Knockdown of BRCA2 enhances cisplatin and cisplatin-induced autophagy in ovarian cancer cells

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Abstract

Clinical implications of the BRCA2 expression level on treatments of ovarian cancer are controversial. Here, we demonstrated that platinum-resistant cancer had a higher percentage of high BRCA2 level (87.5% vs 43.6%, \( P = 0.001 \)), and that patients with a low BRCA2 level in cancer tissues had longer progression-free survival (with a median time of 28.0 vs 12.0 months, \( P < 0.001 \)) and platinum-free duration (with a median time of 19.0 vs 5.0 months, \( P < 0.001 \)) compared with those with a high BRCA2 level. In human ovarian cancer cell lines CAOV-3 and ES-2, cisplatin induced an upregulation of the RAD51 protein, which was inhibited after silencing \( BRCA2 \); silencing \( BRCA2 \) enhanced the action of cisplatin \( in vitro \) and \( in vivo \). Knockdown of BRCA2 promoted cisplatin-induced autophagy. Interestingly, the autophagy blocker chloroquine enhanced cisplatin in \( BRCA2 \)-silenced cells accompanied by an increase in apoptotic cells, which did not occur in \( BRCA2 \)-intact cells; chloroquine enhanced the efficacy of cisplatin against \( BRCA2 \)-silenced CAOV-3 tumors \( in vivo \), with an increase in LC3-II level in tumor tissues. Sensitization of cisplatin was also observed in \( BRCA2 \)-silenced CAOV-3 cells after inhibiting ATG7, confirming that chloroquine modulated the sensitivity via the autophagy pathway. These data suggest that a low BRCA2 level can predict better platinum sensitivity and prognosis, and that the modulation of autophagy can be a chemosensitizer for certain cancers.

Key Words: BRCA2 silence, DNA repair, ovarian cancer, platinum response, autophagy

Introduction

Ovarian cancer has a high response rate (>80%) to the initially platinum-based chemotherapy; however, the majority of cases will gradually become refractory and eventually lead to treatment failure (Bookman 2012, Li et al. 2016). Platinum compounds deactivate cancer cells via the formation of DNA crosslinks, thereby causing DNA breaks; resistant cells can repair DNA damages resulting in survival.

The BRCA2 protein directly binds to and regulates the RAD51 protein, an essential molecule for DNA repair via homologous recombination (HR) (Fradet-Turcotte et al. 2016, Katsuki & Takata 2016, Yan et al. 2016, Sarwar et al. 2017). BRCA2-deficient cancer cells are hypersensitive to DNA-crosslinking agents such as cisplatin (CDDP), and therefore BRCA2 may be a target for the management of platinum resistance (Sakai et al. 2008,
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Rytelewski et al. 2014, Katsuki & Takata 2016). As to the relationship between the status of BRCA2 and therapeutic outcome of platinum in ovarian cancer, there yet lacks a consensus: BRCA2 mutation is associated with improved sensitivity to platinum and longer survival in certain trials, but other trials have demonstrated that mutation leads to platinum resistance (Liu et al. 2012, De Picciotto et al. 2016). Furthermore, mechanisms of BRCA2 impacting on the responses to platinum have not been thoroughly elucidated.

Autophagy is commonly considered a survival pathway, but can be cytotoxic when the damage is critical (Arun et al. 2015, Fan et al. 2016). CDDP can induce autophagy, inhibition of autophagy can enhance the action of CDDP because autophagy favors DNA repair, and autophagy may play an important part in chemoresistance in certain cancers (e.g., ovary and skin) (Claerhout et al. 2010, Yu et al. 2011, Zhang et al. 2012, Wu et al. 2016). These data suggest that BRCA2 can modulate the cells’ sensitivity to CDDP via the autophagy pathway. However, effects of silencing BRCA2 on CDDP-induced autophagy remain unclear.

In the present study, the association between the expression level of BRCA2 protein and the prognosis in ovarian cancer was clinically analyzed, and then effects of silencing BRCA2 on the sensitivity to CDDP and on autophagy in human ovarian cancer cells CAOV-3 and ES-2 were investigated in vitro and in vivo. Preliminary data suggest that silencing BRCA2 can enhance the action of CDDP and CDDP-induced autophagy.

Materials and methods

Patients and tumor tissues

The use of human tissues was ethically approved by the Institutional Review Board of the Second Affiliated Hospital, Chongqing Medical University (Chongqing, China).

Clinical data of 63 cases of ovarian cancer (i.e., age, pathological type/grade, FIGO stage, treatments and therapeutic responses) were collected. All patients underwent debulking surgery followed by platinum-based chemotherapy in the aforementioned hospital, and were followed up (i.e., clinical examinations, serum CA125 and ultrasound/CT scan) every 3 months for 2 years, every 6 months for the next 3 years and then annually (Ledermann et al. 2013). Tumor tissues received immunohistochemical assays. Platinum response was defined as resistance when relapse or progression occurred within 6 months from the last dose and as sensitivity when tumor relapsed after 6 months (Sun et al. 2013). The therapeutic outcome was reflected with the progression-free survival duration (PFS) and platinum-free duration (PFD). PFS was the interval from the date of initial surgery to the date of progression/recurrence or last contact (censored). PFD was the interval from the end of platinum treatment to the date of progression/recurrence or last contact (censored).

Immunohistochemistry

Immunohistochemical staining was performed with the SP kit (ZSGB-BIO, Beijing, China) using a rabbit polyclonal anti-BRCA2 antibody (Abcam). Sections were observed by 2 independent pathologists. The expression level of BRCA2 was scored using the percentage of positively stained cells: 0: no positive cells; 1: ≤10% positive cells; 2: 11–50% positive cells; 3: 51–90% positive cells; 4: ≥91% positive cells). Scores 0 and 1 were categorized as a low expression level, and scores 2–4 were categorized as a high expression level (Swisher et al. 2009).

Cells, transfection and drug exposure

Human ovarian cancer cell lines SKOV3, CAOV-3, ES-2, A2780 and COC1 (identified by STRS; China Center for Typical Culture Collection, Wuhan, China) were cultured in RPMI 1640 medium (Sigma-Aldrich) enriched with 10% fetal calf serum (Gibco), at 37°C and 5% CO2. The passage number was limited to 30. The basal level of BRCA2 protein was calibrated, and therefore CAOV-3 and ES-2 cell lines were selected for gene-silencing trials (described later).

A lentiviral vector expressing BRCA2- (shBRCA2) or control-shRNA (NC) was constructed (GenePharma, Shanghai, China). The targeting sequences of siRNA were (5′-AACAAAAATTACGAACCAAAC-3′) for BRCA2 and (5′-UUCUGCAACGUGUCAGUTT-3′) for NC, respectively (Sakai et al. 2008). Vectors were transferred into CAOV-3 and ES-2 cells with the Polybrene kit (GenePharma). Puromycin (Sigma-Aldrich) was added into the medium to remove uninfected cells.

For in vitro therapeutic trials, cells were treated with CDDP (Qilu Pharm., Jinan, China) and/or chloroquine (CQ; Sigma-Aldrich) for 6h, and then drugs were washed away. Therefore, both the peak level and the value of ‘concentration × time’ of either drug were within the range of human pharmacokinetics, having clinical relevancy (Yu et al. 2016).
Detecting BRCA2, RAD51, ATG7 and LC3 proteins with Western blot

Proteins were prepared, separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Merck Millipore). Rabbit polyclonal antibodies were used: anti-BRCA2, anti-RAD51 (Abcam), anti-LC3 (Abcam), anti-ATG7 (Cell Signaling Technology) and anti-β-actin (Bioss Biotechnol., Beijing, China). The secondary antibody was a goat anti-rabbit IgG antibody (ZSGB-BIO). β-actin served as the reference.

For detecting BRCA2, RAD51 and LC3 after exposure to CDDP (4 μM) and/or CQ (9.69 μM), CAOV-3 and ES-2 cells were seeded into a 6-well plate for 48 h and then received treatments. Proteins were assayed after 24 h. CQ can inhibit the fusion of autophagosomes and autolysosomes, leading to the LC3-II accumulation (Mizushima et al. 2010).

Cell viability

Cells were seeded into a 96-well plate (5.0 × 10³ cells per well) and exposed to CDDP (0–32 μM). Cell viability was determined with a CCK-8 assay (Dojindo Lab., Kumamoto, Japan) after 48 h, and then the drug concentration used for functional assays was determined based upon the cell survival curve.

When combining CDDP and CQ, the level of CDDP was 2, 4 or 8 μM, and that of CQ was 9.69 μM. Cell viability was determined after 24 h.

Colony formation

Cells were seeded into a 6-well plate (500 cells per well), and then exposed to CDDP (2 μM). The number of colony was counted under a microscope after 14 days.

Detecting DNA double-strand break (DSB) using the neutral comet assay

DSB was detected with the neutral comet assay 0–8 h after CDDP (4 μM) and/or CQ (9.69 μM) exposure, and the percentage of comet-formed cells was used to reflect the DSB degree (He et al. 2014).

Detecting the RAD51 foci

Cells were treated with CDDP (8 μM) and/or CQ (9.69 μM). After 12 h, cells were fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.25% Triton X-100 in phosphate-buffered saline (PBS). After blocking (3% bovine serum albumin/3% goat serum/PBS), a primary antibody against RAD51 was added and incubated at 4°C for 16 h. A secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG antibody; ZSGB-BIO) was added and incubated for 1 h. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (Invitrogen). Images were captured under a fluorescent microscope (Ti-E; Nikon). Cells with ≥5 RAD51 foci were scored as positive (Sakai et al. 2008). 100 cells per experimental point were evaluated.

Monitoring the autophagy flux with tandem mRFP-GFP fluorescence assay

The autophagy flux was monitored in siBRCA2-transfected CAOV-3 cells using a monomeric RFP-GFP tandem fluorescent-tagged LC3 assay (GeneChem, Shanghai, China). Cells were seeded on a coverslip, and then exposed to CDDP (4 μM) and/or CQ (9.69 μM). After 12 h, cells were fixed with 4% formaldehyde, washed with PBS and observed under a confocal microscope (Ti-E). 30 cells were checked; yellow puncta (RFP+GFP+) indicated autophagosomes and red dots (RFP+GFP−) showed autolysosomes (Han et al. 2016). Starvation with EBSS was used as the positive control (Proikas-Cezanne et al. 2004).

Apoptosis

Cells were treated with CDDP (8 μM) and/or CQ (9.69 μM). Apoptotic cells were detected using an Annexin V assay (MultiSciences Biotechnology, Hangzhou, China) after 24 h.

CAOV-3 cells’ response to CDDP after silencing ATG7

This was to confirm that CQ-enhanced CDDP was realized via modulating autophagy, as CQ can also affect other pathways (Zhang et al. 2015). ATG7 (an upstream molecule of LC3) was down-knocked. The infection rate was low when co-transfecting shRNA of BRCA2 and ATG7. siRNA of BRCA2 and/or ATG7 (5’- CAGAAGGAGUACAGCUCUUCCUUA-3’; GenePharma), or of NC was transfected into CAOV-3 cells using the siRNA-Mate kit (GenePharma) (Kim et al. 2007). BRCA2 and ATG7 proteins were detected by Western blot after 48 h, and then cells’ response to CDDP (4 μM) was analyzed.
**In vivo therapeutic trials in CAOV-3 tumors**

The use of animals was ethically and scientifically approved by Chongqing Med. Univ. in compliance with the Guide for the Care and Use of Laboratory Animals. *In vivo* effects were tested in 2 trials. $5.0 \times 10^6$ NC/shBRCA2-transfected CAOV-3 cells were subcutaneously injected into the left armpit of 4-week-old BALB/c nude mice (Ctr. Lab. Anim., Chongqing Med. Univ.), with 5 animals in each group. CDDP ($5 \text{mg/kg}$) was injected i.p. every 4 days, and each animal received it 6 times. CQ ($50 \text{mg/kg}$) was injected i.p. every day for 24 days. Other mice received normal saline. Tumor volume was calibrated every 4 days ($\frac{\text{length} \times \text{width}^2}{2}$) (Sun *et al.* 2013). Mice were euthanized 4 days after the last dose, tumor mass was measured and tumors received examinations.

Trial 1 was performed to determine whether silencing *BRCA2* enhanced the anticancer efficacy of CDDP. NC-transfected cells were injected in groups NC and NC+CDDP, and shBRCA2-transfected cells were injected in groups shBRCA2 and shBRCA2+CDDP. CDDP was administrated in groups NC+CDDP and shBRCA2+CDDP. Tumors received pathological examinations, and BRCA2 and RAD51 proteins in tumor tissues were detected using an immunohistochemical assay.

Trial 2 determined whether CQ enhanced the action of CDDP in BRCA2-silenced tumors. shBRCA2-transfected cells were injected to form tumors. Mice received CDDP in groups CDDP and CDDP+CQ, and CQ was injected in groups CQ and CDDP+CQ. Tumors received pathological examinations, and LC3 in tumor tissues was detected with Western blot.

**Statistics**

Data were processed with the software SPSS 17.0. Analysis of variance and *t*-test were used. The chi-square test was used to compare the association between the BRCA2 expression level and the clinicopathologic variables. PFS and PFD were compared with the Kaplan–Meier method. The critical value was set at $P<0.05$.

**Results**

**A low expression level of BRCA2 had better chemosensitivity, and longer PFS and PFD**

Clinicopathological characteristics and their relationships to the expression level of BRCA2 protein in cancer tissues are summarized in Table 1. There was no association between the expression level and age, pathological type/grade, clinical stage, chemotherapy regimen/cycle, or the size of residual tumor. Platinum-resistant cancer had a higher BRCA2 level compared with sensitive cancer (with a high expression percentage of 87.5% (95% CI: 67.6–97.3%) vs 43.6% (95% CI: 37.8–49.4%).

![Table 1](http://erc.endocrinology-journals.org) Clinicopathological characteristics and their associations with the expression level of BRCA2 in cancer tissues.
27.8–60.4%), \( P = 0.001 \); and a score of 2.72 ± 1.24 vs 1.68 ± 1.54, \( P = 0.007 \) (Fig. 1A and B). Patients with a low level of BRCA2 in cancer tissues had longer PFS (with a median time of 28.0 (95% CI: 18.2–37.8) vs 12.0 (95% CI: 9.6–14.4) months, \( P < 0.001 \)) and PFD (with a median time of 19.0 (95% CI: 4.3–33.7) vs 5.0 (95% CI: 3.0–7.0) months, \( P < 0.001 \)), compared with those with a high level (Fig. 1C and D). The predictive value of a low BRCA2 level for platinum sensitivity was 88.0%, and that of a high level for resistivity was 55.3% (\( P = 0.006 \)). These data demonstrated that a low expression level of BRCA2 in cancer tissues indicated a better response to platinum.

**Silencing BRCA2 suppressed the expression of BRCA2 and RAD51 proteins, and DNA repair**

The basal expression level of BRCA2 in 5 cell lines was assayed to determine appropriate cell lines for gene-silencing trials. Western blot demonstrated a higher level in ES-2, CAOV-3 and SKOV3 cell lines, and therefore ES-2 and CAOV-3 (4.7 and 5.7 times higher than that in COC1, respectively) were selected (Fig. 2A and B).

The level of BRCA2 protein was decreased in shBRCA2-infected CAOV-3 or ES-2 cells (\( P = 0.003 \), \( P = 0.034 \)). CDDP did not affect the level of BRCA2 protein, but increased the level of RAD51 protein in both cell lines (\( P = 0.011 \), \( P = 0.012 \)); this inductive effect was suppressed after shBRCA2 transfection (\( P = 0.004 \), \( P = 0.030 \)) (Fig. 2C, D, E, F, G and H).

The neutral comet assay was performed to detect DSB. The percentage of comet-formed cells decreased gradually after CDDP removal, indicating repair. The value at 2–8h in NC-transfected cells was less than that in shBRCA2-transfected cells (\( P < 0.001 \) in CAOV-3; \( P = 0.001 \) in ES-2). 80% DSB were repaired in NC-transfected cells after 8h, but only 50% DSB were repaired in shBRCA2-transfected cells (Fig. 2J, K and L).

CDDP induced the formation of RAD51 foci, which was decreased after shBRCA2 transfection (\( P = 0.007 \) in CAOV-3; \( P = 0.004 \) in ES-2); in NC- and shBRCA2-transfected cells, values were 46.3 ± 6.5 vs 26.3 ± 1.5 in CAOV-3 cells, and 54.3 ± 1.7 vs 27.7 ± 2.5 in ES-2 cells, respectively (Fig. 2M, N, O and P). These data indicated that silencing BRCA2 inhibited the expression of BRCA2 and RAD51 proteins, and decreased DNA repair.

**Silencing BRCA2 enhanced the action of CDDP in vitro**

CDDP treatment resulted in a less survival percentage in shBRCA2-transfected cells in comparison with NC-transfected cells (\( P < 0.001 \) in CAOV-3; \( P < 0.001 \) in ES-2), with IC\(_{50}\) values of 1.28 vs 2.97 \( \mu \)M in CAOV-3 cells, and 1.37 vs 2.63 \( \mu \)M in ES-2 cells, respectively (Fig. 3A and B).

shBRCA2 transfection did not impact on colony formation in both cell lines. Clone inhibition due to CDDP was enhanced after silencing BRCA2, decreasing the clone number (37.7 ± 2.5 vs 8.0 ± 2.0 in CAOV-3, \( P = 0.010 \); 34.0 ± 7.9 vs 8.3 ± 1.5 in ES-2, \( P = 0.001 \)) (Fig. 3C, D, E and F).
These data indicated that silencing \(\text{BRCA2}\) enhanced the action of \(\text{CDDP}\).

**Silencing \(\text{BRCA2}\) enhanced \(\text{CDDP}\) against \(\text{CAOV-3}\) tumors in vivo**

Tumor volume and mass in group \(\text{shBRCA2} + \text{CDDP}\) were less than those in group \(\text{NC} + \text{CDDP}\), with volumes of \(327.7 \pm 68.4\) and \(125.7 \pm 21.4\) mm\(^3\), and masses of \(0.42 \pm 0.05\) and \(0.24 \pm 0.03\) g, in groups \(\text{NC} + \text{CDDP}\) and \(\text{shBRCA2} + \text{CDDP}\), respectively \((P<0.001, P<0.001)\) (Fig. 4A, B and C).

A much lower level of \(\text{BRCA2}\) protein was detected in tumors originated from \(\text{shBRCA2}\)-transfected cells \((3.40 \pm 0.55\) vs \(1.00 \pm 0.00, P<0.001)\), demonstrating effective inhibition. \(\text{CDDP}\) treatment improved the level of \(\text{RAD51}\) protein in tumors formed from both \(\text{NC}\)- and \(\text{shBRCA2}\)-transfected cells, and this inductive effect was suppressed after \(\text{shBRCA2}\) transfection, with scores of \(1.40 \pm 0.55, 4.00 \pm 0.00, 1.00 \pm 0.00\) and \(1.60 \pm 0.55\) in groups \(\text{NC}, \text{NC} + \text{CDDP}, \text{shBRCA2}\) and \(\text{shBRCA2} + \text{CDDP}\), respectively \((P<0.001)\) (Fig. 4D and E). These data showed that silencing \(\text{BRCA2}\) improved the anticancer effect of \(\text{CDDP}\) in vivo.
Silencing BRCA2 enhanced CDDP-induced autophagy

CQ with a concentration of 9.69 μM caused no cytotoxicity (the percentage of dead cells was <10%), and was therefore used to inhibit autophagy. Basal autophagy was noted in both cell lines, and EBSS-induced autophagy can be blocked by CQ (Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article). CDDP caused autophagy in BRCA2-intact cells (P<0.001 in CAOV-3; P<0.001 in ES-2); silencing BRCA2 enhanced CDDP-induced autophagy, increasing the LC3-II level (P=0.004 in CAOV-3; P=0.008 in ES-2) (Fig. 5A and B).

More RFP+GFP+ and RFP+GFP− puncta were detected after CDDP exposure, and the number of RFP+GFP+ puncta was less than that of RFP+GFP− ones (P<0.001), confirming the autophagy flux. The number of RFP+GFP+ dots was remarkably increased after adding CQ (P<0.001), demonstrating a blockage of the fusion of autophagosomes and autolysomes (Fig. 5C and D). These findings indicated that silencing BRCA2 can enhance CDDP-induced autophagy.

Suppression of DNA repair due to silencing BRCA2 was further exacerbated by CQ

DNA repair was decreased in shBRCA2-transfected cells, which was exacerbated by CQ (P=0.003 in CAOV-3; P=0.043 in ES-2); percentages of comet-formed cells at 8 h were 20.3% and 34.3% in CAOV-3 cells, and 19.0% and 32.7% in ES-F cells, without and with CQ, respectively (Fig. 6A and B). CQ enhanced the decrease in RAD51 foci due to silencing BRCA2 (P<0.001 in CAOV-3; P=0.002 in ES-2); the number was 26.3±1.5 vs 13.3±1.5 in CAOV-3 cells, and 27.7±2.5 vs 12.3±2.5 in ES-2 cells, respectively (Fig. 6C and D). These data suggested that CQ can enhance the suppression of DNA repair due to silencing BRCA2.

CQ sensitized the action of CDDP in BRCA2-silenced cells

CDDP led to a higher percentage of dead cells in shBRCA2-transfected cells compared with NC-transfected cells. CQ enhanced CDDP against shBRCA2-transfected cells, resulting in the highest cell death percentage (P=0.002
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in CAOV-3; \( P = 0.001 \) in ES-2); values at 8\,\mu M CDDP were 53.6\% vs 69.9\% in CAOV-3 cells, and 58.3\% vs 68.5\% in ES-2 cells, without and with CQ, respectively. The enhancement was not detected in NC-transfected cells. Percentages of dead cells at 8\,\mu M CDDP were 34.6\% vs 39.0\% in CAOV-3 cells, and 38.4\% vs 41.2\% in ES-2 cells, without and with CQ, respectively (Fig. 6E and F).

CDDP induced apoptosis. The addition of CQ increased the percentage of apoptotic cells in shBRCA2-transfected cells, which did not occur in NC-transfected cells (\( P = 0.005 \) in CAOV-3; \( P = 0.003 \) in ES-2) (Fig. 6G, H and I). In NC-transfected cells, values at 8\,\mu M CDDP were 10.2\% vs 11.6\% in CAOV-3 cells and 12.0\% vs 12.1\% in ES-2 cells, without and with CQ, respectively; levels were increased to 21.6\% vs 36.6\%, and 23.3\% vs 36.4\% in shBRCA2-transfected cells. These data manifested that CQ enhanced cell death and apoptosis attributable to CDDP in BRCA2-silenced cells.

Silencing ATG7 enhanced CDDP against BRCA2-silenced CAOV-3 cells

BRCA2 and ATG7 proteins were downregulated after siRNA transfection (\( P < 0.001, P < 0.001 \)); silencing ATG7 inhibited CDDP-induced autophagy, decreasing the LC3-II level (\( P < 0.001 \)); ATG7 silence did not improve the percentage of dead cells in BRCA2-intact cells, but the value was increased in BRCA2-silenced cells (\( P = 0.001 \)).
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(Fig. 7). These were consistent with data of using CQ, confirming that CQ modulated the cells’ sensitivity via the autophagy pathway.

**CQ enhanced CDDP against BRCA2-silenced CAOV-3 tumors in vivo**

Trial 1 demonstrated that silencing BRCA2 enhanced the action of CDDP in vivo, and in vitro trials indicated that CQ can sensitize CDDP in BRCA2-silenced cells. Therefore, in vivo efficacy of CDDP combined with CQ on BRCA2-silenced tumors was validated. CQ alone did not inhibit tumor growth; CDDP inhibited tumors; the combination of CDDP and CQ led to the smallest tumors \( (P<0.001, P<0.001) \), with volumes of 293.6±35.7 and 83.6±28.0 mm³ in groups CDDP and CDDP+CQ, respectively (Fig. 8A, B, C and D). The pattern of LC3-II demonstrated intratumoral autophagy in group CDDP+CQ \( (P=0.041) \) (Fig. 8E). These data indicated that CQ enhanced the effect of CDDP against BRCA2-silenced tumors, where an increase in autophagy played an important role.

**Discussion**

The present data manifested that patients with a low level of BRCA2 protein in cancer tissues had longer PFS and PFD compared with those with a high BRCA2 level. The BRCA2 level did not correlate with pathological stage/grade and the burden of residual lesions; however, platinum-resistant cancer had a higher percentage of high BRCA2 level. These suggest that longer PFS/PFD result from better therapeutic responses, and that BRCA2 can be a prognostic factor in treatments of ovarian cancer. A higher predictive value was noted when using a low BRCA2 level to predict sensitivity. Therefore, a low BRCA2 level...
in cancer tissues can provide a better clinical reference, i.e., a low expression level commonly indicated platinum sensitivity but a high level may unnecessarily indicate resistance. The present verdict should be validated in a multicenter trial involving more cases. The mutation status of BRCA2 was commonly considered the determinant of therapeutic outcome (De Picciotto et al. 2016, Milne & Antoniou 2016); thus, association of the BRCA2 mutation
status with the expression level should be investigated in following trials.

Platinum agents induced DSB, and cell death resulted from irreparable DSB. DSB can be repaired by HR or non-homologous end joining, resulting in cell survival. BRCA2 was the pivotal molecule in HR, and RDA51 was the downstream effector of BRCA2 (Dungl et al. 2015, Katsuki & Takata 2016). The present data demonstrated that CDDP induced an increase in the RAD51 protein level in vitro and in vivo (i.e., the signal of DNA damages will initiate repair), which was suppressed in silencing BRCA2. These indicated that HR was the major mode of DSB repair in ovarian cancer. Silencing BRCA2 enhanced the efficacy of CDDP, where a decrease in RAD51 played an important part. A reduction of RAD51 decreased the HR capacity, thereby exacerbating DSB due to CDDP. Consequently, a higher percentage of comet-formed cells was detected, which caused a less colony number and a higher percentage of dead cells. The apoptosis percentage was increased in BRCA2-silenced cells, showing that apoptosis was involved in cell death attributable to DSB. A deeper gap between cell death and apoptosis fractions was noted in both cell lines, suggesting that other modes may also contribute to cell death in BRCA2-silenced cells.

As to the role of autophagy in chemotherapy, data released were controversial (Levine & Kroemer 2008). The present findings showed that CDDP caused autophagy in BRCA2-intact cells, accompanied by upregulation of the RAD51 protein. These implied that autophagy can favor HR, thereby alleviating the cytotoxicity of CDDP. Zhang et al. (2012) manifested that inhibition of autophagy due to knockdown of nucleus accumbens-1 enhanced the action of CDDP in human ovarian cancer cells, where apoptosis was the cell death pathway. However, the present data demonstrated that silencing BRCA2 enhanced CDDP-induced cell killing but with an increase in autophagy, suggesting that autophagy may relate to cell death. Autophagy was cytotoxic when serious insults occurred (Arun et al. 2015, Yang et al. 2016). Therefore, silencing BRCA2 may have a sensitization effect, which decreased the cell death threshold of DSB leading to cell deactivation; those damages can be commonly repaired in BRCA2-intact cells, resulting in cell survival.

How BRCA2 modulated autophagy remained unclear. Knockdown of BRCA2 enhanced cell death induced by
AZ2281 (a PARP inhibitor) in breast cancer cells, where autophagy was involved in cell death via eliminating mitochondria (Arun et al. 2015). DNA damages induced autophagy via the ATM- or PARP1-dependent pathway (Eliopoulos et al. 2016). Intracellular targets of CDDP included both nuclear (i.e., DNA breaks) and cytoplasmic (i.e., production of reactive oxygen species damaging organelles including mitochondria) sites (Brozovic et al. 2010). Silencing BRCA2 impacted on autophagy via modulating the cells’ response to CDDP. Silencing BRCA2 increased the amount of DSB via decreasing HR. The damage signal was transmitted to mitochondria via PARP1, ATM, p53, SIRT1, AMPK and PGC1α, causing the dysfunction of mitochondria (Fang et al. 2016). Reactive oxygen species induced by CDDP can directly attack mitochondria. These effects exacerbated damages to mitochondria, arousing autophagy and/or apoptosis. Silencing BRCA2 can also enhance mitochondrial damages directly due to CDDP.

Interestingly, the autophagy blocker CQ enhanced the anticancer activity of CDDP in BRCA2-silenced cells, but this did not occur in BRCA2-intact cells. This was inconsistent with the aforementioned result that autophagy may involve in cell death. The disagreement may relate to intratumoral heterogeneity of ovarian cancer: a cell line consisted of multiple subpopulations with different biological properties (Konstantinopoulos & Matulonis 2013). Silencing BRCA2 produced a sensitization effect in specific subpopulations, decreasing the DSB threshold to divert autophagy to being cytotoxic. The threshold was not altered in other subpopulations; thus, CQ inhibited DSB repair to enhance CDDP. These effects resulted in synergy, thereby improving the action of CDDP in BRCA2-silenced cells. This deduction can be supported with data in the comet and RAD51 foci assays: the use of CQ increased comets and decreased foci. A remarkable increase in apoptotic cells indicated that apoptosis played an important part in cell death after blocking autophagy. Similar results were observed in chemoresistant ovarian cancer cells COC1/DDP when using ultrasound to reverse resistance: ultrasonic sensitization made certain cells undergo necrosis, although CDDP commonly deactivated cells via apoptosis; concurrent occurrence of apoptosis and necrosis improved the action of CDDP.
(Yu et al. 2015). Enhancement of CDDP was detected in BRCA2-silenced cells after knockdown of Atg7, confirming that CQ-enhanced CDDP was realized via modulating autophagy.

In vivo trials demonstrated that a combination of BRCA2-silenced and CQ improved the anticancer efficacy of CDDP. Therefore, knockdown of BRCA2 combined with CQ may be a strategy to treat resistant ovarian cancers, and CQ can be employed to enhance CDDP against cancers with a low BRCA2 level. A higher level of LC3-II in tumor tissues indicated that autophagy was involved in the anticancer effect. Efficacy of this modality should be validated in an orthotopic cancer model, considering limitations of an ectopic model (Zhang et al. 2016, 2017).

In conclusion, the present data demonstrated that ovarian cancer with a low BRCA2 level had better platinum response and longer PFS/PFD; thus, the BRCA2 level in cancer tissues can predict the therapeutic outcome. Autophagy favored DSB repair in BRCA2-intact cells. Knockdown of BRCA2 can enhance the cytotoxicity of CDDP and can improve autophagy. CQ enhanced CDDP against BRCA2-silenced cells, suggesting that modulation of autophagy can be a chemosensitizer for ovarian cancer with a low BRCA2 level.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0261.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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