Inhibition of miR-141 reverses cisplatin resistance in non-small cell lung cancer cells via upregulation of programmed cell death protein 4

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Abstract. – OBJECTIVE: MicroRNAs are a class of essential regulators in cancer, and previous studies have shown that miR-141 is a tumor suppressor in non-small cell lung cancer (NSCLC). However, it is still unknown whether it regulates chemosensitivity. We aimed to investigate the role of miR-141 in cisplatin resistance in NSCLC cells.

MATERIALS AND METHODS: MiR-141 expression in A549 and A549/DDP cell lines have been quantified by real-time PCR. Protein level of PDCD4 and caspase-3 have been determined by Western blot analysis. Drug sensitivity and apoptosis have been determined by MTT assay and TUNEL assay, respectively. Luciferase activity assay was employed to validate the relationship between 3'UTR of PDCD4 mRNA and miR-141.

RESULTS: We observed that miR-141 expression was significantly up-regulated in cisplatin-resistant A549/DDP cells compared with the parental cell line A549; and PDCD4, an important apoptosis regulator, was found to be down-regulated. Luciferase activity assay and Western blot analysis confirmed that PDCD4 is a direct target of miR-141. Inhibition of miR-141 in A549/DDP cells markedly increased cisplatin sensitivity and apoptosis, which was partially abrogated by PDCD4 inhibition, indicating that PDCD4 is a functional target of miR-141 in of the regulation of cisplatin sensitivity.

CONCLUSIONS: Our data showed that miR-141 participates in regulating cisplatin sensitivity in non-small lung cancer cells via PDCD4 inhibition, and suppression of miR-141 might be a therapeutic method to overcome cisplatin resistance in clinical practice.

Key Words:
 Cisplatin resistance, miR-141, PDCD4, Apoptosis.

Introduction

Platinum-based chemotherapy is commonly applied in the adjuvant treatment of non-small cell lung cancer (NSCLC) after surgical resection. As the most widely used platinum compound in cancer therapy, cisplatin (DDP) functions to eliminate cancer cells by binding to DNA double helix and causes DNA crosslinking, which further leads to apoptosis[1-3]. Despite the high initial responsiveness to cisplatin, NSCLC cancer cells often develop acquired resistance after multiple courses of treatment, which greatly limits the therapeutic efficacy of cisplatin. Methods for reversing the cisplatin resistance are still lacking, which underlines the significance of a better knowledge in understanding the mechanisms of cisplatin resistance. Attenuation in the apoptotic activity is one of the key reasons for drug resistance[3,4], and programmed cell death protein 4 (PDCD4) plays a central role in the regulation of apoptosis. It has been proposed by several studies that PDCD4 is implicated in the development of chemoresistance in multiple cancers[5-8], suggesting that modulation of PDCD4 might be applied to overcome cisplatin resistance in lung cancers. Nevertheless, the regulatory mechanism of PDCD4 is still to be fully investigated.

MicroRNAs are a class non-coding short RNAs that are about 18-25 nt in length and are ubiquitously expressed in eukaryotic cells[9]. The main action of microRNA is through its base-pair binding with the 3'untranslated region (UTR) of the targeted mRNAs, which then fa-

cilitates mRNA degradation or blocks the protein translation process. Aberrant expressions of microRNAs are demonstrated to be broadly implicated in the development and progression of lung cancers[10]. Based on the published literature, the diverging regulatory roles of miR-141 in different cancers suggested the fact that the biological functions of miR-141 are still enigmatic: in gastric cancer, the miR-141 expression is reduced and serves as a potent tumor suppressor[11,12]; in colorectal cancer, the miR-141 reportedly acts as a functional oncogene[13]. Importantly, it was found in a clinical investigation that high miR-141 is associated with poor prognosis in lung adenocarcinoma[14]. *In vitro* study also suggests that miR-141 positively regulates the proliferation of lung cancer cells[15]. However, it is not yet clear whether miR-141 participates in the regulation of cisplatin sensitivity.

In the present study, we analyzed the expression of miR-141 in cisplatin-resistant non-small lung cancer cells, and we also investigated the mechanism of miR-141 in the regulation of cisplatin sensitivity, PDCD4 was identified as a novel functional target of miR-141. Our evidence suggests that miR-141 may confer cisplatin resistance in non-small lung cancer cells, and targeting miR-141 might be a potential therapeutic method.

Materials and Methods

Cell Lines

The human lung adenocarcinoma cell line A549 and the cisplatin-resistant variant A549/DDP was purchased from Keygen Biotechnology Co. Ltd (Nanjing, China). Cells were cultured in DMEM supplemented with 10% fetal calf serum (Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified CO_2 incubator. For the maintenance of the cisplatin-resistant phenotype, A549/DDP was cultured in the above-described medium containing 10 μmol/L cisplatin.

Transfection

Cells grown at 70% confluence were used for transfection. Cells were transfected with negative control (NC), miR-141 mimics or miR-141 inhibitor (Genepharma, Shanghai, China) using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent. Knockdown of PDCD4 was performed by transfection of the specific siRNAs, the scramble control siRNA and the

siRNA for PDCD4 were synthesized by Genepharma. 48h after transfection, the cells were used for further analysis.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

A549/DDP cells were equally plated into a 96-well culture plate, after adherence, cells were transfected with microRNAs or siRNAs for 48h followed by treatment with cisplatin at various final concentrations (0, 10, 20, 40, 80 or 160 μmol/L) for 48h. Next, MTT solution (5 mg/mL) was added to the culture medium, and cells were allowed to incubate with MTT solution for 4h. The purple color of the MTT product was developed by DMSO and the absorbance value at 490nm was acquired using a spectrophotometer. All values were normalized to that of the control group and the half maximal inhibitory concentration (IC50) was estimated by the curves prepared in each experiment. All independent experiments were performed for three times.

Real-time PCR

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The quality of RNA was examined by electrophoresis. The first strand of cDNA was synthesized with a PrimeScript™ RT-PCR Kit (Takara, Dalian, China). The PCR primer sets for miR-141 and U6 snRNA (used as internal control) were provided by Genepharma. And the primer sets for PDCD4 and β-actin (used as internal control) quantification were synthesized by Sangon Biotechnology (Shanghai, China). The detected genes were amplified on an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Premix ExTagTM (Takara). All the experiments were performed according to the manufacturers' protocols.

Apoptosis Assay

Cells transfected as described above were then treated with 15 μmol/L cisplatin. Cells were collected 48h after cisplatin treatment, and the apoptosis rate was determined by an In situ cell death detection kit (TUNEL assay, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Western Blot Analysis

Cells were collected by trypsin and washed with PBS, and were then subjected to SDS-lysis

buffer containing protease inhibitor cocktail (Beyotime, Shanghai, China). Then the proteins were loaded onto the SDS-PAGE gel for electrophoresis. Proteins were then transferred onto PVDF membranes, followed by blocking with 5% skimmed milk for 2h. Membranes were then incubated with primary antibodies which were diluted with PBST. After a series washes with PBST on the following day, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Zhongshanjinqiao, Beijing, China) and then visualized by ECL Western Blotting Detection Reagent (Beyotime). All the antibodies used in this study were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The dilution for anti-PDCD4 or anti-active caspase-3 was 1:500, and the dilution for anti-β-actin was 1:1000. All experiments were independently conducted for three times.

Luciferase Activity Assay

The luciferase reporter containing the putative binding site of miR-141 was constructed by subcloning the PDCD4 3'UTR sequence into the 3'UTR site of the pGL3-basic vector. Luciferase activity assay was performed using A549 cells, cells plated in 24 well plates were co-transfected with pGL3-PDCD4 3'UTR, pRL-TK (expresses renilla luciferase as the internal control) and

miR-141 or miR-141 inhibitor. 24h after transfection, the luciferase activity was measured on a Dual Luciferase Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Data were expressed as means ± SD (standard deviation). The difference between two groups was compared using t-test. For more than three groups, one-way analysis of variance (ANOVA) was used, the difference between two groups in ANOVA analysis was compared by LSD test. $p < 0.05$ was considered as statistical significance.

Results

MiR-141 is Downregulated in A549/DDP Cells and is Inversely Correlated with PDCD4 Expression

In the present study, we compared the expression of miR-141 in normal lung adenocarcinoma cell line A549 and the cisplatin-resistant variant cell line A549/DDP. As shown in Figure 1A, miR-141 expression was significantly up-regulated for nearly 3-fold. PDCD4, a tumor suppressor involved in apoptosis, was found to be down-regulated in A549/DDP cells correspondingly (Figure 1B).
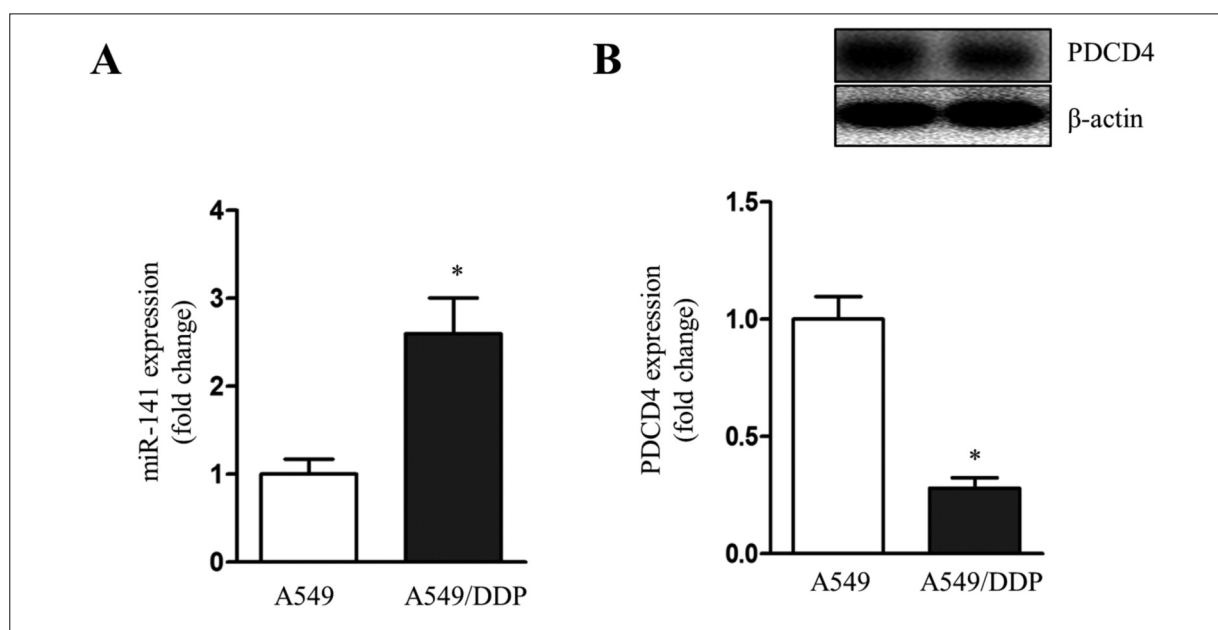

Figure 1. MiR-141 is downregulated in A549/DDP cells and is inversely correlated with PDCD4 expression. Lung cancer cell line A549 and its cisplatin-resistant variant cell lineA549/DDP were used for realtime PCR and Western blot analysis, A549/DDP cells exhibited increased **(A)** miR-141 expression and decreased **(B)** PDCD4 expression. *$p < 0.05$ *vs*. A549.

PDCD4 is a Target of miR-141

To examine the relationship between PDCD4 and miR-141 in A549 cells, we next searched the TargetScan database (www.targetscan.org) and found that PDCD4 is potentially targeted by miR-141 (Figure 2A). Luciferase reporter con- taining the predicted binding site in the PDCD4 3'UTR was constructed, and it was then trans- fected into A549 cells along with miR-141 or miR-141 inhibitor as described above. The over- expression and knockdown of miR-141 were confirmed by real-time PCR (Figure 2B). We
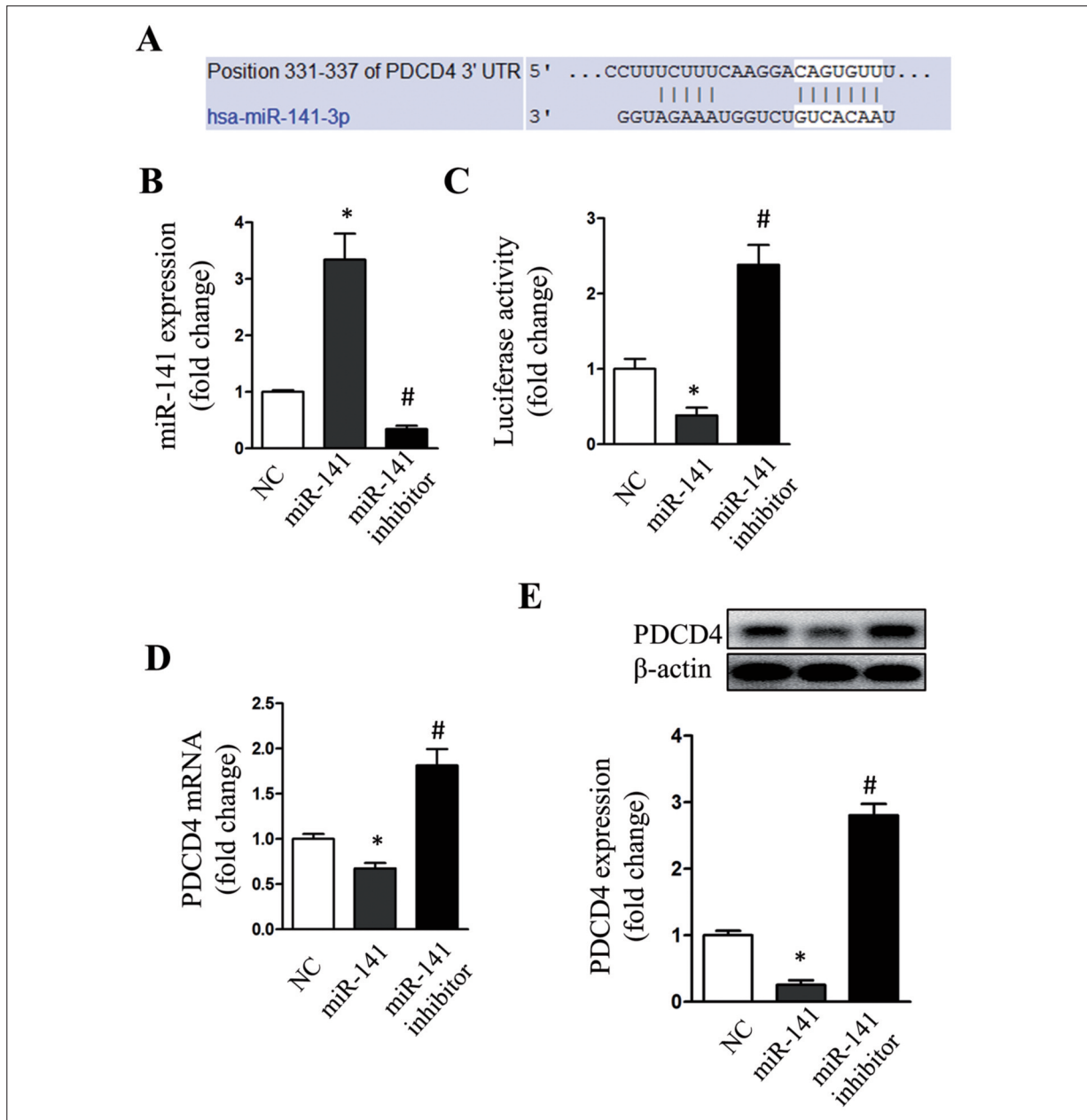

Figure 2. PDCD4 is a target of miR-141. **A,** TargetScan database predicts that PDCD4 is a potential target of miR-141. **B,** The effect of the miR-141 mimics and inhibitor on miR-141 expression. MiR-141 mimics increased miR-141 expression, whereas miR-141 inhibitor decreased miR-141 expression, U6 was used as internal control. **C,** The effect of miR-141 mimics and inhibitor on pGL3-PDCD4 luciferase activity in A549 cells. MiR-141 inhibited luciferase activity, whereas miR-141 in- hibitor exerted an opposite effect. **D,** The effect of miR-141 and miR-141 inhibitor on PDCD4 mRNA level.MiR-141 transfec- tion decreased PDCD4 mRNA level, and miR-141 inhibitor exerted an opposite effect. **E,** Similar results were obtained when comparing the PDCD4 protein level after transfection. β-actin was an internal control. * and #, $p < 0.05$ *vs*. NC.

found that the luciferase activity was significantly inhibited when co-transfection with miR-141, and inhibition of endogenous miR-141 resulted in an increase in luciferase activity (Figure 2C). Importantly, real-time PCR and Western blot analysis revealed that both the mRNA and the protein were inhibited by miR-141, and inhibition of miR-141 resulted in an increase in PDCD4 expression (Figure 2D and E). These data indicate that PDCD4 serves as a direct target of miR-141.

MiR-141 Regulates Cisplatin Sensitivity in A549/DDP Cells

To understand the biological function of miR-141 in mediating cisplatin resistance, A549/DDP cells were transfected with miR-141 inhibitor and were further used for cell viability assay. As shown in Figure 3A and B, cells transfected with miR-141 inhibitor exhibited a much more improved cisplatin sensitivity, which is exemplified by the dramatically reduced IC50. We also found that the apoptosis rate was significantly increased
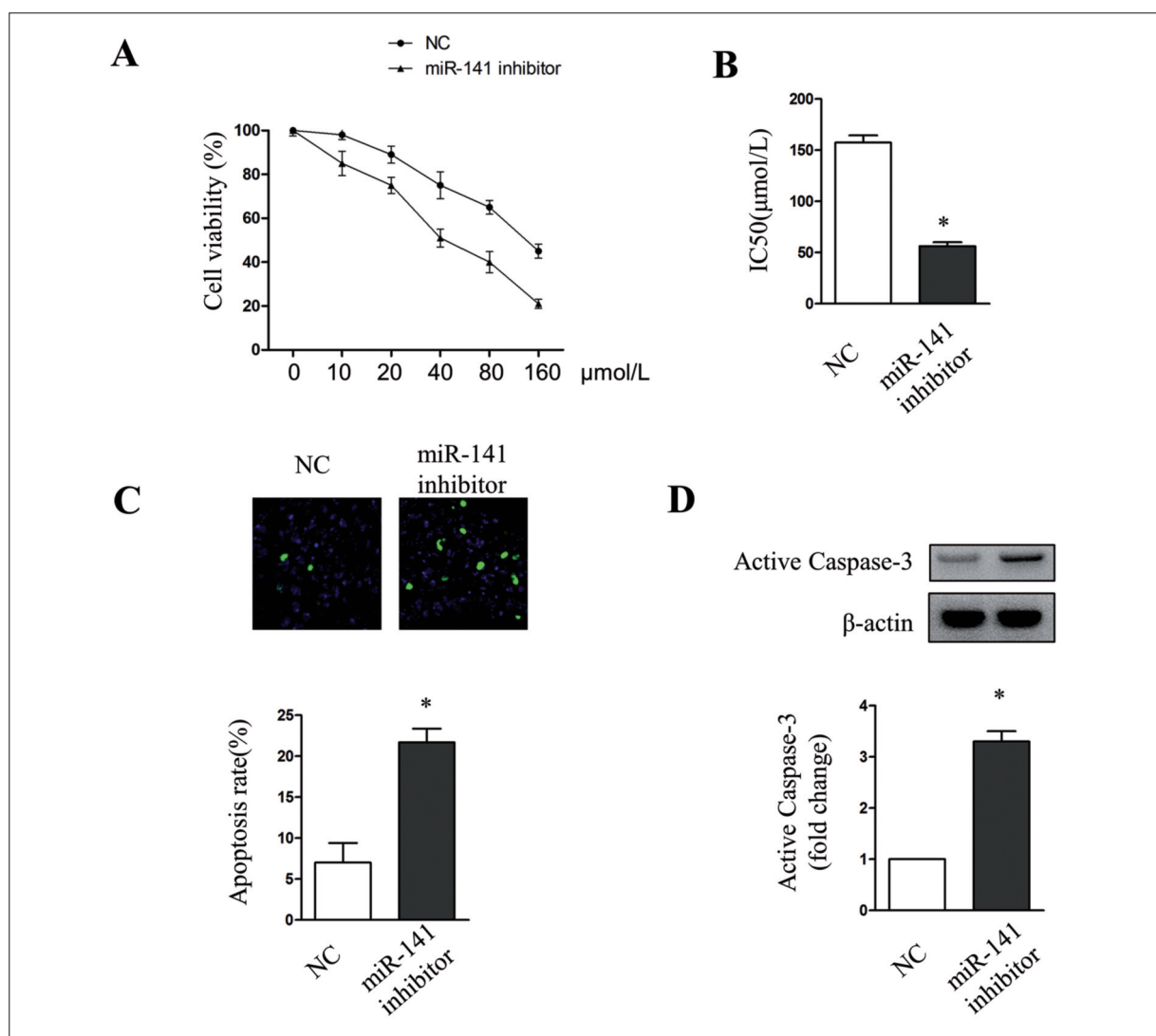

Figure 3. MiR-141 regulates cisplatin sensitivity in A549/DDP cells. **A,** The effect of miR-141 inhibitor on cell survival after cisplatin treatment, A549/DDP cells were transfected with miR-141 inhibitor and then treated with various doses of cisplatin for 48h, cell viability was determined by MTT assay; and **B,** the IC50 value was estimated by the survival curve, miR-141 significantly decreased the IC50 value in A549/DDP cells. **C,** The effect of the miR-141 inhibitor on cisplatin-induced apoptosis after transfection of miR-141 inhibitor in A549/DDP cells, apoptosis was measured by TUNEL assay, and miR-141 inhibitor significantly enhanced apoptosis. **D,** The effect of the miR-141 inhibitor on caspase-3 activation, cells were transfected with miR-141 inhibitor and treated with cisplatin, caspase-3 activation was determined by Western blot analysis, miR-141 inhibitor increased caspase-3 activation. *$p < 0.05$ *vs*. NC.

in miR-141 inhibitor transfected cells after cis-platin treatment (Figure 3C). Moreover, an increase in the expression of apoptosis marker protein (active caspase-3) was detected by Western blot (Figure 3D). These data indicate that miR-141 regulates cisplatin sensitivity by inhibiting apoptosis in human non-small lung cancer cells.

PDCD4 Mediates the Effect of miR-141 on Cisplatin Sensitivity

Given that PDCD4 is a direct target of miR-141, we speculated that the regulatory role of miR-141 in cisplatin resistance is dependent upon PDCD4 inhibition. Cell viability assay revealed that inhibition of PDCD4 by PDCD4 small interfering RNA (siRNA) partially abrogated the effect of miR-141 inhibitor on cisplatin sensitivity (Figure 4A and B), which was accompanied by a decreased expression of the apoptosis marker, active caspase-3, compared with the cells transfected with scramble control (Figure 4C). These data indicate that PDCD4 mediates the effect of miR-141 on cisplatin sensitivity in human non-small lung cancer cells.

Discussion

Increasing evidence has shown that microRNAs are critical regulators in modulating drug sensitivity of cancer cells. To date, a number of microRNAs have been identified to contribute to drug resistance of lung cancer cells. For example, miR-7 regulates paclitaxel sensitivity by modulating EGFR signaling[16]; miR-217 functions as a tumor suppressor and is associated with cisplatin resistance[17]; miR-3127-5p regulates proliferation and invasion and contributes to dasatinib sensitivity in NSCLC cells via Ras/ERK pathway[18]. In the current study, we report for the first time that miR-141 is involved in the regulation of cisplatin sensitivity, and we also established a pro-apoptotic protein, PDCD4, as a novel functional target of miR-141. Therefore, miR-141 might be a novel therapeutic target of cisplatin resistance of lung cancer cells.

Cisplatin is commonly applied in the adjuvant chemotherapy of many cancer types after surgery. However, NSCLC patients often develop low cisplatin sensitivity after a series of drug courses; as a result, larger doses of cisplatin is needed, which constantly yields a high toxicity in these patients. Therefore, it is of great importance to elucidate the underlying molecular mechanism in order to diminish cisplatin resistance. The main anticancer action of cisplatin is to induce programmed cell death via forming DNA adducts within cancer cells. Inability to initiate apoptosis accounts for one of the major reasons for chemoresistance[3]. PDCD4 is a critical regulator in apoptosis initiation; reduced PDCD4 expression was found to be associated with can-
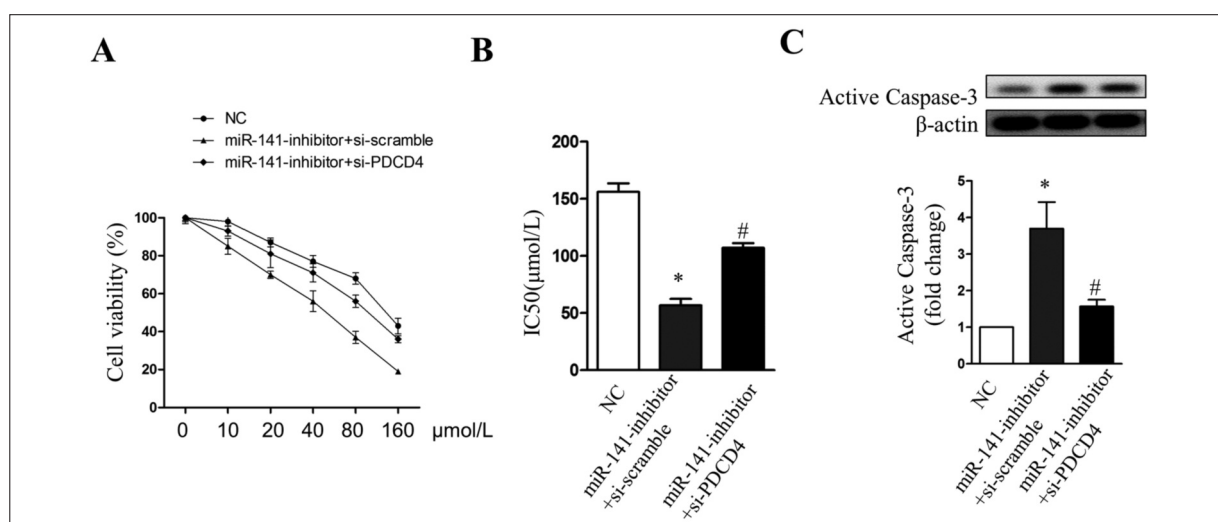

Figure 4. PDCD4 mediates the effect of miR-141 on cisplatin sensitivity. **A,** The effects of miR-141 inhibitor and PDCD4 siRNA on cell survival after cisplatin treatment, cells were treated as described above, PDCD4 inhibition partially abrogated the effect of miR-141 inhibitor. **B,** The IC50 value estimated by the survival curve. **C,** The effect of miR-141 inhibitor and PD-CD4 siRNA on caspase-3 activation, inhibition of PDCD4 partially reduced the activation of caspase-3 by the miR-141 inhibitor. *$p < 0.05$ *vs*. NC, #$p < 0.05$ *vs*. miR-141 inhibitor + si-scramble.

cer clinicopathological parameters and may be used as a prognostic biomarker[19]. In this study, we firstly found that PDCD4 expression was significantly downregulated in cisplatin-resistant variant A549 cell line compared with its parental cell line, suggesting that PDCD4 is involved in regulating cisplatin sensitivity in NSCLC cells. Emerging evidence highlighted the powerful action of the post-transcriptional regulation of apoptosis-related genes by microRNAs in modulating drug sensitivity. Thus, we tested the expression of miR-141 at the same time. Surprisingly, a good correlation between miR-141 and PDCD4 was identified. Target prediction program TargetScan indeed supports the assumption that PDCD4 is regulated by miR-141, which was further experimentally validated using luciferase activity assay and Western blot analysis. Our study reports the first evidence that miR-141 directly regulates PDCD4.

After having evaluated the differential expression of miR-141 in cisplatin-resistant NSCLC cells and established the relationship between PDCD4 and miR-141, we performed functional studies to test whether miR-141/PDCD4 signaling can regulate cisplatin resistance in A549/DDP cells. Previous studies have demonstrated that miR-141 confers cisplatin resistance in ovary cancer cells and esophagus carcinoma cells[20,21] and docetaxel resistance in breast cancer cells[22]. In agreement with these previously published literature, miR-141 exerted a similar function in lung cancer cells. Manipulating the endogenous miR-141 level by miR-141 antisense inhibitor significantly decreased the IC50 value. Using TUNEL assay, we detected an increase in apoptosis rate after cisplatin treatment. Moreover, Western blot analysis revealed a significant activation of caspase-3, which correlated well with the cell viability assay and TUNEL analysis. These results altogether indicated a significant improvement in cisplatin sensitivity after normalization of miR-141 expression, and this effect is most likely due to the enhanced apoptosis. Although the oncogenic role of miR-141 in NSCLC has been recently studied[15], our data reported the effect of miR-141 inhibition on the cellular response to cisplatin for the first time. Intriguingly, a recent clinical study[14] showed that patients with higher miR-141 and miR-200c expression had a shorter overall survival in a cohort of early stage NSCLC patients, this evidence also supported our conclusion that miR-141 has an adverse impact on chemosensitivity and suggested the therapeutic value of targeting miR-141 in clinical practice. The complex miR-mRNA interaction network enables the regulation of multiple mRNAs by a single microRNA. By RNA interfering technology, we further observed that the pro-apoptotic phenotype of miR-141 inhibitor transfected cells was partially abrogated by PDCD4 inhibition, suggesting that PDCD4 acts downstream of miR-141 to contribute to drug resistance in NSCLC. However, it is still to be investigated whether other targets or signaling pathways are involved in the regulatory mechanism of miR-141.

Conclusions

We have showed that miR-141 is up-regulated in cisplatin-resistant NSCLC cells and might contribute to the drug resistance phenotype by suppressing the tumor suppressor PDCD4 in NSCLC. As a consequence, cells were unable to undergo apoptosis properly in response to cisplatin treatment. Normalization of miR-141 by miR-141 inhibitor reverses cisplatin resistance and, thus, may serve as a potential therapeutic method.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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