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Effect of miR-27a-3p on Proliferation and Migration in Non-small Cell Lung Cancer Cells

Zheng Rong-Rong[1], Wu Shao-Feng[2], Jin Xiang-Feng[1], Liu Yu-Hong[1]

[1]The Affiliated Hospital of Qingdao University, 266003, Qingdao, Shandong, China
[2]Medical College of Qingdao University, 266071, Qingdao, Shandong, China

Abstract: Objective To investigate the expression of miR-27a-3p in tumor tissue of patients with non-small cell lung cancer (NSCLC) and explore the effect of miRNA-27a-3p expression on invasion and migration of lung cancer cells. **Methods** The tumor tissues of 21 NSCLC patients and corresponding adjacent normal tissues were collected for this study and the differences of miR-27a-3p expression levels in tumor tissues and adjacent tissues were detected by real-time fluorescence quantitative PCR. The expression of miR-27a-3p in A549 cells was overexpressed and silenced by transfecting miR-27a-3p mimics and miR-27a-3p inhibitor. And the ability of proliferation and migration of A549 cells after transfecting were detected by MTT and cell scratch assaies. **Results** The expression levels of miR-27a-3p in tumor tissues of NSCLC patients were significantly higher than adjacent non-cancerous tissues. The proliferation and migration ability of A549 cells were enhanced significantly after overexpression of miR-27a-3p. The silencing of miR-27a-3p expression in A549 cells produces the opposite effect. **Conclusion** Compared with normal tissues, NSCLC tissues have high expression of miR-27a-3p. Overexpression of miR-27a-3p enhanced the proliferation and migration ability of lung cancer cells. Therefore, miR-27a-3p may be an important regulatory factor in the development of NSCLC.

Keywords: Non-small-cell lung cancer, MiR-27a-3p, Cell proliferation

Introduction

Lung cancer was the malignant tumors with the highest morbidity and mortality around the world (Reck et al., 2017). According to the WHO classification criteria in 2015, the classification of lung cancer pathologically includes small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the predominant type of lung cancer, accounting for 85% of all lung cancers (Reck et al., 2017; Siegel et al., 2017). Due to the lack of effective early diagnosis of lung cancer, it was always found at advanced stage. Furthermore, the overall 5-year survival rate was only up to 15% (Zhang et al., 2016). MicroRNAs (miRNAs) are a class of small (19-24 nucleotides in length) non-coding RNAs which are high conserved . MiRNAs always regulate the degradation or inhibit the translation of miRNAs by binding to specific target mRNAs, thereby depressing the expression of related target gene proteins (Si et al., 2017). Bioinformatics data indicated that a single miRNA can target the function of multiple mRNAs. Therefore, miRNAs are involved in the regulation of various biological activities including development, virus defense, hematopoiesis, organ formation, cell proliferation and apoptosis, fat metabolism, and the

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Liu Yu-Hong (Correspondence)
qdlyuhong @ sohu.com
13963925289

development of tumors (Shen et al., 2008). The relationship between miRNA and tumor has become one of the hot spots in the research of many scientists at present. Different miRNAs may exert stimulative or inhibitory function, which depended on the means of miRNAs, target or cellular environment (Feng et al., 2015). Calin et al demonstrated that deletion or down-regulation of miR-15a and miR-16-1 in the 13q14 region was associated with B-cell chronic lymphocytic leukemia (Calin et al., 2002). Seol et al reported that miR-373 was down-regulated by histone modification in lung cancer cells and inhibited tumor growth by down-regulating IRAK2 and LAMP1 (Seol et al., 2014). Zhou et al discovered that both miR-27a-5p and miR-27-3p were highly expressed in gastric cancer tissues and cell lines, but the expression level of miR-27a-3p was significantly higher than that of miR-27a-5p (Zhou et al., 2016). Further study showed that miR-27a-3p, a mature subtype of miR-27a, can promote the proliferation of gastric cancer cells. At present, it has not been reported that the expression level of miR-27a-3p, the major subtype of miR-27a, in NSCLC patients. And the effect of miR-27a-3p on the biological function of lung cancer cells is not clear. This study was aim to demonstrated the expression of miRNA-27a-3p in NSCLC tissues and cells and detected the effect of miR-27a-3p overexpression or silencing on proliferation and migration of NSCLC cells.

1 Materials and methods

1.1 Clinical information

A total of 21 fresh tumour samples and paired normal tissue samples from NSCLC patients who underwent complete surgical resection in the Affiliated Hospital of Qingdao University were collected from May 2017 to September 2017. All patients had pathologically diagnosed as NSCLC, and did not performed preoperative adjuvant radiotherapy and chemotherapy. All collected clinical specimens were immediately frozen in liquid nitrogen and then frozen in a -80 ° C freezer.

This study was approved by the Affiliated Hospital of Qingdao University Ethics Committee and informed consent was obtained from all subjects.

1.2 Cell culture and reagents

Human lung cancer cell line A549 cell line was purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Biological Industries, Israel. A549 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) with 100 μg/ml penicillin /streptomycin at 37°C with 5% CO_2. MiR-27a-3p mimics, miR-27a-3p inhibitor and theirs corresponding negative controls were designed and synthesized by GenePharma (Shanghai, China) . The PrimeScript ™ RT reagent Kit and the qRT-PCR kit were purchased from TaKaRa, Japan. The MTT kit was purchased from Shanghai Bailey Biotech Co Ltd. Lipofectamine 2000 and RNA extraction trizol were purchased from Life Technologies (Carlsbad, CA, USA).

1.3 RNA extraction and qRT–PCR

According to the instructions of RNA extraction kit, the total RNA of the cancer tissue, corresponding para-cancerous tissue and the A549 cells were extracted with the method of TRIzol. Total RNA was used as the template for reversing transcribe RNA into cDNA adopting miRNA reverse transcription kit. In accordance with qRT-PCR Kit instructions for cDNA amplification, the U6 was used as the internal control for measuring the relative expression of miR-27a-3p. The reaction conditions included an initial step at 95 ° C for 5 min followed by 40 cycles of 95 ° C for 30 s, 58 ° C for 30 s and 72 ° C for 30 s. The primers were listed as followed: miR-27a-3p, F: 5'-CGCCGTTCACAGTGGCTAAG-3' and R: 5'-GTGCAGGGTCCGAGGT-3'; U6, F: 5'-CTCGCTTCGGCAGCACA-3' and R: 5'-AACGCTTCACGAATTTGCGT -3'. The expression levels of miR-27a-3p and U6 were mesured by the threshold period (Ct). Relative expression levels were assessed using the $2^{-\Delta\Delta Ct}$ method. The experiment was

repeated three times.

1.4 Cell proliferation assay

After digestion and centrifugation, A549 cells were resuspended in 1640 medium containing 10% fetal bovine serum. The cells were seeded into 96-well plates (5×10^3 cells/well) directly at 37°C with 5% CO_2 .Then the control group, miR-27a-3p mimics group, miR-27a-3p mimics control group, miR-27a-3p inhibitor group and miR-27a-3p inhibitor control group were duvided. After incubation for 6 h, the cells were replaced with serum-free medium and transfected with miR-27a-3p mimics, miR-27a-3p inhibitor and theirs corresponding negative controls. Then the cells were continued to incubating at 37°C with 5% CO_2. The cells were incubated for 24 h, 48 h, 72 h and 96 h, respectively. After that, A549 cells were incubated with MTT (5 mg/ml) for another 4 h at 37 °C. Finally, 150 μl DMSO was added to each well to resolve the formazan, and the absorbance values at 490 nm were measured. The experimental group and negative control group were set 6 holes, and the experiment repeated 3 times.

1.5 Wound healing assay

A549 cells were seeded into 6-well plates (1×10^5/ well) .The control group, miR-27a-3p mimics group, miR-27a-3p mimics control group, miR-27a-3p inhibitor group and miR-27a-3p inhibitor control group were set up. After transfection for 24 h, the cells were scratched with 200 μl Tip tips and gently swabbed with PBS to remove the scraped cells. The images of cells were taken at 0 h and 24 h after scratched, respectively. Image Pro Plus6 was used to analyze the images and calculated the migration distance.

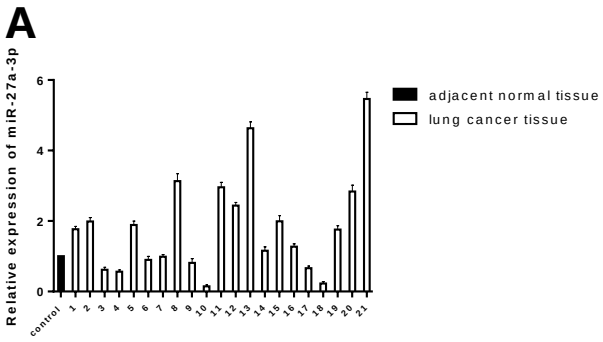
1.6 Statistical analysis

All data were presented as mean±SD and analyzed by Graphpad 6.0 statistical software. The one-way variance analysis were used to determine the differences between groups of tests, and the two groups were compared using t test. $P < 0.05$ was considered statistically significant.

2 Result

2.1 MiR-27a-3p expression in NSCLC tissues and adjacent tissues

Quantitative PCR results showed that the expression of miR-27a-3p was up-regulated (T / N> 1) in 13 of 21 NSCLC tissues (61.90%) compared with matched paracancerous tissues, of which 11 (52.38%) samples showed high expression obviously (T / N> 1.5). No significant changes were found for the miR-27a-3p expression among the three samples (14.29%) of NSCLC samples, while another five samples (23.81%) showed that the expression of miR-27a-3p was down-regulated (Fig. 1A). The comparative analysis showed that the average expression level of miR-27a-3p in NSCLC tissues was significantly higher than that in adjacent non-cancerous tissues ($P < 0.01$) (Fig. 1B). We selected PCR products numbered 8, 13, and 21 for gel electrophoresis. The result of PCR gel electrophoresis showed that the expression levels of miR-27a-3p in NSCLC tissue samples were significantly higher than that in the paracancerous tissues (Fig. 1C).



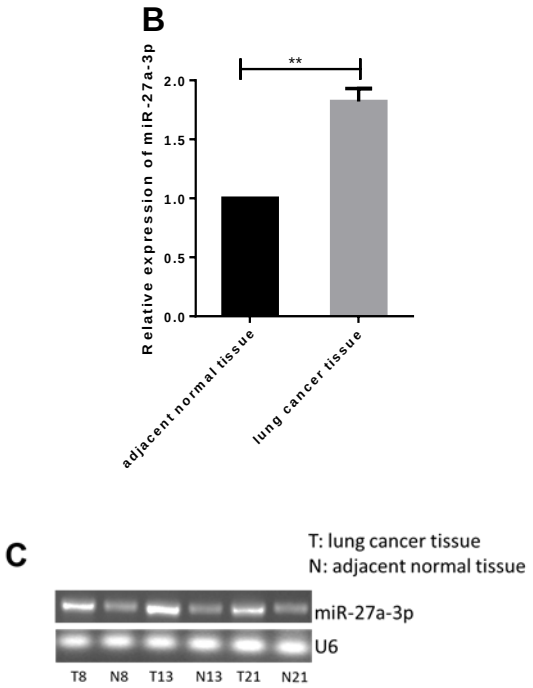


Fig.1 MiR-27a-3p expression levels in human NSCLC tissues and adjacent tissues. **A**. The expression level of miR-27a-3p in 21 NSCLC tissues and corresponding paracancerous tissues was detected by Real-time quantitative PCR (the expression was set to 1 in all paracancerous tissues, and the expression level in cancer tissues was relative to that in adjacent tissues). **B**. miR-27a-3p expression levels in NSCLC tissues and adjacent tissues. **P<0.01 (n=3). **C**. Electropherogram of miR-27a-3p PCR product in NSCLC tissue and paracancerous tissue.

2.2 MiR-27a-3p expression in the A549 cells

The expression levels of miR-27a-3p of A549 cells after transfection were detected by quantitative PCR (Fig. 2). We found that miR-27a-3p expression were significantly increased in cells transfected with miR-27a-3p mimics compared with control group (Fig. 2A), whereas the expression levels of miR-27a-3p in cells transfected with miR-27a-3p inhibitor were obviously decreased compared with control group (Fig. 2B). The difference was statistically significant (P <0.01). This suggested that transfection with miR-27a-3p mimics or inhibitor could achieve overexpression or silencing of miR-27a-3p levels in A549 cells.

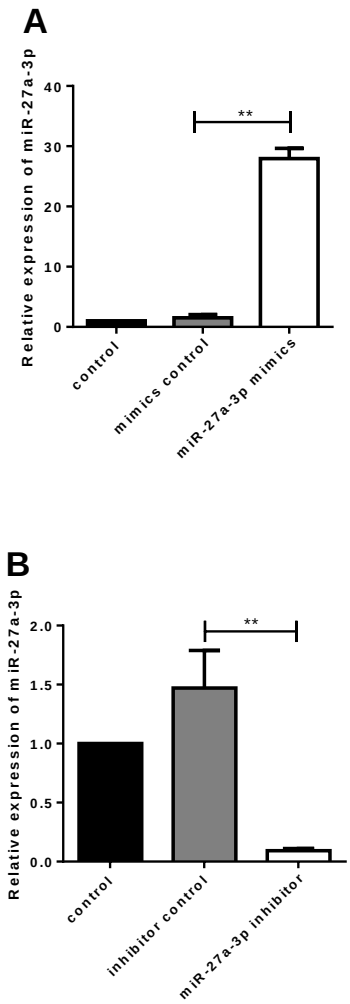


Fig.2 The expression of miR-27a-3p in A549 cells after transfection. **A**. miR-27a-3p expression levels in A549 cells after transfection with miR-27a-3p mimics and their controls. **B**. Expression levels of miR-27a-3p in A549 cells after transfection with miR-27a-3p inhibitor and its control. **P＜0.01（n=3）。

2.3 Effect of miR-27a-3p on Proliferation of A549 Cells

In order to investigate the effect of miR-27a-3p on cell proliferation, we detected the proliferation ability of cells after transfection at different time points by MTT assay. In comparison with the control group, the proliferation ability of miR-27a-3p mimics group was significantly increased at 48 h, 72 h and 96 h (Fig. 3A), which was statistically significant (P <0.01). The

proliferation ability of miR-27a-3p inhibitor group was significantly decreased (Fig. 3B), which was statistically significant as well (P <0.05). The results suggested that the expression level of miR-27a-3p was positively correlated with the proliferation of A549 cells.

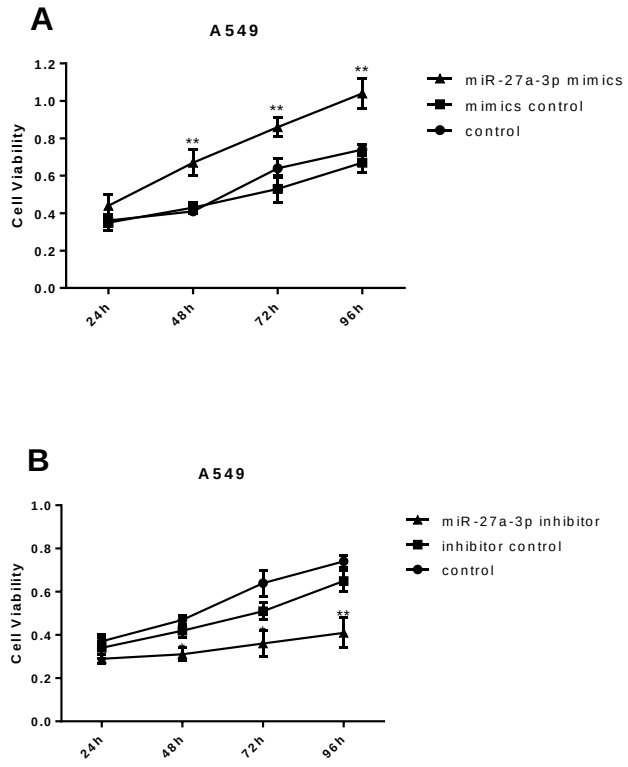


Fig.3 miR-27a-3p promoted the proliferation of A549 cells. **A**. A549 cells were transfected with miR-27a-3p mimics and its negative control, and MTT assay was performed at different time points. **B**. A549 cells were transfected with miR-27a-3p inhibitor and its negative control, and MTT assay was performed at different time points. *P＜0.05（n=3）; ** P＜0.01（n=3）。

2.4 Effect of miR-27a-3p on Migration of A549 Cells

We then investigated the role of miR-27a-3p in the regulation of invasion of A549 cells. Cell wound healing assay was used to examine the migration. And the results displayed that the percentage of healing area in the miR-27a-3p mimics group was significantly increased (P <0.01) (Fig. 4A). Cells transfected with miR-27a-3p showed a significant decreased percentage of the healing area for the scratched cells, which was statistically significant. (Fig. 4B). The migration rate of A549 cells was significantly increased after overexpression of miR-27a-3p. The silencing of miR-27a-3p significantly inhibited the migration of A549 cells. These data suggested an promotional effect of miR-27a-3p in A549 cell migration.

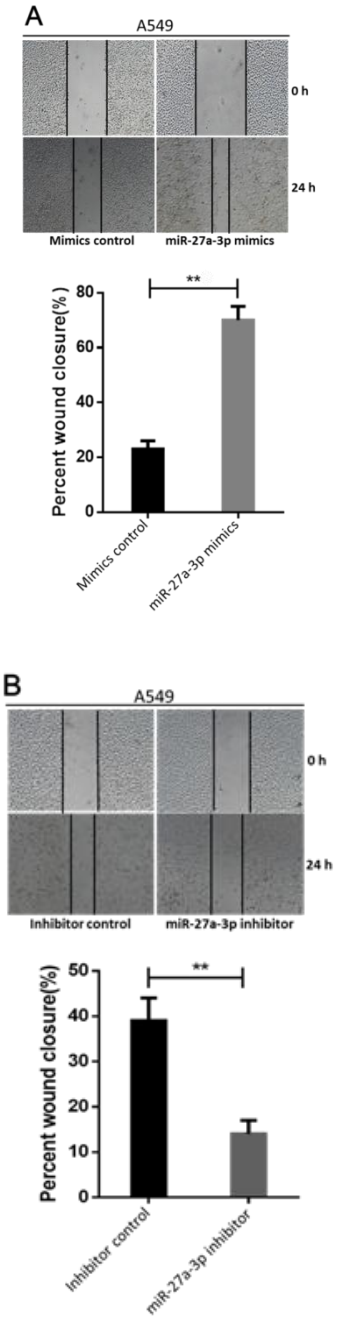


Fig.4 miR-27a-3p promoted the migration of A549 cells in vitro. **A**. The wound healing assay revealed that miR-27a-3p promoted the migration of A549 cells.

Overexpression of miR-27a-3p using mimics promoted wound gap closure obviously. **B**. MiR-27a-3p down-regulation delayed wound gap closure. **P＜ 0.01（n=3）。

Discussion

Lung cancer was the most common malignancy throughout the world. For the lack of effective diagnostic methods at the early stages of lung cancer, most patients were diagnosed at an advanced stage. Accordingly, it was extremely urgent to further investigate the underlying molecular mechanism of lung cancer and explore the molecular targets that could be used for early diagnosis and treatment. In recent years, a large number of miRNAs have been found to be associated with lung cancer through high-throughput miRNA microarray screening and the like. Nevertheless, the function and molecular mechanism of these miRNAs in the development and progression of lung cancer still need to be further developed. The dysregulation or dysfunction of miRNAs may lead to the development of various diseases including tumors. A growing number of studies had shown that miRNAs were dysregulated in human tumors. MiRNAs were involved in the proliferation, apoptosis and differentiation of tumor cells by promoting tumor or tumor suppressor functions (Shen et al., 2014; Chen et al., 2012; Christensen et al., 2014; Li et al., 2014). Hatley et al found that miR-21 overexpression could promote the development of lung cancer (Hatley et al., 2010). Campayo et al discovered that miR-145 inhibits the development of lung cancer (Campayo et al., 2013). Therefore, understanding the expression of miRNAs in tumors and their effects on the biological functions of tumor cells is of great value in exploit cancer treatment strategies. This study aimed to demomstrated the expression of miRNA-27a-3p in NSCLC, as well as the effect of miR-27a-3p expression on the proliferation and migration of lung cancer cells by construct miR-27a-3p overexpression or silencing A549 cell line.

Studies had shown that miR-27a was highly expressed in a variety of cancers containing pancreatic cancer, breast cancer, ovarian cancer, esophageal cancer, renal cell carcinoma, lung cancer and glioma, and was closely related to the biological behavior of cancer cells (Zhou et al., 2016). Tang et al displayed that high expression of miR-27a were in relationship with clinical stage and overall survival time of breast cancer patients (Tang et al., 2012). It has been suggestedthat the overexpression of miR-27a-3p was in correlation with cancer metastasis and invasiveness in oral squamous cell carcinoma and renal cell carcinoma (Qiao et al., 2017; Nakata et al., 2015). Yet studies have also shown that miR-27a-3p expression was significantly suppressed in colorectal cancer and esophageal squamous cell carcinoma, and that miR-27a-3p has tumor suppressive effects in these cancers (Gao et al., 2015; Jiang et al., 2015). These above studies shown that the expression and biological effects of miR-27a-3p in tumors werestill in dispute currently. To investigate the expression levels of miR-27a-3p in NSCLC, we selected tumor tissue and paracancerous tissues from 21 NSCLC patients and found that 13 of them (61.90%) showed high expression for miR-27a-3p, while 5 sample (23.81%) showed low expression. Comparative analysis showed that the average expression level of miR-27a-3p was significantly increased in NSCLC tissues, demonstrating that miR-27a-3p was highly expressed in NSCLC. In this study, the difference of miR-27a-3p expression levels may be related to the pathological type or stage of NSCLC. Therefore, the lack of this study was the small number of cases and the relationship between the expression of miR-27a-3p and the pathological type or staging of NSCLC was not analyzed. In the future, we will expand the sample size and therefore further investigation should be performed.

The mechanism of miR-27a-3p has not been fully studied and multiple downstream targets have been identified. It was found that over-expression of miR-27a-3p in gastric cancer cells can reduce the expression of BTG2 protein and promote the activation

of C-myc through the Ras / MEK / ERK pathway in gastric cancer cells, thereby inhibiting the apoptosis of gastric cancer cells (Zhou et al., 2016). Li et al. confirmed that miR-27a-3p was down-regulated in hepatocellular carcinoma. The cell experiments confirmed that miR-27a-3p expression could down-regulate the expression of DUSP16 in hepatocellular carcinoma to inhibit the growth, migration and invasive ability of cancer cells (Li et al., 2017). In this study, we confirmed that the biological function of miR-27a-3p in lung cancer cells by the overexpression and silencing of miR-27a-3p expression. The molecular mechanism of miR-27a-3p functioning in NSCLC remains to be further studied.

In this study, we found that miR-27a-3p, the major subtype of miR-27a, was significantly elevated in NSCLC tissue samples compared to normal tissues adjacent. This suggested that the up-regulation of miR-27a-3p played an important role in the development of NSCLC. We further up-regulated or silenced miR-27a-3p expression in A549 cells by transfection with miR-27a-3p mimics or miR-27a-3p inhibitor, and observed the changes in cell proliferation and migration after transfection. We found that up-regulation of miR-27a-3p can significantly enhanced the proliferation and migration of tumor cells, suggesting that miR-27a-3p could significantly promote lung cancer cell growth and invasion. In contrast, down-regulation of miR-27a-3p significantly reduced the cell's proliferation and migration capacity. This suggested that miR-27a-3p plays an important role in the invasion and metastasis of NSCLC, but the mechanism of miR-27a-3p that regulating the proliferation and migration of lung cancer cells needs further study. The proliferation and invasive ability of lung cancer cells was an important factor in the rapid progression of lung cancer. Our data showed for the first time that miR-27a-3p was up-regulated in NSCLC and can enhance the proliferation and invasion ability of lung cancer cells.

Therefore, the miR-27a-3p, the major subtype of miR-27a, was an oncogene that played an important role in the development of NSCLC and may provide a potential therapeutic target for the treatment of lung cancer. This study was limited to the detection of clinical samples and cell functions. The molecular mechanism of miR-27a-3p in NSCLC needs further investigation.

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