

## Original Article

# MiR-490-3p sensitizes ovarian cancer cells to cisplatin by directly targeting ABCC2

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Received February 21, 2016; Accepted June 3, 2016; Epub March 15, 2017; Published March 30, 2017

**Abstract:** Cisplatin (CDDP) resistance becomes a large obstacle of the beneficial therapy for patients with ovarian cancer. MicroRNAs (miRNAs) act as post-transcriptional regulators of multiple genes' expression and have been reported to be involved in multi-drug resistance. The purpose of this study was to determine the roles and molecular mechanism of miR-490-3p in the CDDP resistance in ovarian cancer. We found that miR-490-3p was downregulated in CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells, which was due to the hypermethylation of miR-490-3p promoter. Functional studies demonstrated that miR-490-3p increased the cell response to CDDP in OVCAR3, SKOV3 and CDDP-resistant cells, while miR-490-3p inhibition resulted in opposite effects. Luciferase assay, real-time PCR and Western blot as well as immunohistochemistry validated that ABCC2 was a direct target of miR-490-3p and miR-490-3p downregulated ABCC2 expression via binding to its 3'UTR. Importantly, silencing of ABCC2 alleviated CDDP resistance induced by miR-490-3p inhibition, while ABCC2 overexpression restored CDDP resistance inhibited by miR-490-3p. In vivo study showed that miR-490-3p enhanced the cytotoxicity of CDDP. Finally, we found that miR-490-3p was downregulated in CDDP-resistant ovarian cancer tissues, while ABCC2 was upregulated. In conclusion, our data indicate that miR-490-3p enhances CDDP sensitivity of ovarian cancer cells through down-regulating ABCC2 expression, and suggest that delivery of miR-490-3p might be a potential therapeutic strategy for patients with CDDP-resistant ovarian cancer.

**Keywords:** MicroRNA, miR-490-3p, CDDP sensitivity, ABCC2, ovarian cancer

## Introduction

Ovarian cancer is the most important cause of gynecological cancer-related deaths [1, 2]. The main treatment for ovarian cancer is the surgical excision combined with chemotherapy [3]. Platinum is the first line and major therapy for patients with ovarian cancer [4]. However, the overall survival rate and the prognosis remain poor because of the development of platinum resistance [5]. Until now, cisplatin (CDDP) resistance is still a large obstacle for the successful treatment of ovarian cancer. Therefore, it is crucial to investigate and clarify the molecular mechanism of CDDP resistance, which helps to develop beneficial therapeutic strategies and biomarkers for the therapy of patients with ovarian cancer.

MicroRNAs (miRNAs) are a conserved family of endogenous, small non-protein-coding RNA molecules that act as regulators of gene expression at the post-transcriptional level [6]. They can bind to the 3'untranslated region (3'UTR) of target genes through the base pairs between miRNAs and their targets, resulting in the cleavage of target mRNA [7] or repression of gene translation [8] due to the extent of the complementarity between base pairs. A previous study shows that approximately 60% of genes' expression is controlled by miRNAs, suggesting that miRNAs are involved in multiple biological processes [9], including drug chemoresistance. For example, miR-221 enhances cell resistance to cisplatin through PI3K/Akt pathway in human osteosarcoma [10]. MiR-30c regulates the chemoresistance in breast cancer by directly tar-

**Table 1.** The primers for real-time PCR

Gene	ID	Forward (5'-3')	Reverse (5-3')	Amplicon size (bp)
miR-490-3p	574443	TGCGGTTCAAGTAATTCAGGA	CCAGTGCAGGGTCCGAGGT	62
U6	26827	TGCGGGTGCTCGCTTCGGCAGC	CCAGTGCAGGGTCCGAGGT	148
ABCC2	1244	CCCTGCTGTTTCGATATACCAATC	TCGAGAGAATCCAGAATAGGGAC	131
GAPDH	2597	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG	197

getting twinfilin 1 and interleukin-11 [11]. It is reported that miRNAs are abnormally expressed in drug-resistant ovarian cancer [12]. A miRNA array performed by the researchers in our lab shows some up- or downregulated miRNAs in CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells, including miR-93, miR-489 and miR-490-3p. MiR-93 has been validated to be upregulated in CDDP-resistant ovarian cancer and modulates CDDP resistance and cell apoptosis by regulating PTEN/Akt signaling pathway [13]. MiR-489, a downregulated miRNA in CDDP-resistant ovarian cancer cells, has been reported to inhibit CDDP resistance and cell growth by suppressing Akt3 expression [14]. MiR-490-3p has been validated to promote cell growth and epithelial to mesenchymal transition by targeting ERGIC3 in hepatocellular carcinoma [15]. It is also reported that miR-490-3p inhibits tumorigenesis in ovarian cancer by targeting CDK1 [16]. However, the potential roles and molecular mechanism of miR-490-3p in CDDP resistance remain poorly understood.

In the current study, we validated that miR-490-3p was downregulated in CDDP-resistant ovarian cancer cells, which was due to the hypermethylation of its promoter. MiR-490-3p inhibition enhanced CDDP resistance, while miR-490-3p overexpression increased CDDP sensitivity. ABCC2 was confirmed to be a direct target of miR-490-3p, and mediated the effects of miR-490-3p on CDDP response. Importantly, miR-490-3p enhanced CDDP cytotoxicity in vivo. Finally, our data showed that miR-490-3p was downregulated in CDDP-resistant ovarian cancer tissues, while ABCC2 was upregulated. Taken together, the data indicate that miR-490-3p contributes to CDDP chemosensitivity by downregulating ABCC2, suggesting that miR-490-3p has the potential as a therapeutic target for the treatment of patients with CDDP-resistant ovarian cancer.

## Materials and methods

### *Tissue samples, cell culture and transfection*

The CDDP-resistant and -sensitive human ovarian cancer tissues were acquired from Tianjin confirmed by immunohistochemistry, and frozen in liquid nitrogen and stored at -80°C.

Human ovarian cancer cell lines OVCAR3 and SKOV3 were maintained in RPMI1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Sigma) and 100 IU/ml of penicillin, 100 µg/ml of streptomycin (Sigma). The CDDP resistant cell lines, OVCAR3/CDDP and SKOV3/CDDP, were acquired as previously described [13]. The cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

Cell transfections were performed using Lipo-fectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol.

### *RNA isolation and real-time PCR*

Total RNAs (including miRNAs) were extracted using TRIzol reagent (Qiagen) from the transfected cells or tissues. Subsequently, miRNAs were reversely transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Life technologies) with specific miR-490-3p primer according to the manufacturer's instructions. For the cDNA synthesis of ABCC2, Oligo (dT) was used as a common primer. Real-time PCR was finally performed using the SYBR Premix Ex Taq™ Kit (TaKaRa) according to the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. U6 snRNA was used as an internal control to normalize miR-490-3p expression. GAPDH was used as an internal control to normalize ABCC2 expression. Reverse transcription primer for miR-490-3p: 5'-GTCGTATCCA-GTGCAGGGTCCGAGGTGCACTGGATACGACCA-GCATG-3'; U6: 5'-GTCGTATCCAGTGCAGGGTCC-GAGGTATTCGCACTGGATACGACAAAATATGGA-

AC-3'. The primers for real-time PCR were listed in **Table 1**.

## *Cytotoxicity assay (IC<sub>50</sub> analysis)*

The CDDP cytotoxicity was measured using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. Briefly, the cells were seeded in 96-well plates at a density of 4000 cells/well, and transfected when the cell confluence reached approximately 70%. Once the cells were adhesive to the plate, CDDP was added into the media with an increasing concentrations. At 72 h after the CDDP treatment, cell viability was measured using a spectrophotometer at 490 nm. The concentration at which 50% of cell viability was inhibited was considered as IC<sub>50</sub>.

## *Northern blotting assay*

MiRNA was isolated using mirVana™ miRNA Isolation Kit (Ambion) from ovarian cancer cells or CDDP-resistant cells according to the manufacturer's protocol. Next, Northern blotting assay was performed to detect the expression of miR-490-3p as previously described [17]. U6 snRNA was employed as an internal control.

## *Methylation-specific PCR (MSP)*

Genomic DNA was isolated from the culture cells using cultured cell extraction kit (ABigen) according to the manufacturer's protocol. Next, the genomic DNA was subjected to bisulfite treatment using the EZ Methylation Kit (Zymo Research), followed by MSP using primers specific to methylated or unmethylated miR-490-3p promoter.

## *Plasmid construct*

MiR-490-3p mimics, ASO and siRNA against ABCC2 were synthesized. The 3'UTR of ABCC2 containing miR-490-3p binding site was PCR-amplified and inserted downstream of a luciferase reporter gene in the pmirGLO vector. A point mutation was generated within the binding site using a QuikChange® site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's instructions. The coding region of ABCC2 was amplified and cloned into the PCMV6 vector to serve as an ABCC2-overexpressed plasmid.

## *Luciferase assay*

SKOV3 cells were seeded in 24-well plates in triplicates at a density of  $5 \times 10^4$  cells/well. When the cell confluence reached approximately 70%, the cells were co-transfected with miR-490-3p mimics or miR-490-3p ASO and wild-type or mutant luciferase reporter constructs containing either wild-type or mutant ABCC2 3'UTR. Next, the luciferase intensity was measured using a Dual-Glo Luciferase assay (Promega, USA) according to the manufacturer's protocol. Renilla luciferase intensity was employed as an internal control to normalize the firefly luciferase intensity.

## *Western blot assay*

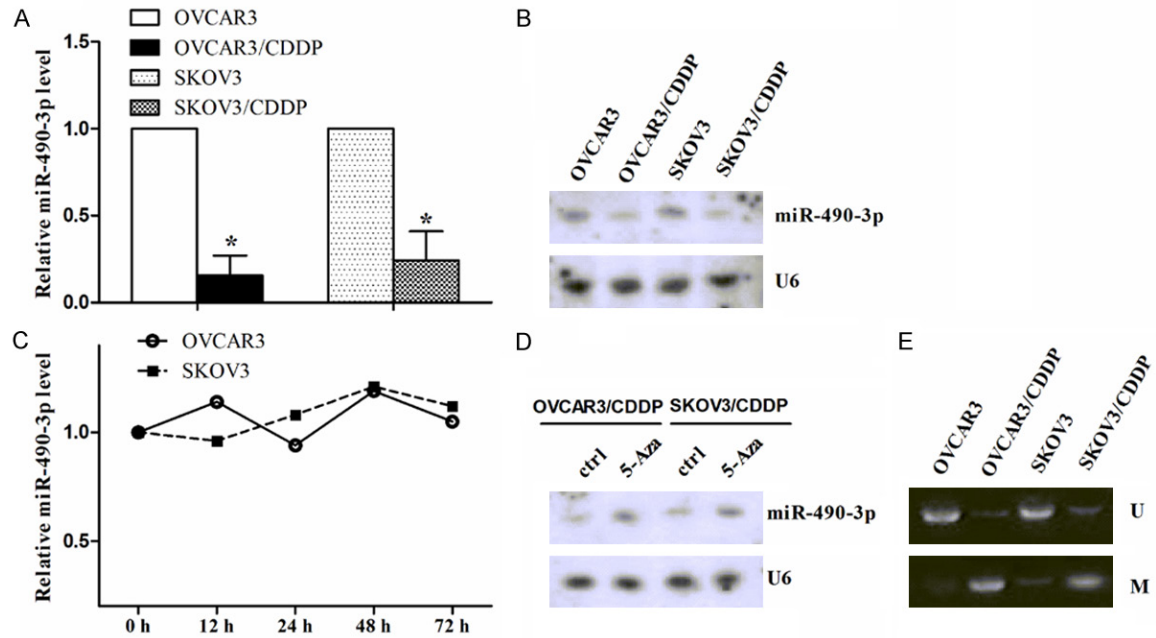
The protein expression of ABCC2 was analyzed using Western blot assay in ovarian cancer cells. Briefly, cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min on ice. Subsequently, 50 µg of proteins were separated on a SDS-PAGE gel, followed by being transferred to a polyvinylidene difluoride (PVDF) membrane and incubated with 5% milk. Rabbit polyclonal to ABCC2 antibody was used as a primary antibody (1:1000, Abcam, USA) and the HRP-conjugated goat anti-rabbit antibody was used as a secondary antibody. Finally, the protein signals were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions. GAPDH was used as a loading control.

## *Immunohistochemical staining*

Immunohistochemistry with a rabbit monoclonal antibody to ABCC2 (1:1000, Abcam, USA) was carried out on archival, formalin-fixed and paraffin-wax-embedded sections of CDDP-resistant or -sensitive ovarian cancer tissue samples as well as ovarian cancer cells. ABCC2 staining criteria was described as follows: according to the expression area, < 5% of staining was considered as (-); 5-20% of staining was considered as (+); 21-50% of staining was considered as (++); and > 50% of staining was considered as (+++).

## *Animal study*

The mice experiments were approved by the Institutional Animal Care and Use Committee of



**Figure 1.** MiR-490-3p is downregulated in CDDP-resistant ovarian cancer cells OVCAR3/CDDP and SKOV3/CDDP. (A, B) The expression of miR-490-3p was analyzed by real-time PCR (A) and Northern blotting assay (B) in OVCAR3, SKOV3 and CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells. U6 was employed as an internal control. \* $P < 0.05$ . (C) The expression of miR-490-3p was examined by real-time PCR at different time points in OVCAR3 and SKOV3 cells after treated with 1  $\mu$ M CDDP. U6 was employed as an internal control. (D) The expression of miR-490-3p was detected by Northern blotting assay in OVCAR3/CDDP and SKOV3/CDDP cells after treated with 1  $\mu$ M 5-aza-2'-deoxycytidine (DNA demethylating reagent, 5-Aza) for 72 h. U6 was employed as an internal control. (E) The hypermethylation of miR-490-3p promoter was determined by MSP in OVCAR3, SKOV3, OVCAR3/CDDP and SKOV3/CDDP cells. U, Unmethylated DNA; M, Methylated DNA.

Tianjin Medical University Cancer Hospital, and performed under specific pathogen-free conditions. Briefly,  $10^6$  SKOV3/CDDP cells were suspended in 100  $\mu$ l of PBS and subcutaneously injected into the flank of 4-5 week-old NOD/SCID mice. On day 13 when the tumor size reached approximately 100 mm<sup>3</sup>, the mice were randomly separated into four groups and then injected with miR-490-3p scramble control or miR-490-3p agomir or CDDP alone or CDDP together with miR-490-3p agomir on day 13, day 16, day 19, day 22 and day 26. The tumor volume was determined by measuring the length (L) and width (W) with calipers and calculated with the formula  $(L \times W^2)/2$ . The tumor xenografts were also used to examine miR-490-3p expression by real-time PCR.

#### Statistical analysis

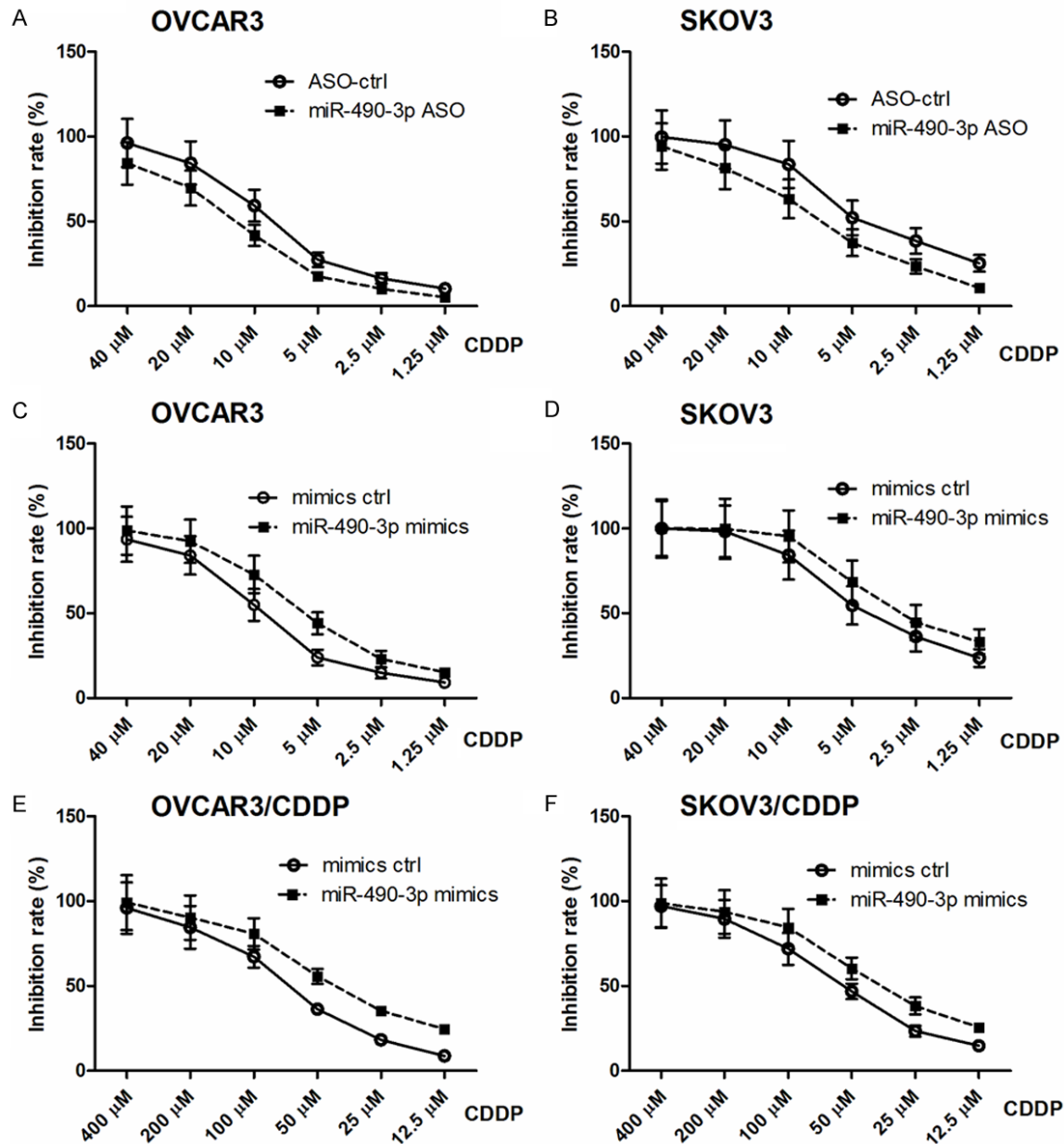
The data were expressed as mean  $\pm$  standard deviation (sd) from three independent experiments in triplicates and one representative data was shown in the study. The statistical

analysis was performed using GraphPad Prism 5 software, and the difference between two groups was analyzed using two tailed Students' t-test. For the differences among three treatment groups in rescue experiment, a one-way analysis of variance (ANOVA) followed by a LSD (least significant difference) test was used. The value of  $P$  less than 0.05 was considered statistically significant.

#### Results

##### *MiR-490-3p is downregulated in CDDP-resistant ovarian cancer cells*

To investigate the potential roles of miR-490-3p in cell response to CDDP, miR-490-3p expression was determined by real-time PCR in OVCAR3, SKOV3 cells and CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells. As shown in **Figure 1A**, we found that miR-490-3p was significantly downregulated in OVCAR3/CDDP and SKOV3/CDDP cells. In agreement with the real-time PCR results, Northern blot-



**Figure 2.** MiR-490-3p modulates cell response to CDDP. A, B. OVCAR3 and SKOV3 cells transfected with miR-490-3p ASO or ASO control were treated with CDDP of different concentrations and cell viability was then measured by MTT assay. The graph represented the inhibition rate. C, D. OVCAR3 and SKOV3 cells transfected with miR-490-3p mimics or mimics control were treated with CDDP of different concentrations and cell viability was then measured by MTT assay. E, F. CDDP resistant OVCAR3/CDDP and SKOV3/CDDP cells transfected with miR-490-3p mimics or mimics control were treated with CDDP of different concentrations and cell viability were then measured by MTT assay.

ting assay indicated that miR-490-3p was less expressed in CDDP-resistant cells (Figure 2B). Next, we aimed to detect the potential molecular mechanism of miR-490-3p downregulation in CDDP-resistant cells. Results from Figure 1C showed that CDDP treatment did not affect the expression of miR-490-3p. A previous study

has demonstrated that the downregulation of miR-490-3p in gastric cancer is due to the hypermethylation of miR-490-3p promoter [18]. Therefore, we treated OVCAR3/CDDP and SKOV3/CDDP cells with 1 μmol/L DNA demethylated reagent 5-aza-2'deoxyctidine (5-Aza). Real-time PCR showed that OVCAR3/CDDP

**Table 2.** The IC<sub>50</sub> of CDDP in OVCAR3 and SKOV3 cells transfected with miR-490-3p ASO or ASO control

IC <sub>50</sub> (μmol/L)	ASO-ctrl	miR-490-3p ASO	Fold change	P value
OVCAR3	9.38 ± 1.43	13.31 ± 3.42	1.41	<0.01
SKOV3	4.71 ± 0.69	7.16 ± 1.14	1.52	<0.01

**Table 3.** The IC<sub>50</sub> of CDDP in OVCAR3 and SKOV3 cells transfected with miR-490-3p mimics or mimics control

IC <sub>50</sub> (μmol/L)	Mimics ctrl	miR-490-3p mimics	Fold change	P value
OVCAR3	9.76 ± 1.62	6.34 ± 1.17	0.65	<0.01
SKOV3	4.46 ± 0.73	3.18 ± 0.42	0.71	<0.01

**Table 4.** The IC<sub>50</sub> of CDDP in CDDP-resistant and SKOV3 cells transfected with miR-490-3p mimics or mimics control

IC <sub>50</sub> (μmol/L)	Mimics ctrl	miR-490-3p mimics	Fold change	P value
OVCAR3/CDDP	69.57 ± 7.48	43.32 ± 4.53	0.62	<0.01
SKOV3/CDDP	53.72 ± 5.95	36.21 ± 5.14	0.67	<0.01

cells treated with 5-Aza had a high miR-490-3p level compared with control cells (**Figure 1D**). Similar results were observed in SKOV3/CDDP cells. These results imply the potential involvement of hypermethylation of miR-490-3p promoter. Using MSP, we found that the hypermethylation of miR-490-3p promoter was observed in CDDP-resistant OVCAR3/CDDP and SKOV3/CDDP cells compared with OVCAR3 and SKOV3 cells (**Figure 1E**).

#### *miR-490-3p enhances the CDDP chemosensitivity in ovarian cancer cells*

Next, we tried to investigate the effects of miR-490-3p on cell response to CDDP using a MTT assay. As shown in **Figure 2A**, we discovered that inhibition of miR-490-3p reduced the inhibition rate of CDDP in OVCAR3 cells, and cells transfected with miR-490-3p ASO had a high IC<sub>50</sub> than that of control cells (**Table 2**). Similar results were acquired in SKOV3 cells transfected with miR-490-3p ASO (**Figure 2B**). In contrast, miR-490-3p-treated OVCAR3 and SKOV3 cells had a high inhibition rate and a low IC<sub>50</sub> of CDDP compared with control cells (**Figure 2C, 2D, Table 3**). We finally determined whether miR-490-3p can restore the CDDP sensitivity in CDDP-resistant cells. As shown in **Figure 2E**

and **Figure 2F**, miR-490-3p increased the inhibition rate of CDDP in OVCAR3/CDDP and SKOV3/CDDP cells, and resulted in a lower IC<sub>50</sub> of CDDP (**Table 4**). Taken together, these data indicate that miR-490-3p could increase the CDDP sensitivity.

#### *ABCC2 is a direct target of miR-490-3p*

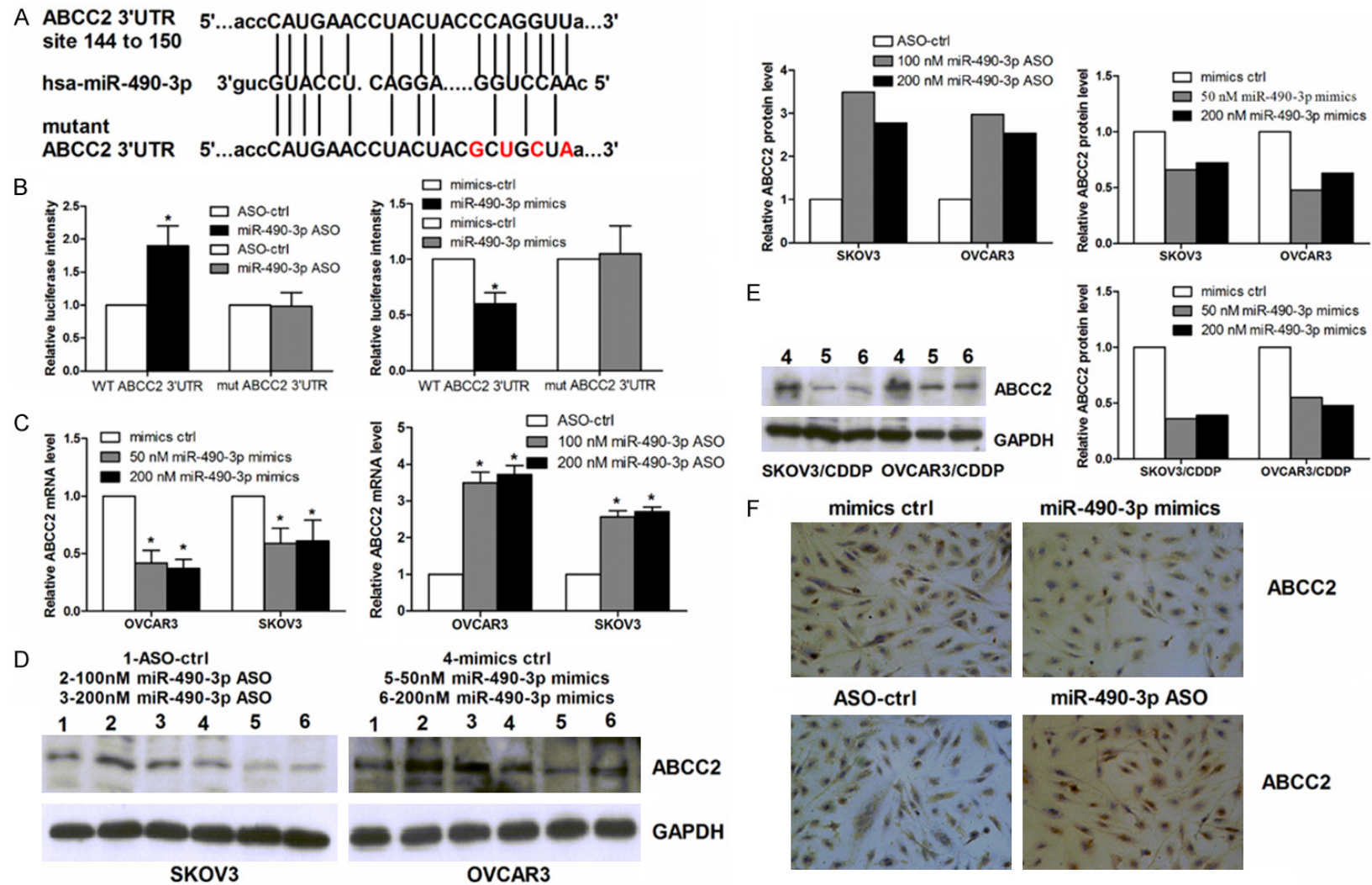
Using TargetScan and miRanda algorithms, we found that there existed a putative binding site for miR-490-3p on ABCC2 3'UTR (**Figure 3A**). Next, the ABCC2 3'UTR containing miR-490-3p binding site was cloned downstream of a luciferase coding gene. Meanwhile, a mutant luciferase reporter construct was generated that several bases were mutated within the binding site (**Figure 3A**). Luciferase assay showed that inhibition of miR-490-3p increased the luciferase intensity of ABCC2 3'UTR in SKOV3 cells, while miR-490-3p overexpression reduced the ABCC2 3'UTR intensity (**Figure 3B**). However, the mutation within the

binding site abrogated the inhibitory effect of miR-490-3p on the intensity of ABCC2 3'UTR (**Figure 3B**). Real-time PCR (**Figure 3C**) and Western blot assay (**Figure 3D**) showed that miR-490-3p overexpression reduced the ABCC2 mRNA and protein levels in both OVCAR3 and SKOV3 cells, while inhibition of miR-490-3p played opposite roles. In addition, similar results were observed in OVCAR3/CDDP and SKOV3/CDDP cells (**Figure 3E**). In addition, immunohistochemical staining indicated that ABCC2 expression was significantly downregulated in miR-490-3p-treated cells (**Figure 3F**). Taken together, these data indicate that miR-490-3p downregulates ABCC2 expression by directly binding to its 3'UTR.

#### *ABCC2 is functionally involved in the effects of miR-490-3p on CDDP response*

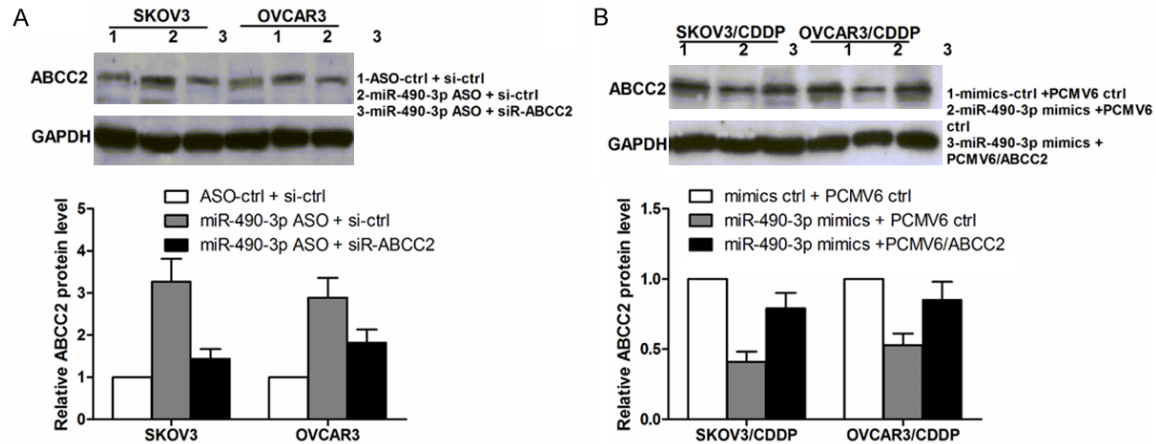
To determine the functional involvement of ABCC2 in miR-490-3p-mediated CDDP resistance, the silencing of ABCC2 was confirmed by Western blot in both OVCAR3 and SKOV3 cells after transfected with ABCC2 siRNA or scramble control (**Figure 4A**). We found that silencing of ABCC2 alleviated the CDDP resistance induced by miR-490-3p ASO, markedly by the reduced IC<sub>50</sub> (**Table 5**). Importantly, overexpres-

# Regulation of ABCC2 by miR-490-3p in CDDP response



**Figure 3.** ABCC2 is a direct target of miR-490-3p. A. The ABCC2 3'UTR existed a binding site for miR-490-3p and several point mutations were generated within the binding site (shown in red). B. Luciferase assay: SKOV3 cells were co-transfected with miR-490-3p ASO or mimics and the luciferase reporter construct containing wild-type or mutant ABCC2 3'UTR, and then luciferase intensity was measured. Renilla luciferase intensity was used as an internal control. \*P<0.05. C. ABCC2 mRNA level was analyzed by real-time PCR in ovarian cancer cells after transfected with miR-490-3p ASO or miR-490-3p mimics. GAPDH was used as an internal control. \*P<0.05. D. ABCC2 protein level was analyzed by Western blot in ovarian cancer cells after transfected with miR-490-3p ASO or miR-490-3p mimics. GAPDH was used as an internal control. The graph represented the relative ABCC2 protein levels. E. ABCC2 protein level was analyzed by Western blot in CDDP resistant ovarian cancer cells after transfected with miR-490-3p mimics or control. F. The expression of ABCC2 was analyzed by immunohistochemical staining in ovarian cancer cells after transfected with miR-490-3p ASO or miR-490-3p mimics.

## Regulation of ABCC2 by miR-490-3p in CDDP response



**Figure 4.** ABCC2 mediates the effects of miR-490-3p on cell response to CDDP. A. The silencing of ABCC2 was confirmed by Western blot in SKOV3 and OVCAR3 cells after transfected with siRNA against ABCC2 or control. GAPDH was employed as an internal control. The graph (below) represented the relative ABCC2 protein level. B. ABCC2 expression was confirmed by Western blot in CDDP resistant cells after transfected with ABCC2-overexpressed plasmid PCMV6/ABCC2. GAPDH was employed as an internal control.

**Table 5.** The  $IC_{50}$  of CDDP in OVCAR3 and SKOV3 cells transfected with miR-490-3p ASO and ABCC2 siRNA or controls

$IC_{50}$ ( $\mu$ mol/L)	ASO-ctrl + si-ctrl	miR-490-3p ASO + si-ctrl	miR-490-3p ASO + siR-ABCC2
OVCAR3	9.82 $\pm$ 1.67	13.64 $\pm$ 2.24 <sup>a</sup>	11.45 $\pm$ 1.95 <sup>b</sup>
SKOV3	4.33 $\pm$ 0.73	6.97 $\pm$ 1.14 <sup>a</sup>	5.57 $\pm$ 1.08 <sup>b</sup>

a:  $P < 0.01$ , ASO-ctrl + si-ctrl group vs miR-490-3p ASO + si-ctrl group; b:  $P < 0.01$ , miR-490-3p ASO + si-ctrl group vs miR-490-3p ASO + siR-ABCC2 group.

**Table 6.** The  $IC_{50}$  of CDDP in OVCAR3 and SKOV3 cells transfected with miR-490-3p mimics and ABCC2 or controls

$IC_{50}$ ( $\mu$ mol/L)	mimics-ctrl + PCMV6 ctrl	miR-490-3p mimics + PCMV6 ctrl	miR-490-3p mimics + PCMV6/ABCC2
OVCAR3/CDDP	63.21 $\pm$ 11.36	38.62 $\pm$ 6.42 <sup>a</sup>	49.77 $\pm$ 8.35 <sup>b</sup>
SKOV3/CDDP	57.34 $\pm$ 10.05	34.58 $\pm$ 6.21 <sup>a</sup>	42.18 $\pm$ 7.49 <sup>b</sup>

a:  $P < 0.01$ , mimics-ctrl + PCMV6 ctrl group vs miR-490-3p mimics + PCMV6 ctrl group; b:  $P < 0.01$ , miR-490-3p mimics + PCMV6 ctrl vs miR-490-3p mimics + PCMV6/ABCC2 group.

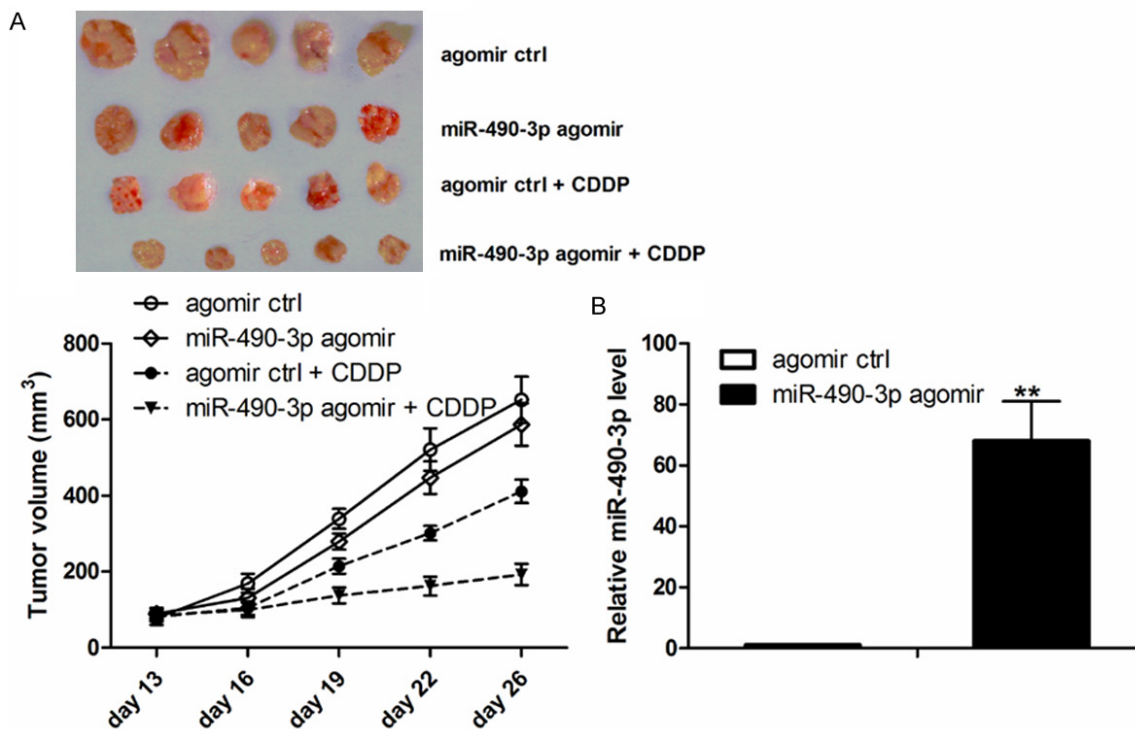
sion of ABCC2 was also confirmed in CDDP-resistant cells after transfected with an ABCC2-overexpressed plasmid PCMV6/ABCC2 (**Figure 4B**). ABCC2 overexpression can enhance the CDDP resistance that was inhibited by miR-490-3p, markedly by the increased  $IC_{50}$  (**Table 6**).

### miR-490-3p enhances CDDP sensitivity in vivo

To determine whether the delivery of miR-490-3p in vivo could enhance the cytotoxicity of CDDP, we established a mice ovarian cancer

model by subcutaneously injecting SKOV3/CDDP cells. When the tumor volume reached approximately 100 mm<sup>3</sup> until day 13, the mice were randomly separated into four group and treated with either miR-490-3p agomir, or agomir control, or CDDP alone, or CDDP together with miR-490-3p agomir. The above treatment was performed every three days until the tumor xenografts were harvested and measured on day 26. As shown in **Figure 5A**, we found that miR-490-3p lightly inhibited tumor volumes compared with control group, and CDDP inhibited the tumor growth significantly. Importantly, CDDP

together with miR-490-3p significantly inhibited the tumor growth compared with CDDP treatment alone. The images of tumor xenografts were shown in **Figure 5A** (above). Next, the expression of miR-490-3p was analyze in tumor xenografts. We discovered that the mice treated with miR-490-3p agomir had a high miR-490-3p level than control group (**Figure 5B**), suggesting that the delivery of miR-490-3p was successful. Overall, the data indicate that miR-490-3p increases CDDP cytotoxicity in vivo.



**Figure 5.** MiR-490-3p enhances CDDP chemosensitivity in vivo. A. NOD/SCID mice were injected with SKOV3/CDDP cells subcutaneously until day 13 when the tumor volume reached approximately 100 mm<sup>3</sup>. On day 13, 16, 19, 22 and 26, mice were treated with CDDP or miR-490-3p agomir alone or miR-490-3p agomir and CDDP together, and tumor volumes were measured until the mice were killed on day 26. (A-above) Representative images of tumor xenografts until the mice were killed on day 26. (A-below) Tumor volumes of xenografts when the mice were killed. B. MiR-490-3p expression was analyzed by real-time PCR in tumor xenografts treated with miR-490-3p agomir or control. U6 was used as an internal control. \*\*P<0.01.

#### *MiR-490-3p and ABCC2 are inversely expressed in ovarian cancer*

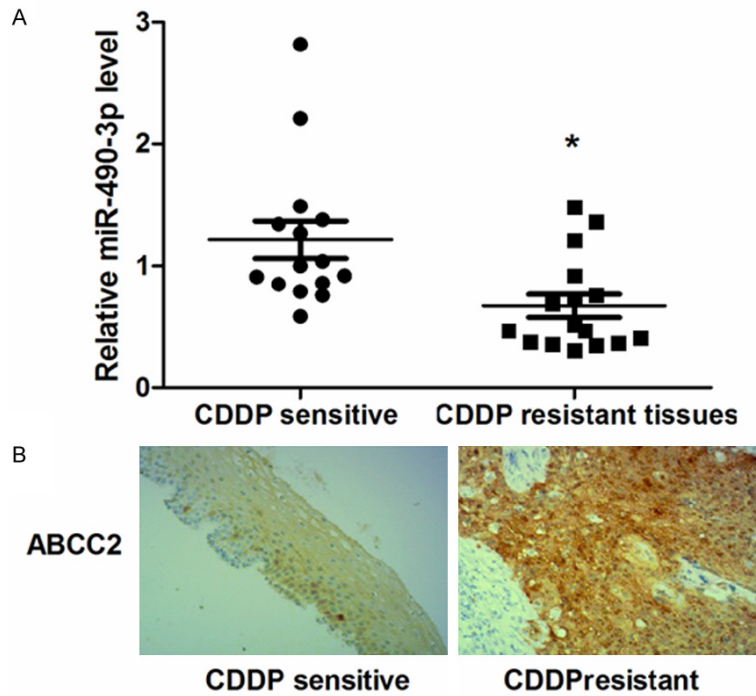
Finally, miR-490-3p was determined by real-time PCR in CDDP-resistant and -sensitive ovarian cancer tissues. As shown in **Figure 6A**, we found that miR-490-3p was downregulated in CDDP-resistant cancer tissues compared with CDDP-sensitive cancer tissues. Immunohistochemistry showed that ABCC2 was mostly expressed in CDDP-resistant cancer tissues (**Figure 6B**, **Table 7**).

#### **Discussion**

Drug-resistance is still a big obstacle for successful therapy of patients with ovarian cancer. Accumulating evidence validates that miRNAs play important roles in the development of drug-resistance in ovarian cancer [12]. For instance, miR-93 contributes to CDDP resistance in ovarian cancer by regulating the PTEN/Akt signaling pathway [13]. MiR-130a and miR-

374a act as key regulators of CDDP resistance in ovarian cancer, and miR-130a may enhance the CDDP resistance through the upregulation of multi-drug resistance protein 1 and down-regulation of PTEN [19]. MiR-497 sensitizes the ovarian cancer cells to CDDP by targeting mTOR/P70S6K1 [20]. In addition, miR-21 [21], let-7e [22], miR-449a [23] and miR-136 [24] also function as important modulators of CDDP resistance in ovarian cancer. However, the studies for miRNAs deregulated in drug resistant ovarian cancer are limited. In this study, we found that miR-490-3p was downregulated in CDDP-resistant ovarian cancer cells and tumor tissues that was due to the hypermethylation of miR-490-3p promoter. MiR-490-3p overexpression enhanced CDDP sensitivity of ovarian cancer in vitro and in vivo. In line with our findings, a previous study shows that the downregulation of miR-490-3p promotes *Helicobacter pylori*-induced gastric carcinogenesis by reactivating the chromatin remodeler SMARCD1 [18], and

## Regulation of ABCC2 by miR-490-3p in CDDP response



**Figure 6.** MiR-490-3p and ABCC2 levels are negatively correlated in ovarian cancer. A. The expression of miR-490-3p was analyzed by real-time PCR in CDDP-sensitive or resistant ovarian cancer tissues. U6 was employed as an internal control. MiR-490-3p was downregulated in CDDP-resistant tissues. \* $P < 0.05$ . B. Protein expression of ABCC2 was determined by immunohistochemical staining in CDDP-sensitive or resistant tissues. ABCC2 was upregulated in CDDP-resistant tissues.

**Table 7.** Immunohistochemical staining of ABCC2 in CDDP-resistant or sensitive tissues

ABCC2 staining	CDDP-sensitive samples	CDDP-resistant samples
-	5/15	1/17
+	6/15	3/17
++	4/15	9/17
+++	0/15	4/17

the downregulation is due to the hypermethylation of its promoter. Inversely, miR-490-3p increases the ovarian cancer cells A2780 and A2780/Taxol resistance to paclitaxel and up-regulates the expression of multi-drug resistance protein 1 [25]. The opposite effects of miR-490-3p on drug-resistance in ovarian cancer may be due to the different mechanism of CDDP and paclitaxel on miR-490-3p expression.

A previous study indicates that DNA methylation contributes to CDDP resistance in ovarian cancer [26]. There are several studies about

the roles of hypermethylation-related genes or miRNAs in drug resistance in ovarian cancer. Epigenetic silencing of miR-130b has been validated to be involved in the development of multi-drug resistance in ovarian cancer by downregulating colony-stimulating factor 1 [27]. Epigenetic downregulation of SFRP5 is associated to chemoresistance of ovarian cancer by regulating Wnt signaling pathway [28]. Moreover, methylation-related miR-199b-5p [29] and miR-9 [30] are related to chemoresistance and paclitaxel in ovarian cancer, respectively. In accord with the previous findings, our study showed that miR-490-3p promoter was hypermethylated by methylation-specific PCR in ovarian cancer cells and 5-Aza treatment restored miR-490-3p expression. Taken together, the current study indicates that CDDP-induced hypermethylation of miR-490-3p enhances CDDP resistance in ovarian cancer.

It is well established that ATP-binding cassette (ABC) transporters play important roles in drug chemoresistance [31, 32]. ABCC2 is one of the ABC transporters, also called multi-drug resistance-associated protein 2 (MRP2) or the canalicular multiple organic anion transporter (cMOAT). ABCC2 is localized in nuclear membranes in ovarian cancer and contributes to the CDDP resistance. In addition, low ABCC2 expression is correlated to longer overall survival [33]. ABC drug transporters (ABCC1, ABCC2 and ABCC3) are involved in the platinum resistance induced by carboplatin therapy in ovarian cancer [34]. ABCC2 is also shown to increase CDDP resistance and controlled by let-7c in non-small cell lung cancer [35]. In line with the effects of ABCC2 in the previous studies, we demonstrated that ABCC2 was a direct target of miR-490-3p and miR-490-3p reduced ABCC2 expression by binding to its 3'UTR. ABCC2 mediated the effects of miR-490-3p on

the ABCC2 was upregulated in CDDP-resistant ovarian cancer tissues.

In conclusion, this study indicated that: 1) miR-490-3p was downregulated in CDDP-resistant ovarian cancer cells and tissues which was due to the hypermethylation of its promoter; 2) miR-490-3p increased cell sensitivity to CDDP by targeting ABCC2. It suggests that miR-490-3p may serve as a therapeutic target to increase the cell response to CDDP in patients with ovarian cancer.

## Acknowledgements

This work was supported by a National Science Foundation from Tianjin Science and Technology Commission (No: 12JCYBJC17000).

## Disclosure of conflict of interest

None.

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