Original Article
MiR-27a down-regulating expression of ZNF139 and inhibiting invasion and migration of lung cancer cells by Rho-ROCK signaling pathway

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Received September 26, 2016; Accepted November 11, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Objective: To investigate the expression of miR-27a in lung cancer tissues at different pathological stages, the effects of miR-27a on invasion, migration and proliferation of lung cancer cells and the related mechanism. Methods: The expression of miR-27a in lung cancer tissues at different pathological stages was measured by qPCR; The overexpression of miR-27a was achieved by miR-27a-mimic and detected by qPCR; luciferase reporter gene was used to detect the interaction between miR-27a and ZNF139; the expression of ZNF139, RhoA and ROCK1 was determined by Western blotting in lung cancer cells treated with miR-27a-mimic; Transwell invasion assay and wound scratch assay were used to detect the effect of miR-27a on the invasion and migration ability; the subcutaneous tumor formation experiment in nude mice was used to detect the effects of miR-27a-mimic on the growth of tumor; and immunohistochemistry was used to measure the expression of ZNF139 and RhoA. Results: the expression of miR-27a was significantly reduced in lung cancer, and it decreased with increase in pathological stages of the disease; the expression of miR-27a increased gradually with decrease in degree of differentiation; and it decreased significantly in lung cancer tissues with lymph node metastasis. miR-27a-mimic could significantly up-regulate the expression of miR-27a; Besides, ZNF139 was the direct target of miR-27a, and miR-27a could down-regulate the expression of ZNF139, RhoA and ROCK1. Moreover, miR-27a could inhibit the invasion and migration of human lung cancer A549 cells. Finally, the in vivo experiment demonstrated that tumor volume and weight were significantly reduced by miR-27a-mimic, and the expression of ZNF139 and RhoA were also decreased significantly by miR-27a-mimic. Conclusion: miR-27a inhibits the invasion and migration of lung cancer by targeting ZNF139 and may through suppressing RhoA/ROCK1 pathway. These results suggest the anti-tumor role of miR-27a in lung cancer.

Keywords: miR-27a, lung cancer, ZNF139, RhoA, ROCK1, invasion, migration

Introduction

Lung cancer is one of the prevalent malignancies in clinical practice. In China, the incidence of lung cancer has been increasing year by year, and it is the top one malignancy in terms of morbidity and mortality [1]. Currently, there is no evident symptom in the early stage of lung cancer, cells are highly aggressive and metastatic. Thus, most of the patients are at advanced stages at diagnosis with multiple metastasis. Tumor recurrence and metastasis are the main reasons for death of patients with lung cancer [2]. The exact mechanism of occurrence, development and metastasis of the disease is not clear at present. Therefore, searching for the molecular target related to and establishing the molecular biological mechanism of tumor metastasis remains the focus in lung cancer treatment.

MiRNA (microRNA) is a class of small RNA molecules widespread in eukaryotes. By inhibiting the functions of ribosome, degrading 5'cap structure and deadenylated poly tail, miRNA negatively regulates mRNA translation [3]. MiRNAs are widely involved in regulation of gene expression, cell cycle and organism development, etc. [4]. Many studies have shown that miRNA is closely associated with a variety of tumors and related with tumor invasion and metastasis, such as breast cancer, hepatoma,
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Colon cancer and lung cancer, etc. [5, 6]. MiR-27a belongs to the evolutionarily highly conserved miRNA family. Many studies have revealed that miR-27a is closely associated with malignant tumors, but there is divergence on whether miR-27a is a tumor promoter or a tumor suppressor. For instance, the expression of miR-27a is significantly increased in cancers of the stomach, pancreas and colon etc., and it is regarded as a tumor promoter [7, 8], but the expression of miR-27a is decreased in melanoma, prostate cancer and oral squamous cell carcinoma, and it is considered as a tumor suppressor [9, 10].

Zinc finger proteins (ZNFs) are transcription factors widely present in eukaryotes, mainly involved in regulation of physiological processes including cell proliferation, differentiation and apoptosis. The study by Jen et al. [11] demonstrated that ZNF139 promoted metastasis of liver cancer by enhancing the invasion and migration of the cancer cells. The study by Jiang et al. [12] indicated that the expression of ZNF139 increased significantly in colon cancer, and down-regulating ZNF139 could inhibit the proliferation and apoptosis abilities of the cancer cells.

This study aims to investigate the expression level of miR-27a in lung cancer tissues and explore the effects of miR-27a on the invasion and migration abilities of the cancer cells and its mechanism.

Materials and methods

Collection and handling of clinical samples

Samples were collected from 115 patients with lung cancer treated in our hospital between September 2014 to November 2015, including 73 males and 42 females aged 64.41±5.31 years. None of the patients received chemotherapy or radiotherapy before surgery. The post-surgical pathological stage of all patients was determined by two pathologists with secondary senior positions or above after reading the images. According to the TNM staging system for lung cancer, there were 43 patients at stage I, 23 at stage II, 40 at stage III, and 9 at stage IV; there were 29 patients with poor differentiation, 36 with moderate differentiation, and 50 with well differentiation. Lymph node metastasis was seen in 48 patients and not in 67 patients. The tumor isolated tissues were divided into two pieces: one was rapidly put into the RNA stock solution, and the other was rinsed using the cold phosphate buffer treated by diethylypyrocarbonate (DEPC) to remove the blood stain and then rapidly put into the liquid nitrogen for cryopreservation.

Cell line and main reagents

The human lung cancer cell line A549 was purchased from ATCC. Cell culture conditions: cultured in RPMI DMEM containing 10% fetal bovine serum at 37°C, 5% CO₂. The fetal bovine serum was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. and RPMI 1640 medium were purchased from Gibco. Primary antibodies ZNF139, RhoA and ROCK1 were from CST. Transwell chambers were obtained from Corning; and Matrigel gel was from BD (US). Nude mice aged 6 weeks were supplied by Laboratory Animal Center, Academy of Military Medical Sciences. Universal immunohistochemical kit was purchased from Beijing Zhongshan Golden Bridge Biotech Co., Ltd. Trizol was purchased from Ambion (US), the reverse transcription kit FSQ-101 from TOYOBIO (Japan), and the PCR kit from Kapa (US). The liposome Lipofectamine 2000 and miR-27a-mimic were purchased from Shanghai Genechem Co., Ltd. The assay kit for luciferase activity was obtained from Promega, which synthesized luciferase reporter vector.

Cell transfection

The A549 cells at the logarithmic growth phase were cultured and transfected with miR-27a-mimic and the negative control according to the instructions of the Lipofectamine 2000 transfection kit. After transfection, the expression of miR-27a was detected by qPCR. After transfection, culture was continued for subsequent experiment.

Transwell invasion assay

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 µl (0.2 µg/µl) Matrigel gel was evenly applied to inner membrane of Transwell chamber, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, the cells were diluted with 2.5×10^4/mL serum-free medium to prepare cell suspen-
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Figure 1. The expression level of miR-27a was measured by qPCR in lung cancer tissues at different pathological stages. Data are represented as the mean ± SD of three experiments. *P<0.05 versus Stage I group.

sion; the cell suspension was added to the upper Transwell chamber at 200 μL each well, and 500 μL of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with formalin, stained by crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields (×40). The experiment was performed in triplicate.

Wound scratch assay

The A549 cells were inoculated into a 6-well plate, scratch from up to bottom using a 200 μl sterile pipette tip, when cell confluence reached 90%; Measure the initial distance of scratch (0 time); the distances of scratch were measured respectively and photographed at 24 h, 48 h and 72 h the cell migration rate were calculated. The experiment was performed in triplicate.

Western blotting

The proteins were extracted from the lung cancer cells transfected with miR-27a-mimic and miR-27a-NC, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 10% SDS-PAGE was prepared, and 20 μg protein sample was transferred to a PVDF membrane, and sealed for 2 h with 5% skim milk, the primary antibodies (ZNF139, RhoA and ROCK1) were diluted 1:600 with TBST overnight at 4°C; then secondary antibody was added for 1:5000 dilution, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

Detection of luciferase activity

The A549 cells were co-transfected with luciferase reporter vector and miR-27a-mimics, and cellsttransfected with pRL-TK were used as standard internal QC. After transfection for 36 h, the cells were harvested. Luciferase activity in the A549 cells was detected according to the instructions of Promega kit. Calculation method: relative luciferase activity = value of firefly luciferase activity/value of Renilla luciferase activity. The experiment was performed in triplicate.

NSCLC xenografts

Lung cancer cells in the logarithmic growth phase in the miR-27a-NC and miR-27a-mimic groups were taken and digested in culture flasks, cell concentration was adjusted to 2×10⁶ cells/ml. to observe subcutaneous tumor growth daily, 0.1 ml of cell suspension was injected subcutaneously into each nude mouse (10 in total) by the left forelimb armpit. One week after inoculation, the tumor grew to about 5–6 mm. The mice were divided into 2 groups, 5 in each group. The animals were sacrificed 28 days after inoculation of tumor cells. The tumor diameter (a) and the vertical orthogonal diameter (b) of tumor-bearing mice were measured in each group. The tumor size V (mm³) = a×b²/2; and the weight was measured. The tumor tissues were fixed in 4% paraformaldehyde.

immunohistochemistry

The tumor tissues were embedded in paraffin and cut into sections of 4 μm in thickness. The immunohistochemical operations were performed according to the immunohistochemical S-P kit (Beijing Zhongshan Golden Bridge Biotech Co., Ltd.): dewaxing of the tissues and then hydrating, Antigen retrieval was performed in microwave with citrate buffer solution for 30 min, cooled down to room temperature, then washed 3 times with PBS, 3 min each time, incubated with 3% H₂O₂ at 37°C for 15 min, then washed 3 times with PBS, 3 min each time, then the primary antibody was placed
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Table 1. Relationship between miR-27a expression and clinical pathological characteristics of lung cancer

<table>
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<th>Low expression of miR-27a</th>
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Results

The expression level of miR-27a was decreased in lung cancer tissues

The results of qPCR (Figure 1) showed that the expression level of miR-27a mRNA was decreased [(1.41±0.31) vs (0.98±0.17) vs (0.49±0.07) vs (0.13±0.02), P<0.05], with statistically significant differences, as lung cancer increased in pathological stage.

The level of miR-27a was related to the pathological stage of lung cancer and lymph node metastasis

It was indicated in statistical analysis (Table 1) that the expression level of miR-27a decreased with increase in pathological stage of lung cancer; it decreased gradually as the degree of differentiation decreased; it decreased significantly in lung cancer tissues with lymph node metastasis; and it was not related to age or sex. The results suggested that the level of miR-27a was related to the pathological stage of lung cancer and lymph node metastasis, and not related to sex or age.

The expression of miR-27a increased in A549 cells transfected with miR-27a-mimic

After the trasfection of miR-27a-mimic into the A549 cells for 48 h, the results (Figure 2) showed that compared to the NC group, the expression of miR-27a was significantly increased in the miR-27a-mimic group [(0.22±0.03) vs (0.89±0.13), P<0.01]. The results suggested that miR-27a-mimic could significantly increase the expression level of miR-27a.

ZNF139 is a target of miR-27a

To clarify whether miR-27a is able to bind to ZNF139 3'UTR, miR-27a-mimic, ZNF139-Wt and ZNF139-Mut were co-transfected into A549 cells. The results showed that miR-27a-mimic could significantly inhibit luciferase activity of ZNF139-Wt (Figure 3); and miR-
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27a-mimic had no remarkable inhibitory effect on the luciferase activity of ZNF139-Mut, suggesting specific binding between miR-27a and ZNF139 3’ UTR.

Figure 2. miR-27a-mimic increased the level of miR-27a. A. Transfection efficiency detected by GFP fluorescence; B. The expression of miR-27a mRNA was measured by qPCR after transfection with miR-27a-mimic. Error bars represent standard error. *P<0.05 versus NC group.

Figure 3. ZNF139 is the direct target of miR-27a. Error bars represent standard error. *P<0.05 versus NC group.
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MiR-27a-mimic inhibiting the expression of ZNF139, RhoA and ROCK1

Western blotting analysis (Figure 4) showed that compared to the NC group, the expression levels of ZNF139, RhoA and ROCK1 were decreased significantly in the miR-27a-mimic group [ZNF139 (81.4±6.8) vs (21.7±2.1); RhoA (91.6±27.3) vs (26.6±3.6), ROCK1 (88.5±7.2) vs (30.9±2.3), P<0.05]. These results suggested that miR-27a-mimic could down-regulate the expression of ZNF139, RhoA and ROCK1, indicating that the RhoA/ROCK1 signaling pathway was involved in the function of miR-27a in A549 cells.

MiR-27a-mimic inhibiting the invasion of A549 cells

The Transwell results (Figure 5) showed that the number of cells passing through the Matrigel gel was 201.57±6.76 in the NC group, significantly more than that in the miR-27a-mimic group (23.92±2.88) (P<0.01), suggesting that miR-27a could inhibit the invasion ability of the human lung cancer A549 cells.

MiR-27a-mimic inhibiting the migration of A549 cells

The width of scratches in each group was measured under a microscope at the time points of
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The migration rate was calculated according to the formula: Migration rate = (D(t = 24 h, 48 h, 72 h) - D(t = 0 h)) / D(t = 0 h).

The results (Figure 6) displayed that compared to the NC group, migration rate in the miR-27a-mimic group was reduced at 24 h, 48 h and 72 h [24 h (0.18±0.02) vs (0.36±0.05), P<0.05; 48 h (0.35±0.05) vs (0.69±0.07), P<0.05; 72 h (0.42±0.05)% vs (0.91±0.08)%]. It was revealed that miR-27a could inhibit the migration ability of A549 cells.

Subcutaneous tumor formation experiment in nude mice indicated that miR-27a-mimic affected tumor growth

The survival time of the tumor-bearing mice was 4-8 weeks, with a median of 6 weeks. The autopsy showed that tumor grew in the left armpit, and the tumor was offwhite, solid, round or oval, with nodular projection on the surface and fish-like sections; and the tumor formation rate was 100%. Tumor growth in nude mice (Figure 7A): The tumor size in the miR-27a-mimic group was significantly reduced compared with that in the NC group.

Compared to the NC group, the tumor volume and weight of the mice were decreased significantly in the miR-27a-mimic group [volume (2.83±0.13) cm³ vs (0.36±0.02) cm³, P<0.05; weight (2.49±0.15) g vs (0.28±0.03) g, P<0.05] (Figure 7B and 7C).

The immunohistochemical (IHC) results (Figure 7D) showed that compared to the NC group, the expression of ZNF139 and RhoA was decreased significantly in the miR-27a-mimic group.

Discussion

MiRNA is a gene regulatory factor, which inhibits the translation of target proteins by binding to the target mRNA, and it also directly participates in occurrence, development, invasion,
metastasis, drug resistance and other biological behaviors of tumors [13]. MiRNAs act as a tumor suppressor as well as a tumor promoter. MiRNA with significantly increased expression
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in tumor plays a cancer promoting effect. On the contrary, the miRNA with significantly decreased expression in tumor plays a tumor suppressing effect [14].

Human miR-27 is located at chromosome 19q13.12, which is classified into miR-27a and miR-27b. Many studies have shown that miR-27a is closely associated with tumor, playing a role of promoting or suppressing tumor growth [15]. Toden et al. [16] investigated the expression of miR-27a in colon cancer tissues and found that it was significantly reduced in the tissues, and miR-27a could play a tumor-suppressing effect by down-regulating Smad2 and STAT3. The study by Zhou et al. [17] has revealed that the expression of miR-27a was significantly increased in breast cancer, and its reduction could lower the risk of breast cancer. In this study, we observed that the expression of miR-27a was reduced with increase in pathological stages of the disease; with decrease in degree of differentiation, the expression decreased gradually; and in the cancer tissues with lymph node metastasis, the expression of miR-27a was significantly reduced, suggesting that miR-27a played a tumor-suppressing effect in lung cancer.

The Zinc finger protein ZNF139 belongs to the Kruppel family, located at chromosome 7q21.3-q22.1. The C-terminal of ZNF includes C2H2 zinc finger motifs which specifically bind to target gene regulatory regions or target proteins. The N-terminal includes SCAN and KRAB domains, which was used to regulate target genes or activity of target proteins [18]. Many studies have demonstrated that ZNF played an important role in tumor occurrence, development and drug resistance. The study by Nie et al. [19] have detected strongly positive expression of ZNF139 in gastric cancer and high expression in paracancer normal gastric mucosa in the proliferative phase, suggesting that ZNF139 was involved in occurrence of gastric cancer. The study by Baoping et al. [20] have indicated that the expression of ZNF139 was significantly higher in the tissues of endometrial carcinoma than that in normal endometrial tissues, and the high expression of ZNF139 was closely related to tumor staging and lymph node metastasis. In this study, by overexpression of miR-27a with miRNA-mimic, the expression of ZNF139 was reduced gradually, and the invasion and migration abilities of lung cancer cells were decreased significantly. By detecting the interaction between miR-27a and ZNF139 using a luciferase reporter gene, we found that miR-27a could bind to ZNF139 directly, suggesting that ZNF139 was involved in regulation of the invasion and migration abilities of lung cancer cells by miR-27a.

ROCK is serine-threonine protein kinase, with two intracellular structures: ROCK-1 and ROCK-2 [21]. The Rho/ROCK signaling pathway plays an essential part in regulation of cell morphology, reconstruction of cytoskeleton and cell migration by regulating the movement of actin. In invasion and migration of tumor cells, changes in cell adhesion ability, reconstruction of cytoskeleton and polarization of motion direction were all closely related to the Rho/ROCK signaling pathway [22]. It was revealed that invasiveness of hepatic cancer cells was strengthened after overexpression of ROCK and weakened after silencing ROCK; it also decreased significantly when Y-27632 (an inhibitor of ROCK) was added, suggesting an important role of ROCK in invasion of hepatic cancer cells [23]. Similar studies on tumors like ovary cancer and urinary bladder cancer also demonstrated that Rho/ROCK had effects in tumor invasion and migration. In this study, the invasion and migration abilities of lung cancer cells were weakened after overexpression of miR-27a, and the expression of RhoA and ROCK1 was decreased remarkably, indicating that a possible involvement of the Rho/ROCK-1 signaling pathway in regulation of invasion and migration of lung cancer cells.

In conclusion, our study found that the expression level of miR-27a was decreased in lung cancer tissues, and the level of miR-27a was related to the pathological stage of lung cancer and lymph node metastasis with no relation to sex or age. Besides, ZNF139 is a target of miR-27a and miR-27a-mimic suppressed the expression of ZNF139, RhoA and ROCK1. Moreover, miR-27a-mimic restrained the invasion and migration of A549 cells. Finally, the NSCLC xenografts experiments offered in vivo evidence that miR-27a-mimic inhibited tumor growth and the expression of ZNF139 and RhoA. Taken together, our research indicated that miR-27a inhibits the invasion and migration abilities of lung cancer cells through...
the Rho/ROCK signaling pathway by targeting ZNF139.

Disclosure of conflict of interest

None.

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