miR-181 regulates cisplatin-resistant non-small cell lung cancer via downregulation of autophagy through the PTEN/PI3K/AKT pathway

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Abstract. A number of miRNAs have been found to be abnormally expressed or mutated in numerous cancers and thus, are considered to act as oncogenes or tumor suppressor genes. The aim of the present study was to investigate the effect of miR-181 on cisplatin-resistant non-small cell lung cancer (NSCLC). In patients with cisplatin-resistant NSCLC, miR-181 expression was found to be markedly decreased. In addition, in the cisplatin-resistant human lung adenocarcinoma cell line A549/DDP, miR-181 downregulation promoted cell growth and metastasis and inhibited cell apoptosis, whereas miR-181 overexpression exerted the opposite effects. Furthermore, miR-181 downregulation suppressed LC3 and ATG5 protein expression in A549/DDP cells through suppression of the PTEN/PI3K/AKT/mTOR pathway, whereas miR-181 overexpression recovered LC3 and ATG5 protein expression by promoting PTEN/PI3K/AKT/mTOR signaling. In turn, PTEN inhibitors reduced the anticancer effects of miR-181 overexpression on A549/DDP cell growth via the regulation of autophagy through the PI3K/AKT/mTOR pathway. Therefore, miR-181 may be a novel and important regulator of cisplatin-resistant NSCLC by serving a role in the regulation of apoptosis, as an established rate-limiting miRNA target.

Introduction

Non-small cell lung cancer (NSCLC) is the most common pathological type of lung cancer, accounting for 80-85% of total lung cancer cases (1). The majority of NSCLC patients have already developed multiple extrapulmonary metastases at diagnosis due to the typically occult nature of disease onset (2). Chemotherapy is the preferred main treatment for such patients (2) and plays an important role in reducing tumor load, relieving patient symptoms, improving quality of life and prolonging survival (3).

As a common cytotoxic antitumor drug, cisplatin has been used as the first-line chemotherapeutic agent for NSCLC over the past several decades (4). Cisplatin promotes cell apoptosis through DNA injury and altering cell metabolic state (4). However, resistance to cisplatin severely affects the effectiveness of chemotherapy. In addition, cisplatin is inevitably associated with common adverse reactions, including alopecia, nausea, vomiting, bone marrow suppression and liver function impairment (5).

Chemoresistance includes primary and acquired resistance. Primary resistance is the manifestation of low sensitivity or insensitivity at the first application of a certain chemotherapeutic drug. This type of chemoresistance may be attributed to the tumor cell characteristics (6). Acquired resistance is described as tumor cells exhibiting lowered sensitivity to a certain chemotherapeutic drug after several applications and is a common type of drug resistance in the clinical setting (7).

The PI3K/AKT/mTOR signaling pathway may stimulate cell growth at multiple phases of the cell cycle. Furthermore, it can control cellular processes through its downstream targets, thus initiating and regulating cancer development (8). Activation of this signaling pathway eventually leads to inhibition of cancer cell autophagy and promotes cancer cell development (9). In addition, the PI3K/AKT/mTOR signaling pathway renders cancer cells resistant to multiple cancer treatments, leading to a poor prognosis for numerous types of cancer (8). The autophagy-associated gene phosphatase and tensin homolog (PTEN) is a key factor in regulating autophagosome formation (10). Namely, PTEN is a positive regulatory molecule of autophagy that blocks the inhibitory effect of PI3K/PKB on autophagy, thereby activating autophagy (10).

In recent years, a new class of small-molecule RNAs termed microRNAs (miRNA/miRs) have been identified in a number of eukaryotes. miRNAs may promote target mRNA
degradation or inhibit protein translation and regulate endogenous gene expression. The key roles of miRNAs in gene regulation are generally achieved through complete or incomplete complementary pairing with the 3' untranslated region of their target mRNA (11) and increasing evidence indicates that miRNAs may regulate gene expression, modification, transcription and translation (12). Additionally, recent studies have uncovered that miRNA polymorphism, abnormal miRNA expression and gene expression that affects drug absorption may result in continuous tumor drug resistance. Furthermore, metabolism and distribution channels, as well as receptors specifically participating in clinical functions, may also exert the aforementioned effects (12). Notably, Liu et al (13) suggested that miR-181b modulates glioblastoma cell growth and apoptosis and Su et al (14) reported that miR-181 regulates myeloid differentiation and the development of acute myeloid leukemia. Therefore, the present study aimed to elucidate the effect of miR-181 on cisplatin-resistant NSCLC.

Materials and methods

RNA extraction and quantitative reverse transcription PCR. Cisplatin-induced NSCLC in patients (n=6) were collected, and cisplatin-induced NSCLC and paracarcinoma tissue were collected and saved at -80˚C. The present study was approved by the Ethics Committee of The People's Hospital of Bozhou (n. PHBZ/L-2015008). All patients provided informed written consents to participate in this study. The characteristics of the patients involved in the present study are shown in Table I. Total RNA was extracted from serum and cell using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription was carried out into cDNA using the M-MLV Reverse Transcription system (Takara Biotechnology Co., Ltd., Dalian, China). The ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) was used to perform qPCR with the SYBR-Green PCR kit (TransGen Biotech, Inc., Beijing, China). PCR primers used were as follows: miR-181: 5'-GCGGCAACATTCAACGCTGTCGGTGAGT-3' and 5'-GTCGTATCCAGTGCGTCGGTGGAGTCGGCAATTG-3'; u6: 5'-CTCGCTTCGGCAACACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. PCR was performed at 95˚C for 10 min, followed by 40 cycles of 95˚C for 25 sec and 60˚C for 30 sec. The relative quantification was calculated using the 2-ΔΔCt method.

Cell culture and transfections. A549/DDP cells were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 800 ng/ml cisplatin at 37˚C in humidified 5% CO2 atmosphere. miR-181: AACA uuCAACGCuGuCGGuGAGu, anti-miR-181: ACCA uCGACC GuuGAuuGuACC and negative control: uuCuCCGAA CGuGuCACGuTT mimics were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Transfection with 100 nM of mR-181, anti-miR-181 and negative control mimics was performed with Lipofectamine 2000 reagent (Invitrogen).

MTT assay and apoptosis assay. After transfections for 24, 48 and 72 h, MTT (5 mg/ml, 20 µl) was added to the cell and incubated for 4 h at 37˚C. Dimethyl sulfoxide (DMSO) was added to the cell and incubated for 20 min at 37˚C. Absorbance was detected at 492 nm using a multi-well plate reader.

After transfections for 72 h, the cells were washed with phosphate-buffered saline (PBS) and resuspended in 100 µl binding buffer. The cells were stained with 5 µl of Annexin V-fluorescein isothiocyanate and 5 µl of propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at room temperature. Apoptosis rate was detected using BD FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis. Total proteins were extracted from cells using RIPA assay (Pierce, Rockford, IL, USA) and quantified using the BCA method (Pierce). Protein (50 µg) was separated on 8-10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Amersham Biosciences, Chicago, IL, USA). The membrane was blocked with 5% skim milk powder in TBST and incubated with Bax, LC3, ATGS, PTEN, PI3K, p-Akt, p-mTOR and GAPDH (1:500; Santa
Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by incubation with secondary antibody (1:2,000; Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were revealed with a chemiluminescence kit (Pierce) and analyzed using Gel-Pro Analyzer software (Version 4.0, Media Cybernetics, Rockville, MD, USA).

**Statistical analysis.** All values are depicted as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).

**Results**

**miR-181 expression in patients with cisplatin-resistant NSCLC.** We first investigated miR-181 expression in patients with cisplatin-resistant NSCLC. As demonstrated in Fig. 1, miR-181 expression was obviously decreased in patients with cisplatin-resistant NSCLC, compared with that in the normal group. These results demonstrated that miR-181 expression may be a significant factor in cisplatin-resistant NSCLC patients.

**Decreased miR-181 expression promotes the growth and metastasis of A549/DDP cells.** We subsequently investigated whether miR-181 affects cell growth and metastasis in the cisplatin-resistant human lung adenocarcinoma cell line A549/DDP. As demonstrated in Fig. 2A, anti-miR-181 mimics decreased miR-181 expression in A549/DDP cells compared with the control group, whereas miR-181 downregulation promoted the growth and metastasis of A549/DDP cells compared with the control group (Fig. 2B and C).

**miR-181 downregulation inhibits apoptosis and caspase-3/9 activity in A549/DDP cells.** Furthermore, miR-181 downregulation inhibited apoptosis and caspase-3/9 activity in A549/DDP cells compared with the control group, whereas miR-181 upregulation promoted caspase-3/9 expression to induce apoptosis in A549/DDP cells (Fig. 3A and B).

**miR-181 overexpression inhibits the growth and metastasis of A549/DDP cells.** Cell growth and metastasis were measured in A549/DDP cells to determine whether they are affected by miR-181 overexpression. As depicted in Fig. 4, miR-181 overexpression inhibited the growth and metastasis of A549/DDP cells.
miR-181 overexpression induces apoptosis and caspase-3/9 activity in A549/DDP cells. miR-181 overexpression was found to induce apoptosis and caspase-3/9 activity in A549/DDP cells compared with the control group, indicating that miR-181 overexpression may suppress cancer cell growth in patients with cisplatin-resistant NSCLC.
compared with the control group (Fig. 5). Therefore, miR-181 overexpression may induce apoptosis of cisplatin-resistant NSCLC cells, although the underlying mechanism requires further investigation.

miR-181 downregulation reduces autophagy in the A549/DDP cells. It was demonstrated that miR-181 downregulation reduced autophagy and suppressed LC3 and ATG5 protein expression in the A549/DDP cells compared with the control group (Fig. 6), which may thus promote tumor progression.

miR-181 downregulation reduces PTEN/PI3K/AKT/mTOR pathway signaling. miR-181 downregulation reduced PTEN protein expression and induced PI3K, p-AKT and p-mTOR protein expression in A549/DDP cells compared with the control group (Fig. 7). These data provide evidence that miR-181 knockdown may promote the proliferation and migration of A549/DDP cells.

miR-181 overexpression promotes autophagy in A549/DDP cells. Overexpression of miR-181 promoted autophagy and induced LC3 and ATG5 protein expression in A549/DDP cells compared with the control group (Fig. 8).

miR-181 overexpression promotes PTEN/PI3K/AKT/mTOR pathway signaling. The overexpression of miR-181 promoted PTEN protein expression and suppressed PI3K, p-AKT and p-mTOR protein expression in A549/DDP cells compared with the control group (Fig. 9).
PTEN inhibitors reduce the anticancer effects of miR-181 on the PTEN/PI3K/Akt/mTOR pathway in A549/DDP cells. To further confirm these results, we used a PTEN inhibitor to reduce the PTEN/PI3K/Akt/mTOR pathway signaling in A549/DDP cells induced by miR-181 overexpression. As depicted in Fig. 10, the PTEN inhibitor reduced the anticancer effects of miR-181 overexpression on the promotion of PTEN protein expression and inhibition of PI3K, p-AKT and p-mTOR protein expression in A549/DDP cells, compared with the miR-181 overexpression group by (A) western blot analysis, and (B-D) by statistical analysis. Control, control negative group; anti-miR-181, miR-181 downregulation group; anti-miR-181 + PTEN inhibitor, miR-181 downregulation + PTEN inhibitor group. *p<0.01 vs. paracarcinoma tissue group; ###p<0.01 vs. miR-181 downregulation group.

PTEN inhibitors reduce the anticancer effects of miR-181 on the growth and metastasis of A549/DDP cells. PTEN inhibitor reduced the anticancer effects of miR-181 on (A) cell growth and (B) metastasis of A549/DDP cells. Control, control negative group; anti-miR-181, miR-181 downregulation group; anti-miR-181 + PTEN inhibitor, miR-181 downregulation + PTEN inhibitor group. *p<0.01 vs. paracarcinoma tissue group; ###p<0.01 vs. miR-181 downregulation group.

PTEN inhibitors reduce the anticancer effects of miR-181 on apoptosis and caspase-3/9 activity in A549/DDP cells. PTEN inhibitors reduced the elevated levels of apoptosis and caspase-3/9 activity in A549/DDP cells induced by miR-181 overexpression, compared with miR-181 overexpression group (Fig. 12).

PTEN inhibitors reduce the anticancer effects of miR-181 on the autophagy of A549/DDP cells. Finally, PTEN inhibitors reduced autophagy and suppressed LC3 and ATG5 protein expression in A549/DDP cells following miR-181 overexpression, compared with the miR-181 overexpression group.
group (Fig. 13). In addition, PTEN/PI3K/AKT/mTOR pathway signaling was inversely correlated with miR-181 levels in patients with cisplatin-resistant NSCLC.

Discussion

NSCLC is the most common pathological type of lung cancer, and due to its typically occult onset, the majority of NSCLC patients present with distant metastasis at the time of diagnosis. As a result, chemotherapy is considered as the main treatment for such patients at present (15). Cisplatin is a classical chemotherapeutic drug used in the first-line treatment of NSCLC, which can effectively reduce tumor load in patients, improve their quality of life and prolong their survival (16). However, a proportion of patients may be sensitive to cisplatin during the first treatment, while others gradually develop drug resistance (16). To overcome this, the dose of cisplatin must be increased, leading to more severe toxic side effects and substantial reductions in patient quality of life and clinical prognosis (2). Therefore, the molecular and biological mechanisms underlying the development of cisplatin resistance must be investigated. In particular, it is crucial to elucidate the cause of tumor tolerance to chemotherapy in order to individualize the treatment of NSCLC patients (17). The present study demonstrated that miR-181 expression was effectively decreased in patients with cisplatin-resistant NSCLC compared with a normal group, suggesting that miR-181 is an important indicator of cisplatin-resistant NSCLC. Pichler et al. (18) reported that miR-181a was associated with poor clinical outcome in patients with colorectal cancer.

Radiotherapy and chemotherapy are considered effective methods for the treatment of NSCLC in clinical practice. Ionizing radiation and chemotherapeutic drugs may induce NSCLC cell death through multiple signaling pathways (19). Research on the role of type I programmed cell death, namely apoptosis, in the combined effects of radiochemotherapy has made progress. However, our understanding of the role of type II programmed cell death, namely autophagy, remains limited (19). Under physiological conditions, autophagy is a process that involves the degradation of autologous long-life proteins and waste organelles through the formation of autophagosomes and participation of lysosomes. In addition, autophagy serves to recycle amino acids as well as proteins to maintain cellular homeostasis (20). Excessive cell stimulation, long stimuli duration and large-scale autophagic degradation products promote programmed cell death (20). In the present study, miR-181 downregulation was found to promote
cell growth and metastasis and to inhibit cell apoptosis in A549/DDP cells, whereas miR-181 overexpression inhibited cell growth and metastasis and induced apoptosis of the cells.

Our understanding of the importance of autophagy and apoptosis in the development and prevention of human disease has improved. In addition, the association between the two has been investigated (21). Notably, it has been observed that apoptosis-regulating genes, such as Bcl-2 family members (Bcl-2 and CASP9) can also regulate autophagy (22), while autophagy-associated proteins, such as Atg5, beclin 1 and Atg4D, are also involved in cell apoptosis (22). Additionally, caspase-8 activation may promote apoptosis and the autophagic degradation of caspase-8 aids to suppress the apoptotic death of mammalian cells. The effect of autophagy on pre-death cells is also associated with apoptosis (23). In other words, autophagy may be an upstream event prior to apoptosis, which may be essential for apoptosis initiation (23). The present study indicated that miR-181 downregulation suppressed LC3 and ATG5 protein expression, whereas miR-181 overexpression restored LC3 and ATG5 protein expression in A549/DDP cells. Wu et al (24) reported that miR-181 inhibits proliferation and promotes apoptosis of chondrocytes in osteoarthritis through suppression of PTEN.

The PI3K/AKT/mTOR signaling pathway is activated in multiple cancers and the activated signaling can promote tumor cell differentiation, growth and survival (9). Mechanisms activating PI3K/AKT/mTOR signaling include a loss of function of the tumor inhibitor PTEN, proliferation and mutation of PI3K and/or AKT, activation of growth factor receptors, and stimulation of carcinogens (25). Signals are transmitted to mTOR, which can regulate protein translation after the signaling pathway is activated. mTOR is a downstream target gene of AKT, which is generally considered to be a regulator of autophagy through regulating a series of autophagy-associated genes (25). Moreover, miR-181 overexpression promoted, while miR-181 downregulation reduced, PTEN/PI3K/AKT/mTOR pathway signaling in A549/DDP cells. Similarly, Strotbek et al (26) reported that miR-181 promotes AKT signaling in luminal breast cancer.

PTEN deficiency can be found in tumor patients. In particular, it was discovered that the expression rate of PTEN in NSCLC tissue was lower compared with that in healthy individuals. Thus, it may be hypothesized that PTEN mutations lead to activation of the AKT pathway and inhibition of autophagy, and play a role in tumor initiation and progression (27,28). Similarly, it has been demonstrated that expression of PTEN protein in NSCLC is lower compared with that in healthy individuals, from which it was suggested that PTEN tumor regulation may be mediated through the regulation of autophagy (29). Thus, alterations in PTEN expression may directly affect tumorigenesis through autophagy. This likely occurs via the catalysis of PI3P to PI2P to inhibit AKT activity, which would thereby relieve the inhibition of the PI3K/AKT/mTOR pathway on autophagy (30).

In the present study, PTEN inhibitors reduced the anticancer effects of miR-181 overexpression on the growth of A549/DDP cells, likely via the regulation of autophagy through the PI3K/AKT/mTOR pathway.

In conclusion, the present study demonstrated that miR-181 overexpression inhibited cell growth and metastasis and induced apoptosis and autophagy in A549/DDP cells through the PTEN/PI3K/AKT/mTOR pathway. Thus, miR-181 may help elucidate the potential molecular mechanisms underlying chemotherapy drug resistance in NSCLC and provide a basis for novel therapeutic strategies for the treatment of NSCLC in the clinical setting.

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