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Identification of a novel *PARP4* gene promoter CpG locus associated with cisplatin chemoresistance

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The protein family of poly (ADP-ribose) polymerases (PARPs) is comprised of multifunctional nuclear enzymes. Several PARP inhibitors have been developed as new anticancer drugs to combat resistance to chemotherapy. Herein, we characterized *PARP4* mRNA expression profiles in cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines. *PARP4* mRNA expression was significantly upregulated in cisplatin-resistant ovarian cancer cell lines, and this upregulation was associated with the hypomethylation of specific cytosine-phosphate-guanine (CpG) sites (cg18582260 and cg17117459) on its promoter. Reduced *PARP4* expression was restored by treating cisplatin-sensitive cell lines with a demethylation agent, implicating the epigenetic regulation of *PARP4* expression by promoter methylation. Depletion of *PARP4* expression in cisplatin-resistant cell lines reduced cisplatin chemoresistance and promoted cisplatin-induced DNA fragmentation. The differential mRNA expression and DNA methylation status at specific *PARP4* promoter CpG sites (cg18582260 and cg17117459) according to cisplatin responses, was further validated in primary ovarian tumor tissues. The results showed significantly increased *PARP4* mRNA expressions and decreased DNA methylation levels at specific *PARP4* promoter CpG sites (cg18582260 and cg17117459) in cisplatin-resistant patients. Additionally, the DNA methylation status at cg18582260 CpG sites in ovarian tumor tissues showed fairly clear discrimination between cisplatin-resistant patients and cisplatin-sensitive patients, with high accuracy (area under the curve = 0.86, P = 0.003845). Our findings suggest that the DNA methylation status of *PARP4* at the specific promoter site (cg18582260) may be a useful diagnostic biomarker for predicting the response to cisplatin in ovarian cancer patients. [BMB Reports 2023; 56(6): 347-352]

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INTRODUCTION

Ovarian cancer is the second most frequently diagnosed gynecological cancer, and has the highest mortality among gynecological cancers worldwide (1). The standard therapy for patients with advanced ovarian cancer is complete cytoreductive surgery, followed by combination chemotherapy, consisting of taxane- and platinum-based regimens. Although patients with advanced ovarian cancer typically respond well to initial treatment with cisplatin-based chemotherapy, the majority of them (over 80%) eventually experience resistance to currently available treatment options, ultimately leading to therapeutic failure (2). Platinum-based anticancer drugs, including cisplatin, are DNA cross-linking agents. Their antitumor activities are mediated by binding with nuclear DNA, resulting in the formation of intra- and inter-strand adducts, which induce DNA damage response pathways that eventually block cell cycle progression and cause apoptotic cell death (3). The mechanisms related to induced resistance to platinum compounds are complex and numerous, and still not fully understood. Several underlying mechanisms of resistance at the cellular and molecular levels have been described, including 1) reduced intercellular drug accumulation or increased efflux of platinum agents mediated by copper-transporting P-type ATPases (ATP7A/7B) and multidrug-associated protein 2, 2) inactivation of intracellular platinum agents by forming adducts with detoxification components and antioxidants, such as glutathione and metallothionein, 3) increased DNA repair processing through five major pathways of nucleotide excision repair (NER), mismatch repair, homologous recombination repair, base excision repair (BER), and translesion synthesis (4). Recently, aberrant DNA methylation changes at promoter cytosine-phosphate-guanine (CpG) sites have been reported in cisplatin-resistant cells. These DNA methylation changes regulate the expression of genes critical for response to chemotherapeutic agents, thereby leading to anticancer drug resistance (5).

Poly (ADP-ribose) polymerases (PARPs) comprise a large protein family of 17 members that possess poly-ADP ribosyltrans-

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ferase activity (6), and are involved in diverse cellular processes, including DNA repair (7). Some isoforms, including PARP1 and PARP2, have been well characterized during their involvement in DNA repair processes, such as BER in response to single-strand DNA breaks (8) and NER (9). Because of their roles in DNA repair, PARP inhibitors are attractive chemotherapy candidates for many cancer types, including ovarian cancer (10). PARP4 is one of the largest members of the PARP protein family, with a molecular mass of 193 kDa, and possesses poly (ADP-ribosyl) transferase activity (11). PARP4 is part of the cytoplasmic ribonucleoprotein complex, also known as the vault, which is known for its involvement in multidrug resistance (12, 13). Although PARP4 is known to be catalytically active, the biological functions of PARP4 remain largely undefined.

Herein, we investigated alterations of *PARP4* mRNA expression and related epigenetic modifications of the *PARP4* promoter region in cisplatin-resistant cell lines, and compared them with cisplatin-sensitive cell lines. Using loss-of-function studies, we examined whether depletion of PARP4 expression sensitized cells to cisplatin and led to a DNA damage response. The differential mRNA expression and DNA methylation status at specific *PARP4* promoter CpG sites, according to cisplatin responses, were further validated in primary ovarian tumor tissues.

RESULTS

Upregulation of *PARP4* in cisplatin-resistant cell lines

Previously, we evaluated cisplatin-induced cytotoxicity in 11 human ovarian cancer cell lines, and classified the results into three groups: sensitive, intermediate, and resistant (14). In the present study, we conducted reverse-transcription quantitative polymerase chain reaction (RT-qPCR) on three cisplatin-sensitive cell lines (PA-1, TOV-112D, and A2780) and three cisplatin-resistant cell lines (OVCAR-3, OV-90, and SK-OV-3). The results showed that *PARP4* mRNA and protein expression were significantly higher in the cisplatin-resistant cell lines, when compared with levels in the cisplatin-sensitive cell lines (Fig. 1A, and Supplementary Fig. 1 of the Supplementary Information (SI)).

Hypomethylation of the *PARP4* promoter region in cisplatin-resistant cell lines

It has been widely observed in various cancer cell lines that aberrant alteration of DNA methylation plays a critical role in the development of chemoresistance (15). We therefore performed epigenome-wide methylation profiling using the Illumina® HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA), to examine differences between cisplatin-sensitive and cisplatin-resistant cell lines in DNA methylation within the promoter region of the *PARP4* gene. The Illumina HumanMethylation450 BeadChip contains 10 CpG sites within the *PARP4* promoter region located on chr13: (25,085,000-25,087,500) (the human GRCh37/hg19 assembly), as shown in Fig. 1B. Of the 10 CpG sites, two CpGs were differentially methylated ($|\Delta\beta| > 0.5$), and both CpG sites were hypomethylated in the cisplatin-resistant

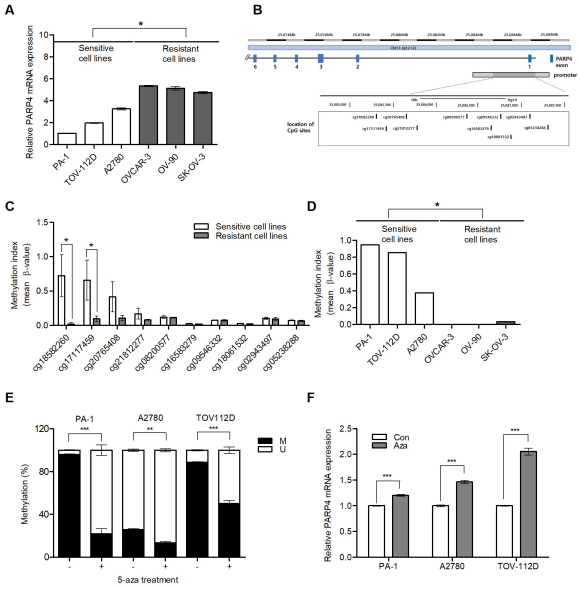

Fig. 1. *PARP4* mRNA expression is upregulated by hypomethylation of promoter CpG sites in cisplatin-resistant cell lines. (A) Relative mRNA expression of *PARP4* in three human cisplatin-sensitive ovarian cell lines (PA-1, TOV-112D, and A2780) and three human cisplatin-resistant ovarian cell lines (OVCAR-3, OV-90, and SK-OV-3). (B) The DNA methylation status of the *PARP4* promoter region was measured using an Illumina HumanMethylation450 BeadChip, which contained 10 CpG sites at position chr13: 25,085,000-25,087,500 (the human GRCh37/hg19 assembly). (C) Each bar denotes the mean β-value of methylation at the corresponding promoter CpG site of *PARP4*. (D) The DNA methylation status of the CpG site (cg18582260) in three human cisplatin-sensitive ovarian cell lines and three human cisplatin-resistant ovarian cell lines. (E) Three cisplatin-sensitive ovarian cancer cell lines were treated with 5-aza-2′-deoxycytidine. The methylation status at the cg 18582260 CpG site was measured by qMSP. (F) After treatment of 5-aza-2′-deoxycytidine, *PARP4* mRNA expression was determined using RT-qPCR. All experiments were carried out in triplicate, and the results are expressed as the mean ± standard deviation (SD). The statistical significance of observed differences was determined using a t-test or Bayesian *t*-statistic (*P < 0.05; **P < 0.01; ***P < 0.001). M, methylated CpG site; U, unmethylated CpG site; Con, control; Aza, 5-aza-2′-deoxycytidine.

cell lines, when compared with the cisplatin-sensitive cell lines (Fig. 1C). Figure 1D shows the DNA methylation status of all six ovarian cancer cell lines at the promoter CpG site (cg18582260). DNA methylation of the promoter CpG site (cg18582260) was significantly decreased (by approximately 38-95%) in cisplatin-resistant cell lines (Fig. 1D).

Regulation of *PARP4* expression by modification of DNA methylation

To determine whether *PARP4* expression was regulated by epigenetic modification, three cisplatin-sensitive cell lines (PA-1, A2780, and TOV-112D) were treated with 5-aza-2′-deoxycytidine (5-aza-dc), an inhibitor of DNA methyltransferase. The DNA me-

thylation status of the CpG site (cg18582260) was determined by quantitative methylation-specific PCR (qMSP), and *PARP4* mRNA expression was quantified by RT-qPCR. After treatment with 5-aza-dC, a reduction in the level of methylation at the CpG site (cg18582260) was confirmed by qMSP (Fig. 1E). In all cisplatin-sensitive cell lines, *PARP4* mRNA expression of 5-aza-dc-treated cells was significantly increased in the range (1.2-2.1)-fold, when compared with that of untreated controls, indicating *PARP4* expression was epigenetically silenced by DNA methylation (Fig. 1F). Increased PARP4 protein expression was also detected in 5-aza-dc-treated cells as determined by western blot analyses (Supplementary Fig. 2 of the SI).

Sensitization of ovarian cancer cells to cisplatin by knockdown of *PARP4* expression

To determine whether *PARP4* knockdown in cisplatin-resistant cell lines sensitized ovarian cancer cells to cisplatin, two cisplatin-resistant cell lines (OVCAR3 and SK-OV-3) were transiently transfected with a small interfering RNA (siRNA) targeting *PARP4* (siPARP4) or a non-targeting control (siNC). After 24 h of transfection, silencing of *PARP4* expression was confirmed by RT-qPCR. Transfection with siPARP4 decreased the levels of *PARP4* expression to 87 and 91% in OVCAR3 and SK-OV-3 cells, respectively, compared with cells transfected with siNC (Fig. 2A).

Cisplatin sensitivity was evaluated in siNC or siPARP4 transfected cells using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Silencing of *PARP4* expression significantly decreased the cisplatin IC_{50} by ~22% in siPARP4-transfected OVCAR3 cells, when compared with siNC-transfected control cells (Fig. 2B). Similar to this result, the cisplatin IC_{50} value was reduced by ~37% in siPARP4-transfected SK-OV-3 cells (Fig. 2C).

Additionally, DNA damage (DNA fragmentation) was detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay by flow cytometry in siRNA-treated SK-OV-3 cells (Fig. 2D). Flow cytometry results revealed knockdown with siPARP4 led to significantly increased (~21%) TUNEL-positive cells, compared to siNC-transfected control cells (Fig. 2E). Collectively, these results showed that *PARP4* knockdown in cisplatin-resistant cell lines significantly reduced cisplatin resistance.

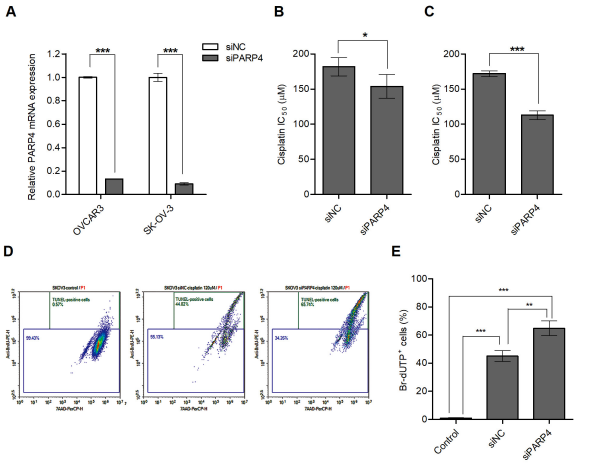

Fig. 2. Depletion of *PARP4* increases sensitivity to cisplatin in cisplatin-resistant cell lines. (A) After 24 h transfection, the knockdown efficiency of siRNA targeting *PARP4* was confirmed by RT-qPCR in OVCAR-3 and SK-OV-3 cells. Cytotoxicities in response to cisplatin in *PARP4*-knockdown OVCAR3 (B) and SK-OV-3 (C) cells were determined after 24 h cisplatin treatment using an MTT assay. (D, E) The TUNEL assay was performed in siNC- or siPARP4-transfected SK-OV-3 cells after cisplatin treatment. The siNC-transfected SK-OV-3 cells without cisplatin treatment served as controls. TUNEL-positive cells were analyzed, shown by a representative flow chart and by quantification of triplicates. The error bars denote the standard deviation from the mean of three independent experiments. Statistical significance was analyzed using a t-test (*P < 0.05; **P < 0.01; ***P < 0.001). The siNC, non-targeting control siRNA; siPARP4, *PARP4* siRNA; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Hypomethylation of the specific promoter CpG site of *PARP4* in cisplatin-resistant ovarian cancer patients

Patients with recurrence within 12 months from the last dose of platinum-based chemotherapy were defined as being cisplatin-resistant, whereas those without recurrence > 12 months from the last dose of platinum-based chemotherapy were defined as cisplatin-sensitive (Table 1).

We examined *PARP4* mRNA expression levels in primary ovarian tumor tissues from 15 cisplatin-sensitive and 9 cisplatin-resistant patients. *PARP4* mRNA expression was significantly upregulated in cisplatin-resistant patients, compared to cisplatin-sensitive patients (Fig. 3A). Receiver operating characteristic curve (ROC) analysis showed acceptable separation of cisplatin-resistant patients from cisplatin-sensitive patients with an area under the curve (AUC) of 0.70 (Fig. 3B). However, the power to evaluate a significant difference between the means of two groups in *PARP4* mRNA expression was 56%, demonstrating that the sample size used for analysis had insufficient power to detect differences between the two groups (Supplementary Fig. 3A of the SI). Furthermore, we performed meta-analysis using fifteen publicly available datasets to assess the association between the expression of *PARP4* and overall survival in patients with serous-type ovarian cancer. The pooled Hazard ratios (HRs) estimate was significantly greater than 1 for overall survival (HR = 1.09, P-value = 0.0034). The association was more significant (HR = 1.12, P-value = 0.0007) when tumor stage was added to the survival analysis as a covariate. The meta-analysis revealed that high expression of *PARP4* was significantly associated with poor prognosis and a higher risk of death in ovarian cancer patients with serous tumor (Supplementary Fig. 4 of the SI).

We also investigated DNA methylation status at specific promoter CpG sites of the *PARP4* gene in primary ovarian tumor tissues from 15 cisplatin-sensitive and nine cisplatin-resistant

Table 1. Clinical characteristics of patients with ovarian cancer who were sensitive or resistant to primary chemotherapy

Patient No.	Chemo-response	Histology	Stage[a]	Grade[b]	PFI (month)
1	R	Serous	IIIC	3	7
2	R	Serous	IIIC	3	12
3	R	Serous	IV	2	0
4	R	Serous	IIIC	3	0
5	R	Serous	IV	2	1
6	R	Serous	IIIC	3	10
7	R	Serous	IIIC	3	9
8	R	Serous	IIIC	3	5
9	R	Serous	IV	2	11
10	S	Serous	IV	2	50
11	S	Serous	IIIC	2	26
12	S	Serous	IV	2	43
13	S	Serous	IIIC	3	>72
14	S	Serous	IIIC	3	25
15	S	Serous	IV	3	>48
16	S	Serous	IIIC2	3	33
17	S	Serous	IIIC	2	>48
18	S	Serous	IIIC	3	>48
19	S	Serous	IIIC	3	>24
20	S	Serous	IIC	2	>48
21	S	Serous	IIIC	3	>48
22	S	Serous	IIIC	3	>48
23	S	Serous	IIIC	3	>48
24	S	Serous	IIIC	3	>48

[a]International Federation of Gynecology and Obstetrics (FIGO) stage.
[b]Histological grade.
R, ovarian tumor tissues from cisplatin-resistant patients; S, ovarian tumor tissues from cisplatin-sensitive patients; PFI, Platinum free interval; the time between the last dose of platinum-based therapy and documented relapse (month).

patients using pyrosequencing analysis. Two specific promoter CpG sites (cg18582260 and cg17117459) on *PARP4* were significantly hypomethylated in cisplatin-resistant patients, when compared with those in cisplatin-sensitive patients (Fig. 3C). The powers to evaluate a significant difference between the means of two groups in methylation at two specific CpG sites were 91 and 68% for cg18582260 and cg17117459, respectively (Supplementary Fig. 3B, C of the SI). The sample size used for the analysis of cg18582260 was therefore sufficient to detect significant differences between cisplatin-sensitive and cisplatin-resistant patients. ROC analysis showed clear discrimination between cisplatin-resistant patients and cisplatin-sensitive patients, with an AUC of 0.86 (P = 0.003845) and 0.80 (P = 0.02539) for cg18582260 and cg17117459, respectively (Fig. 3D). These findings suggest that the DNA methylation status at the specific promoter CpG site (cg18582260) on *PARP4* may be a useful diagnostic biomarker for predicting the response to cisplatin in ovarian cancer patients.

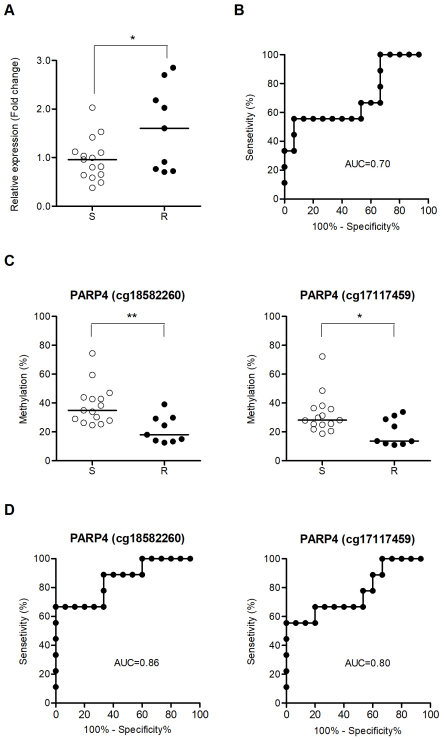

Fig. 3. The association of DNA methylation status at *PARP4* promoter CpG sites during response to cisplatin. (A) *PARP4* mRNA expression was determined using RT-qPCR in ovarian tumor tissues from 15 cisplatin-sensitive and nine cisplatin-resistant patients. (B) Receiver operating characteristic curve (ROC) analysis of *PARP4* mRNA expressions for predicting responses to cisplatin in patients with ovarian cancer. (C) DNA methylation status at two specific *PARP4* promoter CpG sites, cg18582260 and cg17117459, was assessed by pyrosequencing analysis in ovarian tumor tissues from 15 cisplatin-sensitive and nine cisplatin-resistant patients. (D) ROC analysis of DNA methylation status at two specific *PARP4* promoter CpG sites for predicting responses to cisplatin. The lines on the scatter plots denote the median value. Statistical significance was evaluated using a t-test (*P < 0.05; **P < 0.01). S, ovarian tumor tissues from cisplatin-sensitive patients; R, ovarian tumor tissues from cisplatin-resistant patients; AUC, area under the curve.

DISCUSSION

PARP4 is a part of the cytoplasmic ribonucleoprotein complex, also known as the vault, composed of major vault protein (MVP), telomerase-associated protein (TEP1), PARP4, and vault-associated RNA (16). Although upregulation of the vault complex has been implicated in multidrug resistance against a broad spectrum of anticancer drugs, such as doxorubicin and cisplatin, the precise molecular functions of the vault complex remain largely unknown (12, 13). PARP4 loss in mice leads to an increased carcinogen-induced colon tumor incidence, multiplicity, and reduced tumor latency, indicating PARP4 potentially functions as a tumor suppressor. However, PARP4-deficient mice in a lung cancer model did not demonstrate any signi-

ficantly increased carcinogen-induced tumor multiplicity, implying the tumor suppressive role of PARP4 could be cancer-type-dependent (16). PARP4 is thought to be involved in the BER pathway, due to its possession of the BRCA1 carboxy-terminal domain, which is common in other DNA repair pathway proteins, such as PARP1 and X-ray repair cross-complementing 1. Nevertheless, to date, there is no evidence demonstrating that PARP4 is involved in DNA repair processes (17).

In the present study, we investigated the alteration of *PARP4* mRNA expressions and related epigenetic modifications of the *PARP4* promoter region in cisplatin-resistant cell lines, when compared with cisplatin-sensitive cell lines. We showed that *PARP4* expression was upregulated in cisplatin-resistant cell lines by a DNA methylation-dependent transcriptional regulatory mechanism. Using loss-of-function studies, we showed that the depletion of *PARP4* expression in cisplatin-resistant cell lines reduced cisplatin chemoresistance and promoted cisplatin-induced DNA fragmentation. Further studies are warranted to investigate whether PARP4 is directly or indirectly involved in DNA repair pathways, such as NER or BER. The differential *PARP4* mRNA expression and DNA methylation status at the two *PARP4* promoter CpG sites (cg18582260 and cg17117459) in primary ovarian tumor tissues from 15 cisplatin-sensitive and nine cisplatin-resistant patients were further validated. The results showed significantly increased *PARP4* mRNA expressions and decreased DNA methylation levels at specific *PARP4* promoter CpG sites (cg18582260 and cg17117459) in cisplatin-resistant patients. The DNA methylation status at the specific promoter CpG site (cg18582260) showed discrimination between cisplatin-resistant patients and cisplatin-sensitive patients with high accuracy (AUC = 0.86, P = 0.003845).

A previous study examining the expressions of vault proteins, including PARP4, in post-surgery ovarian cancer samples showed that mRNA levels of *MVP*, *TEP1*, and *PARP4* were significantly reduced, with lower expressions in tumors of a higher grade. However, the protein levels of three vault proteins, including PARP4, were increased in tumors of higher grade, indicating possible specific posttranslational regulation of vault complex production (18). In esophageal squamous cell carcinoma (ESCC) tissues, single-cell intratumoral stemness analysis identified PARP4 as a potential novel cancer stemness marker. PARP4 upregulation correlates with poorer outcomes for ESCC patients, including poor prognosis and overall survival (19).

Although the precise mechanisms underlying the protective process against cisplatin-induced DNA damage need to be further investigated, our results suggest aberrant *PARP4* overexpression, which was regulated by epigenetic modification, contributes to cisplatin resistance. We therefore suggest that the DNA methylation status of *PARP4* at the specific promoter site may be a useful diagnostic biomarker for predicting the response to cisplatin in ovarian cancer patients.

MATERIALS AND METHODS

Cell culture

The human ovarian cancer cell lines studied were PA-1, TOV-112D, A2780, OVCAR-3, OV-90, and SK-OV-3. All cell lines were cultured using the complete growth media recommended by the suppliers (Supplementary Table 1) in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO_2.

Tissue specimen collection

Fifteen cisplatin-sensitive patients and nine cisplatin-resistant patients with ovarian cancer were included in this study. Tumor tissue samples were obtained from the Korea Gynecologic Cancer Bank at the time of staging the surgery. The clinicopathological characteristics are shown in Table 1. This study was approved by the Institutional Review Board (Permit Number: EUMC 2014-05-004-001), and written informed consent was obtained from all patients.

The 5-aza-dc treatment

To inhibit DNA methylation, three cisplatin-sensitive ovarian cell lines (PA-1, A2780, and TOV-112D) were treated with 10 μM 5-aza-dc (Sigma-Aldrich, St. Louis, MO, USA) for 3 days at 37°C. The medium was freshly exchanged every day and supplemented with 10 μM 5-aza-dc.

The qMSP

Bisulfite-treated DNA was used as a template for qMSP. The primers of qMSP were designed for detecting the methylated or unmethylated forms of the specific CpG site (cg18582260). The sequences of the methylated/unmethylated-specific primers were as follows: 5′-TGGGAGGTATGGAAAGGC-3′ (methylated forward), 5′-TGGGAGGTATGGAAAGGT-3′ (unmethylated forward), 5′-AAAACATAAACTAACTCTATATTTAA-3′ (reverse). The qMSP was performed using a 7500 Fast Real-time PCR system (Thermo Fisher Scientific) as previously described (21). The percentages of methylation at the specific CpG site were calculated as follows (Ct represents the threshold-cycle): percent of methylation = $100 / [1 + 2(\Delta Ct^{methylation} - \Delta Ct^{unmethylation})]$% (20).

TUNEL assay

The siNC or siPARP4-transfected SK-OV-3 cells were treated with 120 μM of cisplatin after 24 h transfection. The siNC-transfected SK-OV-3 cells without cisplatin treatment served as controls. After 48 h cisplatin treatment, the TUNEL assay (Abcam, Cambridge, MA, USA) was conducted following the manufacturer's protocol. The fluorescent intensity of cells was analyzed using flow cytometry (NovoCyte 3000; Agilent Technologies, Santa Clara, CA, USA): Ex/Em = 488/576 nm for BrdU-Red and Ex/Em = 488/655 nm for 7-AAD.

Pyrosequencing for DNA methylation analyses

The bisulfite pyrosequencing analysis was performed on two CpG sites within the promoter region of the *PARP4* gene

(cg18582260 and cg17117459), for methylation analyses. Each primer was designed using the PyroMark Assay Design SW 2.0 software (Qiagen). The primer sequences were: 5′-GGGGTTA TAGGTGTGAGTTGTT-3′ (forward), and 5′-ATTAACCCAAAAA AAAACTAACATTTTACA-3′ (5′-biotinylated-reverse). Bisulfite-treated DNA was amplified using the PyroMArk PCR kit (Qiagen) in accordance with the instructions of the manufacturer. The biotinylated PCR product was bound to streptavidin Sepharose HP beads (Amersham Biosciences, Little Chalfont, UK) to prepare the ssDNA template for sequencing, following the sample preparation guide. The sequencing reaction was carried out on a PyroMark Q48 Autoprep system using PyroMark Q48 Advanced CpG Reagents (Qiagen) in accordance with the instructions of the manufacturer. The sequences we analyzed used 5′-GGGAGGTATGGAAAG-3′ as the sequencing primer.

Statistical analysis

Results are expressed as the mean ± standard deviation of at least three independent experiments. All statistical analyses were performed using Prism 5 software (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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