Original Article
Effect of siRNA-induced Atg7 gene silencing on the sensitivity of ovarian cancer SKOV3 cells to cisplatin

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Abstract: Ovarian cancer is one of the most common types of gynecological malignant tumors. A proclivity for, or the development of chemoresistance severely affects treatment efficacy for ovarian cancer. Herein we found that as concentrations of cisplatin (DDP) used in SKOV3 cells increased, expression of intracellular reactive oxygen species (ROS) increased as did amounts of proteins of Beclin-1 and Autophagy-Related Gene 7 (Atg7) whereas in contrast, expression of P62 protein decreased gradually. Expression of Atg7 protein in SKOV3 cells in the siRNA-Atg7 transfection treatment group was significantly reduced compared to the negative control group. Post-application of DDP treatments, the apoptotic ratio of SKOV3 cells in the siRNA-Atg7 transfection group increased, and the cell survival rate decreased to a level significantly lower than in the negative control group. Cellular morphological analyses revealed remarkably decreased measures of cell density, as well as shrunk, deformed, and rounded cells with unclear boundaries, and revealed a decreased measures of mitochondrial membrane potential. Taken together, autophagy may be involved in the dynamics and mechanistics underlying DDP resistance in ovarian cancer SKOV3 cells. Thus, inhibition of autophagy through down-regulation expression of Atg7 may have beneficially enhanced the sensitivity of SKOV3 cells to DDP-based chemotherapy which could help improve treatment outcomes for patients afflicted by ovarian cancer.

Keywords: Ovarian cancer, cisplatin, RNA interference, autophagy, Atg7

Introduction

Ovarian cancer is one of the most common gynecological malignant tumors. Because of a present lack of available effective methods for accurate early diagnosis, most afflicted by ovarian cancer are definitively diagnosed only at advanced stages of the disease. With a high degree of malignancy, the prognosis for those afflicted by ovarian cancer is typically poor. The most used and standard treatment regimen for affliction by ovarian cancer is cytoreductive surgery supplemented with platinum-based chemotherapy. However, the development of, or a proclivity to chemoresistance severely affects treatment efficacy and is a main cause of recurrence of ovarian cancer and of a low and decreased survival rate. Thus, approaches are needed that can help to increase sensitivity to treatment with chemotherapy and concomitantly reduce the development of drug resistance, and, these are presently major areas of research in ovarian cancer studies [1]. As a common physiological phenomenon in eukaryotes, autophagy can degrade macromolecular substances in cytoplasm and endogenous substrates in vesicles to achieve recirculation, and both are crucial to maintaining intracellular homeostasis and cellular growth [2]. Recent research has indicated that many types of antitumor drugs induce apoptosis of tumorous cells while also inducing activation of autophagy. Drug-induced autophagy in tumorous cells plays two important and differing roles. One role is to serve as a defense mechanism meant to protect cells and prevent cellular damages induced by environmental changes [3] while the second role is to initiate the cell-autonomous type 2 cell death pathway [4]. However, thus far, no specifics of the dynamics and mechanisms underlying these different patterns have been clearly identified for drug-induced auto-
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phagy. Beclin-1 was the first identified tumor suppressor gene which directly related to the activation of cellular autophagy. Beclin-1 was also involved in autophagosome generation by forming a complex with class III phosphatidylinositol-3-kinase. Autophagy-Related Gene 7 (Atg7) (E1-like enzyme) has been identified as a critical factor needed for autophagosomal membrane extension by way of its ability to help activate Autophagy-Related Gene 12 (Atg12). P62 protein, which is a product of autophagic degradation, is an adaptor protein related to the ubiquitinated protein aggregate and to LC3-II. By way of interacting with LC3-II via its N terminal, P62 is incorporated into autophagosomes during autophagy whereafter it degrades in the autophagic lysosome. Changes in levels of expression of P62 can help to facilitate calculations of corresponding degrees of autophagy and associated functions. For example, as expression of P62 increased, there was found to be correspondingly weaker autophagy and weakening of its associated functions [5, 6]. Because RNA interference (RNAi) silences expression of related genes in a rapid and efficient manner, this method has been successfully applied in examinations of gene function and for tumor treatment. In this study, we sought to use RNAi technology to correspondingly facilitate development of a novel small interfering RNA (siRNA) that could play a specific role of silencing Atg7 gene expression. We aimed to transfect our novel siRNA into ovarian cancer SKOV3 cells, and to measure the subsequent effects upon the sensitivity of these cells to cisplatin-based chemotherapy. Ultimately, our goal was to identify and provide possible novel molecular targets to help improve chemotherapy and gene therapy treatment outcomes for patients afflicted by ovarian cancer.

Materials and methods

Cell culture

Ovarian cancer cell lines (SKOV3) were purchased from Shanghai cell bank of Chinese Academy of Sciences. We cultured SKOV3 cells in Dulbecco's Modified Eagle Medium (DMEM; containing 10% deactivated FCS, 20 mM Hepes, 2.0 glL⁻¹ sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.6 μg/mL insulin). The samples were cultured in a CO₂ incubator (Thermo, USA) set at 37.0°C, with an atmosphere consisting of 95% air and 5% CO₂. Cells we confirmed in the exponential growth phase were divided into different treatment groups for subsequent experiments.

Examination of proliferation of ovarian cancer cell line SKOV3

Single cell suspensions of ovarian cancer SKOV3 cells in exponential growth phase were prepared at a final concentration of 2 × 10⁴ mL⁻¹, and were inoculated in a 96-well tissue culture plate with 190 μL per well. We then incubated the samples for 24 hours (h) at 37.0°C in an atmosphere constituted of 95% air, 5% CO₂, and at a saturated level of humidity. Next, we cultured cells for an additional 24 h in serum-free DMEM medium. After 48 h of total incubation time, we discarded the culture medium by pipetting. We then added 190 μL of DMEM medium containing 5% FBS and cisplatin (DDP) (Solarbio, Beijing, China) into each well. The concentrations of DDP were 2, 4, 7, 9, 10, 20, 40, and 60 μg/mL⁻¹. We used three replicates to confirm each concentration. We then continue the culture, and added 15 μL of 5 mg/mL⁻¹ thiazolyl blue tetrazolium bromide (MTT) (Beyotime, Shanghai, China) solution to each well after 12 h, 18 h, 24 h, and 48 h. We then placed the sampled in the incubator. After 4 h of incubation, we next carefully removed supernatant by pipetting. Then, we added 150 μL of dimethylsulfoxide (DMSO) into each well whereupon tissue culture plates were placed for gentle shaking on an orbital shaker (Thermo, USA) to allow for complete dissolution of formazan. After 30 minutes (min), we measured absorbance values (OD₅₇₀) at 570 nm using an ELISA system.

Protein extraction and western blotting analyses

We collected cells experimental and control group cells, and then cultured them in a 6-well plate at a volume of 2 mL · well⁻¹ and at a concentration of 2 × 10⁵ cells · mL⁻¹ for 24 h before being we applied treatments of 4 μg/mL⁻¹ DDP. Post-washing samples in ice-cold PBS solution, we removed liquid by passing constituents through filter paper. We then added 200 μL of cell lysis buffer into each well and the entire 6-well plate was placed on ice for 30
min. We then scraped off cells and transferred them into EP tubes promptly stored at -80°C. We then subjected samples to three thaw-freeze cycles, and quantified constituents using BCA Protein Assay Kits (Beyotime, Shanghai, China). We adjusted protein concentrations, added loading buffer, and samples were boiled for 3 min. We subjected the cells to SDS-PAGE electrophoresis, and transferred proteins onto a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). We blocked the membrane overnight, and then incubated samples with primary and secondary antibodies for relevant proteins, followed by staining and statistical analyses.

**siRNA interference technology**

We inoculated SKOV3 cells into a 24-well tissue culture plate at $4 \times 10^4$ cells · well$^{-1}$, and cultured the cells until they reached a level of 50% confluence. Following all manufacturer protocols, we prepared siRNA solutions at concentrations of between 50 to 100 nmol·L$^{-1}$ with 5% FCS medium, used different types of siRNAs, and used control siRNA. 24 h post-transfection, we change the cells into 5% FCS culture medium.

**Expression of Atg7 protein in SKOV3 cells transfected with siRNA-Atg7 or siRNA-control by Western blotting**

We collected cells from treatment groups 48 h post-transfection. We then compared relative levels of expression of Atg7 protein in cells in the siRNA-Atg7 group and in the siRNA-control group following the procedures specified above in section 1.3.

**Determination of the effect of cisplatin on survival rates of SKOV3 cells transfected with siRNA-Atg7 or transfected with siRNA-control using MTT analysis**

SKOV3 cells were collected 24 h post-transfection with siRNA-Atg7 or with the siRNA-control, and samples were inoculated in a 96-well tissue culture plate at $6 \times 10^3$ cells · well$^{-1}$. After 24 h of incubation, we treated the cells with 4 μg·mL$^{-1}$ DDP. Cells untreated with DDP were used as the control group, and we used 4 replicate wells for each group. 24 h post-treatment, we calculated survival rates of cells in treatment groups following procedures specified in section 1.2 above.

**Annexin V-FITC/PI staining and flow cytometry analysis**

We transfected SKOV3 cells with siRNA-Atg7 or with the siRNA-control and diluted all samples were diluted to $5 \times 10^6$ cells · mL$^{-1}$. We then inoculated the cells into a 6-well tissue culture plate, and treated the samples with 4 μg·mL$^{-1}$ DDP after incubating for 24 hours. After these culturing steps, we digested collected cells with 0.25% trypsin (without EDTA). We applied pre-cooled 0.1% BSA-PBS to samples, centrifuged them for 5 min, and twice repeated washing steps. We then added 400 μL of buffer to the suspended cells. We then added 5 μL of annexin V-FITC, mixed samples, and placed samples in light free conditions wherein they incubated at 4°C for 15 minutes. Lastly, we added 10 μL of propidium iodide (PI) to sample mixtures. We filtered cells through 400 μm mesh screens and into single-cell suspension preparations. We used preset wavelengths to collect cells, used FITC green fluorescence detection facilitated through the FITC channel (FL1), and used PI red fluorescence, which was detected through the PI channel (FL2). Concurrently, normal cells (with induction for apoptosis) were also used as controls for fluorescence by way of compensation regulation and so as to remove spectral overlap and set the location of the cross gate.

**Statistical analyses**

We performed statistical analyses within SPSS 19.0 software (Version 19.0, Chicago, IL, USA) and GraphPad Prism 8.0.1. We used one-way analysis of variance (ANOVA) to compare means between various treatment groups. For comparisons between 2 groups, unpaired Student’s t tests were used. We used $P$ values < 0.050 as the level of statistical significance at which the null hypothesis of no differences among treatment groups was rejected.

**Results**

**Effect of cisplatin on the proliferation of SKOV3 human ovarian cancer cells**

In order to examine inhibitory effects of the chemotherapy drug DDP on proliferation of human ovarian cancer cell line SKOV3, we used the MTT method. Results indicated effects of DDP on survival rates of ovarian cancer SKOV3 cells changed in a dose-dependent
manner and in a time-dependent manner. According to proliferation inhibition rate of DDP-treated cells in Figure 1A, the optimal effect concentration was determined to be 4 μg·mL\(^{-1}\) and the optimal effect time was 24 h. Therefore, we used a DDP concentration of 4 μg·mL\(^{-1}\) for all subsequent experiments.

**DDP induces oxidative stress in the human ovarian cancer cell line SKOV3**

We used an ROS kits in order to determine measures of changes of the levels of intracellular ROS in human ovarian cancer cell line SKOV3 post-DDP treatment. As viewed Figure 1B, 24 hours post-DDP treatment, expression of ROS as detected by flow cytometry was increased in a dose-dependent manner. This result indicated that the application of DDP induced oxidative stress responses in the human ovarian cancer cell line.

**DDP induced autophagy in human ovarian cancer cell line SKOV3**

Resultant levels of expression of autophagy-related proteins Beclin-1, P62, LC3I, LC3II and Atg7 in SKOV3 cells 24 h post-treatment with different concentrations of DDP were assessed using Western blotting. As viewed in Figure 1C, as concentrations rose from 0 to 2, 4, and up to 8 μg·mL\(^{-1}\), expression of Beclin-1, and Atg7 proteins gradually increased, whereas in contrast expression of P62 protein gradually decreased. The conversion of LC3I to LC3II also gradually increased. These results indicated that DDP could induce the autophagy in SKOV3 human ovarian cancer cells with a dosage dependent manner.

**siRNA-Atg7 effectively silenced expression of Atg7 protein in SKOV3 cells**

Results from western blotting analyses indicated that the levels of expression level of Atg7 protein in SKOV3 cells were significantly downregulated in the siRNA-control group compared with respective results for the siRNA-Atg7 transfection treatment group (\(P < 0.01\)). This result indicated that siRNA-Atg7 can effectively silence expression of Atg7 protein in SKOV3 cells (Figure 2).

**Inhibition of autophagy enhances DDP-induced death of SKOV3 human ovarian cancer cells**

To investigate the role of autophagy in DDP-induced death of SKOV3 ovarian cancer cells,
we measured expression of Atg7, which is a critical autophagy-related protein. We downregulated siRNA-Atg7 expression and examined cell survival rates. As viewed in Figure 3A, when Atg7 expression was down-regulated, the percentage of surviving SKOV3 cells that had been treated with 4 μg·ml⁻¹ of DDP decreased to 54.8%, which was a level that was markedly lower for respective cell survival in the negative control group (78.0%; *P < 0.05). We determined measures of apoptotic ratio by using flow cytometry combined with annexin V-FITC/PI staining. As viewed in Figure 3B, apoptotic ratios significantly increased in the Atg7 down-regulation treatment group. Meanwhile, observed changes in cell morphology using inverted microscopy (Figure 3C). Our results indicated that cells in the control group displayed vigorous adherent growth, a close connection with one another, a characteristically polygonal shape, a distinct cell membrane, and abundant cytoplasm. In contrast, cells in the drug treatment group and the in the Atg7 silenced group were found to have exhibited decreased measures of density. In the DDP/siRNA-Atg7 combined treatment group, our results indicated that measures of cellular density were significantly reduced, that measures of cell-cell gaps were enlarged, that the number of suspended cells were significantly increased, that volumes of cell cytoplasm were reduced, and indicated that cells gradually shrunk, became rounded in shape, and lost their normal morphology with emergent difficult to distinguish and obscure boundaries.

Inhibiting autophagy markedly reduced mitochondrial membrane potential of SKOV3 ovarian cancer cells

We determined measures of mitochondrial membrane potential by applying JC-1 staining. Results from flow cytometry indicated that in the Atg7 silenced treatment group, the ratio between red fluorescence intensity generated by JC-1 complexes and green fluorescence intensity generated by JC-1 monomers had an obvious observable reduction (Figure 3D). This result indicated that inducing inhibition of autophagy resulted in the enhancement of induced reductions in mitochondrial membrane potential for observations of SKOV3 cells and ultimately lead to apoptosis.

Discussion

Autophagy is a protein degradation pathway whereby eukaryotic cells remove intracellular aggregates, damaged cells, and organelles to maintain intracellular homeostasis. Autophagy plays important roles in physiological and pathological cell processes, and it is an indispensable process required for death-related cellular activities. Findings suggest that abnormal autophagy activity is directly related to the initiation, development, and treatment of tumors. As a housekeeping mechanism for cellular homeostasis, autophagy also helps to maintain cell homeostasis by disposing wastes, reducing DNA damage, and minimizing chromosomal instability. Inducing, or otherwise resultant down-regulation of autophagy is known to cause gene mutations, increase instances of malignant phenotypes of cells, and can promote the formation and development of tumors [7, 8]. On the other hand, some research has indicated that autophagy can function as a protective mechanism for tumorous cells. Post-formation of a tumor, cells rely on autophagy as a means to obtain energy, to survive nutrient-and oxygen-deficient environments, and rely on autophagy to protect tumor cells against apoptosis, and ultimately for survival [9]. Likewise to its role in the formation and development of tumors, autophagy also plays dual roles in tumor treatment. Recent research has indicated that many types of antitumor drugs can induce apoptosis while concomitantly increasing measures of autophagy activity in tumor cells. Under different experimental conditions, research has indicated that an increased rate and occurrences of autophagy can result in synergis-
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A

Cell viability (%)

DDP
siRNA-control
siRNA-Atg7

B

Propidium iodide

FITC-A

DDP
siRNA-control
siRNA-Atg7

C

Control
DDP
DDP+siRNA-control
DDP+siRNA-Atg7

Apoptotic cells (%)
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Reactive oxygen species (ROS) is a general term inclusive of reactive oxygen-containing compounds generated by organisms during aerobic metabolism and related processes. Recent research indicated that ROS served as signaling molecules with roles in various intracellular signal transduction pathways. Currently, there are a series of known complex signal transductions and interactions between ROS and autophagy. ROS can be known to be able to participate in the initiation of autophagy, but, in contrast, autophagy may also serve as a buffer system that acts to help control ROS levels [12, 13].

Research has also revealed that anti-tumor drugs can cause stress related responses in tumorous cells, and can stimulate ROS generation through related pathways including such as for endoplasmic reticulum, which in turn, induces autophagy. This enhancement of autophagy as induced by chemotherapy is an adaptive mechanism whereby tumorous cells resist apoptosis in order to prolong survival. In MCF-7 breast cancer cells, ROS induced the dephosphorylation of Ser321 and promoted nuclear transport, consequently thereby promoting protective autophagy, reducing oxidative damage, and enhancing drug resistance [14].

Our novel results indicated that as concentrations of DDP used in SKOV3 ovarian cancer cells increased, fluorescence signal intensity as a proxy for ROS concentrations also gradually increased. Accordingly, these results also indicated that expression of autophagy-related proteins Beclin1 and Atg7 also increased gradually, whereas expression of P62 protein, an autophagic degradation substrate, decreased gradually. Besides, the conversion of LC3I to LC3II also gradually increased. These results indicated that our application of DDP induced oxidative stress and autophagy in SKOV3 ovarian cancer cells, and the effect was found to be dose-dependent.

Figure 3. Effect of inhibition of autophagy on cisplatin-induced apoptosis of SKOV3 cells. A. MTT method detected the survival rate of SKOV3 cells induced by cisplatin after siRNA-Atg7 transfection. B. Annexin V-FITC/PI staining detected apoptosis rate of SKOV3 cells induced by cisplatin after siRNA-Atg7 transfection. C. Observations of morphology of KOV3 cells induced by cisplatin post-siRNA-Atg7 transfection with an inverted microscope. D. JC-1 kit detected mitochondrial membrane potential of SKOV3 cells induced by cisplatin after siRNA-Atg7 transfection. Data are presented as mean ± SD, *P < 0.05, **P < 0.01.
Based upon results from already completed studies, we sought to clarify the effects of DPP-induced autophagy enhancement in SK-OV3 ovarian cancer cells and to clarify resultant impacts upon sensitivities of cells to chemotherapy drugs. Thus, we used siRNA-Atg7 to inhibit autophagy. Our findings indicated that induced silencing of expression of Atg7 increased measures of sensitivity of SKOV3 cells that we treated with cisplatin. Reductions in mitochondrial membrane potential are a signature of early events in the dynamics and mechanisms underlying apoptosis. Thus, we determined mitochondrial membrane potential of SKOV3 cells by using JC-1 staining. Our findings indicated that red fluorescence generated by the JC-1 complexes in the Atg7 down-regulation treatment group was obviously weakened. This finding indicated that autophagy inhibition enhanced DDP-induced reduction of mitochondrial membrane potential in SKOV3 cells, which ultimately lead to apoptosis. In the DDP/siRNA-Atg7 combined treatment group, our finding indicated that the percentage of SKOV3 cells that survived decreased to 54.8%, which was a level significantly lower than we observed in the siRNA control treatment group (78.0%; P < 0.05). Our findings based from flow cytometry analyses with V-FITC/PI staining used to assess apoptosis ratio of cells indicated that the Atg7 downregulation treatment group displayed significantly increased ratios compared to the control treatment groups. In terms of morphological changes, we found that cell density was markedly decreased, that cells shrunk and became rounded in shape, and that cells exhibited abnormal morphology and obscure boundaries. These results further indicated that activated autophagy helped to mediate drug resistance of SKOV3 ovarian cancer cells with respect to applications of DDP, and that autophagy acted as an adaptive responsive measure of cells activated such as to resist chemotherapy induced stress and damages.

Much research has supported the conclusion that inducing autophagy may serve as a new method to enhance the clinical efficacy of chemotherapy. Research by S. Salcher et al. indicated that etoposide and adriamycin induced autophagy in cases of neuroblastoma. The activation of autophagy is known to be related to endoplasmic reticulum stress, which can in turn stimulate the production of ROS. Blocking ROS formation by application of N-acetylcysteine can consequently inhibit autophagy formation. Inhibition of autophagy by application of LC3-knockdown has been found to significantly increase etoposide- and doxorubicin-induced apoptosis [15].

In examinations of neuroblastoma, histone deacetylase (HDAC) 10 promoted autophagy mediated cell survival. Increased expression of HDAC10 resulted in reductions of the efficacy of doxorubicin used to treat neuroblastoma. Inhibition of expression of HDAC10 by MS-275 increased corresponding measures of sensitivity of neuroblastoma cell lines to treatment with doxorubicin [16]. Autophagy of glioma stem cells can subsequently result in the enhancement of KDR/VEGFR-2 phosphorylation and of angiogenesis by way of activating the PI3K-AKT pathway, and this may be a potential mechanism underlying and important in the dynamics of autophagy resistance [17]. Research has indicated that the PI3K-Akt-mTOR pathway participates in dynamics and processes related to survival of cancer cells post-treatment with anti-cancer drugs. After 5-FU treatments applied to SGC7901 gastric cancer cells, researchers found that the number of autophagic vacuoles and that the levels of expression of microtubule associated protein 1 light chain 3 had significantly increased. Following a treatment which combined SiRNA, interfered PI3K III, and 5-FU, research indicated that mitochondrial membrane potentials were significantly reduced, that expression of autophagic markers were significantly downregulated, and documented that proliferation of SGC7901 cells was significantly suppressed. These results suggested that inhibition of autophagy enhanced the killing effect of 5-FU on gastric cancer cells, while also having indicated that autophagy induced by 5-FU alone was protective of autophagy [18]. The combination of LY294002 (LY) and 5-Fu on a single carrier for the treatment of esophageal squamous cell carcinoma (ESCC) had a higher resultant cytotoxicity than did the application of only a single drug. Further, LY induced inhibition of autophagy, and enhanced the sensitivity of cancer cells to 5-FU, ultimately leading to increased measures of cell death [19].

In conclusion, we found that autophagy was involved in the resistance of ovarian cancer
cells to treatment with DDP. Further, our results indicated that inducing the inhibition of autophagy subsequently enhanced the sensitivity of SKOV3 cells to treatments with DDP. Thus, we postulate that during the course of ovarian cancer chemotherapy treatments, monitoring and regulating levels of autophagy in tumorous cells, as well as combining chemotherapy with autophagy related gene targeting may help to improve outcomes for patients undergoing ovarian cancer treatment.

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Disclosure of conflict of interest

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