

Zinc finger domain of p62/SQSTM1 is involved in the necroptosis of human cisplatin-resistant ovarian cancer cells treated with sulfasalazine

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Abstract. Cisplatin resistance in ovarian cancer cells is mainly apoptosis resistant. Although other types of programmed cell death are highly involved in chemoresistance, which type can overcome cisplatin resistance remains unclear. The present study observed that cisplatin-sensitive SKOV3 cells and cisplatin-resistant SKOV3/DDP cells had different levels of sensitivity to sulfasalazine (SAS). The present study aimed to investigate the effect of SAS on necroptosis under the same inhibition rate in these two types of cells. Necroptosis inhibitor Necrostatin-1 (Nec-1) attenuated SAS-induced SKOV3/DDP cytotoxicity. SAS decreased SKOV3/DDP cells survival rate, accompanied by decreased cell adhesion and spreading. SAS treatment activated necrosome formation in SKOV3/DDP cells, suggesting the possibility of necroptosis. p62/sequestosome-1 (SQSTM1)

protein expression levels were also increased over the same time period. The transfection of small interfering (si)-p62 could decrease the ratios of phosphorylated (p)-receptor-interacting serine/threonine kinase 1 (RIP1)/RIP1, p-receptor-interacting serine/threonine kinase 3 (RIP3)/RIP3 and p-mixed lineage kinase domain-like protein (MLKL)/MLKL proteins in SKOV3/DDP cells. Under the si-p62 condition, there was no increase in the rate of cell survival in Nec-1 and SAS combination group compared with SAS. The zinc finger domain deletion of p62/SQSTM1 effectively decreased the expression levels of necroptosis-related p-proteins. Collectively, certain drugs were able to induce necroptosis in SKOV3/DDP, while p62/RIP1/RIP3/MLKL was associated with the induction of necroptosis and with increasing the sensitivity of cisplatin-resistant ovarian cancer cells, which provided evidence for potential as a therapeutic target for overcoming resistance.

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Abbreviations: MLKL, mixed lineage kinase domain-like protein; SQSTM1, sequestosome-1; RIP1, receptor-interacting serine/threonine kinase 1; RIP3, receptor-interacting serine/threonine kinase 3; SAS, sulfasalazine; ZZ, zinc finger

Key words: drug resistance, necroptosis, ovarian cancer, p62/SQSTM1, Sulfasalazine

Introduction

It has been reported that in addition to apoptosis, other types of programmed cell death including ferroptosis, necroptosis and pyroptosis are highly related to chemoresistance (1). Inhibition of necroptosis by application of receptor-interacting serine/threonine kinase 1 (RIP1) activity inhibitor Necrostatin-1 (Nec-1) or knockdown of receptor-interacting serine/threonine kinase 3 (RIP3) significantly inhibits the cisplatin-induced loss of cell viability in apoptosis-resistant esophageal squamous cell carcinoma cells (2), suggesting that induction of necroptosis can increase the sensitivity of chemotherapeutic agents.

Reactive oxygen species (ROS) are considered to be a driving force for necroptosis (3-5). ROS contributes to increasing necroptosis in NF- κ B-deficient cells induced by TNF α by detecting phosphorylated (p)-RIP1, RIP3 and mixed-lineage kinase domain-like (MLKL) (6). ROS production upon TNF α /BV6 treatment is involved in promoting the assembly of the RIP1/RIP3 necrosome in FADD-deficient

Jurkat cells by immunoprecipitation of RIP1/RIP3 (4). Sulfasalazine (SAS), a potent cystine/glutamate transporter cystine-glutamate exchange (xCT) inhibitor that plays an important role in maintaining glutathione levels, impairs the ROS defense system and increases the therapeutic efficacy of anticancer therapies (7-9). Based on the previous results of a close correlation between necroptosis and ROS, a hypothesis of the present study was that SAS might be associated to the induction of necroptosis.

P62/sequestosome-1 (SQSTM1) is a multi-domain adapter protein that is highly expressed in various tumor cells, and it binds to multiple signaling molecules (10,11). However, the results of previous studies highlight that p62/SQSTM1 binds to RIP1 to assemble RIP3/MLKL on the autophagosome membrane in mouse prostate cells lacking Map3k7, suggesting that p62/SQSTM1 not only plays a role as an autophagy adaptor to degrade cellular material, but also may induce necroptosis by recruiting RIP1 (12,13). A previous study has indicated that deletion of the zinc finger (ZZ) domain of p62/SQSTM1 prevents the binding of RIP1 to p62/SQSTM1, and therefore inhibits the activation of the NF- κ B signaling pathway (14). P62/SQSTM1 also exhibits pro-death functions through interaction with caspase-8, which plays a role in the progression of ovarian cancer (15). Notably, RIP1 activates NF- κ B to promote cell survival, and binds to caspase-8 or RIP3 to play a role in the regulation of cell apoptosis and necroptosis (16). Thus the mechanism of necroptosis in SKOV3/DDP cells treated with SAS through p62/SQSTM1 remains to be elucidated.

The present study aimed to determine the effects of SAS on cisplatin-resistant ovarian cancer SKOV3/DDP cells. Another aim was to demonstrate that p62/SQSTM1 regulated the necroptosis of cisplatin-resistant ovarian cancer SKOV3/DDP cells treated with SAS, providing evidence for p62/SQSTM1 as a potential therapeutic target to overcome drug resistance.

Materials and methods

Reagents and antibodies. SAS, necroptosis inhibitor Nec-1 and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich; Merck KGaA. ViaFect™ transfection reagent was purchased from Promega Corporation. Anti-p62/SQSTM1 [cat. no. 66184-1-Ig; 1:5,000 for western blotting (WB)], anti-RIP1 (cat. no. 17519-1-AP; 1:500 for WB) and anti- β -actin (cat. no. 60008-1-Ig; 1:5,000 for WB) were purchased from ProteinTech Group, Inc. Anti-p-RIP1 (cat. no. 31122; 1:1,000 for WB) was purchased from Cell Signaling Technology, Inc. Anti-RIP3 (cat. no. ab305054; 1:1,000 for WB), anti-p-RIP3 (cat. no. ab222320; 1:2,000 for WB), anti-MLKL (cat. no. ab196436; 1:1,000 for WB) and anti-p-MLKL (cat. no. ab279863; 1:1,000 for WB) were purchased from Abcam.

Cell lines and cell culture. Cisplatin-sensitive HOCC SKOV3 cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences (cat. no. 1101HUM-PUMCO00027). SKOV3/DDP were added to the medium containing 0.02 μ g/ml cisplatin, was gradually induced by increasing the concentration of cisplatin, and kept in medium containing 0.2 μ g/ml cisplatin to maintain resistance.

All cells were cultured in RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator.

Cell transfection. p62/SQSTM1-small interfering (si)RNA (si-p62.1, si-p62.2 and si-p62.3) and non-targeting siRNA (scrambled control) were obtained from Shanghai GeneChem Co., Ltd. The target sequences for p62-siRNA were as follows: Si-p62.1 forward, 5'-CAGATGGAGTCGGATAACT-3', and reverse, 5'-AGTTATCCGACTCCATCTG-3'; si-p62.2 forward, 5'-GTGACGAGGAATTGACAAT-3', and reverse, 5'-ATTGTCAATTCTCTCGTCAC-3'; si-p62.3 forward, 5'-GACATCTTCCGAATCTACA-3', and reverse, 5'-TGTAGATTCGGAAGATGTC-3'; and scramble control sequences were forward, 5'-TTCTCCGAACGTGTCACGT-3', and reverse, 5'-ACGTGACAGTTCGGAGAA-3'. The pcDNA3.1- Δ ZZ-p62 plasmid (with a truncated mutation in the ZZ domain) and empty pcDNA3.1 vector (negative control; NC) were constructed by Sangon Biotech Co., Ltd. In total, 25x10⁴ cells were plated into 6-well plates and cultured for 36 h. Following culturing, cells were transfected with 2 μ g siRNA or plasmids per 6-well plate and 200 μ l of total ViaFect™ transfection reagent complex at 37°C in a 5% CO₂ incubator. Subsequent experimentation was carried out within 72 h.

Cell viability assay. In total, 8x10³ cells/per well were seeded in 96-well plates and cultured for 36 h at 37°C with 5% CO₂. Cells were treated with 0.5, 1.0, 2.0, 4.0 and 8.0 mM of SAS or 2.5, 5, 10, 25 and 50 μ M of Nec-1. Cell viability was assessed using an MTT assay which dissolved the purple formazan by DMSO and measured at a wavelength of 570 nm using a microplate reader (Molecular Devices, LLC).

Real-time labeled cell function analysis (RTCA). Cell suspensions were added to 96-well plates at a concentration of 1.5x10⁴ cells in 100 μ l medium per well. The 96-well plates were placed into the RTCA connection instrument (ACEA Bioscience, Inc.; Agilent), and cells were incubated at 37°C in 5% CO₂ according to the manufacturer's protocol. When cells reached the logarithmic growth phase, the program was paused, the 96-well plate was removed and the original solution was discarded. Subsequently, 0.5 mM SAS and 10 μ M Nec-1 were added to SKOV3 wells, and 2.0 mM SAS and 10 μ M Nec-1 were added to SKOV3/DDP wells and the 96-well plate was returned to the RTCA connection instrument. The real-time growth of cells was observed and the program was completed when the pre-planned program ended or when cell proliferation met the requirements. Results were analyzed using the RTCA Data Analysis Software (version, 1.0; ACEA Bioscience Inc.).

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitors (2 mM AEBSF, 0.3 μ M Aprotinin, 0.13 mM Bestatin, 0.014 mM E64 and 0.01 mM Leupeptin). Cells were centrifuged at 1,000 x g for 15 min at 4°C. The protein determination method was BCA assay and 30 μ g protein per lane was separated using 10% SDS-PAGE. Separated proteins were subsequently transferred

onto a PVDF membrane and blocked with 5% skimmed milk for 90 min at room temperature. Membranes were incubated with primary antibodies at 4°C overnight. Following primary incubation, membranes were incubated with HRP-conjugated secondary antibodies (cat. nos. SA00001-1 and SA00001-1; 1:2,000; ProteinTech Group, Inc.) for 2 h at room temperature. Protein bands were visualized using ECL reagent (Thermo Fisher Scientific, Inc.) and protein expression was quantified using a Syngene Bio Imaging system version 4.3.17 (Synoptics).

Immunoprecipitation assay. Cells were lysed in 200 μ l NP40 lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (8 μ M Aprotinin, 0.5 mM Bestatin, 0.15 mM E64 and 0.2 mM Leupeptin) and were centrifuged at 12,000 x g and 4°C for 5 min. Subsequently, 30 μ l of each cell supernatant were conducted as input. Equal amounts 1 mg of each cell supernatants were immunoprecipitated with 2 μ g of the anti-RIP3 primary antibody overnight at 4°C. In total, 25 μ l of protein A and G agarose (Beyotime Institute of Biotechnology) was used in each sample overnight at 4°C. The supernatants were isolated at 1,000 x g and 4°C for 5 min. Beads were washed three times with 1 ml PBS buffer, the magnetic were centrifugated at 1,000 x g and 4°C for 1 min, and eluted proteins were analyzed using anti-RIP1 primary antibody by western blotting.

Statistical analysis. Data are presented as the mean $\pm$ SEM and experimental repeats performed three times. Statistical analysis was performed using SPSS statistical software (version, 20.0; IBM Corp.). Comparisons between groups were conducted using one-way ANOVA followed by a post hoc test. Dunnett's t and Dunnett's T3 tests were used for comparisons using the same control group. LSD tests were used for comparisons between experimental, control and SAS groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SAS treatment activates the necroptosis pathway in SKOV3/DDP cells. The survival rates of SKOV3 and SKOV3/DDP cells significantly decreased in a dose-dependent manner as the concentration of SAS increased, compared with the control group (Fig. 1A and B). Compared with the negative control SKOV3 cells, SKOV3/DDP cells have low sensitivity to SAS. The survival rate for SKOV3 cells treated with 0.5 mM SAS and 2 mM SAS were 81.9 ± 1.4 and $69.3 \pm 2.43\%$ respectively. The survival rate for SKOV3/DDP treated with 0.5 mM SAS and 2 mM SAS were 88.9 ± 1.1 and $80.3 \pm 1.5\%$ respectively. To observe the difference in cell death, in subsequent experiments, 0.5 mM SAS was selected to treat SKOV3 cells, and 2 mM SAS was selected to treat SKOV3/DDP cells with the same cell survival rate.

Notably, it has been reported that Nec-1, a RIP1-specific inhibitor, can block necroptosis (17). The present study tested the effect of Nec-1 on SAS-induced death in SKOV3/DDP cells. The present study intended to observe their cell survival rate, adhesion and spreading and cell morphology when treated with a combination of SAS with Nec-1. Results of the MTT assay demonstrated that, compared with the SAS group, a combination of Nec-1 and SAS did not increase the

survival rate of SKOV3 cells, and a combination of Nec-1 and 5, 10, 25 μ M and 2 mM SAS increased the survival rate of SKOV3/DDP cells (Fig. 1C and D). The cell survival rate was not altered in groups treated with different concentrations of Nec-1. The survival curve of cell index in RTCA indicates the cell number, and the slope of cell index indicates the adhesion and spreading, as well as cell morphology of cells. Compared with the SAS group, the survival curve of SKOV3 cells was low in the 10 μ M Nec-1 and SAS combination group (Fig. 1E). In addition, the survival curve of SKOV3/DDP cells in the 10 μ M Nec-1 and SAS combination group was high compared with the SAS group (Fig. 1F). Results of the RTCA demonstrated that in SKOV3 cells, the slope of the curve was decreased in the SAS group compared with the control. However, there was no significant difference between the slope of the curve in the SAS group, and that in the 10 μ M Nec-1 and SAS combination group (Fig. 1G). In SKOV3/DDP cells, the slope of the curve was decreased in the SAS group, and showed a negative increase compared with the control. Moreover, the slope of the curve was increased in the 10 μ M Nec-1 and SAS combination group compared with the SAS group (Fig. 1H). By contrast, the cell index changes between the Control and SAS groups were 4.7 and 3.7 in SKOV3 cells, respectively, and 4.0 and 0.5 in SKOV3/DDP cells respectively, indicating the adhesion and spreading of SKOV3/DDP cells decreased following SAS treatment in contrast to SKOV3 cells under the same cell survival rate (~80%).

SAS treatment activates necrosome formation in SKOV3/DDP cells. Expression levels of marker proteins associated with the necroptosis pathway were investigated in the present study. The expressions level of necroptosis-associated proteins; namely, p-RIP1, RIP3 and MLKL were significantly increased in SKOV3/DDP cells following SAS treatment for 12 h. On the other hand, in SKOV3 cells treated with SAS, the necroptosis-associated proteins p-RIP1, RIP3 and MLKL were not significantly altered at each observed time point (Fig. 2A-C). Notably, the complex formed following the phosphorylation of MLKL and the combination of RIP1 with RIP3 is the core component of necroptosis. Therefore, the SAS-induced precipitation of RIP3 was investigated in human ovarian epithelial carcinoma cells. Results of the co-immunoprecipitation analysis revealed that the expression of RIP1 and RIP3 complexes were increased in SKOV3/DDP cells compared with SKOV3 cells (Fig. 2D).

P62/SQSTM1 adaptor plays a role in SAS-induced necroptosis in SKOV3/DDP cells. Notably, p62/SQSTM1 protein expression levels were increased in SKOV3/DDP cells, compared with SKOV3 cells (18). During the SAS-induced death of SKOV3/DDP cells, p62/SQSTM1 expression was significantly increased following 12 h of SAS treatment compared with 0 h of treatment. These results are consistent with the increased expression of necroptosis marker proteins observed at the same time point. Results of the present study demonstrated that p62/SQSTM1 protein expression was minimal during the death of SKOV3 cells treated with SAS (Fig. 3A-C). RIP1 interacts directly with p62/SQSTM1 (19), suggesting a mechanism that by which p62/SQSTM1 could play a role in SAS-induced necroptosis. To test this, three si-p62

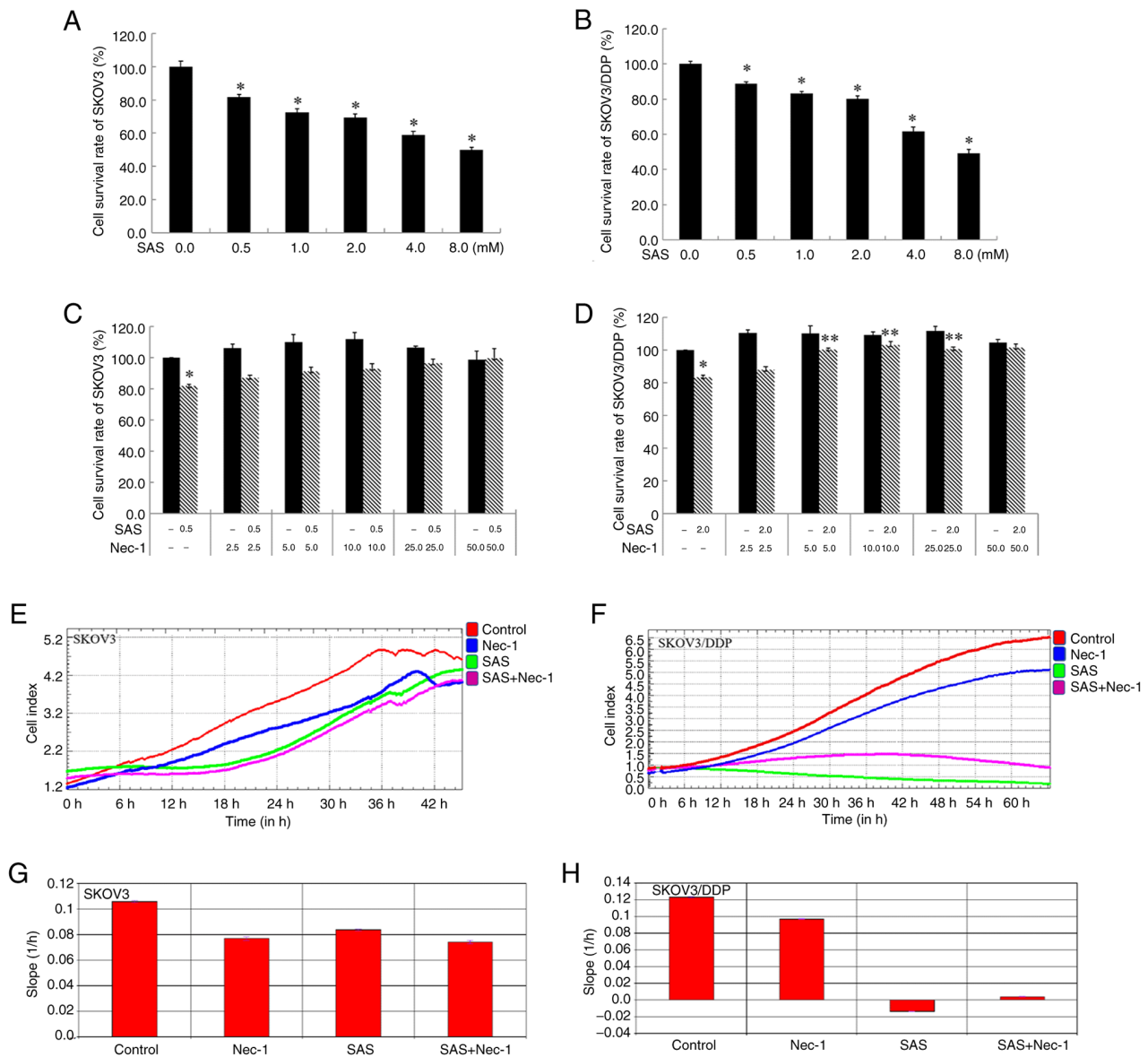


Figure 1. SAS treatment activates the necroptosis pathway in SKOV3/DDP cells. Cell survival rates of (A) SKOV3 and (B) SKOV3/DDP cells treated with different concentrations of SAS. (C) Effects of Nec-1 and 0.5 mM SAS on the survival rate of SKOV3 cells. * $P < 0.05$ vs. Control. (D) Effects of Nec-1 and 2 mM SAS on the survival rate of SKOV3/DDP cells. * $P < 0.05$ vs. Control; ** $P < 0.05$ vs. SAS only. (E) Cell number of SKOV3 cells following treatment with 10 μ M Nec-1 and 0.5 mM SAS. (F) Cell number of SKOV3/DDP cells following treatment with 10 μ M Nec-1 and 2 mM SAS. (G) Slope of SKOV3 cells following treatment with 10 μ M Nec-1 and 0.5 mM SAS. (H) Slope of SKOV3/DDP cells following treatment with 10 μ M Nec-1 and 2 mM SAS ($n=3$). SAS, sulfasalazine; Nec-1, Necrostatin-1.

plasmids were used in transfection experiments, and si-p62.3 was selected for subsequent studies due to the highest level of transfection efficiency (Fig. 3D and E). Then, the present study immunoblotted necroptosis-related proteins after treatment with si-p62/SQSTM1 and found the ratios of p-RIP1/RIP1, p-RIP3/RIP3 and p-MLKL/MLKL proteins were decreased (Fig. 3F and G). Moreover, in contrast to the si-scramble group, which increased the rate of SKOV3/DDP cell survival after treatment with SAS and Nec-1 compared with SAS alone, following transfection with si-p62, there was no change in the rate of SKOV3/DDP cell survival in the 10 μ M Nec-1 and SAS combination group compared with the SAS group (Fig. 3H).

ZZ domain of p62/SQSTM1 regulates necroptosis in SAS-induced SKOV3/DDP cells. The molecular structure of

p62/SQSTM1 contains a ZZ domain that binds to RIP1. In the present study, p62/SQSTM1 protein with a ZZ domain deletion was overexpressed in ovarian carcinoma SKOV3 cells (Fig. 4A and B). Following the aforementioned overexpression, the ratios of p-RIP1/RIP1, p-RIP3/RIP3 and p-MLKL/MLKL were decreased (Fig. 4C and D).

Discussion

The modulation of necroptosis, a type of non-apoptotic programmed cell death, seems to be a promising approach to overcoming apoptotic drug resistance (20,21). To improve the response to acquired resistance, trastuzumab resistance is sensitized through the necroptosis pathway in breast cancer, and the survival rates of patients with breast cancer are affected (22).

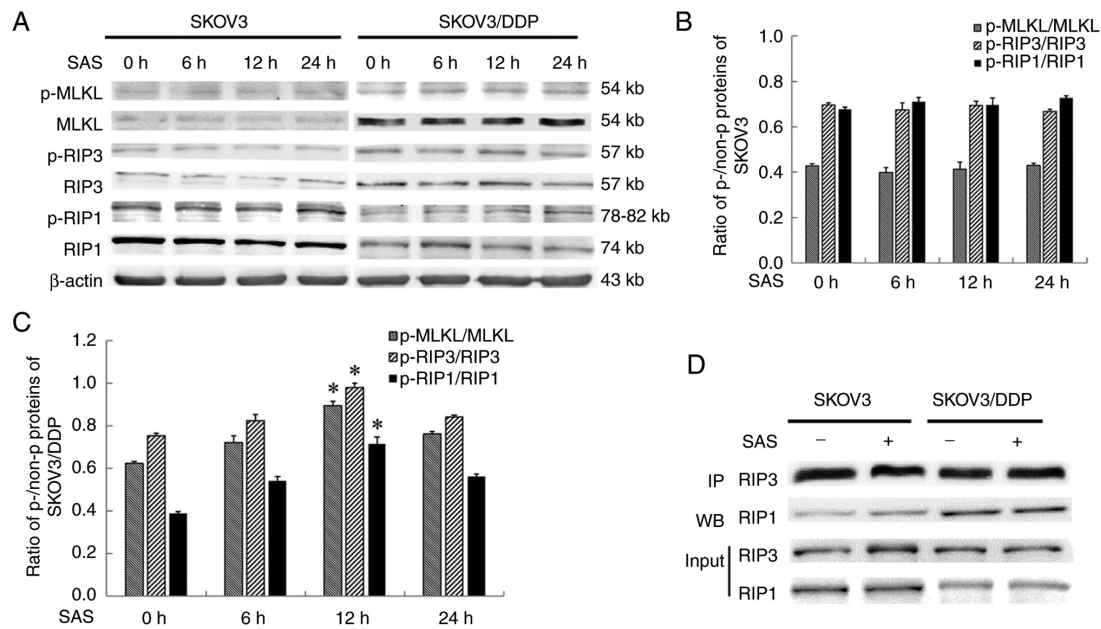


Figure 2. SAS treatment activates necrosome formation in SKOV3/DDP cells. (A) Protein expression levels of p-MLKL/MLKL, p-RIP3/RIP3 and p-RIP1/RIP1 in SKOV3 cells treated with 0.5 mM SAS, and SKOV3/DDP cells treated with 2 mM SAS. Relative protein expression levels of (B) SKOV3 cells treated with 0.5 mM SAS and (C) SKOV3/DDP cells treated with 2 mM SAS (n=3). *P<0.05 vs. the respective treatment groups at 0 h. (D) The combination of RIP1 and RIP3 protein expression was detected using co-immunoprecipitation. SAS, sulfasalazine; p-, phosphorylated; MLKL, mixed lineage kinase domain like protein; RIP, receptor interacting protein kinase.

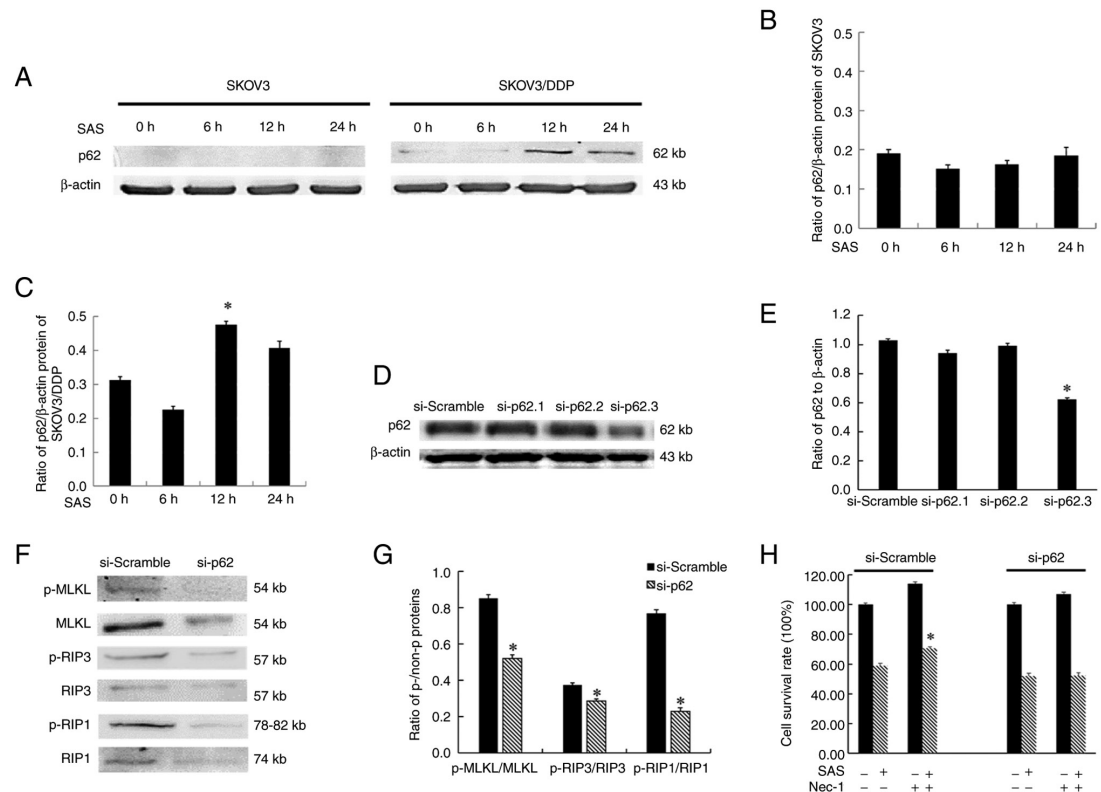


Figure 3. P62/SQSTM1 plays a role in SAS-induced necroptosis in SKOV3/DDP cells. (A) p62/SQSTM1 protein expression in SKOV3 and SKOV3/DDP cells induced by SAS at different time points. Relative protein expression levels of (B) SKOV3 and (C) SKOV3/DDP cells. Protein expression was normalized to the intensity of β -actin and results are expressed relative to the value of the control group. *P<0.05 vs. 0 h group. Values are displayed as the mean $\pm$ SEM (n=3). (D) p62/SQSTM1 protein expression in SKOV3/DDP cells following transfection with siRNA and (E) relative protein expression levels. Protein expression was normalized to the intensity of β -actin and results are expressed relative to the value of the scrambled group. *P<0.05 vs. Scramble group. Values are displayed as the mean $\pm$ SEM (n=3). (F) Protein expression levels of p-MLKL/MLKL, p-RIP3/RIP3 and p-RIP1/RIP1 in SKOV3/DDP cells transfected with siRNA against p62/SQSTM1 and the (G) relative protein expression levels. *P<0.05 vs. si-Scramble group. Values are displayed as the mean $\pm$ SEM (n=3). (H) Survival rate of SKOV3/DDP cells transfected with si-p62 following treatment with SAS and/or Nec-1. Results are expressed relative to the value of the control group. *P<0.05 vs. SAS group. Values are displayed as the mean $\pm$ SEM (n=3). SAS, sulfasalazine; SEM, standard error of the mean; siRNA, small interfering RNA; scrambled control, negative control; p-, phosphorylated; MLKL, mixed lineage kinase domain like protein; RIP, receptor interacting protein kinase.

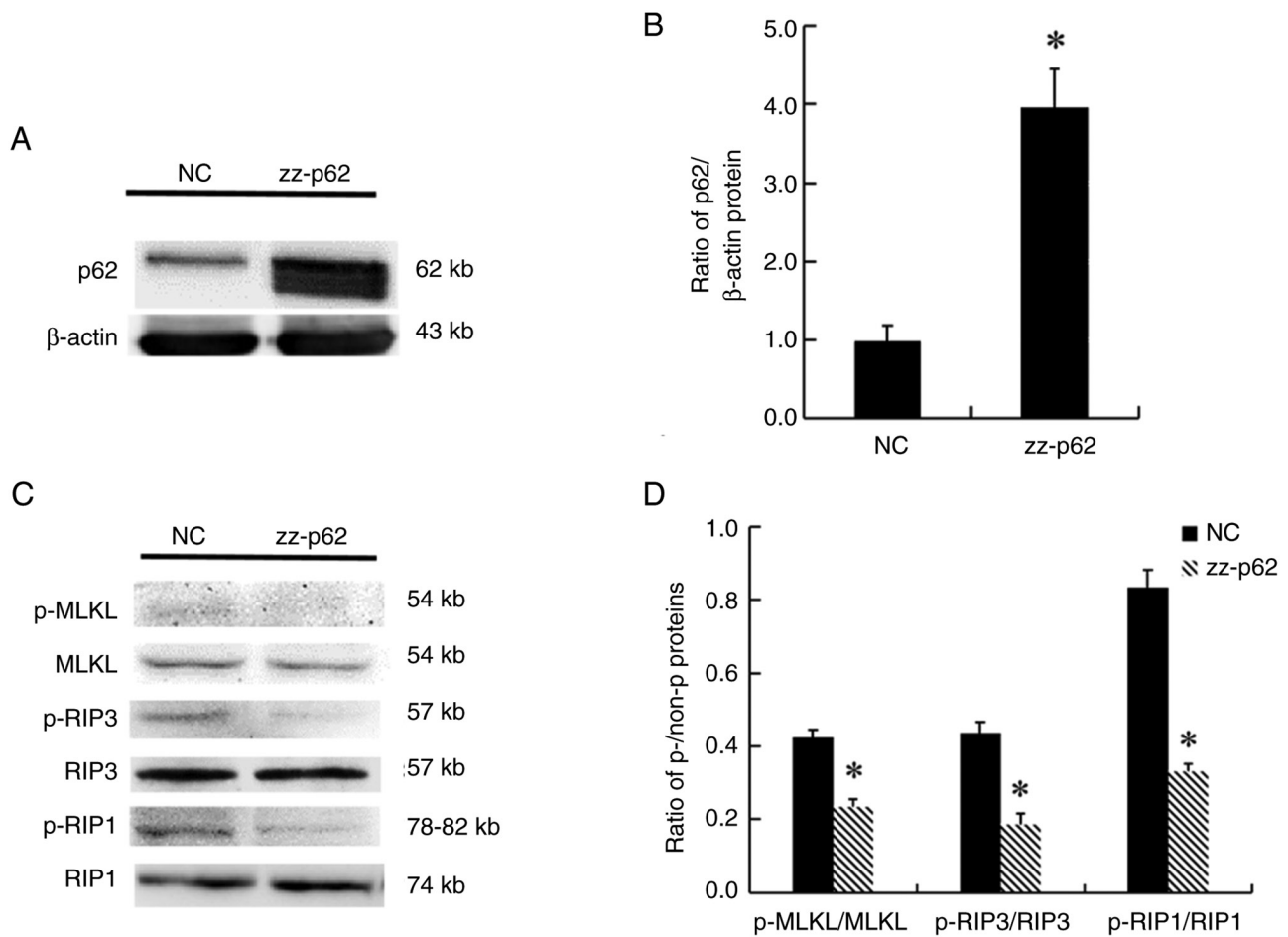


Figure 4. ZZ domain of p62/SQSTM1 regulates necroptosis in SAS-induced SKOV3/DDP cells. (A) p62/SQSTM1 protein expression in SKOV3 cells following overexpression of ZZ domain deletion of p62/SQSTM1. (B) Protein expression was normalized to the intensity of β-actin and results are expressed relative to the value of the NC group. * $P < 0.05$ vs. NC group. Values are displayed as the mean $\pm$ SEM (n=3). (C) Protein expression levels of p-MLKL/MLKL, p-RIP3/RIP3 and p-RIP1/RIP1 in SKOV3 cells with overexpression of ZZ domain deletion p62/SQSTM1 and (D) relative protein expression levels. * $P < 0.05$ vs. NC group. Values are displayed as the mean $\pm$ SEM (n=3). SAS, sulfasalazine; NC, negative control; SEM, standard error of the mean; p-, phosphorylated; MLKL, mixed lineage kinase domain like protein; RIP, receptor interacting protein kinase; ZZ, zinc finger.

Results of a previous study demonstrated that SAS induces the apoptosis of cisplatin-tolerant small cell lung cancer cells (23), and may also induce apoptosis and autophagy in glioma U251 cells (24). In the present study, SKOV3/DDP cells exhibited a higher tolerance to SAS compared with SKOV3 cells. Results of the MTT assay and RTCA demonstrated that the response to Nec-1 also differed between SKOV3 and SKOV3/DDP cells. Notably, the survival rate of SKOV3/DDP cells was increased following treatment with a combination of SAS and Nec-1, while the survival rate of SKOV3 cells was not increased following treatment with a combination of SAS and Nec-1. In SKOV3/DDP cells, p- and non-p-proteins, RIP1 and RIP3, formed complexes, and these were increased following treatment with SAS. These results indicated that the necroptosis could be induced in SKOV3/DDP cells by SAS and increased the sensitivity of SKOV3/DDP cells to SAS.

The results of our previous study revealed that the multi-functional protein p62/SQSTM1 is expressed at low levels in SKOV3 cells, and expressed at high levels in SKOV3/DDP cells (18). Results of the present study demonstrated that the protein expression levels of p62/SQSTM1, RIP1 and RIP3 were increased in SKOV3/DDP cells following SAS treatment

for 12 h. Although autophagy directly inhibits necroptosis through degradation of the kinase RIP1 (25), p62/SQSTM1 also co-immunoprecipitated with RIP1 and provided a scaffold for necroptosis signaling following treatment with tumor necrosis factor-related apoptosis-inducing ligand (19). The results of the present study also revealed that the expression levels of necroptosis-associated proteins, namely, p-RIP1, p-RIP3 and p-MLKL, were decreased following SAS treatment and p62/SQSTM1 knockdown in SKOV3/DDP cells. These results suggested that the p62/SQSTM1 protein may be required for RIP1 localization to autophagy machinery, leading to efficient assembly and activation of the necrosome in SAS-induced SKOV3/DDP cells. The ubiquitination and kinase activity of RIP1 dictates whether TNF signaling leads to activation of NF-κB; thus, protecting cells from apoptosis-mediated death (26), and triggering apoptosis (27) or necroptosis (28). In addition, TNF-α promotes necroptosis and apoptosis through various complexes involving RIP1, and the balance of protein activity controls caspase-dependent and -independent cell death (19). The results of the present study demonstrated that under transfection with si-p62, cell survival induced by Nec-1 and SAS was not increased compared with

the survival of SKOV3/DDP cells following treatment with SAS. These results indicated that p62/SQSTM1 inhibition led to the activation of apoptosis, rather than necroptosis. The p62/RIP1/RIP3/MLKL pathway could be involved in necroptosis in SKOV3/DDP cells treated with SAS, and may increase the sensitivity of cisplatin-resistant ovarian cancer cells.

Notably, the multi-domain adapter protein, p62/SQSTM1, contains rich protein interaction sequences and domains, and plays certain functions in tumor progression depending on the interaction factors recruited. Mutations in the p62/SQSTM1 UBA domain increases the localization of hexokinase 2 on the mitochondria, and increases the phosphorylated ubiquitin form of Parkin (29). This stabilizes the mitophagy process in ovarian cancer A2780 cells, ultimately enabling cell survival. p62/SQSTM1 exhibits pro-death functions through the functional domain, ubiquitin-associated domain and LC3II-interacting region domain interacting with caspase-8 (15). In addition, the ZZ domain of p62/SQSTM1 binds to N-terminally arginylated proteins, and these are considered activators of the autophagic activity of p62/SQSTM1, rather than autophagy cargos (30). The results of a previous study demonstrated that SAS is a classic inhibitor of NF- κ B (24). Notably, the ZZ domain of p62/SQSTM1 regulates K63-associated ubiquitination of RIP1, and loss of the ZZ domain from p62/SQSTM1 leads to poor proliferative capacity and high levels of apoptosis in SKOV3 cells. This ultimately leads to higher levels of cisplatin sensitivity (31). In the present study, the effects of necroptosis were determined using mutant p62/SQSTM1 molecules lacking the ZZ domain required for RIP1 interaction. Notably, the ZZ domain deletion of p62/SQSTM1 significantly inhibited p-RIP1, p-RIP3 and p-MLKL expression levels in SKOV3 cells.

The present study exhibits limitations, for example, there remains to be a lack of experiments on the colocalization of p62/SQSTM1 with the necrosome after the induction of SAS treatment, the interaction of ZZ with the necrosome and the two cells different response when treated with the same concentration of SAS. Moreover, in mammals, there are two autophagy receptors; namely, neighbor of BRCA1 gene 1 and p62/SQSTM1, and these both contain a ZZ domain. These results suggest that activation of the necrosome was regulated by the ZZ domain of p62/SQSTM1; however, the functional significance of the multi-specific binding ability of the aforementioned ZZ domains is yet to be fully established. Further studies should address these questions to fully clarify the function of p62/SQSTM1 by serving as a scaffold for necrosome in ovarian cancer.

Collectively, certain drugs were able to induce necroptosis in SKOV3/DDP, while p62/RIP1/RIP3/MLKL was associated with the induction of necroptosis and increased the sensitivity of cisplatin-resistant ovarian cancer cells. Thus, therapeutic targeting of necroptosis or its associated p62/RIP1/RIP3/MLKL pathway exhibits potential for restoring cisplatin sensitivity as a overcoming resistance therapeutic target.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NL, CY and HY contributed to design, plan and interpret the data. SL, XZ and WT contributed to acquisition of data. HJ, XYe and XYa carried out analysis and interpretation of data. NL and CY confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (including the data) are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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