MIR-19A PARTICIPATES IN PROLIFERATION AND METASTASIS OF NON-SMALL CELL LUNG CANCER CELLS BY REGULATING SMAD4

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ABSTRACT

Objective: *This study was designed to probe into the miR-19a expression in non-small cell lung cancer (NSCLC), and to discuss its function and molecular mechanism.*

Methods: *Thirty-five cases of NSCLC and corresponding normal lung tissues were collected, and the miR-19a relative expression was tested via qRT-PCR. miR-19a-inhibitor, miR-NC and sh-Smad4 were transfected into A549 cells. The miR-19a and Smad4 expression levels were tested by qRT-PCR. Proliferation changes of cells after transfection were assessed by CCK8 test. Cell migration and apoptosis were tested via flow cytometry. The relationship between miR-27a and Smad4 was confirmed via dual-luciferase report. The expression changes of caspase-3 and caspase-9 in transfected cells were detected via Western Blot (WB).*

Results: *The miR-19a expression in NSCLC tissues was obviously higher than that in normal tissues, while the Smad4 expression in lung cancer (LC) tissues was remarkably lower than that in normal ones (P<0.05). Proliferation, migration and invasion of A549 cells were dramatically inhibited and apoptosis was promoted after being transfected with miR-19a-inhibitor. While cell proliferation, migration and invasion were remarkably accelerated and apoptosis was inhibited after being transfected with sh-Smad4. miR-19a was confirmed as a Smad4 target site via dual-luciferase report.*

Conclusion: *The miR-19a expression is up-regulated in NSCLC tissues, which may advance cell proliferation and metastasis by down-regulating the Smad4 expression.*

Keywords: *Non-small cell lung cancer, miR-19a, Smad4, proliferation.*

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Introduction

Lung cancer (LC) is the most familiar tumor in the world[1]. The survey results in 2016 show that there are more than 200,000 new LC patients in the United States alone every year[2]. In China, its morbidity is also rising rapidly, and non-small cell LC (NSCLC) accounts for about 80%[3]. Due to slow progression, the symptoms are hidden and atypical. Therefore, more than 40% were in advanced stage when they were first discovered[4]. For advanced LC, comprehensive treatment mode is generally adopted, including radiochemotherapy, targeted therapy and tumor immunotherapy[5-7]. However, due to tumor drug resistance, off-target effect, tumor microenvironment, immunosuppression and cytokine storm, the efficacy is still not ideal[8]. Thus, it is particularly important to explore new treatment modes. microRNAs (miRNAs) are small non-coding RNA, which can degrade the mRNA of the target gene or induce translation inhibition by incompletely combining with the 3' untranslated region (3'UTR), so as to regulate its expression[9]. A large amount of evidence shows that the expression disorder of miRNAs is related to tumor development and progression. Some miRNAs may be involved in

blocking the cancer process, so they can be used as potential therapeutic targets for cancer[10, 11].

Previous studies have shown that miR-19a is abnormally expressed in various malignancies and takes part in the formation, development and metastasis of cancer[12]. Smad4 is considered as a tumor suppressor gene. Studies have confirmed that Smad4 is lost in pancreatic cancer and other diseases[13, 14]. Ke et al.[15] found that the Smad4 expression in NSCLC tissues was higher than that in normal ones, and it is relevant to lymphatic metastasis. In addition, Smad4 could also inhibit the expression of vascular endothelial growth factor and thrombospondin-1 (TSP-1), which indicated that it might inhibit the metastasis of NSCLC tissues by inhibiting angiogenesis. However, the role and molecular mechanism of miR-19a on Smad4 are vague. Hence, we consider NSCLC A549 cells as a model to probe into the role and molecular mechanism of miR-490-3p, thus providing theoretical basis for finding new therapeutic targets clinically.

Materials and methods

General data

From March 2017 to December 2019, lung lesions and corresponding normal lung tissue specimens (more than 5 cm away from LC tissues) of NSCLC patients were collected, 35 cases in total. All patients were pathologically confirmed as NSCLC. Among them, there were 14 males and 21 females: 6 cases in stage I, 15 in stage II and 14 in stage III; 17 cases of adenocarcinoma, 16 of squamous cell carcinoma and 2 of large cell carcinoma. All of them did not receive any radiotherapy and chemotherapy or molecular targeted therapy before operation, and they all underwent radical surgery. The specimens removed by surgery were stored in liquid nitrogen promptly, and then stored at -80°C refrigerator. The whole collection and storage process was operated according to the non-enzymatic principle.

Main instruments and reagents

Human LC drug-resistant cell line A549 was bought from Shanghai Cell Research Institute, RPMI1640 medium, fetal bovine serum (FBS), trypsin, Lipofectamine®3000, plasmid extraction kit and Trizol reagent were bought from Thermo Fisher Scientific, penicillin-streptomycin mixture was purchased from Sigma, and pGL3 luciferase detection system was purchased from Promega. The fluorescence quantitative kit One Step SYBR® PrimeScript™ RT-PCR Kit was originated from Dalian TaKaRa Biotech Co., Ltd., and the PCR primers were synthesized by Nanjing Genscript Biotechnology Co., Ltd. Rabbit anti-GAPDH, Smad4 antibodies were all purchased from Abcam, SDS/PAGE electrophoresis gel mixture and ECL chromogenic reagent were all purchased from Beyotime Biotechnology, and AnnexinV, FITC/PI apoptosis detection kits were purchased from Nanjing KeyGen Biotech Co., Ltd.

Model 5804R refrigerated desktop centrifuge was purchased from Eppendorf, Germany. Gel electrophoresis instrument, vertical electrophoresis tank and gel imaging system were purchased from Bio-Rad, USA. NanoDrop2000 UV spectrophotometer was bought from Thermo, USA. ABI7300 real-time fluorescence quantitative PCR instrument was bought from Applied Biosystems, USA.

Cell culture and treatment

A549 cells were cultivated in RPMI1640 medium containing streptomycin 100 mg/ml, 10% FBS and penicillin 100 U/ml, and placed in 5% CO2 incubator at 37°C. The culture medium was changed once every two days. After the cells grow to 70%-80%, they were digested with trypsin and then went down to posterity. One day before transfection, the cells were inoculated in a 6-well plate until the cell density reached 80%. miR-19a-inhibitor (inhibition group) and its control vector (miR-NC group) were transfected into A549 cells by Lipofectamine® 3000 transfection kit. Forty-eight hours later, they were digested and collected by trypsin for subsequent experiments. Simultaneously, untransfected A549 cells were enrolled in control group.

RNA extraction and reverse transcription real-time quantitative PCR detection

NSCLC and paracancerous tissues were ground sufficiently, added with Trizol, total RNA was extracted according to Trizol extraction steps, and miR-138 was extracted based on miRNA extraction kit steps. A549 was attached to a 6-well plate, and when the cell density reached 90%, total RNA and miRNA were extracted respectively in the light of the above steps. cDNA was reverse transcripted according to the cDNA first strand synthesis kit, and RT-qPCR detection was performed in view of SYBR Green Mix PCR kit and miRNAPCR kit respectively. miR-19a primer was ordered from Ribo Bio (upstream primer sequence: 5'-AGUUUUGCAUAGUUGCAC-UACA-3', downstream primer sequence: 5'-GCT-

CACTGCAACCTCCTC-3') and internal reference U6 primer (upstream primer sequence: 5'-GCT-TCGGCAGCACATATACTAAAAT-3', downstream primer sequence: 5'-CGCTTCACGAATTTGCGT-GTCAT-3'), which was detected by real-time PCR and amplified by American ABIStepOne real-time quantitative PCR instrument. The total volume of PCR reaction system was 20 μL, which contained 2×miR qPCR Mix (With SYBR Green) 10 μL, Forward Primer 0.4 μL, Reverse Primer 0.4 μL, miR 0.5 μL, and the rest was added with ddH_2O.

Three complex holes were set for each test. Real-time quantitative PCR reaction conditions were as follows: pre-denaturation at 94°C for 30 min, denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s, 40 cycles in total. The miR-19a relative expression in each group was calculated by $2^{-\Delta\Delta Ct}$ method.

Detection of cell proliferation rate

Cell proliferation rate was tested via Cell Counting Kit-8: Twenty-four hours after transfection, 3,000 cells in each group were inserted into the 96-well plate. After they were able to grow adhering to the wall, 10 μl/well CellCountingKit-8 was added at 24, 48, 72, 96 h respectively, and the rate was tested by checking the absorbance at 450 nm.

Detection of cell migration and invasion rate

Transwell test: A549 cells were inoculated into the upper chamber of Transwell plate with matrix membrane at a density of 50×10^3 after being transfected for 24 h, containing 300 μl serum-free DMEM medium. Then, 500 μl DMEM medium containing 10% FBS was placed in the lower chamber. After cells were incubated for 24 h, those passing through the bottom surface of the upper chamber were immobilized with 4% paraformaldehyde. After they were stained with 0.5% crystal violet, the invasion was observed with a microscope.

WB detection

The total protein in transfected cells was extracted and the protein content was determined by BCA method. Protein and 5×loading buffer were mixed at the ratio of 5: 1, and then boiled for 5 min, loading 10 μl per well. Next, it was transferred to PDVF membrane by electrophoresis on SDS-polyacrylamide gel, and PDVF membrane was sealed 2 h with 5% skimmed milk powder. Rabbit anti-human Smad4 antibody (1: 1000) and internal reference β-actin (1: 3000) were added and incubated at 4°C all night. The membrane was cleaned 10 min with TBST, three times in total, incubated 2 h with goat anti-rabbit secondary antibody (1: 4000) at indoor temperature, then cleaned three times with TBST, each time for 10 min, and developed with luminescent liquid. The protein relative expression was represented by gray value of target protein band/gray value of internal reference band.

Apoptosis is detected by flow cytometry

Transfected A549 cells were inoculated into a 6-well plate about 1×10^6 cells per well.

After 48 h, the cells were digested with trypsin and collected. After cells were washed with PBS solution in view of the instructions of AnnexinV-FITC/PI double staining kit, 5 μL AnnexinV and 10 μL PI were added, and they were stained for 15 min, and then 400 μL diluent was added to mix them evenly. All apoptosis rates were detected and analyzed via flow cytometry.

Dual-luciferase reporter gene experiment

miR-19a's potential target was predicted via Targetscan7.2, and the 3'-UTR sequence of the corresponding gene was inserted into the multiple cloning site of pmirGLO vector.

The constructed vector and miR-19a mimics, miR-NC-NC were co-transfected into A549 cells with LipofectAMINE3000, and the activities of firefly luciferase and renilla luciferase were tested via dual-luciferase activity detection kit after 48 h-transfection. The relative luciferase activity was expressed as firefly luciferase activity value/sea kidney luciferase activity value.

Statistical analysis

The collected data were statistically analyzed via SPSS 22.0, and pictures were drawn via GraphPad Prism6.0. The data were expressed as mean±standard deviation (x±s). The comparison of two sample means was conducted by t test, and that of means among multiple samples was conducted by One-way ANOVA. $P<0.05$ was remarkably different.

Results

miR-19a and Smad4 levels in NSCLC tissues

qPCR results showed that the miR-19a level in 30 NSCLC tissues was 2.762±0.565, which was higher than that in paracancerous tissues (1.355±0.325), while the Smad4 expression was 0.692±0.0.213, which was lower than that in paracancerous tissues

(1.204±0.193), and the difference was statistically remarkable (P<0.05).

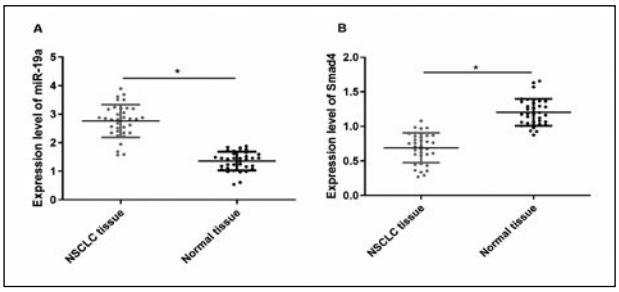

Figure 1: miR-19a and Smad4 expression levels in NSCLC and normal tissues.

*A: The miR-19a expression in NSCLC tissues is dramatically higher than that in adjacent normal ones (P<0.05). B: The Smad4 expression in NSCLC is dramatically lower than that in adjacent normal tissues (P<0.05). Note: *means P<0.05.*

miR-19a's effect on biological function of A549 cells

Compared with the negative control group, the proliferation, migration and invasion of A549 cells transfected with miR-19a-inhibitor decreased obviously, the apoptosis rate increased markedly, and the contents of caspase-3 and caspase-9 increased dramatically (P<0.05). (Figure 2).

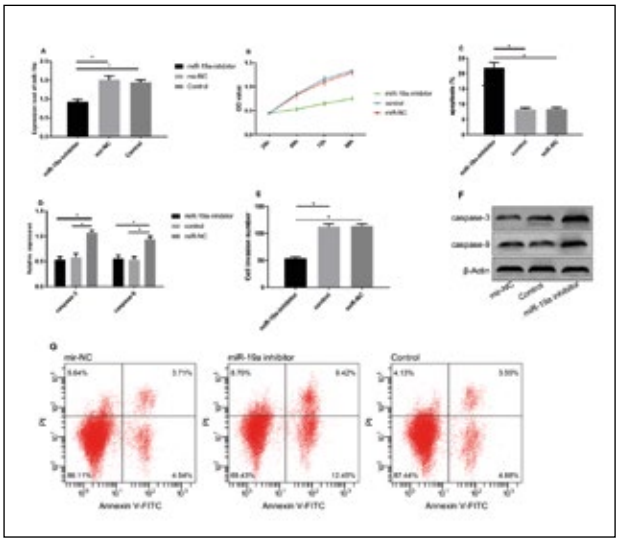

Figure 2: miR-19a's effect on biological function of A549 cells.

*A: Compared with sh-NC and blank control group, the miR-19a-3p expression in A549 cells transfected with miR-19a-inhibitor is markedly down-regulated. B: The proliferation of A549 cells transfected with miR-19a-inhibitors after culture is markedly lower than that of sh-NC group and blank control group. C: The apoptosis rate of A549 cells transfected with miR-19a-inhibitors after culture is markedly higher than that of sh-NC group and blank control group. D: The expression of caspase-3 and caspase-9 in A549 cells transfected with miR-19a-inhibitors is up-regulated after culture. E: The migration of A549 cells transfected with miR-19a-inhibitors after culture is markedly lower than that of sh-NC group and blank control group. F: WB. G: Flow cytometry. Note: *means P<0.05.*

Effect of Smad4 on biological function of A549 cells

The Smad4 expression in A549 cells transfected with sh-Smad4 and miR-NC was markedly higher than those transfected with NC (P<0.05). Compared with NC, the proliferation and invasion of transfected sh-Smad4 cells increased remarkably, and the apoptosis rate decreased markedly (P<0.05). The contents of caspase-3 and caspase-9 in cells transfected with NC decreased markedly after sh-Smad4 transfection (P<0.05). (Figure 3).

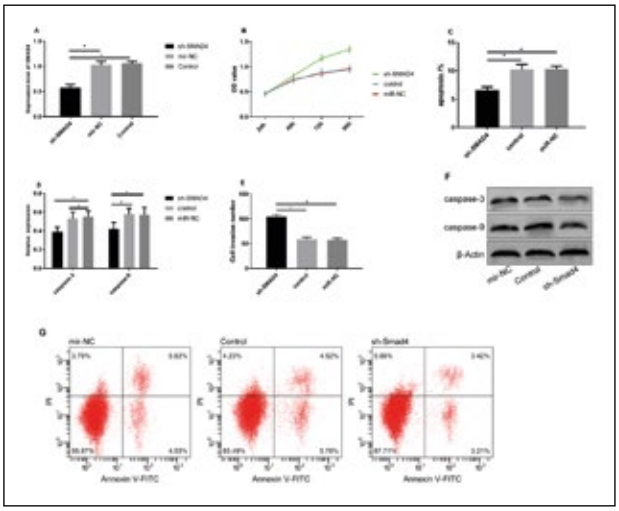

Figure 3: Effect of Smad4 on biological function of A549 cells.

*A: Compared with the cells transfected with sh-NC and blank control group, the Smad4 expression in A549 cells is remarkably down-regulated. B: The proliferation of A549 cells transfected with sh-Smad4 after culture is obviously higher than that of sh-NC group and blank control group. C: The apoptosis rate of A549 cells transfected with sh-Smad4 after culture is obviously lower than that of sh-NC group and blank control group. D: The expression of caspase-9 and caspase-3 in A549 cells transfected with sh-Smad4 is down-regulated after culture. E: The migration of A549 cells transfected with sh-Smad4 after culture is obviously higher than that of sh-NC group and blank control group. F: WB. G: Flow cytometry. Note: *means P<0.05.*

miR-19a gene identification

To probe into the molecular mechanism of miR-19a inhibition in the biological progress of NSCLC cells, miR-19a's downstream target gene was searched through Targetscan website, and miR-19a and Smad4's target binding sites were found. Further detection of dual-luciferase activity showed that over-expressing miR-19a remarkably decreased the luciferase activity of Smad4-WT-3'UTR, but could not inhibit Smad4-MUT-3'UTR. WB identified that the Smad4 protein expression in A549 cells increased markedly after miR-19a-inhibitor transfection, while the expression decreased obviously after miR-19a-3p-minicis transfection. (Figure 4).

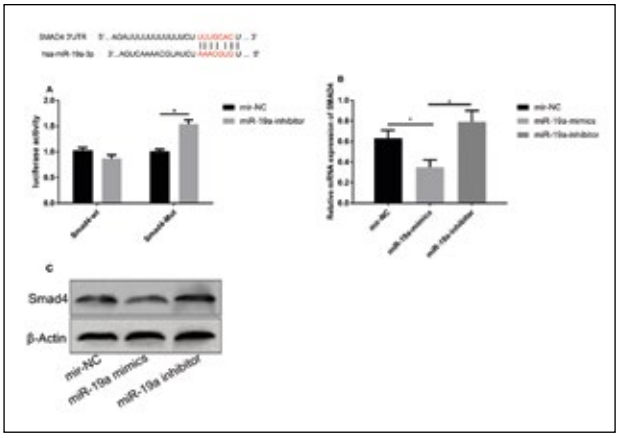

Figure 4: Identification of miR-19a gene.
*A: The luciferase activity experiment reveals that miR-19a over-expression markedly decreases the luciferase activity of Smad4-WT-3'UTR, but can not inhibit Smad4-MUT-3'UTR. B: The Smad4 protein expression in A549 cells increases markedly after miR-19a-inhibitor transfection, but decreases obviously after miR-19a-3p-minicis transfection. C: WB. Note: *means P<0.05.*

Discussion

For decades, with the development of new targeted drugs and immunotherapy, the treatment methods of LC have become more and more diversified, and the efficacy has been improved remarkably. However, the overall prognosis of LC patients is still not optimistic, and the 5-year survival rate is only 15%[15, 16]. Hence, it is necessary to probe into NSCLC's mechanism and find new molecular markers and drug targets to improve the efficacy further[17]. After being binding with the 3' untranslated region of the target gene, miRNA could degrade the target mRNA or inhibit its translation, thus negatively regulating the target gene, adjusting the post-transcriptional level, widely participating in the physiological processes such as proliferation, differentiation and apoptosis of organ development cells, and it also related to the biological behavior and characteristics of complex tumors[18-20]. miRNA-19a belongs to miRNA-17-92 family[21]. Previous studies have shown that it plays a vital role in the development of various cancers. Lin et al.[22] verified that miR-19a expression was low in LC tissues, and its abnormal low expression suggested that the prognosis of patients was poor.

Other studies have shown that miR-19a can promote cell invasion and metastasis by promoting epithelial-mesenchymal transition (EMT) of NSCLC[23]. Smad4 belongs to the signal transduction protein family and is a tumor suppressor gene. Smad4 inactivation has been reported in many types of malignancies including NSCLC[24,25]. Chae et al.[26] found that the Smad4 expression in LC cells was dramatically lower than that in normal lung epithelial cells. While, we tested miR-19a, Smad4 in NSCLC and adjacent normal tissues, and found that the miR-19a expression in NSCLC tissues was remarkably higher than that in paracancerous ones, while the Smad4 expression in LC tissues was remarkably lower than that in paracancerous ones ($P<0.05$). This was similar to the previous research results, which indicated that miR-19a and Smad4 were abnormally expressed in NSCLC, and they might be involved in its occurrence and development. Then, we introduced miR-19a-inhibitor into A549 cells of NSCLC. CCK8, cell scratch and Transwell test found that the proliferation, migration and invasion of A549 cells transfected with miR-19a-inhibitor decreased markedly, the apoptosis rate increased markedly, and the contents of caspase-3, caspase-9 increased dramatically ($P<0.05$).

Compared with NC, the proliferation and invasion of cells transfected with sh-Smad4 increased markedly, and the apoptosis rate decreased markedly ($P<0.05$). After cells were transfected with NC and sh-Smad4, the contents of caspase-3 and caspase-9 reduced markedly ($P<0.05$). It manifested that inhibiting miR-19a could significantly inhibit the proliferation, migration and invasion of A549 cells and boost apoptosis, while inhibiting Smad4 could promote their proliferation, migration and invasion and inhibit apoptosis. It suggested that miR-19a and Smad4 might be a potential target for NSCLC treatment. To find out the specific way that miR-19a and Smad4 affect the biological function of NSCLC cells, we verified the targeted relationship between them. Through the online target gene prediction website (http://www.targetscan.org/vert_72/), we found that miR-19a and Smad4 had binding targets, and Smad4 gene might be the target gene regulated via miR-19a. Therefore, we further proved the regulatory relationship between miR-19a and Smad4 by dual-luciferase report.

The experiment proved that miR-19a over-expression reduced the luciferase activity of Smad4-WT-3'UTR obviously, but could not inhibit Smad4-mut-3'UTR. The Smad4 protein expression in A549 cells increased obviously after miR-19a-inhibitor transfection but decreased dramatically after miR-19a-3p-minicis transfection. The results indicated that miR-19a had a targeted regulation relationship with Smad4, that is, inhibiting miR-19a could increase Smad4 expression, thereby

reducing proliferation and invasion of NSCLC cells and increasing apoptosis rate. We proved that miR-19a could regulate the proliferation and apoptosis of NSCLC cells by targeting Smad4, but whether it could regulate LC cell progression through other signal factors needs further study in subsequent experiments.

To sum up, miR-19a can inhibit proliferation, migration and invasion of NSCLC cells by directly targeting Smad4, which provides an effective target for molecular therapy.

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