# Original Article miR-21 promotes growth, invasion and migration of lung cancer cells by AKT/P-AKT/cleaved-caspase 3/MMP-2/MMP-9 signaling pathway

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**Abstract:** Objective: This study aimed to demonstrate the effects of miR-21 on the growth, migration, and invasion of lung cancer cells A549 in vitro and the possible mechanism. Methods: In vitro cell migration and invasion potential were determined by Transwell chamber assays. FACS was used to assess the effect of miR-21 on A549 cell cycle and apoptosis. 4-6 week-old female mice were utilized to establish a lung cancer model. The pathologic biopsy was processed by H&E staining. The expression of the proteins PTEN, RECK and Caspase 3 were detected through immunohistochemy and tumor cell apoptosis was measured by TUNEL. Results: Transwell chamber assays showed that the cells going through the membrane increased significantly compared to the negative control (P<0.05). The tumor volume resulting from miR-21 mimics was significantly greater than in normal mice. Serum ELISA showed that the protein expression levels of MMP-2 and MMP-9 in miR-21 overexpression group were increased significantly. In addition, H&E staining results showed that in miR-21 overexpression tissue, invasion is more severe and immunohistochemical results proved that the miR-21 overexpression group had high expression of Caspase 3 protein but the expression of PTEN and RECK were decreased. TUNEL experiments show that increased the expression of miR-21 can inhibit the apoptosis of tumor cells. Conclusion: MicroRNA-21 promotes the proliferation of lung cancer cells by the AKT/P-AKT/cleaved-caspase 3/MMP-2/MMP-9 signaling pathway.

Keywords: Lung cancer, miR-21, proliferation, migration, apoptosis, signaling pathway

#### Introduction

Lung cancer remains the leading cause of cancer-related mortality in both males and females. and non-small cell lung cancer (NSCLC) represents the most prevalent lung cancer, which accounts for 85% to 90% of all lung cancers [1-4]. NSCLC has been catalogued into three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [5]. All of the three subtypes indicates poor outcomes, and the 5-year survival rate (17.4%) of lung cancer is much lower than most other leading cancer sites, such as the colon (64.2%), breast (89.4%), and prostate (98.6%) [6]. The high incidence, poor outcomes, and relapses suggest an urgent need for the investigation of lung cancer development, progression, and early biomarkers for rapid, noninvasive, and sensitive detection of recurrent lung tumors, which can lead to prevention strategies.

MicroRNAs (miRNAs) are short (19-22 nucleotides), non-coding RNA molecules that were first identified in Caenorhabditis elegans [7]. They are key regulators of transcription and it has been estimated that miRNAs regulate about 30% of human protein-coding genes [8]. involved in the regulation of a variety of biologic processes, such as differentiation, development, cell cycle and metabolism [9-13]. Importantly, research has reported the roles of miR-NAs in the development of human diseases including neurodegenerative disorders, diabetes, and immune-disorders [13-16]. It has been gradually recognized that they participate in tumorigenesis by regulating the expression of oncogenes and tumor suppressors or by acting as oncogenes and tumor suppressors themselves [17-19], although they are believed to be the biomarkers of tumors [20, 21].

MiRNAs were also reported to be a cause of lung cancer development, progression and metastasis. Takamizawa et al were the first to relate microRNA expression to lung cancer. They reported the reduced expression of let-7 microRNA in human lung cancers and the overexpression of let-7 in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth in vitro [22]. Since then there has been a large number of studies relating microRNA expression to lung cancer. Let-7, miR-126, miR-145, miR-200 and miR-34 were recognized as tumor suppressor microRNAs [23-25]. miR-17-92, miR-21, miR-31, miR-221 and miR-222 were considered to be oncogenic microRNAs [26-29]. Thus, the members of microRNAs superfamily function in different ways in tumor development or progression. Evidence from basic and clinical studies have suggested that miR-21 played a role in promoting proliferation, survival, invasion, and migration in multiple cancer cell lines. But the mechanisms of miR-21 in regulating lung cancer progression have not been fully understood.

In this study, we explored the underlying mechanisms of miR-21 in the development of lung cancer by both in vitro transfection of miR-21 mimics or miR-21 inhibitor and an in vivo metastasis model. In the present study, we show that miR-21 promotes A549 cell proliferation, invasion and metastasis, and suppresses the cell apoptosis by inhibition the expression of PTEN, then activating the PI3K/AKT signaling pathway. miR-21 also promotes tumor invasion and metastasis by inhibiting the expression of RECK, a new matrixmetalloproteinase (MMP) suppressor, then increasing the level of MMP2 and MMP9. Therefore, for the first time, our results disclose the mechanisms by which miR-21 promotes lung cancer metastasis by both the activation of the PI3K/AKT signaling pathway to accelerate tumor cell proliferation, and the inhibition of RECK signaling to increase tumor invasion.

### Materials and methods

### Cell lines and cell culture

The human NSCLC cell line A549 was obtained from ATCC (HTB-38TM, Manassas, VA) and cul-

tured in Dulbecco's modified Eagle's medium/ Nutrient Mixture F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cell cultures were incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### Animals

Nude mice were purchased from Vital River Laboratories (Beijing, China) and maintained in microisolator cages. All animals were used in accordance with institutional guidelines, and the experiments were approved by the Use Committee for Animal Care of Xuzhou Medical University.

### In vivo metastasis model

All cultures used for injection were subconfluent and were fed the day prior to use. The harvested cell suspension was washed twice using the medium containing serum, and then resuspended in medium without serum at 4°C immediately prior to injection. The number of cells to be injected was suspended in 0.1 ml PBS. For intravenous injection of tumor cells, 6-week-old BALB/c nude mice were warmed 10 min away from a 150 W light bulb for 20 to 30 min. Cells were inoculated subcutaneously on the axillary dorsum of the right forelimb in nude mice. The animals were sacrificed 3 weeks after injection. The viscera were removed, and the viscera and carcass were fixed in 10% formalin for subsequent hematoxylin and eosin staining and immunohistochemical analysis.

### Histology and immunohistochemistry

For hematoxylin and eosin staining, tumor samples were fixed in 10% neutral formalin solution for 24 h, dehydrated in increasing concentrations of ethanol, and embedded in paraffin, thereafter, sections of tissue were cut at 3 µm thick. For immunohistochemical staining, paraffin embedded colons sections were deparaffinized, hydrated, and antigen-retrieved, and endogenous peroxidase activity was quenched by 3% H<sub>2</sub>O<sub>2</sub> for 10 min. Sections were then blocked with 5% bovine serum albumin for 20 min, followed by incubation with anti-RECK (sc-9689, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-PTEN (9188, Cell Signaling Technology, Beverly, CA, USA), anti-caspase-3 (9662, Cell Signaling Technology, Beverly, CA, USA)

over night at 4°C. After incubation with secondary antibody for 2 h positive cells were visualized by adding DAB to the sections. Slides were viewed with a Nikon Eclipse 80i microscope equipped with a digital camera (DS-Ri1, Nikon, Shanghai, China).

### TUNEL assay

TUNEL staining was carried out according to the manufacturer's instructions (Promega, Madison, WI). The TUNEL index (%) is the average ratio of the number of TUNEL-positive cells divided by the total number of cells under optical microscopy. For each sample, 10 randomly selected areas of TUNEL-stained slices were counted, and the average values were calculated.

### Transfection

MiR-21 mimics, miR-21 inhibitor, and their negative control oligonucleotides were designed and synthesized by GenePharma (Shanghai, China). A549 cells were seeded in the 6-well plates to 70% confluency in complete medium containing 10% FBS for 24 hours. 15 µl miR-21 mimics (20 µM), miR-21 inhibitor (20 µM) or lipofectamine 2000 were diluted in 500 µl Opti-MEM independently, and then mixed and placed at room temperature to form the complex. Each well was added 1.2 ml Opti-MEM and 800 µl mixture. The transfection reagents were allowed to react with cells for 6 hours, then changed to complete culture medium after washing twice with PBS. After culturing for 48 hours, the cells were obtained for further analysis.

## Cell counting kit 8 (CCK-8) assay

The in vitro effect of miR-21 mimics and miR-21 inhibitor on the viability of A549 cells was determined by CCK8 assay. A549 cells transfected with miR-21 mimics or miR-21 inhibitor were seeded in 96-well plate with a density of  $2 \times 10^3$  ml/well, and to each well was added 100 µl cell medium. Initiation, 12 hour, 24 hour, 48 hour, 72 hour and 96 hour were used as examination times in our investigation. CCK-8 reagent (Beyotime Biotechnology, Shanghai, China) of 10 µl was added to each well with ratio of 1:10 to the medium and then reacted at  $37^{\circ}$ C for 1 hour or 2 hours. Absorbance of each well was read at 450 nm. Each group was averaged from 3 par-

allel wells. The experiment was carried out 3 times.

### RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted using Trizol reagent (cat:15596018, Invitrogen, USA) according to the manufacturer's instruction. A total of 1 µg of total RNA was reverse-transcribed into cDNA. Then, SYBR<sup>®</sup> Green-based quantitative real-time polymerase chain reaction (PCR) was performed with a Bio-Rad CFX 96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH was used as an internal control. For real-time PCR analysis, the relative amount of mRNA or gene to internal control was calculated using the equation  $2^{\Delta CT}$ , in which  $\Delta CT = CT$  gene - CT control.

### Transwell cell invasion assay

Transwell cell invasion assays were performed with BioCoat Matrigel (BD Biosciences, San Jose, CA) and invasion chambers (Millipore, Eschborn, Germany) with an 8 µm pore size according to the manufacturer's instructions. Briefly, Matrigel was added into the upper chamber of Transwell after one night dissolution at 4°C and dilution of 1:3 ratio in serum free medium. After 30 min in the incubator, 1×10<sup>5</sup> transfected cell suspension was seeded into the upper chamber which is serum-free medium. The lower chamber was normal medium containing 10% FCS. The cells were cultured in incubation for 24 hours, embedded with 2% paraformaldehyde for 20 min, and stained by 1% Crystal Violet dye. After washing 3 times with PBS, the cells were examined under the high power lens for a picture. Every sample was calculated under 6 high power fields. The invasion ability was evaluated by the number of cells that penetrated into the matrigel.

# Annexin V/propidium iodide double staining and flow cytometry

Briefly, after transfection, A549 cells were cultured in complete medium containing 10% FBS for different times, then harvested and washed twice, once in PBS and once in Binding Buffer. An Annexin V-FITC Apoptosis Detection Kit (eBioscience, Inc.) was used to detect the



**Figure 1.** miR-21 promoted the proliferation of A549 cell line. In the A549 human lung cancer cells, 48 h and 72 h after the miR-21 transfection, the OD value of the miR-21 mimic group presents an increasing trend compared with other groups, but the OD value of the miR-21 inhibitor group is the lowest in all the groups.

translocation of PS from inner membrane to the outer leaflet of the plasma membrane. Cells were resuspended in Binding Buffer and the concentration was adjusted to  $10^6$ /ml. 5 µL of FITC-conjugated Annexin V was added to 100 µl of the cell suspension. The tubes were gently mixed and incubated for 15 min at room temperature in dark. The unconjugated Annexin V was removed by a wash using Binding Buffer, then 5 µl of Pl was added to 200 µl of the sperm suspension. Flow cytometry analysis was conducted within 2 hours (BD FACSCanto II, BD Biosciences, San Jose, CA), storing at 2-8°C in the dark.

### Flow cytometry analysis of cell cycle

For cell cycle analysis, cells transfected with miR-21 mimics or miR-21 inhibitor were plated in six-well plates at 2×10<sup>5</sup> cells per well for 24 h. The cells were pretreated with 0.3 µM nocodazole (Sigma-Aldrich) for 24 h to synchronize cells at the G2/M boundary. Then, cells were harvested, washed twice with cold PBS, and fixed with cold 70% ethanol overnight at -20°C. The cells were then washed twice with PBS and resuspended with 10 mg/mL RNase A, 400 mg/mL propidium iodide, and 0.1% Triton-X in 1 ml PBS at 37°C for 15 min, and subsequently analyzed by flow cytometry, and DNA content was quantified using ModFit LT software (Verity Software House: Augusta, Topsham, ME, USA).

### Statistical analysis

Statistical analysis of the data was performed using SPSS software (17.0 for Windows, IBM

Inc., Chicago, IL, USA). Comparison between two groups was made using Student's ttest. Comparison among three or more groups was made using one-way ANOVA, followed by the Student-Newman-Keuls test. All data are presented as Mean  $\pm$  SD from at least 3 separated experiments. P<0.05 was considered significant.

#### Results

High expression of miR-21 promoted proliferation of A549 cells

In the A549 human lung cancer cells, 48 h and 72 h after the miR-21 transfection, the OD value of the miR-21 mimic group presents an increasing trend compared with other groups, suggesting that the proliferation capacity of A549 human lung cancer cells with high expression of miR-21 is stronger. On the contrary, the OD value of the miR-21 inhibitor group is the lowest in all the groups, and it was found that the A549 human lung cancer cell proliferation can be inhibited through miR-21 knock-down (**Figure 1**).

# mRNA level of miR-21 in lung cancer cells by q-PCR

As shown in Figure 2, in the A549 human lung cancer cells with high expression of miR-21 in the g-PCR testing, the mRNA levels of PTEN and RECK have a significant decreasing trend compared with the blank group and the no-load control group, while the mRNA level of Caspase 3 represents a significant increasing trend; in the A549 human lung cancer cells with miR-21 knock-down, the mRNA levels of PTEN and RECK have a significant increasing trend compared with the blank group and the no-load control group, while the mRNA level of Caspase 3 represents a decreasing trend. This is basically consistent with the experimental results obtained from western blotting in this project, and also proves that the promotion of proliferation of A549 cells through high expression of miR-21 (the proliferation of A549 cells is inhibited through miR-21 knockdown) may be actually related to the PTEN/Caspase 3 pathway and RECK pathway.



**Figure 2.** miR-21 affected the mRNA expression level of PTEN, RECK and Caspase 3 in A549 cell line. The mRNA levels of PTEN and RECK in A549 human lung cancer cells with miR-21 transfection had a significant decreasing trend compared with the blank group and the no-load control group, while the mRNA level of Caspase 3 represents a significant increasing trend. The mRNA levels of PTEN and RECK had a significant increasing trend in the A549 human lung cancer cells with miR-21 knockdown, compared with the blank group and the no-load control group, while the mRNA level of Caspase 3 represents a significant increasing trend.

# Effect of miR-21 on invasion ability of cancer cells

As shown in **Figure 3**, the invasion ability of the miR-21 high expression group was enhanced compared with the blank group and the no-load control group; on the contrary, the invasion ability of the miR-21 knock-down group was weakened compared with the blank group and the no-load control group.

# Detection of apoptosis and cell cycle in A549 cells after miR-21 transfection

The apoptosis percentages of the blank group, miR-21 high expression plasmid no-load control group, miR-21 high expression group, miR-21 knock-down plasmid no-load control group, and miR-21 knock-down group were 2.706%, 3.133%, 1.257%, 2.929% and 7.267% respectively, suggesting that high expression of miR-21 plays a role in apoptosis inhibition, and on the contrary, the low expression of miR-21 plays a role in apoptosis promotion.

In the cell arrest experiment, the Stage G1 percentages of the blank group, miR-21 high expression plasmid no-load control group, miR-21 high expression group, miR-21 knock-down plasmid no-load control group and miR-21 knock-down group are 48.06%, 47.50%, 47.41%, 55.47% and 68.37% respectively. The Stage G2 percentages are 5.54%, 6.03%, 2.74%, 9.31% and 8.77%, and the Stage S percentages are 46.40%, 46.47%, 49.86%, 35.22% and 22.86% respectively. This suggests that the high expression of miR-21 can enable the Stage G2 cells to be reduced, and the Stage S cells increased, to be able to promote the human lung cancer cell cycle, and facilitate proliferation; while miR-21 knockdown can enable the Stage G1 and Stage G2 cells to be increased, and the Stage S cells to be reduced, to arrest the cells in the Stage G1, and inhibit proliferation of cells.

Effect of miR-21 on tumor formation of lung cancer cells

#### in nude mice

We examined the effect on lung cancer after invivo transfection of miR-21 mimics/inhibitor through the nude mouse tumor transplant experiment. The mice were killed after the lung cancer nude mouse tumor transplant experiment, and the tumor tissue was peeled off, to calculate the volume of the nude mouse transplanted tumor. The results of tumor formation testing are as shown in **Figure 4**. Tumor volume of nude mice in the miR-21 high expression group was significantly higher than nude mice in the control group with the increase of observation days, and the difference had statistical significance (P<0.01). The volume of the nude mouse tumor in the miR-21 knock-down group was significantly less than that in the nude mice in the control group, and the tumor growth rate was significantly slower (P<0.01), which suggests that the high expression of miR-21 of the in vivo level can indeed promote proliferation of cells, and promote growth of tumor, while the miR-21 knockdown can also inhibit growth of tumor.

### MMP2 and MMP9 content in ELISA testing

In the nude mouse serum ELISA testing, we found that the MMP-2 and MMP-9 levels of the



**Figure 3.** miR-21 increased invasion of A549 human lung cancer cells. The invasion of the miR-21 high expression group was enhanced compared with the blank group and the no-load control group; on the contrary, the invasion of the miR-21 knockdown group was weakened compared with the blank group and the no-load control group.



**Figure 4.** Effect of miR-21 on tumor formation of lung cancer cells in nude mice. Tumor volume of nude mice in the miR-21 high expression group was significantly higher than that in the control group with the increase of observation days (P<0.01); the volume of the nude mouse tumor in the miR-21 knockdown group was significantly smaller than that in the control group (P<0.01).

miR-21 high expression group were higher than those of the control group, while the MMP-2 and MMP-9 levels of the miR-21 low expression group were lower than those of the control group. This suggests that MMP-2 and MMP-9 cell signaling pathway may be one of the mechanisms in which miR-21 affects tumor cell growth.

#### PTEN, RECK, and Caspase 3 contents in immunohistochemical testing

The immunohistochemical staining experiment was carried out on the in vivo slices of the formed tumor. The testing results are as shown in **Figure 5**. The PTEN level of the miR-21 high expression group is decreased compared with the control group, while the Caspase 3 level is significantly increased, and the RECK level is significantly decreased; but the PTEN level of the miR-21 interference group is significantly increased compared with the control group. The Caspase 3 level is significantly decreased, while the RECK level has an increasing trend, suggesting that the promotion of tumor formation in nude mice through high expression of miR-21 may be related to the PTEN/Caspase 3 and RECK pathways.

#### Discussion

In recent years, with continuous in-depth research on miR-NAs, extensive experimental research data show that abnormal expression of miRNA plays an important role in occurrence and development of multiple tumor types. Some miR-NAs may become new targets for tumor therapy and progno-

sis markers, which can regulate and control gene expression at the post-transcriptional level through inhibition of translation of the target gene mRNA, or induction of degradation of the target mRNA, and can function as oncogenes or tumor suppression genes, and participate in the occurrence, development, drug resistance, invasion, and metastasis of tumor [30-37].

At present, microRNA-21 shows significant abnormalities in the expression of multiple types of tumor, and participates in the regulation and control of the expression of multiple tumor suppressor genes. Most scholars have considered microRNA-21 as an "onco-microR-NA" similar to an oncogene. By real-time quantitative PCR, gene chip, etc., microRNA-21 has increased expression in multiple types of tumors, such as tissues and cells of leukemia, glioma, prostate cancer, bladder cancer, pancreatic cancer, stomach cancer, colon cancer,



**Figure 5.** miR-21 affected the expression of PTEN, RECK, and Caspase 3 in A549 cell line by immunostaining. Compared to the control group, the expression of PTEN and RECK in miR-21 high expression group were both decreased, while the expression of Caspase 3 was significantly increased. But the expression level of PTEN and RECK of the miR-21 interference group were both significantly increased compared with the control group, while the expression of Caspase 3 was significantly decreased.

and thyroid cancer. Clinical data show that the high expression of microRNA-21 is related to poor prognosis [30-37].

In order to explore the biologic role of miR-21 in lung cancer, we have built the miR-21 overexpression group and the low expression group (A: blank control; B: miR-21 mimic control; C: miR-21 mimic; D: miR-21 inhibitor control; E: miR-21 inhibitor) and subsequently, the mRNA and protein content in the cells are detected through fluorescent quantitative PCR and western blotting. Work on in vitro growth and proliferation, migration and invasion ability, cell cycle and apoptosis, of different cells was done through the CCK8 method, transwell experiments, flow testing, and growth of in vivo transplanted tumor. Results show that the mRNA and protein expression of the miR-21 overexpression group werere significantly increased

compared with the control group; the cell proliferation, migration and invasion ability is significantly enhanced; and the AKT protein level in the cells has no significant change. The phosphorylated AKT level is significantly increased, but PTEN protein level represents a decreasing trend, the Caspase 3 protein level is significantly decreased. The RECK protein level is significantly decreased, and the MMP-9 and MMP-2 protein levels represent a significant increasing trend. The Stage G2 cells of the miR-21 overexpression group are reduced, and the Stage S cells are increased, to be able to promote the human lung cancer cell cycle, and facilitate proliferation, but inhibit apoptosis.

The clinical pathology TNM staging and infiltration depth of lung cancer are closely related to the high expression of micro RNA-21. In order to further discuss the effect of overexpressed miR-21 on in vitro invasion and apoptosis of lung cancer cells A549, in this project, the nude mouse heterotopic implanted tumor model for the human lung cancer A549 cell line was built. A suspension of A549 lung cancer cells in the logarithmic growth stage was vaccinated to the subcutaneous part of the axillary region of the right fore-limb of the nude mouse, to observe the changes of ectopic implanted lung cancer in animals and protein expression of signaling pathways of PTEN/Caspase 3, RECK/MMP-2/ MMP-9, etc. The results showed that the volume of the formed tumor in mice of the overexpression group was greater and the tumor formation and growth rate was significantly quickened compared with the blank control group. H&E staining showed that the tissue invasion of the miR-21 overexpression tumor was severe. By ELISA, the nude mouse serum MMP-2 and MMP-9 protein expression levels showed that the MMP2 and MMP9 contents in the miR-21. overexpression group were significantly increased. PTEN and RECK contents in the immunohistochemical testing tissue were reduced, the Caspase 3 content was increased, and by the TUNEL testing, the tumor cell apoptosis could be inhibited through high expression of miR-21. This means that the growth and proliferation of in vivo cells can be significantly promoted by overexpression of miR-21. The in vivo and in vitro experimental results prove that miR-21 plays a role of a proto-oncogene in lung cancer.

In recent years, attention has been paid to research on the correlation between miRNA and lung cancer. Studies show that miRNA is closely related to the occurrence and development of the non-small-cell lung cancer, miR-21 is expected to be a new biomarker for the diagnosis and treatment of lung cancer and prognosis, but the regulation and control of tumor through miRNA is a very complicated process, so it is not enough to explain the possible mechanism of miR-21 in lung cancer through the confirmation of target gene, and there is still a lot of work to be done.

#### Disclosure of conflict of interest

None.

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