

# Upregulation of p27<sup>Kip1</sup> by demethylation sensitizes cisplatin-resistant human ovarian cancer SKOV3 cells

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**Abstract.** Ovarian cancer has a poor prognosis due to its chemoresistance, and p27<sup>Kip1</sup> (p27) has been implicated in tumor prognosis and drug-resistance. However, the regulatory mechanisms of p27 in drug-resistance in ovarian cancer remain unknown. The current study successfully established chemoresistant cell lines using paclitaxel (TAX), cisplatin (DDP) and carboplatin (CBP) in SKOV3 ovarian cancer cells. The results indicated that the expression levels of p27 were dramatically downregulated in chemoresistant cells. However, 5-aza-2'-deoxycytidine (5-aza) treatment restored p27 expression in DDP-resistant cells, and increased their sensitivity to DDP. In addition, it was observed that the methylation of DDP-resistant cells, which was downregulated by 5-aza treatment, was significantly higher compared with SKOV3 cells. Additionally, the overexpression of p27 arrested the cell cycle in S phase and promoted an apoptotic response to DDP. In conclusion, p27 was involved in chemoresistance of SKOV3 cells. Upregulated p27 expression induced by demethylation may enhance sensitivity to DDP through the regulation of the cell cycle.

## Introduction

Ovarian cancer (OC) is an aggressive tumor and the most lethal gynecologic malignancy (1), and has been shown to be associated

with cell cycle dysregulation (2). As the result of its sensitivity to chemotherapeutic drugs, surgical cytoreduction followed by systemic chemotherapy with platinum and paclitaxel has been the standard treatment for advanced OC (3). However, long-term chemotherapy may result in chemoresistance (4) and a high rate of tumor recurrence in treated patients (5).

The cell cycle normally keeps cells in a balance of growth and death, and certain key proteins serve essential regulatory roles, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) (6,7). However, disorder in cell cycle-related proteins may allow cancer cells to bypass regulatory check-points in the cell cycle and undergo uncontrolled proliferation (8).

p27/Kip1 is a CDKI that results in cell cycle arrest in G<sub>1</sub> phase by inhibiting CDK2-cyclin E and CDK2-cyclin A (9). Previous studies have reported that p27 is downregulated in numerous types of tumor, including carcinomas of the colon, breast, prostate, lung and ovary (8,10,11), and the reduction in p27 expression has been significantly correlated with tumor grade and cancer prognosis in OC (6). Notably, there is growing evidence suggesting that loss of p27 can mediate a drug-resistant phenotype. Chu *et al* (12) demonstrated that p27 inhibition is associated with tamoxifen-resistance. Schmidt and Fan (13) observed that the absence or cytoplasmic localization of p27 is linked to drug resistance. Xing *et al* (14) reported that p27 serves as a regulator of chemoresistance in ovarian tumors. Le *et al* (15) demonstrated that downregulation of p27 by siRNA can reduce the paclitaxel and dasatinib sensitivity of OC cells, and overexpression of p27 led to enhanced sensitivity of OC. Thus, p27 is a candidate biomarker of chemoresistance in OC. However, the mechanism of p27-associated chemoresistance in OC is not fully understood.

In the present study, the effect and mechanism of p27 in the chemoresistance of OC SKOV3 cell lines was investigated, and this may indicate that p27 is a potential therapeutic target for the enhancement of sensitivity to chemotherapy in OC.

## Materials and methods

**Cell culture.** SKOV3 human OC cells were purchased from American Type Culture Collection (Manassas, VA, USA).

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**Abbreviations:** TAX, paclitaxel; DDP, cisplatin; CBP, carboplatin; 5-Aza, 5-aza-2'-deoxycytidine; p27, p27<sup>Kip1</sup>

**Key words:** p27<sup>Kip1</sup>, ovarian cancer, methylation, chemoresistance, cell cycle, SKOV3

SKOV3 cells were cultured as a monolayer in Roswell Park Memorial Institute-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 2 mM GlutaMAX™. Paclitaxel (TAX), cisplatin (DDP) and carboplatin (CBP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemoresistant variants SKOV3/TAX (paclitaxel resistant), SKOV3/DDP (cisplatin resistant) and SKOV3/CBP (carboplatin resistant) were derived by step-wise incubation of the chemotherapeutic agent over a number of weeks, as previously described (16). In brief, the SKOV3 cells were initially exposed to a low concentration of each drug. After 24 h, the drug was replaced with normal medium. Once cells were 80% confluent, a higher concentration of the drug was added to the culture dish. SKOV3/TAX, SKOV3/DDP and SKOV3/CBP lines were maintained at 0.1  $\mu$ M TAX, 1.0 mg/l DDP and 0.39 mg/l CBP, respectively.

*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.* A total of 5,000 cells were plated in 96-well plates. Following 24 h, the medium was replaced with a chemotherapeutic drug at a range of concentrations: TAX, 0, 0.1, 1, 5, 10 and 20  $\mu$ M/l; DDP and CBP, 0, 0.01, 0.1, 1, 10 and 100 mg/l. Following 72 h of culture, 20  $\mu$ l MTT solution (5 mg/ml) was added to each well. After 4 h incubation at room temperature, the MTT solution was replaced by 150  $\mu$ l of dimethyl sulfoxide to dissolve the tetrazolium crystals. The absorption of each well was measured 10 mins later at a wavelength of 570 nm with a microplate reader (Thermo Fisher Scientific, Inc.). The cell viability (%) was calculated as follows: Optical density (OD) of the treated wells/the control wells  $\times$  100. The resistance index (RI) was calculated as follows: IC50 of resistant SKOV3 vs. SKOV3.

*5-aza-2'-deoxycytidine demethylation treatment.* For demethylation studies, all SKOV3 cells were treated with 5  $\mu$ M/l 5-aza-2'-deoxycytidine (Sigma-Aldrich) for 72 h. Cells were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting, MTT assay and bisulfite sequencing PCR (BSP).

*Clonogenic assay.* For colony formation assays, the SKOV3 cells were seeded in dishes (35 mm; 400 cells/well). TAX (5  $\mu$ M), DDP (12.5 mg/l) or CBP (6.25 mg/l) were added into the medium. Following a 4 h exposure, the medium was replaced and cells were fixed in 1% glutaraldehyde (BD Biosciences, San Jose, CA, USA) and stained with 0.5% crystal violet (Sigma-Aldrich) in methanol. Colonies with greater than 30 cells were counted following 7 days growth.

*Flow cytometric analysis of the cell cycle.* Cells were cultured and treated as described above. Subsequently,  $1 \times 10^6$  cells were collected and fixed with 70% ethanol. Cells were washed twice in phosphate-buffered saline (PBS), and propidium iodide (PI) was added to the cell suspension solution and incubated at 4°C for 30 min prior to analysis with a MoFlo cell sorter (Beckman Coulter, Inc., Brea, CA, USA).

*Apoptotic assay.* An annexin V apoptosis detection kit (Thermo Fisher Scientific, Inc.) was used to analyze apoptosis.

Following the indicated treatment, cells were trypsinized, collected and resuspended. A total of  $2 \times 10^5$  cells were harvested and washed twice with cold PBS, then resuspended in 500  $\mu$ l binding buffer. A total of 10  $\mu$ l annexin V-fluorescein isothiocyanate and 10  $\mu$ l PI were added to the solution and agitated. Following 15 min incubation, the cells were analyzed using flow cytometric analysis (BD Biosciences, San Jose, USA).

*Transfection.* SKOV3 cells were cultured as described above for 48 h prior to transfection. A total of 100 pmol of a p27-overexpression plasmid, pCDNA3.1-P27 (Auragene Bioscience, Changsha, China), was diluted in 250  $\mu$ l serum-free medium. A total of 10  $\mu$ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was added to each sample. Following incubation at 37°C for 4 h, the medium was replaced with complete medium for a further 48 h of culture, following which the cells were collected for use.

*Western blotting.* Whole-cell lysates were harvested and washed with PBS, and lysed in a buffer containing 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, NaVO<sub>4</sub>, aprotinin and leupeptin as protease inhibitors, in 50 mM Tris-HCl pH 8.0, 0.2% sodium dodecyl sulfate (SDS) and 1% NP-40. The protein concentrations were determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China). A total of 30  $\mu$ g of protein per sample was resolved on a 12% SDS-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated at 4°C with primary antibody against P27 (1:500; cat. no. BM0447; Abzoom Biolabs, Inc., Dallas, TX, USA) overnight. Following washing, membranes were incubated with goat anti-mouse secondary antibody (1:15,000; cat. no. SA001; Auragene Bioscience) for 1 h at room temperature, followed by enhanced chemiluminescence (ECL) for visualization using an ECL Plus Chemiluminescence detection kit (Auragene Bioscience). For the control, the membrane was stripped and reprobed using an actin antibody (1:2,300; cat. no. SCA01; Auragene Bioscience) following the probing of each membrane with the primary antibody. The ratio of gray on the blots was analysed by Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

*RT-qPCR analysis of mRNA expression.* Total RNA was extracted from SKOV3 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was reverse transcribed by incubating oligo dT (1  $\mu$ l, 0.5  $\mu$ g/ $\mu$ l), total RNA (2  $\mu$ g) and diethylpyrocarbonate water (9  $\mu$ l) at 65°C for 5 min. Then, 5X reaction buffer (4  $\mu$ l; Thermo Fisher Scientific, Inc.), RNase inhibitor (1  $\mu$ l), dNTPs (2  $\mu$ l, 10 mM) and RT (1  $\mu$ l; Thermo Fisher Scientific, Inc.) were added to the reaction and incubated at 42°C for 1 h and 72°C for 10 min. The cDNA was stored at 4°C prior to use in further experiments. RT-qPCR was performed using an ABI 7500 Thermocycler (Thermo Fisher Scientific, Inc.) and SYBR Green Universal PCR Master Mix (BioRad Laboratories, Inc., Hercules, CA, USA). The oligonucleotide sequences of the primer sets used were as follows: Human p27, forward 5'-CAC TGCAGAGACATGGAA-3' and reverse 5'-GCTTCATCA AGCAGTGA-3'; and human  $\beta$ -actin, forward 5'-AGGGGC

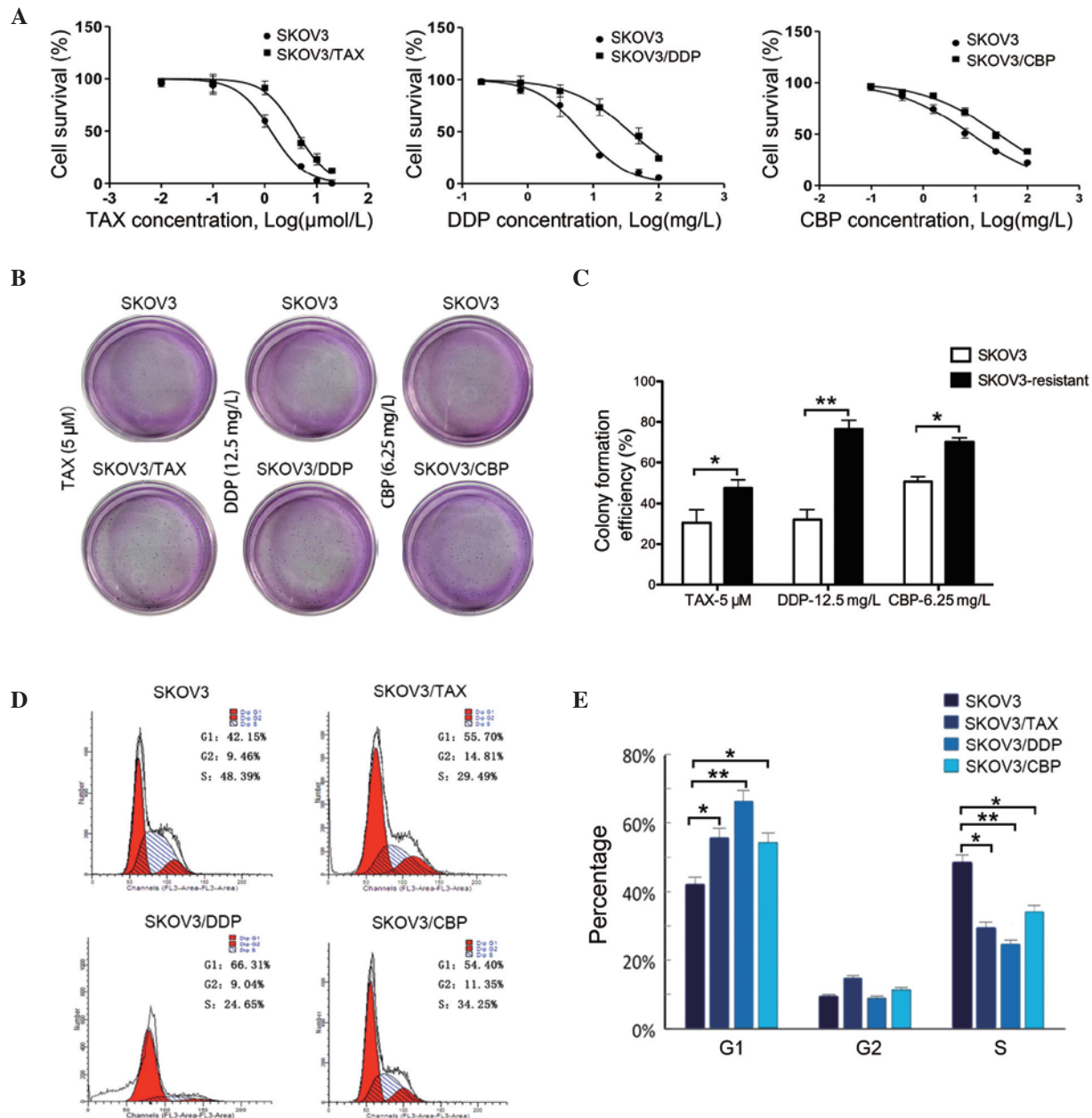


Figure 1. Characteristics of chemoresistant SKOV3 cell lines. (A) Cell survival of chemoresistant SKOV3 cell lines measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. SKOV3 cells were cultured in each drug medium as control and 6 different concentrations of each drug were used in each group. Data were presented as the mean  $\pm$  standard deviation, n=3. (B) Representative images of colonies formed in the different culture medium. (C) Quantification of colony formation of the different cell lines. Data are presented as the mean  $\pm$  standard deviation, n=3. \*P<0.05; \*\*P<0.01. (D) Cell-cycle analysis for SKOV3 cell lines, using flow cytometry. The red peak on the left of each histogram represents G<sub>1</sub> phase, while that on the right indicates the cells in G<sub>2</sub> phase. The blue slashed area indicates cells in S phase. (E) Statistical analysis of cell-cycle distribution in all cell lines. Data are presented as the mean  $\pm$  standard deviation, n=3. \*P<0.05, \*\*P<0.01, comparison indicated by brackets. TAX, paclitaxal; DDP, cisplatin; CBP, carboplatin.

CGGACTCGTCATACT-3' and reverse 5'-GGCGGCACC ACCATGTACCCT-3'). PCR was performed in a total volume of 20  $\mu$ l, which included 10  $\mu$ l of 2X SYBR Green qPCR Mix, 1  $\mu$ l of each forward and reverse primer (10  $\mu$ mol/l) and 40 ng of each cDNA sample (quantified using a Nanodrop). Amplifications were carried out in triplicate in 96-well microtiter plates. The thermal cycling conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 10 sec, and 58°C for 30 sec, and followed by 95°C for 12 sec, 58°C for 50 sec. The  $2^{-\Delta\Delta C_t}$  method was used to analyze the relative changes in gene expression (17).

**DNA methylation analysis using BSP.** Genomic DNA from SKOV3 cell lines was isolated with an Auragene Genomic DNA kit (Auragene Bioscience). Subsequently, Genomic DNA was subjected to bisulfite conversion and purification using the EZ DNA Methylation-Gold™ kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions. The BSP primer was designed by Methprimer Express version 1.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: SKOV3, forward 5'-TTTTTTAGGGAT GGTAGAAATTTTT-3' and reverse 5'-CAACAAACCTAC

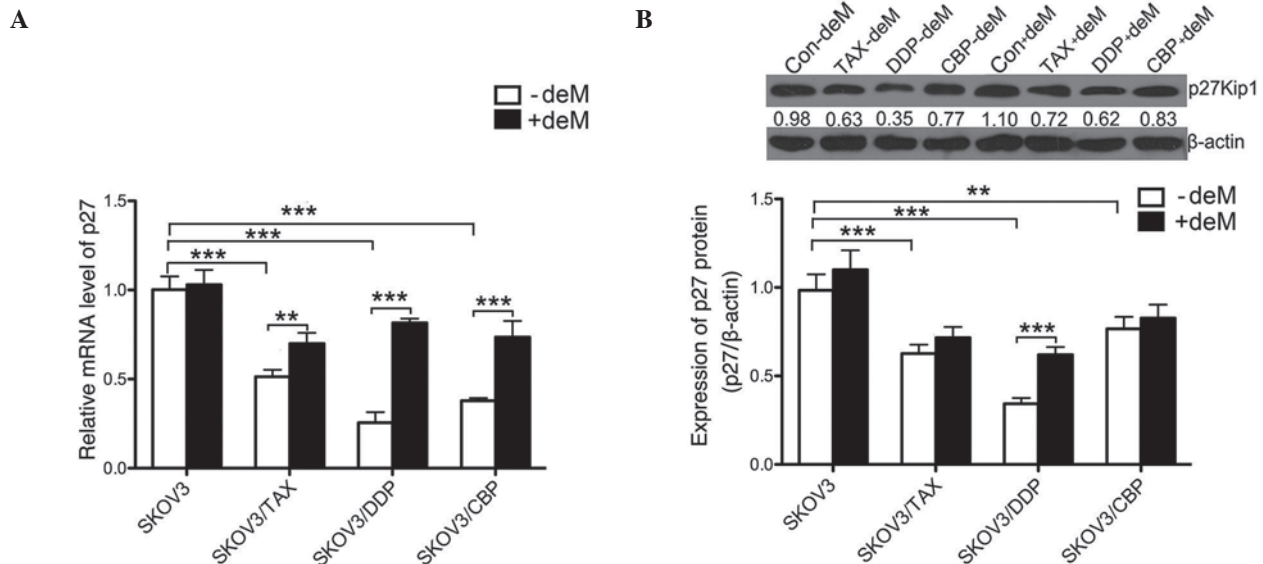


Figure 2. mRNA and protein expression levels of p27 in SKOV3 cell lines. (A) Relative mRNA level of p27 in SKOV3 cells. All cell lines with or without 5-aza treatment were analyzed by reverse transcription-quantitative polymerase chain reaction for the measurement of p27 mRNA. (B) Protein expression of p27 in SKOV3 cells. The expression levels of p27 protein was measured in all SKOV3 resistant cell lines with or without 5-aza treatment by western blotting. Data are presented as the mean  $\pm$  standard deviation,  $n=3$ . \*\* $P<0.01$ , \*\*\* $P<0.001$ , comparison indicated by brackets. 5-aza, 5-aza-2'-deoxyazacytidine; -deM, without 5-aza treatment; +deM, with 5-aza treatment; Con, control; TAX, paclitaxel; DDP, cisplatin; CBP, carboplatin.

TCTAACTAACCTC-3'. PCR was performed in a total volume of 15  $\mu$ l, which included 1.5  $\mu$ l of 10X Lamp Buffer (Beijing Kang Century Biotechnology, Co., Ltd., Beijing, China), 1.2  $\mu$ l of dNTP Mix, 0.5  $\mu$ l of forward and reverse primer (10  $\mu$ M), 0.3  $\mu$ l of Lamp Taq (Beijing Kang Century Biotechnology, Co., Ltd.), 3  $\mu$ l of 5X C'Solution I (Beijing Kang Century Biotechnology, Co., Ltd.), 3  $\mu$ l of template, and 5  $\mu$ l of ddH<sub>2</sub>O. Amplifications were performed in 96-well microtiter plates. The thermal cycling conditions were as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, and followed by 72°C for 5 min. Amplified PCR Products were purified and cloned into pMD19-T (Takara Biotechnology Co., Ltd., Dalian, China). Each cell line colony was sent to Beijing Genomics Institute (Shenzhen, China) for sequencing. The percentage of methylation was calculated comprehensively and comparatively using a CpG viewer ([www.urogene.org/cgi-bin/methprimer/methprimer.cgi](http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)).

**Statistical analysis.** All experiments were repeated three times. All data are presented as the mean  $\pm$  standard deviation. Analysis was performed used SPSS software (version 17; SPSS, Inc., Chicago, IL, USA). Analysis of variance or General Linear model of Single factor Variable was used to compare the differences among multiple groups. Least significant difference and Student-Newman-Keuls methods were used to compared the differences between two means among multiple groups  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Generation of chemoresistant OC cell lines.** To derive cell lines with acquired resistance, SKOV3 cells were cultured in TAX, DDP or CBP by step-wise incubation. The resistant

lines, SKOV3/TAX, SKOV3/DDP and SKOV3/CBP, were obtained several weeks later.

To confirm chemoresistance in the resistant lines, the IC<sub>50</sub> value was calculated in SKOV3/TAX, SKOV3/DDP, SKOV3/CBP and the control group using an MTT assay, and the resulting resistance index observed to be 3.03, 5.989 and 3.32, respectively (Fig. 1A). Additionally, a colony formation assay was used to confirm the proliferation ability of the chemoresistant variants in chemotherapeutic medium (Fig. 1B). It was observed that the colony formation efficiency of SKOV3/TAX ( $0.48\pm0.04$ ,  $P<0.05$ ), SKOV3/DDP ( $0.77\pm0.04$ ,  $P<0.01$ ) and SKOV3/CBP ( $0.70\pm0.02$ ,  $P<0.05$ ) cells were significantly increased compared with the SKOV3 cell line ( $0.30\pm0.07$  in TAX;  $0.32\pm0.05$  in DDP;  $0.51\pm0.02$  in CBP) (Fig. 1C), respectively. Furthermore, the cell cycle was analysed in the chemoresistant cell lines (Fig. 1D). As presented in Fig. 1E, the cell population in G<sub>1</sub> phase was significantly increased, while the proportion of cells in S phase were markedly reduced in all chemoresistant cell lines compared with the SKOV3 group. This indicated that cells were arrested in the G<sub>1</sub> phase. Together, these data demonstrate that the SKOV3/TAX, SKOV3/DDP and SKOV3/CBP cell lines had acquired the resistant phenotype.

**5-aza-2'-deoxyeytidine (5-aza) reverses the low level expression of p27 in SKOV3/DDP cells.** To examine whether the expression levels of p27 were associated with drug resistance, we measured the expression of p27 in chemoresistant and 5-aza-treated cell lines using RT-qPCR and western blotting (Fig. 2). Notably, SKOV3/TAX ( $0.5134\pm0.0389$ ,  $P<0.001$ ;  $0.63\pm0.05$ ,  $P<0.001$ ), SKOV3/DDP ( $0.2552\pm0.0596$ ,  $P<0.001$ ;  $0.35\pm0.03$ ,  $P<0.001$ ), and SKOV3/CBP ( $0.3790\pm0.0150$ ,  $P=0.004$ ;  $0.77\pm0.07$ ,  $P=0.003$ ) showed significant reductions in mRNA and protein expression levels of p27 compared with the control ( $1.0020\pm0.0745$ ;  $0.98\pm0.09$ ). However, this



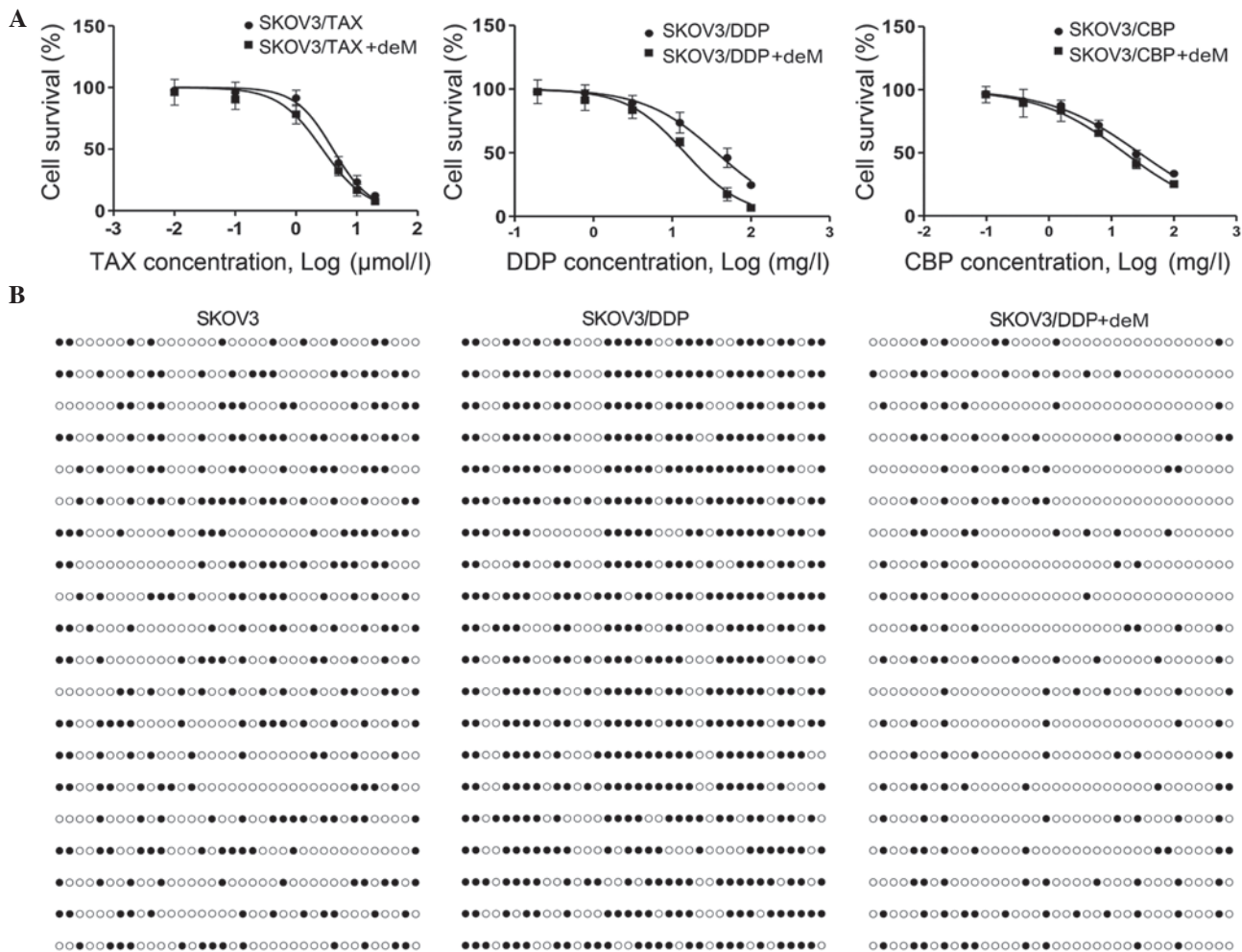


Figure 3. 5-aza treatment sensitizes SKOV3/DDP cells to DDP via the demethylation of p27. (A) Cell survival of cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Chemoresistant cells and their 5-aza treated counterparts were incubated in corresponding drug medium, and 6 different concentrations of each drug were used. (B) Diagrammatic representation of the methylation of SKOV3, SKOV3/DDP and SKOV3/DDP-deM. Each circle represents a CpG repeat. Filled circles represent methylated cytosine. 5-aza, 5-aza-2'-deoxyazacytidine; DDP, cisplatin; +deM, with 5-aza-2'-deoxyazacytidine treatment.

was reversed when 5-aza was added to the chemoresistant cell lines, as presented in Fig. 2A and B. The 5-aza treatment significantly increased p27 mRNA expression levels in SKOV3/TAX ( $0.5134 \pm 0.0389$ ,  $P=0.002$ ), SKOV3/DDP ( $0.8156 \pm 0.0239$ ,  $P<0.001$ ) and SKOV3/CBP ( $0.7360 \pm 0.0908$ ,  $P=0.001$ ) compared with their chemoresistant counterparts, respectively. The protein expression of P27 was increased only in SKOV3/DDP groups ( $0.8156 \pm 0.0239$ ,  $P<0.001$ ) compared with their chemoresistant counterparts. However, no significant differences were observed between 5-aza-treated and untreated SKOV3/TAX cells ( $0.6994 \pm 0.0604$ ,  $P=0.052$ ). Together, these data demonstrated that chemoresistance resulted in the reduction in the expression levels of p27 in OC cell lines, and that 5-aza treatment was able to reverse the reduced expression of p27 in DDP-resistant SKOV3 cells.

*5-aza treatment sensitizes OC SKOV3/DDP cells to DDP by the demethylation of p27.* The effect of 5-Aza-treatment on the sensitivity to chemotherapy was next investigated in the chemoresistant cell lines using an MTT assay. The results indicated that the differences between SKOV3/TAX, SKOV3/CBP and their counterpart 5-aza-treated cells were not significant

(Fig. 3A). By contrast, the  $IC_{50}$  value in the 5-aza-treated SKOV3/DDP cells was markedly reduced compared with the untreated SKOV3/DDP cells. This indicates that the 5-aza treatment resulted in the DDP-resistant OC cells being more sensitive to DDP.

To confirm the mechanism of the sensitizing effect of 5-aza, the level of p27 methylation was investigated in SKOV3, SKOV3/DDP, and 5-aza-treated SKOV3/DDP cells using BSP (Fig. 3B). The data indicated that the frequency of methylation in SKOV3/DDP cells was higher than in SKOV3 cells, with this reduced that following 5-aza treatment. This indicated that drug resistance to DDP resulted in increased methylation of p27 in SKOV3 cells, which may be reduced by 5-aza treatment. Taken together, these data indicate that 5-aza resulted in an increased sensitivity to DDP by downregulating the methylation of P27.

*Overexpression of p27 leads to cell cycle alterations and increased apoptotic response to DDP in SKOV3/DDP.* p27-overexpression plasmids (pCDNA3.1-P27) were transfected into the SKOV3 and SKOV3/DDP cell lines, and the expression levels of p27 protein were measured by western blotting (Fig. 4A).

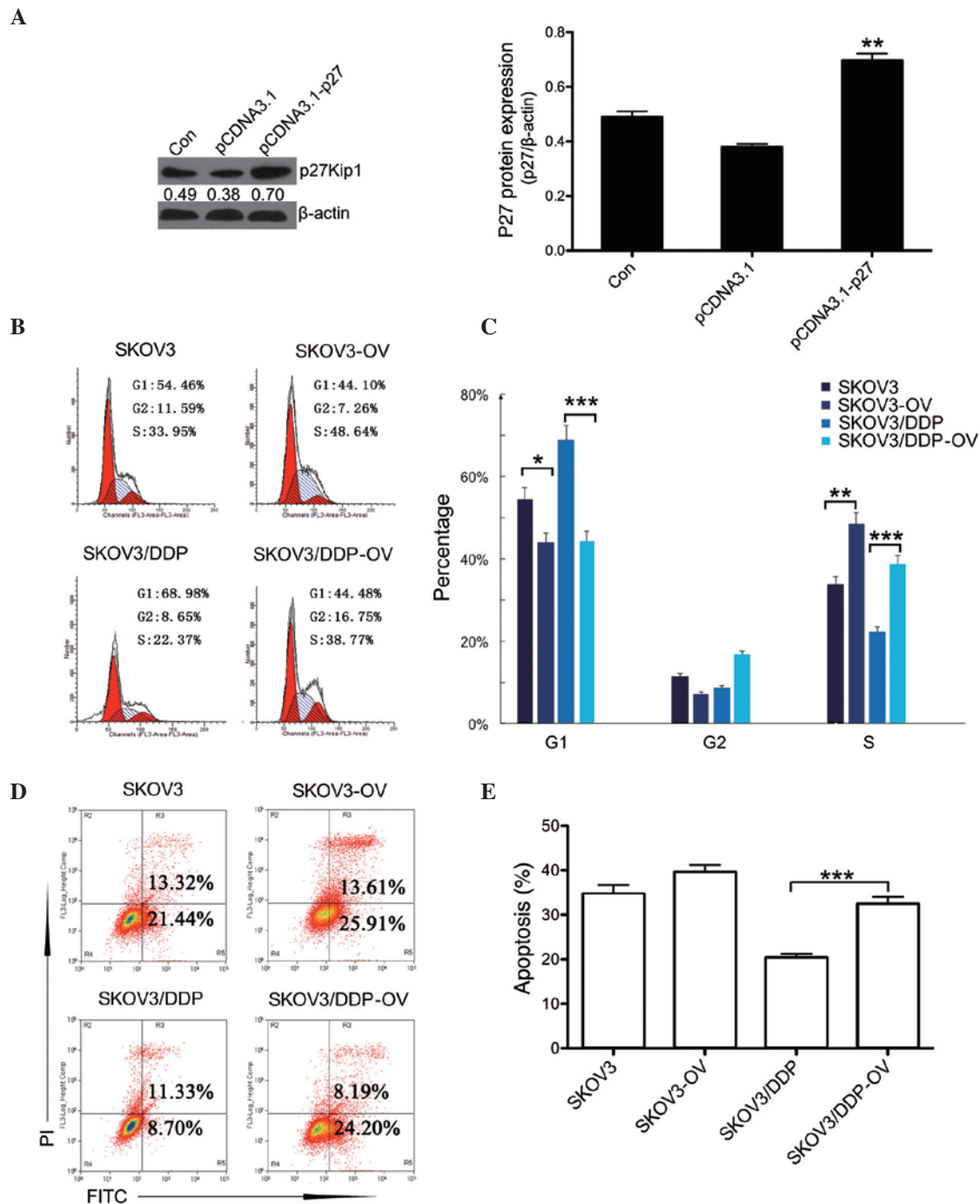


Figure 4. Overexpression of p27 leads to cell cycle alterations and an increased apoptotic response to DDP in SKOV3/DDP cells. (A) Plasmid pCDNA3.1-p27 was transfected into SKOV3 cell lines to establish the p27-OV line. Following transfection, western blotting was used to measure the expression levels of p27 protein.  $^{**}P < 0.01$  vs. pCDNA3.1 group. (B) Cell-cycle analysis for SKOV3, SKOV3/DDP and their p27-OV counterparts was conducted using flow cytometry. The red peak on the left of each histogram represents G<sub>1</sub> phase and the red peak on the right indicates G<sub>2</sub> phase. The blue slashed area indicates S phase. (C) Quantification of cell-cycle distribution. (D) The level of apoptosis in SKOV3, SKOV3-OV, SKOV3/DDP and SKOV3/DDP-OV cells was measured by flow cytometry. The right portion of the quadrant indicates the apoptotic cells. (E) Quantification of the levels of apoptosis. Data are presented as the mean  $\pm$  standard deviation,  $n = 3$ .  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , comparison indicated by brackets. DDP, cisplatin; p27-OV, p27-overexpression; Con, control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Additionally, the cell cycle was analyzed in p27-transfected and untransfected SKOV3 and SKOV3/DDP cells (Fig. 4B). The results indicated that the proportion of cells in G<sub>1</sub> phase were markedly reduced, with an increase proportion of cells in S phase in p27-transfected SKOV3 and SKOV3/DDP cells compared with their untransfected counterparts (Fig. 4C). Furthermore, the apoptotic response to DDP was examined by

flow cytometry (Fig. 4D). The apoptotic rate in p27-transfected SKOV3/DDP cells was significantly increased compared with the untransfected SKOV3/DDP cells. The data indicated that the overexpression of p27 was able to enhance the sensitivity of SKOV3/DDP cells to DDP (Fig. 4E). Together, these results suggest that the overexpression of p27 led to the increased sensitivity of SKOV3/DDP cells to DDP via cell cycle arrest.

## Discussion

In the present study, OC cell lines demonstrated increased survival, enhanced proliferation and an increase of cells in G<sub>1</sub> phase and a reduction in S phase following step-wise incubation-induced chemoresistance. In addition, the reduction in p27 expression was observed in chemoresistant cells. Following demethylation using 5-aza, expression levels of p27 were increased in DDP-resistant cells. These cells also became more sensitive to DDP. p27 overexpression confirmed the enhancing effect of p27 on DDP cytotoxicity.

p27 has been previously observed to be an accurate prognostic marker in OC (2). Using multivariate analysis in 99 primary tumors cases, Belletti *et al* (8) proposed that p27 is a novel potential prognostic marker for patients with advanced epithelial OC, as its loss was significantly associated with a shorter time to progression and a reduced overall survival. These data were confirmed in a study by Hershko (18), which demonstrated that p27 expression was positively associated with overall survival of patients with OC. Furthermore, there is growing evidence suggesting that loss of p27 can mediate a drug-resistance phenotype (7). These previous studies support the results of the current study, which demonstrated that drug resistance resulted in a significant reduction in p27 protein expression, with p27 overexpression in DDP-resistant cell lines leading to an enhanced sensitivity to DDP.

It has been suggested that the silence of tumor suppressor genes, such as p27, is associated with promoter methylation. Li *et al* (19) investigated cisplatin-sensitive and -resistant OC cells, and observed that DNA hyper-methylation may contribute to drug-resistance in OC. In addition, p27 expression in tumors was significantly reduced, however, a number of previous studies have demonstrated that rare methylation of p27 in tumor patients (20,21). Li *et al* (22) observed no hypermethylation in the p27 gene promoter in 5 patients with pancreatic carcinoma. Stanganelli *et al* (23) reported that all the patients with multiple myeloma they tested lacked methylation at the p27 gene. By contrast, the present study observed that the drug-resistant SKOV3 cell lines exhibited high levels of methylation of p27. This indicates that the chemoresistance-induced reduction in p27 in SKOV3 cells may not only occur as the result of p27 methylation. Notably, 5-aza treatment induced demethylation and resulted in upregulation of the expression of p27, similar to the effect of p27 overexpression. This suggests additional mechanisms may lead to the downregulation of p27 in these patients (22).

The drugs investigated in the current study possess different target points in the cell cycle. Paclitaxel binds to microtubules and prevents their depolymerization, blocking cell division at the G<sub>2</sub>/M phase of the cell cycle (24,25). Platinum containing drugs, including cisplatin and carboplatin, react with DNA to form intra- and interstrand crosslinks that also block cell division. Therefore, factors that can reduce the reaction of platinum-containing drugs with DNA will reduce the toxicity of the drug (26). In the present study, it was observed that chemoresistant cells altered their cell cycle distribution, with the proportion of cells in the G<sub>1</sub> phase increased and reduced in S phase. This means that the majority of chemoresistant cell lines reduce the ability of platinum-containing drugs to bind to DNA. With the reduced binding of platinum-containing drugs,

tumor cells may be activated, which may ultimately result in tumor recurrence and poor prognosis (5,26,27). Furthermore, overexpression of p27 restored the cell-cycle distribution in DDP-resistant cells, enabling the cells to divide. This suggests that the target points of the chemotherapeutic drugs were exposed following the return of the cells into the cell cycle, therefore increasing the sensitivity of chemoresistant cells to DDP.

Additionally, it was observed that the protein levels of p27 in SKOV3 cells chemoresistant to TAX and CBP were not significantly restored by 5-aza treatment. This may indicate that the chemoresistance in SKOV3 cells to TAX and CBP is not due to p27 methylation. As mentioned, TAX is a drug that blocks cell division by binding to microtubules and preventing their depolymerization. However, the differential effects on CBP-resistant and DDP-resistant cells implies that different mechanisms are associated with chemoresistance to these drugs. Further research on the different mechanisms may aid in the understanding of the difference between CBP and DDP in clinical applications.

In conclusion, demethylation of p27 was able to enhance the DDP-induced cytotoxicity in human OC cells through the regulation of the cell cycle. In addition, p27 may represent a potential biological marker for OC prognosis, and a potential target for overcoming chemoresistance in OC cells.

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