p62/SQSTM1 as an oncotarget mediates cisplatin resistance through activating RIP1-NF-κB pathway in human ovarian cancer cells

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Platinum (Pt)-based chemotherapies are frequently used as an adjuvant for ovarian cancer treatment. However, drug resistance is still a major obstacle that limits efficacy of Pt-based therapeutic regimens. Since the 1970s, tolerance to Pt has been blamed on effects unrelated to DNA damage. Recently, investigators have paid more attention to these “non-nuclear” effects and the molecular pathways that are involved in the cell survival and apoptotic escape. Our previous study showed that sequestosome-1 (p62/SQSTM1) was highly expressed in cisplatin-resistant ovarian cancer cells. Furthermore, resistance to Pt was removed when we inhibited the p62 expression with the siRNA. These results suggested that p62 may play a key role in the mechanism of platinum-resistance. p62, also known as SQSTM1, was the first identified autophagy adaptor and is important for cellular detoxification and resistance to nutrient stress. However, in contrast to other autophagy adaptors, p62 also functions as a multifunctional hub. It participates in many important cellular events that control proliferation and apoptosis, particularly during tumorigenic conditions. The autophagy-independent role for p62 is linked ubiquitination of RIP1 and inhibited the activation of the NF-κB signaling pathway. Moreover, loss of the ZZ domain from p62 led to poor proliferative capacity and high levels of apoptosis in SKOV3 cells and made them more sensitive to cisplatin treatment. Collectively, we provide evidence that p62 is implicated in the activation of NF-κB signaling that is partly dependent on RIP1. p62 promotes cell proliferation and inhibits apoptosis thus mediating drug resistance in ovarian cancer cells.

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controlled by ubiquitination. The lysine (K) 63-linked polyubiquitin chains serve as a docking site to mediate the formation of a complex upstream of NF-κB that promotes the activation of pro-survival signaling.\textsuperscript{20,23–25} Previous studies have indicated that deletion of the ZZ (Zinc finger) domain of p62 abolished binding of RIP1 to p62 and inhibited the activation of the NF-κB signaling pathway.\textsuperscript{26} However, we still know little about the role of these key molecules in ovarian cancer. Here, we explored the effects of cisplatin on ovarian cancer cells and found that p62 could activate the NF-κB signaling pathway. And this activation was dependent on K63-linked ubiquitination of RIP1 that subsequently mediated drug resistance.

**Materials and Methods**

**Reagents and antibodies.** Cisplatin, trypan blue and 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (State of New Jersey, USA). ViaFect Transfection Reagent was purchased from Promega (Madison, MI, USA). Nuclear and cytoplasmic protein extraction kit were purchased from Sigma-Aldrich (St. Louis, MA, USA). The next day, stained with FITC/Texas Red-conjugated secondary antibody (Proteintech, Chicago, IL, USA); anti-RIP1, anti-K63-linkage Specific Polyubiquitin (Cell Signaling Technology, Beverly, Massachusetts, USA); anti-Lamin A/C, anti-IkB, anti-pIkB (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Cell lines and cell culture.** SKOV3 cells and their cisplatin-resistant clone SKOV3/DDP were purchased from the Chinese Academy of Medical Sciences and Peking Union Medical College. Both cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 culture medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO\textsubscript{2}. SKOV3/DDP cells were cultured in the presence of 1 µg/mL cisplatin to maintain the resistance.

**Cell transfection.** p62-siRNA and non-target siRNA (Scramble) were obtained from Genechem (Shanghai, China). The p62-siRNA (si-p62) sequence was GAC-ATC-TTC-CGAAT C-TAC-A and the non-target siRNA (Scramble) was TTC-TG. The p62-siRNA and non-target siRNA (Scramble) were obtained from Genechem (Shanghai, China). The p62-siRNA (si-p62) sequence was GAC-ATC-TTC-CGAAT C-TAC-A and the non-target siRNA (Scramble) was TTC-TG. The p62-siRNA (si-p62) sequence was GAC-ATC-TTC-CGAAT C-TAC-A and the non-target siRNA (Scramble) was TTC-TG.

**Cell viability assays.** 8 × 10\textsuperscript{3} cells were seeded in 96-well plates in each well. After 36 h cells were treated with different concentrations of cisplatin after transfection. The MTI assay was used to evaluate cell viability. We measured the absorbance at 570 nm using a Vmax Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Trypan blue staining.** Cells were harvested and resuspended in phosphate-buffered saline (PBS). For staining, Trypan blue was used at a concentration of 0.4%. Mix 90 µL cells and 10 µL Trypan blue in 1.5 mL tube for 2 min and count cells with the hemocytometer while dead cells have blue cytoplasm. Finally, we examined the percentage of cells that have clear cytoplasm (viable cells) versus total cells.

**Luciferase assay.** The cells were transfected with the NF-kB-Luc reporter plasmid using transfection reagent (Promega, Madison, WI, USA). After drug treatment luciferase activity was measured using the Dual-Luciferase assay kits (Promega, Madison, WI, USA) and normalized on the basis of Renilla luciferase activity.

**Western blot analysis.** Proteins were subjected to Western blotting as previously described.\textsuperscript{27} Then incubated with primary antibodies overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated (Proteintech, Chicago, IL, USA) according to the manufacturer’s instructions. Then, immunodetection was performed using ECL reagent (Thermo Fisher Scientific) and visualized by a Syngene Bio Imaging (Synoptics, Cambridge, UK).

**Immunoprecipitation assay.** Cells were lysed in NP40 lysis buffer. Equal amounts of lysates were immunoprecipitated with 2 µg of the RIP1 or K63-linkage Specific Polyubiquitin antibody overnight at 4°C. 25 µL of protein A and G agarose (Beyotime, China) were used each sample. Beads were washed with PBS three times with 1 mL each. The eluted proteins were examined by western blot.

**Immunofluorescence staining and confocal laser microscopy.**Cells were washed and fixed in 4% (w/v) paraformaldehyde/PBS for 20 min and subjected to proteinase K digestion for 1 min. Then permeabilized with 0.1% Triton X-100 for 15 min. After blocking with bovine serum albumen for 30 min cells were incubated with primary antibody overnight at 4°C. The next day, stained with FITC/Texas Red-conjugated secondary antibodies (1:200 dilution; Proteintech, Chicago, IL, USA) for 30 min in the dark. Finally, Cells were treated with Hoechst 33342/H2O (1 µg/mL) for 2 min, and Images were acquired by an Olympus FV1000 confocal laser microscope.

**Flow cytometry.** Cells (25 × 10\textsuperscript{4} cells/well) were seeded in 6-well plates. After exposure to different treatment, cells were collected and performed Annexin-V FITC/PI staining according to the manufacturer’s instructions. Samples were analyzed by Accuri C6 Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

**EDU.** Cells were seeded in 96-well plate and exposed to 50 µM of 5-ethyl-29-deoxyuridine (EdU, RiboBio, Guangzhou, China) according to the manufacturer’s instructions. Subsequently, the DNA contents were stained with Hoechst 33342 for 30 min and visualized under a microscope (Olympus, Tokyo, Japan).

**Caspase activity assay (Promega).** Cells were collected and Caspase-Glo 3/7 Assay (Promega, Madison, MI, USA) was used as described in the manufacturer’s instructions. Microplate reader (FLUOstar Omega, MBG LABTECH, German) was used for detecting.

**RNA extraction and quantitative real-time PCR.** Total RNA extracted and First-strand cDNAs synthesized were described as before.\textsuperscript{4} Quantitative real-time PCR was done by using the MX3000 instrument (Agilent, USA) followed by a 2-step PCR protocol. The primers sequences are as follows: primers for TGFB1 5'-CCACAGTGGCTGAGTA-3'; primers for CCL2 5'-CAATCGACGGTCAGGTC-3'; primers for IL-6 5'-TTGTGGCCTTTCCTG-3'; primers for EDU 5'-GACCGGGAGTGGTCG-3'; primers for ACTB 5'-ATATCGGTTC-3'. The relative expression was calculated by ΔCt among different experimental groups normalized to ACTB expression.

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Fig. 1. Cisplatin treatment activates the NF-κB pathway and an increase in RIP1K63-linked ubiquitination in SKOV3/DDP cells. (a) Cells were treated with cisplatin (6 μg/mL) and expression of NF-κB p65 and NF-κB p50 in the nucleus and IκB, p-IκB in whole cell lysates was analyzed by western blotting. (b) SKOV3 and SKOV3/DDP cells were transiently transfected with a pNF-κB-Luc vector. The luciferase activity was assessed and normalized on the basis of Renilla luciferase activity (mean ± SD, n = 3, *P < 0.05, vs control). (c) SKOV3 and SKOV3/DDP cells were stained with Hoechst 33342 and antibodies against p65. They were observed under confocal laser microscopy (scale bar, 25 μm). (d) SKOV3 and SKOV3/DDP cells were immunoblotted (IB) with anti-p62 and anti-RIP1. (e) Immunoprecipitation (IP) was performed using the anti-K63 antibody followed by western blotting using anti-RIP1 antibody.
Statistical analyses. Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, USA). All the data are presented as means ± SD and carried out using the Student’s t-test. P < 0.05 was considered statistically significant.
siRNA markedly induced downregulation of NF-κB transcriptional activity (Fig. 2d). Interestingly, the amount of co-immunoprecipitated K63-polyubiquitinated RIP1 also decreased (Fig. 2c). These data indicated that p62 functioned as a positive regulator for the activation of both the NF-κB pathway and RIP1.
p62/SQSTM1 activates the NF-κB pathway by increasing K63 ubiquitination of RIP1 following cisplatin treatment in SKOV3 cells. A previous study showed that in 293T cells p62 interacted with RIP1 through a specific structure known as the ZZ domain.(17) In this study, to investigate whether p62 upregulated NF-κB through regulating the ubiquitination of RIP1, a ZZ domain truncation mutation (ΔZZ) of p62 and a wild type (wt)-p62 were transfected into SKOV3 cells (Fig. 3a). Co-immunoprecipitation revealed that the K63-linked ubiquitination of RIP1 was downregulated when p62 no longer bound to RIP1 compared with cells overexpressing wt-p62 (Fig. 3b). These results suggested that p62 can truly affect the ubiquitination of RIP1.

We then investigated the activity of NF-κB in ΔZZ-p62 and wt-p62 expressing cells. As expected, deleting the ZZ domain decreased the luciferase activity compared with wt-p62 follow- ing this point, it is reasonable to speculate that loss of p62 ZZ domain may also affect the cell sensitivity to chemother-apy. In the following part, we examined cell viability using an MTT assay. As shown in (Fig. 5a), overexpression of ΔZZ-p62 decreased the sensitivity to cisplatin treatment compared with cells transfected with wt-p62. The trypan blue staining also suggested that transfection of the ΔZZ mutant decreased the number of viable cells (Fig. 5b).

We then determined if the reduction in cell number was the result of apoptosis induction or a failure in proliferation. First, we evaluated apoptosis using flow cytometry. As shown in Figure 5c, overexpression of ΔZZ-p62 in SKOV3 cells increased apoptosis with the treatment of cisplatin. Furthermore, the caspase3/7 activity assay also confirmed this speculation (Fig. 5d). Then we investigated proliferation by examining DNA replication using EdU (5-Ethynyl-2'-deoxy uridine) is incorporated into cellular DNA during replication and newly synthesized DNA is quantitated by the levels of red condensation. 33342 also indicated an increase in apoptotic chromatin condensation.

To further understand the underlying molecular mechanisms of drug resistance induced by NF-κB activation, we then investigated several reported NF-κB target genes(33,34): BCL2 protein regulated by NF-κB activation, we then investigated several reported NF-κB target genes(33,34): BCL2 like 1 (BCL2L1, also known as BCLX) is an anti-apoptotic protein regulated by NF-κB; Interleukin 6 (IL6) belongs to the cytokine/chemokine family; C-C motif chemokine ligand 2 (CCL2) is a regulator of cell proliferation and migration; Transforming growth factor beta 1 (TGFβ1) and colony stimulating factor 3 (CSF3) are both well-studied growth factors.
Our results indicated that compared with wt-p62, SKOV3 cells transfected with ΔZZ-p62 showed lower levels of BCL2L1 expression (Fig. 5e). Consistent with this result deleting the ZZ domain of p62 decreased expression of genes involved in proliferation (Fig. 6b–e). These results indicated that high levels of p62 in cisplatin-resistant ovarian cancer cells may protect them from death by evading apoptosis and promoting proliferation through activation of NF-κB signaling.

Discussion

Chemoresistance has been recognized as one of the crucial features of cancer whereby tumor cells escape the toxicity induced by drugs.\(^{(35)}\) Although the antitumor effects of platinum compounds are related to DNA damage, this chemical feature means that Pt-based drugs also affect a variety of important cellular events including proliferation and apoptosis.\(^{(36,37)}\) Therefore, understanding the role of these pathways may be helpful in combatting resistance. Our study suggests that the p62-RIP1-NF-κB pathway plays an important role in promoting cell survival and downregulating this pathway can significantly increase the sensitivity of ovarian cancer cells to cisplatin (Fig. 7).

We found that basal p62 expression was higher in cisplatin-resistant ovarian cancer cells (Fig. 1d). And p62 was truly related to resistant mechanism, not only cisplatin but other drug, in SKOV3/DDP cells during our previous study (Fig. S1). Although p62 protein levels decreased with cisplatin treatment in SKOV3/DDP cells, which was the result of autophagy, p62 mRNA transcripts still remained constant.\(^{(4)}\) Furthermore, downregulating the expression of p62 increased the sensitivity to chemotherapy (Fig. 2b). Thus we believe that the mechanism of drug resistance in ovarian cancer cells is associated with the high level of p62. However, the precise molecular mechanisms of how p62 contributed to drug resistance in ovarian cancer remained poorly understood. p62 is overexpressed in many tumors and functions as a multi-functional hub that mediates its interactions with many binding partners. These include proteins involved in cellular transformation, proliferation and signaling such as NF-κB and MAPK\(^{(38–40)}\) We observed an increase in the transcriptional activity of NF-κB in drug-resistant SKOV3/DDP ovarian cancer cells when compared with parental SKOV3 cells. This result is in agreement with the previous findings in ovarian cancer cells.\(^{(41,42)}\)
Therefore, it was reasonable to focus on the p62-mediated regulation of the pro-survival signaling complex NF-κB and explore the mechanism of chemoresistance in ovarian cancer cells.

RIP1 is a member of the RIP serine/threonine kinase family and binds with death receptors such as tumor necrosis factor (TNF) and Fas to promote apoptosis. Interestingly, cancer cells also show greater sensitivity to TNF-induced death when RIP1 is absent because of inactivation of the NF-κB survival pathway.\(^{(23,43)}\) Recent studies suggested that the K63-linked polyubiquitin chains of RIP1 can rapidly recruit the I KK complex to promote phosphorylation of NFKBIA and activate NF-κB signaling, which contributed to cell survival in lymphoma glioblastoma, and colorectal cancer cells.\(^{(44–46)}\) Furthermore, Sanz established the relationship between RIP1 and p62 in 293T cells using co-immunoprecipitation. She found that deletion of the ZZ domain of p62 significantly inhibited the binding of RIP1. This interaction increased the expression of pro-survival factors through upregulating the NF-κB signaling pathway.\(^{(17)}\) In the current study, cisplatin treatment resulted in an increase of K63-linked ubiquitination of RIP1 in drug-resistant ovarian cancer cells. When we deleted the ZZ domain that interacts with RIP1, the K63-ubiquitinated form of RIP1 was reduced. Furthermore, the transcriptional activity of NF-κB was also inhibited. We then evaluated sensitivity to cisplatin in SKOV3/DDP cells the escape from apoptosis was partly dependent on the activation of p62-RIPK1-NF-κB survival pathway under the stress of chemotherapy. However, overexpression of the multi-functional p62 protein is likely to have pleiotropic effects.\(^{(48–50)}\) Furthermore, p62 may also function as a switch that regulates opposing pathways depending on its interaction with different molecules in physiological and pathological conditions.

Collectively, our study provides evidence that, in ovarian cancer cells, p62 participates in the mechanism of cisplatin-resistance by activating the NF-κB pro-survival pathway, and this process depends on K63-ubiquitinated RIP1. These data contribute to understanding the role of "non-nuclear" targets in chemotherapy and may aid development of an effective therapeutical strategy to overcome Pt-based drug resistance in ovarian cancer.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CDDP/DDP cisplatin  
NF-κB nuclear factor kappa B  
p50 NFKB1 (nuclear factor kappa B subunit 1)  
p62/SQSTM1 sequestosome-1  
p65 RELA proto-oncogene  
RIP1 Receptor interacting serine/threonine kinase 1  
ZZ Zinc finger

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Fig. S1 p62 participated in the mechanism of VK3-resistance in SKOV3/DDP cells (a) SKOV3 and SKOV3/DDP cells were treated with varying doses of VK3 for 8 and 16 h. Cell viability was determined by MTT assay. Data were presented as a mean ± SD, n = 3. (b) Western blot analysis for the nuclear Nrf2 protein in transfected SKOV3/DDP cells.