MIR-451 INHIBITS PROLIFERATION AND PROMOTES APOPTOSIS OF LUNG CANCER CELLS BY REGULATING TARGET GENE PSMB8-NOS2 AND ACTIVATING PI3K/AKT/MTOR SIGNALING PATHWAY

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ABSTRACT

Objective: *To investigate the effect of miR-451 on inhibiting proliferation and promoting apoptosis of lung cancer cells by regulating target genes PSMB8 and NOS2 and thus activating the PI3K/AKT/mTOR signaling pathway.*

Methods*: Non-small cell lung cancer A549 cells were cultured in vitro, and the expression of miR-451 in A549 cells was detected by real-time quantitative PCR. A549 cells were transfected with the miR-451 mimic and miR-451 inhibitor and were divided into miR-451 over-expression group, the miR-451 inhibitor group, over-expression control group, and inhibitor control group. The cell proliferation rate was measured by the CCK-8 method. The apoptosis rate of each group was determined by flow cytometry, and the mRNA expressions of PSMB8 and NOS2 were detected by real-time quantitative PCR. The levels of PI3K, AKT, and mTOR protein were measured by western blot assay.*

Results*: The mRNA expression of miR-451 in lung cancer A549 cells was lower than that in normal lung cell lines, and the difference was statistically significant ($P < 0.05$). The cell proliferation rate of the miR-451 over-expression group was lower than that of the over-expression control group ($P < 0.05$), and that of the miR-451 inhibitor group was markedly higher than that of the inhibitor control group ($P < 0.05$). The apoptosis rate of the miR-451 over-expression group was remarkably higher than that of the over-expression control group ($P < 0.05$), and that of the miR-451 inhibitor group was significantly lower than that of the inhibitor control group ($P < 0.05$). The mRNA expressions of PSMB8 and NOS2 in the miR-451 over-expression group were lower than those in the over-expression control group ($P < 0.05$), and the mRNA expressions of PSMB8 and NOS2 in the miR-451 inhibitor group were significantly higher than those in the inhibitor control group ($P < 0.05$). The expressions of PI3K, AKT, and mTOR protein in the miR-451 over-expression group were markedly lower than those in the over-expression control group ($P < 0.05$), and the expressions of PI3K, AKT, and mTOR in the miR-451 inhibitor group were significantly higher than those in the inhibitor control group ($P < 0.05$).*

Conclusion*: Overexpression of miR-451 can inhibit the proliferation of lung cancer cells and promote cell apoptosis. Its mechanism may be related to the inhibition of targeted inflammation-related genes PSMB8 and NOS2, which further regulate the PI3K/AKT/mTOR signaling pathway.*

Keywords: *miR-451, PSMB8, NOS2, PI3K/AKT/mTOR signaling pathway, lung cancer, proliferation, apoptosis.*

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Introduction

Lung cancer is the most common cause of malignant tumor-related death, and non-small cell lung cancer accounts for 80 % of all lung cancers. Approximately 75 % of patients are already in the middle and advanced stage when diagnosed, and the 5-year survival rate is relatively low. It has become one of the most threatening malignant tumors[1]. As the population in China ages, lung cancer incidence and mortality are increasing annually, and there is a younger trend. At present, the clinical treatment for non-small cell lung cancer is mostly surgery combined with radiotherapy and chemotherapy. The main method for treating middle and advanced patients is chemotherapy, but the 5-year survival rate is only 5% to 15.8%, which substantially impacts the patients' quality of life[2]. Relevant data show that the mechanism of lung cancer occurrence is closely related to individualized tumor staging and

patient prognosis. Molecular diagnosis and targeted therapy of lung cancer provide a new direction for comprehensive clinical prevention and treatment[3].

miRNA is a small-molecule non-coding RNA, which can be used as an anti-cancer target by negatively regulating target genes involved in various biological processes such as cell proliferation, differentiation, and apoptosis. miR-451 is very conserved evolutionarily, but it is active in cell proliferation and apoptosis of colon cancer, liver cancer, and other cells. Some researchers have found that miR-451 plays an important role in maintaining the self-renewal of pulmonary bronchioles stem cells[4]. Others have found that mir-451 is underexpressed in tissues and lung cancer cells of patients with non-small cell lung cancer, and its mechanism of action may be related to its target genes[5]. Therefore, the purpose of this study was to analyze the effect of miR-451 on inhibiting proliferation and promoting apoptosis of lung cancer cells by regulating the target gene PSMB8-NOS2 and thus activating the PI3K/AKT/mTOR signaling pathway.

Materials and methods

Experimental reagents and instruments

RPMI-1640 medium was purchased from GIBCO (USA), fetal bovine serum and trypsin were purchased from Sigma-Aldrich (USA), and TRIzol reagent was purchased from Invitrogen Life Technologies (USA). The reverse transcription kit and RT-PCR kit were purchased from TaKaRa Bio, Inc. (Japan), the miR-451 primers were purchased from Shanghai Sangon Biological Engineering Co., Ltd. (China), and the CCK-8 kit was purchased from DOJINDO Chemical Research Institute (Japan). Dimethyl sulfoxide was purchased from Hangzhou Chemical Reagent Co., Ltd., protein lysate was purchased from Beyotime Bioengineering (China), and PI3K, AKT, and mTOR antibodies were purchased from Cell Signaling Technology. Ltd. (USA).

The inverted microscope was purchased from the Olympus Corporation (Japan), the PCR thermal cycler was purchased from Bio-Rad Laboratories (USA), and the gel imaging analyzer was purchased from Gene Genius company. The high-speed refrigerated centrifuge was purchased from Eppendorf (USA), and the ultraviolet and visible spectrophotometer was purchased from Shanghai Precision Scientific Instrument Co., Ltd. (China). The vertical electrophoresis apparatus, electrophoretic membrane conversion apparatus, and imager were purchased from Bio-Rad Laboratories (USA). The low-temperature refrigerator was purchased from the Haier Group (China).

Cell culture, transfection, and grouping

The non-small cell lung cancer A549 cell line was purchased from the cell bank of the Chinese Academy of Sciences. The cell lines were conventionally cultured in RPMI-1640 medium in a constant temperature incubator at 37°C with 5% CO2. The culture medium was changed every 2 to 3 days according to the cell growth conditions, and cell passage was performed when the degree of cell fusion reached 80% to 90% on the wall of the cell culture flask. To analyze the different functions of transfected cells, experimental cells were divided into a miR-451 over-expression group (transfected with miR-451 mimic), miR-451 inhibitor group (transfected with miR-451 inhibitor), over-expression control group, and inhibitor control group.

Detection of miR-451 expression

- The expression of miR-451 in the A549 cell line and normal cell lines was detected by RT-PCR. The lung cancer cell line and normal lung cell line were rinsed twice with PBS buffer, and TRIzol reagent was added to extract total RNA. The optical density of 5 μL of the samples at 260 nm and 280 nm were determined by spectrophotometer, and the RNA purity and concentration were calculated. A two-step RT-PCR was used according to manufacturer instructions. A $2^{-\Delta\Delta CT}$ formula was used to analyze the expression of miR-451 mRNA.
- Cell proliferation in each group of cells was detected by the CCK-8 method. Cell precipitate was blowed by a medium containing 10% fetal bovine serum to form the cell suspension. The cell density in the suspension was adjusted to 2×10^4/ml to 4×104/ml, and 100 μl was placed into a 96-well plate. There were 5 replicates for each group. The plates were incubated for 2 to 3 hours to promote the cells' adherence to the wall. After 72 h, the 96-well plate was removed, and the remaining medium in the well was removed by pipet. A mixture of 110 μl of CCK-8 reagent and RPMI1640 medium was added and culturing continued for 2 hours. The absorbance value of each well in the 96-well plate was determined at 450 nm with an enzyme labeling instrument.

• Cell apoptosis in each group was detected by flow cytometry. In a 6-well plate, A549 cells were inoculated and transfected, and the culture medium was changed the next day. The cells were digested 48 hours later and resuspended using fresh medium. The cells were rinsed with PBS buffer twice to collect approximately 1×105/ml to 5×105/ml cells, then 500 μl of binding buffer, 50 μl of annexin V-FITC, and 50 μl of PI were added and the solution mixed. The cells were observed and detected by flow cytometry within 60 min.

• RT-PCR was used to detect the effect of transfection of miR-451 on the expression of PSMB8 and NOS2 mRNA in each group of A549 cells. The methods and steps were the same as in[1].

• Protein expression was detected by western blot assay. A549 cells in each group were collected and total protein extracted according to the instructions of the BCA protein quantitative kit. 5×SDS sample buffer was added to the extracted protein solution, which was centrifuged to denature the protein, and stored at -80°C after packaging. The stacking gel and separating gel were prepared, and the electrophoresis was conducted at a constant voltage. The electrophoresis was stopped when the bromophenol blue dye reached the end of the gel. The protein was transferred to the PVDF membrane after 1 h and sealed at room temperature for 2 h. The primary antibody was diluted with TBST buffer to an appropriate concentration and incubated overnight at 4 °C. The corresponding secondary antibody was added and incubated at room temperature for 2 h. Then the electrochemical luminescent reagent was used for color development and exposure.

Statistical methods

All the data were analyzed by the SPSS 21.0 software package. The measurement data were expressed as mean ± standard deviation (_,x±s), and a Student's t-test was used for comparison between groups. The analysis of variance (ANOVA) was used to compare the data between multiple groups. Statistical significance was designated at $P < 0.05$.

Results

Expression of miR-451 in lung cancer A549 cells and normal cell lines

The results of real-time quantitative PCR showed that the mRNA expression of miR-451 in lung cancer A549 cells was lower than that in normal lung cell lines, and the difference was statistically significant ($P < 0.05$). The results are shown in Figure 1.

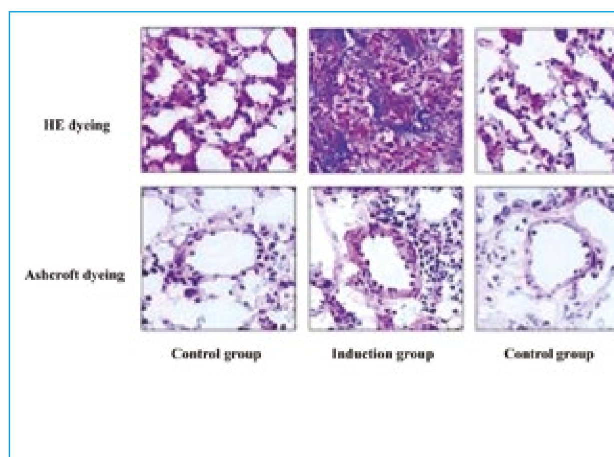

Figure 1: Expression of miR-451 in lung cancer A549 cells and normal cell lines.
*Notes: *The difference is significant at P < 0.05.*

Effects of miR-451 transfection on proliferation and apoptosis of A549 cells in each group

Compared with the over-expression control group, the cell proliferation rate of the miR-451 over-expression group was significantly increased, and the apoptosis rate was reduced ($P < 0.05$). Compared with the inhibitor control group, the cell proliferation rate of the miR-451 inhibitor group was markedly reduced, and the apoptosis rate was remarkably increased, with statistically significant differences ($P < 0.05$). The results are shown in Table 1.

Groups	Sample size	Proliferation rate (%)	Apoptosis rate (%)
miR-451 over-expression group	8	10.23 ± 2.78*	23.02 ± 5.12*
miR-451 inhibitor group	8	51.36 ± 6.74#	5.26 ± 1.74#
Over-expression control group	8	18.32 ± 2.69	15.23 ± 3.20
Inhibitor control group	8	25.12 ± 3.74	10.03 ± 2.36

Table 1: Effects of miR-451 transfection on proliferation and apoptosis of A549 cells in each group ($\bar{x}$±s).
*Notes: Compared with the over-expression control group, *P < 0.05; Compared with the inhibitor control group, #P < 0.05.*

Effects of miR-451 transfection on PSMB8 and NOS2 mRNA expression in each group of A549 cells

The mRNA expressions of PSMB8 and NOS2 in the miR-451 over-expression group were lower than those in the over-expression control group ($P < 0.05$), and the mRNA expressions of PSMB8 and NOS2 in the miR-451 inhibitor group were

significantly higher than those in the inhibitor control group (P < 0.05). The results are shown in Table 2.

Groups	Sample size	PSMB8 mRNA	NOS2 mRNA
miR-451 over-expression group	8	2.32 ± 1.02*	0.72 ± 0.05*
miR-451 inhibitor group	8	7.70 ± 2.03#	4.04 ± 0.16#
Over-expression control group	8	4.18 ± 1.21	2.67 ± 0.52
Inhibitor control group	8	5.33 ± 1.06	2.78 ± 0.36

Table 2: Effects of miR-451 transfection on PSMB8 and NOS2 mRNA expression in each group of A549 cells (x¯±s).

*Notes: Compared with the over-expression control group, *P < 0.05; compared with the inhibitor control group, #P < 0.05.*

Effects of miR-451 transfection on PI3K, AKT, and mTOR protein expression in each group of A549 cells

The results of western blot showed that the expressions of PI3K, AKT, and mTOR protein in the miR-451 over-expression group were markedly lower than those in the over-expression control group (P < 0.05), and the expressions of PI3K, AKT, and mTOR in the miR-451 inhibitor group were significantly higher than those in the inhibitor control group (P < 0.05). The results are shown in Table 3 and Figure 2.

Groups	Sample size	PI3K	AKT	mTOR
miR-451 over-expression group	8	0.68 ± 0.05*	0.77 ± 0.07*	1.02 ± 0.08*
miR-451 inhibitor group	8	5.65 ± 0.32#	4.20 ± 0.18#	5.24 ± 0.28#
Over-expression control group	8	3.38 ± 0.27	3.13 ± 0.19	3.86 ± 0.20
Inhibitor control group	8	1.07 ± 0.04	1.06 ± 0.25	1.13 ± 0.47

Table 3: Effects of miR-451 transfection on PI3K, AKT, and mTOR protein expression in each group of A549 cells (x¯±s).

*Notes: Compared with the over-expression control group, *P < 0.05; Compared with the inhibitor control group, #P < 0.05.*

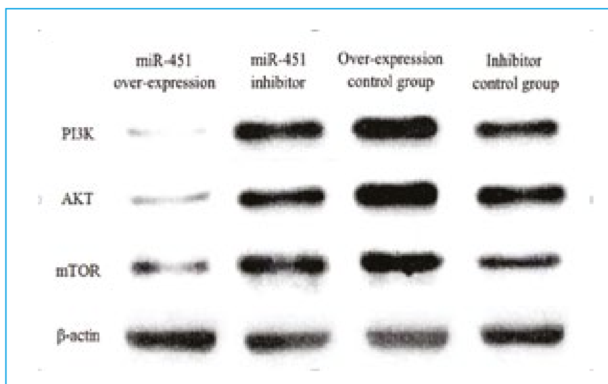

Fig. 2: Effects of miR-451 transfection on PI3K, AKT, and mTOR protein expression in each group of A549 cells.

Discussion

Non-small cell lung cancer (NSCLC) is a malignant tumor with one of the highest mortality rates in the world: 80% of patients die within 1 year after diagnosis, and its morbidity and mortality remain high[6]. Although the clinical efficacy has been improved to a certain extent due to the current optimization model of comprehensive lung cancer treatment, the prognosis is still poor. The infiltration and metastasis of cancer cells are the main factors affecting patient prognosis.

In recent years, with the development of molecular biology and human genomics, targeted molecular targeted has become an emerging treatment method for lung cancer. In addition, researchers have a deeper understanding of the molecular mechanism of the occurrence, invasion, and metastasis of lung cancer and some biological signal transduction pathways, which provides a new direction for developing treatment methods. With the in-depth study of miRNA in tumors and other diseases, an increasing number of studies have revealed that miRNA is involved in tumor invasion and metastasis and has the function of oncogene or tumor suppressor gene[7-8].

miRNAs are a class of single-stranded non-coding RNA molecules composed of 18 to 23 nucleotides, which mainly negatively regulate mRNA expression at the post-transcriptional level and affect the expression of downstream proteins and related functions of proteins. miRNA is involved in many important activities in the body and is peripherally involved in life processes such as blood cell production, insulin secretion, and human cancer cell growt[9]. The first tumor-related miRNA was reported in 2002 when researchers found that both miR-15 and miR-16 were tumor suppressor genes in hematological tumors[10].

At present, miRNAs have become a popular topic in tumor research. However, their regulation of multiple target genes and related mechanisms in the process of tumor formation still require further study. miRNA can induce tumor phenotype changes by promoting gene expression or regulating cell metabolism, differentiation, and expression of apoptotic gene[11]. Relevant data have shown that miR-34, miR-145, and other miRNAs can inhibit or promote lung cancer genes and regulate the pathological state and corresponding gene expression of lung cancer cells[12-13]. In this study, through high-throughput sequencing analysis of miRNA in lung cancer tissues and normal tissues, the expression of miR-451 in lung cancer A549 cells was found to be significantly reduced, suggesting that it may have potential antitumor effects. miR-451 is located

on chromosome 17q11.2 and regulates protein expression in this region by reverse transcription together with miR-144. Researchers have found that over-expression of miR-451 can reduce the proliferation of gastrointestinal tumors, trigger their apoptosis, and increase radiotherapy sensitivity[14]. This study showed that compared with the control group, the cell proliferation rate of the miR-451 over-expression group was significantly increased while the apoptosis rate was reduced. However, the cell proliferation rate of the miR-451 inhibitor group was markedly reduced, and the apoptosis rate was markedly increased ($P < 0.05$), confirming the pathological effect of miR-451 on tumors and revealing its potential therapeutic antitumor effect.

Previous studies have shown that the mechanism of miR-451 inhibition of lung cancer cells' proliferation may be related to inflammatory pathways. PSMB8 is a direct target gene of miR-451. Previous studies have confirmed that the involvement of PSMB8 in the inflammatory response is closely related to biological behaviors such as colorectal cancer proliferation and apoptosis[15]. NOS2 is a key enzyme in BO synthesis, mainly induced by inflammation and tumors. It affects tumor growth, differentiation, and metastasis in specific ways. In this study, RT-PCR detection results showed that the mRNA expressions of PSMB8 and NOS2 in the miR-451 over-expression group were significantly lower than those in the over-expression control group ($P < 0.05$), suggesting that over-expression of miR-451 could inhibit inflammation-related genes PSMB8 and NOS2 in lung cancer cells and participate in the development of lung cancer. The PI3K/AKT/mTOR signaling pathway is widely found in various tumor cells and is involved in tumor angiogenesis and the promotion of cell growth and proliferation (16). In this study, the results of western blot detection showed that the expressions of PI3K, AKT, and mTOR in the miR-451 over-expression group were remarkably lower than those in the over-expression control group ($P < 0.05$), suggesting that over-expression of miR-451 could inhibit cell proliferation and induce apoptosis by regulating the PI3K/AKT/mTOR signaling pathway.

In conclusion, overexpression of miR-451 can inhibit proliferation and promote apoptosis of lung cancer cells. Its relevant action mechanism may be related to the inhibition of targeting inflammation-related genes PSMB8-NOS2, which further regulate the PI3K/AKT/mTOR signaling pathway.

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