

lincRNA-p21 inhibits the progression of non-small cell lung cancer via targeting miR-17-5p

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Abstract. Non-small-cell lung cancer (NSCLC) is well established as one of the major subtypes of human lung cancer. NSCLC is characterized by a high incidence rate and poor patient prognosis. Previous studies have identified that long intergenic non-coding RNA (lincRNA) serves a key role in the development of tumor and malignant metastasis. However, the majority of the underlying mechanisms for lincRNA deregulation in various diseases, including cancer and diabetes, have not been completely elucidated. In the present study, the deregulation of lincRNA-p21 in NSCLC tumor tissues in comparison to adjacent healthy tissues was examined using reverse transcription-quantitative polymerase chain reaction. Furthermore, the effect of lincRNA-p21 overexpression and knockdown on different NSCLC cell lines was further investigated *in vitro*. The association between lincRNA-p21 expression and microRNA (miR)-17-5p level in NSCLC tumor cells was also investigated to clarify the underlying mechanism. The influence of miR-17-5p on different NSCLC cell lines A549 and PC9 were also examined *in vitro* using miR-17-5p mimics and inhibitors. Bioinformatics and luciferase assays were conducted to verify the direct binding sites on lincRNA-p21 for miR-17-5p. The results demonstrated

that there was a significant low-expression of lincRNA-p21 in NSCLC tumor tissues, and lincRNA-p21 effectively inhibited the progression of lung cancer cells by suppressing cell proliferation and migration and promoting cell apoptosis. An evident negative association between lincRNA-p21 and miR-17-5p expression was observed, and the inhibitory effect of overexpressed lincRNA-p21 on lung cancer cells was counteracted by miR-17-5p. Bioinformatics and luciferase reporter analysis results confirmed that miR-17-5p is a direct target for lincRNA-p21. The present study provides evidence for lincRNA-p21 to inhibit the progression of NSCLC via direct targeting of a miR-17-5p associated signaling pathway.

Introduction

Lung cancer is a malignant tumor with a particularly high incidence rate, and its poor prognosis makes it one of the primary causes for cancer-associated mortality in the world (1). Among them, 85% of patients with lung cancer suffer from non-small cell lung carcinoma (NSCLC), which is commonly diagnosed at an advanced stage of disease (2). The 5-year overall survival rates of patients with lung cancer is <15% in spite of the recent advances in targeted therapies (1). Although several studies have reported various protein-coding genes, which are differentially expressed in NSCLC tissues, their relatively low specificity and insufficient sensitivity have presented difficulties with attempting to obtain an early diagnosis, prognosis evaluation, and recurrence prediction. Consequently, there is a requirement to develop novel molecular markers for NSCLC diagnosis and treatment (3,4).

A class of non-coding RNA, termed long non-coding RNAs (lncRNAs) due to their length of >200 nucleotides, have been the focus of research (5). LncRNAs transcriptionally activate genes that do not code for proteins, and these account for >80% of all genes (6). It is well-established that lncRNAs serve essential roles in regulating diverse biological process, including cell proliferation, apoptosis and differentiation, and tumorigenesis and metastasis in tumor tissues, which may predict the overall prognosis in certain types of cancer (7). It has been hypothesized that lncRNAs are a promising source of diagnostic biomarkers and therapeutic targets for human cancer (8).

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Abbreviations: NSCLC, non-small cell lung carcinoma; lincRNA, long intergenic non-coding RNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction; siRNA/si, small interfering RNA; NC, negative control; PI, propidium iodide; miR, microRNA; Bcl-2, B-cell lymphoma-2; MMP9, matrix metalloproteinase; SMO1, SPARC-related modular calcium binding 1; UTR, untranslated region

Key words: apoptosis, non-small cell lung cancer, long intragenic non-coding RNA, cell signaling

A member of the long intergenic non-coding RNA (lincRNA) family, lincRNA-p21, was reported to be able to restrain the invasion and metastasis of various types of cancer, including colorectal cancer and hepatocellular carcinoma, and has been demonstrated to be associated with the enhancement of epithelial-mesenchymal transition (EMT) (9,10). Furthermore, it has been reported that lincRNA-p21 is able to induce EMT by regulating its downstream miRNA, ultimately affecting hepatocellular carcinoma tumor growth (10). For example, lincRNA-p32 is downregulated in human hepatocellular carcinoma (HCC) tissues, and its upregulation remarkably suppresses the migration and invasion of HCC cells (10). Furthermore, lincRNA-p21 was demonstrated to be able to negatively regulate the expression level of microRNA (miR)-9, which directly targets E-cadherin (11). Through this interaction, the progression of HCC is inhibited by lincRNA-p21 via the miR-9/E-cadherin cascade signaling pathway (11). Previous research also identified the abnormal expression of lincRNA-p21 in NSCLC tissues (12). However, the underlying molecular mechanism of lincRNA-p21 in the initiation, development and metastasis of NSCLC remains unclear.

The present study aimed to highlight the important role of lincRNA-p21 in NSCLC tumors. Firstly, the expression of lincRNA-p21 in clinical lung cancer tissues was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and was demonstrated to be negatively associated with the advanced clinical pathology of NSCLC. Furthermore, the potential mechanism of lincRNA-p21 in regulating the proliferation and apoptosis of the lung cancer cells was explored by transfection with lincRNA-p21 small interfering (siRNA) or an overexpression plasmid. Additionally, a panel of factors associated with proliferation, apoptosis, and migration, including B-cell lymphoma-2 matrix metalloproteinase 9, were quantified by western blot analysis and RT-qPCR. Through bioinformatics analysis, it was hypothesized that the downstream miRNA of lincRNA-p21 is miR-17-5p. The role of lincRNA-p21 in regulating miR-17-5p was further demonstrated at a cellular level, and the direct interaction of lincRNA-p21 and miR-17-5p were further validated by a dual luciferase reporter assay. The inhibitory effect of lincRNA-p21 on lung tumor growth was also verified by *in vivo* studies. The results of the present study suggest a novel regulatory function of lincRNA-p21 in NSCLC and provides a potential therapeutic target for the treatment of NSCLC.

Materials and methods

Patients and clinical tissue samples. A total of 40 pairs of lung cancer tissue samples and adjacent tissue samples were obtained from patients with NSCLC in Guangdong General Hospital (Guangzhou, China). Among them, 29 patients were male and 11 patients were female (age range, 25-45 years old; mean age, 36 years old). All the collected cases were diagnosed as NSCLC pathologically in Southern Medical University (Guangzhou, China), and patients did not undergo preoperative radiotherapy and/or chemotherapy prior to resection. All samples were collected with informed consent obtained from each patient and approval from the Southern Medical University Institutional Review Board.

Cell culture and transfection. Human NSCLC cell lines A549 and PC9 (American Type Culture Collection, Manassas, VA, USA) were cultivated in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% of fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO₂ at 37°C. A total of 1x10⁴ A549 and PC9 cells were seeded into 24-well plates, and once cells achieved 85% confluence, they were transfected with 10 nM pcDNA3.1-lincRNA-p21 overexpression plasmid or lincRNA-p21 siRNA (5'-UGAAAAGAGCCGUGAGCUA-3') (both from Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The empty plasmid pcDNA3.1 and lincRNA-p21 scrambled siRNA sequence (5'-AGCCUGCAGGUGAGACCAGAACUG-3') (both from Shanghai GenePharma Co., Ltd.) were used as negative control (NC) groups for the overexpression and knockdown experiments, respectively.

RT-qPCR. Total RNA was first extracted from A549 and PC9 cells or clinical tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total cDNA was reverse transcribed from isolated RNA using the PrimeScript RT Master mix (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions maintained were as follows: 30°C for 10 min, then 42°C for 30 min, followed by 95°C for 5 min. The expression levels of lincRNA-p21 were detected by qPCR on the ABI Biosystems (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). The RT-qPCR primers used were as follows: lincRNA-p21 forward, 5'-CCTGTCCCACTCGCTTTC-3' and reverse, 5'-GGAAGTGGACACGGAATGTC-3'; GAPDH forward, 5'-TGTTTCGTCATGGGTGTGAAC-3' and reverse, 5'-ATGGCATGGACTGTGGTCAT-3'. The thermocycling conditions maintained were as follows: 95°C for 30 sec, then 40 cycles of 95°C for 5 sec followed by 60°C for 30 sec. The relative expression level of lincRNA-p21 was normalized to internal control GAPDH, and quantified using the 2^{-ΔΔC_q} cycle threshold method (13).

Cell proliferation analysis. At 72 h following transfection, the effects of lincRNA-p21 on the proliferation of A549 and PC9 cells were analyzed using a Cell Counting Kit-8 assay (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, A549 cells were washed with PBS buffer (pH 7.4) and harvested by trypsinization. A total of 1x10⁴ cells were reseeded into a 96-well plate. The plate was then incubated in a 5% CO₂ humidified incubator at 37°C. Following the incubation, 10 μl of the CCK-8 solution was added to each well and the plate was incubated for 2 h. The measurements were performed by detecting the absorbance at 450 nm with a microplate reader.

Apoptosis analysis. An Annexin V-FITC and propidium iodide (PI) staining kit (Dead Cell Apoptosis kit with Annexin V Alexa Fluor™ 488 & PI; Thermo Fisher Scientific, Inc.) was used to detect the cell apoptosis according to the manufacturer's protocol. Briefly, A549 and PC9 cells were transfected with lincRNA-p21 overexpression plasmid or lincRNA-p21

siRNA for 72 h, the cells were collected and washed with cold PBS twice. Cells were stained with Annexin-FITC and PI for 5 min at room temperature in the dark, and the cells were subjected to flow cytometric analysis.

Western blot analysis. Total proteins were isolated from A549 cells and PC9 cells using a Protease Inhibitor cocktail (Thermo Fisher Scientific, Inc.). Protein concentrations were quantified using a Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.). A total of 100 μ g of protein/lane was resolved by 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane, followed by blocking in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 with a pH value of 7.4 overnight at 4°C. Subsequently, the PVDF membrane was blotted with the primary antibodies at 4°C overnight against Bcl-2 (1:1,000 dilution; cat. no. 4223; rabbit; Cell Signaling Technology, Inc., Danvers, MA, USA), MMP9 (1:1,000 dilution; cat. no. 13667; rabbit; Cell Signaling Technology, Inc.), SPARC-related modular calcium binding 1 (SMOC1; 1:1,000 dilution; cat. no. ab200219; rabbit; Abcam, Cambridge, UK) and GAPDH (1:1,000 dilution; cat. no. 5174; rabbit; Cell Signaling Technology, Inc.). Chemiluminescence signals were detected incubated with mouse anti-rabbit (1:5,000 dilution; cat. no. ab99696) and rat anti-mouse (1:5,000 dilution; cat. no. ab99616) horseradish peroxidase-conjugated secondary rabbit antibodies (both from Abcam) for 1 h at 4°C. The expression levels of the target proteins were visualized by electrochemiluminescence (cat. no. 32109; Thermo Fisher Scientific, Inc.). The relative protein expression levels were evaluated through the gray value ratio of each protein and GAPDH using ImageJ (version 2.0; National Institutes of Health, Bethesda, MD, USA).

Transwell assay. Transwell chambers were warmed and placed into a 24-well plate at 37°C. The upper and lower chambers were hydrated with 0.5 ml pre-heated RPMI-1640 culture medium with 1% FBS in an incubator for 2 h at 37°C followed by the removal of the solution in the upper and lower chambers. The cells were digested using trypsin and a cell suspension was prepared to make up a total of 5×10^4 cells/ml. The hydrated chambers were transferred into a 24-well plate containing 0.5 ml RPMI-1640 complete medium. The chambers were incubated with 0.5 ml diluted cell suspension for 24 h at 37°C and the liquid in the upper and lower chambers were removed. The upper surface cells on the membrane were gently wiped off and washed three times in PBS. The cells that had migrated to the lower surface were fixed with precooled 100% methanol for 30 min, stained with 1% crystal violet for 1 h at room temperature, and washed with running water. Following the removal the crystal violet by PBS, the dried cells were counted in five randomly selected fields using a high-powered confocal microscope (magnification $\times 200$; Quantity One system; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mean number of cells passing through the basement membrane was recorded and imaged. The experiment was performed in triplicate.

Dual-luciferase reporter analysis. Bioinformatics analysis (miRDB: <http://www.mirdb.org/>; starBase v2.0; <http://starbase.sysu.edu.cn/>) was used to predict targets of miR-17-5p.

To verify whether miR-17-5p was a direct gene target of lincRNA-p21, pmirGLO was used to construct a lincRNA-p21 luciferase reporter with binding site for miR-17-5p (pmirGLO-lincRNA-p21). lincRNA-p21 and its interacting miRNAs were inserted into the luciferase reporters (Promega Corporation, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were transfected with luciferase reporters, internal control and indicating RNAs. At 72 h following transfection, luciferase activities were monitored using the Dual-Luciferase® Reporter assay system (Promega Corporation) according to the manufacturer's protocol. Final results were normalized to *Renilla* luciferase and analyzed statistically. The firefly luciferase acted as the internal reference of 3'UTR wt and the expression of the carrier 3'UTR wt+miR-NC acted as the control.

Tumor xenograft in vivo model. In order to establish an *in vivo* tumor model, a total of 18 BALB/c female nude mice (4-5 weeks old; mean weight, 20 g) were purchased from the Animal Center at Southern Medical University and maintained under pathogen-free conditions according to the protocols. The animals were maintained on a 12/12 h light/dark cycle at a constant temperature of 22°C and humidity, 50-60%. Free access to chow and water were provided. The use of animals in the present study was approved by the Institution of Animal Ethical and Welfare Committee. The size of tumors was measured using calipers and calculated as follows: (Large diameter) \times [2 \times (short diameter)]/2 and expressed in mm³. Twelve mice were randomly divided into three groups (4 mice/group): pcDNA3.1-vector, lincRNA-p21 and lincRNA-p21 plus miR-17 mimics. A549 cells were treated with lentivirus vector or lentivirus lincRNA-p21, using Lipofectamine 3000 as aforementioned. Treated cells were injected into the posterior flank of each mouse subcutaneously. Mice in the vector group were administered with A549 treated with pcDNA3.1-vector, mice in the lincRNA-p21 group were administered with A549 treated with pcDNA3.1-lincRNA-p21, while lincRNA-p21 and miR-17 mimics group was injected with A549 cells treated with pcDNA3.1-lincRNA-p21, followed by injection of miR-17 mimics on the site of tumor after 7 days. To examine the impact of lincRNA-p21 and miR-17 in tumorigenesis, the tumor growth within the mice were monitored every other day. Tumor volumes were measured at using a caliper at the time of sacrifice, 28 days after the cell injection.

Statistical analysis. Experiments were all repeated at least three times. A two-tailed Student's t-test was applied to analysis of two groups and difference between multiple groups was analyzed by one-way analysis of variance followed by Dunnett's test using IBM SPSS Statistics software (version 17.0; SPSS, Inc., Chicago, IL, USA). All data are expressed as mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

lincRNA-p21 expression in NSCLC tumor tissues is significantly decreased. The RT-qPCR results in Fig. 1 illustrate the relative expression levels of lincRNA-p21 in NSCLC tumor tissues and surrounding normal tissues. The data indicated

that lincRNA-p21 exhibited a significantly higher expression level in normal tissues compared with tumor tissues (Fig. 1; $P < 0.01$). Thus, lincRNA-p21 appears to be downregulated in NSCLC tumor tissues. Furthermore, lincRNA-p21 expression was analyzed according to the clinicopathological parameters of 40 patients with NSCLC (Table I). The mean expression value for lincRNA-p21 was used to distinguish low or high expression. Notably, low lincRNA-p21 expression was associated with distance metastasis and advanced TNM stages, and was independent of smoking status, sex, age and lymph node metastasis.

lincRNA-p21 levels in different lung cancer cell lines and expression levels following transfection. As illustrated in Fig. 2A, the expression level of lincRNA-p21 in the human lung cancer cell line A549 was significantly lower compared with that of the human lung cancer cell line PC9. Thus, the A549 lung cancer cells were used as low lincRNA-p21 expression level cells for the transfection of lincRNA-p21, while the PC9 lung cancer cells were used as high lincRNA-p21 expression level cells for the transfection of lincRNA-p21 siRNA to downregulate lincRNA-p21 expression. The RT-qPCR results in Fig. 2B illustrate the lincRNA-p21 expression levels in each cell group following transfection. In Fig. 2C, the A549 cells transfected with the lincRNA-p21 plasmid had a significantly higher lincRNA-p21 expression level, compared with the control group, which was transfected with the vector alone. Furthermore, the PC9 cells transfected with lincRNA-p21 siRNA exhibited a significant reduction in lincRNA-p21 expression when compared with the NC group in PC9 cells, indicating an effective knockdown of lincRNA-p21.

Overexpression of lincRNA-p21 inhibits and knockdown of lincRNA-p21 induces lung cancer cell proliferation. The CCK-8 assay results in Fig. 3A demonstrate the optical density at 450 nm which reflects the cell proliferation rates for different cell groups during the 0, 24, 48 and 72 h intervals. The proliferation rate of A549 cells transfected with lincRNA-p21 was significantly lower compared with the vector-transfected group, demonstrating the inhibitory effect of lincRNA-p21 on human lung cancer cell proliferation. Conversely, the PC9 cell line with si-lincRNA-p21 transfection exhibited a significantly higher proliferation rate, compared with the NC group, providing evidence for the suppressive effect of lincRNA-p21 on human lung cancer cell proliferation.

Cell apoptosis rate is increased in the lincRNA-p21 overexpression group and decreased in the lincRNA-p21 knockdown group. The results demonstrated that the cell apoptosis rate in the A549 lincRNA-p21 group was 16.9%, which was significantly higher compared with that of the A549 vector group (4.1%) ($P < 0.05$), while the cell apoptosis rate in the PC9 si-lincRNA-p21 group was 1.9%, which was significantly reduced compared with that of the PC9 NC group (9.5%) (Fig. 3B). This indicated that the overexpression of lincRNA-p21 may facilitate cell apoptosis in lung cancer cells.

Cell migration is decreased in the lincRNA-p21 overexpression group and increased in the lincRNA-p21 knockdown

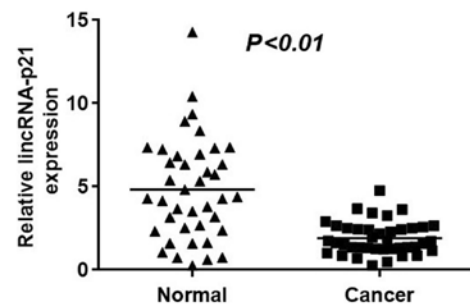


Figure 1. lincRNA-p21 expression in normal and NSCLC tumor tissues. lincRNA-p21 expression levels in 40 pairs of lung cancer tissue samples and adjacent tissue samples were detected by reverse transcription-quantitative polymerase chain reaction. NSCLC, non-small cell lung carcinoma; lincRNA, long intergenic non-coding RNA.

group. The Transwell analysis results as presented in Fig. 3C demonstrated that the A549 lincRNA-p21 group had a significantly reduced number of invading cells (~150 cells) compared with that of the A549 vector group (~300 cells), while the PC9 si-lincRNA-p21 group had a significant increase in the number of invading cells (~400 cells) compared with the PC9 NC group (~170 cells) ($P < 0.005$). Taken together these results demonstrated that the overexpression of lincRNA-p21 sufficiently inhibited the migration of lung cancer cells.

lincRNA-p21 regulates the expression of miR-17-5p in lung cancer cells. Investigations on the association between the expression levels of lincRNA-p21 and miR-17-5p revealed that lincRNA-p21 was able to regulate the expression of miR-17-5p in lung tumor cells. Fig. 4A and B illustrate the effect of lincRNA-p21 on miR-17-5p expression. It was identified that cells with overexpressed lincRNA-p21 (A549 lincRNA-p21 group) had a significantly lower miR-17-5p expression level compared with the low lincRNA-p21 expression cells (A549-vector group), and cells with downregulated lincRNA-p21 expression (PC9 si-lincRNA-p21 group) had a significantly higher miR-17-5p expression level compared with the control group cells (PC9-NC group). The aforementioned observations were further confirmed by the relative miR-17-5p expression results in the A549 lincRNA-p21 and PC9 si-lincRNA-p21 groups following the addition of a miR-17-5p mimic and inhibitor, respectively. The western blot analysis results in Fig. 4C demonstrate that the overexpressed lincRNA-p21 significantly suppressed the expression levels of Bcl-2 and MMP9 compared with the control group, and following the addition of miR-17-5p mimics, the expression levels of Bcl-2 and MMP9 were partially recovered, whereby the expression levels of Bcl-2 and MMP9 were upregulated. Furthermore, the knockdown of si-lincRNA-p21 resulted in a significant increase in Bcl-2 and MMP9 levels compared with the NC group, whereas the addition of miR-17-5p inhibitors caused a reduction in the expression levels of Bcl-2 and MMP9 compared with that in the si-lincRNA-p21+miR-17-5p NC group. These results indicated that lincRNA-p21 affected lung cancer cells through altering the expression of Bcl-2 and MMP9.

lincRNA-p21 affects the proliferation and migration of lung cancer cells via regulating miR-17-5p. The CCK-8 assay results

Table I. Association between lincRNA-p21 expression and clinicopathological parameters of patients with NSCLC.

Parameters	No. of patients	lincRNA-p21	P-value
Smoking status			0.158
Yes	28	1.454±0.908	
No	12	1.912±0.954	
Sex			0.178
Male	29	1.464±0.839	
Female	11	1.930±0.999	
Age, years			0.231
<50	10	1.324±0.655	
>50	30	1.680±1.004	
Lymph node metastasis			0.136
N0	8	1.378±0.647	
N1-N3	32	1.965±0.751	
Distant metastasis			0.046 ^a
M0	29	1.782±0.678	
M1	11	1.023±0.887	
TNM stage			0.034 ^a
0-II	25	1.913±0.789	
III-IV	15	0.987±0.861	

Student's t-test, ^aP<0.05, significant difference. NSCLC, non-small cell lung carcinoma; lincRNA, long intergenic non-coding RNA; TNM, tumor node metastasis.

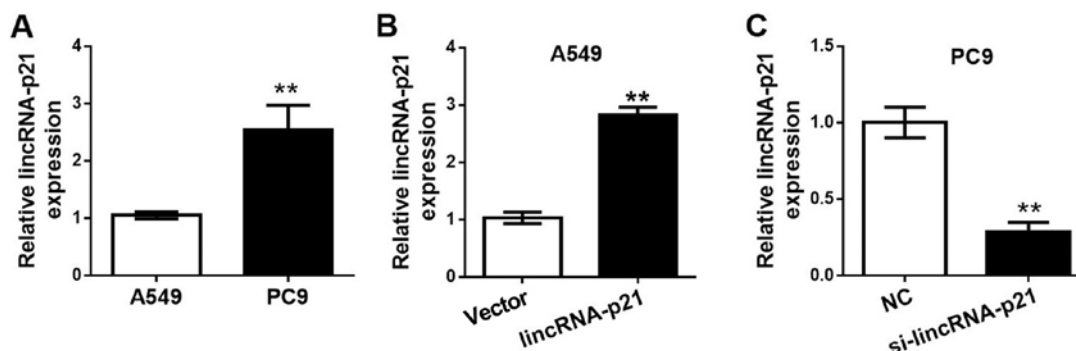


Figure 2. lincRNA-p21 expression in NSCLC cell lines. (A) The expression of lincRNA-p21 in A549 and PC9 cells were detected by RT-qPCR. The lincRNA-p21 expression in A549 cells was set as 1, and the lincRNA-p21 expression in PC9 cells was normalized to that of A549 cells. (B) A549 cells were seeded into 24-well plates and then transfected with empty vector (vector) or lincRNA-p21 overexpression plasmid (lincRNA-p21). The relative lincRNA-p21 expression in overexpression plasmid group was normalized to the empty vector group. (C) PC9 cells were seeded into 24-well plates and then transfected with scrambled siRNA (NC) or lincRNA-p21 siRNA (si-lincRNA-p21). The lincRNA-p21 expression in lincRNA-p21 siRNA group was normalized to the scrambled siRNA group. **P<0.01. RT-qPCR, reverse transcription quantitative polymerase chain reaction; NSCLC, non-small cell lung carcinoma; lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control.

for the A549 lincRNA-p21 group, PC9 si-lincRNA-p21 group and following the addition of a miR-17-5p mimic and inhibitor, respectively are presented in Fig. 5A. Treatment of the A549 lincRNA-p21-transfected cells with miR-17 mimic significantly reduced the cell proliferation rate compared with the NC group. Additionally, treatment of the PC9 si-lincRNA-p21 group with miR-17-5p inhibitors significantly reduced the proliferation of cells compared with the corresponding NC group. The results from cell apoptosis analysis demonstrated that the miR-17-5p mimics significantly suppressed cell apoptosis in

lincRNA-p21-overexpressed A549 cells, whereas the miR-17-5p inhibitor partially recovered the apoptosis rate in the PC9 cell line with si-lincRNA-p21 transfection. The Transwell assay results presented in Fig. 5C confirmed that miR-17-5p significantly accelerated cell migration, while inhibiting miR-17-5p suppressed cell migration, which were opposing to the actions of lincRNA-p21.

miR-17-5p is a direct gene target of lincRNA-p21. Bioinformatics analysis (miRDB: <http://www.mirdb.org/>; starBase

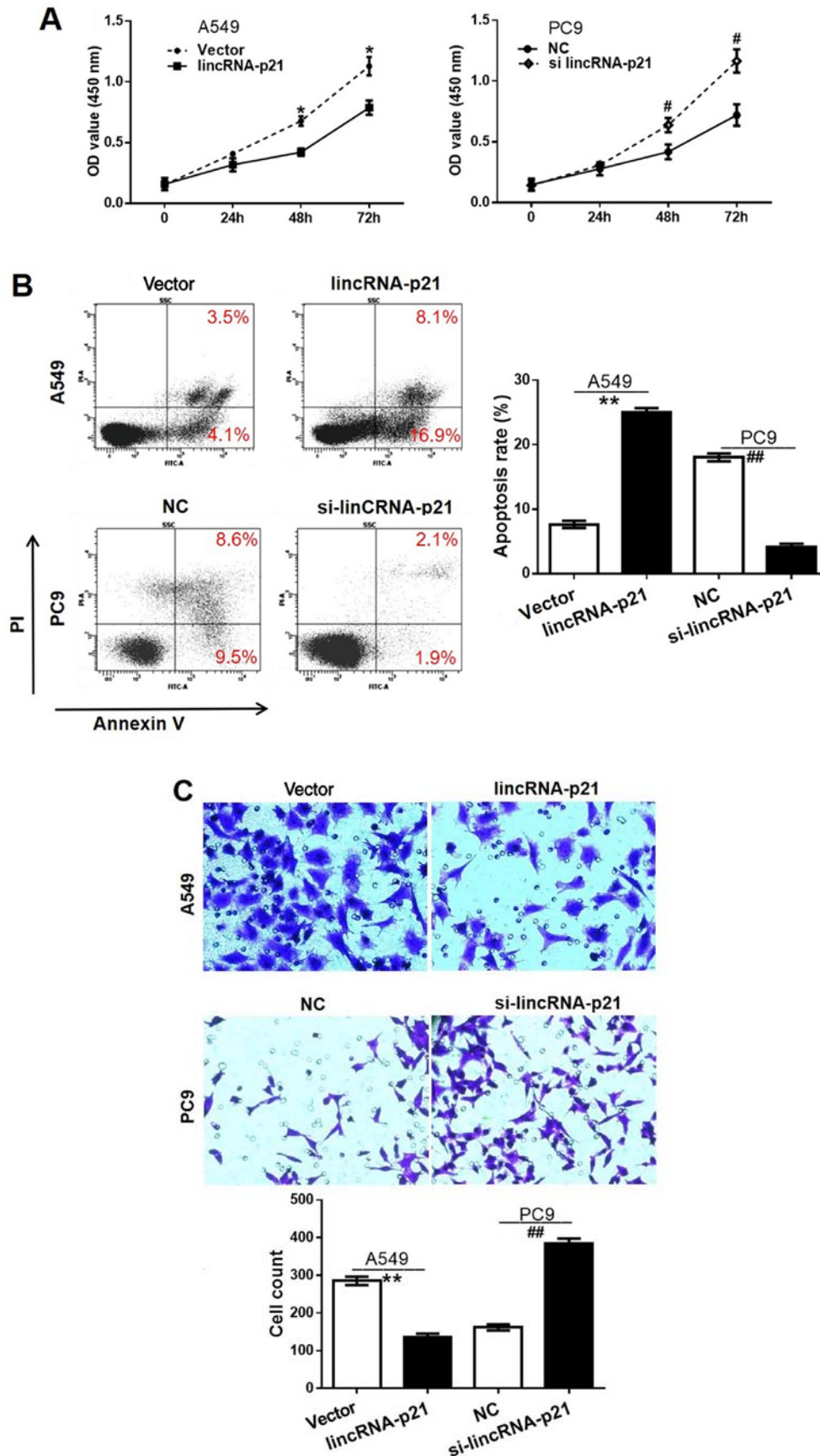


Figure 3. Effects of lincRNA-p21 on lung cancer cell proliferation, apoptosis and migration. (A) A549 and PC9 cells were transfected with lincRNA-p21 overexpression plasmid or lincRNA-p21 siRNA for 72 h, and the cell proliferation was detected using a Cell Counting Kit-8 assay. (B) The cells were transfected with lincRNA-p21 overexpression plasmid or lincRNA-p21 siRNA for 72 h, and cell apoptosis was detected by flow cytometric analysis. (C) The transfected cells were subjected to Transwell analysis for 24 h (magnification x200). * $P < 0.05$ and ** $P < 0.01$ vs. vector group; # $P < 0.05$ and ## $P < 0.01$ vs. NC group. NSCLC, non-small cell lung carcinoma; lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; PI, propidium iodide.

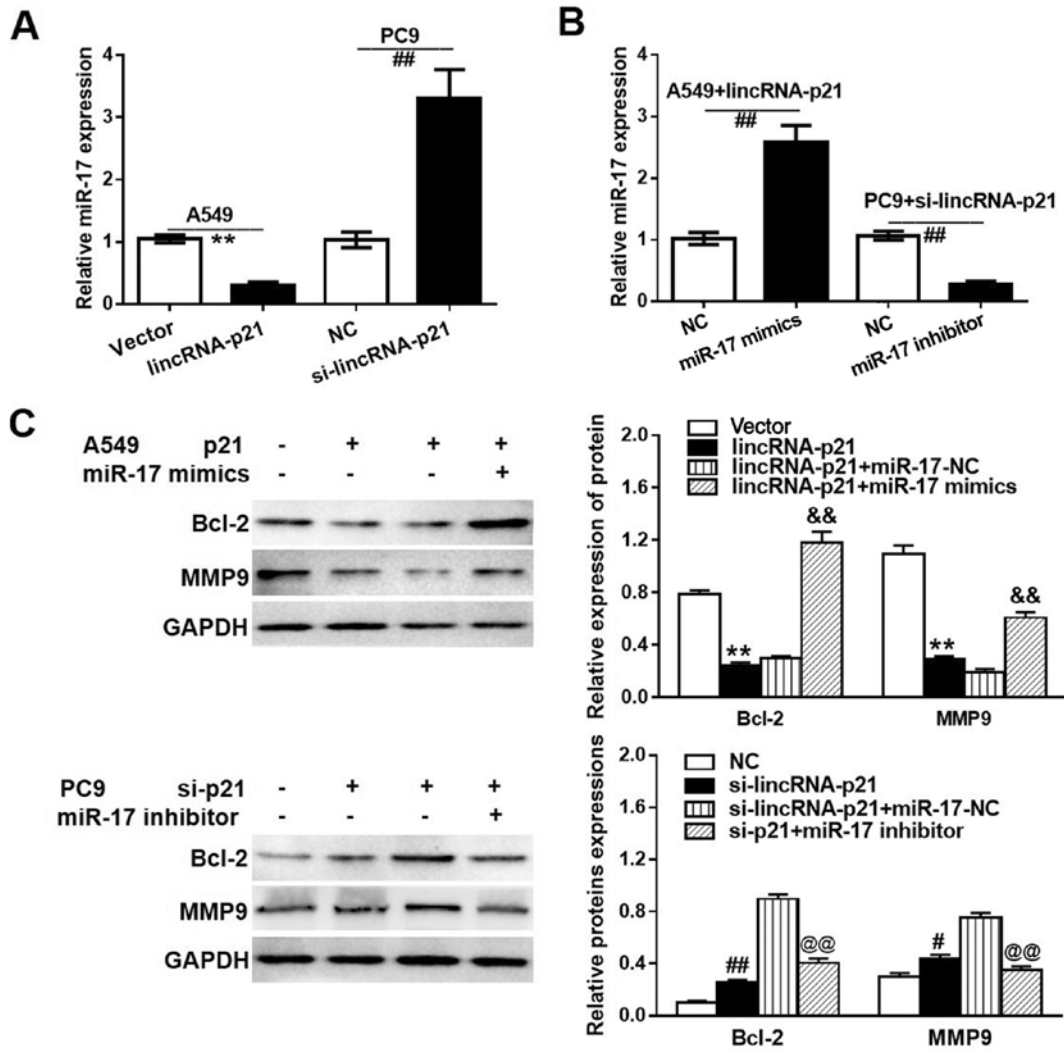


Figure 4. lincRNA-p21 regulates the expression of miR-17-5p in lung cancer cells. (A) miR-17-5p expression levels in different cell groups following transfection with lincRNA-p21 overexpression plasmid or lincRNA-p21 siRNA for 24 h. (B) miR-17-5p expression levels in different cell groups following treatment with miR-17-5p mimics or inhibitor for 24 h. (C) Western blot analysis for Bcl-2 and MMP9 protein expression levels in different cell groups following treatments with miR-17-5p mimics or inhibitor for 24 h. **P<0.01 vs. vector group. #P<0.05 and ##P<0.01 vs. NC group, &&P<0.01 vs. lincRNA-p21+miR-17-NC, @@P<0.01 vs. si-lincRNA-p21+miR-17-NC. lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; miR, microRNA; Bcl-2, B-cell lymphoma-2; MMP9, matrix metalloproteinase.

v2.0; <http://starbase.sysu.edu.cn/>) was first used to generate a lincRNA-p21 recognition sequence and a putative target site for miR-17-5p was revealed (Fig. 6A). To verify whether miR-17-5p was a direct gene target of lincRNA-p21, pmirGLO was used to construct a lincRNA-p21 luciferase reporter with binding site for miR-17-5p (pmirGLO-lincRNA-p21). As shown in Fig. 6B, lincRNA-p21 transfection caused a significant increase in luciferase activity in the pmirGLO-lincRNA-p21 group compared with the pmirGLO control group, and the addition of miR-17-5p mimics reverse this effect. Furthermore, western blot analysis showed that SMOC1 protein, the target gene of miR-17, was significantly increased in A549 cells treated with pcDNA3.1-lincRNA-p21, compared with that in A549 cells treated with pcDNA3.1 vector only, while miR-17 mimics were able to rescue the enhanced expression of SMOC1 triggered by lincRNA-p21 overexpression (Fig. 6C, left panel). Consistently, SMOC1 protein was significantly inhibited in A549 cells treated with si-lincRNA-p21, compared with that in A549 cells treated

with siRNA control, whereas miR-17 inhibitor was able to rescue the reduced expression of SMOC1 induced by knock-down of lincRNA-p21 (Fig. 6C, right panel).

SMOC1 is a direct gene target of miR-17-5p. As presented in Fig. 7A, the bioinformatics analysis results identified the recognition sequence position 127-133 of SMOC1 for miR-17-5p. The dual-luciferase reporter analysis results provided evidence that SMOC1 was a direct gene target for miR-17-5p. As illustrated in Fig. 7B, the relative luciferase activity in the wild type SMOC1 3'untranslated region (UTR) group was significantly suppressed by miR-17-5p, while no significant difference was observed in the relative luciferase activity in the mutant type SMOC1 3'UTR group following miR-17-5p treatment compared with the wild and mutant SMOC1 3'UTR NC groups. Furthermore, SMOC1 expression was significantly reduced in NSCLC tumor tissues compared with adjacent normal tissues as demonstrated by RT-qPCR (P<0.01; Fig. 7C). This result was further validated

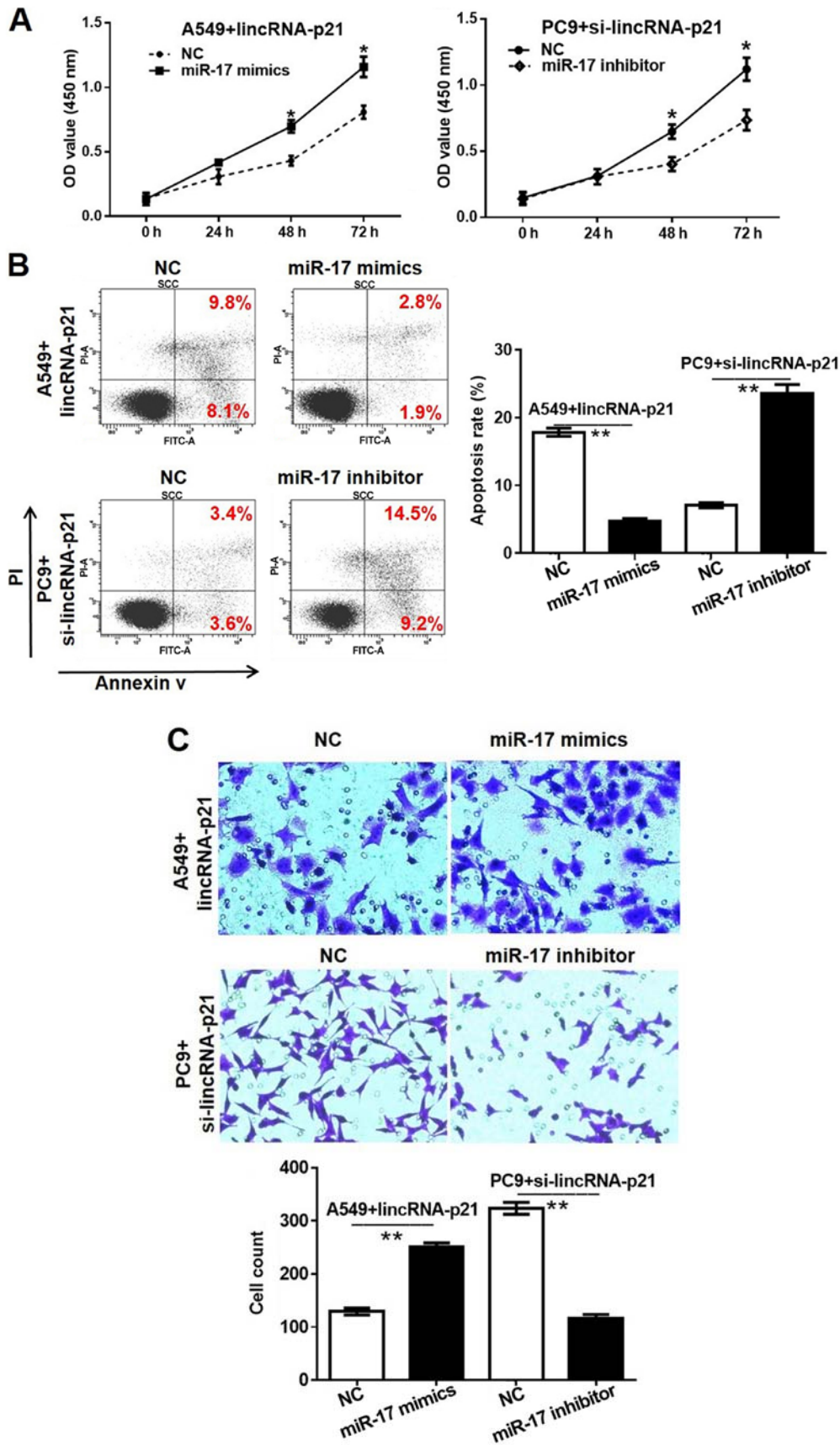


Figure 5. Effects of miR-17-5p on lung cancer cell proliferation, apoptosis and migration. (A) lincRNA-p21 overexpression plasmid- or lincRNA-p21 siRNA-transfected lung cancer cells were treated miR-17-5p mimics or inhibitor for 72 h, and cell proliferation was detected using a Cell Counting Kit-8 assay. (B) lincRNA-p21 overexpression plasmid- or lincRNA-p21 siRNA-transfected lung cancer cells were treated miR-17-5p mimics or inhibitor for 72 h, and the cells apoptosis were detected by flow cytometric analysis. (C) lincRNA-p21 overexpression plasmid- or lincRNA-p21 siRNA-transfected lung cancer cells were treated miR-17-5p mimics or inhibitor for 24 h, and the cells were subjected to Transwell analysis (magnification, x200). * $P < 0.05$ and ** $P < 0.01$ vs. NC group. lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; miR, microRNA; PI, propidium iodide; OD, optical density.

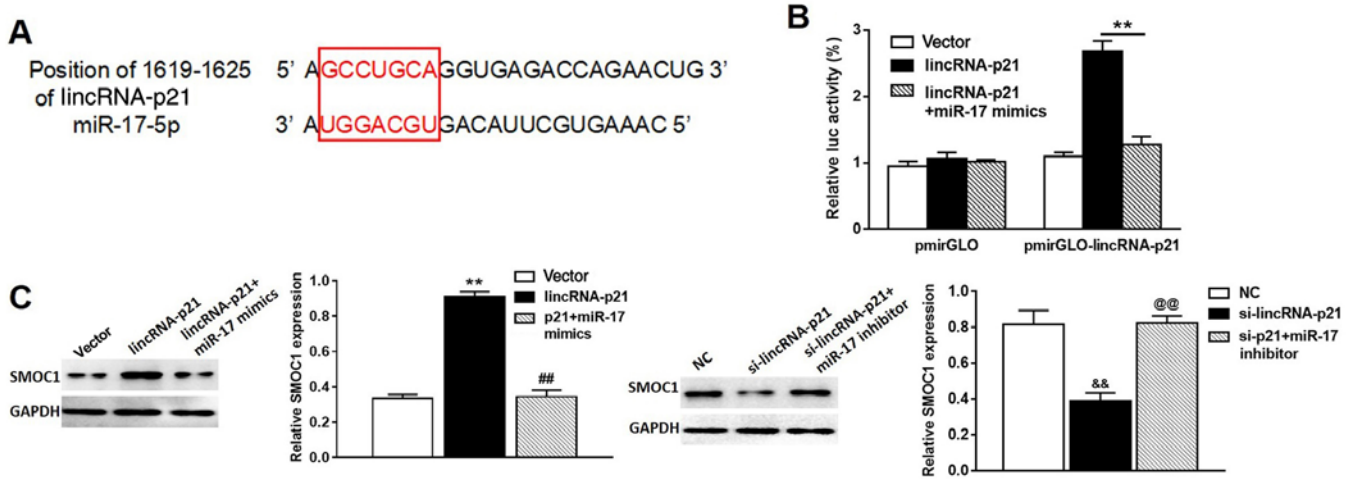


Figure 6. miR-17-5p is a direct gene target of lincRNA-p21. (A) Bioinformatics results for the direct binding sites of lincRNA-p21 and miR-17-5p. (B) Cells in different groups were transfected with luciferase reporters, internal control and indicated RNAs. At 72 h following transfection, luciferase activities were monitored using a luciferase reporter assay system. **P<0.01 (lincRNA-p21 vs. lincRNA-p21+miR-17-5p mimics). (C) lincRNA-p21 overexpression plasmid- or lincRNA-p21 siRNA-transfected lung cancer cells were treated miR-17-5p mimics or inhibitor for 72 h, and the protein SMOC1 expression was detected by western blot analysis. **P<0.01 vs. vector group; ##P<0.01 vs. lincRNA-p21 group; &&P<0.01 vs. NC group; @@P<0.01 vs. si-lincRNA-p21 group. lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; miR, microRNA; SMOC1, SPARC-related modular calcium binding 1.

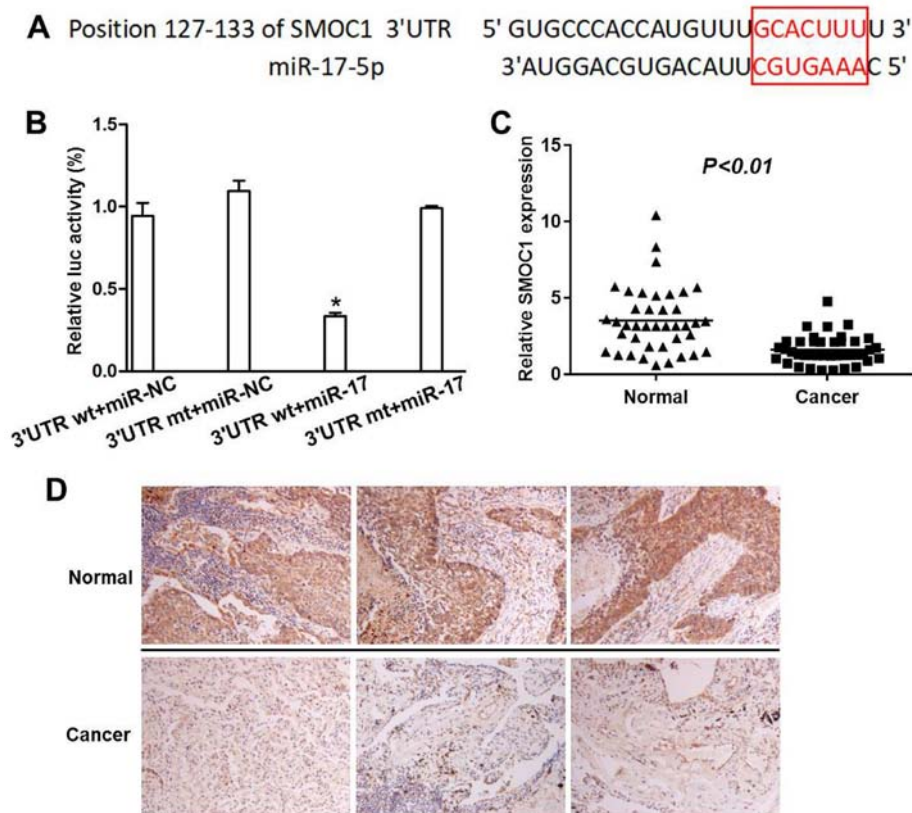


Figure 7. SMOC1 is a direct gene target of miR-17-5p. (A) Bioinformatics results for the direct binding sites position 127-133 on SMOC1 for miR-17-5p. (B) Luciferase reporter analysis for A549 and PC9 cell groups with wild or mutant type SMOC1 3'UTR, and miR-NC or miR-17. (C) Relative SMOC1 expression between 40 pairs of lung cancer tissue samples and adjacent normal tissues. (D) The expression level of SMOC1 demonstrated by representative immunohistochemical staining of lung cancer tissues and adjacent normal tissues. (magnification, x200) *P<0.01 vs. 3'UTR wt+miR-NC. SMOC1, SPARC-related modular calcium binding 1; UTR, untranslated region; lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; miR, microRNA.

using immunohistochemical staining of NSCLC tumor tissues and adjacent normal tissues, which demonstrated a substantial abundance of SMOC1 expression in adjacent normal tissues,

compared with NSCLC tumor tissues (Fig. 7D). All these results suggest that miR-17-5p can directly target the binding sites on SMOC1 in NSCLC tumor.

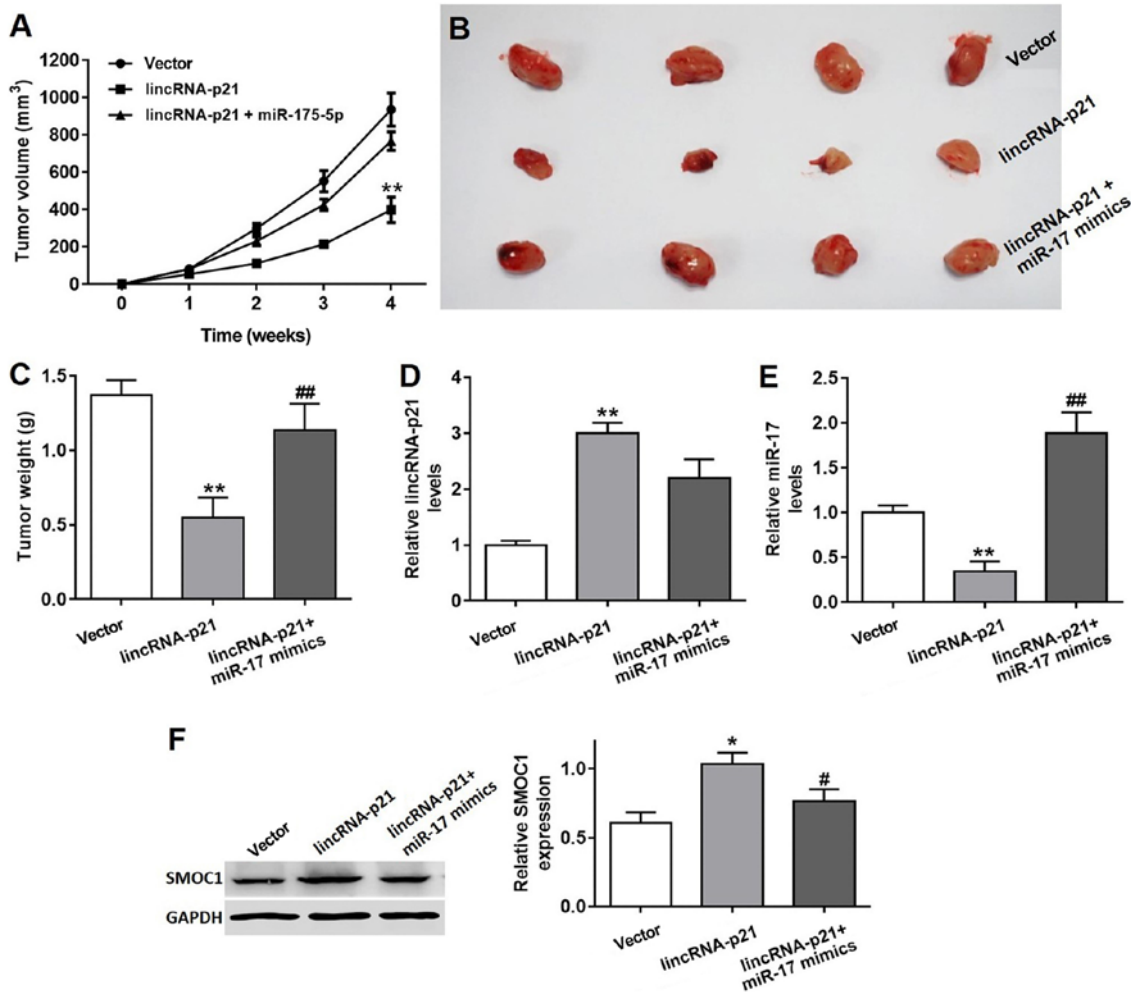


Figure 8. Inhibitory effect of lincRNA-p21 on lung tumor growth *in vivo*. (A) Tumor volumes of different groups were monitored over 4 weeks. ** $P < 0.01$ vs. vector group. (B) Tumor tissues from vector control, lincRNA-p21 treated, lincRNA-p21 and miR-17-5p treated groups were isolated and (C) weighed. The relative levels of (D) lincRNA-p21 and (E) miR-17 in tumors isolated from the vector, lincRNA-p21 and lincRNA-p21+miR-17 mimics groups, as demonstrated by RT-qPCR. ** $P < 0.01$ vs. vector control group, ** $P < 0.01$ vs. lincRNA-p21 group. (F) The SMOC1 protein expression in tumors isolated from the vector, lincRNA-p21 and lincRNA-p21+miR-17 mimics groups, as demonstrated by RT-qPCR. * $P < 0.05$ vs. vector control group, * $P < 0.05$ vs. lincRNA-p21 group. RT-qPCR, reverse transcription quantitative polymerase chain reaction; lincRNA, long intergenic non-coding RNA; siRNA/si, small interfering RNA; NC, negative control; miR, microRNA; SMOC1, SPARC-related modular calcium binding 1.

lincRNA-p21 inhibits lung tumor growth in vivo. Further investigation of the contribution of lincRNA-p21 to lung tumor growth was performed *in vivo*. Nude mice were administered with A549 stably transfected with a vector or lincRNA-p21, or lincRNA-p21 together with miR-17-5p mimics. Subsequently, tumor growth was monitored over time for a total of 4 weeks. The tumor volume (Fig. 8A and B) and tumor weight (Fig. 8C) in the lincRNA-p21 treatment group were significantly decreased compared with the vector group, and the tumor treated with lincRNA-p21 miR-17-5p mimics were smaller compared with the vector group; however, notably larger compared with the lincRNA-p21 treatment group. Furthermore, the expression of lincRNA-p21 was significantly increased in the lincRNA-p21 group, compared with the vector control group ($P < 0.01$; Fig. 8D). Consistently, the expression level of miR-17 was significantly inhibited in the lincRNA-p21 group, compared with the vector control group (Fig. 8E). In addition, the expression of SMOC1 protein was remarkably higher in tumor tissues isolated from the lincRNA-p21 group, compared with the vector control

group. Nevertheless, miR-17 mimics were able to reverse the elevation in SMOC1 expression induced by lincRNA-p21 overexpression in the tumor tissue (Fig. 8F). These results demonstrated that lincRNA-p21 inhibited lung tumor growth via regulating SMOC1 *in vivo*.

Discussion

At present, NSCLC remains difficult to treat with a 5-year survival rate $< 15\%$, which is notably lower compared with that of other common cancer types, for example breast and prostate cancer (12). The principal cause of mortality is tumor invasion and metastasis, which are signs of disease progression and the principal cause of treatment failure. Although a number of studies on tumor invasion and metastasis have been performed, the exact mechanism remains to be elucidated (14,15). Previous studies have reported that the development of lung cancer and malignant metastasis is influenced by genetic factors and small non-coding RNA, in which lincRNAs serve an important role (16-18).

lincRNA-p21 has been recently reported to be associated with pulmonary fibrosis in acute respiratory distress syndrome (19), and is significantly increased in hepatocytes during liver fibrosis (20). However, an evident decrease in lincRNA-p21 in human fibrotic liver and cirrhotic liver was also reported by a recent study (21), indicating the elusive deregulation of lincRNA-p21 in disease. In the present study, an evident reduction in the expression of lincRNA-p21 was identified in NSCLC tumor tissues compared with that in healthy tissues, and the survival percentage of patients in various clinical stages was significantly higher in lincRNA-p21 overexpression cases compared with patients with low lincRNA-p21 expression. In addition, the inhibitory effect of lincRNA-p21 on lung tumor was verified by investigations on different human lung cancer cells. The proliferation and migration of lincRNA-p21-overexpressed cells were significantly inhibited, and conversely, were promoted in lincRNA-p21 knockdown cells compared with the vector group. Furthermore, the overexpression of lincRNA-p21 was observed to significantly increase the rate of lung cancer cell apoptosis, while the knockdown of lincRNA-p21 inhibited the apoptotic rate compared with the vector group. From the results of the present study, it is evident that lincRNA-p21 effectively inhibited the progression of lung cancer.

A previous study reported that under different conditions, the regulation of lincRNA-p21 is associated with various signaling pathways (22). Studies have demonstrated that lincRNA-p21 regulates the proliferation and apoptosis of various cell types, for example vascular smooth muscle cells and keratinocytes, by mediating p53 activity (23-25). It has also been reported that lincRNA-p21 may inhibit the activity of β -catenin signaling to attenuate the tumorigenicity of colorectal cancer stem cells (26). Furthermore, a recent study reported that lincRNA-p21 inhibited the Wnt/ β -catenin signaling pathway by regulating the miR-17-5p level in activated hepatic stellate cells (27). miR-17-5p is reported to be one of most essential miRNAs in lung cancer cells, and the regulation of miR-17-5p contributes to cell proliferation and invasion in NSCLC (28-30). The present study investigated the association between lincRNA-p21 and miR-17-5p expression in NSCLC tumor cells to explore the possible regulating mechanism. An evident negative association between lincRNA-p21 and miR-17-5p expression levels was observed. The miR-17-5p expression level was significantly reduced in lincRNA-p21-overexpressed cells, and significantly increased in lincRNA-p21 knockdown cells; however, these effects were reversed following the addition of a miR-17-5p mimic or inhibitor.

The present study also demonstrated that the inhibitory effect of overexpressed lincRNA-p21 on lung cancer cell proliferation and migration was partially counteracted by treatment with miR-17-5p mimics, and following the addition of miR-17-5p inhibitors the inhibitory effect of lincRNA-p21 was partially reversed in the lincRNA-p21 knockdown group. The upregulated cell apoptotic rate caused by lincRNA-p21 overexpression was suppressed by miR-17-5p mimics and the reduced cell apoptotic rate in lincRNA-p21 knockout cells was increased by miR-17-5p inhibitors. Based on the observed negative association between lincRNA-p21 and miR-17-5p, further studies to verify the direct binding sites for miR-17-5p on lincRNA-p21 are required. The bioinformatics results predicted that miR-17-5p may be a direct target for lincRNA-p21 and the luciferase reporter analysis

confirmed that lincRNA-p21 was able to bind with miR-17-5p directly leading to a mutual inhibiting effect. In addition, *in vivo* studies verified that lincRNA-p21 significantly inhibited lung tumor growth. The mechanism of lincRNA-p21 deregulation in various diseases is primarily imprecise; however, the present study provides, to the best of our knowledge, the first evidence for an inhibitory effect of lincRNA-p21 in the progression of NSCLC via direct targeting of miR-17-5p. The underlying mechanism for the regulation of lincRNA-p21 towards lung cancer cells is suggested to be associated with miR-17-5p associated signaling pathways. However, there is a possibility that other mechanisms may coexist, thus further researches are required for clarification.

In conclusion, the dysregulated expression of lincRNA-p21 in NSCLC tumor tissues was observed, and the functional role in NSCLC tumor cell progression and the underlying signaling pathway have been investigated in the present study. It was demonstrated that lincRNA-p21 is downregulated in NSCLC tumor tissues, and its overexpression has an inhibitory effect on lung cancer cell proliferation and migration while its knockdown accelerates NSCLC cell migration. Furthermore, the inhibitory effect of overexpressed lincRNA-p21 on NSCLC progression was partially counteracted by miR-17-5p mimics. A negative association between the expression levels of lincRNA-p21 and miR-17-5p was observed, and the results demonstrated that miR-17-5p is a direct target of lincRNA-p21. In conclusion, the present study revealed that lincRNA-p21 inhibits the progression of NSCLC via targeting a miR-17-5p associated signaling pathway; this provides a cornerstone for future researches regarding the pathology and therapeutic methods of NSCLC.

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Availability of data and materials

All data sets used in this study are available from the corresponding author on reasonable request.

Authors' contributions

MJ and GC designed the study. MJ, XA, JZ, HL and HX performed the experiments and analyzed the data. HX was the major contributor in developing the first draft of this manuscript. GC reviewed and approved the final draft of the manuscript prior to submission. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval for the study was provided by the Clinical Research and Ethics Committee at Guangdong General Hospital.

Patient consent for publication

All patients provided written informed consent for the publication of all associated data in this study.

Competing interests

The authors declare that they have no competing interests.

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