Silencing of BAG3 promotes the sensitivity of ovarian cancer cells to cisplatin via inhibition of autophagy

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Abstract. Ovarian cancer is the most lethal disease among all gynecological malignancies. Interval cytoreductive surgery and cisplatin-based chemotherapy are the recommended therapeutic strategies. However, acquired resistance to cisplatin remains a big challenge for the overall survival and prognosis in ovarian cancer. Complicated molecular mechanisms are involved in the process. At present, increasing evidence indicates that autophagy plays an important role in the prosurvival and resistance against chemotherapy. In the present study, as a novel autophagy regulator, BCL2-associated athanogene 3 (BAG3) was investigated to study its role in cisplatin sensitivity in epithelial ovarian cancer. However, whether BAG3 participates in cisplatin sensitivity by inducing autophagy and the underlying mechanism in ovarian cancer cells remain to be clarified. Through the use of quantitative real-time PCR, western blot analysis, CCK-8 and immunofluorescence assays our data revealed that cisplatin-induced autophagy protected ovarian cancer cells from the toxicity of the drug and that this process was regulated by BAG3. Silencing of BAG3 increased cisplatin-induced apoptosis. The results also revealed BAG3 as a potential therapeutic target which enhanced the efficacy of cisplatin in ovarian cancer.

Introduction

Ovarian cancer is the most lethal disease among all gynecological malignancies. Although there have been improvements in surgery and chemotherapy in the last several decades, the 5-year survival rate is only 44% (1). More than 80% of patients with advanced ovarian cancer relapse two or three years after the primary therapy, thus, the recurrence rate exceeds 75% (2). The subsequent effectiveness of chemotherapy is limited by the progressive development of drug resistance, and the risk of cumulative toxicity (3). Chemoresistance remains a major limitation for treatment failure and deaths associated with ovarian cancer.

Cisplatin, which is recommended as a first-line chemotherapeutic agent, has dominated the drug therapy of ovarian cancer and other gynecological malignancies during the past three decades since it induces lethal DNA damage (4,5). Its molecular mechanism is complicated and involves a defective DNA repair system, enhanced drug clearance and detoxification, and imbalance between cellular survival and the apoptosis pathway (6,7). However, acquired resistance to cisplatin remains a major limitation of its success clinically in ovarian cancer. Increasing evidence indicates that autophagy plays an important role in the prosurvival and resistance against chemotherapy, however, the underlying mechanisms have not been fully elucidated (8-11).

Autophagy is an evolutionarily conserved process by which cytoplasmic cargo sequestered inside double-membrane vesicles is delivered to the lysosome for degradation (12). It has cytoprotective functions and may also lead to one of the forms of cell death. Beclin-1 is an important autophagy-related gene. The initial discovery of Beclin-1 as a binding partner of B-cell lymphoma-2 (BCL-2) suggests that the Beclin-1/BCL-2 complex may serve as a regulatory complex between autophagy and apoptosis. In addition, a recent study demonstrated that the interaction of BCL-2 with Beclin-1 can inhibit autophagy (13). Another study demonstrated that BCL-2 must detach from Beclin-1 to mediate the induction of autophagy, suggesting the possible involvement of other proteins that physically and/or functionally communicate with BCL-2 in this process (14). BAG3 is a member of the BAG family, which has received increasing attention.
The human BAG3 protein contains a C-terminal heat shock protein 70 (HSP70)-binding BAG domain, which inhibits its chaperone activity (15), along with a WW domain, a proline-rich (PXXP) domain and two conserved isoleucine-proline-valin (IPV) motifs (16). It not only interacts with BCL-2 to inhibit apoptosis (5,17), but also participates in autophagy regulation via interaction with the heat-shock protein family of proteins and heat shock protein family B (small) member 8 (HSPB8) to facilitate the removal of misfolded and degraded proteins (18). In addition, the BAG3-HSP protein-protein interaction is increasingly being recognized as a therapeutic target in the treatment of cancer (19). Recent studies have revealed an association between autophagy and drug sensitivity. Chemotherapy agents, including cisplatin, lead to an autophagic response (20), particularly in apoptosis-defective cells (21), but the survival-supporting and death-promoting roles of autophagy warrant further elucidation. Recent evidence supports that autophagy serves as a potential mechanism for drug resistance since autophagy is an adaptive response to radiation therapy and conventional DNA-damaging chemotherapy, which can protect the cancer cells (22). In ovarian cancer, researchers have endeavored to study the functions of BAG3. However, clarification on whether BAG3 participates in cisplatin sensitivity by inducing autophagy and the underlying mechanisms in ovarian cancer cells have yet to be elucidated.

In the present study, we found that cisplatin exposure induced BAG3 upregulation and autophagic response, consequently conferring cytoprotection from cisplatin. Furthermore, silencing of BAG3 modulated autophagy and altered cisplatin sensitivity in ovarian cancer cells. Thus, our data revealed that BAG3 may play an important role in cisplatin sensitivity through the induction of autophagy. BAG3 may become a potential predictor or target in ovarian cancer therapeutics.

Materials and methods

Cell lines and reagents. The human ovarian cancer cell lines SKOV3 and HO8910 and the normal human ovarian surface epithelial (HOSE) cell line were obtained from The Cell Bank, Chinese Academy of Sciences (Beijing, China). These lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml streptomycin/penicillin at 37°C in a humidified atmosphere containing 95% air and 5% CO2. Antibodies against microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (P62/SQSTM1), caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), cleaved PARP were obtained from the Cell Signaling Technology (Danvers, MA, USA). Antibodies against GAPDH and BAG3 were purchased from Protein Technology (Chicago, IL, USA). Cisplatin, chloroquine dihydrophosphate (CQ) and the secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the cell culture media were purchased from Gibco (Carlsbad, CA, USA).

cDNA constructs, shRNA and transfection. Transfections with the GFP-tagged LC3 cDNA expression construct and BAG3 shRNA were obtained from GenePharma (Shanghai, China) and were performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Cell viability. SKOV3 (1x10⁴) and HO8910 (1x10⁴) cells were seeded into 96-well plates. After adhesion, the cells were pretreated with or without 10 µM of CQ for 2 h, and then treated with cisplatin, CQ or their combination for the indicated concentrations and time-points. Dimethyl sulfoxide (DMSO) was used as the untreated control. In brief, 10 µl of Cell Counting Kit-8 reagent (CCK-8) (Solarbio, Life Sciences, Co., Ltd., Beijing, China) was added to each well and cultured at 37°C for ~1 h. Then, a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to assess the absorbance at 450 nm.

GFP-LC3 plasmid transfection and autophagic flux assay. Cells were transfected with a GFP-LC3 expression construct at ~70-80% confluence using Lipofectamine 3000. Twenty-four hours later, the cells were pretreated with or without CQ. Then, after the designated treatment, the cells were fixed with 3.7% formaldehyde for 15 min and washed with cold phosphate-buffered saline (PBS). Subsequently the cell nuclei were stained with 1 ml of 10 µg/ml 4',6-diamidino-2-phenyindole (DAPI) (Beyotime Biotechnology, Shanghai, China) for 5 min at room temperature in the dark. Then, the distribution and fluorescence of GFP-LC3 were visualized by confocal microscopy. The number of GFP-LC3 puncta were manually counted. For each group, 20 cells in random visual fields were selected for counting.

Transmission electron microscopy (TEM) assay. After the designated treatment, the cells were washed with 0.1 cacodylate buffer (pH 7.4) and fixed with 2% glutaraldehyde in PBS for 24 h at 4°C. Subsequently, the rest of the procedure was conducted using the standard protocol. Zeiss Transmission Electron Microscope was used to examine the sample sections.

Apoptosis assay. The cells were harvested in 0.25% trypsin and washed twice with PBS. After centrifugation, the cells were stained using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD, Shanghai, USA). Analysis of the apoptotic cells was performed on a flow cytometer (Cytomics™ FC 500; Beckman Coulter, Miami, FL, USA).

Quantitative real-time PCR. Total RNA was extracted from cells using TRIzol (Invitrogen). Reverse transcription was carried out using PrimeScript™ RT Master Mix (Takara Bio, Inc., Shiga, Japan). The quantitative real-time PCR (qRT-PCR) experiments were performed using SYBR-Green reagents (Takara Bio Inc.) on an Agilent Mx3005P system (Agilent Technologies, Inc., Santa Clara, CA, USA). Each sample was run in triplicate. Human BAG3 forward primer, 5’-CTCCATTCCGGTGATACACGA-3’ and reverse primer, 5’-TGGTGGGTTCTGGTACTCCC-3’; GAPDH forward primer, 5’-GAAGGTAAGGTGGAGTCGTC-3’ and reverse primer, 5’-GAAGATGGTGATGGGATTTTC-3’. The expression was quantified using the 2−ΔΔCt method.

Immunofluorescence assay. The cells were cultured on cover-slips, fixed with 3.7% paraformaldehyde in PBS for 30 min
and permeabilized with 0.1% Triton X-100 in PBS for 20 min. After blocking with 10% FBS, the cells were incubated with primary antibodies for 2 h, washed three times with PBS, and incubated with secondary antibodies for 1 h at room temperature in the dark. Nuclei were visualized using DAPI (Beyotime Biotechnology). Samples were observed using a Leica TCS SP5 laser confocal microscope (TCS SP5; Leica, Mannheim, Germany).

Western blot analysis. After the designated treatment, the cells were washed with ice-cold PBS and lysed in RIPA buffer and then, the protein concentrations were determined using the BCA protein assay (Beyotime Biotechnology). The protein lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% skimmed milk (BD), and then incubated with primary antibodies (1:1,000) overnight at 4°C, and subsequently with HRP-conjugated secondary antibodies (1:2,000) for 1 h. The membranes were then visualized with ECL reagents.

Statistical analysis. All data and results were confirmed in at least three independent experiments. The data are expressed as the mean values ± SD. Two-sided student's t-tests or analysis of variance (ANOVA) tests were used to assess for statistically significant differences between two groups or among more groups, respectively. P<0.05 was defined as statistical significance.

Results

Cisplatin induces autophagy that protects ovarian cancer cells from apoptosis. We used two human ovarian cancer cell lines, SKOV3 and HO8910, to illustrate whether cisplatin induces autophagy. First, we detected the activity of autophagy in ovarian cancer cells exposed to cisplatin. The treatment of the ovarian cancer cell lines with cisplatin activated autophagy. Western blot analyses revealed that cisplatin treatment led to an increase in the level of LC3-II and a decrease in the quantity of P62, two selective markers of autophagy, in both SKOV3 and HO8910 cells (Fig. 1A). The effect of cisplatin on autophagy was further confirmed by a GFP-LC3 puncta formation assay. Upon transfection of the GFP-LC3 plasmid into both cell lines, we observed the number of puncta fluorescent dots after 24 h of cisplatin treatment, which reflected the conversion from cytoplasmic LC3-I to the phosphatidylethanolamine-conjugated form LC3-II (Fig. 1B). The same result was demonstrated with ultrastructural analysis of characteristic autophagosomes using TEM (Fig. 1C).
To determine whether cisplatin-induced autophagy played a protective role in the drug treatment, we used an autophagy inhibitor, chloroquine (CQ). We treated SKOV3 and HO8910 cells with cisplatin alone or co-treatment with cisplatin and CQ. Western blot analysis indicated that CQ efficiently blocked activation of autophagy, increasing LC3-II and P62 expression level (Fig. 2A). Then, we examined the cell viability using CCK-8 assay. Compared to the co-treatment group, we found that the group treated with cisplatin only was less sensitive to cisplatin in both cell lines (Fig. 2B). Apoptosis assays revealed that co-treatment with CQ enhanced the proportion of SKOV3 and HO8910 apoptotic cells (Fig. 2C). The aforementioned results demonstrated that suppression of autophagy induced an increase of cytotoxicity and apoptosis in SKOV3 and HO8910 cells.

Cisplatin promotes the expression of BAG3 in ovarian cancer cells. BAG3 is a novel autophagy regulator, thus, we wanted to study the potential role of BAG3 in the regulation of cisplatin-induced autophagy. We analyzed BAG3 protein expression and location in ovarian cancer cells exposed to cisplatin for indicated periods of time. Western blot analysis revealed that the endogenous expression level of BAG3 in the HOSE cell line was lower compared to that in the SKOV3 and HO8910 cells without drug treatment (Fig. 3A). Then, we inspected the effects of cisplatin on the expression of BAG3. We treated both cell lines with various concentrations of cisplatin for 24 h. Cisplatin markedly enhanced the level of BAG3 in both cell lines, however this effect was not observed to be in a dose-dependent manner (Fig. 3B). Consistent with the western blotting results, real-time PCR revealed that the mRNA level of BAG3 was increased after treatment with cisplatin for 24 h (Fig. 3C). Meanwhile, confocal microscopic analysis revealed that the green fluorescence of BAG3 was mainly accumulated in the cytoplasm, with small amounts in the nucleus (Fig. 3D) and quantification of the fluorescence intensity was significantly increased in both cell lines after treatment with 20 µM cisplatin (Fig. 3E).

BAG3 negatively regulates cisplatin-induced apoptosis in ovarian cancer cells. To clarify the role of BAG3 in the regulation of sensitivity followed by chemotherapy, we transfected shRNA targeting BAG3 into SKOV3 and HO8910 cells. A significant decrease of the protein level of BAG3 in both cell lines was observed (Fig. 4A). At the end of the 24 h treatment with cisplatin, western blot analyses revealed that knockdown of BAG3 enhanced apoptosis-related protein cleaved-PARP and cleaved-caspase-3 expression level. Cisplatin induced...
Figure 3. Cisplatin promotes the expression of BAG3 in ovarian cancer cells. (A) HOSE, SKOV3 and HO8910 cell lysates were subjected to western blot analysis of the endogenous level of BAG3 and GAPDH (as a loading control). (B) SKOV3 and HO8910 cells were treated with different doses of cisplatin, and whole cell lysates were subjected to western blot analysis for BAG3 and GAPDH expression (as a loading control). (C) SKOV3 and HO8910 cells were treated with or without cisplatin (20 µM) for 24 h and then the mRNA level of BAG3 was assessed by quantitative real-time PCR; *P<0.01 compared with the control group. (D) SKOV3 and HO8910 were treated with or without 20 µM of cisplatin for 24 h, and then the expression level of BAG3 was observed by confocal microscopy. Scale bar, 50 µm. (E) Quantification of the relative fluorescence intensity. Each point or bar represents the mean ± SD of triplicate determinations; **P<0.01. BAG3, BCL2-associated athanogene 3; HOSE, human ovarian surface epithelial.

Figure 4. Inhibition of BAG3 enhances cisplatin-induced apoptosis in ovarian cancer cells. SKOV3 and HO8910 cells were transfected with a control shRNA or a shRNA targeting BAG3, followed by treatment with 20 µM of cisplatin or DMSO for 24 h. (A) At the end of treatment the cell lysates were subjected to western blot analysis for PARP, cleaved-PARP, caspase-3, cleaved-caspase-3, BAG3 and GAPDH expression (as a loading control). (B) Apoptosis was analyzed by flow cytometric analysis with Annexin V/PI staining. The results displayed are the representatives of three independent experiments, and the bars represent the mean ± SD; *P<0.05. BAG3, BCL2-associated athanogene 3.
apoptotic cell death to a great degree after BAG3 knockdown, as revealed by Annexin V and PI staining (Fig. 4B).

Silencing of BAG3 modulates cisplatin sensitivity in ovarian cancer cells via inhibition of autophagy. To determine the connection between BAG3 and cisplatin-induced autophagy in ovarian cancer cells we transfected BAG3 shRNA into SKOV3 and HO8910 cells. At the end of the 24 h treatment with cisplatin (20 μM), western blot analyses revealed that knockdown of BAG3 prevented the formation of LC3-II and the degradation of P62 (Fig. 5A). Then, we assessed the cell viability by CCK-8 assay. It was demonstrated that knockdown of BAG3 expression in these cells significantly enhanced cell sensitivity to cisplatin compared to that in the control group (Fig. 5B). Meanwhile, compared with the control group, SKOV3 and HO8910 cells transfected with BAG3 shRNA exhibited decreased formation of GFP-LC3 puncta fluorescent dots after cisplatin treatment (Fig. 5C). These data revealed a significant role for BAG3 in the regulation of cisplatin-induced autophagy in ovarian cancer cells.

Discussion

Cisplatin is the first-line chemotherapeutic drug used in ovarian cancer and part of the standard treatment for advanced epithelial ovarian cancer (23). However, acquired resistance to cisplatin in cancer cells remains a big challenge for overall survival. Several mechanisms contribute to drug resistance, such as DNA repair mechanisms, drug export transporters and resistance to apoptosis (24). Previous evidence indicated that autophagy plays a prosurvival and resistance role against chemotherapy. It is important to explore key molecules or mechanisms for promoting chemosensitivity in cancers, but it still remains to be elucidated in ovarian cancer.

Previously autophagic cell death, which is called type II programmed cell death, was considered as an alternative form of cell death due to excessive self-digestion in the absence of apoptosis. However, there is evidence that supports the idea that autophagy functions primarily as a cell survival mechanism, particularly when cells are subjected to various stresses associated with cell death (25). In addition, preceding research has shown that various anticancer drugs can induce an autophagic response in cancer cells, which is regarded as a chemoresistant mechanism, and selective inhibition of autophagy regulators may possibly improve the chemotherapeutic response (26). In the present study, we demonstrated that prevention of autophagy promoted cisplatin-induced cell death in SKOV3 and HO8910 cells. A recent study indicated that autophagy plays a protective role in cisplatin resistance (27). Our results demonstrated that cisplatin-induced autophagy protects ovarian cancer cells

Figure 5. Silencing of BAG3 modulates cisplatin sensitivity in ovarian cancer cells via inhibition of autophagy. SKOV3 and HO8910 cells were transfected with a control shRNA or a shRNA targeting BAG3, followed by treatment with 20 μM of cisplatin or DMSO for 24 h. (A) Whole cell lysates were subjected to western blot analysis for LC3, P62, BAG3 and GAPDH expression (as a loading control). (B) The cell viability was analyzed by CCK-8 assay. *P<0.01. (C) SKOV3 and HO8910 cells were co-transfected with either control or BAG3 shRNA and GFP-LC3 plasmid followed by treatment with cisplatin (20 μM) for an additional 24 h. Scale bar, 7.5 μm. Values are reported as the mean ± SD of three independent experiments; *P<0.05. BAG3, BCL2-associated athanogene 3.
from the cytotoxic effects of cisplatin. Collectively, these findings indicate that inhibition of autophagy contributes to the enhancement of cisplatin sensitivity in ovarian cancer cells. Recently, more and more studies have confirmed that activation of autophagy plays a role in chemoresistance in cancer cells, and downregulation of autophagy sensitizes cancer cells to therapeutic drugs. The underlying molecular mechanisms on how to regulate the sensitivity of ovarian cancer cells to cisplatin by BAG3, a novel regulator of autophagy, have yet to be well characterized. BAG3 is constitutively expressed in myocytes and cancer cells derived from myeloid leukemias, neuroblastomas, prostate carcinomas, ovary and breast cancers, glioblastomas and other tumors tissues (28,29). However, in other nontransformed cells (for example epithelial and retinal cells), BAG3 expression can be induced by a variety of stressors, such as heavy metals or HIV infection (30). Previous studies have demonstrated that BAG3 is overexpressed in several epithelial cancers, mainly adenocarcinomas (31). BAG3 dysfunctions are implicated in disorders including cancer, myopathies, and neurodegeneration. Evidence has revealed that the BAG3 protein exerts a relevant role in regulating the balance between cell life and death (32). The discovery of its role in selective autophagy and the description of BAG3-mediated selective macroautophagy as an adaptive mechanism to maintain cellular homeostasis, under stress as well as during aging, makes BAG3 a highly interesting target for future pharmacological interventions (33). In ovarian cancer, scholars have endeavored to study the functions of BAG3. High BAG3 levels in primary carcinomas were significantly related to poor overall survival in ovarian cancer (34). BAG3 increases the invasiveness of uterine corpus and ovarian carcinoma (35,36). Recently, there has been evidence that revealed that downregulation of BAG3 can enhance the efficacy of chemotherapeutic drugs paclitaxel in ovarian clear cell carcinoma cells (37). Induction of BAG3 expression attenuates apoptosis (38), whereas decreasing its expression promotes apoptosis in various human cell models (39). Consistent with other research, the present study confirmed that cisplatin promoted BAG3 expression in ovarian cancer cells, and silencing of BAG3 significantly increased cisplatin-induced apoptosis, as indicated by the increase in the amount of cleaved caspase-3 and PARP present and in the intensity of Annexin V/PI staining. In addition, BAG3 plays a role in the regulation of autophagy (40-44). Increasing evidence has confirmed that autophagy activation participates in chemoresistance in cancer cells, and downregulation of autophagy sensitizes cancer cells to therapeutic drugs (45). In the present study, we transfected BAG3 shRNA into SKOV3 and HO8910 cell lines, which led to a decrease of BAG3. The results revealed an increased sensitivity to cisplatin, a decrease in autophagy-related protein LC3-II, an increase in the expression level of P62 and the amount of GFP-LC3 puncta in the transfection group was lower than that in the control group. These data revealed that autophagy mediated by BAG3 may play a key role in cisplatin sensitivity. Though BAG3 plays a role in ovarian cancer chemotherapy sensitivity, the detailed molecular mechanism underlying the regulation of autophagy via BAG3 may be complex and warrants further investigation.

In conclusion, the present study identified BAG3 as a novel regulator of autophagy and demonstrated its involvement in the modulation of cisplatin sensitivity in ovarian cancer. However, as a regulator of autophagy, BAG3 also affects the apoptosis response. Silencing of BAG3 can enhance the sensitivity of ovarian cancer cells to cisplatin by modulating autophagy and apoptosis. Collectively, BAG3 may be a novel therapeutic target used for the prevention of chemotherapy resistance in cancer cells.

References


