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ORIGINAL ARTICLE

# MiR-532-3p inhibits metastasis and proliferation of non-small cell lung cancer by targeting FOXP3

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## Summary

***Purpose:*** *To investigate the potential effect of microRNA-532-5p (miR-532-3p) on the development of non-small cell lung cancer (NSCLC) and the relevant mechanism.*

***Methods:*** *Thirty-seven patients who underwent primary NSCLC resection were studied. To examine the role of miR-532-3p in NSCLC development, we detected the level of miR-532-3p expression in NSCLC tissues and the para-cancer tissues by qRT-PCR. In order to investigate the potential target of miR-532-3p, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA, to elucidate the putative and possible targets of miR-532-3p. To test the function of miR-532-3p on the proliferation of NSCLC cell, we performed MTT assay to detect the cell proliferation rates. Migration and invasion were also studied.*

***Results:*** *The expression level of miR-532-3p were detected in NSCLC tissues and cells by qRT-PCR, which indicated that the expression of miR-532-3p was low in both tissue and cell levels. Online prediction websites and luciferase reporter assay indicated that FOXP3 is a direct target of miR-532-3p in NSCLC cells. Further results showed that this miR significantly decreased the expression level of FOXP3. MTT assay showed that miR-532-3D remarkably suppressed the proliferation of NSCLC cells. Furthermore, transwell and scratch healing experiments suggested that miR-532-3p inhibited the invasion and migration of NSCLC cells.*

***Conclusions:*** *Our research discovered the suppressive function of miR-532-3p in NSCLC by targeting FOXP3, revealing that miR-532-3p/FOXP3 axis might be a potential therapeutic target for the treatment of NSCLC.*

***Key words:*** *microRNA-532-5p, non-small cell lung cancer, Forkhead box P3*

## Introduction

Lung cancer (LC) is a common malignant tumor, characterized by high morbidity and mortality rates, in which its mortality rate ranks first among all types of malignant tumors, thus posing a great threat to human life and health [1-3]. Surgical treatment has been widely used in previous clinical methods for LC treatment. However, due to the atypical clinical manifestations of most LC patients in the early stage of disease and the limited clinical methods for LC diagnosis at the present phase, 75% of LC patients are diagnosed with regional metastasis in the first visit to hospital, and cancer cells in these patients spread to regional lymph nodes or directly infiltrate the surrounding normal tissues, resulting in a significant reduction (only 20.6%) in the 5-year survival rate. In addition, there are some LC patients with distant metastasis, whose 5-year survival rate is only 2.8%. So far, the overall 5-year survival rate of LC patients is still at a relatively low level (only about 15%), which is due to the fact that most of the patients are with advanced stage when definitely diagnosed, and radiotherapy and chemotherapy efficacy is poor [4-6].

Clinically, NSCLC is the main pathological type of LC, accounting for 85% of the cases [7]. Like other malignant tumors, the main causes of the

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occurrence and development of LC include mutation or loss of activity of tumor suppressor genes and activation of oncogenes. Besides, other biological ways could also exert crucial effects on the prognosis of NSCLC patients and the sensitivity of NSCLC therapeutic drugs [8,9]. Therefore, the study on the occurrence and malignant mechanism of NSCLC contributes to a deeper understanding of the disease, which is of great significance in formulating individualized treatment plans according to actual conditions of different clinical patients and also provide effective reference indicators for evaluating the prognosis of patients.

A micro ribonucleic acid (miRNA) is usually an endogenous non-coding single-stranded RNA containing 20-25 nucleotides. It is ubiquitous in viruses and higher organisms and is highly evolutionarily conserved. About 2% of the genes in the human genome encode miRNAs [10]. On the one hand, miRNAs can promote messenger RNA (mRNA) degradation by binding to the 3' prime untranslated region (3'UTR) of mRNAs to regulate the gene expression at the post-transcriptional level [11,12]. On the other hand, they can also regulate the gene expression by inhibiting the initiation of translation [13]. The 3'UTR of one mRNA could combine with multiple miRNAs, and in turn, one miRNA can also regulate the expression of multiple mRNAs [12]. According to bioinformatics analysis, miRNAs might regulate 30% of human mRNAs [14,15]. In 2002, Calin's et al team first discovered the relationship between miRNAs and tumors [16]. Later, scientists found that more than 50% of miRNA genes were located in gene regions related to tumors or fragile sites on genes [17], suggesting that miRNAs might be closely related to the occurrence and development of tumors. It has been further found in many independent studies that miRNAs were abnormally expressed in different types of cancers [18,19], including NSCLC [20-22].

This study attempted to explore the role of miR-532-3p in NSCLC and tried to find out its possible underlying mechanism.

## Methods

*NSCLC tissues and cells*

Thirty-seven patients who underwent primary NSCLC resection were collected. None of the patients had received radiotherapy or chemotherapy before operation. Tissue specimens included primary NSCLC tissues and cancer-adjacent tissues (they were at >5 cm away from the primary lesion, and there was no tumor cell infiltration confirmed by pathology). After resection, the specimens were quickly frozen in liquid nitrogen and then transferred to a refrigerator at -80°C for further experimentation. This study was approved by the Medical Ethics Committee of Taizhou First People's Hospital, and all patients signed informed consent.

The human lung cancer cell line (A549) together with the normal human lung epithelial cells (BEAS-2B) were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA), containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin and cultured in an incubator with 5% $CO_2$ at 37°C. Passaging began when the cells reached a confluence of 70-80%.

*Transfection*

When A549 cells grew to about 80% confluence, they were transfected with miR-NC, miR-532-3p mimics and FOXP3 using the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Ultimately, the Dulbecco's modified Eagle's Medium (DMEM) was replaced with fresh culture medium after 8 h, and the cells were collected for subsequent experiments after 48 h.

*Luciferase reporter assays*

In TargetScan, miRDB and microRNA websites, it was found that FOXP3 is the target gene of miR-532-3p. The binding sequence of miR-532-3p at the 3'-end of FOXP3 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), For luciferase reporter assay: A549 cells were inoculated into a 24-well plate at $1.5\times10^5$ cells/well until the cells grew to a confluence of 80%. Then, A549 cells were co-transfected with plasmid miR-NC+pGL3-FOXP3-wild type, miR-532-3p mimics+pGL3-FOXP3-wt, miR-NC+pGL3-FOXP3-mutant and miR-532-3p mimics+pGL3-FOXP3-mut, respectively, and the medium was replaced with fresh Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) after 6 h. At 48 h after transfection, cells in each group were lysed according to the Dual-Luciferase Reporter Assay System instructions (Promega, Madison, WI, USA). Then the luciferase activity was detected in a multi-function microplate reader

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

The total RNA was extracted from tissues and cells by TRIzol assay (Invitrogen, Carlsbad, CA, USA), and the TapMan MiRNA Reverse Transcription kit was used for reverse transcription under the reaction conditions of 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Then, the TapMan MiRNA Assays Real-time PCR kit (TaKaRa, Tokyo, Japan) was applied for quantitative amplification. Reaction conditions: amplification at 95°C for 5 min, 95°C for 15 s and 62°C for 30 s for a total of 40 cycles. Besides, three repeated wells were set for each inspection index, with U6 as an internal reference. Finally, the relative expression level of miR-532-3p was quantified by $2^{-\Delta\Delta Ct}$.

*Western blot analysis*

The transfected cells to be tested were washed with phosphate buffered saline (PBS) 3 times, and added with cell lysate containing protease inhibitors to extract the total protein, which was denatured at 100°C for 5 min.

Subsequently, the protein was separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking with 5% bovine serum albumin (BSA) for 1 h, the corresponding primary antibody was added for incubation at 4°C overnight. The next day, the horseradish peroxidase-labeled secondary antibody was added for incubation at room temperature for 1.5 h. Finally, after luminescent liquid was added, the protein was exposed and photographed using a gel imager, and the gray value was measured to calculate the relative expression level. With glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative changes in protein expression were detected.

*Cell proliferation*

When cells grew to the logarithmic growth phase, they were collected, diluted into $1\times10^6$ cell suspension, and added into a 96-well cell culture plate ($5\times10^3$/100 µL per well). The wells only added with medium were used as blank controls. A total of 5 time points was set: 0 (detected immediately after cells were paved onto the plate and adhered to the wall), 24, 48, 72 and 96 h. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). Fifteen µL MTT reagent (500 µg/mL) was added into each well for culture for another 2 h, after which the absorbance (optical density/OD) was measured at 570 nm using an enzyme-labeled spectrophotometer, followed by zero setting using blank wells.

*Cell migration and invasion assays*

Migration assay: the transfected cells to be tested were prepared into a cell suspension with a density of $1\times10^6$/mL, added into a 6-well plate, and cultured overnight to form a monolayer. Then, a transverse line was drawn on the monolayer with a 10 µL spear head, and washed with PBS 3 times to remove the cells dropped due to scratching. Ultimately, photos were taken under a microscope to measure the scratch width, and then these photos were taken out to measure the scratch width after 24 h of culturing.

Invasion assay: each group of cells to be tested was prepared into a suspension with a cell density of $1\times10^6$/mL using a serum-free culture medium, and then the cell suspension was added into a Transwell upper chamber covered with an artificial basement membrane. At the same time, a culture medium containing 20% FBS was added into the lower chamber. After culturing at 37°C for 24 h, the cells at the bottom of the upper chamber were stained with 0.5% crystal violet, and the cells at the inner side of the upper chamber were removed with cotton swabs. Finally, the cells were observed and counted under a microscope.

*Statistics*

Statistical analyses were performed with Student's t-test or F-test. All p values were two-sided and $p<0.05$ was considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

## Results

*MiR-532-3p expression was reduced both in tissues and cells of NSCLC*

To examine the role of miR-532-3p in NSCLC development, we detected the level of miR-532-3p expression in NSCLC tissues and the para-cancer tissues by qRT-PCR. The results showed that the expression of miR-532-3p was pretty low in NSCLC tissues by comparing with para-cancer tissues (Figure 1A). Furthermore, we found the same results at the cellular level (Figure 1B).

*FOXP3 is a direct target of miR-532-3p in NSCLC cells*

In order to investigate the potential target of miR-532-3p, we checked it in three publicly available algorithms (TargetScan, miRDB and microRNA) to elucidate the putative and possible targets of

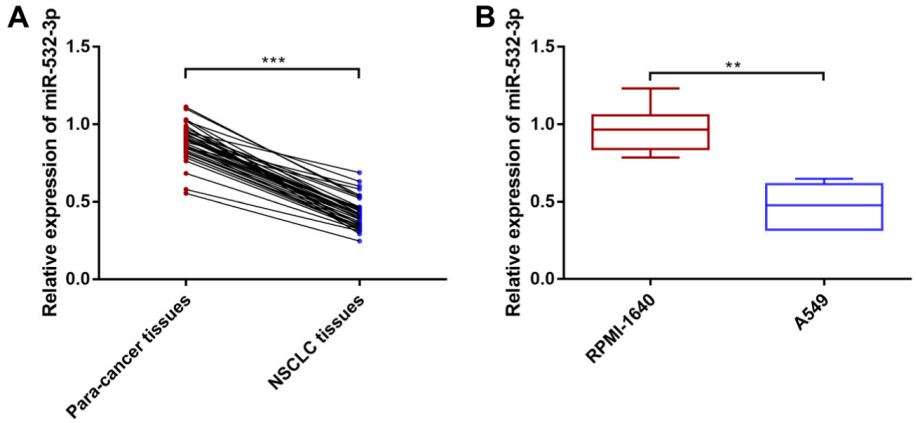


**Figure 1.** The expressions of miR-532-3p in NSCLC tissues and cells. **(A):** Difference in the expression of miR-532-3p between NSCLC tissues and para-cancer tissues (***p<0.001). **(B):** The expression of miR-532-3p in NSCLC cells (A549) and normal human lung epithelial cells (BEAS-2B) (**p<0.01).

miR-532-3p. The FOXP3 was checked as a supposed target of miR-532-3p (Figure 2A). We then established luciferase reporter vectors containing the wild or mutant-type miR-532-3p seed sequences of the FOXP3 3'UTR. The overexpression of miR-532-3p with mimics resulted in the decrease of the luciferase activity of the wide-type of FOXP3, but it had no effect on mutant-type (Figure 2A), which suggested that the expression of FOXP3 could be regulated by miR-532-3p.

Based on this, we further detected the expression of FOXP3 in NSCLC tissues and para-cancer tissues, which was consistent with our expectation. The expression of FOXP3 in NSCLC tissues with low expression of miR-532-3p was significantly restricted. The expression of FOXP3 in normal para-cancer tissues was 2-3 times higher than that in NSCLC tissues (Figure 2B).

*MiR-532-3p decreased the expression level of FOXP3*

We set up three groups to conduct the similar experiments (miR-NC group, miR-532-3p mimics group and the mimics + FOXP3 group) in A549 cells (Figure 3A), and we found the expression of FOXP3 was significantly correlated with the expression of miR-532-3p. When we upregulated miR-532-3p in A549 cells, the expression of FOXP3 decreased significantly (Figure 3A).

*MiR-532-3p suppressed the proliferation of NSCLC cells*

To test the function of miR-532-3p on the proliferation rates of NSCLC cells, we used MTT assay. The MTT results showed that the cell proliferation rates of A549 cells were decreased by upregulated miR-532-3p using mimics transfection, while the proliferation of A549 cells was positively correlated with FOXP3 expression (Figure 3B).

*MiR-532-3p inhibited the invasion and migration of NSCLC cells*

Invasion and migration were the two most key factors in cancer cell metastasis. In the invasion and migration experiments, significant inhibition

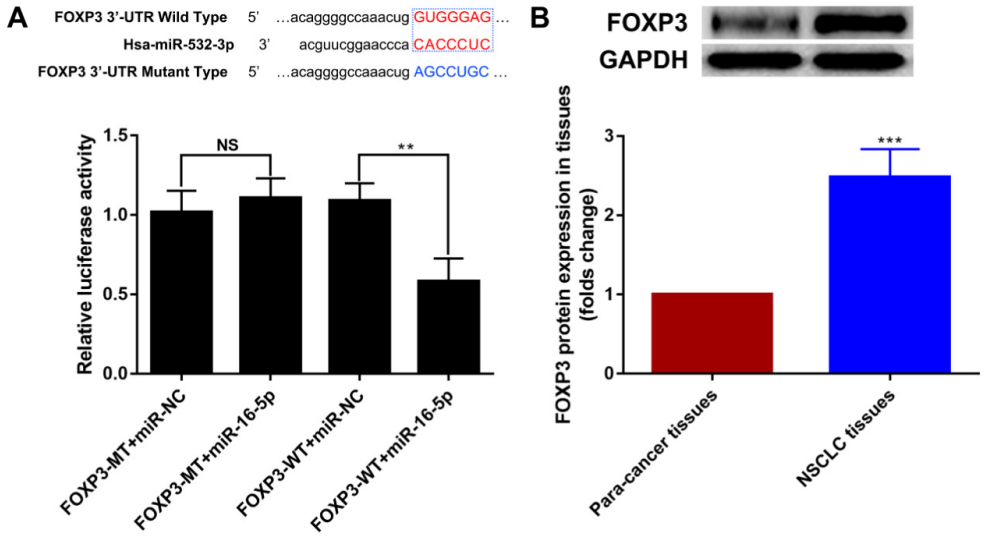


**Figure 2.** FOXP3 is a direct and functional target of miR-532-3p. **(A):** Diagram of putative miR-532-3p binding sites of FOXP3 and relative activities of luciferase reporters (**p<0.01). **(B):** FOXP3 expression level in NSCLC tissues and para-cancer tissues (***p<0.001).

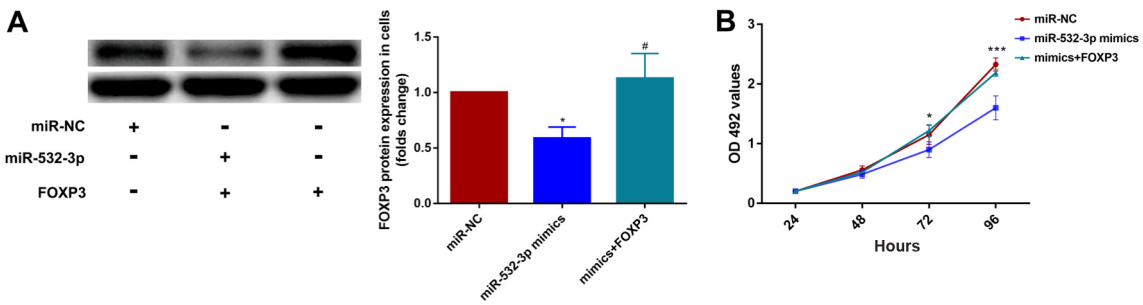


**Figure 3. (A):** MiR-532-3p decreased the expression level of FOXP3 in A549 cells (*p<0.05, *vs.* NC group; #p<0.05 *vs.* Mimics group). **(B):** MiR-532-3p inhibited the proliferation of NSCLC cells (*p<0.05, ***p<0.001).

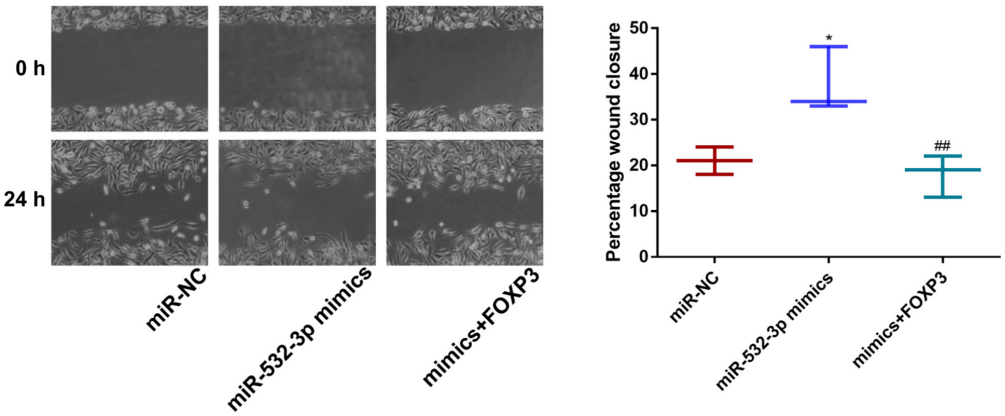


**Figure 4.** The migration of NSCLC cells was analyzed using wound healing assay and detected by microscope (×200) (*p<0.05 *vs.* NC group; ***p<0.001; #p<0.01 *vs.* Mimics group).

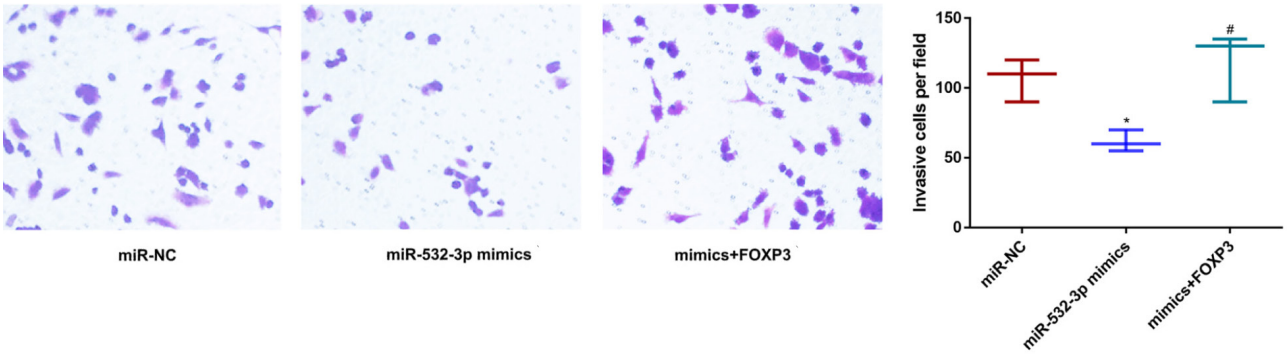


**Figure 5.** The invasion of NSCLC cells was analyzed using transwell assay and detected by microscope (×200) (*p<0.05 *vs.* NC group; #p<0.05 *vs.* Mimics group).

of A549 cell metastasis was observed in the miR-532-3p mimics group. The results indicated that the ability of migration and invasion of A549 cells were restricted by upregulation of miR-532-3p, while the addition of FOXP3 resulted in re-gaining the ability of migration and invasion in NSCLC cell (Figures 4 and 5).

## Discussion

NSCLC has a high mortality rate and is prone to metastasis and recurrence, thus bringing great difficulties to the early diagnosis and treatment of patients. At present, the mechanisms of occurrence and development of NSCLC have not been fully explored. The formation of NSCLC is the result of joint action of a series of internal and external factors. Like malignant tumors from other tissues, the internal factors for the occurrence and development of NSCLC include the upregulation of the oncogene expression level and/or the downregulation of the tumor suppressor gene expression level, so it is a multi-step process involving multiple genes. The miRNA expression can directly regulate the expression of a certain type of genes or even hundreds of types of genes, thus forming a complex regulatory network and further affecting the malignant process of tumors. However, there is accumulating evidence showing that miRNAs are related to the occurrence and development of malignant tumors, including NSCLC. It is believed that the biological functions of miRNAs vary with their target genes and the tissues where cells were located. Some miRNAs were considered as biomarkers for the prognosis of patients with some diseases, including malignant tumors. Some abnormally expressed miRNAs could promote the formation of malignant cells and eventually lead to the occurrence of tumors.

Activation of proto-oncogenes or inactivation of cancer suppressor genes is a key link in the process of tumor occurrence and development, which can cause cell cancerization due to uncontrolled growth. In living organisms, miRNAs regulate the biological behavior of cells by regulating the expression of their corresponding target genes. To study the biological function of miRNAs, the priority is to find and confirm their target genes in the specific biological process.

The human forkhead box P3 (FOXP3) gene consists of 11 exons and 10 introns, which are located on Xp11.23. The cDNA has a full length of

1869 bp and can encode the full-length subtype and the subtype lacking exon 3 (Δ3 subtype), respectively [23]. It belongs to the FOX family and was first found to play a specific role on the surface of cluster of differentiation (CD)$4^+CD25^+$ regulatory T cells (Tregs) [24]. It can reflect to some extent the functional activity and level of Tregs, which is closely related to its development and function. It was revealed that inhibiting the FOXP3 expression could make Tregs lose their regulatory function and enable $CD4^+$T cells to more easily differentiate into T helper 2 (Th2) cells, thus inducing a variety of immune diseases [25]. Although several studies had indicated that FOXP3 was expressed on a small number of $CD4^+CD25^-$T and $CD8^+$T cells [26], it is still considered as the most specific marker of Treg cells. Recently, an ever increasing number of studies have found that Tregs play a key role in tumor immune escape [27,28]. Besides, it was also reported that Tregs were significantly locally increased in breast cancer, ovarian cancer, prostate cancer and pancreatic cancer [29-32]. Hinz et al [33] reported that FOXP3 can be expressed in pancreatic cancer and melanoma cells\, while Karanikas et al [34] observed the expression of FOXP3 in 25 cancer cell lines of different origins *in vitro*. The results showed that FOXP3 was expressed in different degrees in cells, but the role of FOXP3 was different in different cancer cells, and its role in different cancer cells was also not exactly the same.

In our study, the proliferation ability of cells was detected using CCK-8 assay, and the results revealed that the overexpression of miR-532-3p could slow down the proliferation of A549 cells. By virtue of depolarization of epithelial cells, epithelial mesenchymal transition (EMT) makes them lose intercellular adhesion, gain stronger movement ability, separate themselves from the primary site of the tumor, enter blood vessels or lymphatic vessels and transfer to distant places along with circulation to form new metastasis foci, which is the main mechanism of EMT affecting tumor invasion and metastasis [35,36]. Therefore, wound healing assay and transwell assay were adopted to investigate the effects of FOXP3 on the migration and invasion ability of A549 cells. The results of wound healing assay confirmed that the healing rate of scratches in the A549 group was low compared with the control group, and the migration of cells in the former was slower than in the control group, proving that the migration ability of cells in the horizontal direction was weaker. Transwell cell migration assay was further carried out to verify the invasion ability of cells in the vertical direction. The cell invasion ability could be reflected through the comparison of the number of cells passing through the membrane. According to the results, the cell invasion ability in the experimental group was evidently lower than that in the negative control (NC) group. Therefore, the above experiments suggested that FOXP3 can inhibit the migration and invasion of tumor cells.

## Conclusions

It was concluded that overexpression of miR-532-3p could effectively inhibit the proliferation, invasion and migration of NSCLC cells, which was achieved by inhibiting the FOXP3 expression. This study provided a potential therapeutic strategy for the treatment of NSCLC.

## Conflict of interests

The authors declare no conflict of interests.

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