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Original Article

Knockdown of CIP2A sensitizes ovarian cancer cells to cisplatin: an in vitro study

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Abstract: Background: CIP2A is a recently characterized oncoprotein which involves in the progression of several human malignancies. CIP2A is overexpressed in human ovarian cancer and regulates cell proliferation and apoptosis. This study was performed to investigate the role of CIP2A in ovarian cancer (OC) chemoresistance. Methods: Using DDP-resistant SKOV3 cells ($SKOV3^{DDP}$), we first determined the effect of CIP2A silencing by siRNA-mediated knockdown of CIP2A on chemosensitivity in vitro; we then determined the effect of pCDNA3.1-mediated overexpression of CIP2A on chemosensitivity in SKOV3 cells in vitro. To elucidate the molecular mechanisms underlying CIP2A-mediated chemoresistance, the activities of AKT signaling molecules associated with CIP2A were analyzed. Results: Knockdown of endogenous CIP2A in $SKOV3^{DDP}$ cells resulted in the reduction in cell growth and increase in the chemosensitivity of $SKOV3^{DDP}$ cells to DDP in vitro, which may be caused by CIP2A-induced AKT activity inhibition. Notably, CIP2A overexpression could significantly decrease the sensitivities of SKOV3 cells to cisplatin, which might be ascribed to CIP2A-induced activation of the AKT pathway. Conclusions: Taken together, the results suggest that CIP2A contributes to cisplatin resistance in OC. Thus, CIP2A is a potential therapeutic target for OC.

Keywords: Ovarian cancer, chemoresistance, CIP2A

Introduction

Ovarian carcinoma (OC) continues to be the leading cause of death due to gynecologic malignancy in the world because it is usually diagnosed in the advanced stage of the disease [1, 2]. The standard treatment for epithelial ovarian cancer remains surgical debulking and chemotherapy with a platinum and taxane agent. Although many patients with disseminated tumors respond initially to standard combinations of surgical and cytotoxic therapy, nearly 90% of them develop recurrence [3].

Cisplatin (DDP) and its analogues are first-line chemotherapeutic agents for the treatment of human ovarian cancer [4-5]. Cisplatin promotes its cytotoxicity by forming DNA-protein cross-links, DNA mono-adducts, and intrastrand DNA cross-links, which all trigger apoptosis [6, 7]. In ovarian cancer, the majority of tumours acquire drug resistance. Response rates to first-line platinum-based therapy are more than 80%, but most patients with advanced disease will finally relapse and die because of acquired drug resistance [8]. The mechanisms involved in cisplatin resistance are not yet fully understood.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified human oncoprotein that inhibits c-Myc protein degradation in cancer cells. Much evidence have indicated that CIP2A directly promotes malignant transformation, several recent studies have shown that CIP2A inhibition in fully malignant cancer cells results in decreased cell viability and anchorage-independent growth [9-18]. In addition, CIP2A promotes progenitor cell self-renewal and protects cancer cells from therapy-induced apoptosis or senescence induction [9, 19-22]. Furthermore, the role of CIP2A in regulation of cell cycle and mitosis was demonstrated by a recent study identifying PLK1 as a target of CIP2A [23]. Importantly, several independent studies have demonstrated that CIP2A depletion via small interfering RNAs (siRNA) inhibits

the growth of xenografted tumors of various cancers cell types [9, 14, 18]. Choi et al. has found that overexpression of CIP2A has been shown to increase the proliferation of MDA-MB-231 cells and CIP2A expression is associated with sensitivity to doxorubicin [24]. It has recently reported CIP2A depletion in ovarian cancer cell lines inhibited proliferation, blocked cell cycle progression, and increased paclitaxel-induced apoptosis [25], suggesting CIP2A as a new target for breast cancer therapy, however, the mechanism is not very clear.

The AKT signaling pathway is activated in a wide range of tumor types and drives cancer cell proliferation and survival. CIP2A overexpression in liver cancer cells increased AKT phosphorylation, and CIP2A inhibition caused dephosphorylation of serine 473 of AKT in liver cancer and TNBC cells [26, 27]. Moreover, resistance to drugs that act via the AKT pathway seems to occur at least partly because of their effects on CIP2A expression [27]. It is unclear thus far whether CIP2A mediated AKT phosphorylation plays a role in cisplatin resistance, which is the focus of this study.

In the present study, we show that cisplatin could not induce CIP2A expression and AKT activation. Endogenous CIP2A overexpression is an important mediator of chemoresistance in ovarian cancer cells. Overexpression of CIP2A triggers ovarian cell survival and cisplatin resistance through triggering the activity of pAKT. Knockdown of CIP2A decreases ovarian cell survival and increases cisplatin sensitivity through inhibiting the activity of pAKT. We propose that combinatorial targeted inhibition of the CIP2A-AKT-survivin pathway has the potential to enhance the effectiveness of chemotherapy in the treatment of ovarian cancer.

Materials and methods

Cell culture

Ovarian cancer cell lines SKOV3 was obtained from the American Type Culture Collection (ATCC; Shanghai, China). Cisplatin (DDP) resistant SKOV3 cell line ($SKOV3^{DDP}$) was obtained from yiyeqi.cc (Shanghai, China). The cell lines were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS. $SKOV3^{DDP}$ was dissolved in DMSO (Novaplus, Ben Venus Laboratories, Inc.) was added.

Agents

Cisplatin (DDP, cis-diammine-dichloro-platinum II) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (St. Louis, MO). Stock DDP solution was prepared in DMSO (330 mM), stored as aliquots at 20°C, and used within 2 weeks. DDP was further diluted in medium before adding to the cells.

Small interfering RNA transfection

CIP2A siRNA oligonucleotides were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions for 48 hs. The sequence of CIP2A siRNA was 5'-CUGUGGUUGUGUUUGCACUTT-3'. The sequence of scrambled siRNA was 5'-UAACAAUGAGAGCACGGCTT-3'. AKT siRNA that targets AKT was purchased from Cell Signaling Technology. Cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's instructions.

Construction of pcDNA 3.1-CIP2A and transduction of target cells

The CIP2A sequences were amplified by PCR, confirmed by sequencing. The fragment was then inserted into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA) to generate a pcDNA3.1-CIP2A construct which was sequenced commercially (Shenggong, Shanghai, China). The pCDNA3.1-CIP2A and its control pCDNA3.1 plasmid were transfected into the SKOV3 cells to product stably transfected cell populations (SKOV3/CIP2A and SKOV3/pCDNA3.1).

Cytotoxicity assay

The cytotoxicity was measured by the MTT assay. ① $SKOV3^{DDP}$ cells, $SKOV3^{DDP}$/CIP2A siRNA and $SKOV3^{DDP}$/control siRNA (1×10^4 cells/well of 96-well plate) were treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h and thereafter 25 ml of MTT solution (5 mg/ml in PBS) was added. After 2 h of incubation, 100 ml extraction buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was red at 570 nm. ② SKOV3, SKOV3/CIP2A and SKOV3/pCDNA3.1 were treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, or SKOV3, SKOV3/CIP2A and

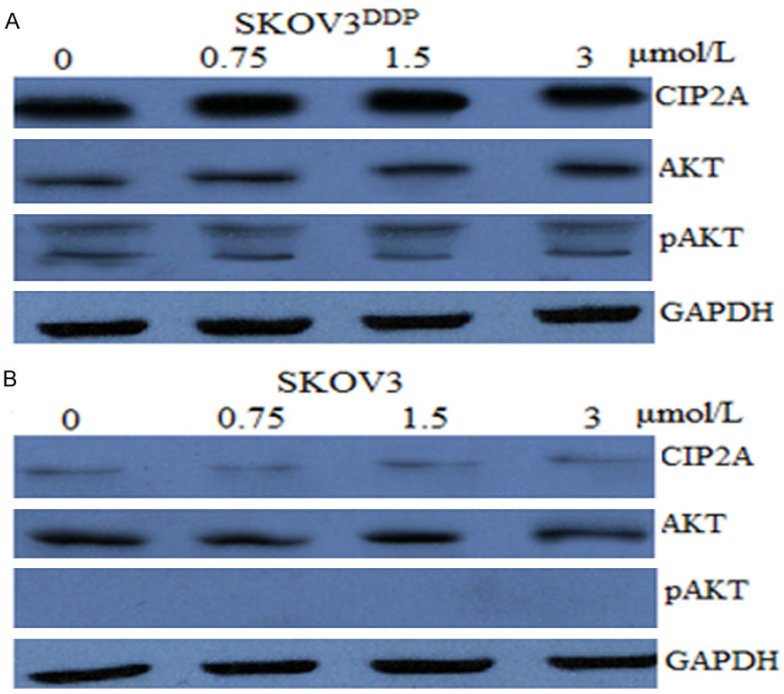

Figure 1. Effect of DDP treatment on the expression of CIP2A and pAKT in breast cancer cells. A. SKOV3^{DDP} cells were treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, western blot was used to detect CIP2A, AKT and pAKT protein in the cells. B. SKOV3 cells were treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, Western blot was used to detect CIP2A, AKT and pAKT protein in the cells.

SKOV3/pCDNA3.1 cells were transiently transfected AKT siRNA for 24 hs first, then treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, and thereafter 25 ml of MTT solution (5 mg/ml in PBS) was added. After 2 h of incubation, 100 ml extraction buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was red at 570 nm.

Quantification of apoptosis by ELISA

The cell death detection ELISA (enzyme linked immunosorbent assay) kit was used for assessing apoptosis according to the manufacturer's protocol. After treatment as above, the cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody for detection of apoptosis.

Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline and lysed in lysis buffer. The samples were pretreated with ultrasound (10 pulses on ice; Sonoplus, Bandelin Electric, Germany) and were centrifuged at 14,000× g for 20 minutes at 4°C. The protein concentration of the cellular extracts was determined using the advanced protein assay reagent. 40 micrograms of protein extract were electrophoresed on 10% NuPage Bis-Tris-Glycine gels (Invitrogen, Germany) for 2 hours at 120 V. Proteins were blotted onto polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany) at 160 mA for 60 minutes using a tank blot system. The membranes were blocked with 1% non-fat dry milk powder in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 (TBST buffer) for 1 hour at 4°C and washed three times with TBST. Immunostaining was performed using antibodies against CIP2A, AKT, pAKT and GAPDH (each Cell Signaling, Shanghai, China). Bound antibody was visualized using the chemiluminescence detection system (Pierce, Rockford, IL) following the supplier's instructions.

Statistical analysis

Data are presented as mean ± SD, and statistical comparisons between groups were performed using one-way ANOVA followed by Student's t test. A value of $P<0.05$ was considered significant.

Results

Comparison of endogenous CIP2A and pAKT status in parental and DDP-resistant human breast cancer SKOV3 cells

To determine if the CIP2A pathway is affected when human SKOV3 cells acquire resistance to cisplatin, we compared the level and activation status of CIP2A and pAKT in parental SKOV3 cells and its cisplatin-resistant variant SKOV3^{DDP} cells. Data shown in **Figure 1**, CIP2A and phosphorylation of AKT (pAKT) pAKT was overex-

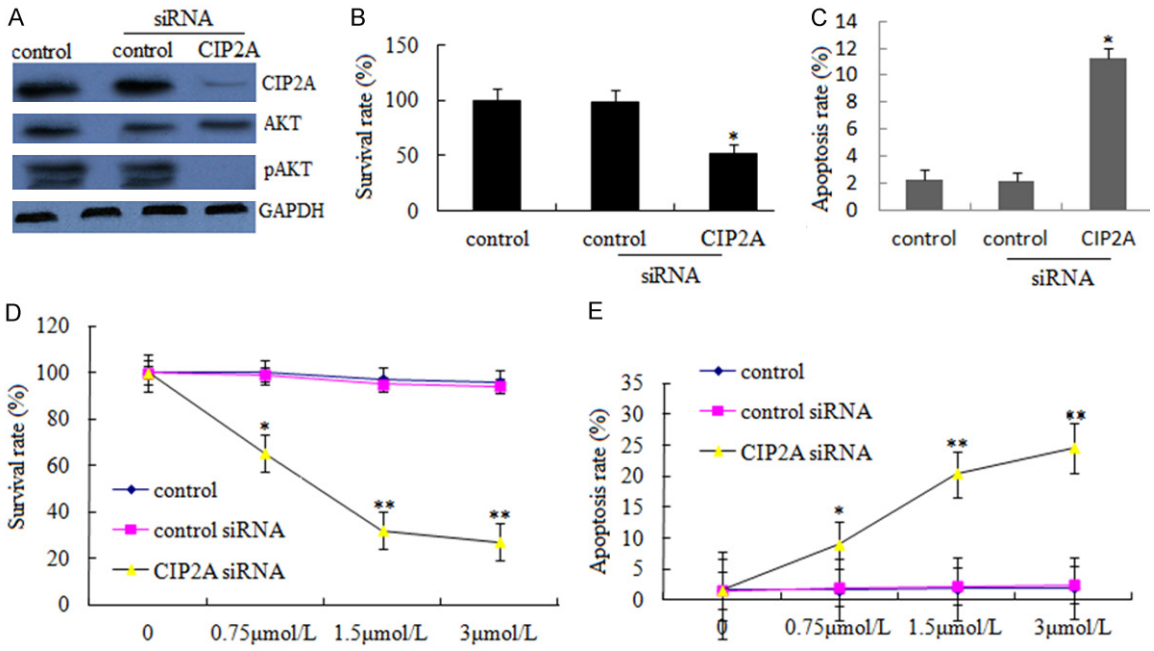

Figure 2. Combination of CIP2A siRNA transfection with DDP efficiently reduces the viability and increases the apoptosis of SKOV3^{DDP} cells. A. SKOV3^{DDP} cells were transient CIP2A siRNA transfection for 48 hs, CIP2A, pAKT and AKT was detected by Western blot assay. B. SKOV3^{DDP} cells were transient CIP2AsiRNA transfection for 48 hs, MTT was used to detect cell viability. Vs control, $^{*}P<0.05$. C. SKOV3^{DDP} cells were transient CIP2A siRNA transfection for 48 hs, ELISA was used to detect cell apoptosis. Vs. control, $^{*}P<0.05$. D. SKOV3^{DDP} cells were transient CIP2A siRNA transfection for 48 hs, then treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, MTT was used to detect cell viability. Vs control, $^{*}P<0.05$, $^{**}P<0.01$. E. SKOV3^{DDP} cells were transient CIP2A siRNA transfection for 48 hs, then treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, ELISA was used to detect cell apoptosis. Vs control, $^{*}P<0.05$, $^{**}P<0.01$.

pressed in the SKOV3^{DDP} cells (**Figure 1A**), CIP2A and pAKT was undetected in the SKOV3 cells (**Figure 1B**).

DDP treatment did not affect CIP2A and pAKT status in parental and DDP-resistant human breast cancer SKOV3 cells

We next investigated whether DDP could activate CIP2A and pAKT in the human breast cancer SKOV3 cells. The results showed when the SKOV3 and SKOV3^{DDP} cells were treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, there was no significant effect on CIP2A and pAKT expression in any of the SKOV3^{DDP} (**Figure 1A**) and SKOV3 (**Figure 1B**) cells analyzed. We therefore suggested that DDP treatment did not affect CIP2A and pAKT level in SKOV3 and SKOV3^{DDP} cells.

Knockdown of CIP2A expression decreased cell viability and increased apoptosis in the SKOV3^{DDP} cells

As shown in **Figure 2A**, CIP2A was significantly inhibited in the SKOV3^{DDP} cells via transient CIP2A siRNA transfection for 48 hs by western blot assay. We also found that pAKT activity was significantly decreased followed by CIP2A inhibition (**Figure 2A**).

SKOV3^{DDP} cells were used to investigate the effect of CIP2A inhibition on cell viability apoptosis as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ELISA.We observed that transient CIP2A siRNA transfection for 48 hs showed a significant decrease in cell survival (**Figure 2B**) and increase in cell apoptosis (**Figure 2C**).

Knockdown of endogenous CIP2A expression abrogate DDP resistance in the SKOV3^{DDP} cells

In order to evaluate the combinatorial effect of CIP2A inhibition with DDP, we measured cell viability and apoptosis after treatment of cells with DDP and CIP2A siRNA transfection.

SKOV3^{DDP} cells treated with DDP (0.75, 1.5, 3 μmol/L) alone for 72 h did not affect cell viability (**Figure 2D**) and apoptosis (**Figure 2E**) of SKOV3^{DDP} cells. However, combined treatment

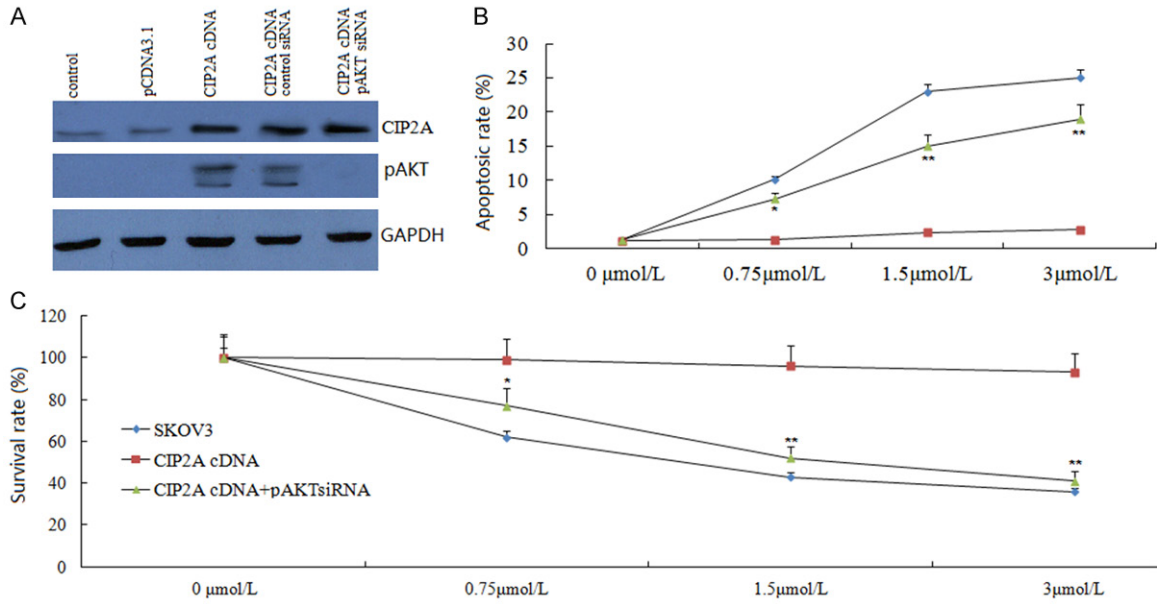

Figure 3. CIP2A overexpression increases DDP resistance in SKOV3 cells via pAKT activity. A. SKOV3 cells was transfected pCDNA3.1CIP2A cDNA or/and pAKT siRNA, CIP2A and pAKT was detected by western blot assay. B. SKOV3 cells was transfected pCDNA3.1CIP2A cDNA or/and pAKT siRNA, then treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, ELISA was used to detect apoptosis. Vs control, $^{*}P<0.05$, $^{**}P<0.01$. C. KOV3 cells was transfected pCDNA3.1CIP2A cDNA or/and pAKT siRNA, then treated with DDP (0.75, 1.5, 3 μmol/L) for 72 h, MTT was used to detect cell viability. Vs control, $^{*}P<0.05$, $^{**}P<0.01$.

of DDP and CIP2A siRNA transfection significantly reduced cell viability (**Figure 2D**). Similarly, combined treatment of CIP2A siRNA transfection with DDP also effectively promoted apoptosis of SKOV3^{DDP} cell compared to single treatment with DDP or CIP2A siRNA transfection (**Figure 2E**).

CIP2A overexpression protects SKOV3 cells from DDP-induced cell death

As shown in **Figure 3A**, CIP2A was significantly increased in the SKOV3 cells via stable pCDNA3.1CIP2A cDNA transfection by western blot assay. We also found that pAKT activity was significantly increased followed by CIP2A overexpression (**Figure 3A**).

SKOV3 cells treated with DDP (0.75, 1.5, 3 μmol/L) alone for 72 h significantly decreased cell viability (**Figure 3B**) and increased apoptosis (**Figure 3C**) in a dose-dependence.

However, in the CIP2A transfected SKOV3 cells, DDP (0.75, 1.5, 3 μmol/L) treatment for 72 h did not significantly affect cell viability (**Figure 3B**) and cell apoptosis (**Figure 3C**). We therefore suggested that CIP2A overexpression increased chemoresistance to DDP in SKOV3 cells.

CIP2A overexpression increases DDP resistance in SKOV3 cells via pAKT activity

As shown above, pAKT activity was significantly increased followed by CIP2A overexpression, furthermore, CIP2A overexpression increased chemoresistance to DDP in SKOV3 cells. However, when the pAKT was inhibited by siRNA transfection via western blot assay (**Figure 3A**), the chemosensitivity of SKOV3/CIP2A cDNA (CIP2A transfected SKOV3 cells) to DDP (0.75, 1.5, 3 μmol/L) was restored (**Figure 3B**, **3C**).

Discussion

Previous reports have indicated that CIP2A expression is common in ovarian cancer, and strong cytoplasmic CIP2A immunopositivity predicted poor outcome in ovarian cancer patients [13]. Our findings show that DDP treatment did not induce increased CIP2A expression in both SKOV3 cells and SKOV3^{DDP} cells; CIP2A was less expressed in the SKOV3 cells, and high CIP2A expression in the SKOV3^{DDP} cells; CIP2A was overexpressed in the DDP-resistant SKOV3 cells (SKOV3^{DDP}), inhibition of CIP2A expression leads to increased apoptosis and decreased cell proliferation and decreased in vitro tumorigenic potential. Furthermore, we

demonstrated that inhibition of CIP2A enhanced sensitivity of SKOV3^{DDP} cells to DDP. We also found that overexpression of CIP2A in the SKOV3 cells decrease the sensitivity of SKOV3 cells to DDP. These results suggest that therapeutic targeting of CIP2A in combination with conventional chemo-therapeutic agents can increase the efficacy of ovarian cancer therapy. We therefore suggested that a high level of CIP2A gene is directly associated with resistance to DDP treatment in human ovarin cancer cells. CIP2A expression is very low in most human tissues and, importantly, undetectable in normal mammary glands [27, 28], thereby creating a potential therapeutic window for CIP2A targeting agents.

The functions of CIP2A in ovarin cancer have not been fully understood yet and its clinical relevance has not yet been established. According to our studies, we found the possible interaction between CIP2A and AKT signaling pathway, which may help to reveal the mechanism for CIP2A enhanced chemoresistance. In the present study, we firstly attempted to address the relationship between CIP2A and AKT phosphorylation. The positive correlation between CIP2A and AKT phosphorylation in our results clearly demonstrated the possible regulation of AKT by CIP2A. We found in the study that CIP2A regulates the phosphorylation status of AKT and finally affects the sensitivity of cancer cells to the treatment. Therefore, CIP2A-mediated DDP sensitivity may be dependent on the regulation of AKT which need further studies to be confirmed.

Collectively, in this context, CIP2A silencing should increase cell death in ovarian cancer cells and sensitized cells to apoptosis induced by cisplatin. Our studies not only establish the correlation between CIP2A and AKT phosphorylation but also reveal novel functions of CIP2A in promoting AKT signaling cascade. Further studies are required to increase the resolution for the identification of CIP2A-targeted AKT substrates well as the role of CIP2A in chemoresistance.

Disclosure of conflict of interest

None.

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