Long intergenic noncoding RNA-p21 inhibits apoptosis by decreasing PUMA expression in non-small cell lung cancer

Tao Yang¹, Wenjun Zhang¹, Li Wang², Chunyan Xiao¹, Bingling Guo¹, Yi Gong¹, Xiping Liang¹, Dehong Huang¹, Qiyong Li¹, Yingyu Nan¹, Ying Xiang¹ and Jianghe Shao¹

Abstract

Objective: Long noncoding RNAs (lncRNAs) are important mediators in tumor progression. Long intergenic noncoding RNA-p21 (lincRNA-p21) participates in multiple biological processes. This study explored the role of lincRNA-p21 in human non-small cell lung cancer (NSCLC) progression and potential regulatory mechanisms.

Methods: LincRNA-p21 expression in NSCLC tissues and cell lines (A549, H1299, H1650, and NCI-H2087) was determined by quantitative real-time PCR. LincRNA-p21 overexpressing and sh-lincRNA-p21 lentiviral were respectively transfected into H1299 and A549 cells. Flow cytometry was used to measure apoptosis. Microarray analysis and RNA pull-down assay were used to predict the target genes of lincRNA-p21. Finally, PUMA siRNA and overexpressing PUMA were transfected into NSCLC cells, and the extent of cell apoptosis was measured. The protein expression levels of the relative genes were confirmed by western blot analysis.

Results: LincRNA-p21 was significantly upregulated in NSCLC tissues and cells. The upregulation of lincRNA-p21 considerably inhibited cell apoptosis while the downregulation of lincRNA-p21 showed the opposite effect. PUMA was a direct target gene of lincRNA-p21 and was negatively correlated with lincRNA-p21 in NSCLC specimens. The anti-apoptotic effect of lincRNA-p21 can be effectively attenuated by the upregulation of PUMA.

¹Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital, Chongqing Cancer Institute, Chongqing Cancer Hospital, Chongqing, China
²Department of Oncology, Chongqing General Hospital, Chongqing, China

Corresponding author:
Jianghe Shao, Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital, Chongqing Cancer Institute, Chongqing Cancer Hospital, No. 181 Hanyu Road, Chongqing 400030, China.
Email: shaojianghe1031@163.com
Conclusion: LincRNA-p21 is aberrantly upregulated in NSCLC and inhibits cell apoptosis by decreasing PUMA expression.

Keywords
Long noncoding RNAs, lincRNA-p21, PUMA, non-small cell lung cancer, apoptosis, p53

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Introduction
Cancers pose a serious threat to human health,1 and lung cancer is the primary cause of cancer-related mortality worldwide.2,3 Non-small cell lung cancer (NSCLC) is the most frequently diagnosed subtype of lung cancer, comprising 85% of all diagnosed lung cancers and 80% of lung cancer-related deaths.4 Although considerable progress has been made in surgery, chemotherapy, radiotherapy, and molecular therapy for lung cancer treatment, the 5-year overall survival is still only 20%.5,6 Therefore, it is of great importance to explore more effective and safe treatment strategies for NSCLC. An understanding of the molecular mechanisms involved in NSCLC progression will provide a basis for novel NSCLC treatment strategies.7

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides (nt) with no protein-coding potential,8,9 which have been found to be pervasively transcribed in the human genome.10 Long intergenic noncoding RNA (lincRNA)-21 is 3100 nt in length and is named for its location on chromosome 17, approximately 15 kb upstream of the p21 gene.11 It was shown to act as a translation suppressor by targeting mRNA or by directing the chromatin location of protein binding partners.12,13 LincRNA-p21 regulates neointima formation, vascular smooth muscle cell proliferation, apoptosis, and atherosclerosis via the upregulation of p53 expression.14 Moreover, growing evidence has revealed the involvement of lincRNA-p21 in the development of various tumors. Wang et al.15 reported that lincRNA-p21 inhibits human prostate cancer progression partly by regulating p53 downstream gene expression and partially by apoptotic activation, while Jia et al.16 revealed that lincRNA-p21 overexpression contributed to the suppression of tumor invasion through mediating Notch signaling-induced epithelial–mesenchymal transition. Additionally, Wang et al.17 found that lincRNA-p21 improves the radiotherapy sensitivity of human colorectal cancer (CRC) by negatively regulating the Wnt/β-catenin signaling pathway. However, the role and mechanisms of lincRNA-p21 remain largely unknown in the progression of NSCLC.

p53 upregulated modulator of apoptosis (PUMA) is a pro-apoptotic member of the Bcl-2 homology 3 (BH3) subgroup of the Bcl-2 family, which can function either in a p53-dependent or p53-independent apoptotic manner.18 Binding of the BH3 domain to inhibitory members of the Bcl-2 family plays a critical regulatory role in the initiation of apoptosis, leading to the displacement of Bax and/or Bak.18 PUMA is expressed and induced by various stimuli, including genotoxic stress, deregulated oncogene expression, toxins, an altered redox status, growth factor/cytokine
withdrawal, and infection. It is also a potent regulator of mitochondrial outer membrane permeabilization through its binding, de-repression, and sensitization of anti-apoptotic Bcl-2 proteins. PUMA transcription can be induced by many transcription factors, including p53, CCAAT-enhancer-binding protein β, the cAMP response element binding protein, c-Jun, and specificity protein 1; it can be negatively regulated by transcriptional repressors including Slug, some alternative splice products of \( p73 \) (\( \Delta Np73 \) and \( p73 \alpha \)) or \( p63 \) (\( \Delta Np63 \)), and microRNAs. LincRNA-p21 is known to be involved in the development and progression of many types of cancer, including CRC, skin tumors, prostate cancer, and chronic lymphocytic leukemia. Moreover, the aberrant expression of lincRNA-p21 was reported to be relevant to CRC stage, tumor tissue invasion, and radiotherapy. However, it is unknown whether PUMA can be regulated by lincRNA-p21 in NSCLC.

In the present study, we investigated the biological role of lincRNA-p21 in the pathogenesis of NSCLC. Interestingly, lincRNA-p21 was found to be upregulated in NSCLC tissues and negatively regulated cell apoptosis by targeting PUMA. Collectively, our data reveal that lincRNA-p21 is an important regulatory molecule in NSCLC development, and could be a useful therapeutic target for NSCLC treatment.

Materials and methods

Tissue specimens

Paired NSCLC and normal adjacent lung tissues were obtained from 31 patients who underwent primary surgical resection of NSCLC between 2013 and 2015 in Chongqing University Cancer Hospital, Chongqing Cancer Institute, Chongqing Cancer Hospital. Tissue specimens were taken from patients who signed written informed consent forms in advance. The fresh specimens were frozen at \(-80^\circ C\) before use. Approval of the study protocol was obtained from the Institute Research Ethics Committee of Chongqing University Cancer Hospital, Chongqing Cancer Institute, Chongqing Cancer Hospital. All experimental methods were strictly performed in accordance with the approved guidelines.

Cell lines and cell culture

NSCLC cell lines A549, H1299, H1650, and NCI-H2087, and the normal bronchial epithelial cell line 16HBE were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, and maintained in a humidified atmosphere at 37°C with 5% CO₂.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s protocol. The RNA concentration and purity were determined by ultraviolet spectrophotometry. cDNA synthesis was performed using a cDNA synthesis kit (Takara Biotechnology, Dalian, China) and cDNA was used as a template for lincRNA quantitative real-time (qRT)-PCR. The primers were as follows: forward, 5'-CCTGTTCCTA CTCGTTTCCA-3' and reverse, 5'-GGA ACTGGAGACGGAATGTC-3' for lincRNA-p21; and forward, 5'-GACCTCT ATGCCAACACAGTGC-3' and reverse, 5'-GTACTCCTGCTTGCTGATCCAC- 3' for \( \beta \)-actin. PCR was performed in a volume of 20 \( \mu \)L with the following
conditions: initial denaturation at 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 minute, then a final extension step at 72°C for 7 minutes. qPCR assays were performed using the Mx3000P QPCR System (Agilent Technologies Inc., Santa Clara, CA, USA). Relative expression levels of lincRNA-p21 were calculated using the 2^−ΔΔCt method.

Establishment of stable cell lines

The lincRNA-p21-overexpressing lentiviral vector and short hairpin (sh)-lincRNA-p21 lentiviral vector were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). An empty lentiviral vector was used as a control. A549 or H1299 cells were seeded into 6-well plates at around 60% confluency 24 hours before transfection. Cells were transfected with a lincRNA-p21-overexpressing lentiviral vector or sh-lincRNA-p21 lentiviral vector lacking an antibiotic resistance gene. After 48 hours, cells were subcultured to 10% confluency in medium containing 1 mg/mL of puromycin (Sigma-Aldrich, St Louis, MO, USA). Antibiotic resistant clones were picked and passaged in medium containing half the concentration of puromycin after the nontransfected cells were killed. The expression of lincRNA-p21 was confirmed by real-time PCR.21

PUMA knockdown and overexpression

PUMA-specific small interfering (si)RNA and an overexpressing PUMA vector were supplied by Shanghai GenePharma Co., Ltd. In brief, cells at 60% confluency were transfected with 200 pmol of siPUMA or overexpressing PUMA using Lipofectamine (Invitrogen Corp.). Nontransfected cells were used as a control.

RNA pull-down assay

The lincRNA-p21 pull-down assay was performed as previously described.22

Western blot analysis

Cells were lysed in ice-cold radioimmunoprecipitation assay buffer, a mammalian protein extraction reagent, containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and phenylmethylsulfonyl fluoride (Roche). Extracted proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen Corp.), then transferred onto nitrocellulose membranes (Sigma-Aldrich). The membranes were first incubated with primary mouse anti-PUMA antibody (1:1000; Cell Signaling Technology; Danvers, MA, USA) at 4°C for 2 hours, then with horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hour. Finally, the protein signals were detected using a chemiluminescence detection system (Pierce Protein Research Products, Rockford, IL, USA), and protein bands were scanned using Image-Pro Plus v. 6.0 software. Results were shown as the expression of the target protein relative to that of glyceraldehyde 3-phosphate dehydrogenase. Western blotting results were quantified by the BCA protein assay reagent (Pierce) via Image J software v. 6.0 (Media Cybernetics, Bethesda, MD, USA).

Apoptosis analysis

An Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to detect the extent of apoptosis in accordance with the
manufacturer’s instructions. Cells seeded in six-well plates were harvested and washed with ice-cold phosphate-buffered saline (PBS). A total of $2 \times 3 \times 10^5$ cells were gently resuspended in 100 μL PBS and 5 μL of fluorescein isothiocyanate (FITC)-Annexin V (20 μg/mL) was added. The tubes were incubated for 30 minutes in the dark at room temperature (RT). Then, 5 μL PI (50 μg/mL) was added for 5 minutes in the dark at RT. Finally, the cells were analyzed within 1 hour using a FACS flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Unstained cells, cells stained with FITC-Annexin V (without PI), and cells stained with PI (no FITC-Annexin V) were used to calculate compensation values and set up quadrant markers. Early apoptotic and late apoptotic cells were respectively distributed in the lower right and upper right quadrants.

Microarray analysis

RNA sequencing analysis was performed in control A549 and sh-lincRNA-p21 A549, or H1299 control and lincRNA-p21 H1299 cells. Microarrays were further analyzed and quantile normalization was performed of the raw data. mRNAs that were differentially expressed between A549 control and A549-sh-lincRNA-p21, or between H1299 control and H1299-lincRNA-p21 groups were confirmed by $P$-value/FDR filtering. Differentially expressed mRNAs between A549 control and A549-sh-lincRNA-p21, or between H1299 control and H1299-lincRNA-p21 groups were identified through fold-change filtering. Venn diagrams were used to identify overlapping differentially expressed mRNAs in the two groups.

Statistical analysis

Data were represented as mean ± standard errors of the mean (SEM). All statistical calculations were performed using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). Each experiment was conducted at least three times independently. Comparisons among groups were analyzed with the Student’s t-test or one-way analysis of variance. $P < 0.05$ was deemed statistically significant.

Results

Expression of lincRNA-p21 in NSCLC tissues and cells

In this study, mRNA expression levels of lincRNA-p21 were determined in 31 paired clinical NSCLC and adjacent normal lung tissues using real-time PCR. The expression of lincRNA-p21 was significantly increased in NSCLC tissues compared with adjacent tissues ($P < 0.001$, Figure 1a). Additionally, the expression of lincRNA-p21 was significantly higher in four NSCLC cell lines than in the normal bronchial epithelial cell line 16HBE ($P < 0.01$, Figure 1b). Of note, lincRNA-p21 expression was highest in A549 cells and lowest in H1299 cells among the four types of NSCLC cells. These results suggested that lincRNA-p21 was upregulated in NSCLC tissues and cells, which might contribute to the progression of NSCLC.

Establishment of stable lincRNA-p21-overexpressing or knockdown cell lines

To better elucidate the role of lincRNA-p21 in NSCLC, we carried out lincRNA-p21 knockdown in A549 cells and lincRNA-p21 overexpression in H1299 cells. A549 cells were treated with control lentivirus or lincRNA knockdown lentivirus vectors (here designated control A549 and sh-lincRNA-p21 A549, respectively); and H1299 cells were treated with control lentivirus and lincRNA-p21 overexpressing lentivirus vectors (here designated control H1299 and linRNA-p21 H1299,
respectively). After selecting for puromycin resistance, we examined the knockdown and overexpression efficiency of lincRNA-p21 in A549 and H1299 cells. Real-time PCR demonstrated that the expression of lincRNA-p21 in sh-linRNA-p21 A549 cells was around 10% of that in control A549 cells, reflecting the successful downregulation of lincRNA-p21 ($P < 0.05$, Figure 1c). Similarly, linRNA-p21 H1299 cells showed an approximate 8-fold increase in lincRNA-p21 expression compared with control H1299 cells ($P < 0.01$, Figure 1d). Thus, models for investigating the role and mechanisms of lincRNA-p21 in NSCLC development were established.

**Role of lincRNA-p21 in NSCLC cell apoptosis**

To determine the biological function of lincRNA-p21 in NSCLC, we performed gain/loss function studies by examining cell apoptosis in control and lincRNA-p21 knockdown/overexpressing cells. A significant increase in cell apoptosis was observed following lincRNA-p21 knockdown with an observed rate of 0.113% in control A549 cells and 13.494% in sh-linRNA-p21 A549 cells, suggesting that the downregulation of lincRNA-p21 enhanced apoptosis ($P < 0.01$, Figure 2a, 2b). The apoptosis rate was 1.127% in lincRNA-p21 H1299 cells compared with 6.002% in control H1299 cells, suggesting that the upregulation of lincRNA-p21 inhibited apoptosis ($P < 0.05$, Figure 2c, 2d). Taken together, these data suggested that lincRNA-p21 negatively regulates cell apoptosis and might serve as a therapeutic target for NSCLC treatment.

**Identification of PUMA as a target of lincRNA-p21**

Although gain/loss of function analysis showed that lincRNA-p21 was closely associated with cell apoptosis, the intrinsic
mechanisms were unknown. We next compared gene expression profiles in control, lincRNA-p21-overexpressing, and -knockdown cells to determine genes that were differentially expressed between control A549, sh-linRNA-p21 A549, control H1299, and lincRNA-p21 H1299 cells. Our findings are shown in Figure 3a, 3b. Using a Venn diagram to examine overlapping genes from the different profiles identified FOXD1, PUMA, and ZNF60 (ZBTB17) (Figure 3c). We chose PUMA for further study because of its direct role in cell apoptosis.

To confirm that PUMA was a target of lincRNA-p21, we performed an RNA pull-down assay which showed that lincRNA-p21 pull-down significantly increased PUMA mRNA expression level compared with the control (\(P < 0.01\), Figure 3d). We observed that lincRNA-p21 downregulation significantly promoted mRNA and protein expression of PUMA, while lincRNA-p21 upregulation significantly inhibited this (\(P < 0.05\), Figure 3e–g). These findings strongly suggested that PUMA was a direct target of lincRNA-p21. After confirming this in cell lines, we next investigated whether lincRNA-p21 was correlated with PUMA expression in clinical tissues. Comparing PUMA mRNA expression in healthy lung
tissues and NSCLC tissues, we observed lower levels in NSCLC patients, and a significant but negative correlation between PUMA expression and lincRNA-p21 in NSCLC samples by linear regression analysis (R = -0.58, Figure 3h). These data showed that PUMA was negatively regulated by lincRNA-p21, indicating that lincRNA-p21 exerted an apoptosis inhibitory effect by downregulating PUMA.

**Effect of PUMA on the anti-apoptotic role of lincRNA-p21**

To investigate whether the anti-apoptotic effect of lincRNA-p21 was mediated by the downregulation of PUMA, we performed PUMA loss/gain of function experiments by transfecting sh-lincRNA-p21 A549 cells with control siRNA or PUMA siRNA. Following PUMA knockdown (P < 0.01, Figure 4a, 4b), the apoptosis rate in A549 cells treated with both sh-lincRNA-p21 and si-PUMA was significantly reduced compared with that in sh-lincRNA-p21 A549 cells infected with sh-lincRNA-p21 alone (P < 0.05, Figure 4c, 4d). This demonstrated that the pro-apoptotic effect of lincRNA-p21 downregulation could be attenuated by PUMA knockdown, revealing a key role for PUMA in this process. This was further confirmed by the overexpression of PUMA in lincRNA-p21 H1299 cells (P < 0.05,
Figure 4e, 4f). PUMA upregulation significantly attenuated the apoptosis inhibitory effect of the upregulation of lincRNA-p21, with apoptosis rates of 13.02% in H1299 cells, 3.82% in lincRNA-p21 H1299 cells, and 5.87% in lincRNA-p21 H1299 cells with upregulated PUMA \((P < 0.05, \text{Figure 4g, 4h})\). Collectively, our results revealed that PUMA is a key molecule in the anti-apoptotic process involving lincRNA-p21 in NSCLC cells.

**Discussion**

In this study, we observed the aberrant increase of lincRNA-p21 in NSCLC tissues compared with healthy lung tissues. Moreover, the upregulation of lincRNA-p21 significantly inhibited cell apoptosis while its downregulation enhanced apoptosis. We also performed RNA-seq analysis and identified PUMA as a potential target of lincRNA-p21 by analyzing the overlapping and differentially expressed genes between A549 and H1299 cell RNA-seq arrays. An RNA pull-down assay confirmed that PUMA was a direct target gene of lincRNA-p21, and a negative correlation was detected between PUMA and lincRNA-p21 using clinical NSCLC specimen analysis. Interestingly, the upregulation of PUMA significantly reversed the anti-apoptotic effect mediated by lincRNA-p21 overexpression.

Lung cancer has the highest mortality rate among all cancers in most developed countries.\(^{31}\) NSCLC is the most prevalent type of lung cancer, accounting for
80%–85% of the disease, and can be further divided into two subgroups: adenocarcinoma and squamous cell carcinoma.2,4 Previous studies have demonstrated that an early diagnosis of lung cancer dramatically increased the 5-year survival to 45%–53% compared with 3%–4% for late diagnosis or advanced stage disease.32 Identifying key molecules might be beneficial for the early diagnosis or clinical treatment of NSCLC.

Several lines of evidence have revealed that lincRNAs are involved in the progression of many types of cancer.33,34 LincRNA-p21 was shown to act as a component of the p53 pathway by binding with the p53 repressive complex to decrease p53 target mRNAs, and to function as a translation suppressor by directly interacting with target mRNAs.14,35 The present study shows, for the first time to our knowledge, that lincRNA-p21 could promote tumor development in NSCLC. This conflicts with other reports that observed the downregulation of lincRNA-p21 in CRC and CRC cell lines,17 which might reflect the individuality of different types of cancer.

The inhibition of cancer cell apoptosis contributes to tumor progression, and lincRNAs have been shown to affect cancer cell apoptosis. Therefore, in this study, we tested lincRNA-p21 gain of function by overexpressing it in lincRNA-p21 low-expression H1299 cells, and loss of function by downregulating it in lincRNA-p21 high-expression A549 cells. FACS analysis demonstrated that upregulation of lincRNA-p21 inhibited apoptosis while downregulation of lincRNA-p21 enhanced it. This anti-apoptotic role for lincRNA-p21 is consistent with our hypothesis that lincRNA-p21 negatively regulates cell apoptosis and thus contributes to tumor progression in NSCLC.

LincRNA-p21 can either directly interact with target mRNAs or act as suppressor of p53-mediated pathway through its p53-binding sites, thus contributing to cell apoptosis. To analyze the lincRNA-p21-associated genes, we applied RNA-seq sequencing to assess the gene expression profiles of sh-lincRNA-p21 A549 and control A549 cells, and of lincRNA-p21 H1299 and H1299 cells. We first identified the differentially expressed genes in the different groups. To exclude the possible genes associated with NSCLC cell lines, we checked the overlapping genes of the differentially expressed genes between the two gene expression profiles. The top three overlapping genes were thus identified, FOXD1, PUMA, and ZNF60 (ZBTB17). FOXD1 is a member of the forkhead family, which is upregulated in breast cancer and increases cell proliferation and chemoresistance in MCF-7 cells.28 Zinc-finger and BTB-domain-containing protein 17 (ZBTB17) is a member of the poxvirus and zinc-finger domain/zinc-finger transcription factor family. It induces cell cycle arrest by upregulating the expression of cyclin-dependent kinase inhibitor 1A or cyclin-dependent kinase inhibitor 2B, and also promotes cell proliferation or transformation by repressing the expression of these cyclin-dependent kinase inhibitors.29 PUMA, an important member of the BH3-only Bcl-2 family, is a downstream target of p53 that plays an important role in stress-induced apoptosis.30 PUMA is a pro-apoptotic protein and can be activated by p53, which is reported to inhibit all the five anti-apoptotic proteins (Mcl-1, Bcl-2, Bcl-XL, Bcl-W, and A1) and directly triggers apoptosis.36 For these reasons, we focused on PUMA in further study and evaluated whether lincRNA-p21 is involved in the regulation of PUMA expression. We verified the correlation of PUMA with lincRNA-p21 in NSCLC tissues. Actually, PUMA was negatively correlated with lincRNA-p21, which was inconsistent with the RNA-seq analysis and RNA pull-down assay. These data revealed that
lincRNA-p21 negatively regulates PUMA expression. On the basis of the regulatory mechanism of lincRNA-p21 on other genes,\textsuperscript{35} we may test a hypothesis that lincRNA-p21 suppresses PUMA expression by increasing its association with PUMA mRNAs and lowering PUMA translation.

Induction of p53-mediated apoptosis is usually executed through the induction of pro-apoptotic proteins from the Bcl-2 family.\textsuperscript{36} Of note, in this study, lincRNA-p21 contains p53-binding sites and PUMA is capable of inhibiting all the five major anti-apoptotic Bcl-2-like proteins (Mcl-1, Bcl-2, Bcl-XL, Bcl-W and A1).\textsuperscript{19} Therefore, it is reasonable to question whether PUMA was involved in the lincRNA-p21 mediated anti-apoptotic effect. To investigate this, we determined the apoptosis in A549 cells that were treated with a double knockdown of linRNA-p21 and PUMA, and in H1299 cells that were treated with a double overexpression of lincRNA-p21 and PUMA. The pro-apoptotic effect of the downregulation of lincRNA-p21 could be attenuated by the knockdown of PUMA, while the anti-apoptotic role of overexpression of lincRNA-p21 was decreased by PUMA upregulation. Our results firmly indicated that PUMA, negatively regulated by lincRNA-p21, played a key role in the apoptosis process of NSCLC cells.

In conclusion, the present study demonstrates that lincRNA-p21 is aberrantly upregulated in NSCLC and inhibits cell apoptosis by decreasing PUMA expression in NSCLC cells. Our data reveal that lincRNA-p21 might serve as a potential therapeutic target for NSCLC.

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ORCID iD
Jianghe Shao http://orcid.org/0000-0002-8110-1166

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