GADD45α alleviates the CDDP resistance of SK-OV3/cddp cells via redox-mediated DNA damage

QI-ZHU ZHANG[1*], FANG WEN[1,2*], HAN-LIN YANG[1], YAN-YAN CAO[1], REN GUO PENG[1], YUAN-MEI WANG[1], LEI NIE[1], YUAN-KUN QIN[1], JIN-JIAN WU[1], XING ZHAO[3] and DAN ZI[2-4]

[1]Department of Obstetrics and Gynecology, Affiliated Hospital of Guizhou Medical University;
[2]Department of Obstetrics and Gynecology, Guizhou Provincial People's Hospital;
[3]National and Guizhou Joint Engineering Laboratory for Cell Engineering and Biomedicine Technique,
Center for Tissue Engineering and Stem Cell Research, Guizhou Province Key Laboratory of Regenerative Medicine,
[4]Key Laboratory of Medical Molecular Biology, Guizhou Medical University, Guiyang, Guizhou 550004, P.R. China

Abstract. Epithelial ovarian cancer has the highest mortality rate of all malignant ovarian cancer types. Great progress has been made in the treatment of ovarian cancer in recent years. However, drug resistance has led to a low level of 5-year survival rate of epithelial ovarian cancer, and the molecular mechanism of which remains unknown. The aim of the present study was to identify the role of redox status in the cisplatin (CDDP) resistance of ovarian cancer. CDDP-resistant SK-OV3 (SK-OV3/cddp) cells were prepared and their reactive oxygen species and glutathione levels were investigated. The effects of hydrogen peroxide on the CDDP sensitivity of the SK-OV3/cddp cells and their expression levels of the redox-associated protein growth arrest and DNA damage 45a (GADD45α) were also investigated. In addition, the impact of GADD45α overexpression on cell viability was evaluated *in vitro* and *in vivo*, and the levels of Ser-139 phosphorylated H2A histone family member X (γ-H2AX), which is associated with DNA damage, were detected. The results suggested that redox status affected the drug resistance of the ovarian cancer cells by increasing the expression of GADD45α. The overexpression of GADD45α reversed the CDDP resistance of the SK-OV3/cddp cells and increased the level of γ-H2AX. In conclusion, GADD45α alleviated the CDDP resistance of SK-OV3/cddp cells via the induction of redox-mediated DNA damage.

Correspondence to: Professor Dan Zi, Department of Obstetrics and Gynecology, Guizhou Provincial People's Hospital, 83 Zhongshan East Road, Guiyang, Guizhou 550004, P.R. China
E-mail: zidangy08@163.com

*Contributed equally

Introduction

Annually, 230,000 women are diagnosed with ovarian cancer and 150,000 succumb to this disease worldwide and the 5-year survival rate of ovarian cancer is only 46% (1). Epithelial ovarian cancer has the highest mortality rate among ovarian cancer types with a 5-year relative survival rate of 29% (2). It is characterized by a lack of clear symptoms at onset, late initial diagnosis, early spread and metastasis, recurrence after surgery, drug resistance and high mortality (3). Although great progress has been made in the treatment of ovarian cancer with the introduction of tumor reduction surgery and platinum chemotherapy combined with paclitaxel, drug resistance remains an urgent issue to be resolved (4). Therefore, it is necessary to uncover the molecular mechanism of drug resistance.

Cisplatin (CDDP) has been widely used for the treatment of a variety of tumors, including head and neck, testicular, bladder, lung, colorectal and ovarian cancers (5,6). It is a non-specific cytotoxic drug that inhibits the cell cycle and distributes non-selectively to tumor tissues. Its main action is the inhibition of tumor growth and induction of tumor cell death via the inhibition of DNA replication and transcription (7). However, drug resistance seriously limits the clinical use of CDDP (8). DNA damage and repair systems serve a vital role in the response to cancer treatment. The upregulated functioning of this system results in an antagonistic response of tumor cells to chemotherapeutic drugs and chemotherapy failure (9). It is well known that redox reactions are important for oxidative stress-induced DNA damage (10). Oxidative stress results in intracellular levels of reactive oxygen species (ROS) being elevated, which causes damage to lipids, proteins and DNA (11). In response to the accumulation of ROS induced by certain chemotherapies, cancer cells commonly produce reducing substances to alleviate oxidative stress, which gradually results in drug resistance (12). Glutathione (GSH) is a reducing substance containing a sulfhydryl group bound to the cysteine moiety of a tripeptide formed from glutamic acid, cysteine and glycine, which has an ROS-scavenging effect (13). Therefore, the drug resistance of cancer cells may be closely

associated with an imbalance of GSH and ROS, resulting in a reduction in the DNA damage induced by CDDP.

Growth arrest and DNA damage 45a (*GADD45α*) is a downstream target gene of p53, which is involved in growth arrest, apoptosis and DNA damage repair (14). GADD45α is closely associated with the occurrence, development and prognosis of tumors, as it is able to maintain genetic stability and inhibit the occurrence and development of tumors (15). However, some tumor cells have been revealed to avoid programmed death by decreasing the expression of GADD45α (16). As a downstream target gene of p53, GADD45α may be a key mediator of ovarian cancer chemosensitivity, which can arrest the cell cycle to increase damaged DNA repair (17). It has also been reported that the decreased ability of cells to repair DNA caused by GADD45α deletion is highly associated with DNA damage-induced tumorigenesis (18). In addition, GADD45α has been shown to alleviate multidrug resistance in tumor cells (19). In one study, the upregulation of GADD45α expression increased the sensitivity of prostate cancer cells to docetaxel (20). In another study, GADD45α expression was induced by platinum-based chemotherapy, which promoted the apoptosis of lung cancer cells and increased their sensitivity to chemotherapy (21). Previous studies have also reported that GADD45α can be upregulated by CDDP treatment in ovarian cancer cells (22,23). It is thus very important to further study the role of GADD45α in ovarian cancer in order to find a new therapeutic target for its treatment. The present study constructed a CDDP-resistant ovarian cancer cell line and performed RNA sequencing, which revealed a difference in GADD45α gene expression in the CDDP-resistant cell line compared with the parental cell line. However, whether GADD45α increases the sensitivity of ovarian cancer to CDDP has not yet been reported.

H2A histone family member X (H2AX) is a sensitive marker for DNA damage that serves a crucial role in molecular and cellular responses to DNA damage and in genome stability maintenance (24). H2AX has been demonstrated to be important for the inhibition of tumor growth, as it increases genomic stability and reduces susceptibility to tumorigenesis (25). H2AX prevents the aberrant repair of programmed and general DNA breakage and functions as a dosage-dependent suppressor of genomic instability and tumors in mice (26). However, the mechanism underlying its effects remains unknown.

The present study investigated the effect of redox status on the drug resistance of ovarian cancer cells, and its association with GADD45α. The effect of the overexpression of GADD45α in CDDP-resistant ovarian cancer cells was investigated *in vitro* and *in vivo*, and whether the role of GADD45α in the CDDP resistance of ovarian cancer cells is mediated via redox-mediated DNA damage was evaluated.

Materials and methods

Cell culture and reagents. SK-OV3 human ovarian cancer cells were purchased from American Type Culture Collection. The cells were cultured in complete RPMI-1640 medium (Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc.) and no antibiotics at 37°C and 5% humidified CO_2. Cells in the logarithmic growth phase were used for the experiments. Thiazolyl blue tetrazolium bromide (MTT) was obtained from Abcam (cat. no. ab211091). The primary antibodies GADD45α (cat. no. ab203090), Ser-139 phosphorylated H2AX (γ-H2AX; cat. no. ab26350), H2AX (cat. no. ab229914) and β-actin (cat. no. ab8226), all 1:1,000, were purchased from Abcam. CDDP was acquired from Sigma-Aldrich (Merck KGaA).

CDDP-induced SK-OV3/cddp cells. SK-OV3 cells were cultured in suspension in complete RPMI-1640 medium containing 10% fetal bovine serum and passaged every 3 days. Cells in the logarithmic growth phase were inoculated in RPMI-1640 medium containing CDDP. Starting at a CDDP concentration of 0.1 μg/ml and with the maintenance of positive drug pressure, sensitive cells gradually died and drug-resistant cells continued to be cultured for 3-4 weeks. The high-concentration drug treatment was initiated when the number of cells reached 1×10^7/ml. After repeated medium replacement, passage and a step-wise increase in CDDP concentration (0.1, 0.15, 0.2, 0.25, 0.375 and 0.5 μg/ml) over the course of 6 months, an SK-OV3/cddp cell line that was able to grow well in 0.5 μg/ml CDDP medium was obtained. The cells were frozen, stored for 3 months, and then recovered or grown in CDDP-free medium for nearly 6 months while maintaining their original drug resistance.

Treatment of cells. SK-OV3 and SK-OV3/cddp cells were treated with CDDP (0, 1, 5, 10, 20, 40 and 80 μM) or H_2O_2 (0, 5, 10, 25 and 50 μM) at 37°C for 24 h for the cell viability assay. The SK-OV3 and SK-OV3/cddp cells were treated with 80 μM CDDP and 50 μM H_2O_2 at 37°C for 24, 48 and 72 h for the cell viability assay. The SK-OV3/cddp cells were treated with 80 μM CDDP and 50 μM H_2O_2 at 37°C for 2 weeks for the soft agar colony formation assay. The SK-OV3/cddp cells were treated with 50 μM H_2O_2 for 24 h at 37°C in RT-qPCR and western blotting assays.

Xenograft experiments in mice. Female BALB/c nude mice of specific pathogen-free (SPF) grade, aged 6 weeks and weighing 18-20 g were used in the study. The 50 nude mice were purchased from Beijing Sibeifu Biotechnology Co., Ltd. The animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) 7th Edition and approved by the Animal Ethics Committee of Guizhou Medical University (approval no. 2000470). The mice were provided with food and water freely available and kept in a SPF-level environment with a temperature of 22±2°C, a humidity of 40-60% and a 12-h light/dark cycle. The mice were divided into five groups: Two control groups (untreated SK-OV3 and SK-OV3/cddp cells, respectively); CDDP group (CDDP treatment and SK-OV3/cddp cells); GADD45α group (GADD45α-overexpressing SK-OV3/cddp cells) and CDDP + GADD45α group (CDDP treatment and GADD45α-overexpressing SK-OV3/cddp cells), each containing 10 mice. The mice were subcutaneously injected in the left axillary region with a 100 μl single-cell suspension of 1.5×10^6 cells in saline, containing SK-OV3, SK-OV3/cddp or GADD45α-overexpressing SK-OV3/cddp cells as appropriate. The mice were continuously observed for 6 weeks. Once the tumor volume reached 50 mm³, the mice in the CDDP group

and CDDP + GADD45α group were intraperitoneally injected with CDDP (4 mg/kg), while the mice in the other groups were injected with saline. The injection frequency was once every 3 days, for a total of five injections. Measurements of the tumor were taken weekly, and tumor volumes were calculated using the following formula: Tumor volume = $(A \times B^2)/2$, where A and B are the tumor length and width (in mm), respectively. When the maximum volume of the transplanted tumor reached 1,500 mm^3, the mice were euthanized by cervical dislocation and tumor samples were taken. The tumor tissues were frozen with liquid nitrogen and stored in a refrigerator at -80°C.

Cell viability assay and 50% inhibitory concentration (IC$_{50}$) calculation. When cells reached 70% confluency in a 96-well microplate, they were cultured in a medium containing CDDP (0, 1, 5, 10, 20, 40 and 80 μM) or H$_2$O$_2$ (0, 5, 10, 25 and 50 μM) at 37°C for 24 h. Then, 10 μl MTT, dissolved in PBS, was added and the cells were incubated for 2 h at 37°C. The purple formazan was dissolved in PBS. The absorbance values of the cells were detected at 490 nm using a microplate reader (Omega Bio-Tek, Inc.). The IC$_{50}$ was calculated with GraphPad Prism v.5.01 software (GraphPad software, Inc).

RNA sequencing. Total RNA from the CDDP-resistant ovarian cancer cell line and its parental cell line was analyzed using Hiseq. Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc.) to assess its quantity and quality. The differences of readcount data were analyzed after being standardized by DESeq v.1.12.0 (Illumina Inc.). Differentially expressed genes for CDDP-resistant SK-OV3 cells compared with the parental cell line were defined as genes with an absolute log-transformed fold change [abs(log2FC)] >1 (P<0.005).

Intracellular GSH content. Intracellular GSH content was measured using 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB). SK-OV3 and SK-OV3/cddp cells were washed twice with PBS and the cell pellet (1x10^6 cells) was lysed with 100 μl cell lysis buffer (Beyotime Institute of Biotechnology). Thereafter, 15 μl HCl (0.1 N) and 15 μl 50% sulfosalicylic acid were added. Xenograft tissue homogenate was prepared by perfusing the tissue with phosphate buffer saline solution (pH 7.4) containing 0.16 mg/ml heparin to remove red blood cells and clots. Then, the tissue was homogenized in 5-10 ml cold buffer (50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA) per gram of tissue. Supernatants were collected after centrifugation for 15 min at 12,000 x g at 4°C. A total of 25 μl cell lysate and 100 μl DTNB in sodium phosphate buffer containing EDTA were mixed and the optical density at 412 nm was measured immediately using a spectrophotometer (Varian Medical Systems, Inc.). The protein level was measured using the Bradford method (27).

ROS level analysis in cells. Cellular ROS levels were measured using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology). SK-OV3 and SK-OV3/cddp cells were washed twice with cold PBS, treated with 10 μM DCFH-DA and incubated for 30 min at 37°C in a light-protected humidified chamber. Following treatment with the probe, the cells were washed at least twice with ice-cold PBS. The fluorescence emitted by the cells was measured with a fluorescence detection instrument (DTX800; Beckman Coulter, Inc.) using an excitation wavelength of 502 nm, and detection wavelength of 530 nm. The fluorescence intensity was recorded and analyzed with GraphPad Prism v.5.01 (GraphPad software, Inc.).

ROS detection in dissected tumor tissue. PBS was used to wash the excised tumor twice. Ophthalmic scissors were used to cut the tumor tissue into pieces, which were then treated with type I collagenase (1 mg/ml) to destroy the extracellular matrix in the tissue at 37°C for 10 min. The digestion fluid was filtered with a 200-mesh filter to obtain a single-cell suspension. Then, 2x10^4 cells/well were seeded in a 96-well plate, treated with 10 μM DCFH-DA and incubated for 30 min at 37°C. The cells were washed with precooled PBS at least twice and then examined using a fluorescence detection instrument (DTX800). The excitation wavelength was 502 nm, and the detection wavelength was 530 nm. The fluorescence intensity was recorded and analyzed with GraphPad Prism v.5.01 (GraphPad software, Inc.).

Soft agar colony formation assay. The lower gel was prepared from 2 ml RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc.) with 0.4% agar. The upper cell-containing layer was prepared by suspending 1x10^3 cells in 4 ml RPMI-1640 medium with 0.2% agar and pouring it onto the lower gel. After 2 weeks, colonies were stained with 0.5% crystal violet at room temperature for 1 h and counted with an inverted microscope (CX43; Olympus Corporation).

Preparation and transfection of lentiviral-GADD45α vector. Lentiviral vector (GV287; 4 μg) (Shanghai Genechem Co., Ltd) containing the GADD45α gene was double digested using *Age*I (5 U/μl) and *Eco*RI (20 U/μl). Virus packaging plasmid Mix:1 μg/μl (Mix=pMDL: VSV-G: REV=5:3:2). The product was recovered, purified and mixed with T4 DNA ligase (1 μl) for 6 h at 16°C. It was then transformed into competent DH5α cells (Shanghai Ji Kai Biotechnology Co., Ltd) with Lipofectamine 2000® (Thermo Fisher Scientific, Inc.). Recombinant positive clones were preliminarily identified using PCR and restriction analysis. Plasmid sequencing was performed by Shanghai Ji Kai Biotechnology Co., Ltd. The primer sequences used were as follows: GADD45α forward, 5'-AGUCGCUACAUGGAUCAAUTT-3' and reverse, 5'-AUUGAUCCAUGUAGCGACUTT-3'; GAPDH forward, 5'-GCAGGGGGGGAGCCAAAAGGGT-3' and reverse, 5'-TGGGTGGCAGTGATGGCATGG-3'. SK-OV3/cddp cells in the logarithmic growth phase were seeded in 6-well plates at a concentration of 2x10^5 cells/ml, cultured for 24 h, mixed with the 4ug lentiviral-GADD45α vector with a multiplicity of infection value of 80 for 12 h, and then replenished with fresh medium. Transfection efficiency was detected using western blotting and reverse transcription-quantitative PCR (RT-qPCR) after 6 days of transfection. GFP lentivirus control plasmid (Shanghai Ji Kai Biotechnology Co., Ltd.) was used as control for the GADD45α vector.

RT-qPCR. The primers used for RT-qPCR validation are as described above for PCR. Total cell RNA samples were

isolated using Total RNA Kit I (Omega Bio-Tek, Inc.). Then, 500 ng RNA was reverse transcribed to cDNA using the Takara PrimeScript™ RT Reagent kit with gDNA Eraser (Takara Bio, Inc.) in a total reaction volume of 20 μl. The RT conditions used were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min followed by storage at 4°C. The cDNA (2 μl) was used as a template for qPCR conducted using a Real-Time PCR System (Bio-Rad Laboratories, Inc.). The qPCR steps were conducted in triplicate using SYBR Green Supermix according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 3 min at 95°C followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Following amplification, an additional thermal denaturizing cycle (temperature range, 65-95°C in 0.5°C increments) was performed to obtain the melting curves of the RT-qPCR products and verify amplification specificity. The relative expression of the gene of interest was normalized to GAPDH expression in each sample. The target gene expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (28) and the values for each gene are expressed as fold changes.

Western blot analysis. SK-OV3 cells were treated with lysis buffer containing 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% Triton X-100 and protease and phosphatase inhibitors on ice for 30 min. The supernatant was harvested after centrifuging the cell lysis products for 10 min at 12,700 x g and 4°C. The total protein concentration was measured using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Total protein samples were boiled for 5 min. Then, 30 μg/lane total protein was electrophoresed on an 8% SDS-PAGE gel, transferred onto PVDF membranes and blocked with 5% BSA for 2 h at room temperature before incubation with the aforementioned primary antibodies overnight at 4°C. The membranes were then washed with Tris-buffered saline containing 0.1% Tween-20, and incubated with goat anti-mouse IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.), for 2 h at room temperature. Protein immunoreactivity was visualized using Immobilon Western Chemilum HRP substrate (EMD Millipore) and quantified using Quantity One software 6.0 (Bio-Rad Laboratories, Inc).

Immunofluorescence assay. Precooled PBS was used for washing SK-OV3 and SK-OV3/cddp cells (10,000 cells/well) cultured in 6-well plates for 3 min each time for 3 times. Subsequently, 4% paraformaldehyde was used to fix cells at room temperature for 15 min. Next, the cells were washed twice with PBS, and permeabilized for 15 min at room temperature with 0.5% Triton X-100 to and blocked with 3% BSA to block the cells at room temperature for 40 min. The blocked SK-OV3 and SK-OV3 /cddp cells were incubated with primary antibody against γ-H2AX (1:200; Abcam; cat. no. ab81299) at 4°C for 12 h. The cells were washed 3 times with PBS prior to incubation with a Goat polyclonal Secondary Antibody to Rabbit IgG (1:100; Abcam; cat. no. ab150077) dilution for 40 min at room temperature. Subsequently, the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific Inc.) for 5 min at room temperature. The cells were observed using a fluorescence microscope at (CX43; Olympus Corporation) (magnification, x400).

Immunohistochemistry (IHC). IHC analysis was performed using an SP-HRP kit (Santa Cruz Biotechnology, Inc.). The tumor tissue was cut into 3-μm thick sections. Sections of tumor tissue were blocked with 5% Goat Serum (Beyotime Institute of Biotechnology) for 30 min at 37°C, incubated with anti-KI67 primary antibody (1:100; cat. no. sc-23900; Santa Cruz Biotechnology, Inc.) at 4°C overnight, then incubated with secondary antibody at dilution (1:5,000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.), for 2 h at room temperature, developed with 3,3'-diaminobenzidine and counterstained with hematoxylin for 5 min at room temperature and observation was performed using a light microscope (CX43; Olympus Corporation).

Statistical analysis. All experiments were performed in triplicate and data are expressed as the mean ± SD. Statistical significance was analyzed with one-way analysis of variance or two-way analysis of variance followed by Dunnett's test when groups were compared with a single control group or Tukey's test when analyzing the differences for all pairs of groups. Student's t-test was used to compare the differences between the SK-OV3 group and SK-OV3/cddp group or the NC group and GADD45α group. GraphPad Prism version 5.01 was used to perform the statistical analysis (GraphPad software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Redox status affects the drug resistance of ovarian cancer cells. To determine the drug resistance of ovarian cancer cells, the 50% inhibitory concentration (IC_{50}) values of SK-OV3 and SK-OV3/cddp cells were detected. The IC_{50} of the SK-OV3 cells was lower than that of the SK-OV3/cddp cells (Fig. 1A). Then, the ROS and GSH levels in the SK-OV3 and SK-OV3/cddp cells were detected. Significantly lower ROS and higher GSH levels were observed in the SK-OV3/cddp cells compared with the SK-OV3 cells (Fig. 1B and C). In order to verify the role of GSH in SK-OV3/cddp cells, different concentrations of H_2O_2 were added to SK-OV3/cddp and SK-OV3 cells for 24 h to deplete GSH. Cell viability was then detected. The viability of SK-OV3/cddp cells was significantly decreased following H_2O_2 treatment (Fig. 1D). It was hypothesized that a high level of reduction and low level of oxidation in SK-OV3/cddp cells may contribute to their drug resistance. To investigate this hypothesis, the effect of H_2O_2 on CDDP resistance was detected. Cell viability and colony formation were detected in SK-OV3/cddp cells treated with CDDP alone or in combination with H_2O_2. H_2O_2 combined with CDDP significantly inhibited the viability and colony formation of SK-OV3/cddp cells compared with that of CDDP alone (Fig. 1E and F), indicating that redox status affected the drug resistance of these ovarian cancer cells.

Redox regulation of ovarian cancer cell resistance is associated with GADD45α. Differentially expressed genes between the SK-OV3 and SK-OV3/cddp cells were detected using RNA sequencing. The two genes presented in red were upregulated 2-fold, and those in green were downregulated 2-fold (P<0.05), and GADD45α exhibited the most notable reduction, as it
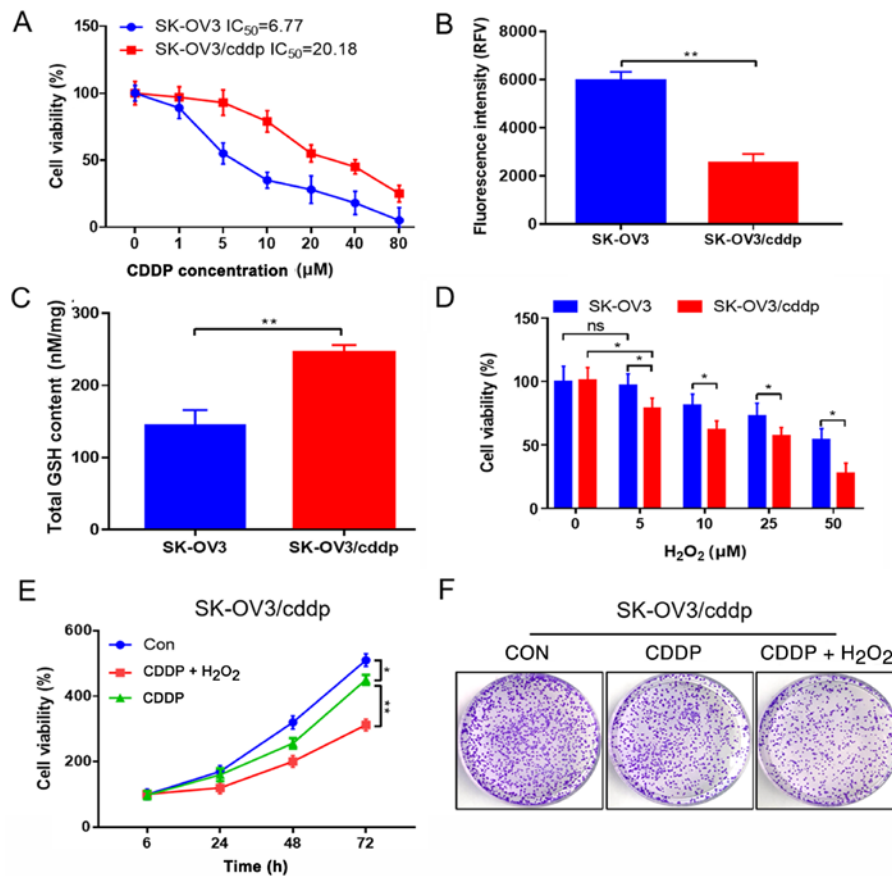
Figure 1. Redox status affects the drug resistance of ovarian cancer cells. (A) SK-OV3 and SK-OV3/cddp cells were assayed for cell viability after treatment with different concentrations of CDDP for 24 h. (B) 2,7-Dichlorodihydrofluorescein diacetate probe results for the detection of reactive oxygen species in living cells. (C) Changes in GSH levels in SK-OV3/cddp cells compared with SK-OV3 cells. (D) MTT assay results demonstrating the viability of SK-OV3/cddp and SK-OV3 cells treated with different concentrations of H_2O_2 for 24 h. (E) Cell viability was detected in SK-OV3/cddp cells treated with CDDP alone or in combination with H_2O_2. (F) Colony formation assay results revealing the effect of hydrogen peroxide on CDDP resistance. *P<0.05 and **P<0.01. ns, not significant; SK-OV3/cddp, Sk-OV3 cells resistant to CDDP; CDDP, cisplatin; IC_{50}, 50% inhibitory concentration; RVF, relative fluorescence value; H_2O_2, hydrogen peroxide; GSH, glutathione.

had the smallest P-value and lowest negative FC (Fig. 2A). To investigate the mechanism by which redox status regulates drug resistance in ovarian cancer cells, the expression of GADD45α, which is involved in the redox process, was detected (29). GADD45α mRNA and protein expression levels were significantly lower in SK-OV3/cddp cells compared with SK-OV3 cells. However, H_2O_2 significantly increased the expression of GADD45α in the SK-OV3/cddp cells (Fig. 2B and C). In order to explore the role of GADD45 in drug resistance, GADD45α was overexpressed in SK-OV3 cells (Fig. 2D), which led to a reduction in the viability of the cells (Fig. 2E). Cell viability and colony formation were also detected in GADD45α-overexpressing SK-OV3/cddp cells following CDDP treatment. Treatment with CDDP significantly inhibited GADD45α viability and markedly reduced colony formation in the GADD45α-overexpressing SK-OV3/cddp cells (Fig. 2F and G). The results indicated that the overexpression of GADD45α reversed the CDDP resistance of SK-OV3/cddp cells, which is associated with redox regulation.

GADD45α regulates DNA damage repair in SK-OV3/cddp cells. GADD45α is involved in DNA damage repair (18), while γ-H2AX is a sensitive molecular marker for monitoring DNA damage initiation (30). Whether GADD45α has a role in the regulation of H2AX is unclear. To clarify this, the expression of GADD45α and H2AX and the levels of γ-H2AX were detected in SK-OV3 and SK-OV3/cddp cells. Western blot and immunofluorescence assays indicated that GADD45α expression and γ-H2AX levels in SK-OV3/cddp cells were markedly lower compared with those in SK-OV3 cells (Fig. 3A and B). Then, GADD45α-overexpressing SK-OV3/cddp cells were treated with CDDP to determine the role of GADD45α in DNA damage repair. Western blotting results revealed that CDDP treatment significantly increased the expression of GADD45α in the SK-OV3/cddp cells. Moreover, the level of γ-H2AX in the SK-OV3/cddp cells was also significantly increased after CDDP treatment, GADD45α overexpression and CDDP combined with GADD45α overexpression. CDDP and GADD45α cooperated in increasing the level of γ-H2AX (Fig. 3C and D). These results indicate that GADD45α overexpression increased DNA damage in SK-OV3/cddp cells, which may be involved in the alleviation of drug resistance.

Regulation of CDDP resistance by GADD45α is associated with redox status in vivo. To investigate the role of GADD45α in CDDP resistance *in vivo*, SK-OV3, SK-OV3/cddp and GADD45α-overexpressing SK-OV3/cddp cells were implanted in the axillary subcutaneous tissue of nude mice, and the tumor volume and weight were subsequently evaluated. The results
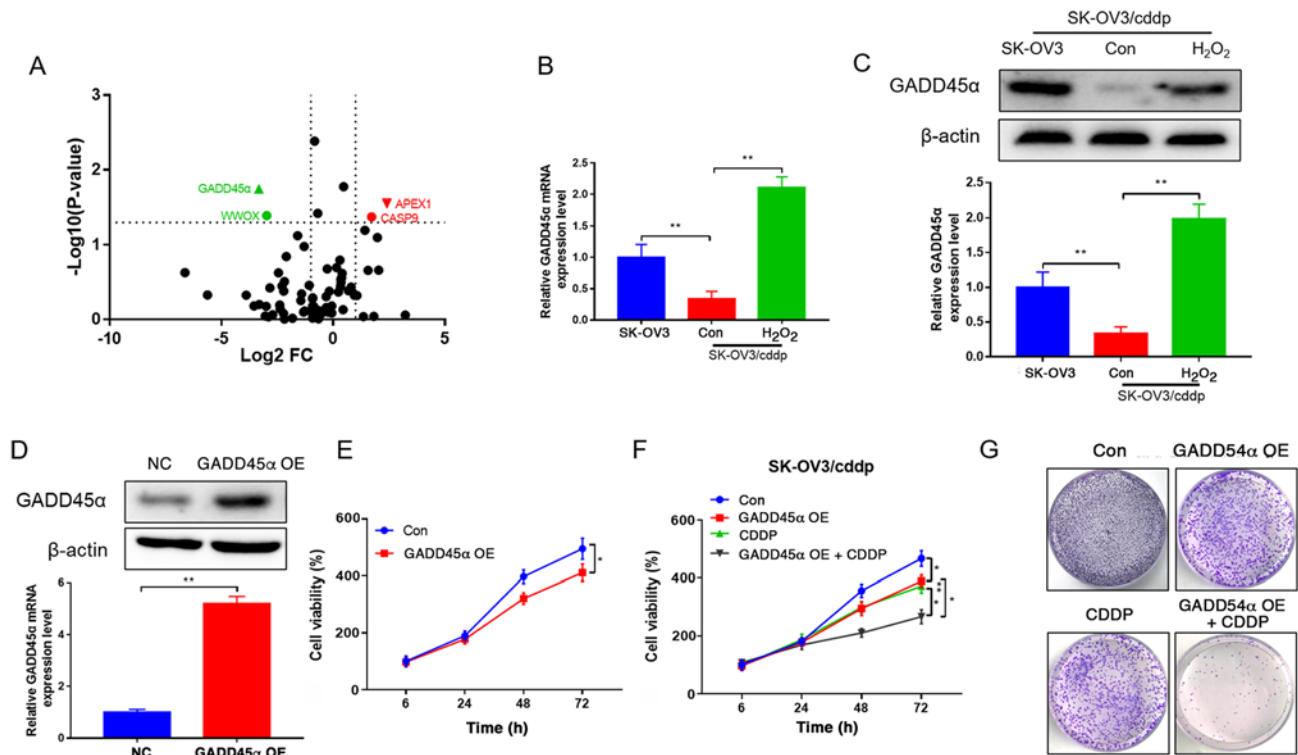
Figure 2. Redox regulation of the CDDP resistance of ovarian cancer cells is associated with GADD45α. (A) Analysis of differences in cell resistance to CDDP based on sequencing results. The horizontal coordinate is the difference multiple (base 2 logarithmic transformation) and the vertical coordinate is the significant false discovery rate (base 10 logarithmic transformation). Red indicates upregulation and green indicates downregulation. Changes in GADD45α (B) mRNA or (C) protein expression in SK-OV3, SK-OV3/CDDP (control) and H_2O_2 treated SK-OV3/CDDP cells. **Significance in the SK-OV3 or H_2O_2 treated SK-OV3/CDDP group when compared with SK-OV3/CDDP. (D) Verification of GADD45α overexpression in transfected SK-OV3 cells. (E) Cell proliferation decreased following GADD45α overexpression. (F) Cell proliferation decreased in GADD45α-overexpressing cells following treatment with CDDP. (G) Colony formation assay revealed that the overexpression of GADD45α reversed the CDDP resistance of SK-OV3 cells. *P<0.05 and **P<0.01. SK-OV3/cddp, Sk-OV3 cells resistant to CDDP; CDDP, cisplatin; GADD45α, growth arrest and DNA damage 45a; H_2O_2, hydrogen peroxide; OE, overexpression; NC, negative control (cells transfected with empty vector); Con, control.

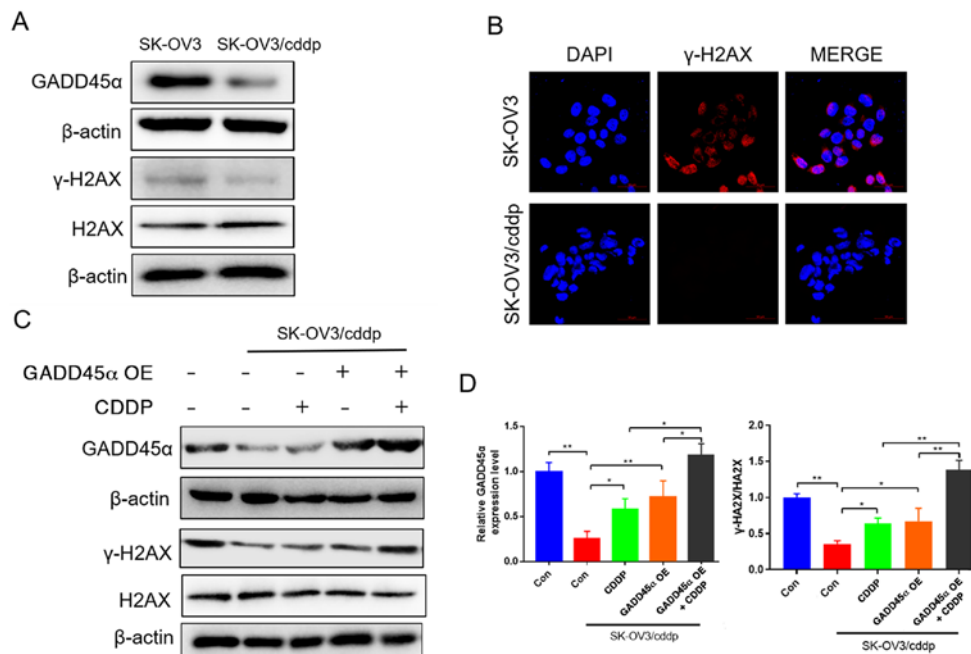Figure 3. GADD45α regulates DNA damage repair. (A) Western blotting results showing the expression of GADD45α and H2AX, and the phosphorylation of H2AX in SK-OV3 and SK-OV3/cddp cells. (B) Immunofluorescence images of γ-H2AX (magnification, x400) to assess cell damage. (C and D) Western blotting results showing the expression of GADD45α, and H2AX, and phosphorylation of H2AX in SK-OV3 and SK-OV3/cddp cells with CDDP treatment, GADD45α overexpression or CDDP combined with GADD45α overexpression; (C) representative blots and (D) quantified data. *P<0.05 and **P<0.01. GADD45α, growth arrest and DNA damage 45; H2AX, H2A histone family member X; γ-, Ser-139 phosphorylated; SK-OV3/cddp, Sk-OV3 cells resistant to CDDP; CDDP, cisplatin; OE, overexpression; Con, control.

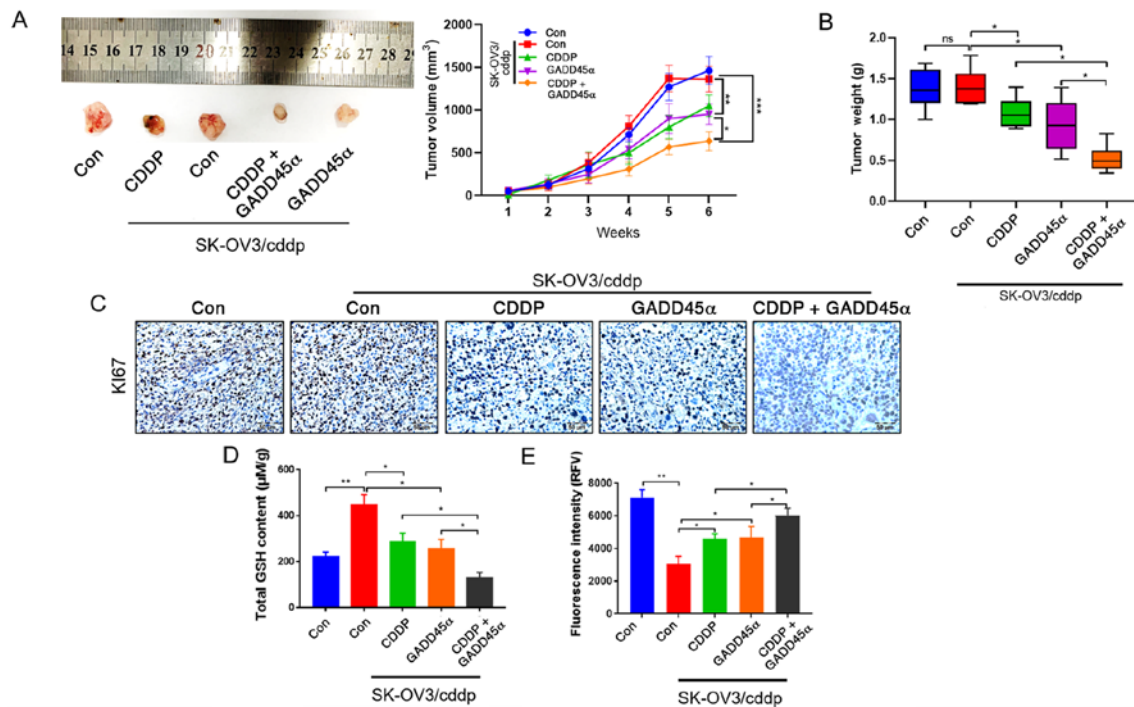Figure 4. Regulation of CDDP resistance by GADD45α is associated with redox status *in vivo*. Effect of GADD45α on xenograft tumor (A) volume and (B) weight. (C) Immunohistochemical images of KI67 (magnification, x400) demonstrating the effect of GADD45α on CDDP resistance *in vivo*. (D) GSH levels in the SK-OV-3/CDDP cell xenograft model. (E) 2,7-dichlorodihydrofluorescein diacetate probe results showing the redox status of the xenografts. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. CDDP, cisplatin; SK-OV3/cddp, Sk-OV-3 cells resistant to CDDP; GADD45α, growth arrest and DNA damage 45; GSH, glutathione; RVF, relative fluorescence value; con, control.

revealed that the tumor volume (Fig. 4A) and weight (Fig. 4B) for the SK-OV3/cddp cell-based tumors were significantly decreased by GADD45α overexpression, and further decreased by GADD45α overexpression combined with CDDP. IHC analysis was performed to detect the expression of the cell proliferation marker KI67 in the nude mouse tumor tissue. KI67 expression in the SK-OV3/cddp-based tissue was markedly decreased by CDDP treatment and further decreased by GADD45α overexpression combined with CDDP treatment (Fig. 4C), indicating that GADD45α alleviated the CDDP resistance of the SK-OV3/cddp cells *in vivo*. As the reversal of CDDP resistance by GADD45α was demonstrated to be associated with redox regulation (Fig. 2), the effect of redox on CDDP resistance *in vivo* was verified by detecting the GSH and ROS levels in the SK-OV3/cddp cell xenograft model. The GSH level was significantly decreased and the ROS level was increased by CDDP treatment, and these changes were significantly stronger when the CDDP treatment was administered in combination with GADD45α overexpression CDDP (Fig. 4D and E). The results indicated that the regulation of CDDP resistance by GADD45α is associated with the redox status *in vivo*.

Mechanism by which reduction-induced CDDP resistance in ovarian cells is regulated by GADD45α expression. The present study explored the mechanism of redox reactions in normal and CDDP-resistant ovarian cells by regulating GADD45α expression, leading to the following hypothesis: In normal ovarian cancer cells, CDDP promotes the oxidation of GSH to glutathione disulfide (GSSG) which increases ROS levels, and thereby upregulates the expression of GADD45α, induces DNA damage and increases tumor cell death.
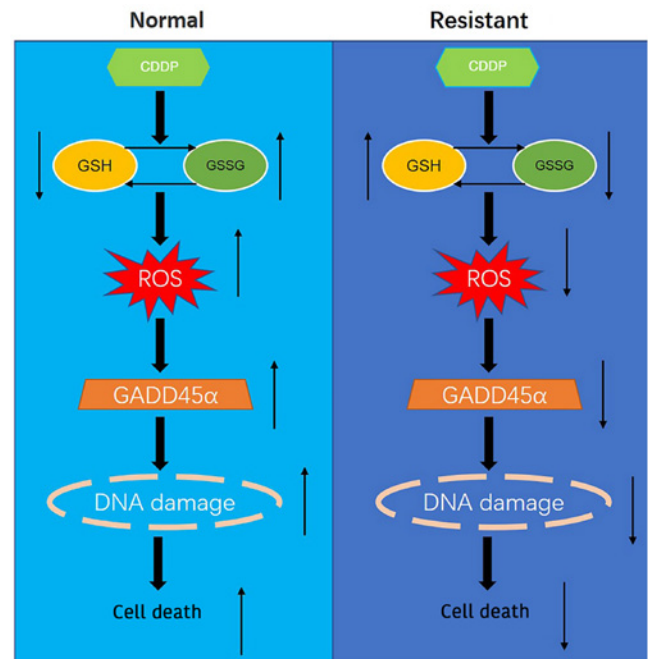

Figure 5. Mechanism of reduction-induced CDDP resistance in ovarian cells via the regulation of GADD45α expression. CDDP, cisplatin; GSH, glutathione; SSSG, glutathione disulfide; ROS, reactive oxygen species; GADD45α, growth arrest and DNA damage 45.

However, in CDDP-resistant ovarian cells, CDDP promotes the reduction of GSSG to GSH, which inhibits the production of ROS, and thereby downregulates the expression of GADD45α, decreases DNA damage and reduces tumor cell death (Fig. 5).

Discussion

The present study demonstrated that the effects of redox status on drug resistance are associated with GADD45α, which regulates DNA damage repair in SK-OV3/cddp cells. The effect of GADD45α on CDDP resistance was also verified *in vivo*. The results indicated that the regulation of CDDP resistance by GADD45α was associated with the redox status of the cells.

CDDP resistance is the primary cause of chemotherapy failure in ovarian cancer. GSH is a major intracellular regulator of redox conditions (31), which is present at high levels in numerous CDDP-resistant cell lines. For example, a recent study reported that elevated GSH levels are associated with the CDDP resistance of non-small cell lung cancer cell lines (32). High cellular GSH levels have also been reported in CDDP-resistant head and neck cancer cells (33). Furthermore, intracellular GSH content has been demonstrated to be much higher in CDDP-resistant human ovarian cancer cells than in CDDP-sensitive cells (34). The present study observed that lower ROS and higher GSH levels were present in CDDP-resistant human ovarian cancer cells (SK-OV3/cddp) compared with parental SK-OV3 cells. Lan *et al* (32) demonstrated that exogenous GSH promotes the CDDP resistance of A549 lung cancer cells. In addition, Cadoni *et al* (35) revealed that CDDP is able to react with GSH, which results in deactivation of the drug. In the present study, SK-OV3 and SK-OV3/cddp cells were treated with H_2O_2, which would be reduced by GSH, resulting in GSH depletion (36). The viability and colony formation of SK-OV3/cddp cells were significantly inhibited by combined CDDP and H_2O_2 treatment compared with CDDP treatment alone. The results indicated that GSH levels serve an important role in the drug resistance of SK-OV3/cddp cells, which is in accordance with previous research.

Yang *et al* (37) reported that H_2O_2 leads to the accumulation of ROS in cells, which increases the expression of GADD45α. GADD45α is involved in DNA repair, cell cycle arrest and apoptosis in response to physiological or environmental stresses (38). Grossi *et al* (29) demonstrated that GADD45α serves an important role in promoting the removal of ROS and sustaining redox balance. There is evidence indicating that GADD45α is involved in the resistance to anticancer drugs. For example, Liu *et al* (38) revealed that the downregulation of GADD45α increased the sensitivity of melanoma to CDDP. However, Wang *et al* (39) demonstrated that overexpression GADD45α attenuated the drug resistance of hepatocellular carcinoma cells. The present study demonstrated that GADD45α mRNA and protein expression levels were lower in CDDP-resistant SK-OV3 cells than in the parental cell line. However, these levels were increased by H_2O_2 treatment, and the overexpression of GADD45α-enhanced the CDDP chemosensitivity of SK-OV3/cddp cells. The dual role of GADD45α in drug resistance may be attributed to differences in the drug resistance mechanism among different types of tumors.

The γ-H2AX protein is involved in the regulation of DNA damage repair (40). The expression of H2AX is increased upon the initiation of DNA damage, which serves as an indicator of DNA damage (41). It has been reported that H2AX serves a role in the drug resistance of cancer cells. Wang *et al* (42) reported that the gemcitabine-induced drug resistance of pancreatic cancer cells was associated with the inhibition of DNA repair protein γ-H2AX. The present study demonstrated that the expression of GADD45α and phosphorylation level of H2AX in SK-OV3/cddp cells was significantly lower compared with that in SK-OV3 cells. As mentioned above, GADD45α is also involved in DNA damage repair (14). The current results are in accordance with the viewpoint that increased DNA repair ability contributes to chemotherapy resistance (43). The present study observed that CDDP treatment significantly increased the expression of GADD45α and the phosphorylation of H2AX. Moreover, the phosphorylation level of H2AX was significantly increased following GADD45α overexpression or treatment with CDDP combined with GADD45α overexpression in SK-OV3/cddp cells. The results demonstrated that GADD45α increases DNA damage and alleviates CDDP resistance in SK-OV3/cddp cells, and these *in vitro* findings were verified in the *in vivo* experiment.

In conclusion, the present study demonstrated that GADD45α alleviated the CDDP resistance of SK-OV3/cddp cells, which was associated with redox-mediated DNA damage. Therefore, GADD45α may be a potential therapeutic target for CDDP-resistant ovarian cancer treatment.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QZZ and FW performed experiments and the data analysis. HLY, YYC, RGP, YMW, LN, YKQ, JJW and XZ contributed to performing experiments and data analysis. DZ contributed to study design. QZZ and DZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Guizhou Medical University (ethics approval no. 2000470).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Lheureux S, Gourley C, Vergote I and Oza AM: Epithelial ovarian cancer. Lancet 393: 1240-1253, 2019.
2. Reid BM, Permuth JB and Sellers TA: Epidemiology of ovarian cancer: A review. Cancer Biol Med 14: 9-32, 2017.
3. Deng J, Wang L, Chen H, Hao J, Ni J, Chang L, Duan W, Graham P and Li Y: Targeting epithelial-mesenchymal transition and cancer stem cells for chemoresistant ovarian cancer. Oncotarget 7: 55771-55788, 2016.
4. Feng RM, Zong YN, Cao SM and Xu RH: Current cancer situation in China: Good or bad news from the 2018 Global Cancer Statistics? Cancer Commun (Lond) 39: 22, 2019.
5. Chu YH, Sibrian-Vazquez M, Escobedo JO, Phillips AR, Dickey DT, Wang Q, Ralle M, Steyger PS and Strongin RM: Systemic Delivery and Biodistribution of Cisplatin in Vivo. Mol Pharm 13: 2677-2682, 2016.
6. Raudenska M, Balvan J, Fojtu M, Gumulec J and Masarik M: Unexpected therapeutic effects of cisplatin. Metallomics 11: 1182-1199, 2019.
7. Trimmer EE and Essigmann JM: Cisplatin. Essays Biochem 34: 191-211, 1999.
8. Qi R, Xiao H, Wu S, Li Y, Zhang Y and Jing X: Design and delivery of camplatin to overcome cisplatin drug resistance. J Mater Chem B Mater Biol Med 3: 176-179, 2015.
9. Telli ML, Stover DG, Loi S, Aparicio S, Carey LA, Domchek SM, Newman L, Sledge GW and Winer EP: Homologous recombination deficiency and host anti-tumor immunity in triple-negative breast cancer. Breast Cancer Res Treat 171: 21-31, 2018.
10. Kurz T, Leake A, von Zglinicki T and Brunk UT: Lysosomal redox-active iron is important for oxidative stress-induced DNA damage. Ann N Y Acad Sci 1019: 285-288, 2004.
11. Schieber M and Chandel NS: ROS function in redox signaling and oxidative stress. Curr Biol 24: R453-R462, 2014.
12. Jiang P, Du W and Wu M: Regulation of the pentose phosphate pathway in cancer. Protein Cell 5: 592-602, 2014.
13. Kwon DH, Lee H, Park C, Hong SH, Hong SH, Kim GY, Cha HJ, Kim S, Kim HS, Hwang HJ, et al: Glutathione Induced Immune-Stimulatory Activity by Promoting M1-Like Macrophages Polarization via Potential ROS Scavenging Capacity. Antioxidants 8: E413, 2019.
14. Li J, Dong J, Li S, Xia W, Su X, Qin X, Chen Y, Ding H, Li H, Huang A, et al: An alternative microRNA-mediated post-transcriptional regulation of GADD45A by p53 in human non-small-cell lung cancer cells. Sci Rep 7: 7153, 2017.
15. Liu LQ, Tian FJ, Xiong Y, Zhao Y and Song JB: Gadd45a gene silencing by RNAi promotes cell proliferation and inhibits apoptosis and senescence in skin squamous cell carcinoma through the p53 signaling pathway. J Cell Physiol 233: 7424-7434, 2018.
16. Zhan Q, Chen IT, Antinore MJ and Fornace AJ Jr: Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding. Mol Cell Biol 18: 2768-2778, 1998.
17. Wingert S, Thalheimer FB, Haetscher N, Rehage M, Schroeder T and Rieger MA: DNA-damage response gene GADD45A induces differentiation in hematopoietic stem cells without inhibiting cell cycle or survival. Stem Cells 34: 699-710, 2016.
18. Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC and Fornace AJ Jr: Dimethylbenzanthracene carcinogenesis in Gadd45a-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res 61: 2487-2491, 2001.
19. Chen H, Shan J, Chen D, Wang R, Qi W, Wang H, Ke Y, Liu W and Zeng X: CtIP promotes G2/M arrest in etoposide-treated HCT116 cells in a p53-independent manner. J Cell Physiol 234: 11871-11881, 2019.
20. Zhang Y, Yuan Y, Liang P, Zhang Z, Guo X, Xia L, Zhao Y, Shu XS, Sun S, Ying Y, et al: Overexpression of a novel candidate oncogene KIF14 correlates with tumor progression and poor prognosis in prostate cancer. Oncotarget 8: 45459-45469, 2017.
21. Krushkal J, Zhao Y, Hose C, Monks A, Doroshow JH and Simon R: Concerted changes in transcriptional regulation of genes involved in DNA methylation, demethylation, and folate-mediated one-carbon metabolism pathways in the NCI-60 cancer cell line panel in response to cancer drug treatment. Clin Epigenetics 8: 73, 2016.
22. De Feudis P, Debernardis D, Beccaglia P, Valenti M, Graniela Siré E, Arzani D, Stanzione S, Parodi S, D'Incalci M, Russo P, et al: DDP-induced cytotoxicity is not influenced by p53 in nine human ovarian cancer cell lines with different p53 status. Br J Cancer 76: 474-479, 1997.
23. Delmastro DA, Li J, Vaisman A, Solle M and Chaney SG: DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines. Cancer Chemother Pharmacol 39: 245-253, 1997.
24. Tarrade S, Bhardwaj T, Flegal M, Bertrand L, Velegzhaninov I, Moskalev A and Klokov D: Histone H2AX Is Involved in FoxO3a-Mediated Transcriptional Responses to Ionizing Radiation to Maintain Genome Stability. Int J Mol Sci 16: 29996-30014, 2015.
25. Celeste A, Difilippantonio S, Difilippantonio MJ, Fernandez-Capetillo O, Pilch DR, Sedelnikova OA, Eckhaus M, Ried T, Bonner WM and Nussenzweig A: H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell 114: 371-383, 2003.
26. Bassing CH, Suh H, Ferguson DO, Chua KF, Manis J, Eckersdorff M, Gleason M, Bronson R, Lee C and Alt FW: Histone H2AX: A dosage-dependent suppressor of oncogenic translocations and tumors. Cell 114: 359-370, 2003.
27. Banerjee K, Ganguly A, Chakraborty P, Sarkar A, Singh S, Chatterjee M, Bhattacharya S and Choudhuri SK: ROS and RNS induced apoptosis through p53 and iNOS mediated pathway by a dibasic hydroxamic acid molecule in leukemia cells. Eur J Pharm Sci 52: 146-164, 2014.
28. Chen H, Liao K, Cui-Zhao L, Qiang-Wen F, Feng-Zeng X, Ping-Wu F, Liang-Guo S and Juan-Chen Y: Cigarette smoke extract induces apoptosis of rat alveolar Type II cells via the PLTP/TGF-β1/Smad2 pathway. Int Immunopharmacol 28: 707-714, 2015.
29. Grossi V, Forte G, Sanese P, Peserico A, Tezil T, Lepore Signorile M, Fasano C, Lovaglio R, Bagnulo R, Loconte DC, et al: The longevity SNP rs2802292 uncovered: HSF1 activates stress-dependent expression of FOXO3 through an intronic enhancer. Nucleic Acids Res 46: 5587-5600, 2018.
30. Mah LJ, El-Osta A and Karagiannis TC: gammaH2AX: A sensitive molecular marker of DNA damage and repair. Leukemia 24: 679-686, 2010.
31. Chen HHW, Song IS, Hossain A, Choi MK, Yamane Y, Liang ZD, Lu J, Wu LY, Siddik ZH, Klomp LW, et al: Elevated glutathione levels confer cellular sensitization to cisplatin toxicity by up-regulation of copper transporter hCtr1. Mol Pharmacol 74: 697-704, 2008.
32. Lan D, Wang L, He R, Ma J, Bin Y, Chi X, Chen G and Cai Z: Exogenous glutathione contributes to cisplatin resistance in lung cancer A549 cells. Am J Transl Res 10: 1295-1309, 2018.
33. Tonigold M, Rossmann A, Meinold M, Bette M, Märken M, Henkenius K, Bretz AC, Giel G, Cai C, Rodepeter FR, et al: A cisplatin-resistant head and neck cancer cell line with cytoplasmic p53(mut) exhibits ATP-binding cassette transporter upregulation and high glutathione levels. J Cancer Res Clin Oncol 140: 1689-1704, 2014.
34. Okuno S, Sato H, Kuriyama-Matsumura K, Tamba M, Wang H, Sohda S, Hamada H, Yoshikawa H, Kondo T and Bannai S: Role of cystine transport in intracellular glutathione level and cisplatin resistance in human ovarian cancer cell lines. Br J Cancer 88: 951-956, 2003.
35. Cadoni E, Valletta E, Caddeo G, Isaia F, Cabiddu MG, Vascellari S and Pivetta T: Competitive reactions among glutathione, cisplatin and copper-phenanthroline complexes. J Inorg Biochem 173: 126-133, 2017.
36. Mailloux RJ, Craig Ayre D and Christian SL: Induction of mitochondrial reactive oxygen species production by GSH mediated S-glutathionylation of 2-oxoglutarate dehydrogenase. Redox Biol 8: 285-297, 2016.
37. Yang Y, Su Y, Wang D, Chen Y, Wu T, Li G, Sun X and Cui L: Tanshinol Attenuates the Deleterious Effects of Oxidative Stress on Osteoblastic Differentiation via Wnt/FoxO3a Signaling. Oxid Med Cell Longev. 2013: 351895, 2013.
38. Liu J, Jiang G, Mao P, Zhang J, Zhang L, Liu L, Wang J, Owusu L, Ren B, Tang Y, et al: Down-regulation of GADD45A enhances chemosensitivity in melanoma. Sci Rep 8: 4111, 2018.
39. Wang X, Zou F, Zhong J, Yue L, Wang F, Wei H, Yang G, Jin T, Dong X, Li J, et al: Secretory Clusterin Mediates Oxaliplatin Resistance via the Gadd45a/PI3K/Akt Signaling Pathway in Hepatocellular Carcinoma. J Cancer 9: 1403-1413, 2018.

40. Sekhar SC, Venkatesh J, Cheriyan VT, Muthu M, Levi E, Assad H, Meister P, Undyala VV, Gauld JW and Rishi AK: A H2AX-CARP-1 Interaction Regulates Apoptosis Signaling Following DNA Damage. Cancers (Basel) 11: E221, 2019.

41. Salzano M, Sanz-García M, Monsalve DM, Moura DS and Lazo PA: VRK1 chromatin kinase phosphorylates H2AX and is required for foci formation induced by DNA damage. Epigenetics 10: 373-383, 2015.

42. Wang Y, Kuramitsu Y, Kitagawa T, Tokuda K, Baron B, Akada J and Nakamura K: The Histone Deacetylase Inhibitor Valproic Acid Sensitizes Gemcitabine-Induced Cytotoxicity in Gemcitabine-Resistant Pancreatic Cancer Cells Possibly Through Inhibition of the DNA Repair Protein Gamma-H2AX. Target Oncol 10: 575-581, 2015.

43. Tang XH, Li H, Zheng XS, Lu MS, An Y and Zhang XL: CRM197 reverses paclitaxel resistance by inhibiting the NAC-1/Gadd45 pathway in paclitaxel-resistant ovarian cancer cells. Cancer Med 8: 6426-6436, 2019.