ABBS

Original Article

Berberine sensitizes ovarian cancer cells to cisplatin through miR-21/PDCD4 axis

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Recent studies have shown that microRNA-21 (miR-21) contributes to tumor resistance to chemotherapy. Interestingly, we have found that berberine could inhibit miR-21 expression in several cancer cell lines. In this study, we investigated whether berberine could modulate the sensitivity of ovarian cancer cells to cisplatin and explored the mechanism. The cisplatin-resistant SKOV3 cells that were incubated with berberine combined with cisplatin had a significantly lower survival than the cisplatin alone group and enhanced cisplatin-induced apoptosis. Berberine could inhibit miR-21 expression and function in ovarian cancer, as shown by an enhancement of its target PDCD4, an important tumor suppressor in ovarian cancer. The results suggested that berberine could modulate the sensitivity of cisplatin via regulating miR-21/PDCD4 axis in the ovarian cancer cells.

Introduction

Epithelial ovarian cancer is one of the most common malignant diseases in women, and the first leading cause of death from gynecological cancers [1,2]. According to statistics, 85–90% of ovarian cancers are epithelial, and more than two-thirds are diagnosed at an advanced stage. Currently, the standard treatment for advanced-stage ovarian cancer is primary cytoreductive surgery, followed by platinum and paclitaxel combination chemotherapy [3]. Although many tumors initially respond to chemotherapy, patients with metastatic and/or relapsed disease continue to have extremely poor survival outcomes [4,5]. So, new treatment modalities need to be developed.

Berberine, the main alkaloid component in Huang Lian and other medicinal herbs, is the most commonly used medicine for centuries in China. Berberine is used as a strong inhibitory drug in the treatment of inflammation because of its antimicrobial effects and anti-inflammatory activities. A number of laboratory studies have shown that berberine has antitumor activity for a wide variety of cancer cells. It exhibits antitumor effects by inhibiting the growth, invasion, and metastatic ability of several cancers, including lung cancer, Ehrlich ascites carcinoma, epidermoid carcinoma, breast cancer, and leukemia [6,7].

Several studies have shown that berberine can modulate the sensitivity of cancer cells to chemotherapeutic drugs or radiation [8,9]. This study was therefore undertaken to explore the reversal effect of berberine on the drug resistance in human ovarian cancer cell lines and its mechanisms.

Materials and Methods

Cell lines and cell culture

Human epithelial ovarian cancer cell lines SKOV3 and OVCAR3 were described previously [10,11]. They were maintained in Dulbecco's modification of Eagle's medium (DMEM; Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin.

Cell viability assay

Cells (1×10^4) were seeded into 96-well plates in DMEM medium containing 10% FBS. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to a final concentration of 0.5 mg/ml at 72 h after treatment, and cells were incubated for another 4 h at 37°C. The optical density was read at 570 nm with a microplate spectrophotometer. Each experiment was carried out in triplicate and repeated three times.

Apoptosis assay

Cells were plated in 6-well plates (5×10^5 cells/well). In the presence or absence of 10 μM berberine, SKOV3 cells were treated by cisplatin, with final concentration of 10 μg/ml, respectively. Twenty-four hours after the treatment of cisplatin, flow cytometry was used to detect apoptosis of

SKOV3 cells by determining the relative amount of AnnexinV-FITC-positive-PI-negative cells with Apoptosis Detection Kit I (BD Biosciences, San Diego, USA) as previously described [12].

Quantitative reverse transcription-polymerase chain reaction for miR-21

Total RNA was extracted from the cultured cells using Trizol (Invitrogen, Carlsbad, USA). Expression of miR-21 was measured using stem-loop reverse transcription (RT) followed by real-time polymerase chain reaction (PCR) analysis as previously described [13]. The primers used for stem-loop RT-PCR for miR-21 and U6 are listed in **Table 1**. PCR cycles were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. The relative amount of each miRNA was normalized to U6 snRNA. The fold-change for each group cells relative to the control was calculated using the $2^{-\triangle\triangle CT}$ method [14]. PCR was performed in triplicate.

Vector construction

To construct a luciferase reporter with the wild-type programmed cell death 4 (PDCD4) 3′UTR, we placed the 3′UTR of PDCD4 (amplified by PCR from genomic DNA using the following primers: sense, 5′-AATATAAGAACTCTTGCAGTC-3′, anti-sense: 5′-GAAGATACATTCCAATCTTGC-3′) downstream of a firefly luciferase cassette in a pCDNA3 vector. The mutated PDCD4 3′UTR was amplified by PCR using the following primers: sense, 5′-AGTGGAATATTCTAATTTCGTACCTTTTGTAAGTG-3′, anti-sense, 5′-GCACTTACAAAAGGTACGAAATTAGAATATTCCAC-3′.

Oligonucleotides transfections

miR-21 inhibition was achieved by transfecting cells with 100 nmol/well of antisense oligonucleotides (ASO) by Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. ASO sequences were as follows: 5′-AUCGAAUAGUCUGACUACAACU-3′ (miR-21 inhibitor) and 5′-CCCCCCCCCCCCCCCCCCCCC-3′ (negative control, NC). MiR-21 overexpression was achieved by transfecting cells with miR-21 mimics. miR-21 mimics

sequences were as follows: 5′-UAGCUUAUCAGACUGAUGUUGA-3′.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis

Extraction and detection of protein was done as previously described [15]. Protein (60 μg) was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, USA). Membranes were then blocked with 1% bovine serum albumin in 0.05% Tween20-containing TBS buffer containing 0.1% Tween-20 for 1 h. The membranes were then incubated with antibodies against PDCD4 (1:1000; Cell Signaling Technology, Beverly, USA), and GAPDH (1:2000; Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:10,000; Santa Cruz) for 1 h at room temperature. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Beyotime Biotechnology, Shanghai, China). The protein bands were quantified using ImageJ 1.33 software, and the data were normalized to GAPDH.

siRNA transfection

The designed siRNA targeting PDCD4 and non-specific negative control were purchased from Ambion (Austin, USA). The sequences of PDCD4-siRNA were as follows: sense, 5′-GAGAUGGAAUUUUAUGUAATT-3′, and antisense, 5′-UUACAUAAAAUUCCAUCUCCA-3′. The siRNAs were transfected into OVCAR3 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were prepared for further analysis 48 h after transfection. The transfection efficiency was evaluated by fluorescence microscopy by calculating the percentage of fluorescein-labeled cells. The transfection efficiency was approximately 75%.

Statistical analysis

All statistical analyses were performed using SPSS12.0 software (Chicago, USA). Each experiment was repeated at least three times. Numerical data were presented as mean ±

Table 1 Primers used for real-time RT-PCR assay

Target	Primer	Sequence
hsa-miR-21	RT	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAGCCCA-3
	Forward primer	5-GCGGCAACACCAGTCGATG-3
	Reverse primer	5-TGCGTGTCGTGGAGTC-3
U6	RT	5-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGACAAA-3
	Forward primer	5-GCTTCGGCAGCACATATACTAAAAT-3
	Reverse primer	5-CGCTTCACGAATTTGCGTGTCAT-3

standard deviation. The difference between different groups was analyzed with Student's *t*-test. Statistical significance was set as $P < 0.05$.

Results

Effects of berberine on the growth of SKOV3 cells

Firstly, the effect of berberine on SKOV3 and OVCAR3 cells growth were determined with MTT assay. The viability of the cells was evaluated as described in Materials and Methods. **Figure 1(A)** showed that SKOV3 cells were resistant to cisplatin compared with OVCAR3 cells. Berberine was dissolved in DMSO to get the stock solution with 1 mM concentration. As shown in **Fig. 1(B)**, berberine at concentration of 1 μM and 10 μM had no significant inhibitory effects on the growth of SKOV3 cells, while the anti-proliferative effect was observed at higher concentrations (100 μM). To minimize the effect of berberine itself on the growth of resistant cell, we chose lower concentrations of berberine (10 μM) in the reversal experiments.

Berberine partially sensitizes the SKOV3 cells to cisplatin

We further investigated the effects of berberine on cisplatin-induced cytotoxicity in SKOV3 cells. The SKOV3 cells were incubated with different concentrations of cisplatin in the presence or absence of 10 μM berberine to examine the reversal effects of berberine on SKOV3 cells. As shown in **Fig. 2(A)**, at cisplatin concentrations greater than 6.25 μg/ml, SKOV3 cells that were incubated with berberine had a significantly lower survival than the control group ($P < 0.05$), suggesting that berberine altered cisplatin sensitivity in SKOV3 cells. As shown in **Fig. 2(B–D)**, when cisplatin was combined with 10 μM of berberine, the mean apoptotic population of SKOV3 cells was increased almost 2 folds, compared with 10 μg/ml of cisplatin treatment alone. The result suggested that the increased inhibitory effect on SKOV3 cells from the combination of berberine with cisplatin is achieved through the action of berberine, which enhances the cisplatin-induced apoptosis.

Effects of berberine on the expression of miR-21 and its target PDCD4

Increasing evidence indicated that miR-21 plays a role in tumor cell resistance and/or sensitivity to chemotherapeutic agents [16]. To demonstrate the effect of berberine on miR-21 expression and function, quantitative PCR and western blot analysis were performed for miR-21, and PDCD4 which has been predicted to be a miR-21 post-transcriptional target. After 24 h of berberine treatment, SKOV3 cells showed a significant reduction in miR-21 expression [**Fig. 3(A)**]. In parallel, PDCD4 protein levels were increased significantly ($P < 0.05$) in SKOV3 lines [**Fig. 3(B,C)**]. The OVCAR3 cells were co-transfected with PDCD4 3′ UTR-wild luciferase reporter and miR-21 mimics. Transfections with control vector were performed in parallel. As shown in **Fig. 3(D)**, miR-21 markedly decreased the activity of the PDCD4 3′ UTR-wild reporter ($P < 0.05$). However, miR-21 did not affect the PDCD4 3′ UTR-mut reporter activity ($P > 0.05$). The luciferase assay results confirmed that PDCD4 was a direct miR-21 target in ovarian cancer cells. These results suggested that berberine could inhibit miR-21 expression and function in ovarian cancer, as shown by a reduction of its target PDCD4, an important tumor suppressor in ovarian cancer.

Involvement of miR-21 in the resistance to cisplatin

We further investigated the effects of miR-21 on cisplatin-induced cytotoxicity in SKOV3 cells that were transfected with a miR-21 inhibitor. The cells were transfected with either the miR-21 inhibitor or a NC, and were subsequently incubated with various concentrations of cisplatin. As shown in **Fig. 4(A)**, the miR-21 inhibitor effectively reduced the expression of miR-21 ($P < 0.05$). On the contrary, SKOV3 cells that were transfected with the miR-21 inhibitor had a significantly higher PDCD4 protein levels [**Fig. 4(B,C)**]. At cisplatin concentrations greater than 6.25 μg/ml, SKOV3 cells that were transfected with the miR-21 inhibitor had a significantly lower survival than the control group [**Fig. 4(D)**, $P < 0.05$], suggesting that the decrease of miR-21 expression alters cisplatin sensitivity in SKOV3
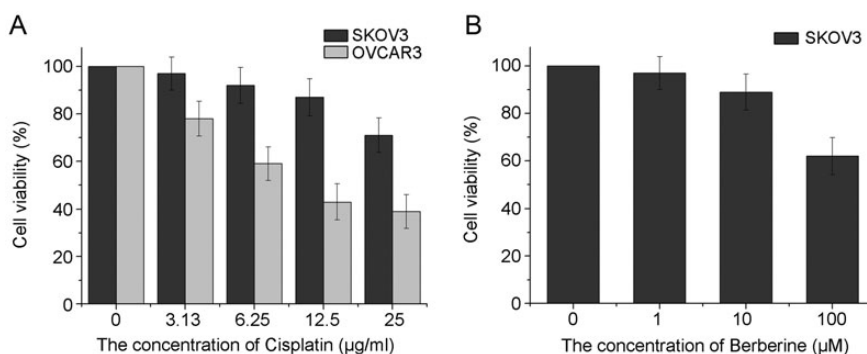
Figure 1 Effects of cisplatin and berberine on the growth of ovarian cancer cells   (A) Effects of cisplatin on the growth of SKOV3 and OVCAR3 cells. (B) Effects of berberine on the growth of SKOV3 cells.
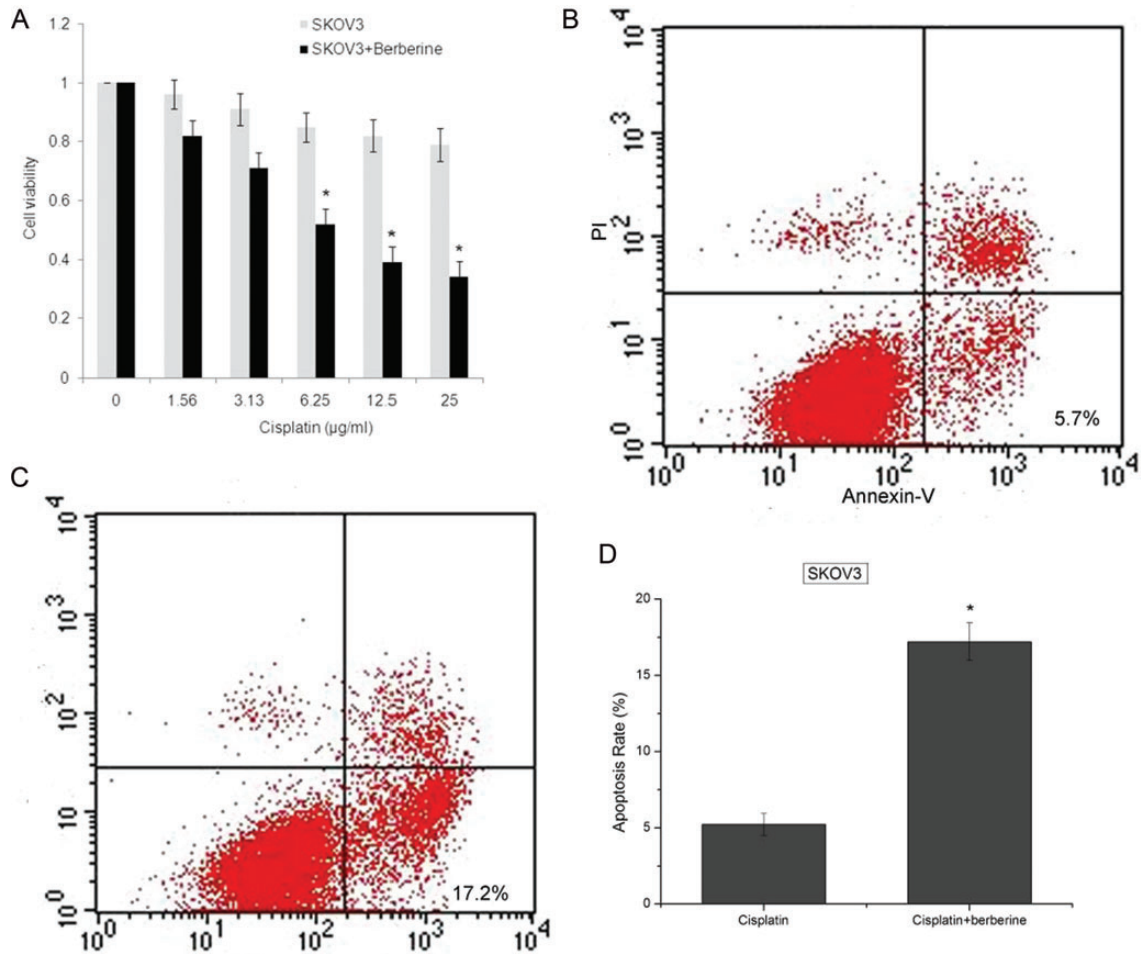
Figure 2 Berberine enhanced the cisplatin-induced cytotoxicity in SKOV3 cells (A) The combination of berberine with cisplatin decreased the survival rate in SKOV3 cells. (B) Cisplatin (10 μg/ml)-induced apoptosis in SKOV3 cells. (C) Cisplatin (10 μg/ml) combined with berberine (10 μM)-induced apoptosis in SKOV3 cells. (D) The apoptosis rate of two groups of cells.*$P < 0.05$.
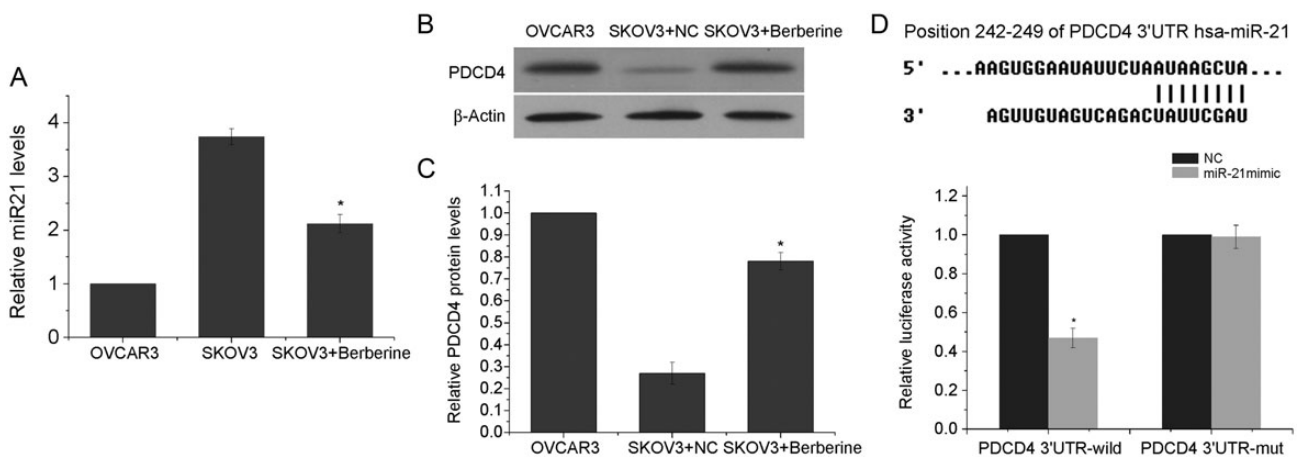

Figure 3 Effects of berberine on the expression of miR-21 and PDCD4 in SKOV3 cells (A) Berberine inhibited the expression of miR-21. (B, C) Berberine enhanced the expression of PDCD4. (D) PDCD4 was a direct target gene of miR-21. *$P < 0.05$.

cells. In order to confirm that berberine could modulate cisplatin sensitivity through targeting miR-21, we transfected miR-21 mimics into SKOV3 cells. As shown in **Fig. 4(E)**, we found that berberine could enhance the cisplatin chemosensitivity, which could be partly alleviated by the transfection of miR-21 mimics. These results indicated that miR-21 plays a role in modulation of cisplatin sensitivity by berberine.
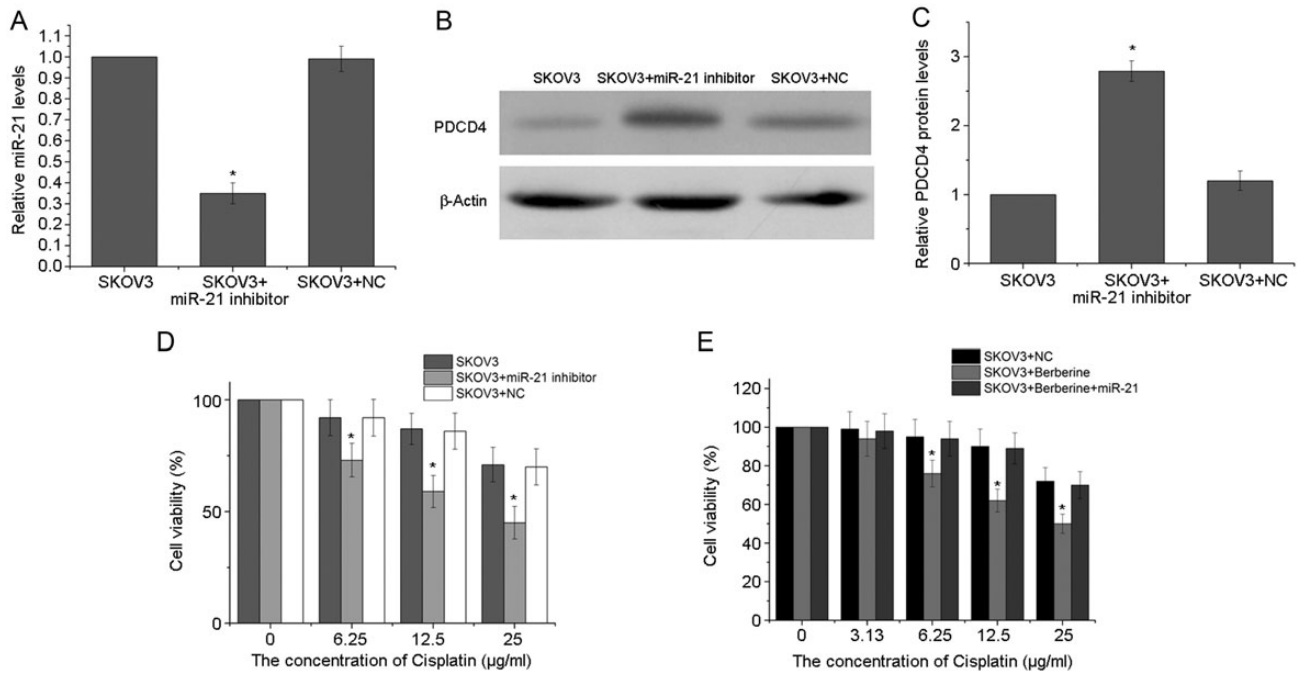
Figure 4 Involvement of miR-21 in the resistance to cisplatin in SKOV3 cells (A) The miR-21 inhibitor reduced the expression of miR-21. (B, C) The miR-21 inhibitor induced the expression of PDCD4. (D) The miR-21 inhibitor increased the cisplatin-induced cytotoxicity. (E) The enhancement of chemosensitivity induced by bereberine was partly alleviated by the miR-21 mimics.*$P < 0.05$.
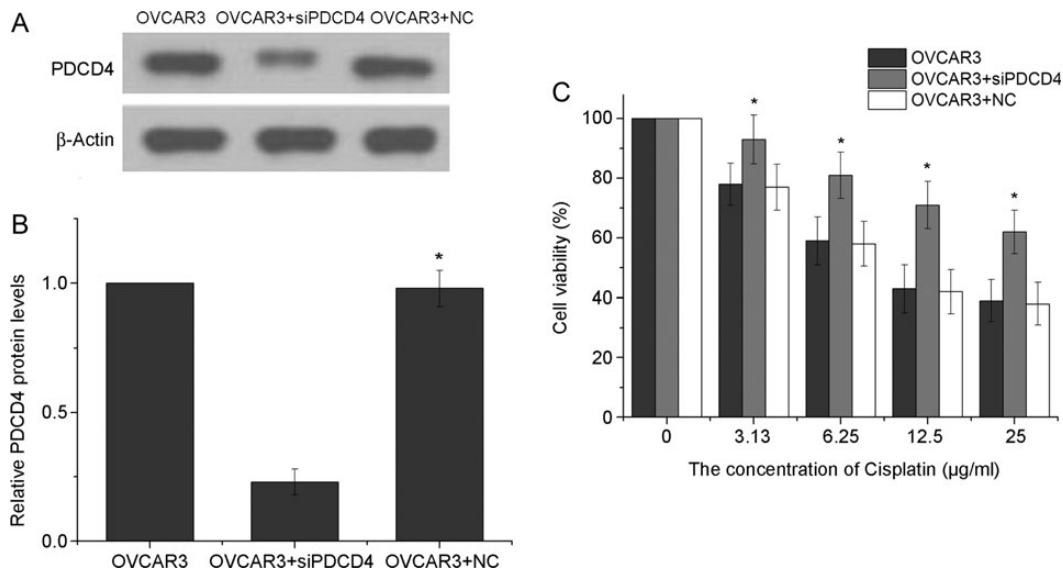

Figure 5 PDCD4 is a key signal molecule in the resistance to cisplatin in ovarian cancer cells (A, B) siPDCD4 decreased the PDCD4 protein levels in OVCAR3 cells. (C) siPDCD4 increased the survival rate in OVCAR3 cells. *$P < 0.05$.

PDCD4 is a key signal molecule in cisplatin resistance in ovarian cancer cells

Previous studies have shown that PDCD4 is involved in drug resistance in several types of cancer. To explore the relationship between PDCD4 and cisplatin-induced cytotoxicity, we transfected PDCD4 siRNA or a NC into OVCAR3 cells, followed by treatment with various doses of cisplatin. PDCD4 siRNA effectively reduced the PDCD4 protein level [**Fig. 5(A,B)**]. Furthermore, OVCAR3 cells that were treated with PDCD4 siRNA had increased survival compared with the control group [**Fig. 5(C)**]. These results suggested that berberine modulates the sensitivity of cisplatin via regulating PDCD4 in the ovarian cancer cells.

Discussion

One major mechanism of drug resistance in cancer cells is the defective apoptosis pathway [17,18]. Recently, more

and more studies have suggested that miRNAs modulate drug resistance of cancer cells, at least in part, through this mechanism [19].

In our study, it was found that the proapoptotic protein PDCD4 was down-regulated, whereas miR-21 was up-regulated in reisitant SKOV3 cells, compared with OVCAR3 cells. The mechanistic connection of miR-21 dysregulation with the establishment of drug resistance in SKOV3 cells was associated with PDCD4 protein levels.

Ovarian cancer exhibits a high rate of platinum sensitivity in the first-line setting, but resistance frequently develops in recurrent disease [20]. As such, understanding the signaling networks that regulate drug resistance is critical for successful treatment. This can be suitable to any cancer commonly treated with cisplatin such as head and neck cancer, small cell lung cancer, hepatocelluar cancers, and colorectal cancers. Recently, some investigators have reported a correlation between miRNAs expression and chemoresistance in several types of cancers. For example, Fujita *et al*. [21] have reported that the expression of miR-34a attenuated chemoresistance to an anticancer drug in prostate cancer cells. Among these reports on the correlation of miRNAs expression with chemoresistance, miR-21, which is reported to be increased in many cancers including hepatocellular carcinoma, is one of the most common miRNAs related to chemoresistance [22,23]. For example, it has been reported that miR-21 reduced the sensitivity to gemcitabine in cholangiocarcinoma cells [24]. Also in glioblastoma cells, miR-21 was reported to contribute to VM-26 resistance [25]. Furthermore, several studies have reported a significant association between miR-21 expression and chemoresistance to gemcitabine in pancreatic cancer cells [26]. In this study, we found that miR-21 was up-regulated in SKOV3 cells compared with OVCAR3 cells, indicating that miR-21 is involved in ovarian cancer cisplatin resistance.

Since mature miR-21 is over-expressed in most cancerous cell lines, it has been used as a model for studying miRNA expression and maturation [27]. An analysis of the consensus sequences within the miR-21 promoter region identified several conserved enhancer elements, including the binding sites for activation protein 1 (AP-1) and nuclear factor kappa B (NF-κB) [28]. Oncogenic transformation is frequently associated with the enhancement of endogenous AP-1 activity through various signal transduction pathways, and AP-1 activation strongly contributes to the oncogenic potential. Therefore, up-regulated miR-21 expression in SKOV3 cells may reflect the elevated AP-1 activity in ovarian cancer. Kuo *et al*. [29] have found that the berberine-mediated suppression of MMP2 and MMP9 involves in the inhibition of the Akt/NF-κB and AP-1 signaling pathways. So we hypothesized that berberine may inhibit the expression of miR-21 through modulating AP-1 activation. The results of RT-PCR confirmed our hypothesis. Berberine inhibited the expression of miR-21 in SKOV3 cells.

PDCD4 is a suppressor of tumorigenesis. Tumor PDCD4 interacts with the translation initiation factors, eIF4A and eIF4G, to inhibit translation in mRNA-specific fashion [30]. Consequently, PDCD4 has been found to inhibit pro-oncogenic events, such as activation of AP-1, anchorage-independent growth, and invasion [31]. Recently, PDCD4 was also shown to be involved in the sensitivity of chemotherapy by cisplatin and paclitaxel [32]. The effects of PDCD4 expression on drug sensitivity are through the down-regulation of Y-box-binding protein 1 expression. Here, we found that berberine inhibited the expression of miR-21 and increased the PDCD4 protein levels in SKOV3 cells. Other studies have found that miR-21 can directly down-regulate the expression of PDCD4 by targeting its 3′UTR in several cancers cells. Our results showed that silencing of PDCD4 expression increased the cell viability and resistance to cisplatin in SKOV3 cells, suggesting that PDCD4 is a functional target for miR-21-induced chemoresistance to cisplatin in ovarian cancer cells.

In conclusion, our data showed that berberine inhibited miR-21 expression and increased PDCD4 protein levels in the ovarian cancer SKOV3 cells. Increased miR-21 expression played important roles in the resistance to cisplatin in ovarian cancer cells. Meanwhile, PDCD4, a direct and functional target of miR-21, may mediate miR-21-induced chemoresistance to cisplatin in SKOV3 cells. The miR-21/PDCD4 axis will provide new clues toward understanding the mechanism of drug resistance and may help to develop potential therapeutics against ovarian cancer.

Acknowledgements

Funding

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