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Inhibition of pulmonary carcinoma proliferation or metastasis of miR-218 via down-regulating CDCP1 expression

X.-J. ZENG[1], Y.-H. WU[2], M. LUO[3], P.-G. CONG[4], H. YU[5]

[1]Department of Cardiac Surgery, GuiZhou Province People's Hospital, Guiyang, China
[2]Guangdong Cardiovascular Institute of Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China
[3]Department of Thoracic Surgery, GuiZhou Province People's Hospital, Guiyang, China
[4]Department of Anesthesiology, Second Hospital Affiliated to Guiyang Traditional Chinese Medicine College, Guiyang, China
[5]Department of Cardiothoracic Surgery, Second Hospital Affiliated to Guiyang Traditional Chinese Medicine College, GuiYang, China

Abstract. – OBJECTIVE: Pulmonary carcinoma is one common malignant tumor with a high risk of recurrence and metastasis. Non-small cell lung cancer (NSCLC) is the most common subtype. As one tumor biomarker, microRNA (miR) has tissue sensitivity and can facilitate oncogene or inhibit tumor suppressor gene. MiR-218 has abnormal expression and can work as one molecular marker for tumors. However, its expression and function mechanism in lung cancer cells have not been fully illustrated.

MATERIALS AND METHODS: *In vitro* cultured pulmonary adenoma A549 cells and normal bronchial epithelial cell line 16HBE were tested for miR-218 expression. A549 cells were transfected with miR-218 mimic or negative controls, followed by real-time PCR quantifying for miR-218. MTT method was used to test cell proliferation, whilst Transwell chamber was adopted for measuring cell invasion. Dual luciferase reporter gene assay (DLRGA) was used to test target relationship between miR-218 and CDCP1. Western blot was used to test CDCP1 expression.

RESULTS: MiR-218 was down-regulated in A549 cells compared to 16HBE ($p<0.05$). Transfection of miR-218 mimic significantly facilitated miR-218 expression, inhibited tumor proliferation or invasion. As the target gene of miR-218, CDCP1 expression was suppressed by miR-218 over-expression ($p<0.05$ compared to control group).

CONCLUSIONS: MiR-218 inhibits NSCLC proliferation or metastasis via down-regulating CDCP1, and can work as one novel molecular target for lung cancer diagnosis.

Key Words:
MicroRNA-218, CDCP1, Non-small cell lung cancer, Proliferation, Metastasis

Introduction

Both incidence and mortality of lung cancer are among leading tumors over the world. Lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the later of which occupies more than 80% of all cases[1,2]. As one malignant tumor that severely threatens human health, more than million people die from lung cancer, making it one of the most deadly cancers. The mortality rate of lung cancer is the highest all over the world[3,4]. Various treatment methods including surgery, radiotherapy, chemotherapy, immune therapy and intervention therapy can be used for lung cancer[5]. The treatment efficiency of lung cancer is still unfavorable at current stage with a high rate of metastasis and recurrence, leading to shorter survival span, worse life quality, and unfavorable prognosis, causing heavy burdens for global economy[6,7]. As the most common, and easy for recurrence and metastasis tumor in the respiratory tract, the molecular mechanism for lung cancer is still unclear. The identification of a precise molecular target for lung cancer and further regulation of tumor proliferation or metastasis have become a novel research focus[8]. Therefore it is worth study for the pathogenesis, progression, diagnosis and treatment of lung cancer[9].

MicroRNA (miR) is one type of small molecule that regulates biological features of animals and plant cell via base pairing for negative regulation on downstream transcription of a target gene via degrading mRNA and regulating protein

Corresponding Author: Xiangjun Zeng, MD; e-mail: xiangjunzeng07@163.com

expression, thus mediating gene expression at the post-transcriptional level[10,11]. Prediction by Targetscan software showed that miR regulated 1%-5% of all genes, with each miR regulating over 200 target genes on average, indicating that at least one-third of human functional protein coding genes are mediated by miR[12]. As one tumor biological marker, miR has tissue sensitivity as it can exert functions via facilitating oncogenes or inhibiting potential tumor suppressor genes[13]. MiR-218 has been shown to be abnormally expressed in tumors, making it one candidate for tumor molecular marker[14]. MiR-218 is down-regulated in liver cancer and osteosarcoma, and it is believed to be one tumor suppressor gene[15, 16]. However, the expression or functional mechanism of miR-218 in lung cancer has not been fully illustrated.

Materials and Methods

Major Reagents and Equipment

NSCLC A549 cell line (CAT number: CRM-CCL-185) and normal human bronchial epithelial cell line 16HBE were purchased from ATCC cell bank (US). DMEM medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (Logan, UT, USA). DMSO and MTT powders were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). Trypsin-EDTA lysis buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). PVDF membrane was purchased from Pall Life Sciences Inc. (Pensacola, FL, USA). EDTA was purchased from Hyclone (Logan, UT, USA). The Western blotting reagent was purchased from Beyotime (Beijing, China). ECL reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human NF-κB monoclonal antibody (Catalogue No. 13586; 1:2000), rabbit anti-human EGFR monoclonal antibody (Catalogue No. 2646; 1:2000), and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody (Catalogue No. 7074; 1:1000) were all purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Transwell chamber was purchased from Corning (Corning, NY, USA). RNA extraction kit, reverse transcription kit and lipo2000 transfection reagent were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). TaqMan microRNA reverse transcription kit was purchased from Thermo Electron Corp (Burlington, Ontario, Canada). MiR-218 mimic and negative control (NC) were synthesized by GenePharma (Shanghai, China). Other common reagents were purchased from Sangon (Shanghai, China). ABI7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). HEARcell CO_2 incubator was purchased from Thermo Electron Corp (Burlington, Ontario, Canada). Lab systems Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

A549 Cell Culture and Grouping

A549 cells stored in liquid nitrogen were resuscitated in 37 °C water-bath until fully thawing. Cells were centrifuged at 1000 r/min for 3 min, and were re-suspended in 1 ml fresh medium and were removed from 5 ml culture flask containing 2 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO_2 at 37 °C for 24~48 h. A549 cells were seeded in 6-well plate at 1×10^6 per cm^2. The culture medium contained 10% FBS, and 90% high-glucose DMEM medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin). Cells were kept in a humidified chamber with 5% CO_2 at 37 °C. Cells at log-phase with 3rd to 8th generation were randomly divided into control group, negative control (NC) group and miR-218 group, the latter of which received miR-218 NC or miR-218 mimic transfection.

Liposome Transfection of miR-218 Mimic into A549 Cells

MiR-218 mimic (5'-AGGUC GGUGA AUCGU UCA-3') or miR-218 negative control (5'-AUUCA GGUCA AUCGG UGG-3') oligonucleotides were transfected into A549 cells. In brief, cells were cultured until reaching 70%~80% confluence. MiR-218 mimic or negative control liposome were mixed with 200 μl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with miR-218 mimic or negative controlled dilutions for 30-min room temperature incubation. Serum was removed, followed by PBS rinsing gently and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO_2 at 37 °C for 6 h, followed by the application of 10% FBS-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for miR-218 Expression in A549 Cells

Trizol reagent was used to extract RNA from A549 cells. Reverse transcription was performed according to the manual instruction of test kit,

Table I. Primer sequences.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
miR-218	CTCCCACAGCTGTACTAAG	GCACGATTGCTGTACTGGATT

using primers designed by PrimerPremier 6.0 and synthesized by Invitrogen, Shanghai (China) as shown in Table I. Real-time PCR was performed on target genes under the following conditions: 55 °C for 1 min, followed by 35 cycles each containing 92 °C for 30 s, 58 °C for 45 s and 72 °C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the reference. A standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by the $2^{-\Delta Ct}$ method.

MTT for Cell Proliferation

A549 cells at log-phase were seeded into 96-well plate which contained DMEM medium with 10% FBS at 5×10^3 density. After 24 h incubation, the supernatant was removed. 20 µl sterile MTT was added into each test well in triplicates. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 µl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values were measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group.

Transwell Chamber Assay for Cell Invasion

48 h after transfection, cells were cultured in DMEM medium with 10% FBS. Serum-free culture medium was used for another 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4 °C air-dry. Excess liquid was removed from the plate. 50 µl serum-free culture medium containing 10 g/L was added into each well for 30 min incubation at 37 °C. The Transwell chamber was placed in a 24-well plate. 500 µl DMEM medium containing 10% FBS was added outside the chamber. Inside the chamber 100 µl tumor suspension was added using serum-free culture medium. Each group was tested in triplicate. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber to remove membrane-fixed cells. Chambers were then fixed in cold ethanol and stained by crystal violet for 20 min. Cells at the lower surface of the micro-pore membrane were observed and counted under an inverted microscope. Each sample was observed for 10 fields to get the average value. Each experiment was repeated for more than three times.

Western blot for CDCP1 Protein Expression

Total proteins were extracted from A549 cells. In brief, cells were mixed with lysis buffer on ice for 15-30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 ×g for 15 min, the supernatant was saved, quantified by Bradford method and was stored at -20 °C for Western blot assay. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method (100 mA, 1 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-Bcl-2 monoclonal antibody (1:2 000) was added for 4 °C overnight incubation. After PBST washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by X-ray exposure. The film was scanned and analyzed by protein imaging system and Quantity One software for measuring band density. Each experiment was replicated for four times (n=4) for statistical analysis.

Targeted Relationship Between miR-218 and CDCP1

Targetscan (http://www.targetscan.org/) gene prediction software was used to predict the target gene of miR-218. Dual luciferase reporter gene assay (DLRGA) was used to analyze the targeted relationship between miR-218 and CDCP1. A mutant form of CDCP1 (mt) that contains potential binding sites for miR-218, and wild-type CDCP1 (wt) were sub-cloned into psiCheck2 dual luciferase reporter gene for transfection into A549 cells. DLRGA system was used to analyze luciferase activity at 48 hours after transfection.

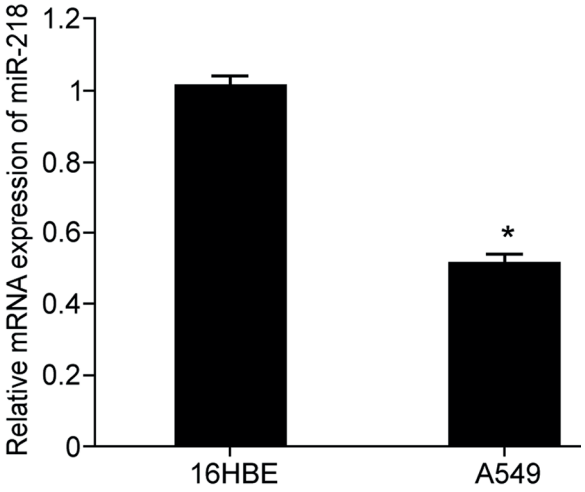

Figure 1. Expression of miR-218 in lung cancer A549 cells and normal bronchial epithelium. *, $p<0.05$ compared to normal 16HBE cells.

Statistical Analysis

All data were presented as mean±standard deviation (SD). Comparison of means between two groups was performed by t-test. SPSS 11.5 software was used for statistical analysis. Between-group difference was tested by using Tukey's post hoc test. Enumeration data were analyzed by chi-square test. A statistical significance was defined when $p<0.05$.

Results

Expression of miR-218 in A549 and Normal Bronchial Epithelial Cells

Real-time PCR was used to test the expressional profile of miR-218 in lung cancer A549 cells and normal bronchial epithelial cells. Results showed significantly decreased miR-218 mRNA in A549 cells ($p<0.05$ compared to normal 16HBE cells, Figure 1).

Effects of miR-218 Mimic Transfection on miR-218 Expression in A549 Cells

Real-time PCR was used to test the effect of miR-218 mimic on miR-218 expression in lung cancer cell line A549. Results showed significant facilitation of miR-218 mRNA expression in lung cancer A549 cells by miR-218 mimic transfection ($p<0.05$ compared to control or empty group, Figure 2).

Effects of miR-218 on A549 cell Proliferation

MTT assay was used to describe the effect of miR-218 mimic transfection on the proliferation of lung cancer cell A549. Results showed that transfection of miR-218 mimic for 48h significantly inhibited cell proliferation ($p<0.05$ compared to control group, Figure 3). These results indicated that miR-218 up-regulation had significant inhibition on A549 cell proliferation.

Effects of miR-218 Modulation on A549 cell Invasion

Transwell chamber assay was used to test the effect of miR-218 mimic transfection on invasion potency of A549 cells. Results showed that, after 48 hours of miR-218 mimic transfection, miR-218 was up-regulated, with inhibited A549 cell invasion ($p<0.05$ compared to control group, Figure 4). These results indicated that miR-218 up-regulation might affect tumor cell invasion potency.

Identification of miR-218 Downstream target genes

Online prediction using bioinformatics software package Targetscan (http://www.targetscan.org/) found 3'UTR of CDCP1 mRNA as the target gene of miR-218 (Figure 5A). In those cells expression 3'UTR of CDCP1 wt, transfection of miR-218 mimic up-regulated miR_218 expression and depressed luciferase activity ($p<0.05$ compared to control group). Transfection of 3'UTR of CDCP1 mt up-regulated miR-218 level but did not change luciferase activity significantly (Figu-

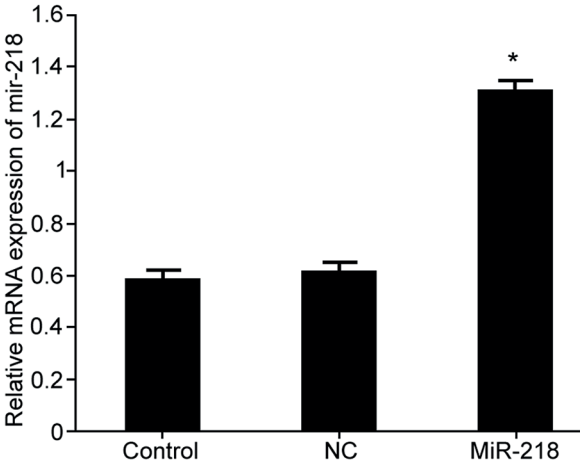

Figure 2. Effects of miR-218 mimic transfection on miR-218 expression in A549 cells. *, $p<0.05$ compared to control cells.

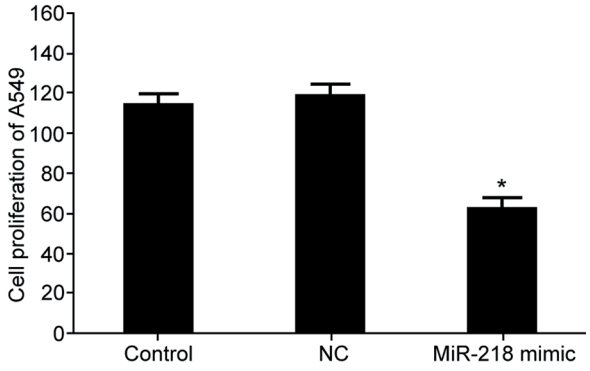

Figure 3. Effects of miR-218 on A549 cell proliferation. *, $p<0.05$ compared to control cells.

re 5B), indicating that miR-218 exerts its role via mediating 3'UTR of CDCP1.

Effects of miR-218 Modulation on CDCP1 Expression in A549 Cells

Western blot was used to test the effect of miR-218 regulation on CDCP1 protein in A549 cells. Results showed that 24 hours after transfecting miR-218 mimic, the up-regulation of miR-218 significantly down-regulated CDCP1 protein ($p<0.05$ compared to control group, Figure 6). These results showed that over-expression of miR-218 in A549 cells helped to inhibit proliferation of lung cancer cells and suppressed tumor cell invasion, probably related with regulation on downstream gene CDCP1 protein expression.

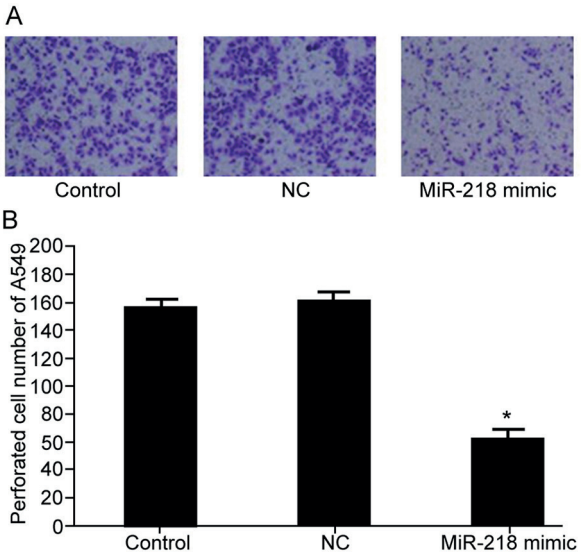

Figure 4. Effects of miR-218 modulation on A549 cell invasion. ***(A)*** Effects of miR-218 on A549 cell invasion; ***(B)*** Analysis of miR-218 effects on A549 cell invasion. *, $p<0.05$ compared to control group.

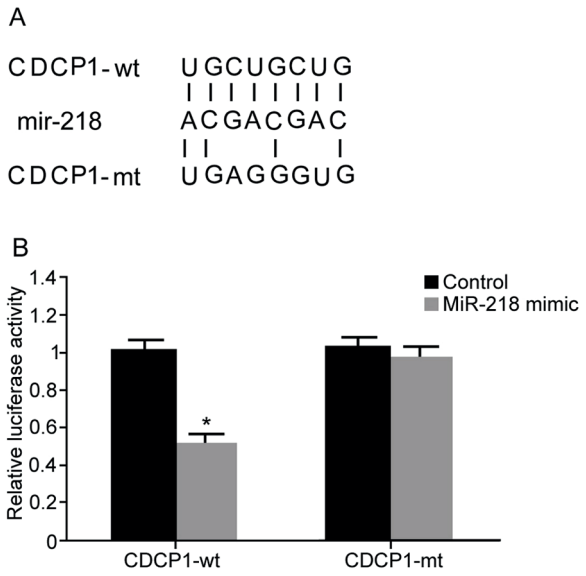

Figure 5. Identification of miR-218 downstream target genes. ***(A)*** Bioinformatics software prediction of miR-218 target gene; ***(B)*** Dual luciferase reporter gene assay. *, $p<0.05$ compared to control group.

Discussion

Lung cancer is one common malignant tumor. NSCLC is one common subtype having high in-

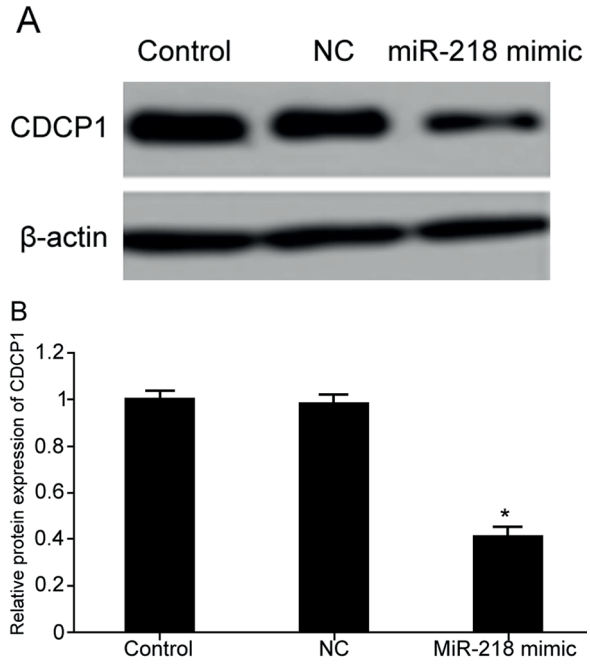

Figure 6. Confirmation of downstream target gene of miR-218. ***(A)*** Bioinformatics software predicting miR-218 target gene; ***(B)*** Dual luciferase reporter gene assay. *, $p<0.05$ compared to control group.

cidence, recurrence, and worse prognosis, making it one major health concern[17]. Although progression has been made, the long-term efficiency is still unfavorable. Lung cancer is susceptible for recurrence, as a result of a complex process involving multiple factors and steps[18]. During tumor onset and progression, invasion and metastasis are major reasons causing tumor recurrence, unfavorable prognosis or even death. NSCLC has a high rate of metastasis and infiltration, making its 5-year survival rate at relatively lower level[19]. Therefore, the identification of bio-markers with high sensitivity and specificity for early diagnosis and prognostic prediction of NSCLC is of great clinical significance[20].

Abundant evidences show that multiple biological regulations including animal/plant growth, cell proliferation or apoptosis, cell growth and differentiation/mutation or cell cycle regulation are correlated with abnormal expression of miR[20,21]. MiR can regulate post-transcriptional level, and mediates cell death, proliferation, differentiation, development and metabolism via regulating certain signal molecules including cell growth factors, transcriptional factors and death gene expression[22]. This study showed lower miR-218 expression in lung cancer cell line A549 compared to normal 16HBE cells, as consistent with previous reports showing lower miR-218 in other tumors[15, 16]. These results further supported the tumor suppressor role of miR-218. By transfection of miR-mimic for its over-expression in lung cancer cells, we demonstrated that miR-218 over-expression inhibited tumor cell proliferation, and suppressed invasion potency. These data indicated that miR-218 inhibited proliferation and metastasis of lung cancer.

Further study using bioinformatics software to predict miR-218 target gene demonstrated the regulatory relationship between miR-218 and CDCP1. As one cell surface glycoprotein, CDCP1 can be expressed in pluripotent stem cells and hematopoietic cells[23]. It is one transmembrane glycoprotein, with multiple interactions between its cytoplasmic domain with various cytosolic proteins[24]. CDCP1 is found to be up-regulated in various tumor cells, and is correlated with over-proliferation of lung cancer, pathology subtyping, TNM stage and lymph node metastasis[25]. In summary, this work showed that mediation of miR-218 expression can inhibit pulmonary carcinoma cell proliferation and metastasis in lung cancer cells.

Conclusions

We observed that miR-228 can inhibit proliferation or metastasis of lung cells via down-regulating CDCP1, and thus may work as one biological target for diagnosis and treatment of lung cancer.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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