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Influence of miR-199a on rats with non-small cell lung cancer *via* regulating the HIF-1α/VEGF signaling pathway

L.-M. WANG[1,2], L.-L. ZHANG[3], L.-W. WANG[4], L. ZHU[5], X.-X. MA[6]

[1]Interventional Therapy Department, Shandong Provincial Chest Hospital, Jinan, China
[2]Cheeloo College of Medicine, Shandong University, Jinan, China
[3]Thoracoscopic Ward, Shandong Provincial Chest Hospital, Jinan, China
[4]Department of Radiology, Tai'an City High-Tech Zone Tianbao Central Health Center, Tai'an, China
[5]Magnetic Resonance Imaging Room, Beihai Hospital of Yantai, Yantai, China
[6]Department of Radiology, Qilu Hospital of Shandong University, Jinan, China

Abstract. – OBJECTIVE: Micro-ribonucleic acids (miRNAs) are involved in the occurrence of various cancers, and the hypoxia-inducible factor 1-α (HIF-1α) is the main regulator of cell proliferation induced by hypoxia. The relationships of miR-199a and HIF-1α expressions with non-small cell lung cancer (NSCLC) remain unclear, so they were explored in this work.

MATERIALS AND METHODS: On the basis of establishing the rat model of NSCLC, the messenger RNA (mRNA) expressions of miR-199a, HIF-1α and the vascular endothelial growth factor (VEGF) were analyzed in NSCLC rats, and the correlations of miR-199a with the mRNAs of HIF-1α and VEGF and cancer staging were investigated.

To further study the role of miR-199a in NSCLC cell proliferation via the HIF-1α/VEGF signaling pathway, NSCLC cells were treated with the signaling pathway inhibitor and transfected with miR-199a mimics, respectively. Also, the roles of the inhibitor PX-478 and miR-199a mimics in the expressions of miR-199a, HIF-1α, and VEGF proteins, as well as their influences on cell proliferation ability were detected.

RESULTS: In NSCLC rats, the expression of miR-199a was substantially decreased ($p<0.01$), but the expressions of HIF-1α and VEGF were notably raised ($p<0.01$). MiR-199a was negatively correlated with the expression of VEGF. As cancer developed, the expression of miR-199a was gradually lowered, but the expressions of HIF-1α and VEGF were gradually increased. Both HIF-1α/VEGF signaling pathway inhibitor PX-478 and miR-199a mimics significantly reduced the expressions of HIF-1α and VEGF proteins ($p<0.01$) and suppressed the cell proliferation activity.

CONCLUSIONS: MiR-199a prevents the proliferation of NSCLC cells via the targeted down-regulation of the HIF-1α/VEGF signaling pathway.

Key Words:
MiR-199a, HIF-1α/VEGF signaling pathway, Non-small cell lung cancer, Influence.

Introduction

Non-small cell lung cancer (NSCLC), the most common type of lung cancer, is a leading cause of cancer deaths, and its pathogenesis has still been unclear with an unsatisfactory late prognosis[1,2]. Hence, probing into the occurrence and progression of NSCLC at a molecular level may help develop the therapeutic methods of this disease.

The roles of micro-ribonucleic acids (miRNAs) in human cancers have been discovered, and was showed that they serve as oncogenes or tumor suppressors through targeting different regulatory factors[3,4]. MiR-137 can promote the occurrence and development of cancer in patients with lung cancer, and it can be used as the survival marker for NSCLC patients[5]. Scholars[6,7] have revealed that miR-199a is maladjusted in bladder and ovarian cancers. The role of miR-199a in the progression of NSCLC remains unclear.

Hypoxia-inducible factor 1-α (HIF-1α) is an endogenous hypoxia marker[8]. It has been reported that the up-regulation of HIF-1α is correlated with the tumor necrosis and overall survival in NSCLC[9,10], and its protein synthesis is regulated by the activation of the phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) signaling pathway[11]. Mack et al[12] have demonstrated that the suppressing the activity of HIF-1α can significantly inhibit the tumor growth in animal models[12]. The

Corresponding Author: Xiangxing Ma, BM; e-mail: xiangxingma2006@163.com

miRNA which regulates the expression of HIF-1α in a targeted manner is still unknown. Tumor angiogenesis is a complex process triggered by the paracrine signals of tumor cells and peripheral matrices. The vascular endothelial growth factor (VEGF) mainly drives the progression of cancers under hypoxic conditions, and its inhibitor is currently applied to chemotherapies for cancers[13].

Therefore, the present study explored the relationships of the expressions of miR-199a and HIF-1α with NSCLC and revealed the influence of miR-199a on NSCLC rats through regulating the HIF-1α/VEGF signaling pathway.

Materials and Methods

Experimental Reagents

Superscript III reverse transcription kit was purchased from Invitrogen (Carlsbad, CA, USA), SapphireAmp® Fast polymerase chain reaction (PCR) Master Mix from Wuhan U-Me Biotech (Wuhan, China), TaqMan MicroRNA Assay and messenger RNA (mRNA) Assay from Applied Biosystems (Foster City, CA, USA), PX-478 from Selleck China (Shanghai, China), anti-HIF-1α, anti-VEGF and anti-β-actin antibodies from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and methyl thiazolyl tetrazolium (MTT) cell proliferation kit from Shanghai Weiao Biotech Co., Ltd. (Shanghai, China).

Laboratory Animals and Cell Line

A total of 30 female Wistar rats weighing (200 ± 30) g, purchased from the Experimental Animal Center of Shandong University, were utilized for the experiments in this study. This investigation was approved by the Animal Ethics Committee of the Shandong University Animal Center. The lung squamous cell carcinoma cell line NCI-H520 was provided by Shanghai Y-S Industrial Co., Ltd. (Shanghai, China).

Establishment of Rat Models of NSCLC

In this work, the lung squamous cell carcinoma cell line NCI-H520 cultured *in vitro* was transplanted into rats to prepare the rat model of NSCLC. In brief, the concentration of NCI-H520 cells cultured *in vitro* was adjusted to 1×10^6 cells/mL using Hank's solution, and 0.2 mL of the resulting cells were intravenously injected into rats. After one week of normal feeding, rats exhibited a squamous bronchial mucosa, which indicated that the modeling was successful.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The NSCLC tissues and normal adjacent tissues were taken to extract the total RNAs with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the total RNAs were reversely transcribed using the Superscript III reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Then real-time qPCR was conducted with the SapphireAmp® Fast PCR Master Mix, and the mRNA expressions of miR-199a, HIF-1α, and VEGF were determined *via* the TaqMan MicroRNA Assay and mRNA Assay. The expression levels of miRNA and mRNA were standardized as those of U6 small nuclear RNA (snRNA) and β-actin, respectively. MiR-199a primer: (sense) GGTGGTGGAAAATGATATTTATTTG and (anti-sense) GGTGGTGGAAAATGATATTTATTTG, VEGF primer: (sense) CCATGCAGATCATGCGGATCA and (anti-sense) CCTTGGCTTGTCACATCTGCAA, HIF-1α primer: (sense) ACCTATGACCTGCTTGGTGCTGAT and (anti-sense) CAGTTTCTGTGTCGTTGCTGCCAA, and β-actin primer: (sense) ACCATTGGCAATGAGCGGT and (anti-sense) GTCTTTGCGGATGTCCACGT.

Cell Separation and Culture

The lung squamous cell carcinoma tissues in model group were digested to single cells and cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640: Gibco, Grand Island, NY, USA) medium. The cell lines subjected to the passage at a stable proliferation rate were established as the cell line of NSCLC.

Experimental Grouping and Treatment

NSCLC cell lines isolated from the rats in model group were cultured *in vitro* and divided into 4 groups: blank control group, PX-178 group, mimic control group, and miR-199a mimic group. Blank control group: lung squamous cell carcinoma cells were normally cultured without any treatment. PX-178 group: lung squamous cell carcinoma cells were treated with 100 nM PX-178, the inhibitor of the HIF-1α/VEGF signaling pathway. Mimic control group: lung squamous cell carcinoma cells were transfected with 100 nM mimics. MiR-199a mimic group: 100 nM miR-199a mimics were transfected into lung squamous cell carcinoma cells.

Western Blotting

Cell lysates were isolated in 10% dodecyl sulfate, sodium salt-polyacrylamide gel electropho-

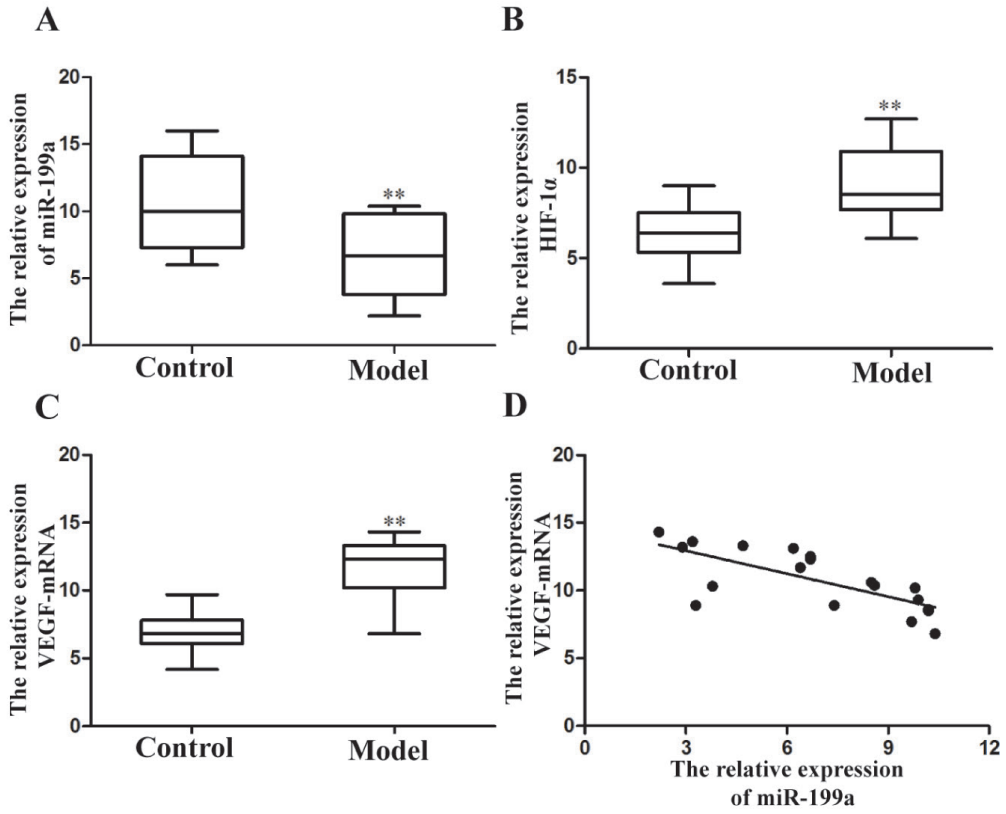

Figure 1. Expressions of miR-199a, HIF-1α and VEGF in rat models of NSCLC **A-C,** MRNA expressions of miR-199a, HIF-1α, and VEGF detected via RT-PCR (**p<0.01) **D,** Correlation of miR-199a with VEGF expression (p=0.0005, r=0.7202).

resis (SDS-PAGE) gel, transferred onto nitrocellulose membranes and sealed in 5% of skimmed milk, followed by the incubation with anti-HIF-1α, anti-VEGF, and anti-β-actin antibodies. Then the cells bound to horseradish peroxidase-conjugated IgG. Finally, the resulting products were developed with a chemiluminescence enhancer and photographed, followed by data processing *via* the analysis of optical density and grayscale.

Detection of Cell Proliferation Activity via MTT assay

A total of 5×10^4 cells treated with different methods were transfected in a 96-well plate (Corning, Corning, NY, USA) for 24 h and grew for 5 d. The cells were incubated in 50 mL MTT (0.1 mg/mL) for 4 h at 37°C, followed by 15 min of lysis in 150 mL dimethyl sulfoxide at room temperature. The absorbency of the wells was read at 580 nm with a microplate reader. Each experiment was done in triplicate.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) and expressed as mean ± standard deviation. The Student's *t*-test was used for the analysis of significance, and p<0.05 represented that the difference was significant.

Results

Expressions of MiR-199a, HIF-1α, and VEGF in Rat Models of NSCLC

First, the rat models of NSCLC were established to detect the expressions of miR-199a, HIF-1α and VEGF in the cancer tissues of 18 models (model group) and adjacent normal tissues (control group), as well as to analyze the correlations of miR-199a with HIF-1α and VEGF. As shown in figures, in model group, the expression of miR-199a was substantially decreased (p<0.01) (Figure 1A), but the expressions of HIF-1α, and VEGF were notably raised (p<0.01) (Figure 1B, 1C) compared with those in control group. The correlation analysis result revealed that there was no significant correlation between miR-199a and HIF-1α expression, while the former was significantly negatively correlated with the expression of VEGF (Figure 1D).

Relationships of MiR-199a, HIF-1α, and VEGF Expressions with NSCLC Staging

To further explore whether the expressions of miR-199a, HIF-1α and VEGF are correlated with the progression of NSCLC, the mRNA expressions of miR-199a, HIF-1α and VEGF were analyzed in the NSCLC rat samples at different cancer stages. The results showed that, as cancer progressed, the expression of miR-199a was gradually lowered (Figure 2A), but the expressions of HIF-1α and VEGF were gradually increased (Figure 2B, 2C).

Influence of the HIF-1α/VEGF Signaling Pathway Inhibitor on NSCLC Cell Proliferation

Furthermore, the activity of HIF-1α in NSCLC cells was suppressed using PX-478, the HIF-1α inhibitor, and the cell proliferation activity was determined *via* the MTT assay to observe the influence of the HIF-1α/VEGF signaling pathway on the proliferation ability of NSCLC cells. The results showed that compared to the control drug, PX-478 substantially decreased the expressions of HIF-1α, VEGF proteins (Figure 3A) and cell activity (Figure 3B).

Influence of MiR-199a on NSCLC Cell Proliferation via Targeting the HIF-1α/VEGF Signaling Pathway

To further study the effect of miR-199a on NSCLC cell proliferation *via* the HIF-1α/VEGF signaling pathway, NSCLC cells were transfected with miR-199a mimics to verify the influence of miR-199a mimics on the expressions of miR-199a, HIF-1α and VEGF, and the cell proliferation ability was measured *via* the MTT assay. Results suggested that miR-199a mimics could significantly promote the expression of miR-199a ($p<0.01$) (Figure 4A), and they substantially decreased the expressions of HIF-1α, and VEGF proteins in NSCLC cells ($p<0.01$) (Figure 4B). Additionally, miR-199a mimics could notably lower the proliferation activity of NSCLC cells ($p<0.01$) (Figure 4C). Results suggested that miR-199a prevents the proliferation of NSCLC cells *via* the targeted down-regulation of the HIF-1α/VEGF signaling pathway.

Discussion

NSCLC is one of the most important causes of death from cancer worldwide, and deaths from it account for most of all lung cancer deaths. The occurrence of NSCLC is closely associated with hypoxia, which is intimately related to tumor proliferation and increases the expression of hypoxia effectors, such as HIF-1α and HIF-2α[14]. The HIF-1α inhibitors, as chemical molecules, have been applied in the researches on clinical treatments. Currently, the detailed relationship of HIF-1α with the progression of NSCLC has not been clarified yet.

Large numbers of studies have discovered that the maladjustment of miRNAs is an important factor for the occurrence and development of cancers, although the mechanisms have still been unclear to a large extent[15]. In the maladjustment of all miRNAs, the down-regulation of miR-199a is closely related to the metastasis, invasion, proliferation, apoptosis, and prognosis of some types of cancers[16-18]. It has been found that miR-199a is expressed in multiple tissues, such as hepatic, vascular and visceral smooth muscles, cerebral, ovary and testicular tissues, endothelial cells and myocardial cells[19]. However, miR-199a is down-regulated in numerous cancer tissues, including gastric cancer, osteosarcoma, and colorectal cancer tissues[20-22].

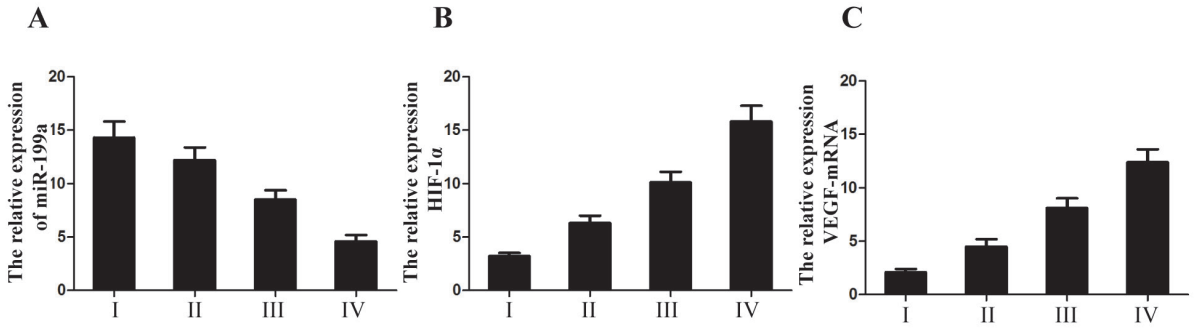

Figure 2. Relationships of miR-199a, HIF-1α and VEGF expressions with NSCLC staging.

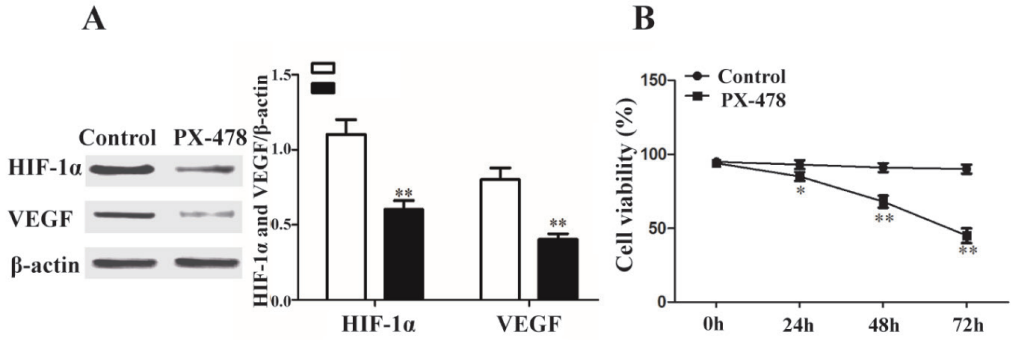

Figure 3. Influences of PX-478 on the expressions of HIF-1α and VEGF proteins and NSCLC cell proliferation **A,** Influences of PX-478 on the expressions of HIF-1α and VEGF proteins detected via Western blotting (**p<0.01) **B,** Cell proliferation activity determined via MTT assay (*p<0.05 and **p<0.01).

In this study, miR-199 was lowly expressed in NSCLC tissues, while the expressions of HIF-1α, and VEGF exhibited a trend of down-regulation, and these results may provide a certain reference for the clinical prognosis of NSCLC. The HIF-1α/VEGF signaling pathway is activated to restore and enhance angiogenesis, which is a compensatory response under hypoxic conditions. The pathway has biological activity under such physiological conditions as wound healing[23,24]. HIF-1α is the major regulatory factor for oxygen homeostasis[25]. Normally, it exists at an undetectable level under normoxic conditions due to rapid hydroxylation, but this process is inhibited under hypoxic conditions[26,27]. Friis et al[28] suggested that salubrinal has influences on the process of angiogenesis, inhibiting the VEGF signal transduction and the proliferation and migration of endothelial cells. In the present study, it was discovered that miR-199a mimics could significantly down-regulate the expressions of HIF-1α and VEGF proteins. These results revealed that miR-199a mimics stop the proliferation of cancer cells through blocking the HIF-1α/VEGF signaling pathway.

In addition, this investigation found that the change trend of miR-199a expression was negatively correlated with the change in HIF-1α expression, suggesting that miR-199a and HIF-1α have opposite effects on the progression of NSCLC. HIF-1α serves as an endogenous hypoxia marker, and the mechanism of its accumulation under hypoxic conditions has been widely researched and relatively clarified clear[29,30]. It was also discovered that the NSCLC staging exhibited certain correlations with the expression changes of miR-199a, HIF-1α and VEGF. With the progression of cancer, the expression of miR-199a was gradually lowered, but the expressions of HIF-1α and VEGF were gradually increased. How miRNA regulates the expression of HIF-1α

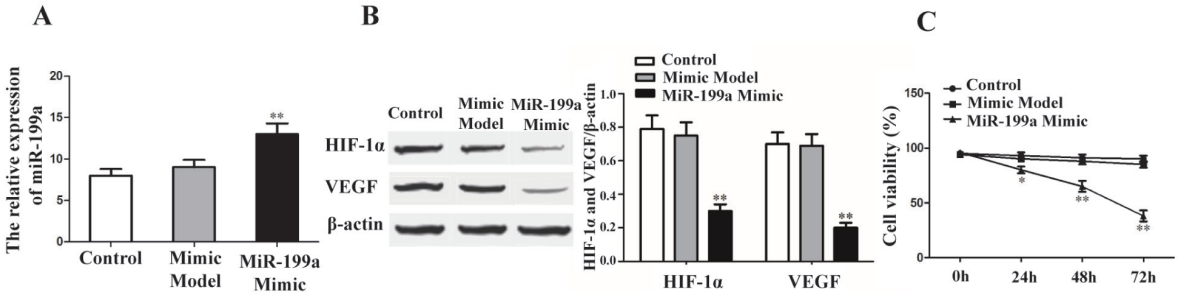

Figure 4. MiR-199a lowers the proliferation of NSCLC cells via the targeted down-regulation of the HIF-1α/VEGF signaling pathway. **A,** Up-regulatory effect of miR-199a mimics on miR-199a verified via RT-PCR (**p<0.01) **B,** Inhibitory effect of miR-199a mimics on the expressions of HIF-1α and VEGF proteins detected via Western blotting (**p<0.01) **C,** Inhibitory effect of miR-199a mimics on the proliferation of NSCLC cells detected via the MTT assay (**p<0.01).

remains unclear in NSCLC. It has been reported that miR-199a may affect the expressions of Sirt1 and HIF-1α[31]. It is inferred that miR-199a probably regulates the expression of HIF-1α as well, thus taking part in the regulation of the hypoxia-induced proliferation of NSCLC cells. To explore the interaction between HIF-1α and miR-199a, PX-478, the HIF-1α inhibitor was applied to the cells which were isolated from the rats and cultured *in vitro*. PX-478 prominently lowered the expressions of HIF-1α and VEGF, and the cell proliferation activity. Moreover, NSCLC cells cultured *in vitro* were transfected with miR-199a mimics to determine the expression levels of HIF-1α and VEGF in cells. According to the results, miR-199 mimics with functions similar to those of PX-478 substantially promoted the inhibition on HIF-1α and VEGF expressions and lowered the cell proliferation activity. These observation results revealed that miR-199a negatively regulates the hypoxia-induced proliferation through targeting the expressions of HIF-1α and VEGF.

Conclusions

We found that the decrease in the miR-199a level and the increase in HIF-1α and VEGF levels were closely correlated with the progression of NSCLC. The study results of the interaction mechanism revealed that miR-199a inhibits the expression of the HIF-1α/VEGF signaling pathway to repress the proliferation of NSCLC. Due to an expression maladjustment, the level of miR-199a in NSCLC tissues is significantly lower than that in normal tissues. As a result, the maladjusted miR-199a regulates the expressions of HIF-1α and VEGF, thereby further promoting the development of lung cancer.

Conflict of interest

The authors declare no conflicts of interest.

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