

Impact Analysis of miR-1253 on Lung Cancer Progression Through Targeted Regulation of ANXA3

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Objective: This study set out to investigate the effect of *miR-1253* on lung cancer progression through targeted regulation of *ANXA3*.

Methods: RT-PCR was employed to detect the *miR-1253* expression levels in lung cancer cells and its targeted gene *ANXA3* mRNA determined by biological information prediction. MTT, invasion and apoptosis rate tests were employed to detect the proliferation, invasion and apoptosis rate of lung cancer cells over-expressing *miR-1253* or those with low expression of *ANXA3* and the expression of related proteins.

Results: RT-qPCR results manifested that the *miR-1253* level was down-regulated in lung cancer tissues and cells, and the *ANXA3* expression increased. The *miR-1253* and *ANXA3* expression levels were negatively correlated. *miR-1253* was correlated with tumor differentiation degree, TNM stage and lymph node metastasis of lung cancer patients. Cell tests confirmed that *miR-1253* played a tumor-inhibiting function, including inhibiting proliferation and invasion of lung cancer cells and promoting apoptosis. Bioinformatics prediction and subsequent experiments proved that *ANXA3* was the direct target of *miR-1253*. Moreover, after the *ANXA3* expression in lung cancer cells was knocked down, proliferation and invasion of those cells were inhibited dramatically, the apoptosis rate increased markedly, and the expression levels of pro-apoptosis-related proteins *Bax* and *caspase-3* were up-regulated, and the anti-apoptosis-related protein *Bcl-2* expression was down-regulated.

Conclusion: *miR-1253* can inhibit the proliferation and invasion of lung cancer cells and promote their apoptosis by targeting *ANXA3*. It can be used as a new potential target for lung cancer treatment.

Keywords: *miR-1253*, lung cancer, *ANXA3*, proliferation, invasion, apoptosis

Introduction

Lung cancer (LC), as one of the most frequent malignancies, is also one of the main types of cancer-induced death.¹ In recent years, with the deterioration of the natural environment and the change of living habits, the morbidity of LC has become higher and higher, posing serious risks to human life and health.² Although good advancement has been made in its diagnosis and treatment with the progress of medical technology recently, the prognosis of LC patients is still poor due to its rapid progression, high metastasis rate, high recurrence rate, etc.^{3,4} Therefore, it is quite significant to explore the pathogenesis and development mechanism of LC for its diagnosis and treatment clinically.

miRNA is a non-coding microRNA with a length of about 18–25 nucleotides, which has been found to be effective in various diseases, especially tumors in the past.⁵ Previous

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studies^{6,7} have shown that miRNA can act as both cancer-promoting gene and tumor-suppressor gene in tumors. They have considered that miRNA imbalance plays a crucial part in the occurrence of tumors, having an impact on cell proliferation, invasion and apoptosis. However, many studies have been discussed on the role of miRNA in LC. For example, research⁸ has found that *miR-330-3p* can directly target *hSOD2b* to advance LC cells' invasion, migration and metastasis. All these signify that miRNA plays an essential part in its occurrence and development. As a member of miRNA, *miR-1253* has also been reported to play a vital role in tumor in the past. For example, some studies⁹ have reported that *miR-1253* can regulate the proliferation and development of prostate cancer by targeting *EZH2*.

The latest research¹⁰ has shown that the *miR-1253* expression level has high predictive value for the prognosis of LC patients. In order to analyze the mechanism of *miR-1253* in LC in depth, we found a targeted relationship between *miR-1253* and *ANXA3* through online website prediction, and further explored the mechanism of *miR-1253*.

Patients and Methods

Clinical Data

One hundred and two LC patients who underwent surgical treatment in our hospital from April 2015 to October 2016 were collected as research objects, including 62 male and 40 female patients. Through PCR test, we found that there were 40 Mycoplasma positive cases and 62 Mycoplasma negative cases. They were (58.31±3.28) years old on average. One hundred and two cases of LC tissue and 102 cases of paracancerous tissue were obtained during the operation with patients' consent. Inclusion criteria: Patients diagnosed as LC by pathological diagnosis and those with an expected survival period over 3 months were included in the research group. Exclusion criteria: Patients with other malignancies, severe liver and kidney dysfunction, infection, blood or immune system disorders and those did not cooperate with the study were excluded. All patients and their families agreed to participate in the test and sign an informed consent form, and this test has been approved by The Third Affiliated Hospital of Peking University. This experiment conforms to the Declaration of Helsinki.

Cell Culture and Transfection

Human LC cell lines A549, H1299, H157, SPC-A-1 and human bronchial epithelial cell line (BEAS-2B) (ATCC) were cultured in RPMI 1640 medium containing 10% fetal

bovine serum (Gibco, USA), and the cells were cultured in a humid atmosphere at 37°C, 5% CO₂. Through the detection of *miR-1253* and *ANXA3* expression in cell lines, we found that the *miR-1253* expression in A549 and H157 cell lines was lower than that in other two groups, so these two cell lines were chosen for transfection and subsequent experiments. The miR-1253-mimics, *miR-1253*-inhibitor, miR-NC, *ANXA3* small interfering RNA (siRNA) (Si-*Anx3*) and its negative control (si-NC) were transfected into cells by Lipofectamine™ 2000 kit (Invitrogen, Carlsbad, USA), and the operation steps were strictly conducted based on the kit instructions.

RT-PCR Detection

The total RNA in the tissue was extracted with Trizol reagent (Invitrogen, USA), and its purity and concentration were detected by ultraviolet spectrophotometer. Then, every 5 µg of total RNA was taken, respectively, to reversely transcribe cDNA based on the instructions of the kit (TaKaRa, Dalian, China). Amplification conditions were as follows: PCR reaction conditions: pre-denaturation at 94°C for 30 s, denaturation at 94°C for 5 s, annealing extension at 60°C for 30 s, a total of 40 cycles. *miR-1253* employed *U6* as internal reference, *ANXA3* employed GAPDH as internal reference, and data were analyzed by $^{-\Delta\Delta Ct1}$. The primer sequences are shown in Table 1.

Cell Proliferation Was Detected via MTT Assay

Based on the instructions of MTT Kit (Beyotime Biotechnology Co., Ltd.), LC cell lines transfected for 48 h were inoculated into 96-well plates, each well was inoculated with about 5000 cells with a cell density of 3×10^4 cell/mL, and then incubated at 37°C. Altogether 10 µL of MTT solution was supplemented to each well for 24, 48, 72 and 96 h, respectively. After the reagents were added, the cells were continuously cultured 4 h in an incubator, and then 150 µL of dimethyl sulfoxide was supplemented. After they were shaken for 10 min, the absorbance was surveyed at 490 nm using an enzyme reader to detect cell proliferation. This test was repeated three times.

Cell Invasion Detection (Transwell)

Cells transfected for 24 h were collected, adjusted to 3×10^5 cell/mL and inoculated on a 6-well plate, and

Table 1 Primer Sequence Table

Factors	Upstream Primer 5'-3'	Downstream Primer 5'-3'
<i>miR-1253</i>	GCTGTAACAGCGGCGGAAGCTCC	ATCCGCAGGAGTGTCAGAG
<i>U6</i>	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTTCAT
<i>ANXA3</i> <i>GAPDH</i>	ACAGCGGCAGCTGATTGTTA GGATATTGTTGCCATCAATGACC	TCACTAGGGCCACCATGAGA AGCCTTCTCCATGGTGGTGAAGA

inoculation and upper chamber were washed with PBS twice. Altogether 200 μ L DMEM culture solution was supplemented to the upper chamber, and 500 mL of DMEM containing 20%FBS was supplemented to the lower chamber. Substrates and cells in the upper chamber that did not cross over the membrane surface were wiped off after culturing at 37°C for 48 h and cleaned 3 times with PBS. Then, they were fixed 10 min by paraformaldehyde, washed 3 times with double-distilled water, and stained by 0.5% crystal violet after drying. Finally, cell invasion was observed by a microscope.

Apoptosis Test

Cells were digested with 0.25% trypsin, cleaned twice with PBS after digestion, supplemented with 100 μ L of binding buffer, prepared into 1×10^6 /mL suspension, sequentially supplemented with AnnexinV-FITC and PI, incubated 5 min at room temperature in dark, and detected with FC500MCL flow cytometer system. We repeated this experiment 3 times and took the average value.

Western Blot Test

Total protein was extracted from the collected and cultured cells of each group via RIPA lysis method (Thermo Fisher, USA). Protein concentration was detected through BCA method and adjusted to 4 μ g/ μ L. It was separated by 12% SDS-PAGE electrophoresis and then transferred to a PVDF membrane. The membrane was dyed with Ponceau S working solution, immersed 5 min in PBST (PBS+0.1%Tween 20) and then washed, blocked 2 h with 5% defatted milk powder, and finally blocked all night long at 4°C with *Bax* (1:500), *Bcl-2* (1:500), *caspase-3* (1:500), *ANXA3* (1:1000) and β -*Actin* primary antibody (1:1000) (Cell Signaling Technology). It was washed to remove primary antibody. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1000) (Abcam, USA) was supplemented to the membrane for a 1-hour incubation at 37°C. Soon afterwards, it was cleaned 3

times with PBS, for 5 min each time. Ultimately, it was developed in a darkroom by the enhanced chemiluminescence reagent (ECL), and its excess liquid was absorbed with a filter paper.

Dual-Luciferase Reporter Enzyme

Dual-luciferase reporter gene assay was conducted to test whether *ANXA3* was a direct target gene of *miR-1253*. *ANXA3* 3'UTR dual-luciferase reporter plasmids (WT and MUT) were constructed via RiboBio. They were co-transfected into cells with *miR-1253* mimics or mimetic control, respectively, via Lipofectamine 2000. Forty-eight hours after incubation, luciferase activity was tested via a dual-luciferase assay system (Promega Corporation).

Statistical Methods

In this research, data were processed by SPSS 20.0 software, and relevant pictures were drawn by GraphPad Prism 6 software. The measurement data were presented as mean \pm standard deviation (SD \pm meas) and checked through *t* test. Inter-group comparison was under independent-samples *t* test and expressed as *t*. Multi-group comparison was under one-way analysis of variance. Post hoc pairwise comparison was under LSD-*t* test. Multi-time point expression was under repeated measures analysis of variance. Backtesting adopted Bonferroni and correlation analysis adopted Pearson. $P < 0.05$ was seen as statistical difference.

Results

Expression and Clinical Significance of *miR-1253* and *ANXA3* in LC

The *miR-1253* and *ANXA3* mRNA levels were detected by qRT-PCR. The results revealed that the *miR-1253* expression in LC tissues was dramatically lower than that in paracancerous tissues ($P < 0.05$), and the *ANXA3* mRNA expression was dramatically higher than that in paracancerous tissues ($P < 0.05$). The *miR-1253* expression in LC cell line was

lower than that of BEAS-2B cell line ($P<0.05$), and the *ANXA3* expression in LC cell line was higher than that of BEAS-2B cell line ($P<0.05$). Correlation analysis manifested that the *miR-1253* and *ANXA3* expression levels in LC tissue were negatively correlated ($P<0.05$). ROC curve analysis revealed that the AUC of *miR-1253* for LC's diagnosis was 0.892. According to the mean expression of *miR-1253*, patients were divided into high expression group (43 cases) and low expression group (59 cases). Further analysis of the relationship between *miR-1253* and pathological data signified that *miR-1253* was relevant to tumor differentiation, TNM stage and lymph node metastasis of LC patients ($P<0.05$). (Figure 1, Table 2)

miR-1253's Effect on Biological Function of LC Cells

To study *miR-1253*' effect on proliferation, invasion and apoptosis of LC cells, A549 and H157 cells were transfected with *miR-1253*-mimics, *miR-1253*-inhibitor and *miR-NC*. Forty-eight hours after transfection, compared with *miR-NC*, *miR-1253* in the two cells transfected with *miR-1253* mimic increased obviously ($P<0.05$), and the *miR-1253* expression in cells transfected with *miR-NC*

decreased obviously ($P<0.05$). In addition, MTT assay, cell invasion and flow apoptosis assay were performed after transfection. The results showed that the *miR-1253* over-expression greatly inhibited proliferation ($P<0.05$) and invasion ($P<0.05$) of A549 and H157 cells, and promoted apoptosis. And WB experiment confirmed that the pro-apoptotic protein *Bax* and *caspase-3* protein expression levels in cells increased, and the anti-apoptotic protein *Bcl-2* expression was down-regulated ($P<0.05$). The proliferation and invasion of cells transfected with *miR-1253*-inhibitor were markedly enhanced, the apoptosis rate decreased dramatically ($P<0.05$), and the pro-apoptotic protein *Bax* and *caspase-3* protein expression levels decreased, while the anti-apoptotic protein *Bcl-2* expression was up-regulated ($P<0.05$). (Figure 2)

Effect of Down-Regulation of ANXA3 Expression on Biological Function of LC Cells

In order to confirm whether the tumor inhibitory effect of *miR-1253* in LC was mediated by *ANXA3*, we knocked down the *ANXA3* expression in A549 and H157 cells. Western blot analysis identified that after transfection

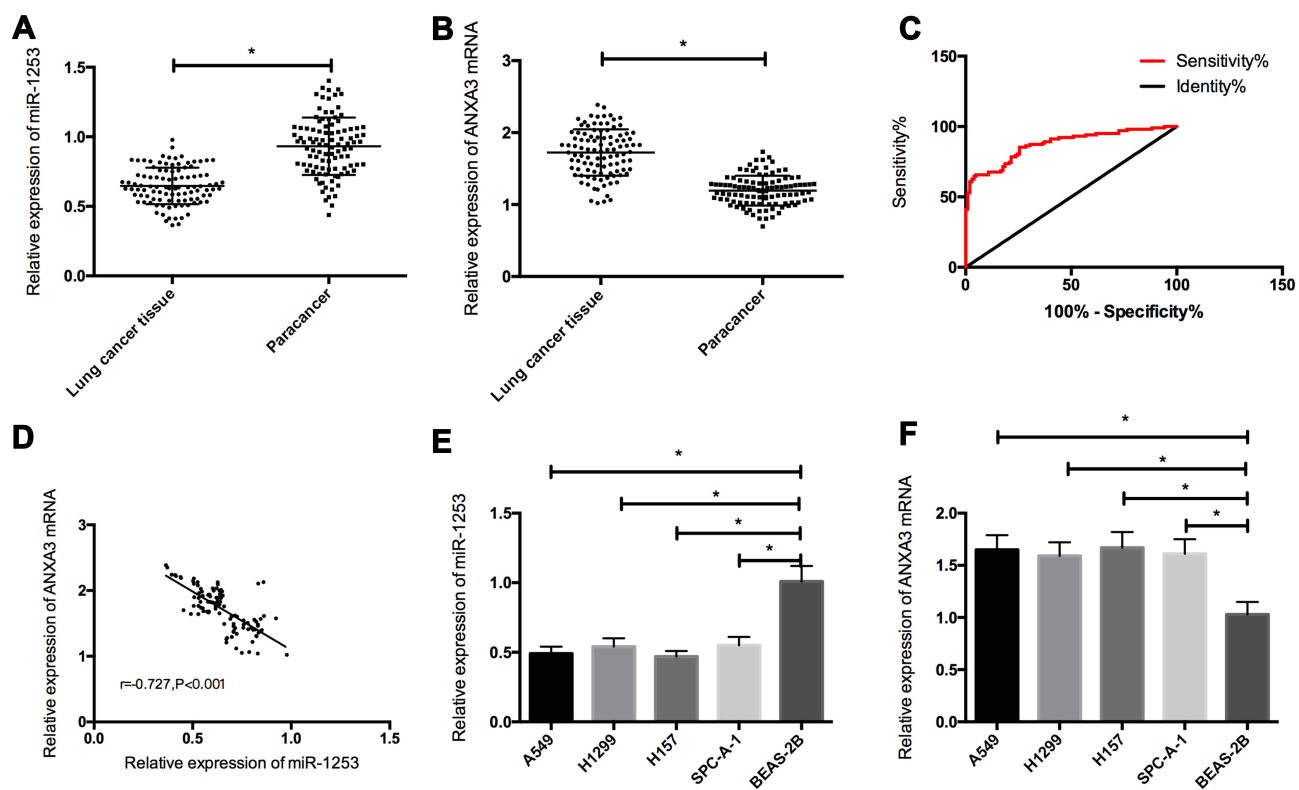


Figure 1 Expression and clinical significance of *miR-1253* and *ANXA3* in LC. (A) *miR-1253* expression in LC tissues. (B) *ANXA3* expression in LC tissues. (C) ROC of *miR-1253* in diagnosing LC. (D) Correlation analysis between *miR-1253* and *ANXA3*. (E) *miR-1253* expression in LC cells. (F) *ANXA3* expression in LC cells. * Indicates $P < 0.05$.

Table 2 Relationship Between *miR-1253* and Clinicopathological Data of LC Patients

Factors		<i>miR-1253</i>		χ^2 value	P value
		Low Expression (n=59)	High Expression (n=43)		
Age	≥ 58 years old (n=53)	31 (52.54)	22 (51.16)	0.019	0.891
	< 58 years old (n=49)	28 (47.46)	21 (48.84)		
Gender	Male (n=62)	37 (62.71)	25 (58.14)	0.218	0.641
	Female (n=40)	22 (37.29)	18 (41.86)		
TNM stage	Stages I–II (n=57)	22 (37.29)	35 (81.40)	19.63	<0.001
	Stages III (n=45)	37 (62.71)	8 (18.60)		
Differentiation	Low differentiation (n=63)	50 (84.75)	13 (30.23)	31.30	<0.001
	Moderate + high differentiation (n=39)	9 (15.25)	30 (69.77)		
Lymphatic metastasis	Metastasis (n=37)	28 (47.46)	9 (20.93)	7.572	0.001
	No metastasis (n=65)	31 (52.54)	34 (79.07)		
Mycoplasma infection	Positive (n = 40)	23(38.98)	17(39.53)	0.003	0.955
	Negative (n = 62)	36(61.02)	26(60.47)		

with si-*ANXA3*, *ANXA3* was down-regulated in A549 and H157 cells ($P<0.05$). After transfection, MTT assay and cell invasion assay manifested that si-*ANXA3* remarkably inhibited the proliferation and invasion of A549 and H157 cells and promoted their apoptosis ($P<0.05$). WB experiment revealed that the pro-apoptotic proteins *Bax* and *caspase-3* expression levels increased and the anti-apoptotic protein *Bcl-2* expression decreased in the cells knocked down the *ANXA3* expression. These results further indicated that *ANXA3* was the direct functional target of *miR-1253* in LC (Figure 3).

Dual-Luciferase Reporter Enzyme

In order to explore *miR-1253*'s potential mechanism in LC, bioinformatics analysis was conducted to predict the target gene of *miR-1253*. *ANXA3* was identified as its target gene. Luciferase reporter gene assay was conducted to check whether the 3'UTR of *ANXA3* could be directly targeted by *miR-1253*. The results revealed that the *miR-1253* over-expression decreased the luciferase activity of

ANXA3 3' UTR Wt ($P<0.05$), but had no effect on *ANXA3* 3' UTR Mut. What is more, Western blot showed that the *ANXA3* protein expression in A549 and H157 cells mock transfected with *miR-1253* was down-regulated, but in LC cells transfected with *miR-1253*-inhibitor, it was markedly up-regulated ($P<0.05$). (Figure 4)

Rescue Experiment

Through co-transfection of *miR-1253*-inhibitor+si-*ANXA3* to A549 and H157 cells, their proliferation, invasion and apoptosis were detected. The results signified that the three of transfected *miR-1253*-inhibitor+si-*ANXA3* cells were not different from those of transfected miR-NC cells, while those were dramatically enhanced and decreased compared with those of transfected si-*ANXA3* cells, which indicated that *miR-1253*-inhibitor could reverse the effect of si-*ANXA3* on LC cells. WB detection revealed that the *Bax*, *caspase-3*, and *Bcl-2* expression levels in transfected *miR-1253*-inhibitor+si-*ANXA3* cells were not different from those in transfected miR-NC cells, and the

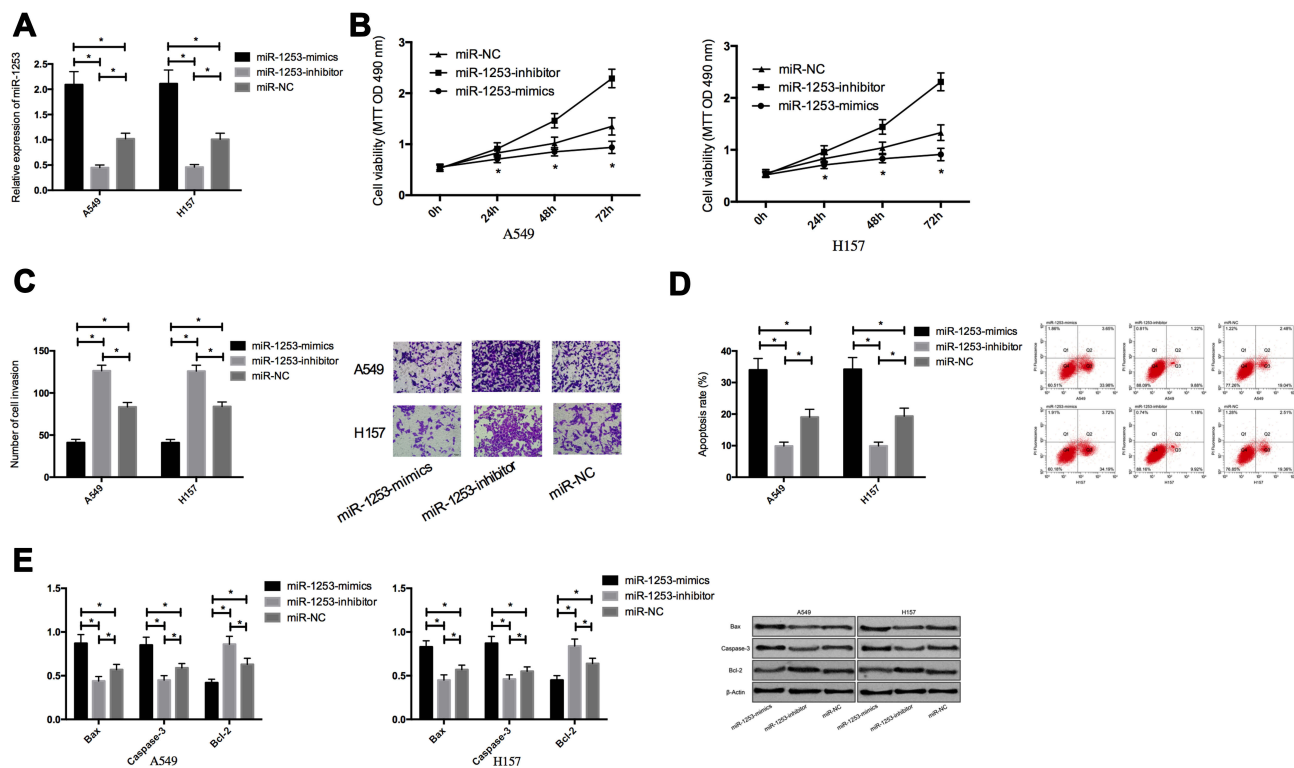


Figure 2 Effect of *miR-1253* on proliferation, invasion and apoptosis of LC cells. (A) *miR-1253* expression in LC cells after transfection. (B) Effect of *miR-1253* on proliferation of LC cells. (C) Effect of *miR-1253* on invasion ability of LC cells. (D) Effect of *miR-1253* on apoptosis of LC cells. (E) Effect of *miR-1253* on apoptosis-related cells in LC cells. * Indicates $P < 0.05$.

Bcl-2 protein expression was obviously higher than that in transfected *si-ANXA3* cells, while the *Bax* protein and *caspase-3* protein expression levels were dramatically lower ($P < 0.05$). (Figure 5)

Discussion

LC is the principal reason for cancer-related death all over the world currently, and the mechanism of its occurrence and development has always been the focus of medical research.¹² Although advances in medical technology in recent years have enabled people to have a certain understanding of the development of LC, it is impossible to accurately evaluate every process of its development.¹³ miRNA's important role in tumors has been continuously verified and confirmed, including LC. For instance, research¹⁴ found that miR-203 could inhibit their proliferation, invasion and migration by down-regulating RGS17. Therefore, the analysis of miRNA's influence on LC progression has momentous clinical significance for the diagnosis and treatment of patients.

miR-1253 has been found to act as a tumor-suppressor gene in some tumors. For example, research¹⁵ found that inhibiting *miR-1253* expression could promote the

development of prostate cancer. In our study, we first found that the *miR-1253* expression in LC tissues and cells was dramatically down-regulated, and further analyzed its clinical significance. We found that *miR-1253* had high diagnostic value for LC, and its expression was bound up with the pathological stage, differentiation degree and lymph node metastasis of patients. This further indicates that *miR-1253* may be crucial in LC's occurrence and development. Rapid development, high recurrence and metastasis of LC usually lead to poor prognosis, so it is quite significant for those patients to know how to inhibit its progression.^{16,17} Therefore, in order to observe *miR-1253*'s effect on the progression of LC, we regulated the *miR-1253* expression in those cells and observed their changes after the regulation of *miR-1253*. We observed that when we over-expressed *miR-1253* in LC cells, their proliferation and invasion were obviously inhibited, but the apoptosis rate increased dramatically. *Bax* and *Bcl-2* belong to the BCL family; previous studies¹⁸ showed that increased *Bax* expression might induce apoptosis, while increased *Bcl-2* expression might inhibit apoptosis. *Caspase-3* belongs to the cysteine protease family, which is the main effector in the process of apoptosis; its

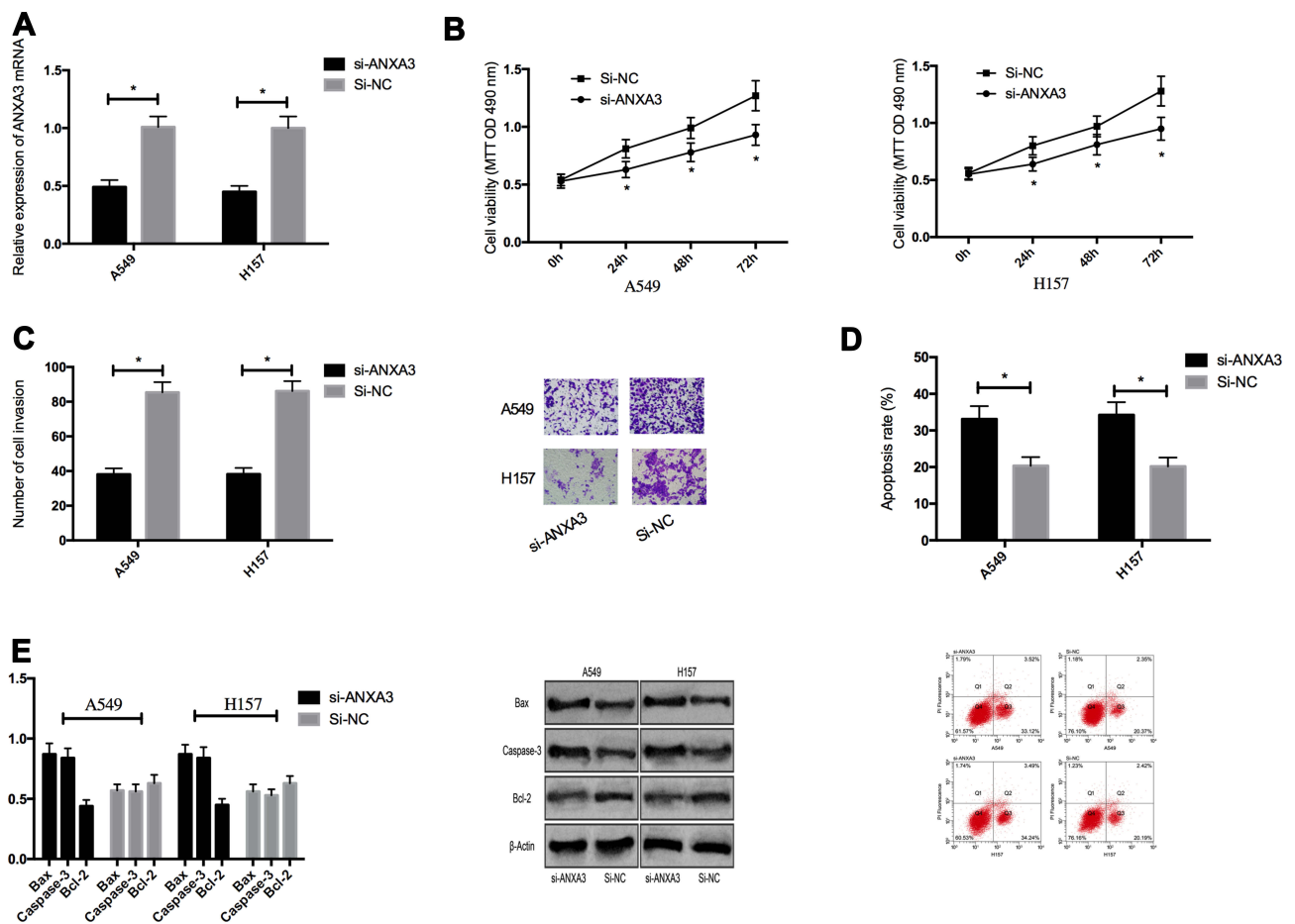


Figure 3 Effect of downregulation of ANXA3 expression on proliferation, invasion and apoptosis of LC cells. (A) ANXA3 expression in LC cells after transfection. (B) Effect of ANXA3 on proliferation of LC cells. (C) Effect of ANXA3 on the invasive ability of LC cells. (D) Effect of ANXA3 on apoptosis of LC cells. (E) Effect of ANXA3 on apoptosis-related cells in LC cells. * Indicates P<0.05.

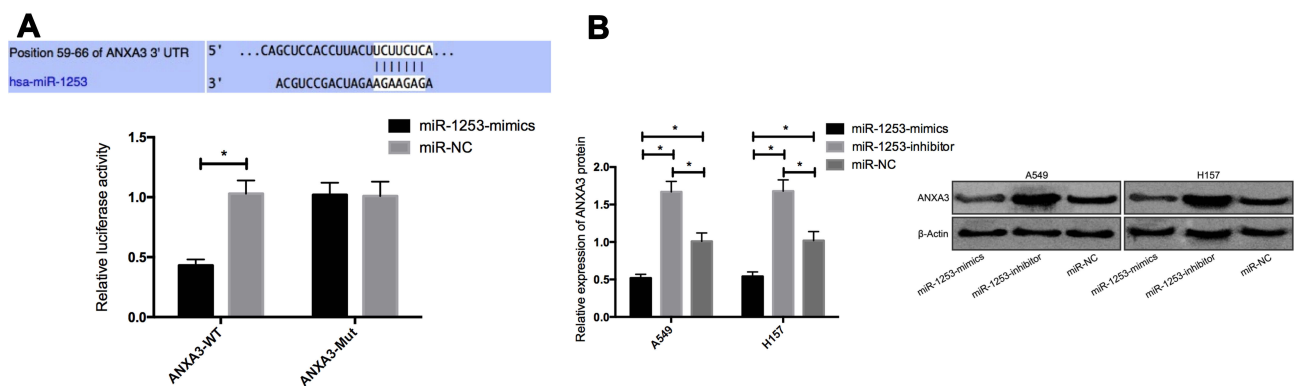


Figure 4 Dual-luciferase reporter enzyme. (A) Effect of miR-1253 on ANXA3 dual-luciferase reporter enzyme activity. (B) Effect of miR-1253 on ANXA3 protein expression. * Indicates P<0.05.

activation marks the irreversibility of apoptosis.¹⁹ The changes of apoptosis-related proteins were consistent with the trend of apoptosis rate. However, when we further inhibited the *miR-1253* expression, we observed the opposite cell phenotype. This suggested that *miR-1253* could

inhibit the development of tumor cells and promote their apoptosis. In the past, its role in tumor was also found to be a tumor-suppressor gene. For example, research²⁰ found that *miR-1253* could inhibit the malignant behavior of osteosarcoma by regulating *FOXF1*. Another study²¹

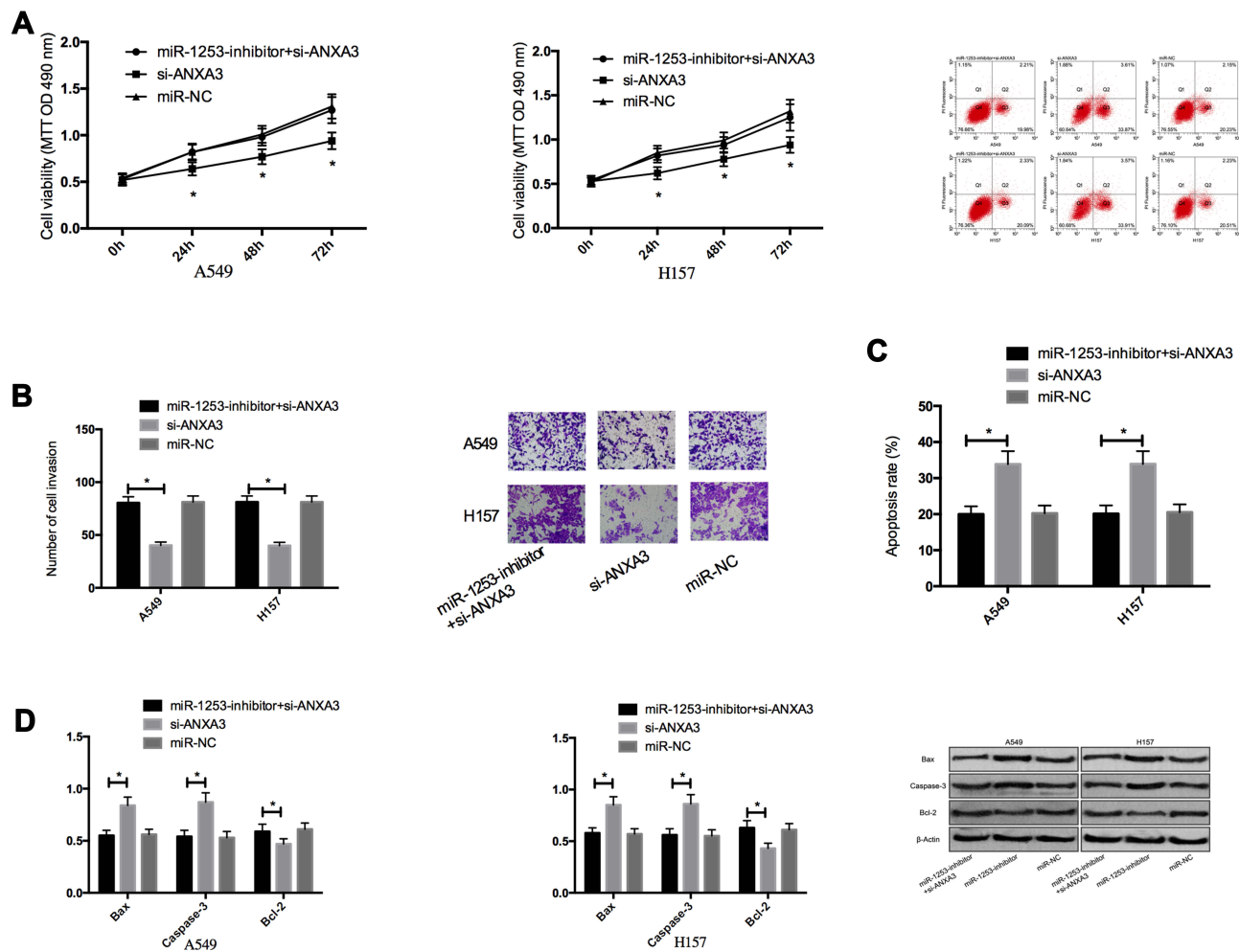


Figure 5 Rescue experiment. (A) Effect of *miR-1253*-inhibitor+si-ANXA3 on proliferation of LC cells. (B) Effect of *miR-1253*-inhibitor+si-ANXA3 on invasion ability of LC cells. (C) Effect of *miR-1253*-inhibitor+si-ANXA3 on apoptosis rate of LC cells. (D) Effect of *miR-1253*-inhibitor+si-ANXA3 on apoptosis-related proteins in LC cells. * Indicates $P < 0.05$.

discovered that inhibiting *miR-1253* could promote the progression of pancreatic ductal adenocarcinoma cells. This was consistent with the role of *miR-1253* found in LC.

It is well known that miRNA affects cell function by binding its target genes.²² Previous studies²³ have also pointed out that *miR-1253* can inhibit proliferation and invasion of non-small cell LC by targeting *WNT5A*, which is consistent with *miR-1253*'s effect on LC cells found in the above studies. However, each miRNA has more than one target gene. We found that *ANXA3* was also the direct target of *miR-1253* through bioinformatics prediction. It is a member of the annexin family, which can be combined with calcium-dependent acidic phospholipids.²⁴ Previous studies have indicated that *ANXA3* is essential in the occurrence and development of tumors.²⁵ For example, it is reported that *ANXA3* is a potential biomarker in bladder cancer²⁶ and gastric cancer.²⁷

In our work, we found that the *ANXA3* expression in LC tissues and cells increased markedly, which signified that it might act as cancer-promoting gene in LC. Previously, the role of *ANXA3* in tumors was also found to be increased in expression, and some studies²⁸ also found that the *ANXA3* expression in LC cells was dramatically higher than that in normal cells, which was consistent with our detection results. Previously, some studies²⁹ have found that RNA interference silencing the expression of *ANXA3* can inhibit the proliferation and invasion of breast cancer cells, and³⁰ other studies have pointed out that the increased expression of *ANXA3* will further enhance the tumorigenicity of liver cancer cells, which is consistent with our observation of the role of *ANXA3* in lung cancer. Subsequently, in order to observe *ANXA3*'s effect on LC cells in depth, we down-regulated the *ANXA3* expression in those cells. The results revealed that when we down-regulated its expression, their proliferation and invasion were

dramatically inhibited, and the apoptosis rate increased dramatically. This also confirmed that *ANXA3* acted as an oncogene in LC. Soon afterwards, in order to verify the targeted relationship between *miR-1253* and *ANXA3*, we conducted dual-luciferase reporter enzyme experiments and rescue experiments. The results confirmed that there was indeed a targeted relationship between *miR-1253* and *ANXA3*, and *miR-1253*-inhibitor could reverse the effect of si-*ANXA3* on LC cells, and further demonstrated that *miR-1253* influenced the development of LC by regulating *ANXA3*.

Conclusion

miR-1253 is low expressed in lung cancer and acts as a tumor-suppressor gene. The specific mechanism may be achieved by regulating the *ANXA3*, which may be a potential target for lung cancer treatment, which also provides a new target. There are still some inadequacies in this study. For example, first of all, we have yet to prove the effect of *miR-1253* on tumor growth through in vivo tumor-forming tests. Secondly, we are still not clear about the downstream mechanism of *ANXA3*. Hence, we will carry out further basic experiments on this in future studies in order to provide more data support for our conclusions.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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