

MiR-411-5p acts as a tumor suppressor in non-small cell lung cancer through targeting PUM1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression of micro ribonucleic acid-411-5P (miR-411-5p) in non-small cell lung cancer (NSCLC), and to explore the effect of miR-411-5p on the biological behavior of NSCLC cells as well as the underlying molecular mechanism.

PATIENTS AND METHODS: Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was used to detect the expression level of miR-411-5p in NSCLC tissues and cells. MiR-411-5p mimics and relevant controls were transfected into NSCLC cells according to the instructions of Lipidosome 2000. Transfected cells were divided into the experimental group and the control group. The transfection efficiency of each group was detected by qRT-PCR. After miR-411-5p overexpression, methyl thiazolyl tetrazolium (MTT) assay, flow cytometry, and transwell assay were used to detect the biological changes of cells in each group. Bioinformatics predicted that pumilio homolog 1 (PUM1) was the target gene of miR-411-5p. Subsequently, the mRNA and protein expression level of PUM1 in each group was detected by qRT-PCR and Western blotting, respectively. Dual-luciferase reporter assay was used to validate the target regulatory relationship between miR-411-5p and PUM1.

RESULTS: The results of qRT-PCR showed that miR-411-5p was relatively lowly expressed in NSCLC tissues and cells. After miR-411-5p overexpression, MTT results revealed that the proliferation of NSCLC cells was decreased. Flow cytometry results indicated that the apoptosis rate of NSCLC cells was increased, and cell cycle was arrested in the G0-G1 phase. Meanwhile, the transwell assay demonstrated that the migration and invasion abilities of NSCLC cells were decreased. Bioinformatics predicted that PUM1 was the target gene of miR-411-5p. After miR-411-4p was overexpressed in NSCLC cells,

qRT-PCR and Western blotting showed that both the mRNA and protein expression levels of PUM1 were up-regulated. Moreover, dual-luciferase reporter assay demonstrated that miR-411-5p could significantly inhibit the luciferase activity of wild-type PUM1-3'-untranslated region (3'-UTR). However, it exhibited no effect on the luciferase activity of cells transfected with mutant plasmids.

CONCLUSIONS: MiR-411-5p may be involved in regulating the biological function of NSCLC cells via targeting PUM1. In addition, miR-411-5p may serve as a potential target for the molecular therapy of NSCLC.

Key Words:

NSCLC, MiR-411-5p, Biological function, PUM1.

Introduction

Lung cancer is the most common pulmonary primary malignant tumor in clinics, among which non-small cell lung cancer (NSCLC) accounts for 80-85% of the total number of lung cancer cases¹. Due to the difficulties in early diagnosis, most lung cancer patients are diagnosed in the middle and late stage. Therefore, radical resection cannot be performed. Chemotherapy has become the primary treatment for lung cancer, however, a considerable number of patients have unsatisfactory treatment outcomes and poor prognosis². Therefore, searching new therapeutic targets for the treatment of NSCLC has become the most urgent problem to be solved nowadays.

Micro ribonucleic acid (miRNA) is a class of non-coding small RNAs with about 20-25 nucleotides in length. MiRNAs can regulate

gene expression at the post-transcriptional level. Nearly 50% of human miRNAs is located in the fragile sites and genomic regions of malignant tumors³. New evidence has suggested that miRNA is closely related to the occurrence and development of human tumors. Meanwhile, miRNA can also play a similar role as tumor promoters or suppressors^{4,5}. It has been reported that miR-106b-5p can promote the proliferation and inhibit the apoptosis of NSCLC cells through regulating B-cell translocation gene anti-proliferation factor 3 (BTG3)⁶. MiR-455-3p is relatively highly expressed in NSCLC tissues and cells, which can be used as an independent prognostic factor for NSCLC patients. Besides, miR-455-3p can promote the invasion and metastasis of NSCLC cells via regulating homeobox B5 (HOXB5)⁷. However, no research has explored the expression level and biological function of miR-411-5p in NSCLC tissues and cells.

MiR-411-5p is located on chromosome 14q32.31. Reports have indicated that in rhabdomyosarcoma, a self-regulatory loop can be formed between transforming growth factor (TGF)-/miR-411-5p/sprouty receptor tyrosine kinase signaling antagonist 4 (SPRY4) and the mitogen-activated protein kinase (MAPK) pathway, eventually mediating the proliferation and differentiation of rhabdomyosarcoma⁸. Zhang et al⁹ have found that the expression of miR-411-5p is down-regulated in breast cancer. Moreover, miR-411-5p can inhibit the proliferation, invasion and metastasis of breast cancer cells through regulating growth factor receptor-bound protein 2 (GRB2). However, no reports have investigated the expression level and biological function of miR-411-5p in NSCLC tissues and cells. We firstly explored the expression level of miR-411-5p in NSCLC tissues and cells, and found that miR-411-5p were down-regulated in NSCLC. Bioinformatics and *in vitro* experiments showed that miR-411-5p acted as a "tumor suppressor" gene by targeting pumilio homolog 1 (PUM1).

Patients and Methods

Tissues and Cells

A total of 67 pairs of surgically resected NSCLC tissues and cancer-adjacent tissues (>5 cm away from cancer) from patients treated in the Shan County Central Hospital were collected in this study from January 2014 to December 2016.

This study was approved by the Ethics Committee of Shan County Central Hospital. Signed written informed consents were obtained from all participants before the study. Inclusion criteria of this study were as follows: 1) patients who received no anti-tumor treatments such as radiotherapy and chemotherapy prior to tissue collection; 2) patients who had complete clinical data; 3) patients definitely diagnosed by histopathology. Tissues resected in the operation were immediately washed with normal saline to remove bloodstains, and were stored in liquid nitrogen at -80°C for quantitative real-time polymerase chain reaction (qRT-PCR) detection.

One 16 human bronchial epithelial cell line (16HBE) and four NSCLC cell lines (A549, SPCA-1, PC-9 and SK-MES-1) were purchased from the Shanghai Institute for Cells, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA). The cells were grown in a 37°C, 5% CO₂ incubator. After grew to 70-80%, the cells were digested with trypsin and subculture was performed.

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to NSCLC tissues and cells for lysis, and total RNA was extracted in accordance with the chloroform and isopropanol method. After washing with 75% ethanol solution for 2 times, total RNA was finally dissolved in diethyl pyrocarbonate (DEPC) water. QRT-PCR was performed on the 7300 Real-Time PCR System using the One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan). Reaction conditions were as follows: 95°C for 30 s, 95°C for 5 s, 60°C for 30s, a total of 40 cycles, and extension at 72°C for 10 s. The relative expression level of genes was expressed by $2^{-\Delta\Delta Ct}$. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference for PUM1, while U6 was used as an internal reference for miR-411-5p. Primers used in this study were: miR-411-5p, F: 5'-CCGGAACCCCTCCTTACTC-3', R: 5'-AATGGGATGTGTCCGAAGGA-3'; PUM1, F: 5'-TTCACAGACACCACCTCCTT-3', R: 5'-CTGGAGCAGCAGAGATGTAT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Methyl Thiazolyl Tetrazolium (MTT) Assay

Transfected NSCLC cells were seeded into 96-well plates. 20 μ L 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well at 0 h, 24 h, 48 h, 72 h and 96 h, respectively, followed by incubation for 4 h. According to the absorption (A) value of each well determined by a microplate reader, the proliferation of cells was calculated and analyzed. Proliferation inhibition rate = $[1 - \text{A value (the miR-411-5p transfected group or the miR-411-5p control group)} / \text{A value (un-transfected group)}] \times 100\%$. The experiment was repeated for three times.

Flow Cytometry

Transfected NSCLC cells were inoculated into 6-well plates at a density of 1×10^6 cells/well. 48 h later, the cells were digested with trypsin and then collected. According to the instructions of the Annexin V-FITC/propidium iodide (PI) double staining kit (Abcam, Cambridge, MA, USA), reagents were added successively. Subsequently, flow cytometry was used to detect and analyze the apoptosis rate of each group. Cells were collected under the same condition with the same method. Then the cells were re-suspended in precooled 75% ethanol and fixed at -20°C overnight. The intracellular DNA concentration was detected by flow cytometry and PI staining. The cell cycle was divided into the G1/G0 phase, S phase and G2/M phase. The percentage of cells in each phase was calculated.

Transwell Assay

Transfected NSCLC cells were seeded into 6-well plates. After the cells grew to 80-90%, they were digested with trypsin and re-suspended with serum-free medium into single cell suspension. Subsequently, cell density was calculated and adjusted to $3 \times 10^5/\text{mL}$. A total of 200 μ L cell suspension was added to the upper transwell chamber (upper chamber surface coated with a bottom membrane of transwell chamber with 50 mg/L BD Matrigel diluted at 1:8), while 600 μ L 1640 medium containing 20% FBS was added in the lower chamber, followed by incubation under normal conditions for 24 h. 3-5 replicates were set in each group. Finally formaldehyde fixation and crystal violet staining were conducted.

Western Blotting Assay

48 h after transfection, the cells were harvested and protein lysate was added. After centrifugation at room temperature for 30 min, the cells were centrifuged at 12,000 rpm for 10 min. The concentration of proteins was determined by the bicincho-

nic acid (BCA) method (Beyotime, Shanghai, China). Loading buffer was added to each protein sample, followed by water boiling for 5 min. Each well was loaded with 20 μ L sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis for protein separation. Then the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by using the wet transfer method. Subsequently, the membranes were incubated with 5% skim milk powder for blocking at room temperature for 2 h, followed by incubation with primary antibodies of (dilution at 1:1000) PUM1 and GAPDH at 4°C overnight. Corresponding secondary antibodies were added for incubation at room temperature for 1 h. Finally, enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA) color development reagents were used for protein visualization.

Dual-Luciferase Reporter Assay

Bioinformatics software predicted that PUM1 was a target gene of miR-411-5p. Then the reporter gene plasmids of wild-type and mutant PUM1-3'-untranslated region (3'-UTR) were constructed. The reporter gene plasmid, promoter-less Renilla-thymidine kinase (pRL-TK) plasmid, miR-411-5p mimics and its negative control plasmids were transfected into HEK293T cells, respectively. Dual-luciferase assay specification was performed 48 h later according to the manufacturer's instructions. Relative fluorescence intensity of different treatment groups was calculated by the ratio of fluorescence intensity of firefly to the fluorescence intensity of kidney.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 Software (IBM, Armonk, NY, USA) was used for statistical analysis. Data were expressed as mean $\pm$ standard deviation. One-way analysis of variance was applied for comparisons among multiple groups, followed by Post-Hoc Test (Least Significant Difference). *t*-test was used to compare the difference between two groups. $p < 0.05$ was considered as statistically significant.

Results**Expression of miR-411-5p in NSCLC Tissues and Cells**

We first used qRT-PCR to detect the expression level of miR-411-5p in 63 pairs of NSCLC and can-

cer-adjacent tissues as well as 4 cell lines. Results showed that miR-411-5p was down-regulated in 49 NSCLC tissues (Figure 1A), and it was down-regulated in all four NSCLC cell lines (Figure 1B). To study the biological function of miR-411-5p in NSCLC, miR-411-5p mimics was synthesized and transiently transfected into NSCLC cells. Then, the efficiency of overexpression was detected 48 h later transfection (Figure 1C and 1D).

Effect of miR-411-5p Overexpression on the Proliferation of NSCLC Cells

To explore the effect of miR-411-5p on the proliferation ability of NSCLC cells, miR-411-5p mi-

mics was transfected into NSCLC cells. MTT assay was performed at 0 h, 24 h, 48 h, 72 h and 96 h, respectively. The results revealed that compared with the control group, the inhibition rates of the miR-411-5p transfected group at 72 h and 96 h were significantly increased, and the differences were statistically significant ($p < 0.05$) (Figure 2A and 2B).

Effect of miR-411-5p Overexpression on the Apoptosis and Cell Cycle of NSCLC Cells

We then studied the effect of miR-411-5p on the apoptosis and cell cycle of NSCLC cells. Flow

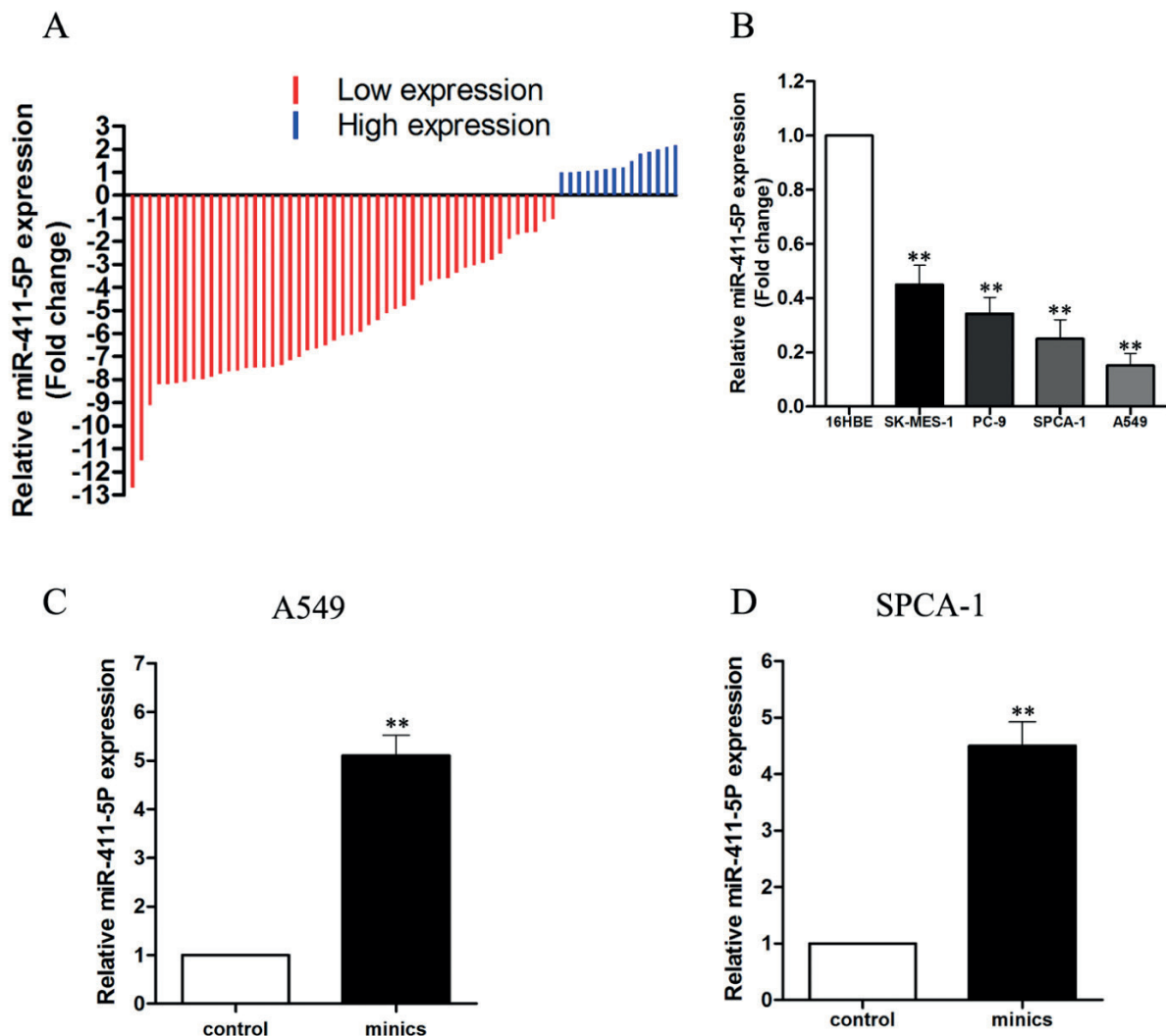


Figure 1. Expression of miR-411-5p in NSCLC tissues and cells. *A*, In 63 pairs of NSCLC tissues and adjacent tissues, qRT-PCR showed that miR-411-5p was relatively lowly expressed in NSCLC tissues. *B*, QRT-PCR results revealed that the expression of miR-411-5p was decreased in 4 NSCLC cell lines when compared with that in 16HBE. *C&D*, The mimics and control sequences of miR-411-5p were transiently transfected into NSCLC cells according to the instructions of liposomal Lip3000, and the transfection efficiency was detected by qRT-PCR assay 48 h later (**: $p < 0.01$).

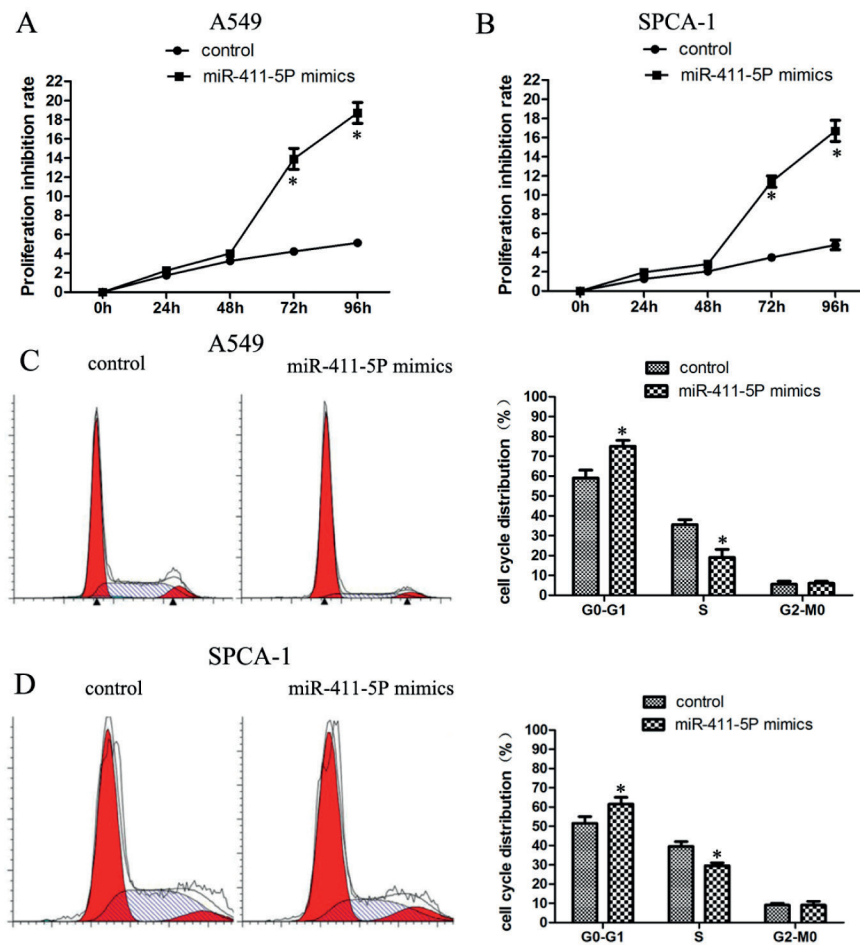


Figure 2. Effect of miR-411-5p overexpression on the proliferation of NSCLC cells. *A-B*, The mimics and control sequences were transiently transfected into NSCLC cells. MTT assay indicated that miR-411-5p overexpression inhibited the proliferation of NSCLC cells. *C-D*, The mimics and control sequences were transiently transfected into NSCLC cells. Flow cytometry showed that cell cycle was arrested in the G0-G1 phase in NSCLC cells. (*: $p < 0.05$).

cytometry indicated that compared with the control group, cell cycle of NSCLC cells was arrested in the G0-G1 phase after miR-411-5p overexpression (Figure 2C and 2D). Subsequently, the same method was performed to detect the apoptosis rate of NSCLC cells. Results demonstrated that the apoptosis rate of the miR-411-5p overexpression group at 48 h after transfection was significantly higher than that of the control group ($p < 0.05$) (Figure 3A and 3B).

Effect of miR-411-5p Overexpression on the Metastasis and Invasion of NSCLC Cells

Afterwards, transwell assay was conducted to measure the invasion and metastasis abilities of NSCLC cells. Cells in the overexpression group and the control group were planted in Transwell cham-

bers, and the number of cells that passed through the chambers was counted 48 h later. Results indicated that compared with the control group, the cell invasion and migration abilities of the overexpression group were inhibited (Figure 3C and 3D).

Target Regulation of miR-411-5p on PUM1

Bioinformatics (TargetScan, miRDB) predicted that PUM1 was the target gene of miR-411-5p. QRT-PCR and Western blotting indicated that the mRNA and protein expression levels of PUM1 in the miR-411-5p transfected group after 48 h were both down-regulated (Figure 4A and 4B). To further verify that PUM1 was a direct target of miR-411-5p, a fluorescent reporter plasmid containing wild-type and mutant PUM1-3'UTR as well as miR-411-5p mimics and its control plasmid

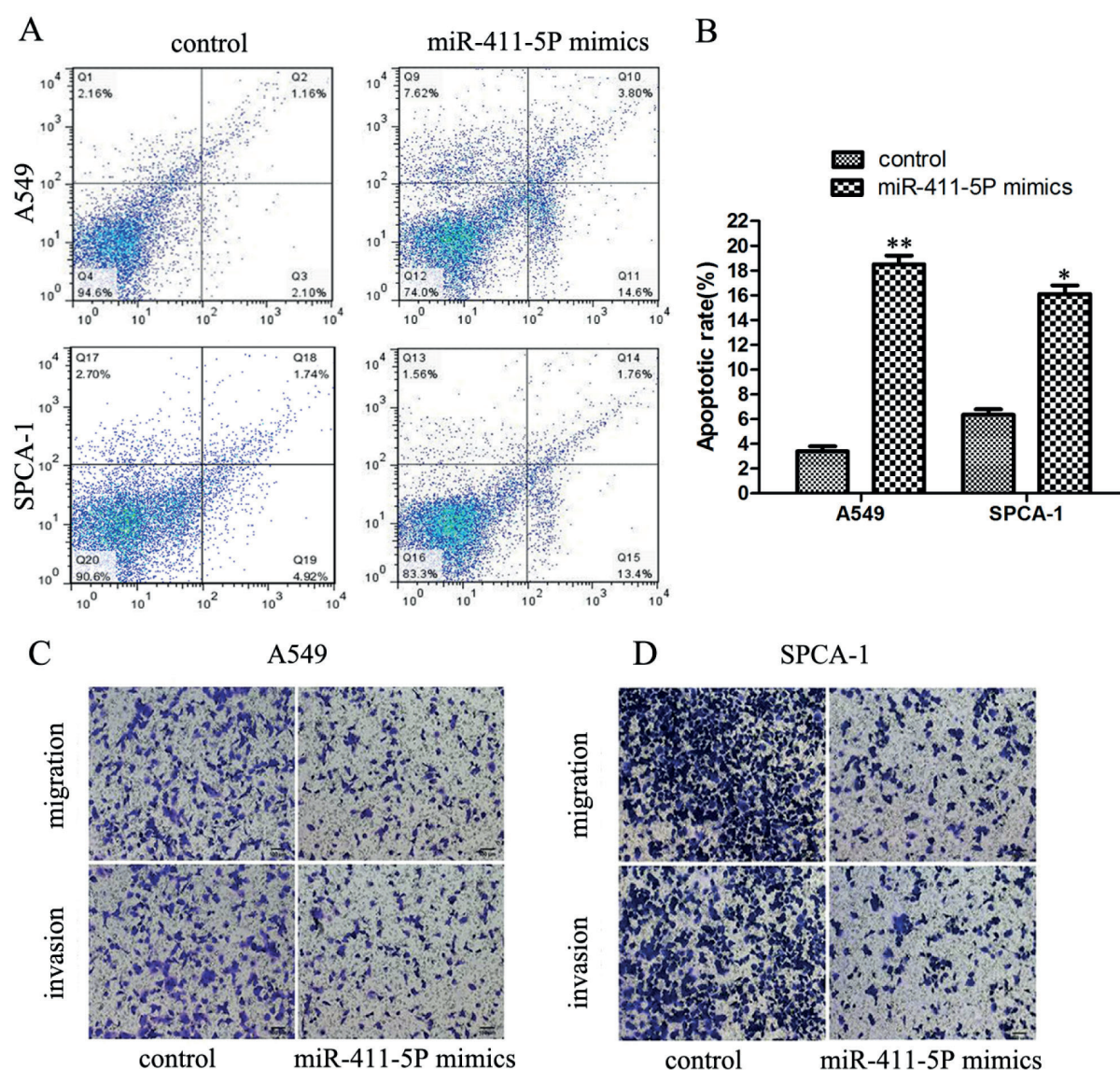


Figure 3. Effect of miR-411-5p overexpression on the apoptosis and metastasis of NSCLC cells. **A-B**, Flow cytometry was used to detect the effect of miR-411-5p overexpression on the apoptosis rate of NSCLC cells. **C-D**, Transwell assay revealed that miR-411-5p overexpression inhibited the migration (containing no matrigel) and invasion (containing matrigel) of NSCLC cells. (**: $p < 0.01$, *: $p < 0.05$).

were transfected into HEK293T cells. The results showed that miR-411-5p mimics significantly inhibited the luciferase activity of cells transfected with wild-type PUM1-3'UTR, while it exerted no effect on the luciferase activity of mutant plasmid transfected cells (Figure 4C and 4D).

Discussion

Lung cancer is now the most common malignant tumor in the world with highest morbidity

and mortality rates. There are about 2.2 million new lung cancer cases in China each year. Moreover, an average about 1.6 million cases may die from lung cancer every year, of which over 4/5 are NSCLC¹⁰. In the recent decade, certain progresses have been made in the individualized treatment of lung cancer based on different characteristics. However, the 5-year survival rate is still only about 10% with no significant improvement^{11,12}.

Current dilemmas of NSCLC are still the lack of effective diagnosis in the early stage and effective targeted treatment in the advanced sta-

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