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p62/SQSTM1 involved in cisplatin resistance in human ovarian cancer cells by clearing ubiquitinated proteins

Huimei Yu [a,c], *Jing Su* [a,c], *Ye Xu* [a,b], *Jinsong Kang* [a], *Hongyan Li* [a], *Ling Zhang* [a], *Haowei Yi* [a], *Xiyan Xiang* [a], *Fei Liu* [a], *Liankun Sun* [a,*]

[a] *Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, Changchun, Jilin 130021, PR China*
[b] *Medical Research Lab, Jilin Medical College, Jilin 132013, PR China*

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ABSTRACT

Mechanisms of cisplatin resistance in cancer cells are not fully understood. Here, we show a critical role for the ubiquitin-binding protein p62/SQSTM1 in cisplatin resistance in human ovarian cancer cells (HOCCs). Specifically, we found that cisplatin-resistant SKOV3/DDP cells express much higher levels of p62 than do cisplatin-sensitive SKOV3 cells. The protein p62 binds ubiquitinated proteins for transport to autophagic degradation, reducing apoptosis induced by endoplasmic reticulum (ER) stress in SKOV3/DDP cells. Knockdown of p62 or inhibition of autophagy using 3-methyladenine resensitises SKOV3/DDP cells to cisplatin. Collectively, our data indicate that p62 acts as a receptor or adaptor for autophagic degradation of ubiquitinated proteins, and plays an important role in preventing ER stress-induced apoptosis, leading to cisplatin resistance in HOCCs.

1. Introduction

Ovarian cancer, the deadliest gynaecological malignancy,[1] is typically treated in its advanced stages with cytoreductive surgery and platinum-based chemotherapy. Cisplatin (cis-diamminedichloroplatinum) is widely used as a chemotherapeutic agent for ovarian cancers. However, cisplatin resistance limits its use in cancer patients;[2,3] some cancers develop cisplatin resistance over time, while others are intrinsically resistant. Several mechanisms of cisplatin that confer drug resistance have been proposed.[4,5] Recent studies have shown that cisplatin-induced apoptotic signalling can occur through endoplasmic reticulum (ER) stress, suggesting that the ER is a cytosolic target of cisplatin. This indicates that stress tolerance in the ER is possibly involved in the development of cisplatin resistance.[6,7] Physiological and pathological conditions, including anticancer therapies, viral infections, hypoxia and oxidative injury, may interfere with protein folding, leading to accumulation of misfolded proteins in the ER lumen, a condition called 'ER stress'. Parts of soluble misfolded proteins are mainly degraded by ubiquitin-proteasomes,[8,9] while most misfolded proteins that include polymers and aggregates are degraded by autophagy-lysosomes.[10,11] Roles of production and degradation of misfolded proteins in cisplatin resistance are unclear.

The ubiquitin-binding protein, p62/SQSTM1 (sequestosome 1) is multifunctional; it promotes survival–critical

* *Corresponding author*: Address: Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, PR China. Tel.: +86 431 85619485; fax: +86 431 85619402.
E-mail address: sunlk@jlu.edu.cn (L. Sun).
[c] These authors contributed equally to this study.
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signals, including proliferation, differentiation and induction of anti-apoptotic genes.[12] The protein p62 has unique features: an N-terminal Phox, and a Bem1p domain (capable of self-oligomerisation), and a C-terminal ubiquitin-associated domain, which interacts with ubiquitinated proteins.[13–15] These properties imply p62's involvement in aggregate formation. In autophagy-impaired mice and flies, additional p62 loss is linked to reduced formation of ubiquitin inclusions.[16,17] p62 is selectively degraded by autophagy. It can act as a receptor or adaptor for ubiquitinated substrate autophagy,[18–20] which is mediated through microtubule-associated protein 1 light chain 3 (LC3). The LC3 is drawn to phagophore/isolation membranes and remains coupled to completed autophagosomes.[15] Bjorkoy et al. showed that p62 may affect ubiquitinated proteins' linkage to autophages through LC3, thus maintaining cell homeostasis and survival.[18]

We found p62 to be associated with cisplatin resistance in human ovarian cancer cells (HOCCs), and that cisplatin-resistant SKOV3/DDP cells express much higher levels of p62 than do cisplatin-sensitive SKOV3 cells. The p62 binds and targets ubiquitinated proteins for degradation through autophagy, thus maintaining cell homeostasis and causing cisplatin resistance in HOCCs.

2. Materials and methods

2.1. Cell lines

Cisplatin-sensitive HOCCs SKOV3 and their cisplatin-resistant clones SKOV3/DDP were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College. Both cells were cultured in Roswell Park Memorial Institute – 1640 culture (RPMI-1640, GIBCO, Carlsbad, CA), supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C, 5% CO_2 with high humidity. According to the recommendation from the Chinese Academy of Medical Sciences and Peking Union Medical College, cisplatin-resistant SKOV3/DDP cells were maintained in RPMI-1640 10% foetal bovine serum medium containing 1 μg/ml cisplatin (Sigma–Aldrich, St. Louis, MO) to maintain resistance.

2.2. Cell viability assays

Cell viability was determined by MTT assays. Cells were plated at 1×10^4 per well in 96-well plates. The following day, different concentrations of cisplatin were added to wells and incubated for 24 h and 48 h. Each treatment was repeated in three wells. To each well was added 20 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (Sigma–Aldrich, St. Louis, MO), and incubated for 4 h; 150 μl dimethylsulphoxide was then added to dissolve the formazan crystals. Absorbance was measured with a V_{max} Microplate Reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm.

2.3. Immunofluorescence staining and confocal laser microscopy

Apoptotic nuclear changes were assessed with Hoechst 33258 (Sigma–Aldrich, St. Louis, MO). After treatment with 6 μg/ml cisplatin for 0 h and 24 h, cells were fixed with 4% paraformaldehyde, stained with Hoechst 33258 (2 μg/ml) for 30 min, washed with phosphate-buffer solution (PBS), and examined using Olympus FV1000 confocal laser microscopy to reveal the cell chromatin condensation.

Cells were cultured on coverslips overnight, then treated with 6 μg/ml cisplatin for 12 h, and rinsed with PBS for 3 times. After incubation, cells were fixed for 20 min with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 for 5 min, blocked with bovine serum albumen (BSA), incubated with primary antibodies p62 and LC3(1:100 dilution) overnight at 4 °C, FITC/Texas Red-conjugated secondary antibodies (1:400 dilution) (all antibodies, Santa Cruz Biotechnology, CA) for 0.5 h, then stained with Hoechst 33258 (2 μg/ml) for 2 min, washed with PBS for 3 times, and cells were examined by confocal fluorescence microscopy to reveal the colocalisation of p62 and LC3.

2.4. RNA extraction and reverse-transcriptase PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. First-strand cDNAs were generated by reverse transcription of RNA samples with SuperScript preamplification system (Promega, Madison, MI). Absolute gene transcription was normalised to glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Primers for GAPDH were 5′-GGG-TGA-TGC-TGG-TGC-TGA-GTA-TGT-3′, 5′-AAG-AAT-GGG-AGT-TGC-TGT-TGA-AGT-3′. Primers for p62 were 5′-GAA-CTC-CAG-TCC-CTA-CAG-AT-3′, 5′-CGA-TGT-CAT-AGT-TCT-TGG-TC-3′. The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide and visualised by Tanon-1600 figure gel image processing system and analysed by GIS 1D gel image system software (Tanon, Shanghai, China).

2.5. Western blot analysis

Cells were harvested following the different treatments described above, washed with cold PBS, and then incubated in ice-cold RIPA buffer. Cell lysates were sonicated for 30 s on ice and then lysed at 4 °C for 60 min. Cell lysates were centrifuged for 30 min at 12,000*g*. Protein concentration was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA). For western blot analysis, lysate proteins (30–50 μg) were separated by 12% w/v SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose transfer membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in buffer (10 mM Tris–HCl [pH 7.6], 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, incubated with the desired primary antibody overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated secondary antibody (Thermo, Waltham, MA) at 1: 2000 dilution for 1 h at room temperature. Immunoreactive bands were visualised using the DAB (Sigma, St. Louis, MO) colouration method. Protein levels were quantified by densitometry using Quantity One software (Bio-Rad).

2.6. P62 knockdown by small interfering RNA

Small interfering RNA (siRNA) sequences targeting human p62/SQSTM1 (GenBank Accession NM_003900) and a non-target

sequence were constructed by Genechem (Shanghai, China). The p62 siRNA (si-p62) sequence was GAC-ATC-TTC-CGA-ATC-TAC-A, and that of non-target siRNA (Scramble) was TTC-TCC-GAA-CGT-GTC-ACG-T. Transfections with siRNA were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Briefly, cisplatin-resistant SKOV3/DDP cells were placed into 6-well plates, and transfected the next day with 4 μg si-p62 or si-Scramble, using 10 μl (1 μg/μl) LipofectAMINE 2000. Cells were harvested 2 days after transfection; whole cell lysates were isolated for western blots. For MTT assay, transfected cells were treated with cisplatin for 24 h, followed by MTT assay to determine cell viability.

2.7. Flow cytometry

After exposure to different experimental conditions, cells were trypsinised and incubated with propidium iodide (PI, 1 μg/ml) and Annexin V-FITC (1 μg/ml; Invitrogen, Carlsbad, CA) for 15 min at 37 °C. Samples were then analysed for apoptosis by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.8. Statistics

Experiments were performed in triplicate, and are presented as means ± SD. Comparisons were made between treatments using paired Student's *t*-test, or one-way ANOVA for multiple group comparisons to single controls; differences between treatment means were examined with Dunnett's test. We used SPSS version 16.0 (SPSS/IBM, Chicago, Illinois). $^{*}P < 0.05$ was considered statistically significant.

3. Results

3.1. Cisplatin inhibits growth and induces apoptosis in ovarian cancer cells

We treated cisplatin-sensitive SKOV3 cells and cisplatin-resistant SKOV3/DDP cells with increasing doses of cisplatin for 24 h and 48 h, and then examined growth inhibition using MTT assays. While cisplatin inhibited growth of both cell lines, SKOV3 cells were more sensitive to cisplatin than SKOV3/DDP cells (Fig. 1A).

Based on these MTT results and previous studies,[21] we treated both cell lines with 6 μg/ml cisplatin, and examined apoptotic chromatin condensation with Hoechst 33258 staining by fluorescence microscopy (Fig. 1B). Compared with controls at 24 h, cisplatin-induced apoptotic chromatin condensation was obviously seen in cisplatin-sensitive SKOV3 cells, but rare in cisplatin-resistant SKOV3/DDP cells. Meanwhile, we assessed its apoptotic effects through activation of caspase-3 by western blot, using an antibody that specifically recognises cleaved caspase-3. Cisplatin enhanced expression of cleaved caspase-3 in SKOV3 cells at 12 h and 24 h (Fig. 1C and D). These results indicate that cisplatin can efficiently induce apoptosis in SKOV3 cells but not in SKOV3/DDP cells.

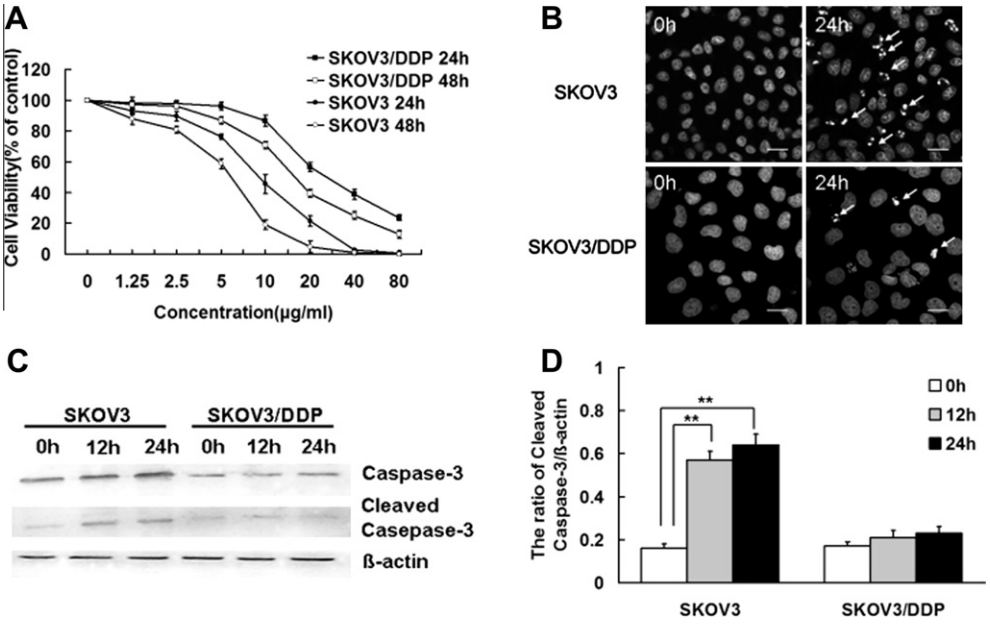

Fig. 1 – Cisplatin inhibits the growth of human ovarian cancer cells. (A) SKOV3 and SKOV3/DDP cells were treated with varying doses of cisplatin for 24 h and 48 h. Cell viability was determined by MTT assay. Data were presented as a mean ± SD, *n* = 3. (B) Cells were treated with 6 μg/ml cisplatin for 0 h and 24 h, stained with Hoechst 33258. Cell morphology was observed by fluorescence microscopy (Bar, 20 μm, arrows, apoptotic cells). (C) Western blot analysis for the expression of Caspase-3 and cleaved caspase-3 protein in SKOV3 and SKOV3/DDP cells treated with 6 μg/ml cisplatin. (D) Quantitation of cleaved caspase-3 protein level. Data were presented as a mean ± SD, *n* = 3, $^{**}P < 0.01$.

3.2. *Cisplatin induces ER stress-mediated apoptosis*

To further evaluate cisplatin's action in HOCCs, and whether the resulting apoptosis is induced by ER stress, we assessed ER stress proteins expression in cells treated with cisplatin. Glucose-regulated protein-78 (Grp78) is an ER chaperone protein, which is up-regulated by ER stress.[22] In cisplatin-sensitive SKOV3 cells, significantly increased Grp78 protein expression was seen at 12 h and 24 h after cisplatin treatment, but not in cisplatin-resistant SKOV3/DDP cells (Fig. 2A and B). To determine the relevance of ER stress to cisplatin-induced apoptosis, we investigated whether ER-resident caspases are activated by cisplatin. Caspase-4 is an ER-resident caspase, processed in response to ER stress and required for ER stress-induced apoptosis (similar to caspase-12 in murine cells).[23] We detected caspase-4 activation, reflected in a cleavage of caspase-4. In SKOV3 cells, this cleavage was significantly increased at 12 h after cisplatin treatment, and confirmed at 24 h (Fig. 2A and B). We also examined expression of the growth-arrest-and DNA-damage-inducible gene 153/C/EBP homology protein (GADD153/CHOP), which is involved in ER stress-induced cell death.[24] Results showed GADD153 to be induced in SKOV3 cells, but not in SKOV3/DDP cells (Fig. 2A and B). These results indicate that cisplatin-induced apoptosis is mediated by ER stress in SKOV3 cells.

Accumulated misfolded proteins in the ER lumen induce ER stress.[8] We compared the levels of ubiquitinated proteins after cisplatin treatment in both cell lines. Cisplatin-sensitive SKOV3 cells significantly accumulated ubiquitinated proteins at 12 h and 24 h after treatment, while cisplatin-resistant SKOV3/DDP cells did not, demonstrating that cisplatin can lead to accumulation of ubiquitinated proteins, and consequently to ER stress-mediated apoptosis in cisplatin-sensitive SKOV3 cells (Fig. 2C and D).

3.3. *Cisplatin-resistant SKOV3/DDP cells express more p62*

Reportedly, p62 recruits ubiquitinated proteins to autophagosomes for degradation.[19] To investigate whether the absence of accumulated protein was due to autophagy or proteasome degradation in SKOV3/DDP cells, we examined p62 protein and mRNA expression. Surprisingly, cisplatin-resistant SKOV3/DDP cells express much higher p62 levels than do cisplatin-sensitive SKOV3 cells. Also, after 12 h and 24 h treatment with 6 μg/ml cisplatin, p62 protein levels in SKOV3/DDP cells decreased gradually while p62 mRNA transcripts remained constant, indicating that cisplatin decreases p62 at the protein level. (Fig. 3A–D)

We used fluorescence microscopy to locate p62 in cisplatin-treated SKOV3/DDP cells. Reportedly, p62 can recruit and transport ubiquitinated proteins for autophage degradation.[19] The autophagy microtubule-associated protein LC3 is the mammalian equivalent of yeast Atg8. When autophagy is activated, LC3-I is cleaved to proteolytic-derived LC3-II and LC3-II aggregates in autophagosomal membranes.[25] After 12h treatment with cisplatin, colocalisation of p62 and LC3 indicated that p62 could be degraded through autophagy (Fig. 3E). Also, LC3 significantly accumulated in SKOV3/DDP cells. Ratios of LC3-II to LC3-I in SKOV3/DDP cells treated with

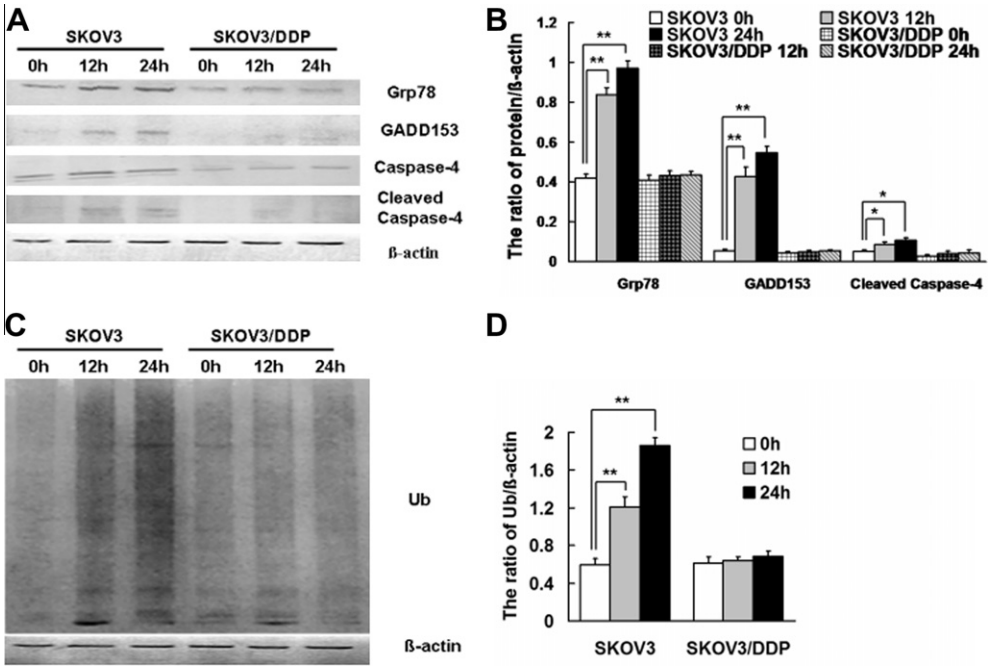

Fig. 2 – Cisplatin triggers ER stress-induced cell death in SKOV3 cells, but not in SKOV3/DDP cells. (A) Western blot analysis for the expression of ER stress proteins in SKOV3 and SKOV3/DDP cells treated with 6 μg/ml cisplatin. (B) Quantitation of ER stress protein levels. Data were presented as a mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$. (C) Western blot analysis for the expression of ubiquinated proteins in SKOV3 and SKOV3/DDP cells treated with cisplatin. (D) Quantitation of ubiquinated proteins level. Data were presented as a mean ± SD, $n = 3$. $^{**}P < 0.01$.

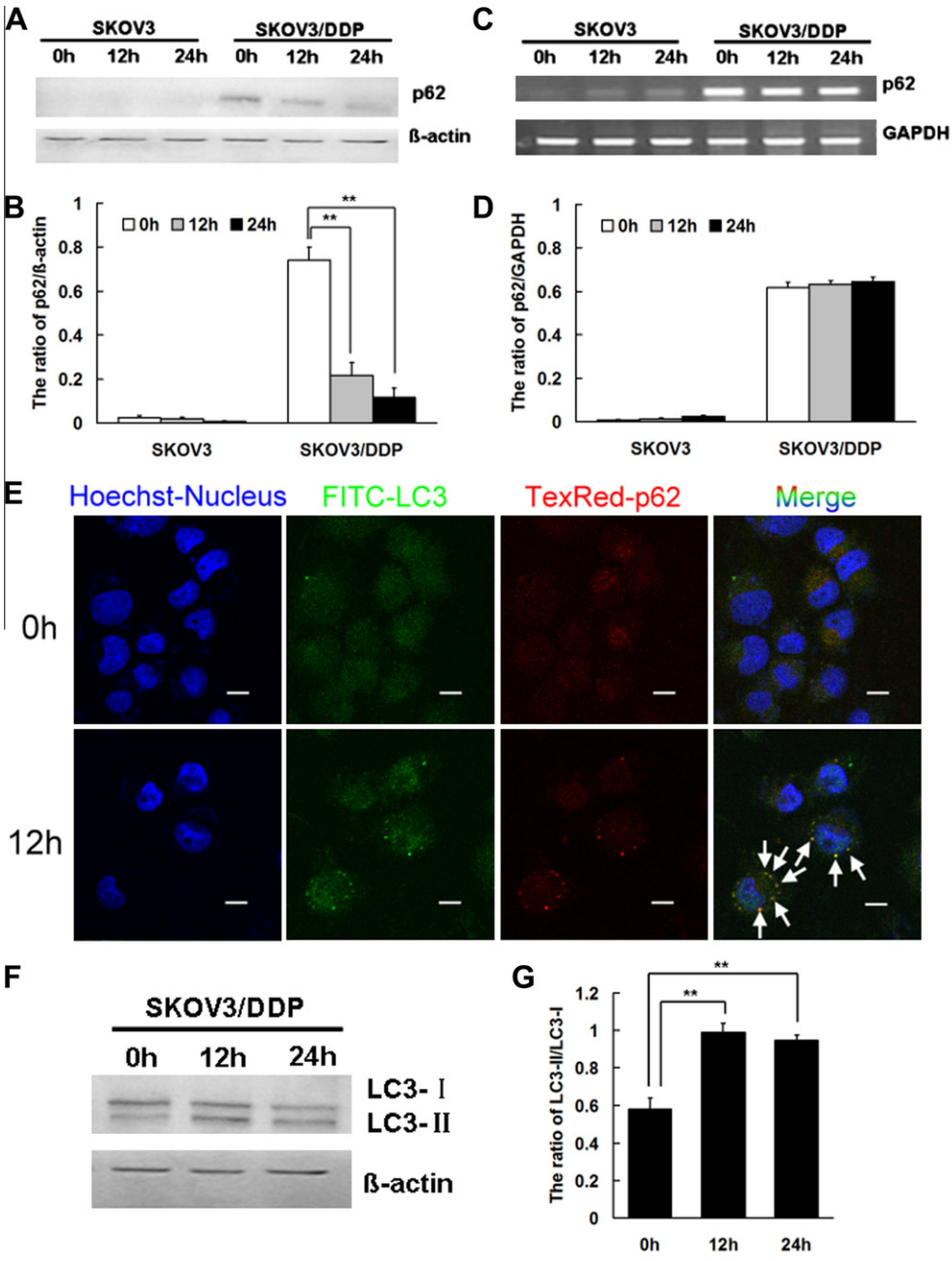

Fig. 3 – The expression of p62 in SKOV3 and SKOV3/DDP cells treated with cisplatin. (A) Western blot analysis for the protein expression of p62 in SKOV3 and SKOV3/DDP cells treated with 6 μg/ml cisplatin. (B) Quantitation of p62 protein. Data were presented as a mean ± SD, $n = 3$. $^{**}P < 0.01$. (C) RT-PCR analysis for the mRNA expression of p62 in SKOV3 and SKOV3/DDP cells treated with cisplatin. (D) Quantitation of p62 mRNA level. Data were presented as a mean ± SD, $n = 3$. (E) The colocoalisation of p62 and LC3 in SKOV3/DDP cells treated with 6 μg/ml cisplatin for 12 h. (Bar, 10 μm, arrows, the colocoalisation of p62 and LC3). (F) The accumulation of LC3 was analysed by western blot in SKOV3/DDP cells treated with cisplatin. (G) Quantitation of the ratio of LC3-II to LC3-I. Data were presented as a mean ± SD, $n = 3$. $^{**}P < 0.01$.

cisplatin for 12 h and 24 h were significantly higher than those in the control group (Fig. 3F and G).

3.4. *Inhibited degradation of misfolded protein enhances cisplatin-induced apoptosis in resistant cells*

Our next goal was to determine whether degradation of the p62 and ubiquitinated proteins was dependent on the autophagy pathway in cisplatin-resistant SKOV3/DDP cells. Cells were treated with 10 mM of the autophagy-specific inhibitor 3-MA, which has no significant toxic effect on cells. While MTT assays indicated that 3-MA alone had no significant effect on cell viability, 3-MA treatment enhanced the cytotoxic effect of cisplatin (Fig. 4A). As shown in Fig. 4B and C, compared with cells treated only with cisplatin, cells treated with cisplatin combined with 3-MA enhanced ubiquitinated

proteins accumulation and inhibited p62 degradation. In addition, Grp78, GADD153 and cleaved caspase-4 increased in cisplatin-resistant SKOV3/DDP cells treated with cisplatin combined with 3-MA (Fig. 4D and E). This evidence indicates that cisplatin induces generation of misfolded proteins, while p62 transport those proteins to autophagic degradation.

Reportedly, p62 recruits ubiquitinated proteins to proteasomes for degradation.[26,27] Lactacystin, a proteasome inhibitor, was used. In MTT assays, 10 μM lactacystin alone had no significant toxic effect on cell viability. Treatment with lactacystin combined with cisplatin had no significant toxic effect compared to cisplatin treatment alone (Fig. 4A). We analysed p62 degradation and ER stress proteins activity in cisplatin-resistant SKOV3/DDP cells treated with cisplatin in combination with lactacystin, and found no significant difference in p62 degradation and ER stress proteins activity between cells treated with cisplatin alone and those treated with combined cisplatin and lactacystin (Fig. 4B–E). However, compared with cells treated with cisplatin only, SKOV3/DDP cells treated with cisplatin and lactacystin had somewhat greater accumulation of ubiquitinated proteins (Fig. 4B and C), suggesting that some ubiquitinated proteins are degraded by the proteasome pathway. This does not seem to be a dominant pathway compared to autophagy. These results indicate that, while ubiquitinated proteins induced by cisplatin in SKOV3/DDP cells are degraded through both autophagy and proteasomes, the autophagy pathway predominates and p62 is involved in it.

3.5. *Knockdown of p62 resensitises cisplatin-resistant SKOV3/DDP cells to cisplatin*

To investigate the direct role of p62 in cisplatin resistance, we used siRNA to knockdown p62 expression, and then examined the effects of p62 knockdown on cisplatin-induced growth inhibition. We transfected cisplatin-resistant SKOV3/DDP cells with siRNA against p62 or non-target sequence and showed that p62 was effectively knocked down in p62 siRNA transfected cells (sip62-SKOV3/DDP cells), compared to cells transfected with control siRNA. Both mRNA and protein expression of p62 significantly decreased in sip62-SKOV3/DDP cells (Fig. 5A and B). Importantly, p62 knockdown

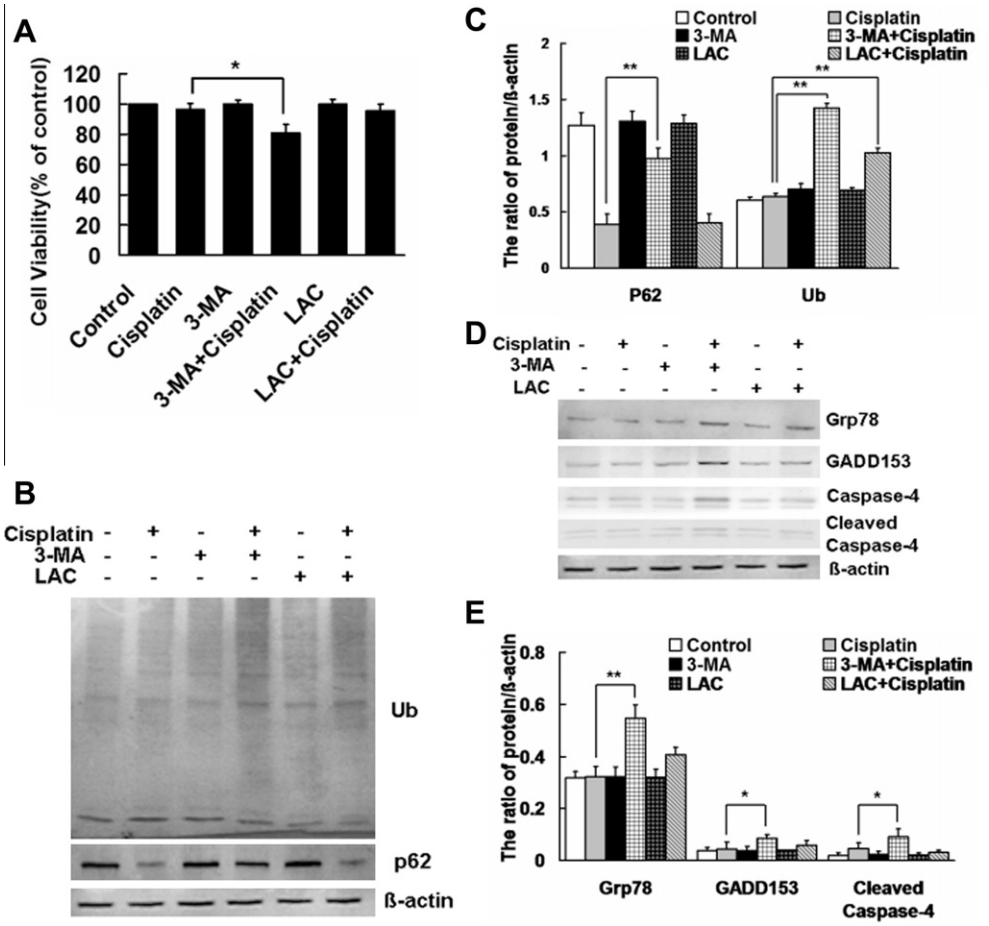

Fig. 4 – p62 binds the ubiquitinated proteins for degradation through autophagy but not proteasome pathway. (A) SKOV3/DDP cells were treated with cisplatin(6 μg/ml) and/or 3-MA(10mM) or LAC(10 μM) for 24 h. Cell viability was determined by MTT assay. Data were presented as a mean ± SD, $n = 3$. (B) Western blot analysis for the expression of p62 and ubiquinated proteins in SKOV3/DDP cells treated with cisplatin and/or 3-MA or LAC for 12 h. (C) Quantitation of p62 and ubiquinated proteins level. Data were presented as a mean ± SD, $n = 3$. $^{**}P < 0.01$. (D)Western blot analysis for the expression of ER stress proteins. (E) Quantitation of ER stress proteins level. Data were presented as a mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

increased cisplatin-induced growth inhibition compared to the same cells transfected with control siRNA (Fig. 5C). After 12 h treatment with 6 μg/ml cisplatin, ubiquitination, ER stress proteins and cleaved caspase-3 significantly increased in sip62-SKOV3/DDP cells (Fig. 5D–G). Additionally, after 24 h, we examined apoptotic chromatin condensation with Hoechst 33258 staining (Fig. 6A). Cisplatin-induced apoptotic chromatin condensation was easily seen in sip62-SKOV3/DDP cells. Meanwhile, sip62-SKOV3/DDP cells were subjected to PI and Annexin V-FITC staining, and flow cytometry analysis to quantify apoptotic cell populations. After 24 h, 6 μg/ml cisplatin induced apoptosis of cells transfected with non-target siRNA to 10.6% ($P < 0.05$). In sip62-SKOV3/DDP cells, cisplatin resulted in an increase in the apoptotic cell population of 20.7% ($P < 0.05$; Fig. 6B).

4. Discussion

Cisplatin is widely used to treat ovarian cancer, as a monotherapy or in combination with other anticancer agents.[28,29] While about 70% of ovarian cancer patients respond to cisplatin initially, most relapse as resistance to cisplatin develops.[2,3] Better understanding of the mechanism of cisplatin resistance is, therefore, critical. Mechanisms of cisplatin resistance in ovarian cancer involve alteration of the gene and also some anti-apoptotic pathway.[30,31] Recently, the ER

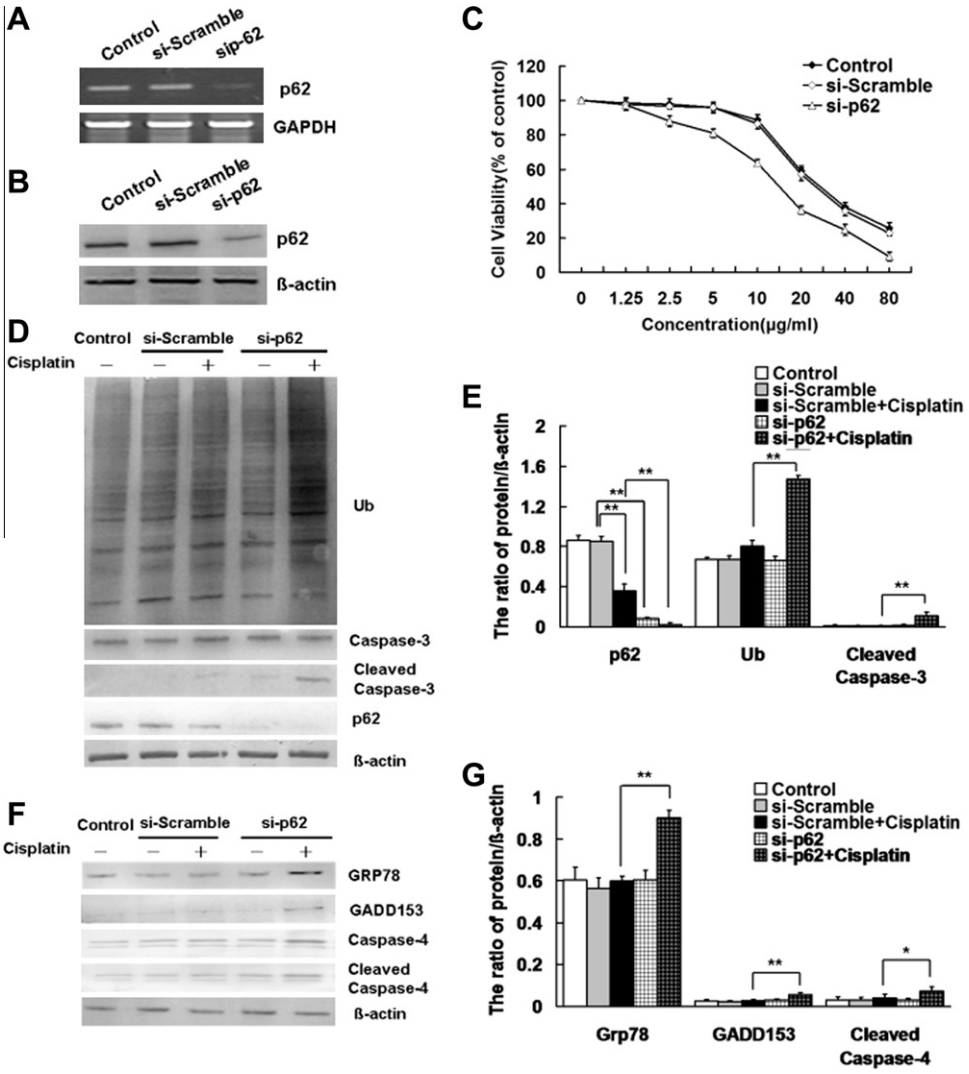

Fig. 5 – Knockdown of p62 increases cisplatin-induced cell death. (A) SKOV3/DDP cells were transfected with p62 siRNA (si-p62) and non-target sequence siRNA(Scramble). Western blot analysed the knockdown efficiency of p62. (B) RT-PCR analysed the knockdown efficiency of p62. (C) Transfected SKOV3/DDP cells treated with varying doses of cisplatin for 24 h. Cell viability was determined by MTT assay. Data were presented as a mean ± SD, $n = 3$. (D) Western blot analysis for the expression of ubiquinated proteins, caspase-3, cleaved caspase-3 and p62 in transfected SKOV3/DDP cells treated with 6 μg/ml cisplatin. (E) Quantitation of p62, ubiquinated proteins and cleaved caspase-3 protein level. Data were presented as a mean ± SD, $n = 3$. $^{**}P < 0.01$. (F) Western blot analysis for the expression of ER stress proteins in transfected SKOV3/DDP cells treated with cisplatin. (G) Quantitation of ER stress proteins level. Data were presented as a mean ± SD. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

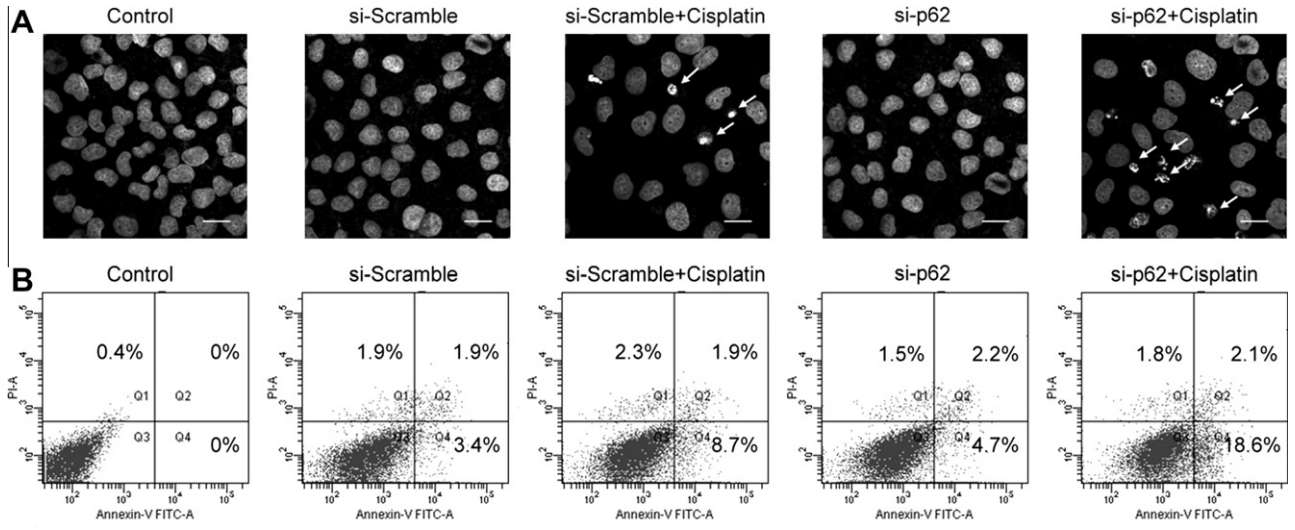

Fig. 6 – Cisplatin induces apoptosis in transfected SKOV3/DDP cells. (A) Transfected SKOV3/DDP cells were treated with 6 μg/ml cisplatin for 24 h, stained with Hoechst 33258. Cell morphology was observed by fluorescence microscopy (Bar, 20 μm, arrows, apoptotic cells). (B) Transfected SKOV3/DDP cells were treated with 6 μg/ml cisplatin for 24 h, and then stained with PI and Annexin V-FITC. The positive stained cells were counted using FACScan. Data were presented as a mean ± SD, *n* = 3.

was reported to be a cytosolic target of cisplatin-induced apoptosis via the ER stress pathway, suggesting that ER stress tolerance is involved in cisplatin resistance in some cancers.[6,7] ER stress is caused by alterations of ER homeostasis. Initial ER stress results in the translation suppression of misfolded protein and increases autophagy, thus enhances cell survival. However, sustained and unabated ER stress induces caspase-mediated apoptosis.

Here, we chose SKOV3/DDP cells as an *in vitro* model to examine its mechanism of cisplatin resistance. SKOV3 cells are relatively resistant to cisplatin because of an altered mitochondrial pathway,[30] SKOV3/DDP cells are more resistant than SKOV3 cells. The mechanism is not clearly understood.

p62 has been characterised as an immediate early response gene, which has an important function in promoting survival signals, including proliferation, differentiation and induction of anti-apoptotic genes.[32] Abnormal expression has been documented in various neoplasms including gastrointestinal, prostate and breast cancers.[33–35] In this study, we found that in cisplatin-resistant SKOV3/DDP cells, p62 can bind and target ubiquitinated proteins and aggregates to autophagosomes to escape ER stress-induced apoptosis. These results also show that p62 knockdown resensitises cisplatin-treated SKOV3/DDP cells to ER stress-induced apoptosis, causing aggregation of ubiquitinated proteins and ER stress-induced apoptosis in cisplatin-treated sip62-SKOV3/DDP cells. The multifunctional protein p62 thus contributes to cisplatin resistance in HOCCs.

Autophagy regulates cell survival or death in both physiological and pathophysiological conditions, extensive autophagy or inappropriate activation of autophagy results in cell death.[36] Some studies suggest that autophagy can provide a cytoprotective role in renal tubular cells as well as in tumour cells treated with cisplatin.[37–39] Our results showed that the cisplatin-induced cell death was increased and ubiquitinated proteins were also accumulated in the SKOV3/DDP cells after autophagy inhibitor 3-MA treatment. Therefore, cisplatin-induced autophagy may provide a prosurvival role in SKOV3/DDP cells.

The protein p62 conveys ubiquitinated proteins to both proteasome and autophagy degradation systems. Ubiquitinated proteins are degraded by proteasomes through the ER-associated degradation pathway, and by autophagy through the ER-activated autophagy pathway.[8] Both of these pathways are activated in response to ER stress caused by ubiquitinated proteins. Removing ubiquitinated proteins protects the ER and mitigates ER stress. In particular, p62 was the first protein shown to bind directly to LC3 to facilitate autophage degradation of ubiquitinated protein aggregates.[13] As a ubiquitin-binding protein, p62 plays an important homeostatic role in clearing cells of misfolded proteins, while performing a critical function in the formation of signalling complexes that activate NF-kB, p38 MAPK and PI3K.[40] In our study, we found that p62 levels were much higher in cisplatin-resistant SKOV3/DDP cells than in cisplatin-sensitive SKOV3 cells, and that p62 degraded gradually in SKOV3/DDP cells treated with cisplatin. We did not find abundant ubiquitinated proteins in cisplatin-resistant SKOV3/DDP cells after 12 h and 24 h treatment with cisplatin. However, when autophagy was blocked with 3-MA, p62 levels significantly increased, ubiquitinated proteins accumulated, and ER stress-mediated apoptosis was activated in SKOV3/DDP cells treated with cisplatin. At the same time, blocking the proteasome pathway with lactacystin did not significantly affect p62 degradation itself. Ubiquitinated proteins increased somewhat and ER stress-mediated apoptosis was only slightly activated in cisplatin-treated SKOV3/DDP cells. These results indicate that SKOV3/DDP cells treated with cisplatin can produce some ubiquitinated proteins. Ubiquitinated protein clearance depends on autophagy and proteasome pathways, especially the former, and p62 is involved in autophagic degradation of ubiquitinated proteins. These data indicate that p62 enhances

the cells' ability to alleviate ER stress, and thus plays a very important role in cisplatin resistance in HOCCs.

Our experiments showed p62 knockdown to enhance about 20% sensitivity of cisplatin-resistant SKOV3/DDP cells to cisplatin. Knockdown of p62 caused accumulation of ubiquitinated proteins in SKOV3/DDP cells, resulting in ER stress-mediated apoptosis, indicating that p62 was involved in the degradation of ubiquitinated proteins and maintained stability in the endoplasmic reticulum, which was partially contributed to apoptotic cell death of ovarian carcinoma cells in response to cisplatin.

In summary, we found that p62 efficiently transports cisplatin-induced misfolded proteins for degradation in cisplatin-resistant HOCCs, allowing cells to avoid ER stress-mediated apoptosis and become drug resistant; and that p62 knockdown resensitises cisplatin-resistant SKOV3/DDP cells to cisplatin. This evidence indicates that cisplatin-resistant cells effectively avoid ER-mediated apoptosis because p62 transports ubiquitinated proteins for degradation by autophagy, thus maintaining cell homeostasis and survival, and providing cisplatin resistance. Protein p62 could be therapeutically targeted for improvement of cisplatin efficacy.

Conflict of interest statement

None declared.

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