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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: Quantitati al Time Polymerase Chain Reaction (q was used to detect the expression level R-411-5p in NSCLC tissues and cells. MiR-4 mimics and relevant controls were transfe into NSCLC cells according to the instructio of Lipidosome 2000. Transfect were di vided into the experimental he control group. The transfectio ficien of each group was detected by q CR. Aft -411-5p overexpression, met azoly (MTT) assay, flow cy me nswell assay were used to d the b al changes of cells in each p. Bioinfo predicted that pumilio g 1 (PUM1) he target gene of mi Subsequently, the mRNA and prote xpres vel of PUM1 in each group was etected by CR and Western blotting, pectively. Dual rase reporter assay used to validate the arget regulatory rel ship h een miR-411-5p and PUM1.

R LTS: T results of qRT-PCR showed that 411 was re ely lowly expressed in NS ues a ells. After miR-411-5p expre MT esults revealed that the ation C cells was decreased. Flo tometry ults indicated that the apoptosi e of NSCLC cells was increased, and cell cy ed in the G0-G1 phase. Meanw , the well assay demonstrated that the ration and invasion abilities of NSCLC cells decreased. Bioinformatics predicted that P as the target gene of miR-411-5p. After miR -4p was overexpressed in NSCLC cells, CR and Western blotting showed that the mRNA protein expression levels of M1 were up-r ated. Moreover, dual-luciferase reporter ass demonstrated that miR-411-5 ld signif tly inhibit the luciferase activi PUM1-3'-untranslated region (3'-UT ver, it exhibited no effect on the luciferase activity of cells transfected with mu- plasmids.

CONCLUSIONS: MiR-411-5p may be involved reg ting 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 cases[1]. 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 prognosis[2]. 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

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gene expression at the post-transcriptional level. Nearly 50% of human miRNAs is located in the fragile sites and genomic regions of malignant tumors[3]. 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 promotors 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 kinase signaling antagonist 4 (SPRY4) and the mitogen-activated protein kinase (MAPK) pathway, eventually mediating the proliferation and differentiation of rhabdomyosarcoma[8]. Zhang al[9] 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 by regulating growth factor receptor bound protein 2 (GRB2). However, 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 was down-regulated in NSCLC. Bioinformatics and *in vitro* experiments showed that miR-411-5p acts as a "tumor suppressor" gene by targeting pumilio homolog 1 (PUM1).

Materials and Methods

Tissues and Cells

A total of 47 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 informed consents were obtained from all participants before the study. Inclusion criteria of this study were as follows: 1) patients received no anti-tumor treatments such as radiotherapy and chemotherapy prior to tissue collection; 2) patients who had complete clinical data; 3) definitely diagnosed by histopathology. Tissues resected in the operation were immediately washed with normal saline to remove blood stains, and were stored in liquid nitrogen at -80°C for quantitative real-time polymerase chain reaction (qRT-PCR) detection.

One 16HBE bronchial epithelial cell line (16HBE) and four NSCLC cell lines (A549, SPCA-1, PC-9 and H446) 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% CO_2 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'-CCGGAACCCCCTCCTTACTC-3', R: 5'-AATGGGATGTGTCCGAAGGA-3'; PUM1, F: 5'-TTCACAGACACCACCTCCTT-3', R: 5'-CTGGAGCAGCAGAGATGTAT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Methyl Thiazolyl Tetrazolium (MTT) Assay

Transfected NSCLC cells were seeded into 96-well plates. 20 μL 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 - A value (the miR-411-5p transfected group or the miR-411-5p control group) / A value (un-transfected group)] × 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 VFITC/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% [illegible] nol and fixed at -20°C overnight. The intr[illegible] DNA concentration was detected by flow cy[illegible]try and PI staining. The cell cycle was divide[illegible] the G1/G0 phase, S phase and G2/M phase. [illegible] percentage of cells in each phase w[illegible] calculated.

Transwell Assay

Transfected NSCLC c[illegible] were s[illegible]ed into 6-well plates. After the[illegible] grew [illegible] they were digested w[illegible] tr[illegible] re-suspended with serum-fre[illegible]dium [illegible]gle cell suspension. Subseq[illegible]y, cell densit[illegible] calculated and adjuste[illegible]^5/mL. A total [illegible]200 μL cell suspensi[illegible]as a[illegible] the upper transwell chamber (u[illegible]per chamber[illegible]e coated with a bottom m[illegible]brane of transw[illegible]mber with 50 mg/L M[illegible]atrigel diluted at 1:8), while 600 μL 1640 [illegible]ium c[illegible]ining 20% FBS was added in the [illegible] cha[illegible], follo[illegible]d by incubation under norma[illegible]ns for 2[illegible]3-5 replicates were set i[illegible]each g[illegible]. Finall[illegible]rmaldehyde fixation and [illegible] violet [illegible]were conducted.

Western Blotting Assay

[illegible]sfection, the cells were harvested [illegible]protein lysate was added. After centrifugation [illegible]om temperature for 30 min, the cells were centri[illegible]d at 12,000 rpm for 10 min. The concentration [illegible]proteins was determined by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Loading buffer was added to [illegible] sample, followed by water boiling fo[illegible]min. E[illegible] well was loaded with 20 μL sodium [illegible]decyl sulfate (SDS)-polyacrylamide gel electr[illegible]sis for protein separation. Then the proteins [illegible]nsferred to polyvinylidene difluorid[illegible]PVDF) [illegible]ranes (Millipore, Billerica, MA[illegible]A) by using [illegible] transfer method. Subseq[illegible]y, the [illegible]mbranes [illegible] incubated with 5% sk[illegible]milk p[illegible]r for blocking at room temperature[illegible]h, [illegible]wed b[illegible]ubation with primar[illegible]tibo[illegible](dilution[illegible]1:1000) PUM1 and GA[illegible]1 at 4°C [illegible]igh[illegible]rrespondding second[illegible]tibodies wer[illegible] for incubation at roo[illegible]ture for 1 h. F[illegible]ly, enhanced chemilu[illegible]scen[illegible]) (Thermo Fisher Scientific, Waltham, MA, U[illegible]r development reagents w[illegible]d for protein v[illegible]ation.

Dual-Luciferase Reporter Assay

Bioinformati[illegible]ftware predicted that PUM1 [illegible]a target ge[illegible]f miR-411-5p. Then the re-p[illegible]gene pl[illegible]ds of wild-type and mutant PUM[illegible]slated 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 ± 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-411-5 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 mimics was transfected into NSCLC cells. MTT assay was performed at 0 h, 24 h, 48 h, 7 h, respectively. The results revealed that compared with the control group, the inhibition rates of the miR-411-5p transfected groups 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-411-5 Overexpression on the Apoptosis and Cell Cycle of NSCLC Cells

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

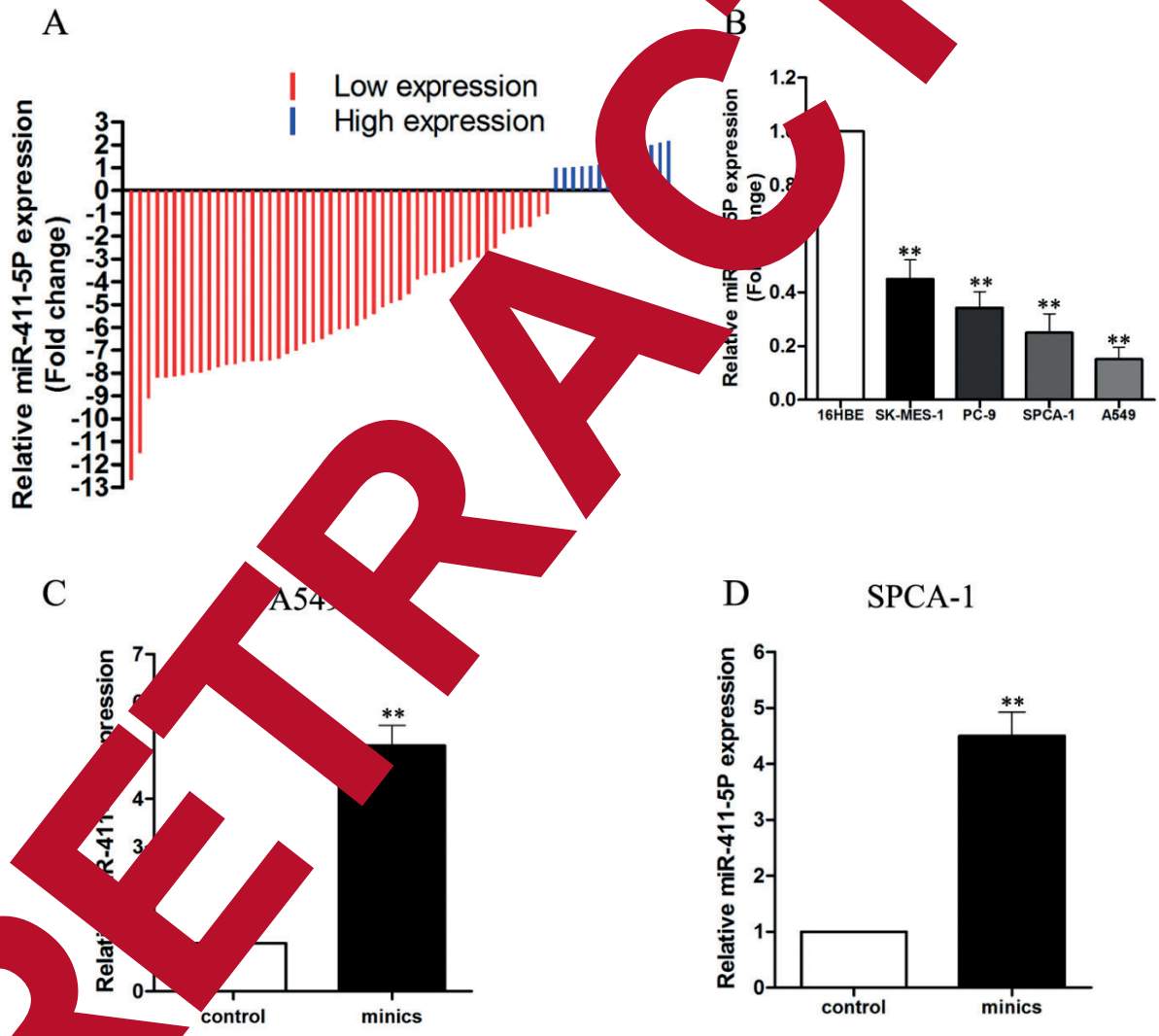

Figure 1. Expression of miR-411-5p in NSCLC tissues and cells. *A,* In 63 pairs of NSCLC tissues and adjacent tissues, qRT-PCR showed that miR-411-5p was relatively lowly expressed in NSCLC tissues. *B,* QRT-PCR results revealed that the expression of miR-411-5p was decreased in 4 NSCLC cell lines when 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 (**: $p<0.01$).

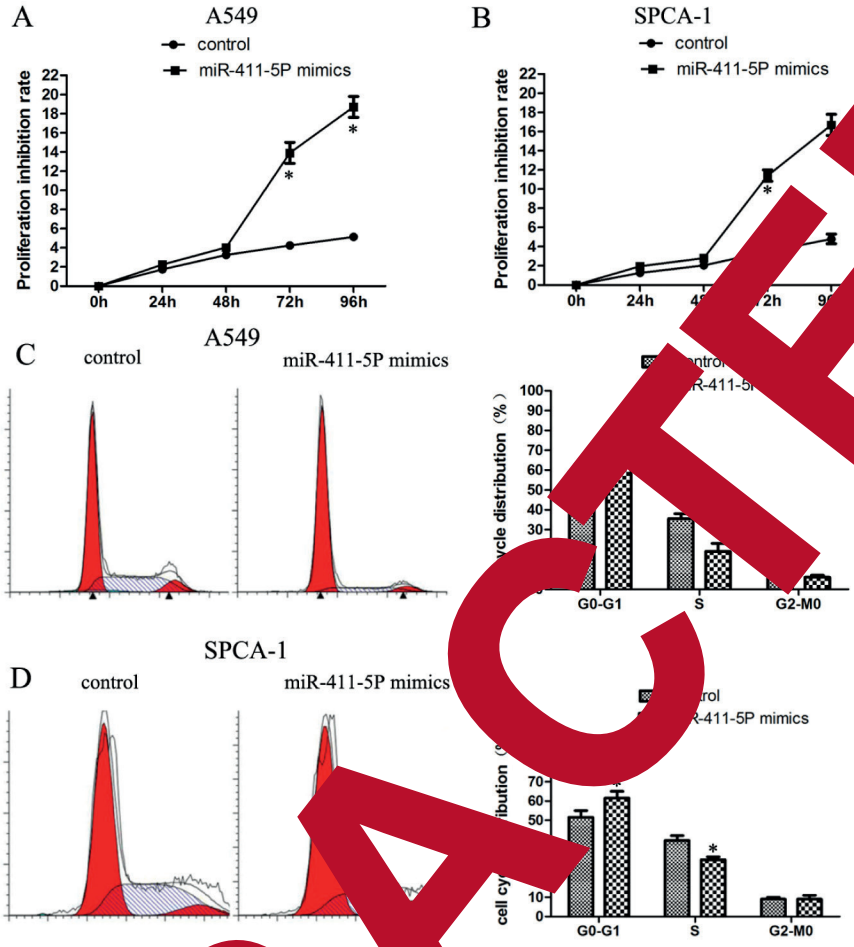

Figure 2. Effect of miR-411-5p ove n the proli n of NSCLC cells. ***A-B,*** The mimics and control sequences were transiently transfected into N C cel TT assay i ed that miR-411-5p overexpression inhibited the proliferation of NSCLC cells. ***C-D,*** The s and co sequences re transiently transfected into NSCLC cells. Flow cytometry showed that cell cycle was arr n the G LC cells. (*: $p<0.05$).

cytometry indicat at compare he control group, cell SCLC cells w rrested in the G0-G1 e a R-411-5p overexpression (Figur 2C and 2D). quently, the same method w erformed to det apoptosis rate of NSC cells. Results demo strated that the apop rate miR-411-5p overexpression gr 48 h r trans tion was significantly highe of the ol group ($p<0.05$) (Fi- re 3A a).

Eff of miR- -5p Overexpression on the tastasis and Invasion of NSCLC C

terwards, transwell assay was conducted to ure the invasion and metastasis abilities of cells. Cells in the overexpression group and the c ol group were planted in Transwell chambers, 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

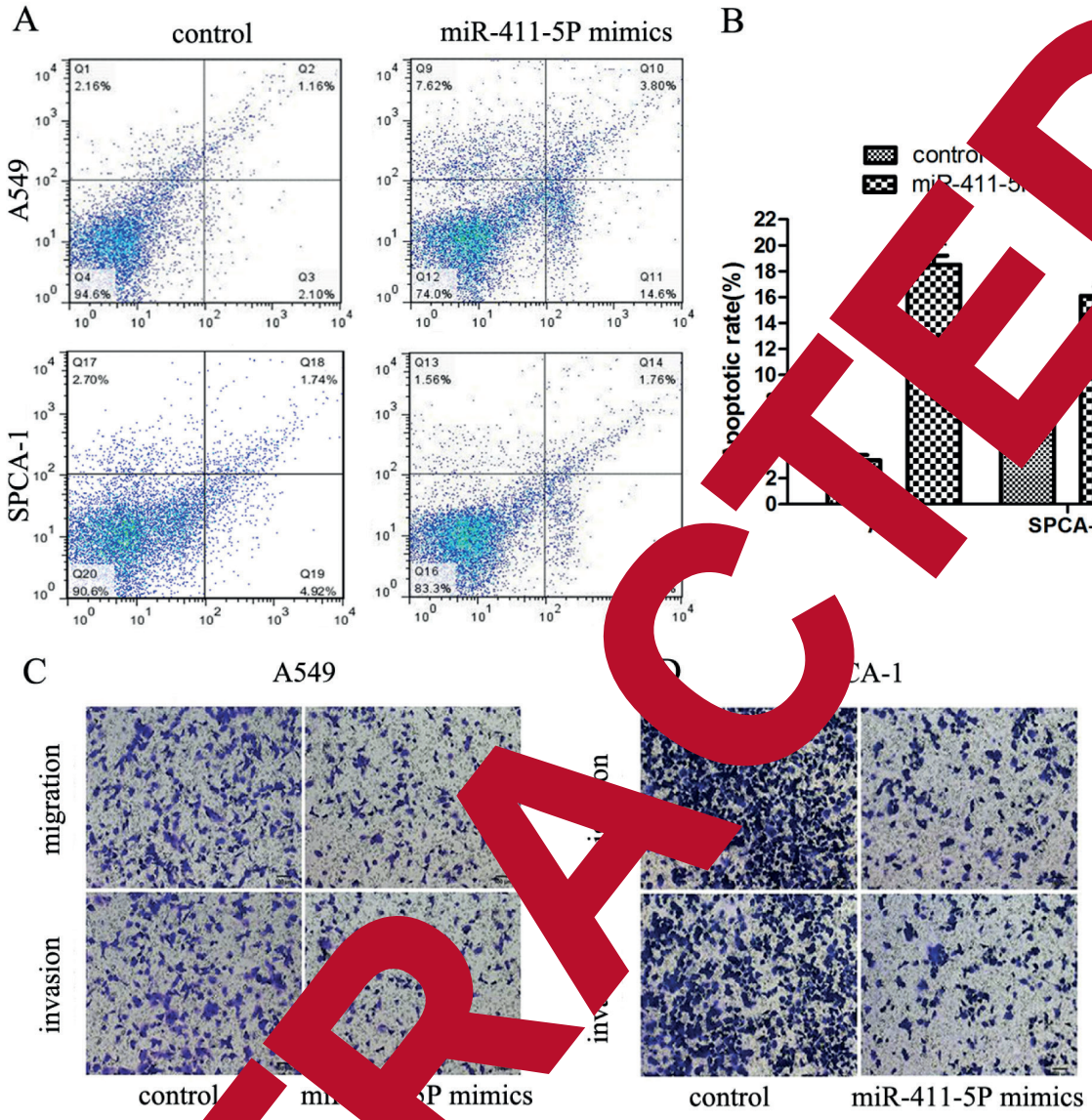

Figure 3. Effect o[illegible]-5p overexpress[illegible] the apoptosis and metastasis of NSCLC cells. ***A-B,*** Flow cytometry was used to detect th[illegible]ct [illegible]411-5p overexpression on the apoptosis rate of NSCLC cells. ***C-D,*** Transwell assay revealed that miR-411-5p [illegible]expres[illegible]hibited the migration (containing no matrigel) and invasion (containing matrigel) of NSCLC cells. (**: $p<$[illegible]; *: $p<0.05$).

were [illegible]sfected [illegible]o HEK293T cells. The results [illegible]d th[illegible]R-411[illegible] mimics significantly inhibi[illegible]fferase [illegible]ity of cells transfected [illegible]ith wild [illegible]PUM[illegible]UTR, while it exerted no [illegible]n the [illegible] activity of mutant plasmid tran[illegible]ed cells ([illegible]ure 4C and 4D).

Discussion

[illegible] cancer is now the most common malignan[illegible]mor 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 NSCLC[10]. 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-

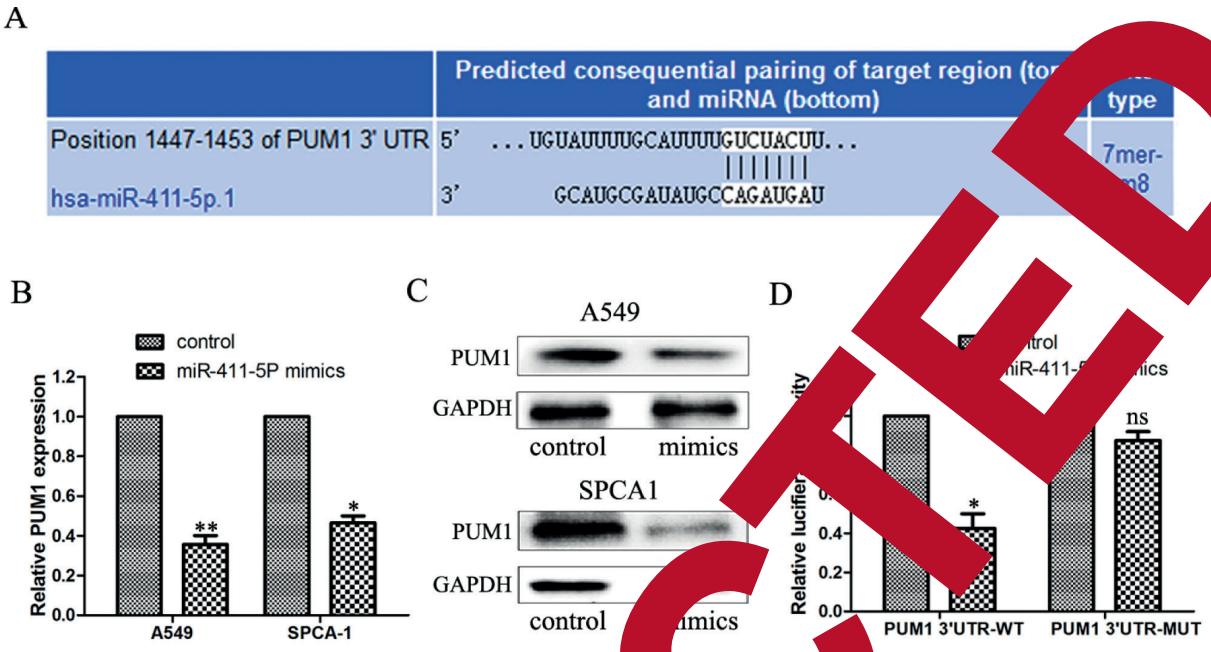

Figure 4. Targeted regulation of miR-411-5p on PUM1. ***A,*** Bioinformatics predicted PUM1 was the direct target of miR-411-5p. ***B,*** After miR-411-5p overexpression, qRT-PCR was applied to detect the mRNA expression changes of PUM1. ***C,*** After miR-411-5p overexpression, the protein expression of PUM1 was detected by Western blotting. ***D,*** Dual-luciferase reporter assay confirmed that PUM1 was the direct target of miR-411-5p.

ge[13]. Recently discovered miRNAs are a class of endogenous, non-coding, small molecule single-stranded RNAs related to the regulation of gene expression, which are widely existed in animal genes[14,15]. Most human miRNAs may inhibit mRNA translation by complementing base-sic groups in the 3'-UTR target mRNA in the form of complete or incomplete pairing, and induce the cleavage and degradation of mRNAs. This may further affect the growth and development of the body from cell to individual level. At the same time, miRNAs are involved in the occurrence of various diseases including malignant cancer[16-18]. Currently, the expression of miRNAs in NSCLC tissues is different from that in normal lung tissues, which can serve as a guide for the diagnosis and treatment of NSCLC.

Few studies have investigated the biological function of miR-411-5p as well as its role in tumorigenesis. However, the specific underlying mechanism of miR-411-5p in NSCLC remains unclear. This study showed that miR-411-5p overexpression in NSCLC cells could inhibit cell proliferation and invasion, and promote apoptosis. The above results suggested that miR-411-5p may play an inhibitory role in the development of NSCLC. MiRNA plays a major role in the biological behavior of cancer cells by regulating the expression of target genes. The currently reported target genes of miR-411-5p include FOXFO1, SP1 and ITCH[19-21]. Bioinformatics revealed that in NSCLC, PUM1 might be the target of miR-411-5p, which was further confirmed by qRT-PCR, Western blotting and dual-luciferase reporter assay in our study.

Conclusions

We demonstrated that miR-411-5p regulates the proliferation, invasion, metastasis and apoptosis of NSCLC cells by target regulating PUM1 in NSCLC, which may provide an effective target for the molecular therapy of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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