European Review for Medical and Pharmacological Sciences 2019; 23: 4807-4814

MiRNA-621 inhibits the malignant progression of non-small cell lung cancer *via* targeting SIX4

M. ZHANG[1], H. SHI[1], C. ZHANG[2], S.-Q. ZHANG[1]

[1]Department of Thoracic Surgery, Shengzhou Renmin Hospital, Shengzhou, China
[2]Department of Thoracic Surgery, the First Hospital of Zhejiang University, Hangzhou, China

Abstract. – OBJECTIVE: To clarify the role of microRNA-621 (miRNA-621)/SIX4 axis in regulating the malignant progression of non-small cell lung cancer (NSCLC) and the potential mechanism.

PATIENTS AND METHODS: MiRNA-621 expression in NSCLC tissues and paracancerous tissues (n=50) was examined by quantitative Real-time polymerase chain reaction (qRT-PCR). We further analyzed the correlation between miRNA-621 expression and pathological indexes of NSCLC patients. By transfection of miRNA-621 mimics, its expression was upregulated. Regulatory effects of miRNA-621 on proliferation and apoptosis of H1299 and SPC-A1 cells were evaluated by cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU) assay and flow cytometry, respectively. Finally, the potential mechanism of miRNA-621 in regulating target gene SIX4 was explored by Western blot and rescue experiments.

RESULTS: MiRNA-621 was lowly expressed in NSCLC tissues relative to paracancerous tissues. NSCLC patients with low-level miRNA-621 were expected to have a worse clinical grade and shorter overall survival compared with those with a high level. Transfection of miRNA-621 mimics in H1299 and SPC-A1 cells markedly suppressed proliferation, but induced apoptosis. SIX4 was found to be highly expressed in NSCLC and negatively regulated by miRNA-621. MiRNA-621 overexpression could downregulate protein levels of SIX4, CD31, Ki-67, c-Myc, MMP-2 and MMP-9 in H1299 and SPC-A1 cells. Overexpression of SIX4 partially reversed the role of miRNA-621 in the malignant progression of NSCLC.

CONCLUSIONS: MiRNA-621 is closely related to pathological grade and poor prognosis of NSCLC. Besides, miRNA-621 can inhibit the malignant progression of NSCLC by regulating SIX4 expression.

Key Words:
MiRNA-621, SIX4, NSCLC, Proliferation.

Introduction

Lung cancer is a common malignant tumor in the world, which is characterized by high morbidity and mortality. The mortality of lung cancer ranks first in all types of malignancies, posing a great threat to human life and health[1-3]. In males, the morbidity and mortality of lung cancer account for 17% and 23% of malignancies, respectively. Females are also endangered by lung cancer, ranking the second place only to breast cancer[4-6]. Small cell lung cancer (SCL) and non-small cell lung cancer (NSCLC) are the two major pathological types[7]. NSCLC accounts for 85% of all subtypes of lung cancer[7,8]. Tumor severity and metastasis are varied in different subtypes of lung cancer[9]. Similar to malignant tumors, mutation or loss-of-function of tumor-suppressor genes, and activation of oncogenes are the major pathological factors of NSCLC[9,10]. In addition, some biomarkers present significant influence on prognosis and drug-sensitivity of NSCLC[10]. Therefore, researches on the malignant mechanism of NSCLC help to develop individualized therapeutic strategies, and provide effective prognostic hallmarks[10,11]. At present, microRNAs (miRNAs) have been identified to be related to the tumorigenesis of NSCLC and other malignant tumors[8]. MiRNAs are extensively present in plants and animals. They are non-coding, single-stranded RNAs containing 18-24 nucleotides derived from protein-coding gene sequences or intergenic sequences. Multiple biological functions of miRNAs rely on the recognition of seed sequences at miRNA 5' terminal and target gene 3'-Untranslated Regions (3'-UTR). Subsequently, miRNAs induce the degradation of target mRNAs by complementary pairing[12-14]. Although miRNAs account for a small proportion in the human genome, they exert regulatory effects on a series of

Corresponding Author: Shaoqin Zhang, BM; e-mail: zhangsq230@163.com

physiological processes. Nearly one-third of the genes involved in cell growth, proliferation, and cell cycle are regulated by miRNAs[14,15]. MiRNA-621 is abnormally expressed in solid malignant tumors, serving as an oncogene to promote tumor cell proliferation[16,17]. Recent studies have determined the potential of miRNAs to be therapeutic targets for tumors. MiRNA-621 is capable of regulating tumorigenesis by mediating SIX4. We believed that NSCLC-specific miRNAs could be utilized as targets by acting on SIX4. In this paper, we focused on the potential function of miRNA-621/SIX4 axis in the progression of NSCLC, and our results provide new directions in the diagnosis and treatment of NSCLC.

Patients and Methods

Patients and NSCLC Samples

Tumor tissues and paracancerous tissues were harvested from 60 NSCLC patients undergoing radical resection. None of patients were preoperatively treated with chemotherapy or radiotherapy. Clinical stage of NSCLC was evaluated based on the criteria proposed by UICC (Union for International Cancer Control). Patients who participated in this study gave their consent. This study was approved by Ethic Committee of Shengzhou Renmin Hospital.

Cell Lines and Reagents

NSCLC cell lines (A549, H1299, PC-9, H358, SPC-A1) and bronchial epithelial cell line (BEAS-2B) provided by American Type Culture Collection (ATCC) (Manassas, VA, USA), were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA). Cells were maintained in a 37°C, 5% CO_2 incubator.

Transfection

MiRNA-621 mimics and negative control (NC) were provided by GenePharma (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 70%, followed by plasmid transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested at 48 h for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assay

Transfected cells for 48 h were seeded into the 96-well plate with 2000 cells per well. At 24 h, 48 h, 72 h and 96 h, cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan) was supplied in each well. After 2 h of culture, absorbance of each well at 490 nm was recorded using a microplate reader.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

Transfected cells for 24 h were labeled with 50 μM EdU (Beyotime, Shanghai, China) for 2 h, dyed with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) in dark for 30 min. EdU-positive rate was calculated as the ratio of EdU-positive cells (red) and DAPI-stained cells (blue) under a fluorescent microscope.

Flow Cytometry Analysis of the Cell Apoptosis

Cells were washed with phosphate-buffered saline (PBS) twice, digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin and resuspended at the density of 1×10^6/mL. Subsequently, cells were incubated with 1× binding buffer and 1.25 μL of Annexin V-FITC (fluorescein isothiocyanate) for 15 min in dark. After centrifugation at 1000×g for 5 min, the precipitate was incubated with 10 μL of Propidium Iodide (PI). Apoptosis was determined within 1 hour by flow cytometry (Partec AG, Arlesheim, Switzerland).

QRT-PCR

We extracted total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into the complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR reaction was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: MiRNA-621: forward: 5'-TTAGCTCAGGATCATCATCATTTACATAGATAGGG-3', reverse: 5'-AACACTCGAGTGAGAGAAGAGAGTGCCTAGA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; SIX4: forward: 5'-TCTCGGGGTGATCGACAAGAA-3', reverse: 5'-CCCTTTGTTCATTCGTTCCTGG-3'; β-actin: forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-GCTGATCCACATCTGCTGGAA-3›.

Western Blot

Total protein was extracted using the cell lysate, and qualified using the protein determination kit (Pierce, Rockford, IL, USA). Protein samples were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred on the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 Software (IBM, Armonk, NY, USA) was utilized for statistical processing. Univariate analysis was performed using χ^2 test and Fisher's exact test; multivariate analysis was performed by COX regression analysis. Kaplan-Meier was introduced for survival analysis, followed by Log-rank test for intergroup curve determination. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). $p<0.05$ was considered statistically significant.

Results

Expression Pattern of miRNA-621 in NSCLC

Expression pattern of miRNA-621 in NSCLC tissues and cell lines was determined by qRT-PCR. Compared with paracancerous tissues, miRNA-621 was lowly expressed in NSCLC tissues (Figure 1A, 1B). Identically, miRNA-621 expression was lower in NSCLC cell lines relative to BEAS-2B cells, especially in H1299 and SPC-A1 cell lines (Figure 1C). Based on miRNA-621 level, NSCLC patients were divided into high-level group and low-level group. Kaplan-Meier curve indicated shorter overall survival in NSCLC patients with low level of miRNA-621 than those with high level (Figure

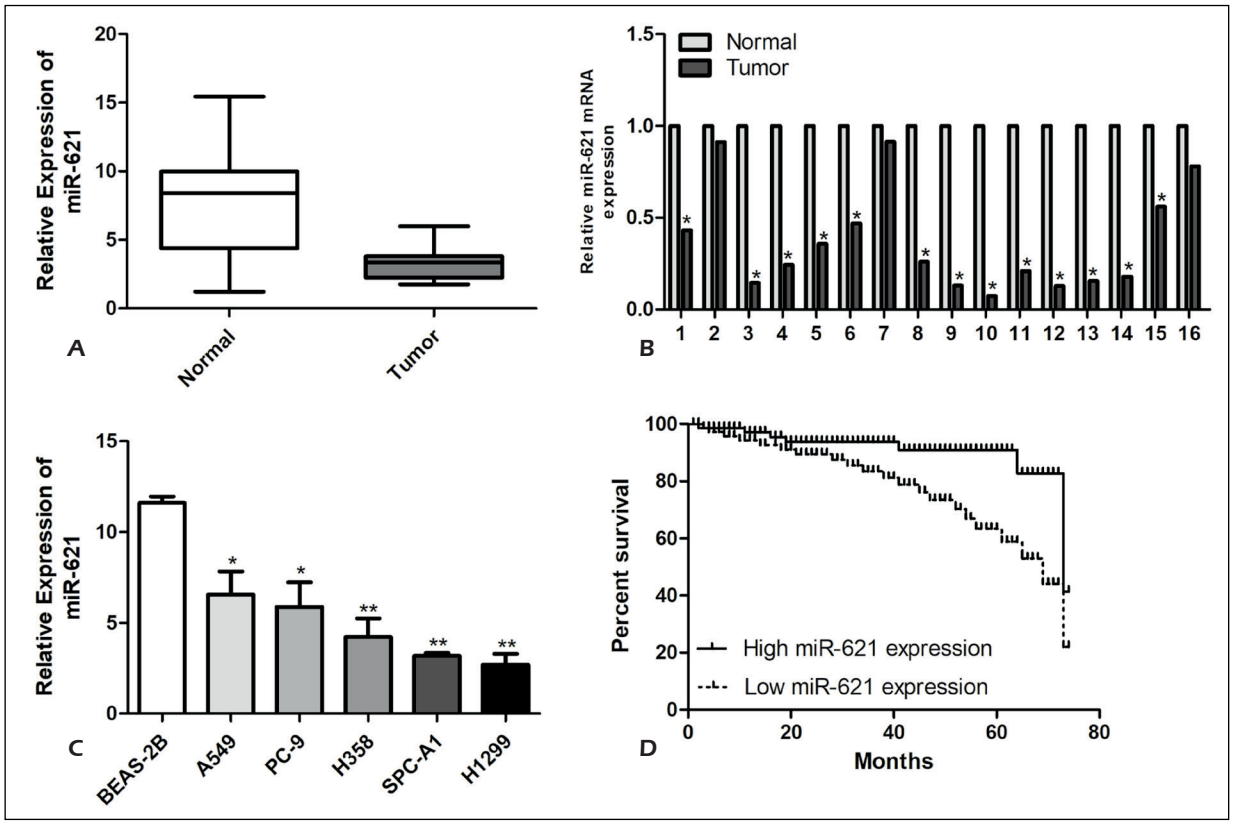

Figure 1. Expression pattern of miR-621 in NSCLC. **A-B**, Compared with paracancerous tissues, miR-621 was lowly expressed in NSCLC tissues determined by qRT-PCR. **C**, MiR-621 expression was lower in NSCLC cell lines relative to BEAS-2B cells, especially in H1299 and SPC-A1 cell lines. **D**, Kaplan-Meier curve indicated shorter overall survival in NSCLC patients with low level of miR-621 than those with high level.

Table I. Association of miR-621 expression with clinicopathologic characteristics of lung cancer.

Parameters	No. of cases	miR-621 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.812
<60	20	12	8	
≥60	30	19	11	
Gender				0.514
Male	24	16	8	
Female	26	15	11	
T stage				0.009
T1-T2	30	23	7	
T3-T4	20	8	12	
Lymph node metastasis				0.190
No	32	22	10	
Yes	18	9	9	
Distance metastasis				0.171
No	37	25	12	
Yes	13	6	7	

1D). Subsequently, we analyzed the correlation between miRNA-621 expression and pathological indexes of NSCLC patients. As the data revealed, miRNA-621 expression was positively correlated to clinical grade of NSCLC patients, rather than age, gender, lymph node metastasis and distant metastasis (Table I). We believed that miRNA-621 may be a novel hallmark for predicting the malignant progression of NSCLC.

Upregulation of miRNA-621 Inhibited Proliferative Rate and Induced Apoptosis of NSCLC

To explore cellular regulations of miRNA-621 on NSCLC, we first constructed miRNA-621 mimics and verified its transfection efficacy (Figure 2A). Proliferative and apoptotic changes in H1299 and SPC-A1 cells influenced by miRNA-621 were evaluated. As CCK-8 assay indicated, transfection of miRNA-621 mimics markedly inhibited proliferative rate of NSCLC cells (Figure 2B). EdU assay showed fewer proliferative cells after miRNA-621 overexpression relative to those transfected with NC, indicating the inhibited proliferative capacity of NSCLC (Figure 2C). In addition, transfection of miRNA-621 mimics markedly increased the number of apoptotic cells (Figure 2D).

Upregulation of miRNA-621 Inhibited Relative Genes in SIX4 Pathway

Next, we focused on the specific mechanism of miRNA-621 in regulating the malignant progression of NSCLC. Western blot was performed to determine relative gene expressions in SIX4 pathway. Overexpression of miRNA-621 downregulated protein levels of SIX4, CD31, Ki-67, c-Myc, MMP-2 and MMP-9 in NSCLC cells (Figure 3).

SIX4 was Highly Expressed in NSCLC

SIX4 expression was downregulated by transfection of miRNA-621 mimics in H1299 and SPC-A1 cells (Figure 4A). QRT-PCR data showed higher level of SIX4 in NSCLC tissues relative to controls (Figure 4B). Similarly, SIX4 was also highly expressed in NSCLC cell lines (Figure 4C). Correlation analyzed revealed a negative correlation between expressions of SIX4 and miRNA-621 in NSCLC tissues (Figure 4D).

MiRNA-621 Directly Inhibited SIX4 Expression

Rescue experiments were performed to verify whether miRNA-621 exerted its function in NSCLC *via* mediating SIX4. Transfection efficacy of pcDNA-SIX4 was first verified in NSCLC cells (Figure 5A). Downregulated SIX4 by miRNA-621 overexpression was partially reversed after co-overexpression of SIX4 and miRNA-621 (Figure 5A). Interestingly, regulatory effects of miRNA-621 on proliferation and apoptosis of NSCLC cells were reversed by SIX4 overexpression (Figure 5B-5D).

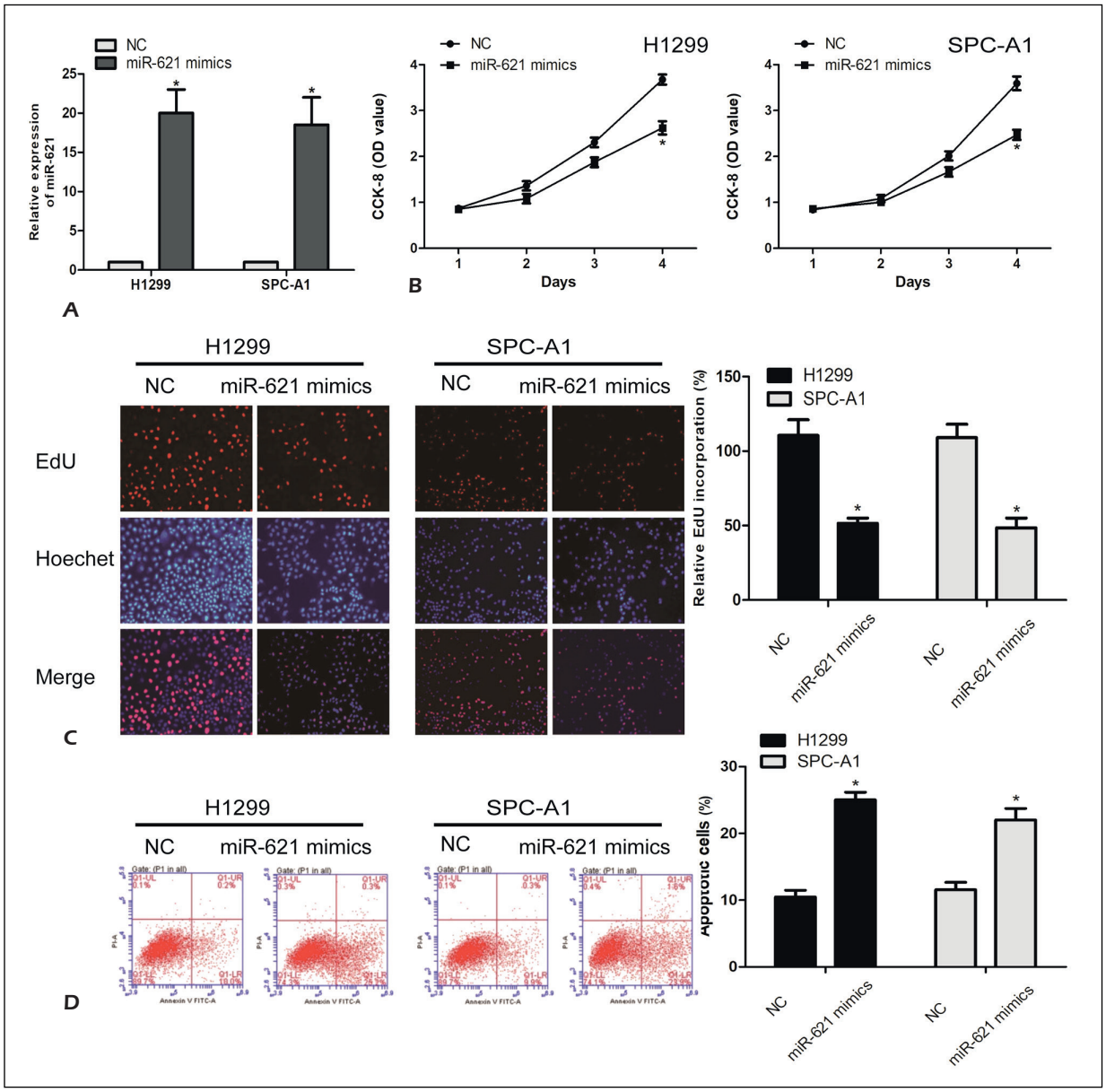

Figure 2. Upregulation of miR-621 inhibited proliferative rate and induced apoptosis of NSCLC. **A**, Transfection efficacy of miR-621 mimics in H1299 and SPC-A1 cells. **B**, CCK-8 assay showed that transfection of miR-621 mimics markedly inhibited proliferative rate of H1299 and SPC-A1 cells. **C**, EdU assay showed that transfection of miR-621 mimics markedly decreased the number of proliferative cells. **D**, Flow cytometry showed that transfection of miR-621 mimics markedly induced apoptosis of H1299 and SPC-A1 cells.

Discussion

NSCLC is a high-grade malignant tumor in humans. The 5-year survival of NSCLC is lower than 15%, and its mortality ranks first among all malignant tumors[1-4]. It is reported that the mortality of NSCLC is remarkably higher than that of breast cancer, colorectal cancer and prostate cancer in North America and developing countries[4,5]. As a highly metastatic tumor, NSCLC is prone to brain metastases and severely threatens the prognosis[6,7]. Abnormal proliferation and apoptosis of tumor cells are fatal factors for carcinogenesis[7,8]. The progression of NSCLC is a complex process involving various factors, relying on a series of internal environmental factors[9-11].

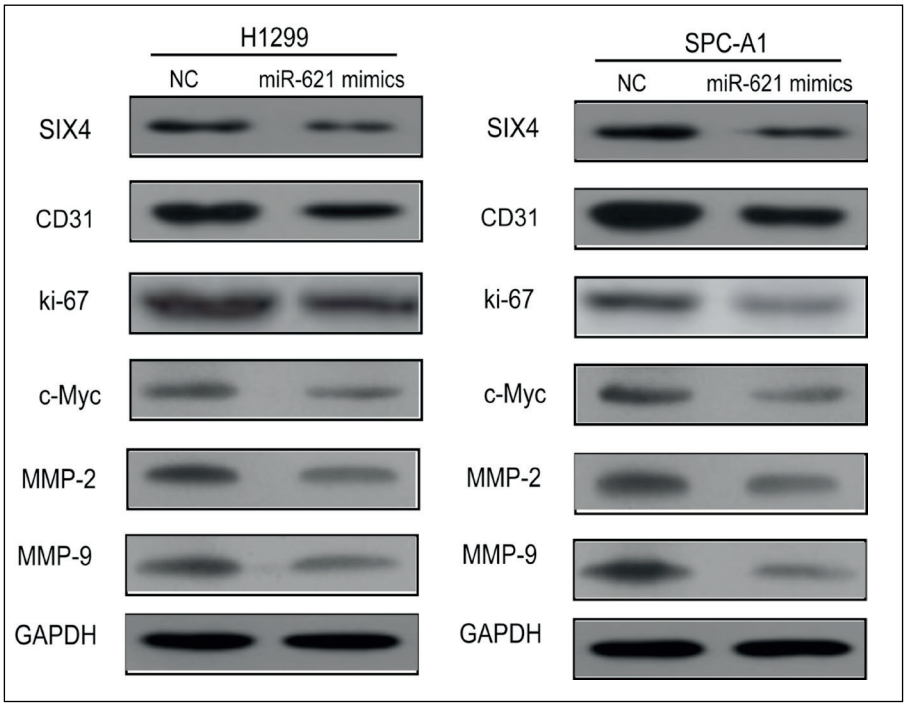

Figure 3. Upregulation of miR-621 inhibited relative genes in SIX4 pathway. Western blot analyses of SIX4, CD31, Ki-67, c-Myc, MMP-2 and MMP-9 in H1299 and SPC-A1 cells transfected with miR-621 mimics or NC.

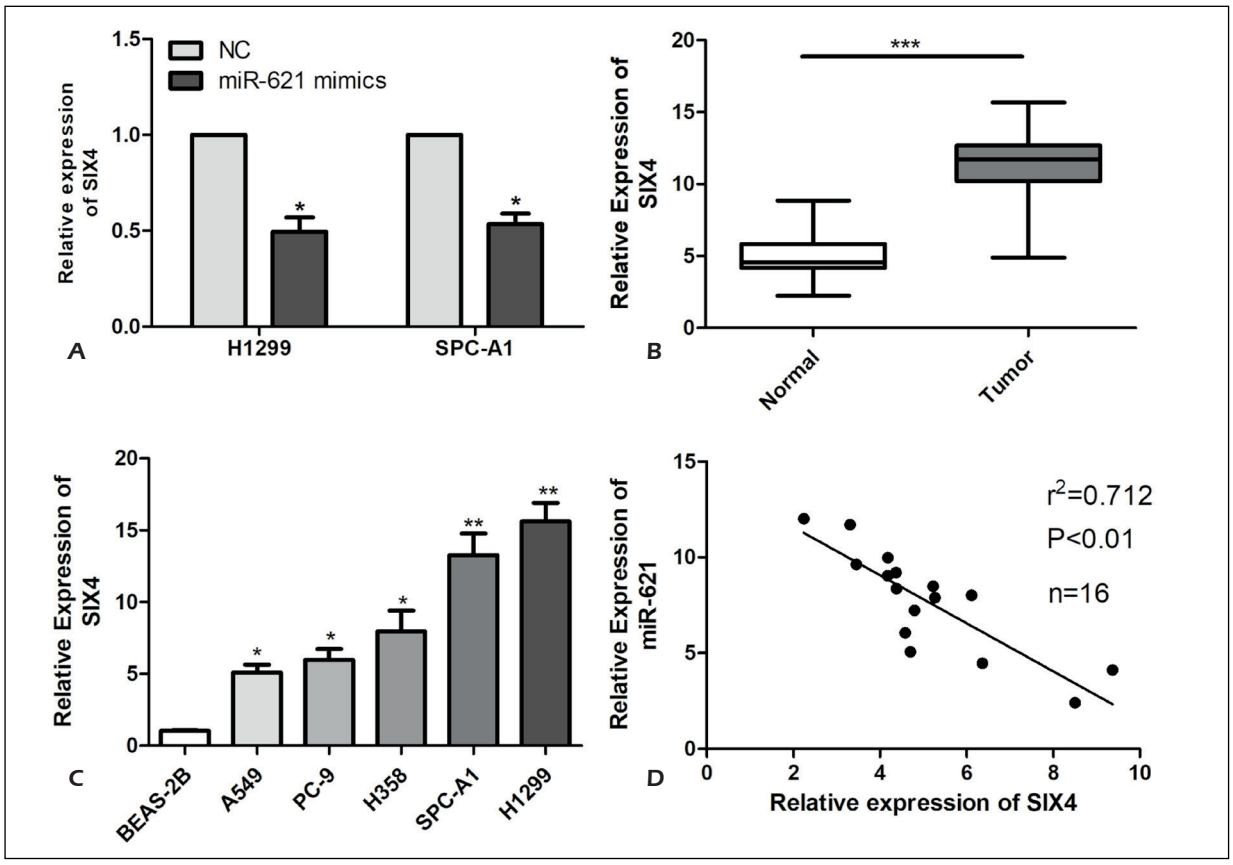

Figure 4. SIX4 was highly expressed in NSCLC. **A**, SIX4 expression was downregulated after transfection of miR-621 mimics in H1299 and SPC-A1 cells. **B**, QRT-PCR data showed higher level of SIX4 in NSCLC tissues relative to controls. **C**, QRT-PCR data showed higher level of SIX4 in NSCLC cell lines. **D**, Correlation analyzed revealed a negative correlation between expressions of SIX4 and miR-621 in NSCLC tissues.

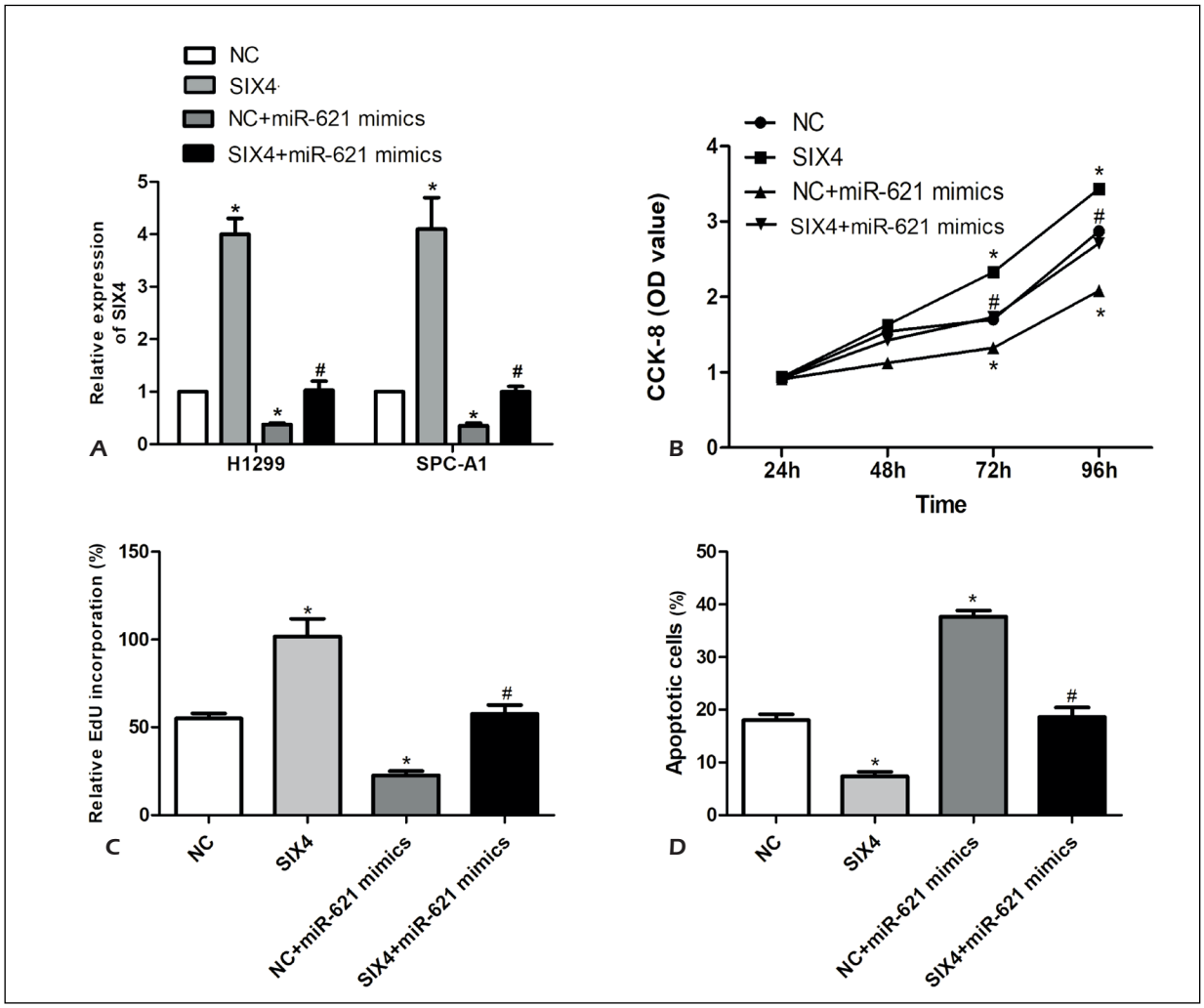

Figure 5. MiR-621 directly inhibited SIX4 expression. H1299 and SPC-A1 cells were transfected with NC, pcDNA-SIX4, NC+miR-621 mimics or pcDNA-SIX4+miR-621 mimics, respectively. **A**, Relative level of SIX4 detected by qRT-PCR. **B**, CCK-8 assay determined viability. **C**, EdU assay determined the number of proliferative cells. **D**, Flow cytometry determined the percentage of apoptotic cells.

Although the oncology of NSCLC has been well explored, there are many unknown aspects need to be resolved[10,11]. Abnormal changes in miRNA expressions usually indicate the occurrence and development of diseases. Investigations of regulatory effects of miRNAs on diseases are significant to drug development[12, 13]. Recently, a variety of methods have been discovered to effectively regulate miRNA expression, including development of mimics, inhibitors, transgenic regulation, and point mutations[14]. Among the above methods, transfection of miRNA mimics or inhibitor could successfully and effectively alter miRNA expressions. They have been widely applied in experiments due to simple procedures and high successful rate[15-17]. MiRNA-621 is lowly expressed in several tumors, and its expression is closely correlated to pathological performances and prognosis. It is suggested that miRNA-621 serves as an oncogene in malignant tumors[16, 17]. To explore the role of miRNA-621 in the development and progression of NSCLC, its expression was first determined in 60 cases of NSCLC tissues and paracancerous tissues. QRT-PCR data showed downregulated miRNA-621 in NSCLC, and its expression was correlated to clinical stage of NSCLC patients. We speculated the tumor-suppressor effect of miRNA-621 on NSCLC. Subsequently, transfection of miRNA-621 mimics markedly inhibited proliferative rate, but in-

duced apoptosis of NSCLC cells. Overexpression of miRNA-621 downregulated levels of SIX4, CD31, Ki-67, c-Myc, MMP-2 and MMP-9, thus inhibiting the progression of NSCLC. Expression pattern of SIX4 in NSCLC was determined as well, which was highly expressed in NSCLC. An interaction between miRNA and its target genes has been revealed to regulate disease progression[13-15]. Our study showed that SIX4 promoted the malignant behaviors of NSCLC cells. SIX4 expression was negatively regulated by miRNA-621, indicating a potential interaction between them. Of note, the regulatory effects of miRNA-621 on proliferation and apoptosis of NSCLC cells were reversed by SIX4. Thus, our results identified the function of miRNA-621/SIX4 axis in mediating the malignant progression of NSCLC.

Conclusions

In summary, miRNA-621 is closely related to pathological grade and poor prognosis of NSCLC; Besides, miRNA-621 can inhibit the malignant progression of NSCLC by regulating SIX4 expression.

Conflict of Interests

The authors declared no conflict of interest.

References

1) Pisapia P, Malapelle U, Troncone G. Liquid biopsy and lung cancer. Acta Cytol. 2018 Dec 19:1-8. doi: 10.1159/000492710. [Epub ahead of print] Review.
2) Beardslee T, Lawson J. Alectinib and brigatinib: new second-generation ALK in-hibitors for the treatment of non-small cell lung cancer. J Adv Pract Oncol 2018; 9: 94-101.
3) Huang Q, Tan Q, Mao K, Yang G, Ma G, Luo P, Wang S, Mei P, Wu F, Xu J, Guo M, Lv Z, Fan J, Zhang S, Wang X, Jin Y. The role of adrenergic receptors in lung cancer. Am J Cancer Res 2018; 8: 2227-2237.
4) Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ, Wu YL, Paz-Ares L. Lung cancer: current therapies and new targeted treatments. Lancet 2017; 389: 299-311.
5) Rivera GA, Wakelee H. Lung cancer in never smokers. Adv Exp Med Biol 2016; 893: 43-57.
6) Nanavaty P, Alvarez MS, Alberts WM. Lung cancer screening: advantages, con-troversies, and applications. Cancer Control 2014; 21: 9-14.
7) Wang LL, Zhang M. miR-582-5p is a potential prognostic marker in human non-small cell lung cancer and functions as a tumor suppressor by targeting MAP3K2. Eur Rev Med Pharmacol Sci 2018; 22: 7760-7767.
8) Lamichhane SR, Thachil T, De Ieso P, Gee H, Moss SA, Milic N. Prognostic role of MicroRNAs in human non-small-cell lung cancer: a systematic review and meta-analysis. Dis Markers 2018; 2018: 8309015.
9) Prelaj A, Tay R, Ferrara R, Chaput N, Besse B, Califano R. Predictive biomarkers of response for immune checkpoint inhibitors in non-small-cell lung cancer. Eur J Cancer 2019; 106: 144-159.
10) Penault-Llorca F, Radosevic-Robin N. Tumor mutational burden in non-small cell lung cancer-the pathologist's point of view. Transl Lung Cancer Res 2018; 7: 716-721.
11) Hendriks LE, Rouleau E, Besse B. Clinical utility of tumor mutational burden in patients with non-small cell lung cancer treated with immunotherapy. Transl Lung Cancer Res 2018; 7: 647-660.
12) Tutar Y. miRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.
13) Eyileten C, Wicik Z, De Rosa S, Mirowska-Guzel D, Soplinska A, Indolfi C, Ja-strzebska-Kurkowska I, Czlonkowska A, Postula M. MicroRNAs as diagnostic and prognostic biomarkers in ischemic stroke-a comprehensive review and bioin-formatic analysis. Cells 2018; 7: 249.
14) Bernardo BC, Ooi JY, Lin RC, McMullen JR. miRNA therapeutics: a new class of drugs with potential therapeutic applications in the heart. Future Med Chem 2015; 7: 1771-1792.
15) Jin RH, Yu DJ, Zhong M. MiR-1269a acts as an onco-miRNA in non-small cell lung cancer via down-regulating SOX6. Eur Rev Med Pharmacol Sci 2018; 22: 4888-4897.
16) Li DY, Chen WJ, Luo L, Wang YK, Shang J, Zhang Y, Chen G, Li SK. Prospec-tive lncRNA-miRNA-mRNA regulatory network of long non-coding RNA LINC00968 in non-small cell lung cancer A549 cells: a miRNA microarray and bi-oinformatics investigation. Int J Mol Med 2017; 40: 1895-1906.
17) Xue J, Chi Y, Chen Y, Huang S, Ye X, Niu J, Wang W, Pfeffer LM, Shao ZM, Wu ZH, Wu J. MiRNA-621 sensitizes breast cancer to chemotherapy by suppress-ing FBXO11 and enhancing p53 activity. Oncogene 2016; 35: 448-458.