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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

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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,

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: $\leq 10\%$ positive cells; 2: 11–50% positive cells; 3: 51–90% positive cells; 4: $\geq 91\%$ 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'-AACAACAATTACGAACCAAAC-3') for BRCA2 and (5'-UUCUCCGAACGUGUCACGUTT-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 $\times$ 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 μ M) and/or CQ (9.69 μ M), 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 $\times$ 10³ cells per well) and exposed to CDDP (0–32 μ M). 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 μ M, and that of CQ was 9.69 μ M. 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 μ M). 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 μ M) and/or CQ (9.69 μ M) 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 μ M) and/or CQ (9.69 μ M). 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 μ M) and/or CQ (9.69 μ M). 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 μ M) and/or CQ (9.69 μ M). 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'-CAGAAGGAGUCACAGCUCUCCUUA-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 μ M) 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. 5.0×10^6 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 mg/kg) was injected i.p. every 4 days, and each animal received it 6 times. CQ (50 mg/kg) was injected i.p. every day for 24 days. Other mice received normal saline. Tumor volume was calibrated every 4 days ($(\text{length} \times \text{width}^2)/2$) (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:

Table 1 Clinicopathological characteristics and their associations with the expression level of BRCA2 in cancer tissues.

Clinicopathological variables	Case no.	BRCA2 expression level		P value
		Low (n=25)	High (n=38)	
Age (year)				
<50	26	8 (30.8%)	18 (69.2%)	0.225
≥50	37	17 (45.9%)	20 (54.1%)	
Histological type				
Serous	54	22 (40.7%)	32 (59.3%)	0.958
Clear cell	9	3 (33.3%)	6 (66.7%)	
Pathological grade				
2	20	11 (55.0%)	9 (45.0%)	0.090
3	43	14 (32.6%)	29 (67.4%)	
FIGO stage				
I/II	20	10 (50.0%)	10 (50.0%)	0.254
III/IV	43	15 (34.9%)	28 (65.1%)	
Residual tumor				
≤1 cm	51	20 (39.2%)	31 (60.8%)	1.000
>1 cm	12	5 (41.7%)	7 (58.3%)	
Chemotherapy regimen				
Platinum+paclitaxel	56	23 (41.1%)	33 (58.9%)	0.820
Platinum+cyclophosphamide	7	2 (28.6%)	5 (71.4%)	
Cycle		6.0 (1–12)	5.8 (2–12)	0.671
Platinum response				
Sensitive	39	22 (56.4%)	17 (43.6%)	0.001
Resistant	24	3 (12.5%)	21 (87.5%)	

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 2–8 h 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 8 h, but only 50% DSB were repaired in shBRCA2-transfected cells (Fig. 2I, J, 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 ± 7.1 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 μM in CAOV-3 cells, and 1.37 vs 2.63 μM 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).

Figure 1

Correlation between the BRCA2 expression level in cancer tissues and the clinical outcome in ovarian cancer patients. Representative immunohistochemical images of BRCA2 expression in cancer tissues (A); the scale was 100 μm . Scores of the BRCA2 level in platinum-resistant and -sensitive cancer (B): resistant cancer had a higher BRCA2 level. A Kaplan-Meier analysis of progression-free survival (C) and platinum-free duration (D): cases with a low BRCA2 level in cancer tissues had longer progression-free survival and platinum-free duration. $\blacktriangle P<0.05$.

Figure 2

Effects of silencing *BRCA2* on the expression of *RAD51* and on DNA repair. Basal level of the *BRCA2* protein in 5 cell lines (A and B): a higher level was noted in ES-2 and CAOV-3, and therefore these two cell lines were employed for silencing trials. Silencing *BRCA2* decreased the level of *BRCA2* and *RAD51* proteins in CAOV-3 (C, D and E) and ES-2 (F, G and H) cells: CDDP induced upregulation of *RAD51*, which was suppressed after silencing *BRCA2*. DSB detected with the neutral comet assay after CDDP exposure (4 μ M) in CAOV-3 (I and J) and ES-2 (K and L) cells: silencing *BRCA2* decreased DNA repair, increasing the percentage of comet-formed cells. *RAD51* foci after CDDP exposure (8 μ M) in CAOV-3 (M and N) and ES-2 (O and P) cells: CDDP induced the formation of *RAD51* foci, which was suppressed in *BRCA2*-silenced cells. Images were captured under 200 $\times$ field. Values were mean $\pm$ standard deviation for 3 independent experiments. $\blacktriangle P < 0.05$.

These data indicated that silencing *BRCA2* enhanced the action of CDDP.

Silencing *BRCA2* enhanced CDDP against CAOV-3 tumors *in vivo*

Tumor volume and mass in group shBRCA2+CDDP were less than those in group NC+CDDP, with volumes of 327.7 ± 68.4 and 125.7 ± 21.4 mm³, and masses of 0.42 ± 0.05 and 0.24 ± 0.03 g, in groups NC+CDDP and shBRCA2+CDDP, respectively ($P < 0.001$, $P < 0.001$) (Fig. 4A, B and C).

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

Figure 3

Silencing *BRCA2* enhanced the action of CDDP *in vitro*. Survival percentages after CDDP exposure in CAOV-3 (A) and ES-2 (B) cells: a lower survival fraction occurred in *BRCA2*-silenced cells. Clone-forming assay in CAOV-3 (C and D) and ES-2 (E and F) cells: less colony number occurred after silencing *BRCA2*. Values were mean $\pm$ standard deviation for 3 independent experiments. $\blacktriangle P < 0.05$.

Silencing *BRCA2* enhanced CDDP-induced autophagy

CQ with a concentration of 9.69 μ M 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 autolysosomes (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-2 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 ($P = 0.002$

Figure 4

Silencing *BRCA2* enhanced the anticancer effect of CDDP on transplanted CAOV-3 tumors in mice ($n=5$). Gross morphology of tumors (A). Tumor volume and mass (B and C): CDDP treatment led to the smallest size in *BRCA2*-silenced tumors. *BRCA2* and *RAD51* proteins in tumor tissues (D and E): *RAD51* was upregulated after CDDP treatment, which was suppressed after silencing *BRCA2*; the scale was 50 μm. Values were mean ± standard deviation. ▲ $P<0.05$.

in CAOV-3; $P=0.001$ in ES-2); values at 8 μM 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 μM 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 μM 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 ($P=0.001$)

Figure 5

Silencing *BRCA2* enhanced CDDP-induced autophagy. Autophagy detected by the LC3-II accumulation in CAOV-3 (A) and ES-2 (B) cells: a higher level was noted in *BRCA2*-silenced cells. The autophagy flux in CAOV-3 cells observed using the tandem mRFP-GFP fluorescence assay (C and D): both yellow (RFP+GFP+) and red (RFP+GFP-) dots were increased after CDDP exposure, and the number of yellow dots was less than that of red dots, demonstrating the occurrence of autophagy flux; a remarkable increase in yellow dots occurred when using CQ, demonstrating the blockage of fusion of autophagosomes and autolysosomes. Images were captured under 600× field. Values were mean ± standard deviation for 3 independent experiments. ▲*P* < 0.05.

(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

Figure 6

CQ enhanced the action of CDDP in *BRCA2*-silenced cells *in vitro*. DNA break detected with the neutral comet assay in CAOV-3 (A) and ES-2 (B) cells: DNA repair was suppressed by silencing *BRCA2*, which was further exacerbated when adding CQ. RAD51 foci in CAOV-3 (C) and ES-2 (D) cells: the foci number was decreased in *BRCA2*-silenced cells, and adding CQ led to less foci. Percentages of dead cells in CAOV-3 (E) and ES-2 (F) cells: silencing *BRCA2* increased the cell death fraction, with the highest value noted when adding CQ. Apoptosis in CAOV-3 (G and H) and ES-2 (G and I) cells: CDDP-induced apoptosis was enhanced after silencing *BRCA2*, and CQ further increased the percentage of apoptotic cells. Values were mean $\pm$ standard deviation for 3 independent experiments. $\Delta P < 0.05$.

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

Figure 7 CAOV-3 cells' response to CDDP after inhibiting ATG7. BRCA2 and ATG7 proteins (A): levels were decreased after transferring siRNA. Autophagy detected by the LC3 assay (B): silencing *BRCA2* enhanced CDDP-induced autophagy, increasing the LC3-II level; this was inhibited after knockdown of ATG7. Cell death percentages after CDDP exposure (4 μ M) (C): knockdown of ATG7 enhanced CDDP in *BRCA2*-silenced cells. Values were mean $\pm$ standard deviation for 3 independent experiments. $\blacktriangle P < 0.05$.

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 *RAD51* 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 the 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

Figure 8

CQ enhanced the anticancer effect of CDDP against *BRCA2*-silenced CAOV-3 tumors in mice ($n=5$). Gross morphology of tumors (A). Tumor volume (B) and mass (C): CDDP treatment reduced the tumor, with the smallest tumor when combining CDDP and CQ. Pathological examinations of tumor xenografts (D); the scale was 50 μ m. Autophagy detected by LC3-II accumulation in tumors (E): a higher level was detected in group CDDP+CQ. Values were mean $\pm$ standard deviation. $\blacktriangle P < 0.05$.

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 PGC1 α , 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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