shRNA-mediated AMBRA1 knockdown reduces the cisplatin-induced autophagy and sensitizes ovarian cancer cells to cisplatin

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ABSTRACT — Recent research has revealed a role for Ambra1, an autophagy-related gene-related (ATG) protein, in the autophagic pro-survival response, and Ambra1 has been shown to regulate Beclin1 and Beclin1-dependent autophagy in embryonic stem cells and cancer cells. However, whether Ambra1 plays an important role in the autophagy pathway in ovarian cancer cells is unknown. In this study, we hypothesized that Ambra1 is an important regulator of autophagy and apoptosis in ovarian cancer cells. We firstly confirmed autophagic activity in ovarian cancer OVCAR-3 cells which were treated with cisplatin by assessing endogenous microtubule-associated protein 1 light chain 3 (LC3) localization and the presence of autophagosomes and LC3 protein levels in OVCAR-3 cells. Cell apoptosis and viability were measured by annexin-V and PI staining and MTT assays. We then knocked down Ambra1 expression with transfection with the plasmid expressing the small hairpin RNA (shRNA) targeting AMBRA1, then re-evaluated autophagy in the OVCAR-3 cells subject to cisplatin treatment, and re-determined the sensitivity of OVCAR-3 cells to cisplatin. Results demonstrated that cisplatin treatment induced autophagy in OVCAR-3 cells in association with Ambra1 upregulation in the ovarian cancer cells. When Ambra1 expression was reduced by shRNA, the ovarian cancer cells were more sensitive to cisplatin. In conclusion, Ambra1 is a crucial regulator of autophagy and apoptosis in ovarian cancer cells subject to cisplatin to maintain the balance between autophagy and apoptosis. And the Ambra1-targeting inhibition might be an effective method to sensitize ovarian cancer cells to chemotherapy.

Key words: Ambra1, Cisplatin, Autophagy, Cisplatin sensitivity, Ovarian cancer

INTRODUCTION

Ovarian cancer (OC) is the most lethal gynecological malignancy (Jemal et al., 2011), with 90% of the primary tumor as epithelial cell in origin (Cho and Shih, 2009; Bryant et al., 2011), whichever is classified as serous, endometrioid, mucinous or clear cell tumor (Tummala and McGuire, 2005). Though the current combined option with debulking surgery and cisplatin-based chemotherapy has been widely used (Kim et al., 2012), the chemoresistance acquisition eclipses the prognosis of OC patients, with the five-year survival rate for OC patients as low as 30% (Siddik, 2003). Thus, accumulating studies have focused on the molecular mechanism underlying the cisplatin-resistance of OC cells (Siddik, 2002, 2003; Lee et al., 2014; Rosano et al., 2010; Galluzzi et al., 2012). However, it remains to be elucidated with regard to the markers and the signaling pathways promoting cisplatin resistance in ovarian cancer cells.

Autophagy is a highly conserved auto-degradation process by which cytoplasmic components are sequestered in autophagosomes and degraded in lysosomal compartments (Klionsky, 2007). Autophagy is carried by a number of autophagy-related genes (Atgs) (Klionsky, 2007), which are responsible for the autophagosome formation, fusing autophagosome with lysosomes for deg-

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radation or reuse of macromolecules (Mizushima, 2007). And accumulating reports have identified the molecular markers and pathways underlining the autophagy regulation, such as mitogen-activated protein kinase (MAPK)/c-Jun NH2-terminal kinase (JNK) signalling (Zhou et al., 2015), membrane-trafficking small GTPases (Bento et al., 2013), nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Sciarretta et al., 2015), AMP-activated protein kinase (AMPK)- mammalian target of rapamycin (mTOR)-Unc-51 like autophagy activating kinase 1 (Ulkl/2) (Alers et al., 2012) and even microRNAs (Frankel and Lund, 2012). Autophagy is promoted under multiple stress conditions, such as starvation and drug treatment (Beljanski et al., 2015). In particular, the cisplatin treatment induces autophagic resistance in various types of cancers including lung cancer (Wu et al., 2015), ovarian cancer (Bao et al., 2015; Wang and Wu, 2014). Hypoxia robustly augments cisplatin-induced autophagy activation, whereas suppresses the cisplatin-induced BNIP3 death pathways (Wu et al., 2015). NF-E2-related factor 2 (Nrf2) induces cisplatin resistance in ovarian carcinoma via regulating the expression of Atg3, Atg6 and Atg12 (Bao et al., 2015). And the cisplatin treatment activates MAPK and subsequently promotes autophagy and counteracts cisplatin-induced cell death in ovarian cancer cells (Wang and Wu, 2014).

Activating molecule in Beclin1-regulated autophagy 1 (Ambra1) is a novel ATG gene, and the crucial regulator role of it in autophagy has been recognized (Strappazzon et al., 2011; Tooze and Codogno, 2011). Ambra1 interacts with Beclin1 through the target lipid kinase type III phosphoinositide 3-kinase (PI3KC3/Vps34) and thus regulates the formation of autophagosomes (Fimia et al., 2011). And the dynamic interaction between Ambra1 and BCL-2 in mitochondria potentially regulates Beclin1-dependent autophagy and apoptosis (Strappazzon et al., 2011). And what’s more, Ambra1 has been confirmed to be upregulated by cisplatin in breast cancer cells and contributes to the cisplatin resistance (Shen et al., 2015). Up to now, the role of Ambra1 ovarian cancer cells in response to cisplatin treatment has not been identified.

In the present study, we examined the involvement of Ambra1 in the cisplatin-induced autophagy in ovarian cancer OVCAR-3 cells, and then investigated the role of Ambra1 in the cisplatin-resistance in OVCAR-3 cells. Our study firstly identified the crucial role of Ambra1 in the cisplatin-induced autophagy and in the sensitivity in ovarian cancer cells.

MATERIALS AND METHODS

Reagents, cell culture and treatment

Cisplatin was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), was dissolved in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with a concentration of 1 mM, and was stored at -80°C before use. Ovarian cancer OVCAR-3 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and was cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone, Pittsburgh, PA, USA), with 100 U/mL penicillin, and 100 mg/mL streptomycin (CSPC Pharmaceutical Group Limited, Shijiazhuang, China) at 37°C in a humid incubator (Thermo Fisher Scientific, Inc., Waltman, MA, USA) under 5% CO2. Ambra1 shRNA plasmid (shRNA-Ambra1, Cat no. sc-96257-SH) and the Control shRNA Plasmid-A (shRNA-Control, Cat no. sc-108060) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were utilized for the Ambra1 knockdown. 1x10^5 cells/well OVCAR-3 cells with 85% confluence were treated with cisplatin for 12, 24 or 48 hr, with or without transfection with sc-96257-SH or with sc-108060. Then the incubation medium was replaced with 50 μL DMSO was added to dissolve the precipitate completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer. The cell viability was expressed as relative viable cells (%) to control OVCAR-3 cells.

Cell viability assay

Cellular viability of OVCAR-3 cells post treatment was determined by MTT assay. The OVCAR-3 cells which seeded in 96-well plates, with 85% confluence, were treated with cisplatin for 12, 24 or 48 hr, with or without transfection with sc-96257-SH or with sc-108060. Then the incubation medium was replaced with 50 μL 1 × MTT solution for incubation for 2 hr at 37°C. The MTT solution was updated with 150 μL DMSO was added to dissolve the precipitate completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer. The cell viability was expressed as relative viable cells (%) to control OVCAR-3 cells.

Visualization of GFP-positive autophagic vesicles with GFP-LC3 reporter

To visualize the autophagic vesicles formed in OVCAR-3 cells, the quantitative Green Fluorescence Protein (GFP)-LC3 light microscopy autophagy assay was
performed with the GFP-LC3 reporter. GFP-LC3-pcDNA3.1 (+), were performed in OVCAR-3 cells. Post various treatments, 1 x 10^5 OVCAR-3 cells with more than 85% confluence were transfected with 2 μGFP-LC3-expressing plasmids with Lipofectamine 2000 (Invitrogen) for another 24 hr; then the GFP-positive autophagic vesicles were visualized and were analyzed under fluorescence microscopy (Olympus, Tokyo, Japan).

**Western blot analysis**

OVCAR-3 cells for western blotting assay were collected with a cell scraper (Corning Inc., Corning, NY, USA) and were homogenized with an ice-cold Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA, USA). The cell lysate was centrifuged at 10,000 rpm for 30 min at 4°C to remove the cellular debris. Then the supernatant was collected and stored at -70°C before use. Protein samples were successively separated with SDS-PAGE, were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA), and then were detected by western blot using rabbit polyclonal antibody against human LC3 (Abcam, Cambridge, UK), against Ambra 1 (Santa Cruz Biotechnology), against Beclin 1 (Sigma-Aldrich), or against β-actin (Sino Biological, Beijing, China). Goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) and ECL detection systems (Super Signal West Femto, Pierce) were used for detection.

**mRNA isolation and real-time PCR analysis**

Total cellular mRNA from OVCAR-3 cells was prepared with TRIzol reagent (Life Technologies, Grand Island, NY, USA), and reverse transcription (RT) was performed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) as follows: 42°C for 5 min and 95°C for 10 sec for the reverse transcription. And the real-time PCR assay was performed at 95°C for 5 sec and 60°C for 20 sec for the PCR reaction, with 40 cycles. Results were normalized to internal control β-actin, with the ΔΔCt method (Schmittgen and Livak, 2008).

**Apoptosis analysis**

Apoptosis of OVCAR-3 cells post treatment was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Abcam) according to the product’s manual. In brief, OVCAR-3 cells post transfection with sc-96257-SH or with sc-108060 were treated with 10 μM cisplatin for 24 or 48 hr and then were harvested and resuspended in binding buffer. Cells were mixed with annexin V-FITC and PI for an incubating for 15 min in the dark. The apoptotic cells were assayed with a flow cytometry.

**Statistical evaluations**

Results were presented as mean ± S.E. The analysis of GFP-LC3 vesicle number, of the relative mRNA or protein level, of the colony number or of the apoptotic cell number was performed using the Student’s t test.

**RESULTS**

**Cisplatin reduces cellular viability and promotes autophagy and Ambra1 in OVCAR-3 cells**

To investigate the sensitivity or resistance of ovarian cancer OVCAR-3 cells to cisplatin, we treated OVCAR-3 cells with 0, 10 or 50 μM cisplatin for 12, 24 or 48 hr, and then examined the cellular viability. It is indicated in Figs. 1A and 1B that the cisplatin with 10 or 50 μM significantly reduced the cellular viability at either 24 (p < 0.05 or p < 0.01) or 48 (p < 0.001 or p < 0.0001) hr post treatment (H.P.T). And there was a marked difference in such reduction between the 10 and 50 μM treatment at 24 (p < 0.05) or 48 (p < 0.01) H.P.T. To identify the possible role of autophagy in the sensitivity or resistance to cisplatin, we then determined the autophagy induction in the cisplatin-treated OVCAR-3 cells. The OVCAR-3 cells which were treated with 10 μM cisplatin were transfected with the GFP-LC3 reporter for another 24 hr, and then the autophagic vesicles, which were inserted with LC3-II-GFP, were observed under a fluorescence microscopy. Results demonstrated that, compared to the control OVCAR-3 cells (Fig. 1C), there were plenty of GFP-positive dots diffusing in the cytosol of the cisplatin-treated OVCAR-3 cells (Fig. 1D), with a significance (p < 0.001, Fig. 1E).

To reconfirm the autophagy induction by the cisplatin treatment in OVCAR-3 cells, we then analyzed the autophagy-characterized molecular marker, converted LC3-II from LC3-I, with western blotting assay. As shown in Figs. 2A and 2B, compared to the blank OVCAR-3 cells, the treatment with 100 nM rapamycin, as the autophagy inducer, promoted significantly high level of converted LC3-II from LC3-I in the OVCAR-3 cells (p < 0.001). Moreover, the treatment with 10 or 50 μM cisplatin for 24 hr also markedly promoted the conversion of LC3-I to LC3-II (p < 0.01 or p < 0.001), dose-dependently (p < 0.001). Taken together, the cisplatin treatment induces autophagy in OVCAR-3 cells.

Additionally, Ambra1 is a novel autophagy-associated marker (Strappazzon et al., 2011; Tooze and Codogno, 2011), via interacting with Beclin1 (Fimia et al., 2011), and has been recently indicated to be promoted by cis-
Fig. 1. Cellular viability and autophagic vesicles in the cisplatin-treated OVCAR-3 cells. A: Cellular viability of OVCAR-3 cells which were treated with 0, 10 or 50 μM cisplatin for 12, 24 or 48 hr; B: Statistical analysis of the different cellular viability of the cisplatin-treated OVCAR-3 cells; C and D: Representative images of GFP-positive autophagic vesicles (LC3 puncta) in the control (C) and the cisplatin-treated (D) OVCAR-3 cells (10 μM) after GFP-LC3 transduction; E: Quantification of the LC3 puncta in the OVCAR-3 cells with or without the 10 μM cisplatin treatment. Quantitative data was averaged for triple independent results. H.P.T.: Hours post treatment. Statistical significance was shown as *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001.

Fig. 2. Western blot analysis of the LC3-II conversion and the expression of Beclin 1 and Ambra1 in the cisplatin-treated OVCAR-3 cells. A and B: Western blot analysis of the conversion of LC3-I to LC3-II in the OVCAR-3 cells which were treated with 0, 10 or 50 μM cisplatin or with 100 nM Rapamycin for 24 hr; C and D: Western blot analysis of Beclin1 and Ambra1 in the OVCAR-3 cells which were treated with 0, 10 or 50 μM cisplatin or with 100 nM Rapamycin for 24 hr; Experiments were repeated independently in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001.
platin in breast cancer cells (Shen et al., 2015). We also investigated the promotion of Ambra1 and Beclin1 in the cisplatin-treated OVCAR-3 cells. Western blotting assay demonstrated that both Ambra1 and Beclin1 were markedly upregulated in the treatment with 10 or 50 μM cisplatin for 24 hr (p < 0.05, p < 0.01 or p < 0.001, Figs. 2C-D), dose-dependently (p < 0.05). Therefore, we confirmed the promotion of both Ambra1 and Beclin1 by cisplatin in OVCAR-3 cells.

shRNA-mediated Ambra1 abrogation reduces the cisplatin-induced autophagy and beclin1 in OVCAR-3 cells

To examine the regulatory role of Ambra1 in the cisplatin-induced autophagy in OVCAR-3 cells, we abrogated Ambra1 with shRNA-Ambra1 in OVCAR-3 cells, and then re-evaluated the autophagy promotion by cisplatin in the OVCAR-3 cells. Fig. 3A indicates that the Ambra1 mRNA level was significantly reduced in the OVCAR-3 (Ambra1-) cells, which were transfected with shRNA-Ambra1, compared to the OVCAR-3 (Ctrl) cells, which were transfected with control shRNA (p < 0.05 for 6 H.P.T, p < 0.01 for 12 or 24 H.P.T). And the cisplatin-induced Ambra1 was also reduced in protein level in the OVCAR-3 (Ambra1-) cells (p < 0.01 for 12 or p < 0.001 for 24 H.P.T). We then examined the autophagic vesicle formation in both OVCAR-3 (Ambra1-) and OVCAR-3 (Ctrl) cells. As shown in Fig. 3C, the GFP-positive autophagic dots also markedly decreased in the OVCAR-3 (Ambra1-) cells compared with the OVCAR-3 (Ctrl) cells (p < 0.01). And the LC3-II conversion from LC3-I was also markedly reduced in the OVCAR-3 (Ambra1-) cells compared with the OVCAR-3 (Ctrl) cells. As shown in Fig. 3C, the GFP-positive autophagic dots also markedly decreased in the OVCAR-3 (Ambra1-) cells compared with the OVCAR-3 (Ctrl) cells (p < 0.01 for 24 H.P.T). Moreover, the LC3-II conversion from LC3-I was also markedly reduced in the OVCAR-3 (Ambra1-) cells compared with the OVCAR-3 (Ctrl) cells (p < 0.01 for 24 or p < 0.001 for 48 H.P.T, Fig. 3E). In addition, the Beclin1 level was also markedly reduced in the OVCAR-3 (Ambra1-) cells (p < 0.05 or p < 0.01).

shRNA-mediated Ambra1 abrogation deteriorated the cytotoxicity of cisplatin in OVCAR-3 cells

To further investigate the regulatory role of Ambra1 on the cytotoxicity of cisplatin in OVCAR-3 cells, we adopted the colony assay for this analysis. As shown in Fig. 4A, the cisplatin treatment with 10 μM significantly reduced the colony forming of OVCAR-3 cells (p < 0.01, column 3 vs column 1 in Fig. 4B). And such reduction of colony forming was also confirmed in the OVCAR-3 (Ambra1-) cells (p < 0.01, Fig. 4A and column 4 vs column 2 in Fig. 4B). More interestingly, there was a marked difference in the colony forming between the OVCAR-3 (Ctrl) and OVCAR-3 (Ambra1-) cells. The Ambra1 abrogation cells significantly deteriorated the colony reduction in OVCAR-3 (Ambra1-) (p < 0.05, column 4 vs column 3 in Fig. 4B). Thus, we confirmed that the cisplatin-induced Ambra1 posed a promotion effect to the growth of OVCAR-3 cells.

In addition, we measured the viability and the apoptosis levels in the cisplatin-treated OVCAR-3 cells with or without Ambra1 abrogation. Fig. 5A indicated that transfection with shRNA-Ambra1 or control plasmid exerted no different influence on the viability of OVCAR-3 cells. However, there was a significant difference in the cisplatin-mediated cellular viability reduction of OVCAR-3 cells, the Ambra1 abrogation deteriorated the viability reduction markedly (p < 0.05 for 24 H.P.T or p < 0.01 for 48 H.P.T, Fig. 5B). Moreover, the cisplatin-induced apoptosis was also markedly aggravated by the Ambra1 abrogation (p < 0.01 for 24 or 48 H.P.T, Fig. 5C). Therefore, Ambra1 abrogation deteriorated the cytotoxicity of cisplatin in OVCAR-3 cells.

DISCUSSION

Beclin1 is a principal regulator in autophagosome formation (Sun et al., 2009), via which, various markers could interact with Beclin1 and regulate autophagy. B-cell lymphoma 2 (Bcl-2) inhibits starvation-induced autophagy by binding to Beclin1 (Maiuri et al., 2007). Moreover, along with other autophagy-related genes, Beclin1 is associated with multidrug resistance in cancers (Shuhua et al., 2015; Sun et al., 2015), via regulating autophagy. However, its mechanism is not clear. Recently, Ambra1 has been identified to interact with Beclin1 and regulate autophagy in embryogenesis (Fimia et al., 2007), and then been found to promote autophagy in numerous cell types (Miki et al., 2015; Cianfanelli et al., 2015a), but the role of Ambra1 in the chemoresistance of cancer cells, such as ovarian cancer cells, remains unknown.

In this study, we firstly confirmed the autophagy induction in ovarian cancer OVCAR-3 cells, along with the promotion of both Ambra1 and Beclin1. Then we examined the role of Ambra1 in the cisplatin-induced autophagy in OVCAR-3 cells via the Ambra1 knockdown with the shRNA targeting Ambra1. Our results demonstrated that the Ambra1 knockdown markedly reduced autophagy induction and the Beclin1 promotion in the OVCAR-3 cells, subject to cisplatin. Moreover, the Ambra1 knockdown markedly sensitized OVCAR-3 cells to cisplatin, by reducing the colony forming and cellular viability, whereas increasing the apoptosis induction in the cisplatin-treat-
ed OVCAR-3 cells. Our study firstly indicated the regulatory role of Ambra1 in the resistance of ovarian cancer OVCAR-3 cells to cisplatin, via upregulating autophagy. 

Autophagy-promoting proteins and stimuli are often associated with inhibition of cell proliferation. And Ambra1 has been indicated to regulate the stability of the oncprotein and pro-mitotic factor c-Myc, and to affect both cell proliferation rate and tumorigenesis via the Ambra1-Beclin1 interaction (Cianfanelli et al., 2015b). In the present study, we confirmed the reduced proliferation of OVCAR-3 cells post the Ambra1 abrogation, in the presence of cisplatin, implying the regulatory role of Ambra1 in the ovarian cancer cells.

In conclusion, Ambra1 is a crucial regulator of autophagy and apoptosis in ovarian cancer cells subject to cisplatin to maintain the balance between autophagy...
and apoptosis. And the Ambra1-targeting inhibition might be an effective method to sensitize ovarian cancer cells to chemotherapy.

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Fig. 4. Ambra1 abrogation deteriorates the cisplatin-medicated colony forming reduction of OVCAR-3 cells. A: Representative images of the colony forming in the OVCAR-3 (Ambra1-) or OVCAR-3 (Ctrl) cells which were treated with 0 or 10 μM cisplatin; B: Quantification of the colonies formed by the cisplatin-treated OVCAR-3 (Ambra1-) or OVCAR-3 (Ctrl) cells. Each quantitative data was averaged for triple independent results. Statistical significance was shown as *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 5. Cellular viability and apoptosis induction in the cisplatin-treated OVCAR-3 (Ambra1-) or OVCAR-3 (Ctrl) cells. A: MTT assay of OVCAR-3 cells which were transfected with shRNA-Ambra1 (OVCAR-3 (Ambra1-)) or with control shRNA Plasmid-A (OVCAR-3 (Ctrl)) for 0, 24 or 48 hr; B: MTT assay of OVCAR-3 (Ambra1-) or OVCAR-3 (Ctrl) cells which were treated with 10 μM cisplatin; C: Apoptosis induction in the OVCAR-3 (Blank), OVCAR-3 (Ambra1-) and OVCAR-3 (Ctrl) cells, both of which were treated with 10 μM cisplatin for 24 or 48 hr. All experiments were performed in triplicate. H.P.T.: Hours post treatment. Statistically significant was showed as ns: no significance, * p < 0.05 or **p < 0.01.
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Conflict of interest---- The authors declare that there is no conflict of interest.

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