

## Original Article

# MicroRNA-21 regulates non-small cell lung cancer cell proliferation by affecting cell apoptosis via COX-19

Qi Guo<sup>1</sup>, Hui Zhang<sup>1</sup>, Ling Zhang<sup>2</sup>, Yang He<sup>1</sup>, Shaobo Weng<sup>3</sup>, Ziping Dong<sup>4</sup>, Jianmin Wang<sup>1</sup>, Pingfang Zhang<sup>1</sup>, Ribu Nao<sup>4</sup>

<sup>1</sup>Orthopedics Department I, General Hospital of North China Petroleum Administration, Renqiu 062552, P.R. China; <sup>2</sup>Orthopedics Department II, General Hospital of North China Petroleum Administration, Renqiu 062552, P.R. China; <sup>3</sup>Department of Urology, General Hospital of North China Petroleum Administration, Renqiu 062552, P.R. China; <sup>4</sup>Department of Emergency, General Hospital of North China Petroleum Administration, Renqiu 062552, P.R. China

Received February 15, 2015; Accepted April 23, 2015; Epub June 15, 2015; Published June 30, 2015

**Abstract:** Aims: This study is to investigate the regulatory effect of microRNA-21 (miR-21) on bone metastasis of non-small cell lung cancer (NSCLC). Methods: In this study, 18 patients were diagnosed with vertebral column metastasis of NSCLC. MiR-21 or small interfering RNAs were transfected into H2170 cells using Lipofectamine 2000. Real-time PCR was performed to detect miR-21 expression. Western blotting was used to measure the expression of COX-19 protein. Enzymatic activity tests were performed to measure the activity of cytochrome C oxidase. Flow cytometry was used to monitor changes in cell apoptotic rate. MTT assay was used to determine the capability of cell proliferation. Results: Bone metastasis of NSCLC enhanced the levels of miR-21 in NSCLC patients. Proliferation capability of cells with high expression of miR-21 was greater than that of cells with the inhibition of miR-21 expression. High expression of miR-21 promoted cell proliferation by inhibiting cell apoptosis. COX-19 was a key factor in the inhibition of apoptosis by miR-21. Inhibition of COX-19 expression reduced cell proliferation by enhancing cell apoptosis. Conclusions: This study demonstrates that inhibition of miRNA-21 suppresses NSCLC cell proliferation by promoting cell apoptosis via the decrease of COX-19 expression.

**Keywords:** Bone metastasis, non-small cell lung cancer, microRNA-21, cytochrome C oxidase, COX-19, apoptosis

## Introduction

Bone is a good target site for the metastasis of lung cancers. Non-small cell lung cancer (NSCLC) is the commonest type of lung cancer that metastasizes to bones, with its molecular mechanisms of occurrence and development remaining unclear [1]. Bone metastasis is not the direct cause that threatens NSCLC patients, but metastasis to weight-bearing bones such as cervical vertebrae, thoracic vertebrae or lumbar vertebrae can lead to paralysis that severely affects patients' life spans. It is necessary to perform timely treatments for patients with bone metastasis of NSCLC. The incidence of bone metastasis of NSCLC is usually related to the positions and pathology of NSCLC. Therefore, it is of great value to understand the molecular mechanisms of the occurrence and development of NSCLC, and to find cytokines that regulate relevant signaling pathways.

MicroRNA (miRNA or miR), a type of non-coding RNA molecule, can inhibit the expression of target genes at multiple stages [2-5]. There are about 800-1000 known miRNAs in the human genome that play important roles in cell proliferation, differentiation, apoptosis and migration [6-8]. Researches show that the expression of miR-21 is up-regulated in several malignant tumors [9-11], and that miR-21 participates in the occurrence and development of tumors via complex regulations. In NSCLC patients, enhanced expression of miR-21 affects the proliferation and metastasis of cancer cells through regulating EGFR-Akt pathway activity, tropomyosin activity, and the expression of programmed cell death 4 and apoptosis-related proteins [12-19].

Studies reveal that the activity of cytochrome C oxidase (COX) is closely related to cell apoptosis [20-22]. Reduction of COX activity increases

the content of cytochrome C, which activates cell apoptosis signaling pathway and finally leads to apoptosis [23-25]. Reversely, enhanced COX activity inhibits cell apoptosis. COX-19 is an important protein that participates in the assembly of COX subunits and the expression of COX-19 affects COX activity [26]. By software screening, we found that COX-19 is a potential binding target of miR-21. In this study, we investigated whether COX-19 participates in the process in which miR-21 regulates cell apoptosis, as well as the molecular mechanism of action of miR-21 on NSCLC cell apoptosis.

### Materials and methods

#### Patients

In this study, 18 patients (11 males and 7 females; aged 50-66; average age of 61.4) were diagnosed with vertebral column metastasis of NSCLC by clinical manifestations, radiology and laboratory tests. In the control group, 20 patients with bone tuberculosis were included (11 males and 9 females; aged 50-87; average age of 69.5). Osseous tissues of femur, thoracic vertebra, and cervical vertebra with lung cancer metastasis were obtained by biopsy. All procedures were approved by the Ethics Committee of General Hospital of North China Petroleum Administration. Written informed consents were obtained from all patients or their families.

#### Cells and treatments

H2170 cells, a type of NSCLC cells, were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) under 37°C at 5% CO<sub>2</sub>. The cells were transfected with miR-21 (Lipo miR-21 group) or small interfering RNAs (siRNA) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's manual. siRNAs (30 nM; RiboBio, Guangzhou, China) was used to target-silence COX-19, while mismatched siRNAs served as negative control (mismatched siRNA group). The sequences of siRNAs that silence COX-19 were: i) 5'-GAGGGCAUGUAGACGGUUA dTdT-3' and 3'-dTdT CUCCCGUACAUCUGCCAAU-5'; ii) 5'-ACAAUUGCUACCUCCAAA dTdT-3' and 3'-dTdT UGUUUAUCGAUGGAGGUUU-5'; and iii) 5'-GACUGGAUCCAUACAAUUAU dTdT-3' and 3'-dTdT CUGACCUAGGUAUGUUUAU-5'.

The sequences of mismatched siRNA were 5'-GAGGGCAUGUAGACGGUUA dTdT-3' and 3'-dTdT CUCCCGUACAUCUGCCAAU-5'.

Intracellular miRNA-21 expression was silenced by co-transfection with its specific inhibitor using Lipofectamine according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA).

#### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

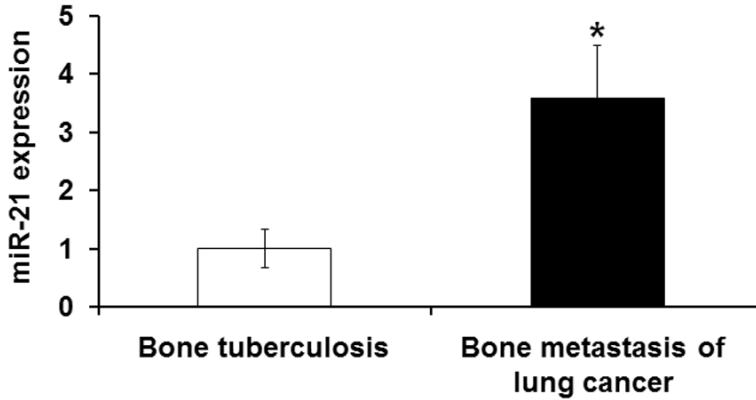
At 48 h after transfection, medium was discarded from each well of the 96-well plate, followed by the addition of 200 µL MCDB131 medium (Invitrogen, USA). Then, 20 µL MTT was added into each well, followed by shaking. After 3.5 h of incubation, medium was discarded, followed by the addition of 100 µL dimethyl sulfoxide in each well. After incubation for 5-10 minutes, supernatants were transferred to another clean 96-well plate for optical density measurement under 490 nm. Each sample test was repeated for 5 times in 5 individual wells.

#### Flow cytometry

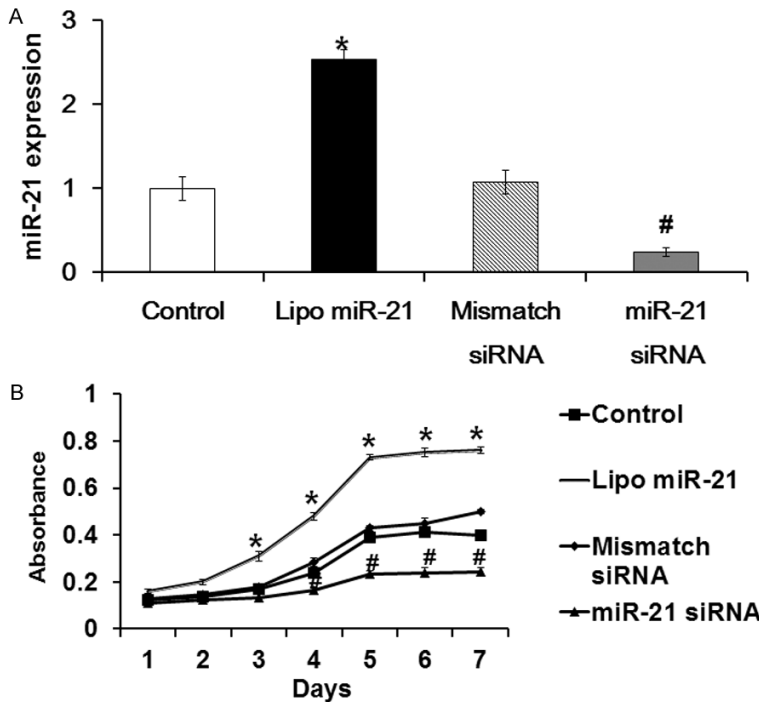
The cells were trypsinized before centrifugation at 200 ×g for 5 minutes, followed by washing with phosphate-buffered saline. The cells were incubated with 100 µl Annexin-V-FLUOS staining reagent containing 2% propidium iodide (Roche, Basel, Switzerland) under room temperature for 10-15 minutes. Cell apoptosis was detected using flow cytometry. Apoptotic index was obtained from the percentage of cells double-stained by Annexin-V/propidium iodide. Each test was performed in triplicate.

#### COX activity detection

Cardiomyocytes were trypsinized and collected in Eppendorf tubes, followed by the addition of 1 ml mitochondrial separation medium. The cells were then transferred to Dounce homogenizer for homogenization of 200 times on ice. The homogenized cells were transferred to another 2 ml Eppendorf tube for centrifugation at 600 ×g and 4°C for 5 minutes. The supernatant was then transferred to another 1.5 ml Eppendorf tube for centrifugation at 1000 ×g and 4°C for 5 minutes. Then, the supernatant was collected for another centrifugation at 7000 ×g and 4°C for 10 minutes, followed by



**Figure 1.** Expression of miR-21 in patients with bone tuberculosis and bone metastasis of lung cancer. Histograms represent the levels of miR-21. The mean values are normalized to that in bone tuberculosis group. The data are presented as means ± standard deviation. \*, P < 0.05 compared with bone tuberculosis group.



**Figure 2.** Effect of miR-21 expression on H2170 cell proliferation. A. Expression of miR-21 in H2170 cells in normal control, Lipo miR-21, mismatched siRNA and miR-21 siRNA groups. Histograms represent the expression levels of miR-21. The mean values are normalized to that in normal control. The data are presented as means ± standard deviation. \*, P < 0.05 compared with normal control group; #, P < 0.05 compared with Lipo miR-21 group. B. H2170 cell absorbance curve in normal control, Lipo miR-21, mismatched siRNA and miR-21 siRNA groups. Higher absorbance value corresponds to higher proliferation level. \*, P < 0.05 compared with control group; #, P < 0.05 compared with Lipo miR-21 group.

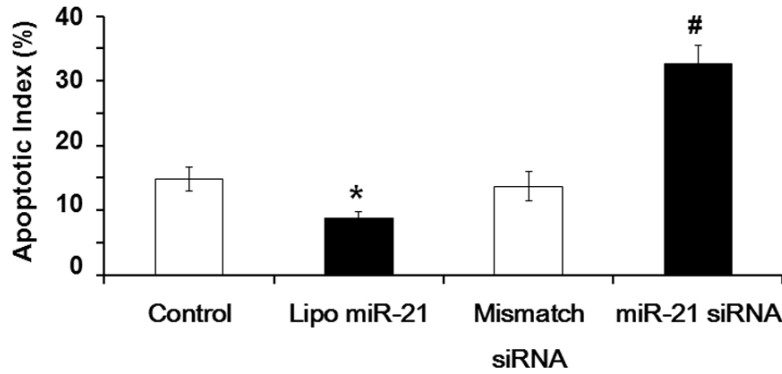
aration medium, followed by centrifugation at 7000 ×g and 4°C for 10 minutes. After the supernatant was discarded again, the sediments were resuspended by 1 ml mitochondrial separation medium, followed by the final centrifugation at 10000 ×g and 4°C for 10 minutes before the collection of mitochondria.

Collected mitochondria samples were mixed with 40 μl 1% sodium dodecyl sulfate and placed on ice. The Eppendorf tube was vortexed for 10 seconds every 5 minutes for consecutive 6 times, before centrifugation at 12000 ×g and 4°C for 10 minutes. The supernatant contained the extracted mitochondrial proteins. The activity of COX was determined using COX activity detection kit (Genmed, Shanghai, China) according to the manufacturer's manual.

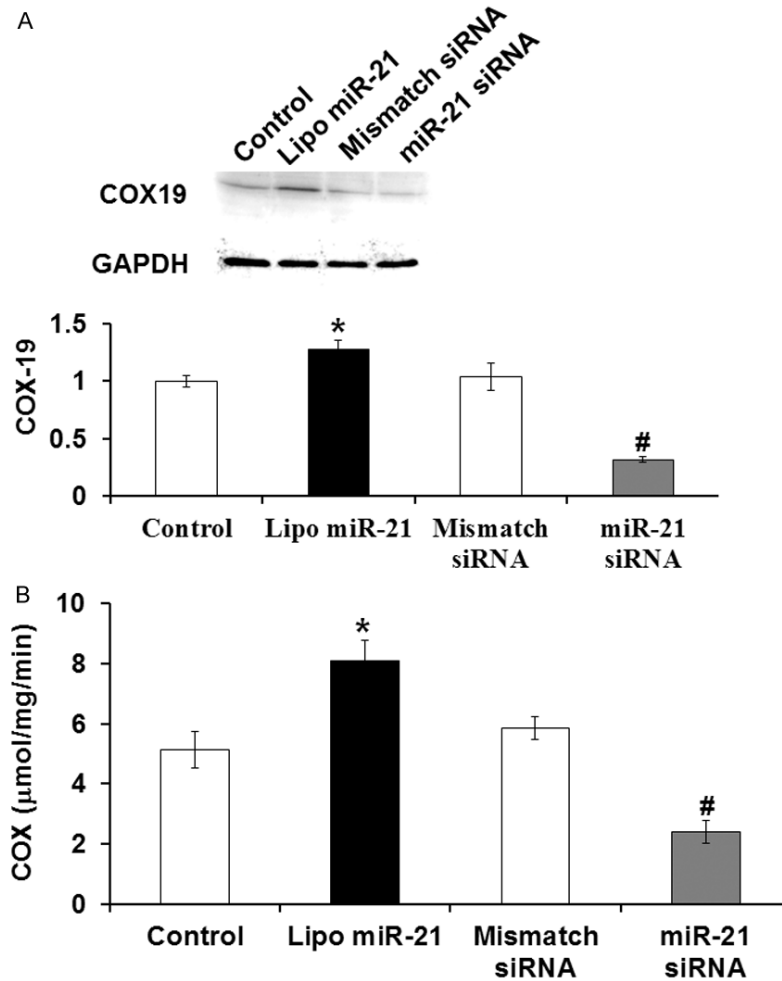
*Real-time RT-PCR*

The cDNA synthesis was performed using 1 μg total RNA and M-MLV Reverse Transcriptase kit (Takara, Dalian, China). Reverse transcription system and protocol were in accordance with the manufacturer's manual. For real-time RT-PCR, the primer sequences for miR-21 were: forward, 5'-TCGCTCGAGATTTTATCAAGAGGG-3'; reverse, 5'-TCGGCGGCCGCA-CAAGAATGAGACTTTAATC-3'. PCR reaction system contained 12.5 μl SYBR Premix Ex Taq, 1 μl forward primer, 1 μl Uni-miR primer, 2 μl template, 8.5 μl ddH<sub>2</sub>O (total 25 μl). Each test was performed in triplicate. PCR amplification conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 seconds and an-

the discard of supernatant. The sediments was resuspended by adding 1 ml mitochondrial sep-



**Figure 3.** The effect of miR-21 expression on H2170 cell apoptosis. Flow cytometry was used to measure and calculate apoptotic index. Histograms represent apoptotic index. The data are presented as means  $\pm$  standard deviation. \*,  $P < 0.05$  compared with normal control; #,  $P < 0.05$  compared with Lipo miR-21 group.



**Figure 4.** Effect of miR-21 on (A) COX-19 expression and (B) COX activity. All data are means  $\pm$  standard deviation and presented as histograms and error bars. \*,  $P < 0.05$  compared with normal control; #,  $P < 0.05$  compared with Lipo miR-21 group.

nealing at 60°C for 20 seconds. The  $2^{-\Delta\Delta C_t}$  method was used to calculate the relative expression of miR-21.

*Western blotting*

Briefly, 30 mg protein samples were subjected to 10% standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis with pre-stained molecular weight markers being run in parallel to identify COX-19 protein. Afterwards, the resolved proteins were transferred to polyvinylidene difluoride membranes. The membranes were then incubated with anti-COX-19 and anti-b-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After extensive washing, the membranes were incubated with anti-mouse IgG-horse-radish peroxidase conjugate antibody for 1 h at room temperature and developed with a Luminol chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA).

*Statistical analysis*

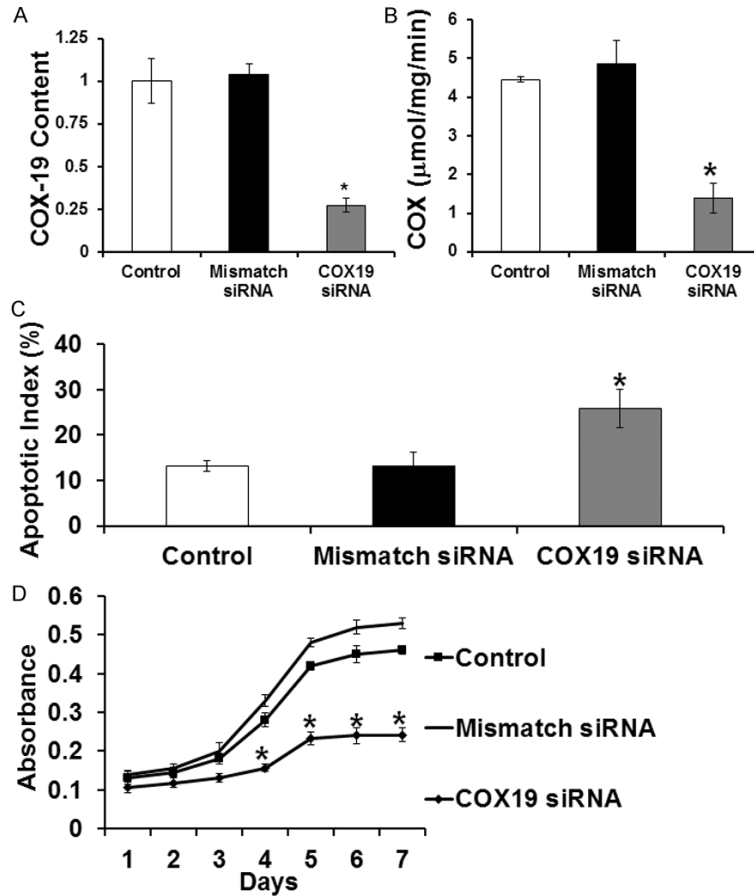
All data were analyzed using SPSS 13.0 statistical software (IBM, Chicago, USA). Differences between groups of data were compared using t-test.  $P < 0.05$  was considered statistically significant.

**Results**

*Bone metastasis of NSCLC enhances the levels of miR-21 in NSCLC patients*

To measure the expression of miR-21, real-time PCR was performed. The results showed that the expression

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**Figure 5.** Effect of COX-19-silencing siRNA on (A) COX-19 expression, (B) COX activity, (C) apoptotic index, and (D) cell proliferation. All data are means  $\pm$  standard deviation and presented as histograms and error bars. \*,  $P < 0.05$  compared with control.

of miR-21 in patients with bone metastasis were significantly higher than that in patients with bone tuberculosis (Figure 1) ( $P < 0.05$ ). These results suggest that bone metastasis of NSCLC enhances the levels of miR-21 in NSCLC patients.

*Proliferation capability of cells with high expression of miR-21 is greater than that of cells with the inhibition of miR-21 expression*

To examine the relationship between miR-21 expression and cell proliferation, cultured H2170 cells were transfected with miR-21 or siRNAs. Cell proliferation was detected by MTT assay. H2170 cells were divided into normal control, Lipo miR-21, mismatched siRNA and miR-21 siRNA groups. The results showed that the expression of miR-21 in H2170 cells transfected with miR-21 was significantly higher than that in normal control (Figure 2A) ( $P < 0.05$ ). In addition, transfection with siRNA that

target-silence miR-21 inhibited the expression of miR-21, while transfection with mismatched siRNA had no effect on the expression of miR-21 (Figure 2A). MTT assay showed that the absorbance of H2170 cells with high expression of miR-21 was higher than that of normal control ( $P < 0.05$ ). Moreover, siRNA silencing of miR-21 significantly decreased the absorbance of H2170 cells compared with cells transfected with miR-21 ( $P < 0.05$ ) (Figure 2B). These results indicate that the proliferation capability of cells with high expression of miR-21 is greater than that of cells with the inhibition of miR-21 expression.

*High expression of miR-21 promotes cell proliferation by inhibiting cell apoptosis*

To investigate whether miR-21 expression was related to cell apoptosis, flow cytometry was performed. H2170 cells were divided into normal control, Lipo miR-21, mismatched siRNA and miR-21 siRNA groups. The results showed that the apoptotic index of H2170 cells with high expression of miR-21 was significantly lower than that in normal control ( $P < 0.05$ ) (Figure 3). By contrast, inhibition of miR-21 expression significantly increased apoptotic index (Figure 3). This data suggests that high expression of miR-21 promotes cell proliferation by inhibiting cell apoptosis.

*COX-19 is a key factor in the inhibition of apoptosis by miR-21*

To study the mechanism by which miR-21 inhibit cell apoptosis, Western blotting was used to measure the expression of COX-19. H2170 cells were divided into normal control, Lipo miR-21, mismatched siRNA and miR-21 siRNA groups. The results showed that the expression of COX-19 was significantly higher in Lipo miR-21 group than control group ( $P < 0.05$ ), while inhibition of miR-21 expression in miR-21 siRNA group sig-

nificantly reduced COX-19 expression ( $P < 0.05$ ) (Figure 4A). In addition, high expression of miR-21 significantly enhanced the COX activity, and inhibition of miR-21 expression decreased COX activity (Figure 4B). These results indicate that COX-19 is a key factor in the inhibition of apoptosis by miR-21.

### *Inhibition of COX-19 expression reduces cell proliferation by enhancing cell apoptosis*

To investigate the effect of COX-19 on cell proliferation, H2170 cells were divided into normal control, mismatched siRNA and COX-19 siRNA groups. The results showed that siRNA that silenced COX-19 significantly inhibited the expression of COX-19 ( $P < 0.05$ ), while mismatched siRNA did not affect COX-19 expression (Figure 5A). Furthermore, inhibition of COX-19 expression significantly reduced COX activity, increased apoptotic index, and finally inhibited cell proliferation compared with normal control ( $P < 0.05$ ). By contrast, mismatched siRNA had no effect on COX activity, cell apoptosis, and proliferation (Figure 5B-D). These results further demonstrate that inhibition of COX-19 expression reduce cell proliferation by enhancing cell apoptosis.

### Discussion

In recent years, researches show that miRNAs play important roles in the occurrence and development of tumors [3, 4]. Of note, miR-21 is a key factor in the proliferation, differentiation and apoptosis of tumor cells, with its molecular mechanism of action unclear yet.

The present study showed that the proliferation rate of NSCLC cells with high expression of miR-21 was higher than that of normal NSCLC cells. By software prediction, COX-19 that was closely related to COX activity was found to be a potential target of miR-21. COX-19 is an important protein that affects COX subunit assembly, which subsequently influences COX activity [26]. Alteration of COX activity induces cell apoptosis [20-22]. Our results demonstrated that reduced apoptosis of lung cancer cells was induced by elevated COX-19 expression, and increased COX activity. This study demonstrated for the first time that miR-21 regulates COX activity by inducing COX-19 expression. Subsequent studies showed that, consistent with the effect of inhibiting miR-21 expression,

cell apoptosis was promoted by inhibiting COX-19 expression. These results further demonstrated the regulatory effect of miR-21 on COX-19.

In this study, we did not elucidate the molecular mechanism of the inhibition of COX-19 expression by miR-21, which will be investigated in our future work. COX activity plays important roles in cell apoptosis, but miR-21 might affect COX activity through other possible pathways, which will also be further studied. In addition, the molecular mechanism of the regulation of apoptosis by miR-21 is meaningful for the development of miR-21-related anti-tumor drugs. In conclusion, this study demonstrated that miRNA-21 promoted NSCLC cell proliferation by inhibiting cell apoptosis via the increase of COX-19 expression.

### Acknowledgements

This work was directed and supported by Dr. Zhenjia Zhang of General Hospital of North China Petroleum Administration and Dr. Faming Tian of Hebei United University.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Hui Zhang, Department of Orthopedics, General Hospital of North China Petroleum Administration, No. 1 Huizhan Road, Renqiu City 062552, Hebei Province, P.R. China. Tel: 86-317-2725331; Fax: 86-317-2725331; E-mail: hbsy555@126.com

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