**.

#### Invasion and migration ability of NCI-H1299 cells

Wound healing and Transwell invasion assays were conducted to explore the functional roles

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**Figure 2.** Expression levels of LINC00961 and miR-3127 in NSCLC cells. A: Expression levels of LINC00961 in BEAS-2B and NCI-H1299 cell lines; B: Expression levels of miR-3127 in BEAS-2B and NCI-H1299 cell lines; C: Expression levels of LINC00961 in NCI-H1299 cells before and after transfection; D: Expression levels of miR-3127 in NCI-H1299 cells before and after transfection; 2-tailed Student's t-test between two groups: \*\*\* $P < 0.001$ ;  $n = 3$ .

of LINC00961 and miR-3127 in NSCLC cell migration and invasiveness. Overexpression of LINC00961 or knockdown of miR-3127 significantly inhibited invasion (Figure 4A, 4B) and migration (Figure 4C, 4D) of NCI-H1299 cells.

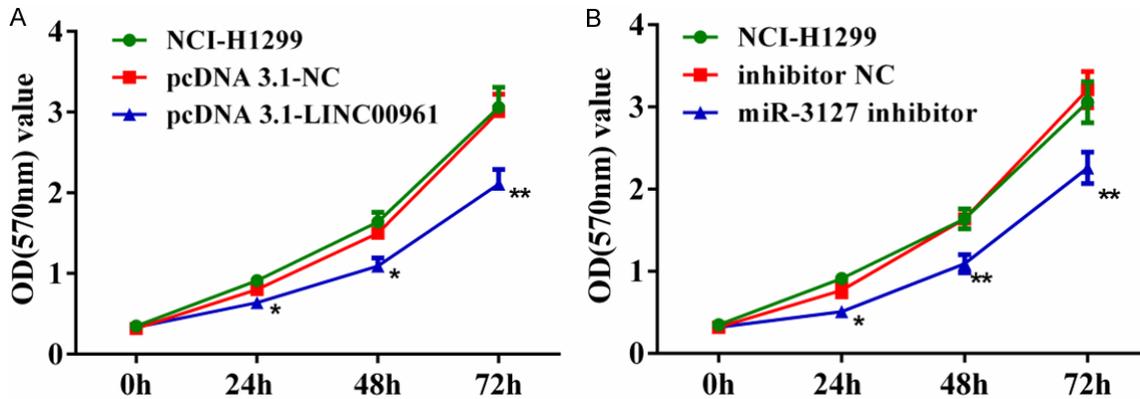
### Expression of proteins associated with invasion and apoptosis of NCI-H1299 cells

Western blot results identified that LINC00961 overexpression or miR-3127 knockdown significantly increased E-cadherin and inhibited MMP-2 and MMP-9 protein levels ( $P < 0.05$ ), Figure 5B, 5C. Overexpression of LINC00961 or knockdown of miR-3127 elevated Bax and Caspase-3 protein levels, while reducing Bcl-2 protein expression, Figure 5D, 5E.

### LINC00961 targeted and regulated miR-3127 expression

A schematic diagram of the interaction between LINC00961 and miR-3127 is shown in Figure 6A. Through online tools, miR-3127 was shown to be LINC00961's potential target, Figure 6B. Later on, NCI-H1299 cells were subjected to con-transfection of miR-3127 mimic and mimic NC with either pmirGLO-WT-LINC00961 or pmirGLO-MUT-LINC00961. miR-3127 significantly inhibited the luciferase activity of NCI-H1299 cell transfected with wild-type vector but had no effect on the luciferase activity of cells transfected with mutant vector ( $P < 0.01$ ), Figure 6C. The results indicated that LINC00961 can bind to miR-3127 and reduce the expression of miR-3127.

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**Figure 3.** Cell proliferation. A: NCI-H1299 cell proliferation after transfection with pcDNA 3.1-LINC00961; B: NCI-H1299 cell proliferation after transfection with miR-3127 inhibitor; 2-tailed Student's t-test between two groups: \* $P < 0.05$ , \*\* $P < 0.01$  vs the NC group;  $n = 3$ .

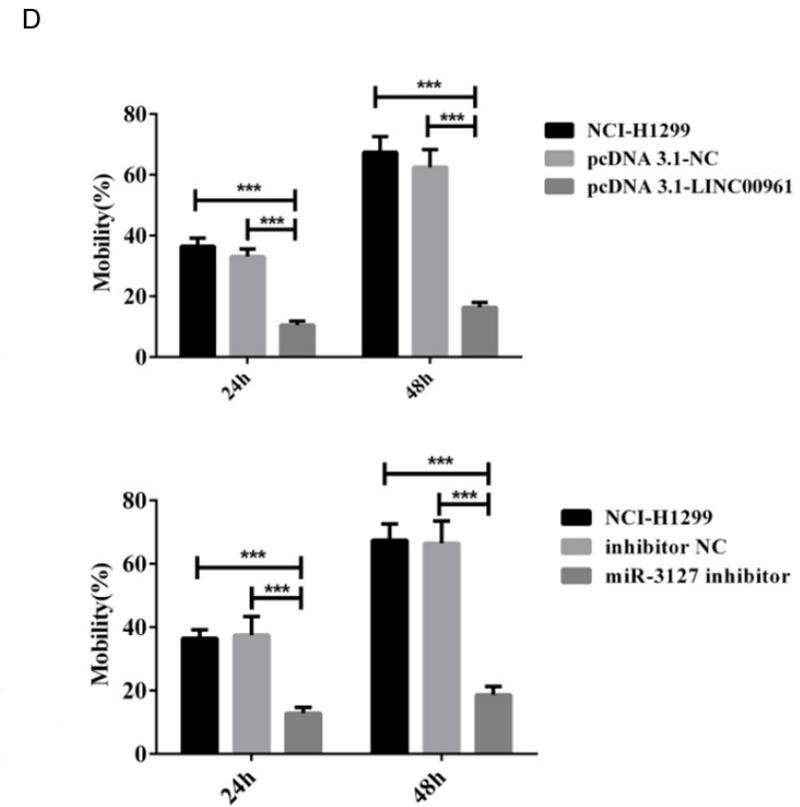
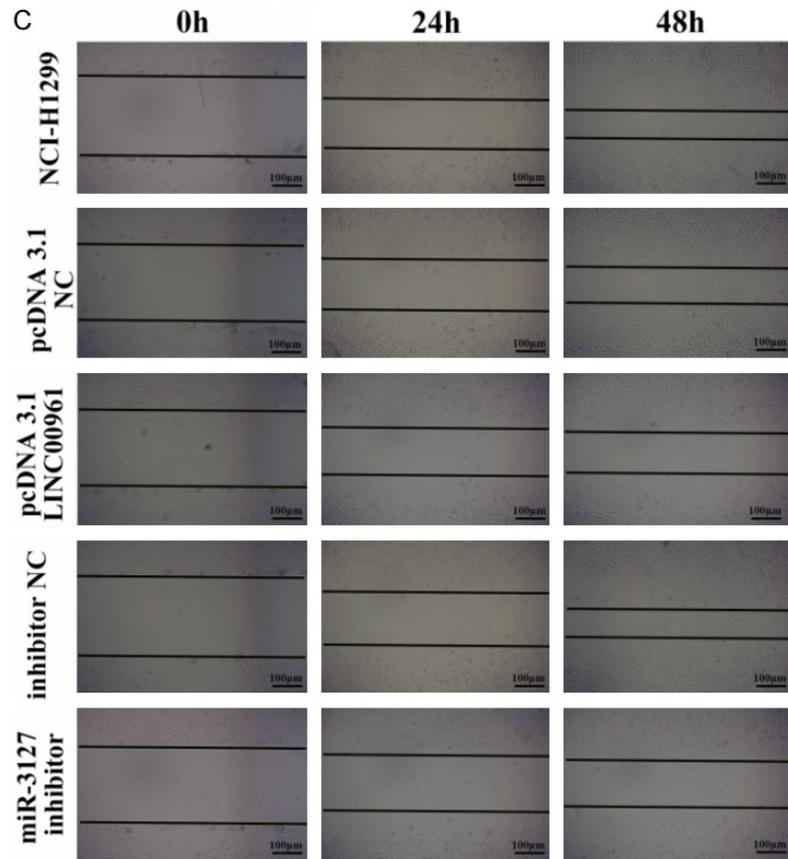
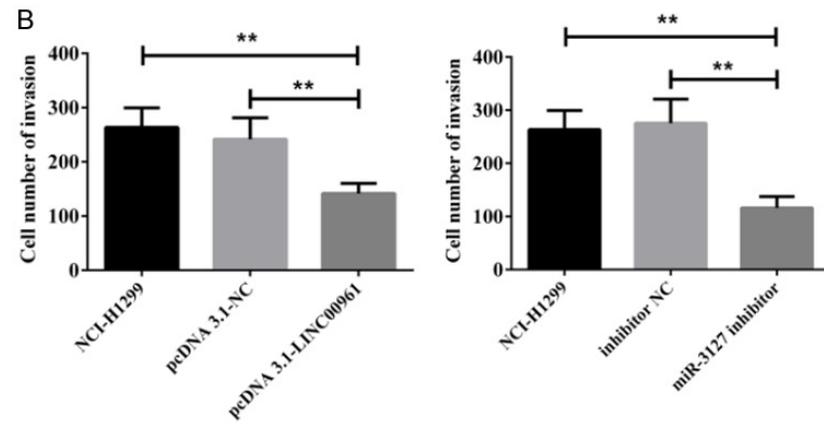
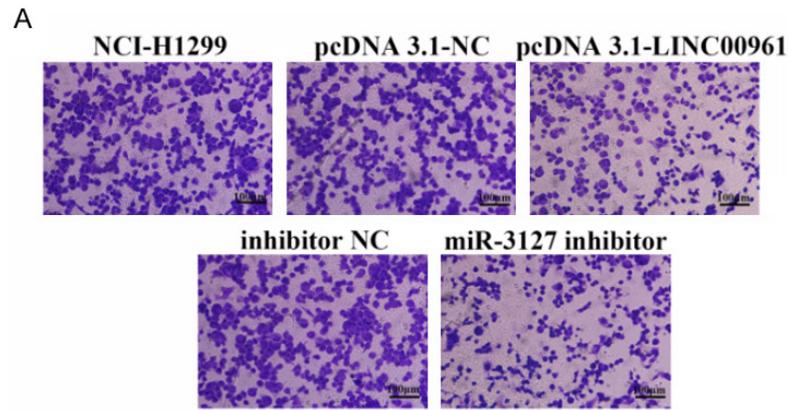
### Discussion

Non-small cell lung carcinoma (NSCLC) has high morbidity and mortality [23]. Early intervention and screening for markers that have a connection with the pathogenesis and prognosis of NSCLC are of significance for improving the survival of patients. LncRNAs can regulate inflammatory response and chromosome remodeling, and are involved in cell proliferation and apoptosis, which are closely related to chronic obstructive pulmonary disease and lung cancer [24, 25]. LncRNAs and miRNAs are key molecules of the endogenous RNA competition mechanism, and their interactions play an important role in gene regulation [26]. Li [27] reported that lncRNA HCP4 promoted NSCLC cell multiplication by up-regulating survivin through miR-320 down-regulation. Therefore, more lncRNA-miRNA regulatory mechanisms are yet to be discovered.

Our study mainly studied the function of miR-3127 and LINC00961 in NSCLC. The results showed that in clinical NSCLC tissue samples, LINC00961 was down-regulated while miR-3127 was up-regulated. Overexpression of LINC00961 and knockdown of miR-3127 significantly attenuated NCI-H1299 cell migration, invasiveness and multiplication. These results were consistent with the findings of Huang [28] who revealed that LINC00961 expression was decreased in NSCLC tissues, and downregulating LINC00961 could promote cell multiplication. Another study showed that LINC00961 was under-expressed in glioma samples, and

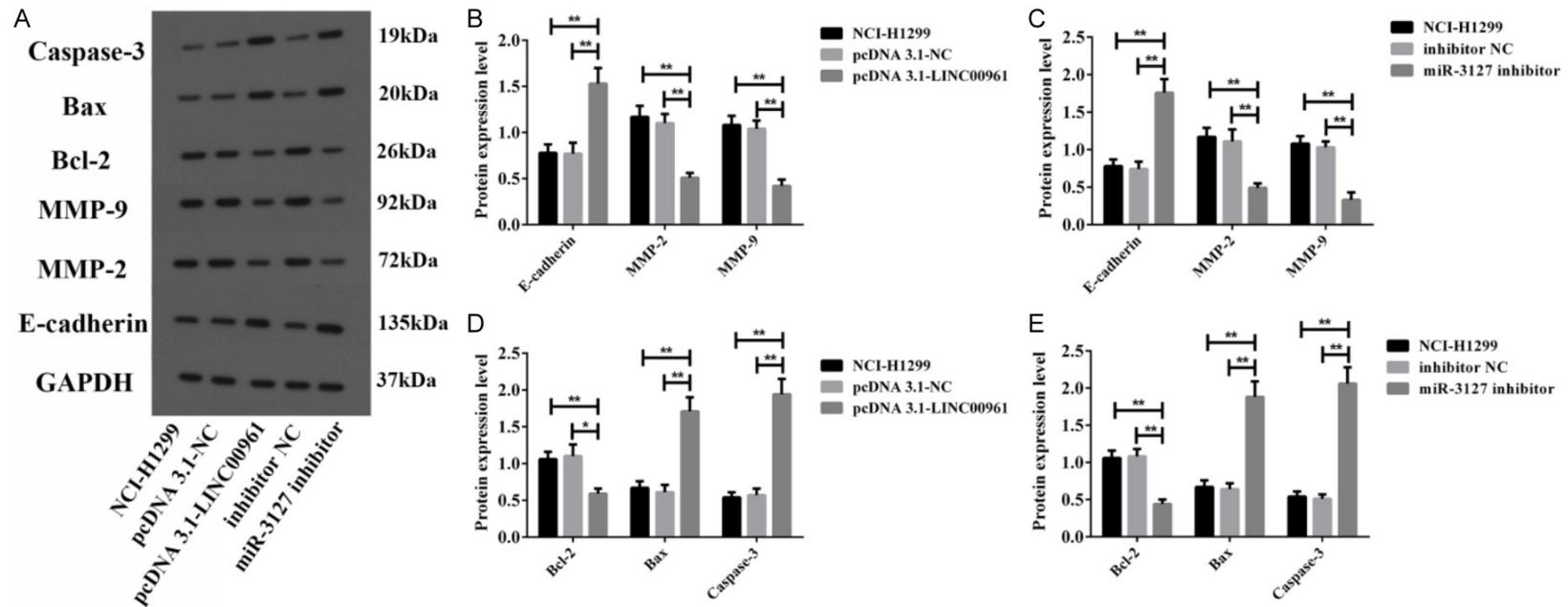
its ectopic expression could weaken glioma cell invasiveness, multiplication, migration, and epithelial mesenchymal transition (EMT) [29]. Furthermore, Sun [30] showed that in NSCLC, miR-3127-5p down-regulation promoted EMT by FZD4 regulation of Wnt/ $\beta$ -catenin axis. In this study, overexpression of LINC00961 and knockdown of miR-3127 reduced MMP-2, MMP-9, and Bcl-2 protein levels, and increased E-cadherin, Bax, and Caspase-3, with statistically significant differences. In previous clinical studies, E-cadherin was shown to have a connection with tumor differentiation and lymph node metastasis [31]. Reduced E-cadherin expression in NSCLC patients is correlated with lymph node metastasis, tumor differentiation, and unfavorable prognosis [32]. Major energy efficiencies of the MMP family, which indicates efficient degradation of basement membranes and extracellular matrix, can also promote tumor infiltration and metastasis through nascent capillaries [33]. Caspase-3 in the caspase cascade is a key step in many biologic processes inducing apoptosis [34]. Activation of Caspase-3 can induce the cleavage of specific substrates, which is essential for apoptosis [35, 36]. Besides, alterations of the ratio of Bcl-2 family proteins are shown to have a connection with mitochondrial homeostasis disequilibrium, leading to apoptosis. Increased level of pro-apoptotic protein Bax and/or decreased level of anti-apoptotic protein Bcl-2 lead to loss of mitochondrial membrane potential, which is a key process to initiate apoptosis [37, 38]. It is worth mentioning that LINC00961 acts on a polypeptide named Small Regulatory

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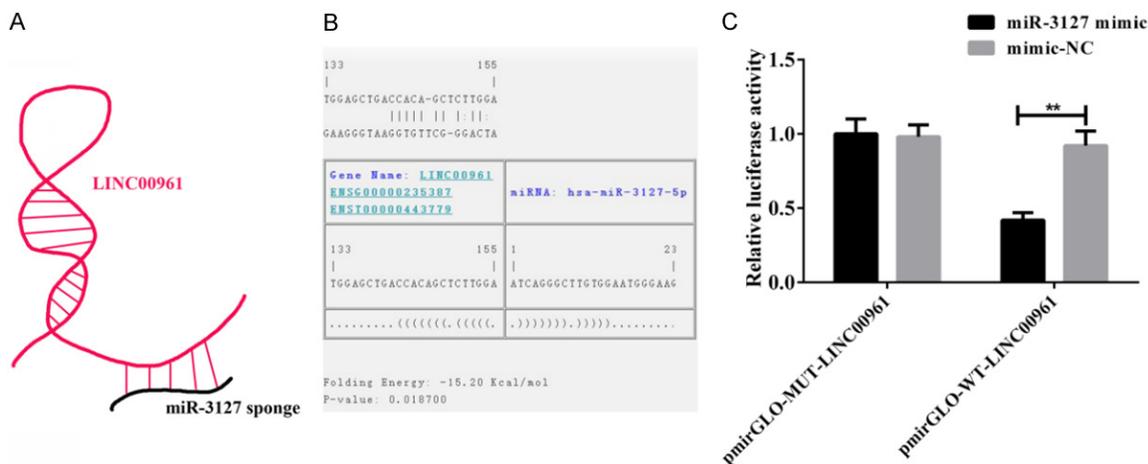
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**Figure 4.** Invasion and migration ability of NCI-H1299 cells. A: Cell invasion; B: NCI-H1299 cell invasion ability after transfection; C: Wound healing; D: NCI-H1299 cell migration ability after transfection; 2-tailed Student's t-test between two groups: \*\*P<0.01, \*\*\*P<0.001; n=3.



**Figure 5.** Expression levels of proteins related to invasion and apoptosis in NCI-H1299 cells. A: Protein expression; B: Expression levels of invasion-related proteins in NCI-H1299 cells after transfection with pcDNA 3.1-LINC00961; C: Expression levels of invasion-related proteins in NCI-H1299 cells after transfection with miR-3127 inhibitor; D: Expression levels of apoptosis-related proteins in NCI-H1299 cells after transfection with pcDNA 3.1-LINC00961; E: Expression levels of apoptosis-related proteins in NCI-H1299 cells after transfection with miR-3127; 2-tailed Student t-test between two groups: \*P<0.05, \*\*P<0.01; n=3.

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**Figure 6.** Dual luciferase reporter assay. A: Schematic diagram; B: The binding site of LINC00961 and miR-3127; C: Detection of double luciferase activity in each group; \*\* $P < 0.01$ ;  $n = 3$ .

Polypeptide of Amino Acid Response [39], which inhibits mTORC1 and affects mTORC1 activity. This complex can also control protein and ATP production and consumption of electron transport chain complexes in mitochondria. Thus, LINC00961 could regulate Bcl-2 and Bax. Finally, we verified through the DLR assay, that LINC00961 could target miR-3127. In the study of Mu [40], LINC00961 was found to suppress cutaneous melanoma multiplication and invasiveness by targeting the miR-367/PTEN axis. In patients with coronary heart disease, down-regulation of LINC00961 promoted vascular smooth muscle cell multiplication and inhibited cell apoptosis by sponging miR-367 [41]. In cervical carcinoma, LINC00319 enhanced cervical carcinoma cell migration, invasiveness and EMT process by modulating miR-3127-5p/RPP25 signaling [42]. LINC00174 accelerated colorectal carcinoma cell multiplication and migration through the miR-3127-5p/E2F7 axis [43]. In our study, we first demonstrated that LINC00961 could target miR-3127 in NSCLC.

In conclusion, lncRNA LINC00961 was down-regulated and miR-3127 was up-regulated in NSCLC, both of which played a role in the onset and progression of NSCLC and are candidate markers for disease assessment of NSCLC patients. Nevertheless, there is still room for improvement in this study. The pathologic data and follow-up data of the clinical samples were lacking in this study, and the mechanism of LINC00961 and miR-3127 in NSCLC needs fur-

ther exploration. Therefore, it is crucial to further expand the sample size and carry out basic research in combination with patient prognosis information to provide new targets for future NSCLC diagnosis and treatment.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Gao-Wa Jin, Department of Oncology, Ordos Central Hospital, 23 Ijinholuoxi Street, Dongsheng District, Ordos 017-000, Inner Mongolia, China. Tel: +86-13327060498; E-mail: jvrd72@163.com

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