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Original Paper

Berberine Sensitizes Human Ovarian Cancer Cells to Cisplatin Through miR-93/PTEN/Akt Signaling Pathway

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Key Words
Ovarian cancer cells • Cisplatin • Berberine • miR-93

Abstract
Background: Berberine, a well-known component of the Chinese herbal medicine Huanglian, has wide range of biochemical and pharmacological effects, including antineoplastic effect, but the exact mechanisms remain unclear. The aim of the present study was to evaluate the potential chemo-sensitization effect of berberine in ovarian cancer cell line A2780. ***Methods:*** The expression of miR-93 was measure by RT-PCR. The target of miR-93 was confirmed by luciferase activity assay. Hoechst 33258 staining, Annexin V and PI double staining were used for apoptosis analysis. ***Results:*** In this study, we found A2780/DDP cells that were incubated with berberine combined with cisplatin had a significantly lower survival than the control group. Berberine enhanced cisplatin induced apoptosis and induced G0/G1 cell cycle arrest in A2780 cells. Next, we observed that the miR-93 levels in cisplatin resistant cell lines were higher than that in cisplatin sensitive cell lines. Furthermore, our study found berberine could inhibit miR-93 expression and function in ovarian cancer, as shown by an increase of its target PTEN, an important tumor suppressor in ovarian cancer. A2780 cells that were treated with PTEN siRNA had increased survival compared to NC group and this could be partly alleviated by the AKT inhibitor Triciribine. More importantly, A2780 cells that were treated with PTEN siRNA had a survival pattern that is similar to cells with miR-93 overexpression. ***Conclusion:*** The results suggested that berberine modulated the sensitivity of cisplatin through miR-93/PTEN/AKT signaling pathway in the ovarian cancer cells.

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Introduction

Ovarian cancer remains the most lethal form of all gynecological malignancies [1]. Approximately 23,000 women are diagnosed with ovarian cancer each year, and the 5-year survival rate is 30% [2]. Due to the lack of an efficient method for early diagnosis, ovarian cancer is often detected in an advanced stage. In this situation, the patients usually have to undergo surgery to remove the tumor and receive chemotherapy using anticancer drugs, such as cisplatin and its platinum derivatives. Most patients are responsive to chemotherapy at first; however, recurrent ovarian tumors are more aggressive, metastasize to secondary target tissues, and acquire resistance to conventional chemotherapeutics [3]. Therefore, it is important to explore new types of drugs that reverse chemotherapy resistance and enhance sensitivity to platinum-based chemotherapy drugs.

Berberine, a clinically important natural isoquinoline alkaloid derived from Huang Lian and other medicinal herbs, has been reported to exhibit multiple pharmacological activities including anti-cancer effect [4]. A number of laboratory studies have shown that berberine has antitumor activity for a wide variety of cancer cells, including gastric cancer [5], hepatoma [6], oral cancer [7], prostate cancer [8], leukemia [9], osteosarcoma [10], glioblastoma [11] and ovarian cancer [12]. In most cases, berberine was found to inhibit cell cycle progression and to induce apoptosis. In addition, berberine may impair tumor growth by inhibiting angiogenesis [13]. Berberine was also reported to have a radiosensitizing effect on lung cancer cells by inducing autophagy [14]. A recent study showed that berberine could modulate the anticancer effects of doxorubicin in mutliple human cancer cells [15]. But the mechanisms of berberine enhance sensitivity to chemotherapy drugs remain unclear.

Based on these findings, we hypothesized that combining cisplatin with berberine as a novel strategy for ovarian cancer therapy. The present study was therefore performed to test this hypothesis in ovarian cancer cell line A2780. Our study revealed that berberine enhances the antitumor effects of cisplatin in ovarian cancer cells through miR-93/PTEN/AKT signaling pathway.

Materials and Methods

Cell lines and culture

The human ovarian cancer cell line A2780 was purchased from the Institute of Shanghai Cell Biology. The CDDP-resistant cell line A2780/DDP was induced using progressive concentration of CDDP as described previously [16]. A2780 and A2780/DDP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. All of the cell culture media contain 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Both resistant and sensitive cells were seeded into 96 well plates at a concentration of 4000 cells/well in six replicates with complete culture medium. At 70% to 80% confluence, cells were treated with DMSO alone (0.1% v/v; control), different doses of berberine alone, cisplatin alone, or berberine and cisplatin in combination. After 72 h of these treatments, the number of viable cells was determined using the MTT dye as described elsewhere [17].Then cells were treated with cisplatin in different dose from 1 μg/ml to 16 μg/ml. 48 hours after treatment, wells were added 20 μl of MTT solution and incubated for 4 h, and 150 μL dimethyl sulfoxide (DMSO) was added to each well to dissolve formed formazan crystals. The optical density was measured at 490 nm on a microplate reader.

Real time RT-PCR analysis

For the detection of miR-93 level, the stem-loop quantitative RT-PCR was performed [18]. The miR-93 reverse transcription (RT) primer (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAC CT-3') and real time RT-PCR primers (Forward: 5'-AGG CCC AAA GTG CTG TTC GT-3'; Reverse: 5'-GTG CAG GGT CCG AGG T-3') were synthesized by Sangon Biotech. SYBR Green–based real time RT-PCR was

Table 1. Sequence of interferents

Target	Sequence (5'-3')
has-miR-93 mimic	CAAAGUGCUGUUCGUGCAGGUAG
NC mimic	UUCUCCGAACGUGUCACGUTT
has-miR-93 inhibitor	CUACCUGCACGAACAGCACUUUG
NC inhibitor	CAGUACUUUUGUGUAGUACAA

performed using SYBR PrimeScript miRNA RT-PCR Kit (Takara) to measure the expression of mature miR-93 in cells by a CFX-96 Real-Time PCR Detection System(Bio-Rad, Hercules, CA, USA). The reactions were incubated in a 96-well plate at 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. U6 was used as endogenous control.

Transfection with miR-93 mimic and inhibitor

5×10^5 cells were seeded in six-well plates and grown to 70% confluence. Human has-miR-93 inhibitors or its negative control was directly transfected into A2780/DDP cells at a final concentration of 50 nmol/l according to the manufacturer's protocol. Human has-miR-93 mimics or its negative control was allowed to form transfection complexes and transfected into A2780 cells with Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in free of serum Opti-MEMH (Invitrogen) at a final concentration of 40 nmol/l. Sequences of miR-93 mimic, inhibitor or NC were shown in Table 1.

Annexin V and PI double staining for apoptosis analysis

Cells were harvested by trypsinization and washed twice with cold PBS. 200 μl of the solution was incubated with 5 μl of FITC-conjugated Annexin V antibody and 5 μl of PI for 20 min at room temperature in the dark. Subsequently, 400 μl of binding buffer was added to each sample and the samples were analyzed by FACScalibur system (Becton Dickinson, Mountain View, CA) using CellQuest Research Software. Annexin V-positive and PI-negative cells were considered to be early apoptosis. Apoptosis was calculated as the number of apoptotic cells/the number of total cells in each group. Each measurement was repeated three times independently.

Luciferase reporter assay

To construct a luciferase reporter vector, the PTEN 3-UTR fragment containing putative binding sites for miR-93 was amplified by PCR using the following primers: sense 5'-TCG CTC GAG ATT TTT TTT TAT CAA GAG GG-3' and reverse 5'-TCG GCG GCC GCG ACA AGA ATG AGA CT TTA ATC-3' and inserted into downstream of the luciferase gene in the the psiCHECK2 vector (Promega) and named PTEN 3'-UTR-wild. Site-directed mutagenesis of the miR-93 target-site in the PTEN-3'-UTR was performed using the Quick-change Mutagenesis Kit (Stratagene, Heidelberg, Germany) and named PTEN 3'-UTR-mut. For the mutated construct, the miR-93 target site AAAGTGC was substituted with a TTTCACG fragment. Transfection was performed in triplicate with miR-93 mimics and PTEN 3'-UTR-wild or PTEN 3'-UTR-mut. Luciferase assays for both firefly and renilla luciferase was performed 48 h after transfection with a Dual-Glo Luciferase assay kit (Promega, Madison, WI, USA). The renilla luciferase readings were normalized to the firefly luciferase activity in the corresponding well.

Western blot

Cells were washed twice with ice-cold PBS and then lysed in Lysis Reagent (Sigma–Aldrich, St. Louis, MO). Cell lysates (approximately 30 μg of protein) were loaded on an 8% SDS–PAGE gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). Primary antibodies against PTEN, phospho-AKT1, AKT1 were purchased from Santa Cruz Biotechnology (Santa Cruz, *CA*, USA).Antibodies to PTEN, AKT1, phosphorylated AKT1 or the endogenous control GAPDH were incubated with blots overnight at 4 °C in the buffer recommended. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (BeyoECL Plus; Beyotime Institute of Biotechnology, Shanghai, China). The protein bands were normalized to GAPDH.

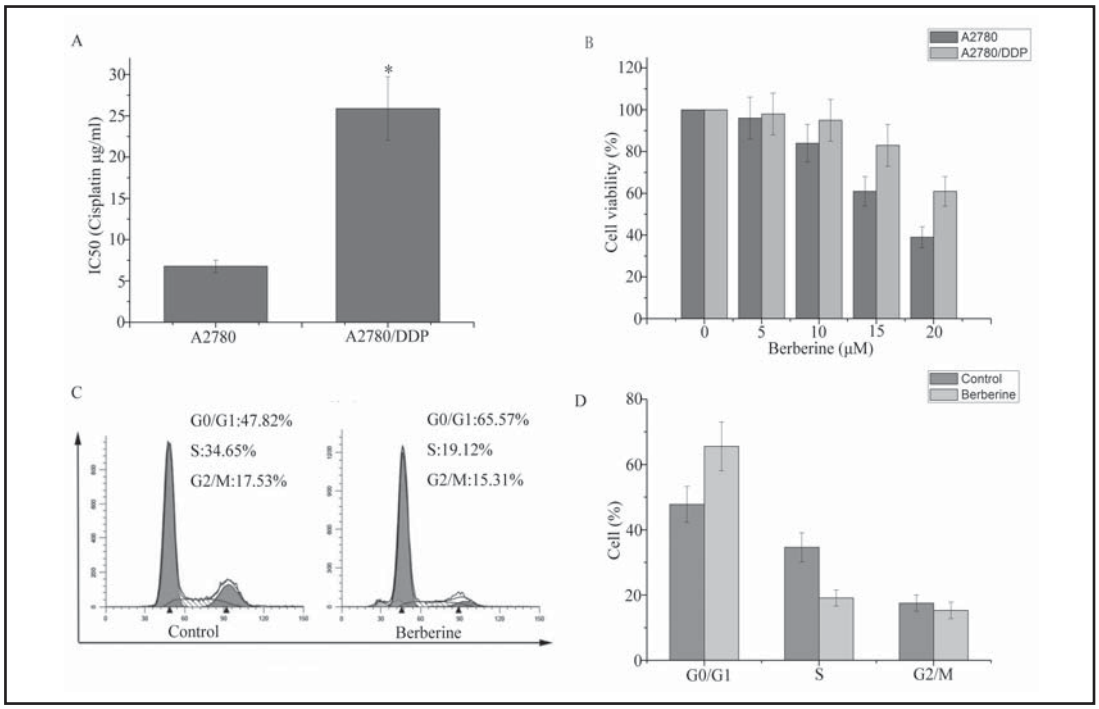

Fig. 1. Berberine inhibits the proliferation of ovarian cancer cells. A: A2780/DDP (AD) and A2780 cells were exposed to various concentrations of cisplatin for 48 h, and the IC50 of cisplatin was examined by the MTT assay. B: The cell viability was detected by MTT assay. The ovarian cancer cells were treated with berberine in different dose. C, D: The cell cycle was measured by FCM in A2780 cells treated with berberine or control. * $p<0.05$.

siRNA transfection

A SignalSilence PTEN siRNA kit was purchased from Cell Signaling Technology (CST). A2780 cells were transfected with PTEN siRNA and negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was evaluated by fluorescence microscopy by calculating the percentage of fluorescein–labelled cells. At 48 h after the transfection of the siPTEN, the cells were then subjected to viability and western blot assays for PTEN, pAkt.

Statistical Analysis

Results were expressed as mean ± S.E. Comparisons between groups were made by a Student's t test. Scheffe test were used for multiple comparisons. The significance level was set at $p<0.05$.

Results

Berberine inhibits the proliferation of ovarian cancer cells

We confirmed the cisplatin resistance of the A2780/DDP cell line compared with their cisplatin-sensitive parental SKOV3 cells using an MTT assay. As shown in Fig. 1A, the 50% inhibitory concentration (IC50) of cisplatin in the A2780/DDP cells was four-fold than the parental A2780 cells. As previously reported for other types of cancer cells, berberine treatment alone had a growth-inhibitory effect on ovarian cancer cells in a dose-dependent manner (Fig. 1B). Berberine at concentraton 5 and 10 μM had no significant inhibitory effects on the growth of A2780 and A2780/DDP cells, while the anti-proliferative effect was observed at higher concentrations. To further probe the regulatory mechanisms of berberine, we conducted a cell-cycle assay. A higher proportion of A2780 cells treated with berberine mimics were in the G0/G1 phase compared with those transfected with a negative control (Fig. 1C and 1D).

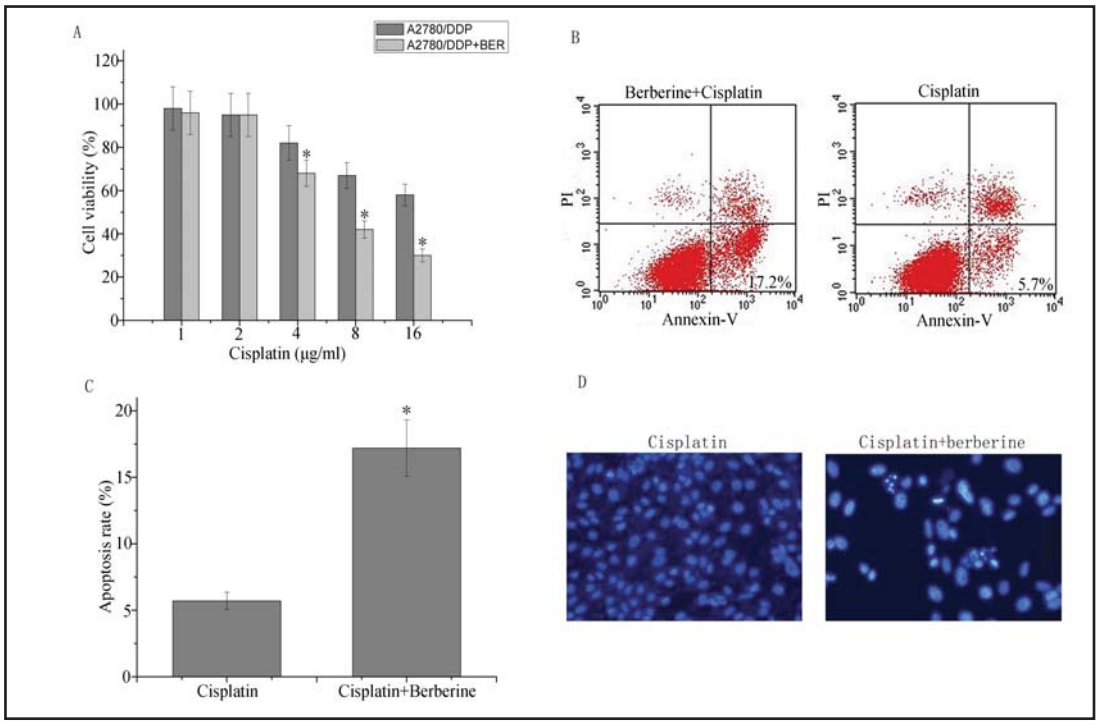

Fig. 2. Berberine sensitizes ovarian cancer cells to cisplatin. A: The effects of berberine on cisplatin-induced cytotoxicity were measured by MTT. C: The apoptosis in A2780/DDP cells treated with cisplatin alone or combination of berberine and cisplatin was detected by FCM. D: The apoptosis in A2780/DDP cells was determined by Hoechst Dye 33258 assay. * $p<0.05$.

Berberine sensitizes ovarian cancer cells to cisplatin

To minimize the effect of berberine itself on the resistant cell growth, we chose lower concentrations of berberine (10 μM) in the reversal experiments. We further investigated the effects of berberine on cisplatin-induced cytotoxicity. The cells were incubated with different concentrations of cisplatin in the presence or absence of 10 μM berberine to examine the reversal effects of berberine. As shown in Fig. 2A, at cisplatin concentrations greater than 4 μg/ml, A2780/DDP cells that were incubated with berberine had a significantly lower survival than cisplatin treatment alone ($P<0.05$), suggesting that berberine alters cisplatin sensitivity in A2780/DDP cells. As shown in Fig. 2B and 2C, when cisplatin was combined with 10 μM of berberine, the mean apoptotic population of A2780/DDP cells was increased almost three times, compared with 5 μg/mL of cisplatin treatment alone. The hoechst 33258 dye stain assay confiemed that treatment with berberine increased the apoptosis induce by cisplatin in A2780/DDP cells (Fig. 2D). The result suggested that the increased inhibitory effect on A2780/DDP cells from the combination of berberine with cisplatin was achieved through the action of berberine, which enhanced the cisplatin-induced apoptosis.

Berberine inhibits the expression of miR-93 in ovarian cancer cells

Accumulating evidence indicates that miR-93 is closely associated with both cancer development and chemotherapy resistance [19]. Next, we tested whether berberin enhances cisplatin-induced cytotoxicity through inhibiting miR-93 expression. As shown in Fig.3A, the miR-93 levels in A2780/DDP cells were higher than A2780 cells and decreased significantly after berberine treatment. We further investigated the effects of miR-93 on cisplatin-induced cytotoxicity in A2780 cells that were transfected with a miR-93 mimics. As is shown in Fig. 3B, the miR-93 mimics effectively increased the expression of miR-93 ($P < 0.05$). A2780 cells that were transfected with the miR-93 mimics had a significantly higher survival than the control group (Fig. 3C. $P < 0.05$), suggesting that increasing miR-93 expression alters cisplatin sensitivity in A2780 cells.

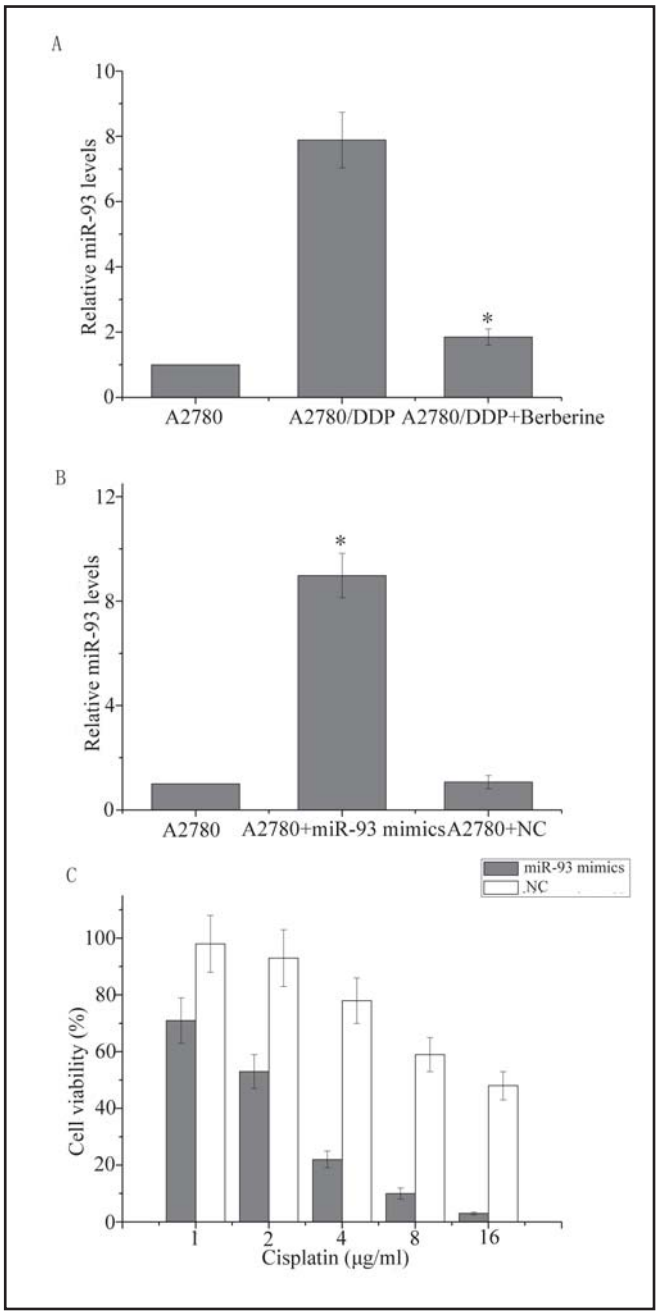

Fig. 3. miR-93 confers cisplatin resistance in A2780 cells. A: The miR-93 levels were detected in A2780 cells, A2780/DDP cells and A2780/DDP cells treated with berberine. B: The miR-93 mimics enhanced the expression of miR-93 in A2780 cells. C: A2780 cells were transfected with miR-93 mimics, and were subsequently treated with various doses of cisplatin. Cell viability was determined using a MTT assay. *P < 0.05.

PTEN is a direct target of miR-93 in ovarian cancer cells

To further clarify the molecular mechanisms of miR-93 in cisplatin sensitivity, we used a online tool, TargetScan, to predict the putative targets of miR-93. The 3′-UTR of PTEN mRNA contained a complementary site for miR-93 (Fig. 4A). We thus hypothesized that PTEN was a direct miR-93 target in A2780 cells. To confirm this, the A2780 cells were co-transfected with PTEN 3'-UTR-wild or PTEN 3'-UTR-mut luciferase reporter and miR-93 mimics. As shown in Fig. 4B, miR-93 markedly decreased the activity of the PTEN 3'-UTR-wild reporter ($P < 0.05$). The luferase of reporter vector with the mutated PTEN 3'-UTR was not affected by the change of miR-93. Furthermore, RT-PCR and western blot analysis showed that the mRNA levels and protein levels of PTEN decreased after transfection of miR-93 mimics compared with those in the NC group (Fig. 4C, 4D and 4E). These data indicated that PTEN is directly and negatively regulated by miR-93.

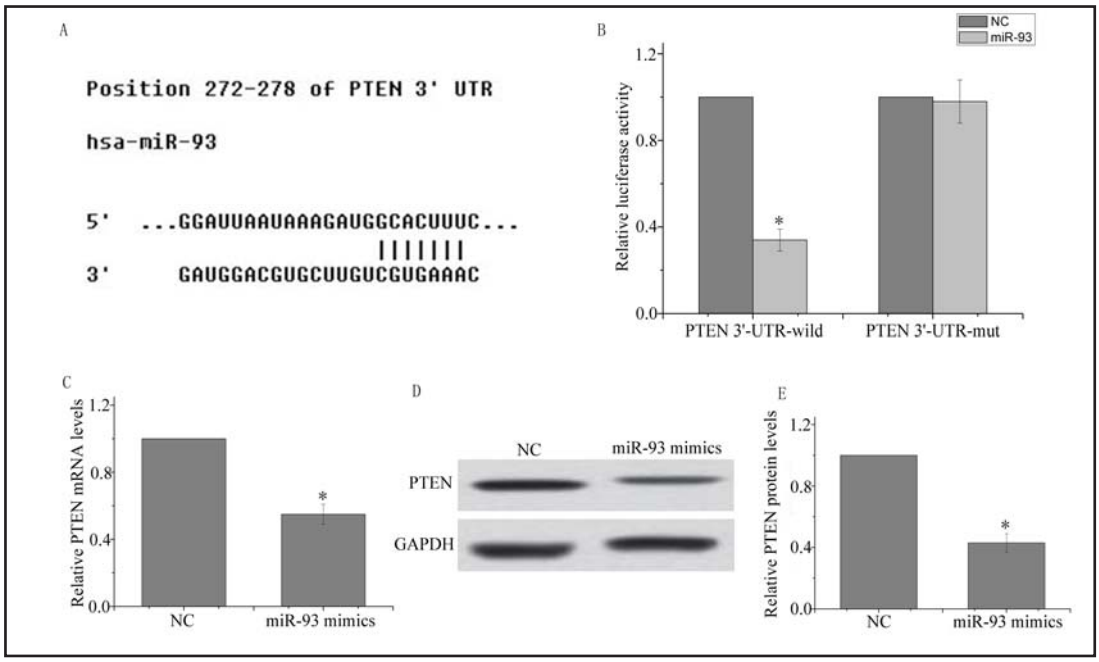

Fig. 4. PTEN is a direct target of miR-93 in ovarian cancer cells. A: TargetScan predicted that the 3′-UTR of PTEN mRNA contained a complementary site for miR-93. B: miR-93 decreased the luciferase activity of the PTEN 3'-UTR-wild reporter. The luferase of reporter vector with the mutated PTEN 3'-UTR was not affected by the change of miR-93. C: Overexpression of miR-93 decreased the PTEN mRNA levels. D, E: Overexpression of miR-93 decreased the PTEN protein levels. *P < 0.05.

PTEN/AKT is a key pathway in cisplatin resistance in ovarian cancer cells

To explore the relationship between PTEN and resistance-induced cytotoxicity, we transfected PTEN siRNA or a NC into A2780 cells, followed by treatment with various doses of cisplatin. PTEN siRNA effectively reduced the PTEN protein level (Fig. 4A, 4B). PTEN siRNA increased the ratio of the phosphorylated AKT/total AKT, and this could be partly alleviated by the AKT inhibitor Triciribine (Fig. 5A, 5B, 5C). Furthermore, A2780 cells that were treated with PTEN siRNA had increased survival compared to NC group and this could be partly alleviated by the AKT inhibitor Triciribine (Fig. 5D). More importantly, A2780 cells that were treated with PTEN siRNA had a survival pattern that is similar to cells with miR-93 overexpression, suggesting that miR-93 confers resistance resistance via regulating PTEN/AKT in the A2780 cells.

Discussion

One major mechanism of drug resistance in cancer cells is the defective apoptosis pathway [20, 21]. Recently, more and more studies have found that miRNAs modulate drug resistance of cancer cells, at least in part, through this mechanism [22]. In our study, we found that berberine could sensitize human ovarian cancer cells to cisplatin via down-regulating miR-93.

Ovarian cancer exhibits a high rate of platinum sensitivity in the first-line setting, but resistance frequently develops in recurrent disease [23]. As such, understanding the signaling networks that regulate drug resistance is critical for successful treatment. A growing body of evidence indicates miRNAs play an important regulatory role in biological processes including proliferation, differentiation, apoptosis, metastasis, and drug resistance [24, 25]. Thus far, there are several published miRNAs that were reported to be associated with cisplantin resistance in varies tumors include miR-451, miR-21, miR-214, miR-23a and miR-

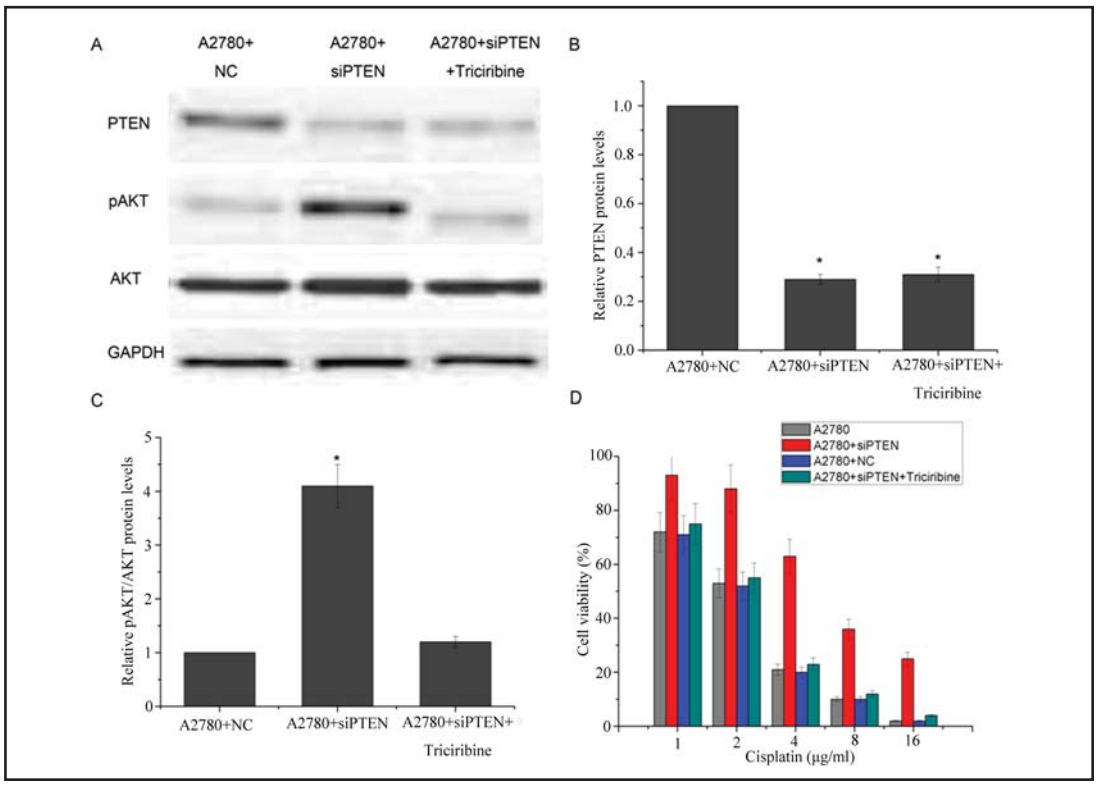

Fig. 5. PTEN/AKT is a key pathway in cisplatin resistance in ovarian cancer cells. A, B: PTEN siRNA effectively reduced the PTEN protein level. A, C: PTEN siRNA increased the ratio of the phosphorylated AKT/total AKT, and this could be partly alleviated by the AKT inhibitor Triciribine. D: A2780 cells that were treated with PTEN siRNA had increased survival compared to NC group and this could be partly alleviated by the AKT inhibitor Triciribine. *P < 0.05.

141 [26-28]. MiR-93 is a typical member of miR-106b-25 clusters. Fu et al. reported that miR-93 is over-expressed in the cisplatin-resistant OVCR3/CDDP and SKOV3/CDDP cells compared with cisplatin-sensitive parental cell lines [16]. In the present study, we found that miR-93 was upregulated in A2780/DDP cells compared to A2780 cells, indicating that miR-93 is involved in ovarian cancer cisplatin resistance. Berberine could sensitize the A2780/DDP cells to cisplatin via inhibiting miR-93 expression. MiR-93 may be a target of berberine.

Of miR-93 targets, we focused on the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), because it is an important gene that is involved in drug resistance. Studies have shown that transfection of PTEN in the drug-resistant AML cell line (HL60AR) or ALL cell line (EU21) causes chemosensitivity to anti-cancer agents [29, 30]. Consistent with this finding, we demonstrate here that PTEN is a target of miR-93 and that it plays a role in cisplatin resistance in the A2780 cell line. Moreover, knockdown of PTEN significantly increased cell survival and had an overall effect that was similar to miR-93 overexpression. It has been well reported that constitutive activation of AKT contributes to chemoresistance in different types of tumors, including ovarian carcinoma [31]. Recent studies have demonstrated BIM as a key regulator participating in the AKT pathway in cisplatin-sensitive and resistant ovarian cancer cells [32]. Yang et al. have reported that miR-214 induces cisplatin resistance by targeting PTEN [33]. In our study, we confirmed that miR-93, a family member of PTEN regulator, blocks PTEN translation leading to activation of the AKT pathway and played an important role in regulating cisplatin chemosensitivity pathway in ovarian cancer. We can nevertheless confirm that there are some limitations of our study. As a result of the limitation of time and experimental conditions, only one cell line used in this

study. Therefore cell line specific effects cannot be ruled out. We should confirm the role of Berberine/miR-93 axis in other ovarian cancer cell lines in the later study.

In conclusion, our data indicated that berberine inhibited miR-93 expression and increased PTEN protein levels in the ovarian cancer A2780/DDP cells. Increased miR-93 expression plays important roles in the resistance to cisplatin in ovarian cancer cells. Meanwhile, PTEN/AKT pathway may mediate miR-93-induced chemoresistance to cisplatin in A2780 cells. The miR-93/PTEN/AKT axis provides a new avenue toward understanding the mechanism of drug resistance and may help us develop potential therapeutics against ovarian cancer.

Disclosure Statement

The authors declare no conflict of interest.

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