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ORIGINAL ARTICLE

MicroRNA-122-5p promotes the development of non-small cell lung cancer via downregulating p53 and activating PI3K-AKT pathway

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Summary

Purpose: *To investigate the role of microRNA-122-5p in the pathogenesis of non-small cell lung cancer (NSCLC) and its underlying mechanism.*

Methods: *A total of 72 pairs of NSCLC tissues and paracancerous tissues were collected. The expression level of microRNA-122-5p in NSCLC tissues and paracancerous tissues were detected by qRT-PCR (quantitative real-time polymerase chain reaction). The relationship between microRNA-122-5p expression and the clinical prognosis of NSCLC patients was then analyzed. Bioinformatics prediction and luciferase activity assay were performed to validate the direct binding of microRNA-122-5p and p53. Cell cycle, proliferation, and apoptosis were detected after microRNA-122-5p knockdown in NSCLC cells. The regulatory effect of microRNA-122-5p on promoting NSCLC development was detected by Western blot.*

Results: *MicroRNA-122-5p was more overexpressed in NSCLC tissues than in paracancerous tissues. MicroRNA-122-5p expression was negatively correlated with survival rate of NSCLC patients. Besides, microRNA-122-5p knockdown remarkably inhibited the proliferation and cell cycle advancement and increased apoptosis of NSCLC cells. Luciferase reporter gene assay and Western blot results indicated that microRNA-122-5p downregulated p53 and activated PI3K-AKT pathway, thereby promoting NSCLC development.*

Conclusion: *MicroRNA-122-5p is overexpressed in NSCLC. Overexpression of microRNA-122-5p promotes NSCLC development by downregulating p53 and activating PI3K-AKT pathway.*

Key words: *microRNA-122-5p, non-small cell lung cancer, P53, PI3K-AKT*

Introduction

Lung cancer is the most common primary lung malignancy. It is also known as bronchial lung cancer, reflecting its origin from bronchial epithelium. Lung cancer is classified into two main types based on the morphology of cancer cells as follows: Non-small cell lung cancer (NSCLC) and Small cell lung cancer (SCLC), of which about 80-85% are NSCLC [1]. Adenocarcinoma and squamous cell carcinoma are the most common types in NSCLC [2], which has now become a global cancer killer. The latest data released by the World Health Organization in September 2011 showed that the morbidity and mortality of NSCLC ranked first in all types of malignant tumors. The death toll from NSCLC per year exceeds the sum of deaths resulted from breast cancer, prostate cancer and colorectal cancer, making NSCLC a serious public health problem [3]. High incidence, rapid growth, high mortality, and poor prognosis are significant epidemiological features of NSCLC [4]. Therefore, effective diagnosis and treatment of NSCLC have long been important issues that are needed to be urgently explored.

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MicroRNAs are non-coding small RNAs with 22 nucleotides in length. MicroRNAs act as post-transcriptional modifications by binding to the 3'UTR of mRNAs [5,6]. Differentially expressed microRNAs are associated with the development of many diseases including tumors and cardiovascular diseases [7,8]. Calin et al. found that microRNA-15 and microRNA-16 were located on chromosome 13q14. Over 60% of this chromosome region was absent in chronic lymphocytic leukemias, resulting in the downregulation of microRNA-15 and microRNA-16 [9]. Subsequent studies have also identified a series of dysregulated microRNAs in tumors, which are served as oncogenes or tumor suppressor genes [10,11].

In recent studies, certain microRNAs have been shown to be involved in the carcinogenic process of NSCLC. Salim et al. found that microRNA-214 was associated with invasiveness of lung cancer cells and directly regulated the expression of α-protein kinase 2 [12]. Kim et al. reported that the expressions of microRNA-126 and microRNA-122-5p were related to the prognosis of NSCLC patients [13]. Geng et al. considered that microRNAs could serve as diagnostic biomarkers of NSCLC [14]. However, the molecular mechanism of microRNAs in NSCLC development remains unclear. In-depth studies of differentially expressed microRNAs and their regulatory roles in NSCLC contribute to better improvement of diagnosis and treatment of NSCLC.

Methods

Specimen collection

A total of 72 patients with resected NSCLC were included in this study. Fresh tissues were resected by specialized doctors, immediately rinsed with diethyl pyrocarbonate (DEPC) water, and stored in liquid nitrogen. Subsequently, clinical data of the enrolled patients were collected from the Department of Pathology and Medical Record Room, Tongji Hospital. All patients consented for sample collection in this study and which was approved by the Ethics Committee.

Cell culture

BEAS-2B, A549, HCC823, NCL-H23, and NCL-H358 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640), containing 10% FBS (fetal bovine serum), 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were maintained in a 5% CO_2 incubator at 37°C. For cell transfection, the medium was removed and the cells were washed with PBS (phosphate buffer saline) when cell confluency was up to 50-60%. Transfection of microRNA mimic or inhibitor was performed according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was verified by qRT-PCR (quantitative real-time polymerase chain reaction). The microRNA mimic and inhibitor used in this study were provided by GenePharma Co. Ltd. (Shanghai, China).

CCK-8 (cell-counting kit-8) assay

Cells in the logarithmic growth phase were selected to seed in 96-well plates at a density of 1×10^6/mL. After cell culture for 24 hrs, 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan) were added to each well and then the incubation was continued at 37°C in a 5% CO_2 incubator for 4 hrs. Absorbance of each well at the wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated three times and the average value was adopted.

Transwell assay

Cells in the logarithmic growth phase were digested with trypsin and washed once with PBS and serum-free medium. After the cells were re-suspended in serum-free medium, the concentration was adjusted to 2×10^5/mL. Six hundred μL of medium containing 10% FBS were added to the lower chamber and 100 μL of the cell suspension were added to the upper chamber. After cell culture for 24 hrs, the upper chamber medium was removed and the cells were fixed with formaldehyde for 30 min. Crystal violet was used to stain the fixed cells for another 15-30 min. Cell invasion and growth were observed under microscope (Olympus, Tokyo, Japan).

Cell cycle detection

Cells in the logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5/mL. After cell culture for 24 hrs, cells were digested with trypsin, washed 3 times with pre-cooled PBS, and fixed with ethanol at 4°C overnight. Propidium iodide (PI) was then added to stain cells for 25 min in the dark and flow cytometry was used to detect the cell cycle. The experiment was repeated three times and the average value was adopted.

Cell apoptosis detection

The cells were digested with trypsin, pipetted into single-cell suspension, and washed twice with PBS. Cell density was adjusted to 1×10^6/mL. The cells were centrifuged and re-suspended in 100 μL of buffer prior to transfer to a flow tube. After 10 μL of Annexin-V were added to each tube, cells were incubated in the dark for 15 min at 4°C. Subsequently, 380 μL of buffer and 10 μL of PI were added per flow tube and immediately analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Plasmid construction

The binding sites of microRNA-122-5p and p53 were predicted by Target Scan. Wild-type and mutant-type plasmids were conducted by Nanjing, Ruizhen, Co., Ltd. (Nanjing, China). MicroRNA-122-5p mimic was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The relative fluorescence after plasmid transfection was then measured by a standardized method. The

experiment was repeated three times and the average value was adopted.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The rapid extraction of total RNA was performed by the TRIzol method (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, the TRIzol and cell mixture were centrifuged at 4°C and the upper aqueous phase was kept. Isopropanol was used to precipitate RNA. After being air-dried at room temperature, the total RNA was dissolved in 20-30 μL of DEPC (Diethyl Pyrocarbonate) and stored at -80°C in a freezer. Reverse transcription was performed according to the instructions of TaKaRa OneStep PrimeScript® miRNA cDNA (complementary Deoxyribose Nucleic Acid) Synthesis Kit (Tokyo, Japan). Amplification was conducted using the SYBR Green I fluorescent dye method (Invitrogen, Carlsbad, CA, USA). The p53 primer sequence

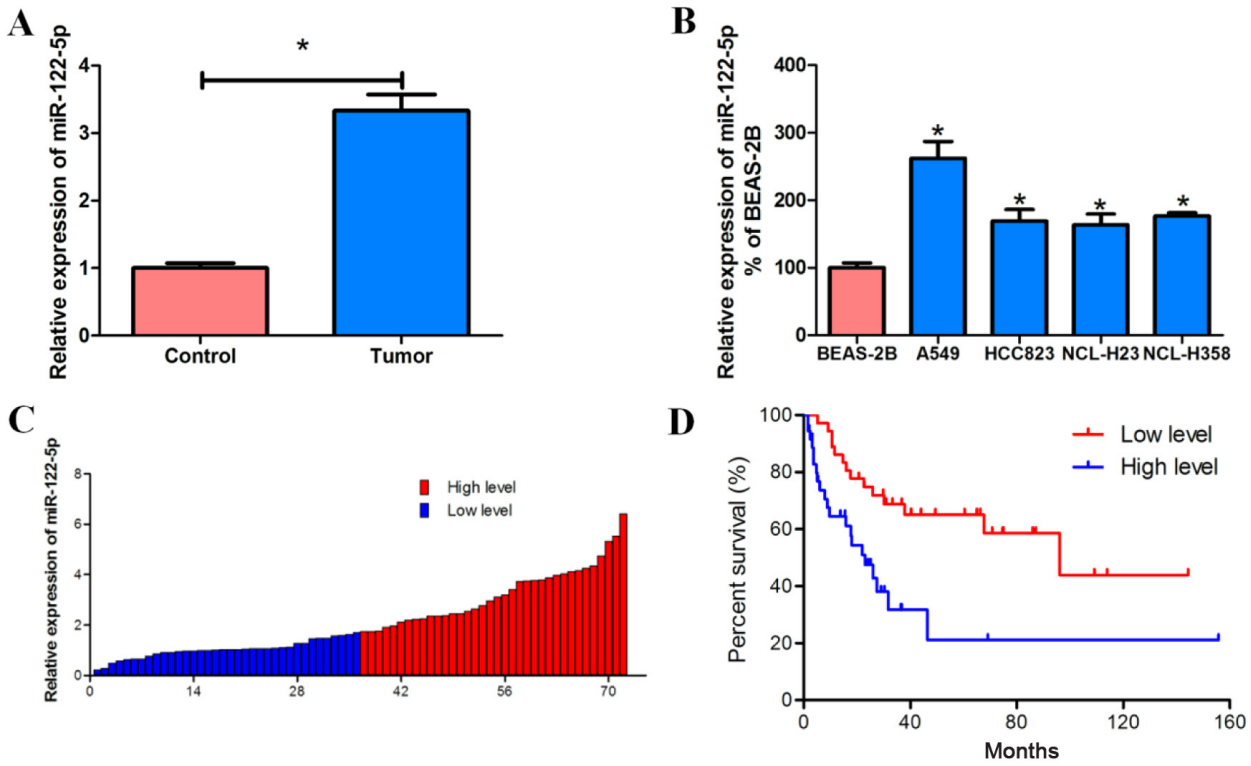

Figure 1. MicroRNA-122-5p was overexpressed in NSCLC. **A:** MicroRNA-122-5p was overexpressed in NSCLC tissues. *p<0.05 compared with control. **B:** MicroRNA-122-5p was overexpressed in NSCLC lines. *p<0.05 compared with BEAS-2B cells. **C:** MicroRNA-122-5p expression in paracancerous tissues. **D:** Survival of NSCLC patients with higher expression of microRNA-122-5p was significantly lower compared with those with lower expression (p<0.05).

Table 1. Clinicopathological data of the included patients

Clinicopathological data	*Number of cases*	*miR-122-5p expression*		*p value*
		Low (n=36)	*High (n=36)*	
Age (years)		36	36	0.6326
≤60	30	16	14	
>60	42	20	22	
Tumor size				0.0277
T1-T2	38	16	12	
T3-T4	34	10	24	
N stages				0.0477
N0	39	16	13	
N0-N3	33	10	23	
Metastasis				0.0043
Yes	21	5	16	
No	51	31	20	

was as follows; F: 5'-CAGCACATGACGGAGGTTGT-3', R: 5'-TCATCCAAATACTCCACACGC-3'. The results were calculated by the $2^{-\Delta\Delta CT}$ method. Each experiment was repeated three times and the average value was adopted.

Colony formation assay

After transfection for 24-48 hrs, the cells were washed with PBS and digested with trypsin. The transfected cells were centrifuged at 1000 r/min for 3 min and then seeded in 6-well plates with 500 cells per well. After culturing for 2 weeks, the cells fixed with methanol were stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA) and photographed using an inverted microscope.

Western blot

Total protein was extracted from treated cells by RIPA (radio-immunoprecipitation) solution (Beyotime, Shanghai, China). Protein sample was separated by 10% SDS-PAGE electrophoresis (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). After the membranes were blocked with skimmed milk, they were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by chemiluminescence.

Statistics

SPSS 20.0 (IBM, Armonk, NY, USA) and Graphpad statistical software (La Jolla, CA, USA) were used for data analysis. The chi-square test was used for the data classification and the Student's t test was used for the data measurement. All measurements were expressed as mean±standard deviation. P value <0.05 was accepted as statistically significant.

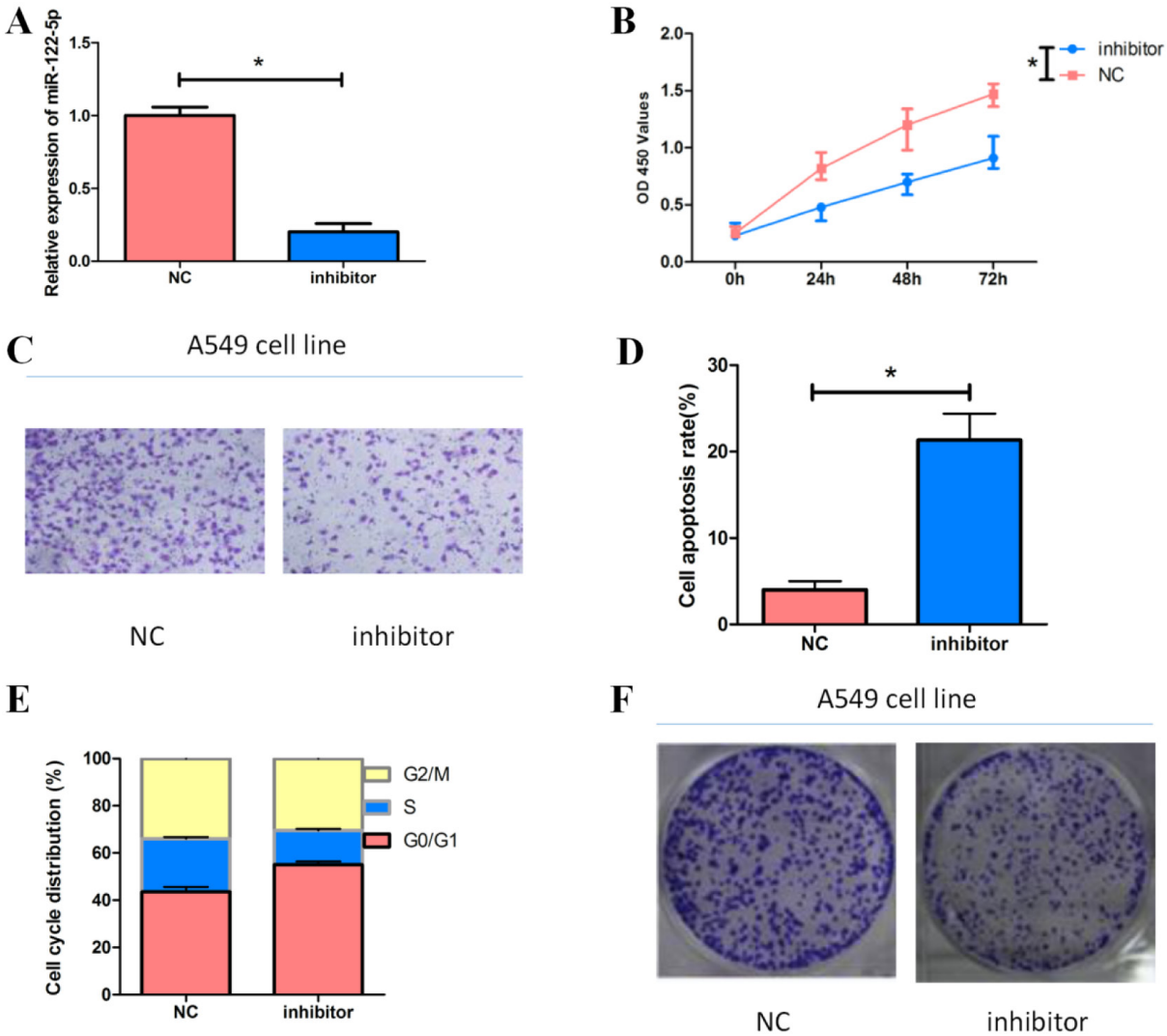

Figure 2. MicroRNA-122-5p knockdown inhibited cell proliferation and migration. **A:** MicroRNA-122-5p was significantly downregulated by microRNA-122-5p inhibitor. **B:** Downregulated microRNA-122-5p could inhibit cell proliferation. **C:** Downregulated microRNA-122-5p could inhibit cell colony formation. **D:** Downregulated microRNA-122-5p could inhibit cell migration. **E:** Downregulated microRNA-122-5p could promote apoptosis. **F:** Downregulated microRNA-122-5p could arrest cells in G0/G1 phase. *p<0.05 compared to NC (NC: microRNA-122-5p inhibitor native control).

Results

MicroRNA-122-5p was overexpressed in NSCLC

MicroRNA-122-5p was more overexpressed in NSCLC tissues than in paracancerous tissues (Figure 1A). Shorter survival time was found in NSCLC patients with higher expression of microRNA-122-5p compared to those with lower expression (Figure 1B and 1C). NSCLC patients with higher expression of microRNA-122-5p also showed higher tumor grade compared to those with lower expression (Table 1). Subsequently, we detected microRNA-122-5p level in different lung cancer cell lines. The results indicated that microRNA-122-5p was significantly upregulated in NSCLC cell lines compared with the normal lung cell line BEAS-2B, (Figure 1D). In particular, A549 cells showed the highest expression of microRNA-122-59, which were selected for the following experiments.

MicroRNA-122-5p knockdown inhibited cell proliferation and migration

By transfection of microRNA-122-5p inhibitor, the expression of microRNA-122-5p in A549 cells was knocked down (Figure 2A). MicroRNA-122-5p knockdown exhibited a remarkable reduction in cell proliferation and migration, while promoting cell apoptosis (Figure 2B-D). Meanwhile, cell cycle was arrested in the G0/G1 phase when microRNA-122-5p expression was inhibited (Figure 2E). Colony formation assay also showed that cell colony formation ability was significantly inhibited (Figure 2F).

MicroRNA-122-5p knockdown upregulated p53 expression

Results of dual-luciferase reporter assay demonstrated that microRNA-122-5p directly bound to p53 (Figure 3A). Downregulation of p53 has been reported to be involved in many cancers [15]. MicroRNA-122-5p knockdown remarkably decreased both protein and p53 mRNA levels (Figure 3B and 3C). The data given above suggested that microRNA-122-5p may promote NSCLC development by targeting p53.

MicroRNA-122-5p knockdown suppressed the activity of PI3K-AKT pathway

After downregulation of microRNA-122-5p in A549 cells, we found that the phosphorylation lev-

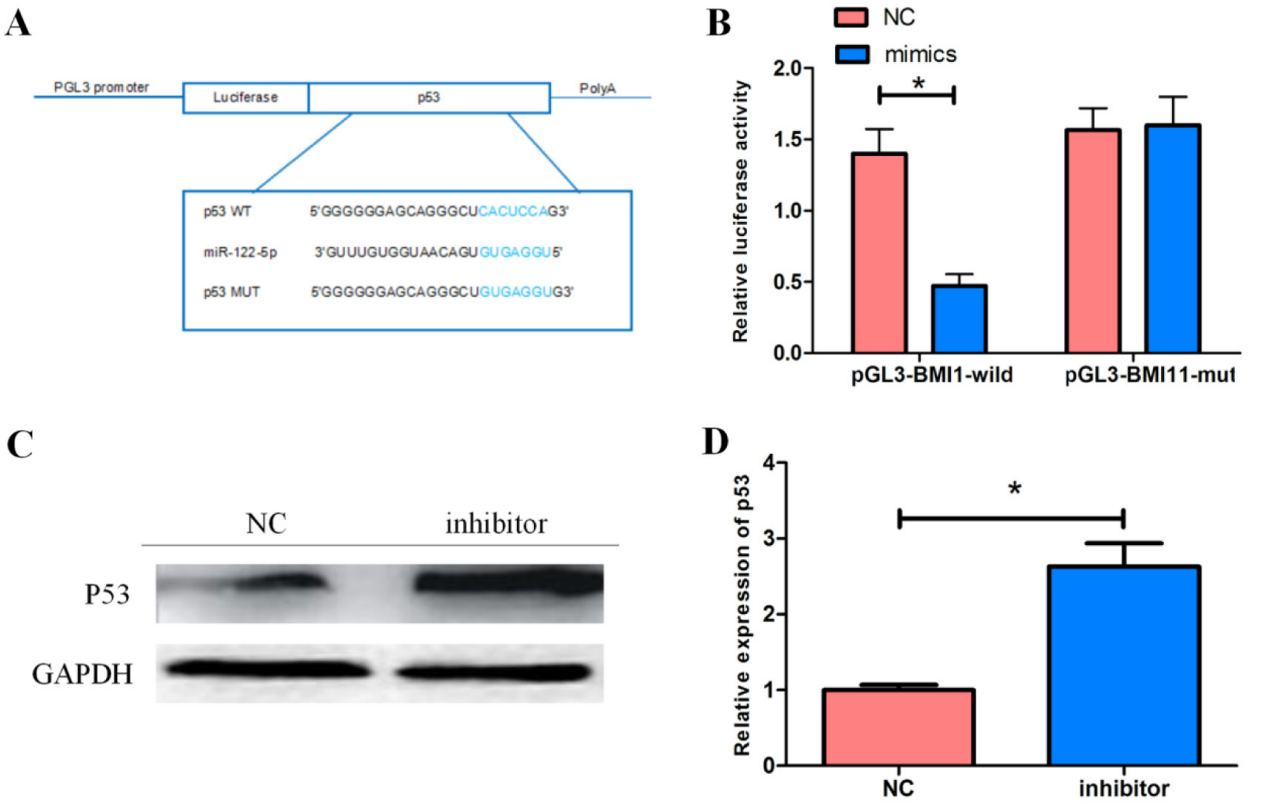

Figure 3. MicroRNA-122-5p knockdown enhanced the p53 expression. **A:** Predicted binding sites of microRNA-122-5p and p53. **B:** Luciferase reporter gene assay confirmed that microRNA-122-5p directly bound to p53. **C:** Downregulated microRNA-122-5p significantly upregulated protein expression of p53. **D:** Downregulated microRNA-122-5p significantly upregulated mRNA expression of p53. *p<0.05 compared with NC (NC: microRNA-122-5p inhibitor native control).

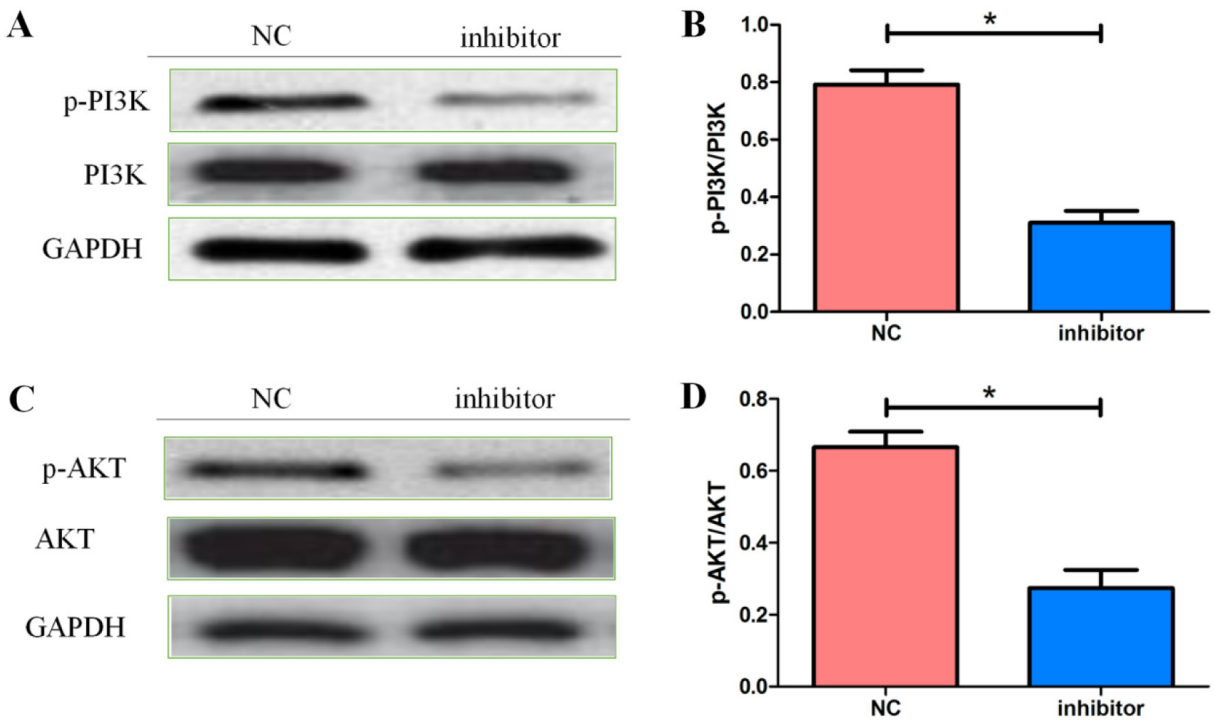

Figure 4. MicroRNA-122-5p knockdown suppressed the activity of PI3K-AKT pathway. **A,B:** Downregulated microRNA-122-5p significantly reduced the phosphorylation of AKT. **C,D:** Downregulated microRNA-122-5p significantly reduced the phosphorylation of PI3K. *p<0.05 compared with NC (NC: microRNA-122-5p inhibitor native control).

els of AKT and PI3K were remarkably decreased (Figure 4A-4D), indicating that microRNA-122-5p may promote NSCLC development by activating the PI3K-AKT pathway..

Discussion

Accumulating evidence has shown that microRNAs exert a crucial role in maintaining of normal cell growth and function. Differentially expressed microRNAs are closely related to various types of tumors [16]. For example, let-7 expression was negatively correlated with postoperative survival in NSCLC patients [17]. MicroRNA-21 inhibited cell apoptosis in breast, prostate, lung, colon, pancreas, and gastric cancers, thus promoting tumor growth [18]. Disordered microRNAs may have an important effect on cell homeostasis.

This study aimed at the investigation of the role of microRNA-122-5p in the development of NSCLC. We downregulated microRNA-122-5p in NSCLC cells and studied its *in vitro* function. Infinite proliferation and clonality are the most prominent features of tumor cells. In the present study, microRNA-122-5p knockdown inhibited the proliferation and clonality of tumor cells *in vitro*.

Tumor cells also have a strong migration ability that leads to tumor metastasis. Transwell assay confirmed that microRNA-122-5p knockdown could effectively decrease tumor cell migration, suggesting that overexpression of microRNA-122-5p in NSCLC patients might promote tumorigenesis. MicroRNA-122-5p knockdown also led to cell-cycle arrest and increased apoptosis in NSCLC cells.

We initially focused on p53 as the potential target gene of microRNA-122-5p based on the results from REGRNA, TargetScan, and other bioinformatics software. P53 is a tumor suppressor gene, which is mutant in over 50% of malignant tumors. As a transcription factor, p53 controls the initiation of cell cycle and cell division. If damaged cells are not repaired on time, p53 protein regulates apoptosis of damaged cells. However, p53-deficient cells continue to be divided even under adverse conditions. Under normal circumstances, p53 monitors cell division. P53 promotes cellular self-repair if the degree of DNA variability is smaller, while it induces apoptosis if the degree of DNA variability is greater [19]. It has been demonstrated that p53 is associated with a variety of tumors and regulated by some certain microRNAs [20-23]. We verified the direct binding of microRNA-122-5p and p53 by dual-luciferase reporter gene assay. Inhibition of microRNA-122-5p resulted in a remarkable upregulation of p53.

A large number of studies have shown that PI3K-AKT pathway is closely related to NSCLC occurrence [24,25]. Phosphatidylinositol-3-kinase (PI3K) consists of a regulatory subunit (p85) and a catalytic subunit (p110). The SH2 and SH3 do-

mains in the regulatory subunits can interact with target proteins containing the corresponding binding sites. There are four catalytic subunits as follows: p110α, β, δ, and γ [26]. AKT is an important downstream molecule of PI3K pathway. AKT includes at least three forms as follows: AKT1, AKT2, and AKT3. Functionally, AKT is capable of regulating cell growth, proliferation, migration, survival, and glucose metabolism [27]. Our results showed that microRNA-122-5p activated the PI3K-AKT pathway, thereby promoting NSCLC development.

In conclusion, microRNA-122-5p is overexpressed in NSCLC. Overexpression of microRNA-122-5p promotes NSCLC development by downregulating p53 and activating the PI3K-AKT pathway.

Conflict of interests

The authors declare no conflict of interests.

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