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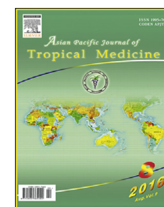

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Effect and molecular mechanism of mir-146a on proliferation of lung cancer cells by targeting and regulating MIF gene

Wu-Ming Wang[1,2], Ji-Chun Liu[3*]

[1]*Medical College of Nanchang University, Nanchang 330006, Jiangxi Province, China*

[2]*Department of Thoracic Surgery, Jiangxi Chest Hospital, Nanchang 330006, Jiangxi Province, China*

[3]*Department of Thoracic & Cardiovascular Surgery, The First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China*

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ABSTRACT

Objective: To discuss the effect and molecular mechanism of miR-146a on the proliferation of lung cancer cells by targeting and regulating the macrophage migration inhibitory factor (MIF) gene.

Methods: RT-PCR was employed to detect expression of miR-146a; immunohistochemistry was used to detect the expression of MIF. The luciferase reporter gene technique was adopted to verify that MIF was the specific reverse target gene of miR-146a and the liposome Lipofectamine™2000 was employed to transfer the modeled miR-146a mimics, and miR-146a negative control (NC) in NSCLC cells to detect the expression of MIF mRNA and protein. MTT assay was used to detect cell viability, cloning technique to detect cell proliferation ability, AnnexinV-PI to detect cell apoptosis, UV spectrophotometry to detect viability of cysteinyl aspartate specific proteinase 3 (Caspase 3), and western blot to detect expression of nuclear factor-κB (NF-κB) in cells.

Results: The expression of miR-146a in NSCLC lung tissues was lower than that in the normal lung tissues besides the lung cancer; while the expression of miR-146a in NSCLC cells was lower than that in normal human embryonic lung tissues. It was chosen as the subsequent cell line for its appropriate expression in A549. The expression of MIF protein in NSCLC lung tissues was higher than that in the normal lung tissues besides the lung cancer. The luciferase reporter gene proved that MIF was the reverse target gene of miR-146a. The miR-146a mimics were transfected into A549 cells through the liposome. Compared with NC group, the expression of MIF protein and mRNA was significantly decreased ($P < 0.01$), with the decrease in the cell viability ($P < 0.01$), the decrease in the number of clones ($P < 0.01$), cell apoptosis ($P < 0.01$), the increase in the activity of Caspase 3 ($P < 0.01$), and decrease in the phosphorylation of NF-κB p65 ($P < 0.01$).

Conclusions: miR-146a has low expression in NSCLC tissues and cell lines, while MIF has the over expression in NSCLC tissues. The increased expression of miR-146a can inhibit the expression of MIF via the gene targeting and thus inhibit the proliferation of A549 cells and induce the apoptosis of cancer cells, which may be realized through NF-κB signaling pathway.

1. Introduction

Lung cancer is a common malignant tumor in the respiratory tract, and the disease caused the greatest harm to human health in the 21st century. The lung cancer can be divided into 2 types according to the pathological pattern, namely the small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), where the latter one occupies about 85% of the lung cancer [1,2]. NSCLC can be further divided into the squamous cell carcinoma, adenocarcinoma and large cell lung cancer according to the histological type. Presently, the treatment of lung cancer is mainly surgery, accompanying comprehensive treatments of chemotherapy, radiotherapy and targeted therapy, but the five-year survival rate was only 15% [3,4]. It is always the terminal stage when the lung cancer is diagnosed, as there

*Corresponding author: Ji-Chun Liu, Department of Thoracic & Cardiovascular Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi Province, China.
Tel: +86 13907913502
E-mail: liujichun2213@163.com
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are no obvious symptoms in the early stage. Therefore, to identify markers for early diagnosis of lung cancer will be helpful for the treatment of lung cancer.

A great number of researches have proved that MicroRNA (miRNAs) had the abnormal expression in many tumors and had the close relationship with the occurrence and development of many tumors such as the lung cancer [5–7]. Where, miR-146a is located at LOC285628 gene of No. 5 chromosome and the mature sequence is in the second exon. According to the previous researches, the expression of miR-146a in patients with NSCLC was lower than that in patients with the benign lung cancer and its expression in the lung cancers was lower than that in normal lung cells. The low expression of miR-146a was closely related to the high TNM staging, lymph node metastasis and low survival rate [8–10]. It indicated that there was close relationship between miR-146a and the occurrence and development of NSCLC, its specific mechanism has been unknown yet. The macrophage migration inhibitory factor (MIF) is some kind of highly conserved protein from T-cell, which can also be secreted by the monocytes/macrophages and anterior pituitary cells. Many researches reported that MIF was involved in the occurrence and development of lung cancer, especially NSCLC. Kamimura *et al* [11] adopted the immunohistochemistry to study the overexpression of MIF in the lung tissues of patients with NSCLC, which was clearly related to the prognosis. Liu *et al* [12] and Howard *et al* [13] also reported that the expression of MIF in lung cancer tissues was significantly higher than that in the paracarcinoma tissues, which indicated that miR-146a and MIF had the abnormal expression in NSCLC. It is assumed that MIF might be one of reverse target proteins of miR-146a. Therefore, on that basis, RT-PCR and immunohistochemistry were employed to detect the expression of miR-146a and MIF in NSCLC lung tissues, respectively. The luciferase reporter gene was used to analyze whether MIF is the reverse target gene of miR-146a. Furthermore, miR-146a mimics was transfected into NSCLC cells through the liposome to discuss its effect on the expression of MIF and cell proliferation in NSCLC cells and its specific mechanism as well.

2. Materials and methods

2.1. Materials

Twenty samples of NSCLC lung tissues and 20 samples of normal lung tissues besides the lung cancer were collected from patients who received the surgery in Jiangxi Chest Hospital from July 2014 to December 2014, including 12 cases with the lymph node metastasis and 8 cases without the lymph node metastasis. Patients were clinically staged according to 2009 UICC TNM Staging System, including 13 cases of squamous cell carcinoma and 7 cases of adenocarcinoma; TEM staging: 14 cases at Ⅰ–Ⅱ stage and 6 cases at Ⅲ–Ⅳ stage. Patients were not given the chemotherapy or radiotherapy before the operation, but they were diagnosed pathologically.

2.2. Reagents and instruments

NSCLC cells HCC827, A549, NCI-H1650, NCI-H258 and human embryonic lung cells WI-38 were purchased from Cell Bank, Chinese Academy of Sciences, with the item No. of TCHu153, TCHu150, TCHu152, TCHu151 and GNHu42 respectively; the rabbit anti-NF-κB p65 and pp6 monoclonal antibody from Epitmics; the rabbit anti-MIF monoclonal antibody from Cell Signaling Technology; the methyl thiazolyl tetrazolium from Gibco; the fetal bovine serum, RPMI-1640 medium from Hyclone; Caspase 3 activity assay kit from Nanjing KeyGen Biotech Co., Ltd.; the trizol, RT-PCR kit, wild-type MIF 3′ untranslated region (3′UTR) luciferase vector pGL3-MIF 3′UTR-Wt and mutant-type MIF 3′UTR luciferase vector pGL3-MIF 3′UTR-Mut from Invitrogen; miR-146a mimics and negative control (NC) were synthesized by Guangzhou RiboBio. The dual mini vertical electrophoresis apparatus, mini transfer electrophoresis apparatus and Chem-iDocTM XRS gel imaging system were purchased from Bio-Rad; the luciferase activity assay kit from Promega.

2.3. MTT to detect the cell viability of MCF-7

NSCLC cells were seeded onto 96-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-146a mimics and NC with the transfection concentration of 50 nmol and 50 nmol, respectively. Fourty-eight hours after the transfection, MTT was added for the continuous culture of 4 h and then the supernatant were removed. Afterwards, DMSO was added and CD value was measured at 560 nm of analyzer of enzyme-linked immunosorbent assay, which was regarded as the cell viability [14].

2.4. Colony formation assay

NSCLC cells were seeded on 6-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-146a mimics and NC with the transfection concentration of 50 nmol and 50 nmol, respectively. Fourty-eight hours after the transfection, the staining solution with 10% formaldehyde and 0.1% crystal violet was used for the fixed staining. It was placed at room temperature for 30 min. Afterwards, the staining solution was gently flung off and each well was washed with the distilled water. The culture plate was inverted on the absorbent paper to suck out the water. Then the photo was taken and the analysis was performed.

2.5. AnnexinV-PI doubles staining flow cytometry to detect the cell apoptosis

NSCLC cells MCF-7 were seeded onto 6-well plate. When the confluency of cells reached to 50%, Lipofectamine™2000 was transfected with miR-146a mimics and NC with the transfection concentration of 50 nmol and 50 nmol, respectively. The operation procedure should be in accordance with the manual of kit and it was tested on the machine.

2.6. RT-PCR

The total RNA was extracted according to the manual of trizol kit. The primers were as follows. miR-146a forward primer: 5′-CAGTGCGTGTCGTGGAGT-3′, reverse primer: 5′-GGGTGAGAACTGAATTCCA-3′, length: 158 bp. MIF forward primer: 5′-ACTAAGAAAGACCCGAGGC-3′, reverse primer: 5′-GGGGCACGTTGGTGTTTAC-3′, length: 366 bp. GAPDH forward primer: 5′-AGCCACATCGCTCAGACA-3′, reverse primer: 5′-TGGACTCCACGACGTACT-3′, length:

314 bp. The RT-PCR kit was employed to reversely transcribe RNA into cDNA and then perform the PCR amplification. The collected amplification product was used for the further testing with agarose gel. The primers were added in 25 μL PCR system. The reaction conditions included the denaturation at 94 °C for 45 s, renaturation at 59 °C for 45 s and extension at 72 °C for 60 s, with 35 cycles in total.

2.7. Western blot

The RIPA lysis buffer was added in the collected cells. The supernatant obtained from the centrifugal was the total protein. The protein concentration was measured with BCA kit. After the denaturation, the protein loading buffer was treated with SDS gel electrophoresis for (1–2) h and then it was transferred with the wet method for (30–40) min. Then the film was incubated into the primary antibody solution at 4 °C over night. Afterwards, it was incubated into the secondary antibody solution at the room temperature for (1–2) h. Then it was exposed in the gel imaging system. Statistics was performed on the gray value of each antibody band using 'Quantity one' software.

2.8. Immunohistochemistry to detect the expression of MIF protein in lung tissues

Samples were fixed with 10% formalin. After being embedded and sliced into sections, they were deparaffinaged with dimethylbenzene and dehydrated with anhydrous, 95% and 80% ethanol. They were washed with the running water. After the antigen retrieval, the horse serum was employed for the antigen blocking. After being blocked with the primary antibody and secondary antibody, they were immersed in the hematoxylin and hydrochloric acid alcohol. They were washed with the running water to be antiblue and then dehydrated with the gradient ethanol. They were clarified with xylene and the neutral balsam for mounting and results were observed under the microscope. The positive staining of MIF was light yellow and brown yellow, which was at the cytoplasm.

2.9. Expression of luciferase reporter gene

miR-146a and MIF recombinant plasmid were transfected into NSCLC cells, with the grouping as follows: miR-146a mimics + Wt MIF, miR-146a mimics NC + Wt MIF, miR-146a mimics + Mut MIF and miR-146a mimics NC + Mut MIF. The dual luciferase assay system was employed to detect the viability of cell luciferase. Firstly, cells were washed with PBS. After adding PLB lysis buffer for the lysis of 10 min, LAR solution was added and the fluorescence value was read. The stopping solution was then added and the fluorescence value was read again. The relative fluorescence value was then calculated (relative fluorescence value = fluorescence value of firefly luciferase/fluorescence value of renilla luciferase).

2.10. Statistical analysis

All data were analyzed with SPSS 17.0 and expressed as mean ± SD. The t test was employed and $P < 0.05$ was considered to have significant difference.

3. Results

3.1. Expression of miR-146a in NSCLC lung tissues and lung tissues beside the cancer

The expression of miR-146a in NSCLC tissues was significantly lower than that in the normal tissues beside the cancer, with the statistical difference ($P < 0.05$). Expression of miR-146a in NSCLC lung tissues and lung tissues beside the cancer was (0.68 ± 0.05) and (0.23 ± 0.00), respectively; and expression of MIF in NSCLC lung tissues and lung tissues beside the cancer was (0.89 ± 0.02) and (5.49 ± 0.48), respectively.

3.2. Expression of miR-146a in NSCLC cell lines and normal lung cell lines

The relative expression of miR-146a in NSCLC cell lines of HCC827, A549, NCI-H1650 and NCI-H258 was significantly lower than that in the human embryonic lung cell WI-38. Because of the appropriate expression, A549 could be chosen as the research object. Expression of miR-146a in NSCLC cell lines and normal lung cell lines was as follows, HCC827 (0.20 ± 0.02), A549 (0.24 ± 0.04), NCI-H1650 (0.51 ± 0.05), and WI-38 (0.75 ± 0.02).

3.3. Expression of MIF in NSCLC lung tissues and lung tissues beside the cancer

Compared with the normal lung tissues besides the lung cancer, the positive expression of MIF in NSCLC lung tissues was significantly higher, with the statistical difference ($P < 0.01$).

3.4. Expression of luciferase reporter gene

The miR-146a mimics, NC and wild-type vector pGL3-MIF 3′UTR-Wt and mutant-type vector pGL3-MIF 3′UTR-Mut were transfected in A549 cells. The results indicated that the fluorescent intensity in the cotransfection group of miR-146a mimics and wild-type vector pGL3-MIF 3′UTR-Wt was significantly weaker than that in other transfection groups, with the statistical difference ($P < 0.01$). For the mutant-type vector pGL3-MIF 3′UTR-Mut, there was no significant difference in the fluorescent intensity among groups ($P > 0.05$). Expressions of Luciferase Reporter Gene in pGL3-MIF 3′UTR-Wt group were: miR-146a NC (1.03 ± 0.10) and miR-146a mimics (0.54 ± 0.06); in pGL3-MIF 3′UTR-Mut group were: miR-146a NC (0.99 ± 0.09) and miR-146a mimics (0.99 ± 0.11).

3.5. Effect of miR-146a mimics on expression of MIF and mRNA in A549 cells

After transfecting miR-146a mimics and NC in A549 cells, the expression of MIF protein and mRNA was significantly decreased ($P < 0.01$).

Effect of miR-146a NC on expression of MIF and mRNA in A549 cells were: MIF protein (1.12 ± 0.10) and MIF mRNA (1.40 ± 0.13); Effect of miR-146a mimics on Expression of MIF and mRNA in A549 Cells were: MIF protein (0.35 ± 0.03) and MIF mRNA (0.52 ± 0.05).

3.6. Effect of miR-146a mimics on viability of A549 cells

After transfecting miR-146a mimics in A549 cells, the results of MTT assay indicated that, compared with the control group, the cell viability of transfection group was significantly decreased, with the statistical difference ($P < 0.01$) (Table 1).

Table 1
Effect of miR-146a mimics on viability of A549 cells, number of clones and apoptosis.

Type	Cell viability (%)	Number of clones	Cell apoptosis rate (%)	
			Early apoptosis	Late apoptosis
miR-146a NC	100.00 ± 10.10	68.34 ± 8.23	1.38 ± 0.14	13.08 ± 1.31
miR-146a mimics	60.42 ± 6.43**	35.49 ± 3.65**	2.54 ± 0.25	38.12 ± 3.81

Compared with NC group, $^{**}P < 0.01$.

3.7. Effect of miR-146a mimics on colony formation of A549 cells

After transfecting miR-146a mimics in A549 cells, the results of cloning technique indicated that, compared with the control group, the cloning ability of cells in the transfection group was significantly decreased, with the statistical difference ($P < 0.01$).

3.8. Effect of miR-146a mimics on apoptosis of A549 cells

After transfecting miR-146a mimics in A549 cells, the results of AnnexinV-PI assay indicated that, compared with the control group, the number of cell apoptosis in the transfection group was significantly increased ($P < 0.01$).

3.9. Effect of miR-146a mimics on NF-κB signaling pathway in A549 cells

After transfecting miR-146a mimics in A549 cells, the results of western blot indicated that, compared with the control group, the NF-κB p65phosphorylation in the transfection group was significantly decreased ($P < 0.01$).

Effect of miR-146a NC on NF-κB signaling pathway and Caspase 3 activity in A549 cells were: pp65/p65 (0.43 ± 0.04) and relative activity of Caspase 3 (1.39 ± 0.13); miR-146a mimics were: pp65/p65 (0.43 ± 0.04) and relative activity of Caspase 3 (1.39 ± 0.13).

3.10. Effect of miR-146a mimics on Caspase 3 activity in A549 cells

After transfecting miR-146a mimics in A549 cells, compared with the control group, the phosphorylation of Caspase 3 in the transfection group was significantly increased ($P < 0.01$).

4. Discussion

The development and progression of NSCLC tumor is a process that involves multiple factors and steps, being presented as the activation of oncogenes and inactivation of tumor suppressor genes finally. A great number of researches have proved that miRNAs serve as the cancer suppressor or cancer promoter in different tumors and play a critical role in the proliferation and apoptosis of tumor cells, signal transduction and the regulation of tumor pathogenesis [5–7]. As some kind of tumor suppressing gene, the expression of miR-146a was down-regulated in many types of cancer cells [15–19]. Wang *et al* [20] reported that the expression of miR-146a in the serum of NSCLC was higher than that in cells of normal persons. Jia *et al* [9] found that the expression of miR-146a in patients with NSCLC was lower than that in the benign lung cancer tissues. According to Wu *et al* [10], the low expression of miR-146a in NSCLC was closely related to the high TNM staging, lymph node metastasis and low survival rate and the high expression of miR-146a in NSCLC was related to the high overall response rate and high survival rate. Chen *et al* [14] proved that the low expression of miR-146a in NSCLC was closely related ot the high TNM staging and distant metastasis. Cornett *et al* [8] reported that the expression of miR-146a in the lung cancer cells was significantly lower than that in the normal lung cells, which fully indicated that there was the close relationship between miR-146a and the occurrence and development of NSCLC. The results of this study were in accordance with above findings. The expression of miR-146a in NSCLC lung tissues was lower than that in the lung tissues beside the cancer and its expression in NSCLC cell lines of HCC827, A549, NCI-H1650 and NCI-H258 was also lower than that in the human embryonic lung cell WI-38. Because of the appropriate expression of miR-146a in A549, it was chosen as the cell line of subsequent experiment.

Furthermore, the previous researches have proved that miRNAs could regulate the biological behavior of tumor cells by targeting some molecules [21–23]. The analysis of luciferase reporter gene proved that MIF was the reverse target gene of miR-146a. MIF is some kind of cytokine from T lymphocytes, which can inhibit the migration of macrophages and cause the aggregation and infiltration of macrophages in the delayed type hypersensitivity to be involved in the inflammatory response. Besides, MIF is regulated by the hypothalamus and pituitary system, which plays an important role in the apoptosis and proliferation of tumor cells and the development of malignant tumors. The expression of MIF was relatively high in the cancer tissues of melanoma, NSCLC, prostate cancer and colorectal cancer [24]. In this study, the results of immunohistochemistry also proved that the expression of MIF protein and mRNA in NSCLC was higher than that in the tissues beside the cancer. In addition, according to the immunohistochemistry by Kamimura *et al* [11], MIF was overexpressed in the lung tissues of patients with NSCLC and it was related to the prognosis. Howard *et al* [13] also found that the expression of MIF in the lung cancer tissues was significantly higher than that in the tissues beside the cancer. White *et al* [25] reported that the high expression of MIF in NSCLC lung tissues was positively related to the high expression of VEGF in the blood and the microvessel density of tumor tissues. It fully indicated that MIF was overexpressed in NSCLC and it was closely related to the occurrence and development of NSCLC. Meanwhile, by up-regulating the expression of miR-146a, it could significantly inhibit the expression of MIF protein and mRNA in A549 cells, which indicated that MIF was the reverse target molecule of miR-146a protein. Accordingly, on that basis, it would further discuss the mechanism of miR-146a that

regulated the proliferation and apoptosis of A549 cells by targeting MIF molecule.

The infinite proliferation of tumor can result in the continuous division and proliferation of tumor cells, which makes the anabolism of protein higher than the catabolism in the tumor cells. It can even grab the products of protein metabolism in normal cells to make the organism in the condition of cachexia and thus aggravate the illness. Thus to induce the apoptosis of tumor cells would be an effective measure to inhibit the infinite proliferation of tumor. Chen *et al* [14] transfected miR-146a mimics in NSCLC cell lines of H358, H1650, H1975, HCC827 and H292 and found that it could significantly inhibit the cell proliferation and induce the cell apoptosis. Mawhinney *et al* [26] reported that the growth of primary tumor in the mouse with Lewis lung cancer that knocked out MIF or lacked the MIF activity was slower than that in the wild-type mouse. Thus in this study, by transfecting miR-146a mimics in A549 cells, it found that the viability of tumor cells and cloning ability were decreased and the apoptosis rate was increased, which indicated that the up-regulated expression of miR-146a could target inhibiting the expression of MIF and thus induce the apoptosis of A549 cells. The proliferation and apoptosis of tumor cells were regulated by the apoptosis signaling pathway. NF-κB signaling pathway is one of common signaling pathways. In the condition without the stimulation or in normal physiological conditions, it can be bound with IκB. But in the condition with the stimulation or in pathological conditions, IκB will be degraded and phosphorylated to release NF-κB p65 and transfer it from the cytoplasm to the nucleus, in order to regulate the expression of apoptosis-related genes such as Caspase 3. According to previous researches, the activity of NF-κB p65 was significantly increased in NSCLC and the interference against its activity to the certain extent could inhibit the proliferation of tumor cells [27–30]. In addition, the promoter region of miR-146a has the binding site of NF-κB. miR-146a could negatively regulate NF-κB signaling pathway and thus affect the process of tumor formation [31,32]. Paik *et al* [31] found that in the cell lines of NK/T cell lymphoma, miR-146a could interfere with TRAF6/NF-κB pathway to inhibit the cell proliferation, promote the cell apoptosis and increase the sensitivity of chemotherapy. The knockout of miR-146a gene in C57BL/6 mice could cause the malignant tumor of myelocytes, with the high expression of NF-κB p65 in the spleen and nucleus of bone marrow of mice and the increase in the transcription of reverse genes [32]. It fully indicated that miR-146a could make NF-κB p65 lose the activity and then significantly inhibit the proliferation of tumor cells and induce the apoptosis of tumor cells. Meanwhile, it also found that during the stroke of mice, the over expression of MIF would also reduce the activity of Caspase 3 and inhibit the apoptosis of neurons, which was realized through NF-κB signaling pathway [33]. siRNA MIF could significantly induce the apoptosis of H460 tumor cells and increase the activity of Caspase 3 and Caspase 4 [34]. It indicated that the down-regulated expression of MIF could inhibit the activity of NF-κB to increase the activity of Caspase 3 and thus induce the apoptosis of tumor cells. It could be assumed that the up-regulated expression of miR-146a could target inhibiting the expression of MIF in A549 cells to induce the apoptosis of A549 cells, which could be realized by inhibiting NF-κB signaling pathway. The results of this study also proved such assumption. To be specific, after the up-regulated expression of miR-146a, the apoptosis of A549 cells was increased, with the decreased phosphorylation of NF-κB p65 and the increased activity of Caspase 3.

In conclusion, miR-146a had the low expression in NSCLC lung tissues and cell lines and MIF had the overexpression in NSCLC lung tissues. The up-regulated expression of miR-146a could target inhibiting the expression of MIF to inhibit the proliferation and induce the apoptosis of A549 cells, which could be realized by inhibiting NF-κB signaling pathway. In addition, miR-146a/b could negatively regulate the activity of NF-κB p65 in MDA-MB-231 cells of breast cancer to inhibit the metastasis of tumor cells [35]. Therefore, the further experiment can discuss the effect of miR-146a on the biological behavior such as the invasion and metastasis of NSCLC cells through such signaling pathway.

Conflict of interest statement

We declare that we have no conflict of interest.

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