MicroRNA-185-5p modulates chemosensitivity of human non-small cell lung cancer to cisplatin via targeting ABCC1

K. PEI¹, J.-J. ZHU², C.-E. WANG¹, Q.-L. XIE³, J.-Y. GUO⁴

¹Department of Pharmacy, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, China
²Department of Gastroenterology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, China
³Department of Ophthalmology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, China
⁴Department of Nephrology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, China

Abstract. – OBJECTIVE: MicroRNA-185-5p (miR-185-5p) dysregulation is found in various human cancers. Our purpose is to investigate the association of miR-185-5p expression with the sensitivity of non-small cell lung cancer (NSCLC) to cisplatin.

MATERIALS AND METHODS: Real-time PCR or Western blot assay was performed to detect the expression of mature miR-185-5p and ATP-binding cassette, subfamily C, member 1 (ABCC1) protein. Cell lines with abnormal expression of miR-185-5p were generated using miR-185-5p inhibitor and mimics. The viabilities of treated cells were analyzed using MTT assay. Cell apoptosis was evaluated by TUNEL assay. Apoptosis-related protein expressions were tested by Western blot. Dual-luciferase assay was applied to assess the target gene of miRNA.

RESULTS: The expression level of miR-185-5p in A549 cell line was significantly higher than that in A549/DDP cell line \( (p < 0.05) \). Transfection of miR-185-5p mimics increased the sensitivity of A549 cells to cisplatin and the expression of an apoptosis-related factor, and restrained cell proliferation. MiR-185-5p inhibitor promoted cisplatin resistance and cell growth in A549 cells, and declined apoptosis-related factor levels. ABCC1 was verified as the target gene of miR-185-5p. MiR-185-5p exhibited negative correlation with ABCC1 in A549/DDP cells.

CONCLUSIONS: The results of the present study demonstrated that inhibition of miR-185-5p was involved in chemo-resistance of NSCLC cells to cisplatin via down-regulating ABCC1.

Key Words
miR-185-5p, ABCC1, Chemosensitivity, A549; NSCLC.

Introduction

Lung cancer is one of the most common malignancies worldwide with a five-year survival rate of 15%. Approximately 85% of lung cancer cases belong to non-small-cell lung cancer (NSCLC). At present, chemotherapeutic agents are widely used in the treatment of lung cancer in spite of the quick development of targeted therapy. As a platinum-based compound, cisplatin (DDP) is one of the first-line chemotherapeutic agents for the treatment of NSCLC. However, its efficacy is usually limited by the development of acquired drug resistance. Thus, investigation of the molecular mechanisms of DDP resistance may help the clinician to monitor the resistance in advance thus elevating the efficacy of lung cancer therapeutics.

MicroRNAs (miRNAs) are small non-coding RNAs at the length of 20-22 nucleotides. MiRNAs suppress gene expression through complementary binding with 3’ untranslated regions (UTRs) of target mRNA. MiRNAs are predicted to targeted bind with over 50% of all human protein-coding genes. MiRNAs facilitate the target genes to play numerous regulatory roles in various physiological and developmental processes, such as cell development, differentiation, apoptosis and proliferation, through mediating the mRNA expression at transcriptional or post-transcriptional level. MiR-185-5p is one of the miRNAs which were usually found to be dysregulated in many kinds of cancers, such as hepatocellular carcinoma, breast cancer, and renal cancer. Moreover, miR-185-5p functions as an oncomiRNA to enhance prolifera-
tion of cancer cells\cite{12}. Recent studies\cite{13} suggested that miR-185-5p plays an important role in drug resistance. However, there is still a lack of report about the role of miR-185-5p in cisplatin sensitivity. Therefore, we hypothesized that the downregulation of miR-185-5p might be associated with chemotherapeutic resistance in NSCLC, but the molecular mechanism remains unclear.

As an important tumor suppressor, ATP-binding cassette, subfamily C, member 1 (ABCC1) affects transcription and translation of multiple genes, and modulates different signaling pathways\cite{14}. However, the upstream regulation of ABCC1 is still unknown. Up to now, the mainly identified miRNAs that directly target ABCC1 involve miR-7, miR-133a, miR-134, and miR-326\cite{15-17}. MiR-7 has been reported to negatively regulate ABCC1 expression in NSCLC\cite{16}. However, other potential ABCC1-targeting miRNAs needs to be defined. We speculated that miR-185-5p might play an important role in chemoresistance of NSCLC cells by downregulating ABCC1.

**Materials and Methods**

**Cell Line**

The human lung adenocarcinoma cell lines A549 and cisplatin-resistant A549/DDP were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in DMEM containing 10% fetal bovine serum (Gibco\textregistered, Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 humidified incubator before harvesting the cells.

**Drugs and Reagents**

Cisplatin was purchased from QiLu Pharmaceutical (Jinan, China). MiR-185-5p inhibitor, mimics, and their negative control oligonucleotides were obtained from Shanghai GeneChem Co., Ltd (Shanghai, China). They were transfected into A549 cells using Lipofectamine\textregistered 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction. Monoclonal rabbit anti-human ABCC1, p21, p27, FASL, Bim, and GAPDH antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA) were used for Western blot analysis.

**Cell Transfection**

Cells seeded in a 6-well plate (2.5×10{\textsuperscript{4}}/well) were transfected at 50% confluence using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) with Opti-MEM\textsuperscript{TM} MI (Gibco, Rockville, MD, USA) according to the manufacturer’s instructions. After 48 h, the transfected cells were harvested for downstream analyses or measured for cell wound healing assay.

**MTT Assay**

Cells transfected with miR-185-5p inhibitor or mimics were seeded into 96-well plates at 5×10{\textsuperscript{3}} cells/well and incubated for different times. Total of 20 ul MTT reagent (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the cells and then incubated in the dark for 4 h. The absorbance of the plate was tested using a microplate reader at the wavelength of a 570 nm reference (BMG Lab Technologies, Germany). The results were presented as the percentage of absorbance about untreated controls. Each treatment was carried out in triplicate.

**TUNEL Assay**

A549/DDP cells plated on glass slides were washed with ice-cold PBS three times for 5 min and fixed with 4% paraformaldehyde, followed with acetic acid/ethanol (1:3) for postfixation. The terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay was carried out with the In Situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. TUNEL-positive cells were identified as apoptotic cells. The percentage of apoptotic cells was calculated by the ratio of TUNEL-positive cells to total cells. At least 10 different high-power fields of a fluorescent microscope in duplicate wells and five different experiments were selected for the calculation.

**Real-time PCR**

For miRNA expression detection, reverse transcription (RT) reaction was performed with PrimeScript\textsuperscript{TM} RT reagent Kit (Takara Biotechnology co., LTD., Dalin, China). Real-time PCR was performed using SYBR\textsuperscript{®} Premix Ex Taq\textsuperscript{TM} II (Takara Biotechnology co., LTD., Dalin, China) according to the protocol provided by the manufacturer. For mRNA expression detection, reverse transcription reaction was performed with PrimeScript\textsuperscript{®} RT reagent Kit (Takara Biotechnology co., LTD., Dalin, China) and RT-PCR was performed using SYBR\textsuperscript{®} Premix Ex Taq\textsuperscript{TM} II (Takara Biotechnology co., LTD., Dalin, China). The expression of the target miRNA was normalized relative to that of the internal control U6. The expression of the target gene was normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an
MicroRNA-185-5p modulates chemosensitivity

Western Blot

The proteins were separated on an SDS denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. The primary antibodies were incubated with the membranes overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein expression was evaluated by enhanced chemiluminescence and exposure to chemiluminescent film. LabWorks™ Image Acquisition and Analysis Software (UVP, Upland, CA, USA) were used to quantify the band intensities.

Dual-luciferase Assay

The predicted binding site of miR-185-5p on 3'-UTR of ABCC1 was amplified through conventional RT-PCR from the cDNA library of A549 cells. A recombinant mutant 3'-UTR of ABCC1 was generated by a Site-Directed Mutagenesis Kit (SBS Genetech). The amplified wild-type and mutant ABCC1 3'-UTRs were then inserted into a pmiRREPORT luciferase reporter vector (Ambion, Austin, TX, USA) to generate Luc-PTEN and Luc-mPTEN (mutant) vectors according to the manufacturer’s instruction. A negative control luciferase vector, Luc-C was also generated. Then the three vectors (Luc-PTEN, Luc-PTEN, Luc-C) were co-transfected with β-galactosidase and miR-26b-mimic (RiboBio) into HEK293T cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. HEK293T cells were then cultured at 37°C with 5% CO₂ for another 24 h. The dual-luciferase activities were measured by a luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer’s instruction, and normalized to the β-galactosidase activity of the cells transfected with Luc-C.

Statistical Analysis

All data were presented as the mean ± standard deviation of the mean. Statistical analysis was performed with SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Two-tail unpaired Student’s t-test was used for statistical analysis. Pearson correlation analysis was adopted to evaluate the relationship between miR-185-5p and ABCC1. p < 0.05 was considered significant difference. All experiments were repeated at least three times.

Results

MiR-185-5p was Downregulated in A549/DDP Cells

To define the role of miR-185-5p in human lung cancer cisplatin resistance, the expression levels of miR-185-5p in human lung cancer cell line (A549 cells) and cisplatin-resistant cell line (A549/DDP cells) were detected by real-time PCR. As shown in Figure 1, the expression level of miR-182 in A549 cells was significantly higher than that in A549/DDP cells (p < 0.05).

MiR-185-5p Suppressed A549/DDP Cell Proliferation

To assess the effect of miR-185-5p, miR-185-5p inhibitor and the negative control oligonucleotides were transfected into A549/DDP cells. Transfection of cells with miR-185-5p inhibitor obviously suppressed miR-185-5p level and promoted cell proliferation compared with the control cells. MiR-185-5p mimics and related negative control were transfected into A549/DDP cells. The results showed that miR-185-5p mimics significantly upregulated miR-185-5p expression and weakened cell growth compared with control group (Figure 2).

MiR-185-5p promoted A549/DDP cell apoptosis induced by cisplatin

To evaluate the effect of miR-185-5p on cell apoptosis, miR-185-5p mimics or inhibitor were transfected with A549/DDP cells. TUNEL

Figure 1. MiR-185-5p was down-regulated in A549/DDP cell line compared to that in A549 cell line. *p < 0.05, compared with A549 cells.
MiR-185-5p Targeted ABCC1 in A549/DDP Cells

MiR-185-5p showed complimentary pairing with the 3'-UTR of ABCC1 (Figure 4A). We designed a sequence with 3'-UTR mutation of ABCC1. A549/DDP cells were transfected with miR-185-5p inhibitor or mimics. Dual-luciferase assay revealed that the relative luciferase activity representing 3'-UTR of ABCC1 was markedly enhanced after transfection with miR-185-5p inhibitor, and was declined in A549/DDP cells transfected with miR-185-5p mimics (Figure 4B). The ABCC1 protein level was increased in miR-185-5p-suppressed cells, and reduced in miR-185-5p-overexpressed cells compared with control (Figure 4C). Moreover, ABCC1 expression exhibited significant negative correlation with miR-185-5p level (p < 0.05, Figure 4D).

Discussion

Although chemotherapeutic agents are widely used in the treatment of lung cancer, the efficacy is often limited by the congenital or acquired drug resistance. As one of the first-line chemo-
MicroRNA-185-5p modulates chemosensitivity

Cisplatin is a platinum-based compound that induces cell apoptosis via binding with DNA. Cisplatin treatment usually causes the development of drug resistance, resulting in therapeutic failure. However, the molecular mechanism of cisplatin chemoresistance is poorly understood despite tremendous efforts. Factors that mediate the sensitivity of NSCLC cells to cisplatin may be treated as the biomarkers of drug response or targets for therapy.

MiRNAs are considered to be either tumor suppressors or oncogenes through targeting oncogenes or tumor suppressor genes during tumorigenesis and development of cancers. MiR-185-5p has been thought to play an important role in multiple cell regulatory process. It was found that miR-185-5p is involved in the regulation of sterol regulatory element-binding protein (SREBP2) by the core protein in liver cancer. Also, miR-185-5p has been considered as a biomarker for various tumors, and it could be detected in plasma or peritoneal fluid. Furthermore, the introduction of miR-185-5p into cisplatin-resistant squamous cell carcinoma, which is unable to phosphorylate ΔNp63α, render these cells more sensitive to cisplatin treatment, indicating the potential role of miR-185-5p in cisplatin resistance. However, there is still a lack of investigation about the influence of miR-185-5p in NSCLC.

Previous studies demonstrated that the acquired drug resistance of cancer cells is related to the deregulation of some miRNAs, such as miR21, miR-503, miR-181a, miR134 and miR-620. Besides, a recent study has been proved that the tumor suppressor in NSCLC participates in regulating cisplatin sensitivity in non-small lung cancer cells via PDCD4 inhibition.
suggesting that suppression of miR-141 might be a therapeutic method to overcome cisplatin resistance in clinical practice. In this study, to explore whether the downregulated miR-185-5p was involved in NSCLC cells resistance to cisplatin, we transfected miR-185-5p inhibitor and mimics into A549/DDP cells. MTT assay showed that the cell growth of miR-185-5p-overexpressed cells was significantly slower than control cells. Moreover, TUNEL assay and apoptosis-related factors detection demonstrated that miR-185-5p transfection obviously enhanced A549/DDP cell apoptosis induced by cisplatin, suggesting that downregulation of miR-185-5p may be involved in chemoresistance of NSCLC cells to cisplatin.

In NSCLC tissues, many onco-miRs/tumor suppressor target or tumor suppressor-miRs/onco-target pathways have been reported to participate in the tumorigenesis of lung cancer, such as miR-99b/FGFR3 axis, miR7/BCL2 axis, miR-192/RB1 axis, miR-101/EZH2 axis, and miR-196/HOXA5 axis 28-32. However, miRNA/target network was so complex that more and more miRNA/target axis needs to be clarified in NSCLC. In the present study, A549 cells were transfected with miR-185-5p inhibitor or mimics. The ABCC1 mRNA and protein were increased in miR-185-5p-suppressed cells, whereas reduced in miR-185-5p-upregulated cells compared with control cells, indicating that miR-185-5p was a negative regulator of ABCC1. Furthermore, we found that ABCC1 expression was negatively correlated with the miR-185-5p level in A549/DDP cells. These results suggested that miR-185-5p in combination with cisplatin therapy may be a method to reverse chemotherapeutic resistance. However, further research is needed to investigate whether the expression level of miR-185-5p in tumor tissue and plasma might be used to predict platinum-based chemotherapy response in NSCLC patients.

Conclusions

The results of the present study demonstrate that downregulation of miR-185-5p may participate in chemoresistance of NSCLC cells to cisplatin via suppressing ABCC1. This finding indicates that overexpression of miR-185-5p may be a useful therapeutic strategy for the treatment of NSCLC.
MicroRNA-185-5p modulates chemosensitivity

Conflicts of Interest
The Authors declare that they have no conflict of interests.

References


4703

