Life Sciences 232 (2019) 116561

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

ABT737 enhances ovarian cancer cells sensitivity to cisplatin through regulation of mitochondrial fission via Sirt3 activation

Lin Hou[a], Ruobing Wang[b], Haifeng Wei[c], Shouqing Li[c], Lei Liu[c], Xiaodan Lu[c], Huimei Yu[d,*], Ziling Liu[a,**]

[a] Department of Oncology, the First Hospital of Jilin University, Changchun, Jilin 130021, China
[b] Department of Hepatobiliary and Pancreatic Surgery, the First Hospital of Jilin University, Changchun, Jilin 130021, China
[c] Tumor Biotherapy Center, Jilin Province People's Hospital, Changchun, Jilin 130021, China
[d] Department of Pathophysiology, Basic College of Medicine, Jilin University, Changchun, Jilin 130021, China

ARTICLE INFO

Keywords:
ABT737
Cisplatin
Sirt3
Mitochondria
Ovarian cancer

ABSTRACT

Aims: The poor prognosis of ovarian cancer is mainly caused by chemotherapy resistance. Studies show that the Bcl-2 inhibitor ABT737 can significantly improve the effect of cisplatin and induce mitochondrial pathway apoptosis. However, the mechanism of ABT737 increases sensitivity to cisplatin by regulating mitochondrial function remains unclear in ovarian cancer cells. Sirt3, as a histone deacetylase, is involved in the regulation of mitochondrial function in cancers. In this study, we intend to explore the mechanistic link between Sirt3 and mitochondrial dysfunction induced by ABT737 and cisplatin in ovarian cancer cells.

Main methods: Apoptosis was examined by flow cytometry following Annexin V and PI staining. Sirt3 activity was assessed using Sirt3 deacetylase fluorometric assay. The mitochondrial membrane potential was examined by flow cytometry following JC-1 staining. Overexpression and knock-down of Sirt3 were confirmed by western blot analysis. Mitochondrial fission/fusion dynamics were detected by immunofluorescence staining or western blot analysis.

Key findings: Cisplatin accompanied with ABT737 promoted apoptosis and decreased mitochondrial membrane potential. ABT737 enhanced the sensitivity of ovarian cancer cells to cisplatin, which was partly achieved by activating Sirt3 to regulate the mitochondrial fission process.

Significance: This study identified the activation of Sirt3 played an important role in increasing sensitivity of ovarian cancer cells to cisplatin induced by ABT737. Furthermore, Sirt3 might represent a potential therapeutic target for ovarian cancer.

1. Introduction

Ovarian cancer is a global issue for women, which has a poor prognosis with just 40% of five-year survival rate [1]. This kind of disease is usually diagnosed at an advanced stage as there is no effective early screening strategy. Standard treatments for ovarian cancer include platinum-based chemotherapy and cytoreductive surgery. For the former, the drug resistance after long-term treatment limits the efficacy for chemotherapy [2]. Therefore, understanding the mechanism of platinum resistance and finding approaches to overcome them are the key directions of ovarian cancer research.

Cisplatin, as one of the platinum analogues, shows high activity on tumor killing. This therapeutic agent has been used in chemotherapeutics for a long time, but it has the same problem of resistance like other platinum-based chemotherapy drugs. The mechanism of cisplatin resistance is considered to relate with promoting drug efflux, increasing DNA damage repair, reducing cellular uptake, and regulating MLH1 hypermethylation [3]. Some studies show that cisplatin resistance is associated with changing mitochondrial dynamics and prompting the expression of Bcl-2 family protein, which can be reversed by the Bcl-2 inhibitor [4].

B-cell lymphoma 2 (Bcl-2) family proteins are main regulators of the apoptosis process which can be classified as pro-apoptotic and anti-apoptotic proteins [5]. The Bcl-2 family proteins are highly expressed in many cancers [6]. Moreover, the overexpression of anti-apoptotic members of the Bcl-2 family such as Bcl-2, Mcl-1, or Bcl-xL, can

* Correspondence to: H. Yu, Department of Pathophysiology, Basic College of Medicine, Jilin University, No. 126 Xinmin Street, Changchun, Jilin 130021, China.
** Correspondence to: Z. Liu, Department of Oncology, the First Hospital of Jilin University, No. 71 Xinmin Street, Changchun 130021, China.
E-mail addresses: yuhuimei@jlu.edu.cn (H. Yu), drzilingliu@163.com (Z. Liu).

https://doi.org/10.1016/j.lfs.2019.116561
Received 20 February 2019; Received in revised form 10 June 2019; Accepted 10 June 2019
Available online 24 June 2019
0024-3205/

enhance resistance to cisplatin in various other cancer cells [7]. Therefore, Bcl-2 is regarded as the target of cancer treatment. One strategy is to use small molecule inhibitors to interfere with the interaction between Bcl-2 and pro-apoptotic components [8]. By using nuclear magnetic resonance (NMR) screening, ABT737 was found as a small molecule inhibitor of Bcl-2. Mechanistic studies indicated that the function of ABT737 was realized by regulating the signals of apoptosis rather than by directly initiating the apoptotic process [9].

Recently, increasing evidence has shown that Bcl-2 family proteins can regulate the link between cancer and mitochondrial fission/fusion dynamics [10,11]. The pro-apoptotic members of the Bcl-2 family cause mitochondrial fission during the apoptosis process by inhibiting anti-apoptotic Bcl-2 protein-mediated fusion [12]. Moreover, studies demonstrate that the overexpression of BAX and BAK protein can induce mitochondrial fission [13]. Together, these researches show that the Bcl-2 family plays a role in regulating mitochondrial dynamics. However, it still remains unclear how Bcl-2 family proteins influence mitochondrial dynamics at the molecular level.

Sirt3 belongs to the sirtuin family of NAD^+-dependent histone deacetylases, which participates in eliminating reactive oxygen species (ROS) and the regulation of mitochondrial proper function [14,15]. It was reported that Kaempferol induced apoptosis by decreasing Bcl-2 and increasing Sirt3. In addition, the mitochondrial localization of Sirt3 can be observed, which suggests there is a role for Sirt3 in the apoptotic mitochondrial pathway [16]. Recent studies show that Sirt3 is required for apoptosis induced by silencing Bcl-2, which means Sirt3 is an essential pro-apoptotic mediator for Bcl-2 regulated apoptosis [17].

Considering both Sirt3 and Bcl-2 play important regulatory roles in regulating mitochondrial function, we intend to explore whether Sirt3 involved in the regulation of mitochondrial dysfunction which associated with the function of Bcl-2.

2. Materials and methods

2.1. Cell line and cell culture

Human ovarian cancer cells (SKOV3) were obtained from Chinese Academy of Sciences Shanghai Institute for Biological Sciences Cell Resource Center. SKOV3 cells were grown in RPMI Medium 1640 (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (TBD Science, Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Ameresco, USA), at 37 °C in humidified atmosphere of 5% carbon dioxide.

2.2. Antibodies and reagents

Antibodies against Bcl-2 (sc-130,307), BAX (sc-23,959), GAPDH (sc-166,574) and Tom 20 (sc-136,211) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Sirt3 (ab217319), DRP1 (ab184248) and MFN1 (ab57602) were obtained from Abcam (Cambridge, MA). Anti-rabbit IgG HRP-linked antibody (7074), anti-mouse IgG HRP-linked antibody (7076) and antibody against Cleaved caspase 3 (9664S) were purchased from Cell Signaling Technology (Beverly, MA). DAPI (4,6-diamidino-2-phenylindole) (P0131) was purchased from Beyotime (Shanghai, China). Cisplatin (P4394)and MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide] (M2128) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ABT737 (S1002) was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO).

2.3. Transfection assay

SKOV3 cells were plated into 6-well plates the day before transfection to achieve 70%–80% confluency, and then transiently transfected with pcDNA3-Sirt3 plasmid prepared in the laboratory using Lipofectamine 2000 (Invitrogen, NY) according to the manufacturer's instructions.

2.4. Cell viability by MTT assay

SKOV3 cells were seeded at 10000 cells per well in 96-well plates and allowed to grow for 24 h. Cells were treated with cisplatin and/or ABT737 for 6 h, 12 h, 24 h, 48 h. To measure cell viability after the treatment, cells in each well were incubated with 20 μl of MTT dissolved in phosphate buffered saline (PBS) solution at the concentration of 5 mg/mL at 37 °C. After the incubation for 4 h, the medium was removed, and 100 μl dimethyl sulfoxide (DMSO) was added into each well to dissolve formazan in the live cells. Then the absorbance was measured at 570 nm using an automated Microplate Reader (Bio-Rad, CA, USA). Cell viability of each treatment group was expressed as a percentage of the untreated control. All determinations were performed in triplicate.

2.5. Colony formation assay

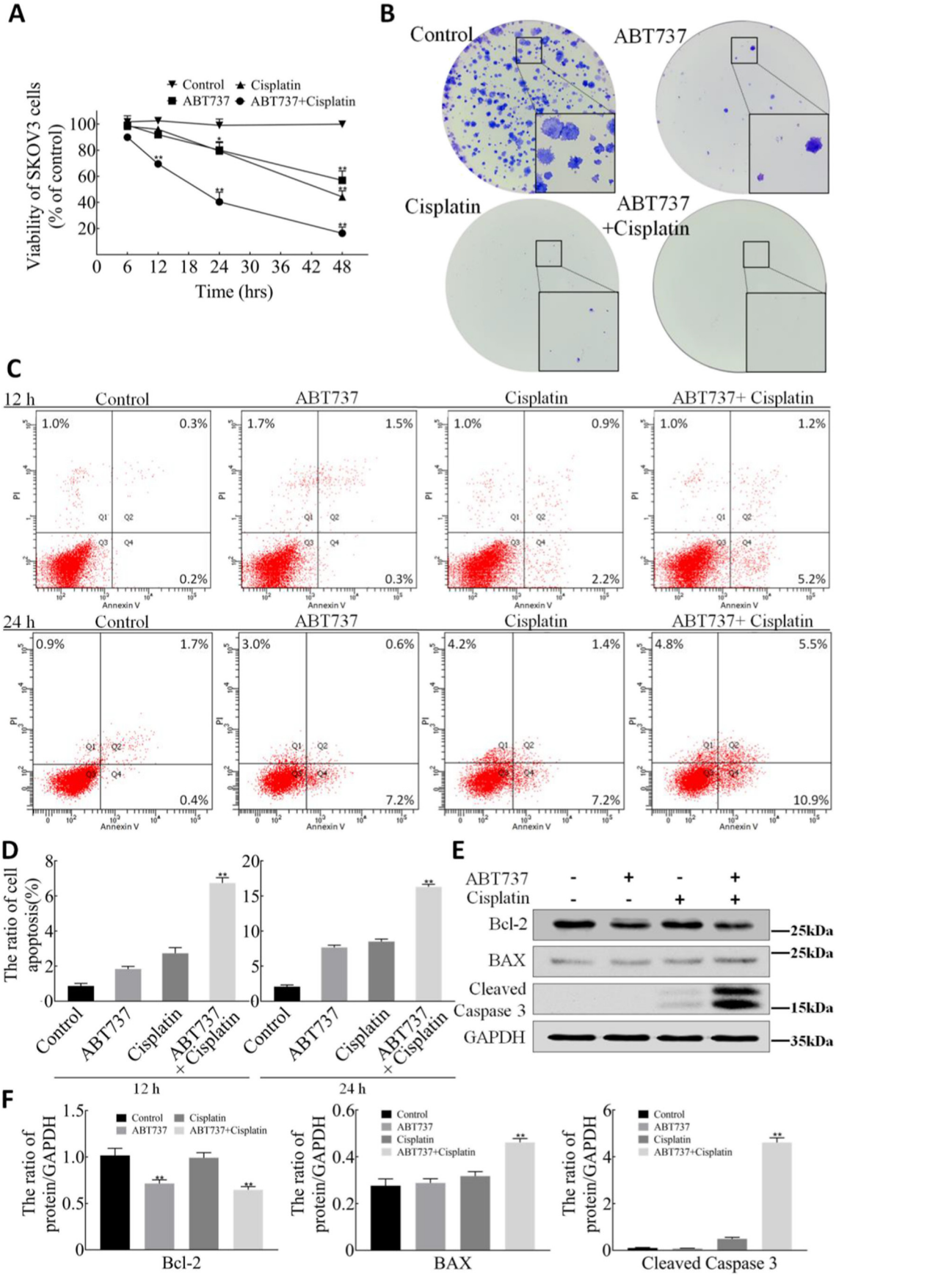
SKOV3 cells were seeded at 1000 cells per well in 6-well plates. After the treatments with cisplatin or/and ABT737, cells were allowed to continue growing. Plates were placed in an incubator and left them there until cells in control dishes have formed sufficiently large clones. After the treatment for 2 weeks, medium was removed, and cells were gently washed in PBS once, fixed and stained in a mixture of 6% glutaraldehyde and 0.5% crystal violet for 2 h. The mixture of glutaraldehyde and crystal violet was removed and cells were rinsed several times with water.

2.6. Western blot analysis

SKOV3 cells were treated with cisplatin and/or ABT737 for 12 h. Then cells were lysed in cell lysis buffer (1% Triton X-100, 10 mM Tris-HCl, 0.015 M NaCl, 1 mM PMSF, 1 mM EDTA, 10 μg/mL of each leupeptin and pepstatin A) and incubated on ice for 30 min. The lysates were centrifuged at 12000 *g* for 10 min at 4 °C, the protein levels of supernatant were quantified with a Pierce BCA kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Proteins were separated on 8–10% polyacrylamide gels, transferred onto PVDF membranes, and blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (0.1% TBS-T) for 1 h at room temperature. The membranes were then probed with diluted primary antibodies overnight at 4 °C, followed by the anti-rabbit-HRP or anti-mouse-HRP secondary antibody respectively for 1 h at room temperature. Secondary antibodies were detected with Pierce ECL (Thermo Fisher Scientific) reagents. Signals were examined by a chemiluminescence detection kit (GE Healthcare, USA). All blots were corrected for loading using GAPDH expression.

2.7. Measurements of apoptosis and mitochondrial membrane potential by flow cytometry

SKOV3 cells were seeded at cells 5×10^5 per well in 6-well plates. After 24 h incubation, cisplatin and/or ABT737 were added to the SKOV3 cells to culture for a further 12 h. After the treatments, cells were trypsinized and stained by Annexin V (BD Biosciences, San Diego, CA) and PI (Cell Cycle and Apoptosis Analysis Kit, Beyotime) to measure the cells experiencing apoptosis according to the manufacturer's protocol. The cells were analyzed using a flow cytometer. Mitochondrial membrane potential (MMP) measurement was detected using JC-1 Mitochondrial Potential Sensors (Invitrogen, USA). After dying with JC-1, the decrease of red/green fluorescence intensity ratio indicates the mitochondrial depolarization. The process was followed by manufacturer's instructions using flow cytometry.



(caption on next page)

Fig. 1. ABT737 sensitized SKOV3 cells to cisplatin.
(A) Ovarian cancer cells were treated with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM) for 6 h, 12 h, 24 h, and 48 h. The viability of SKOV3 cells was determined by MTT assay. The data are presented as the percentage of cell number compared with the control group; $*p < 0.05$ vs. control. $**p < 0.01$ vs. control. (B) In colony formation assay, SKOV3 cells were treated with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM) in 6-well plates for two weeks. (C) Cells were treated with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM) for 12 h or 24 h. Apoptosis was analyzed by flow cytometry after stained with Annexin V/PI. (D) Apoptosis data are expressed as mean ± SEM of three independent experiments; $**p < 0.01$ vs. control. (E) Protein lysates were prepared after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). Western blot analysis was performed with Bcl-2, BAX, Cleaved caspase 3, and GAPDH antibodies. GAPDH was used as the loading control. (F) Quantitation of Bcl-2, BAX, Cleaved caspase 3. The data are expressed as mean ± SEM of three independent experiments; $**p < 0.01$ vs. control.

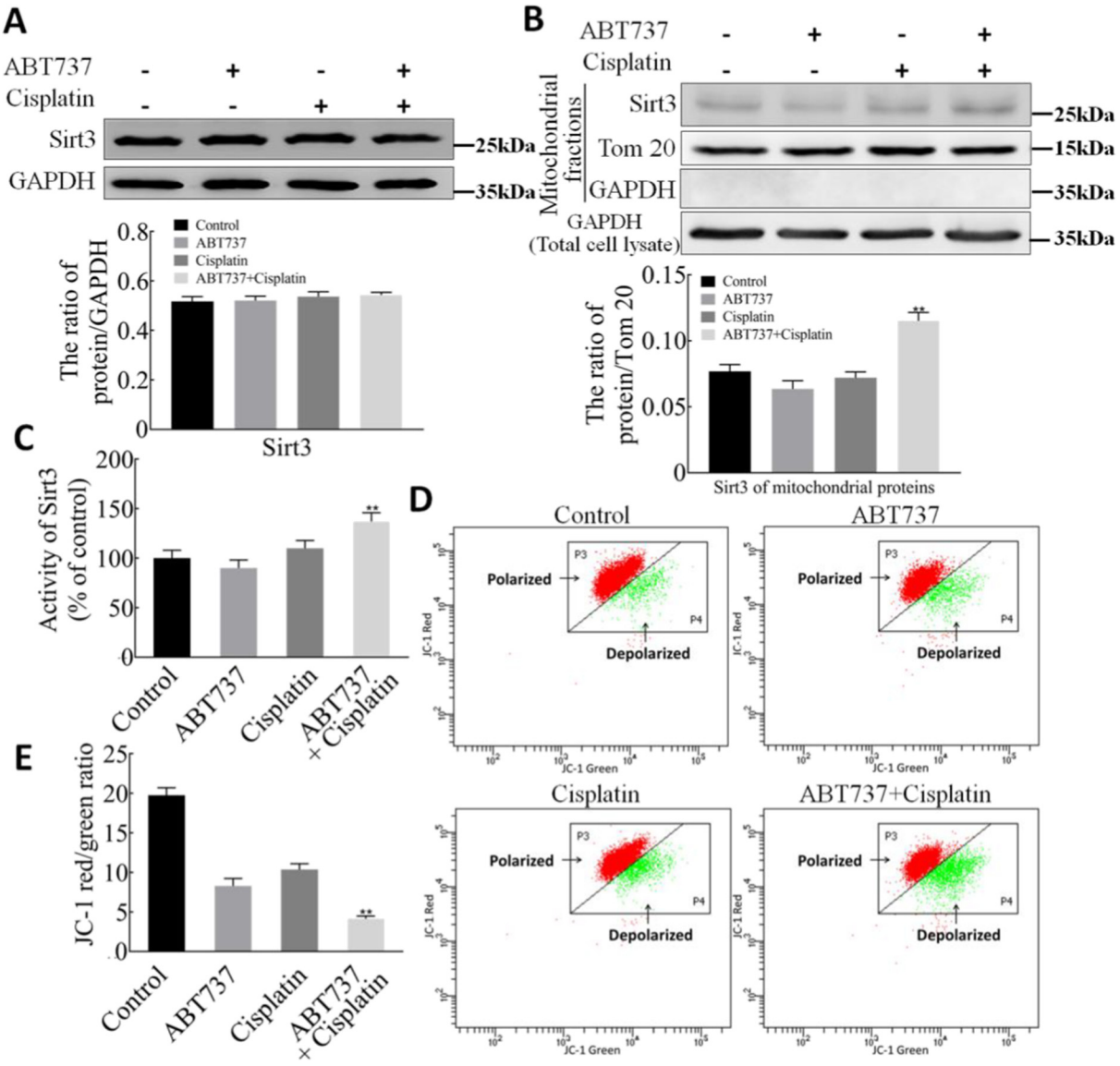


Fig. 2. Cisplatin accompanied with ABT737 up-regulated Sirt3 activity and affect mitochondrial membrane potential.
(A) Protein lysates were prepared after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). Western blot analysis was performed with Sirt3 and GAPDH antibodies. GAPDH was used as the loading control. The data are expressed as mean ± SEM of three independent experiments. (B) Mitochondrial fractions were prepared after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). Western blot analysis was performed with Sirt3, Tom 20, and GAPDH antibodies. Tom 20 was used as the loading control. GAPDH in total cell lysate was used as the positive control. The data are expressed as mean ± SEM of three independent experiments; $**p < 0.01$ vs. control. (C) Sirt3 activity was assessed using a Sirt3 Deacetylase Fluorometric Assay Kit after the treatment of cisplatin (2.5 μg/mL) and/or ABT737 (20 μM) for 12 h. The data are expressed as mean ± SEM of three independent experiments; $**p < 0.01$ vs. control. (D) SKOV3 cells were treated with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM) for 12 h. JC-1 was used to assess mitochondrial membrane potential. The data were obtained by flow cytometry. (E) The MMP data are expressed as mean ± SEM of three independent experiments; $**p < 0.01$ vs. control.

2.8. Mitochondria isolation and measurement of Sirt3 activity

Mitochondria were extracted using the Mitochondria Isolation Kit (Beyotime Biotechnology, Shanghai, China) after SKOV3 cells treated by agents for 12 h. The process of mitochondria isolation was followed by manufacturer's instructions. The mitochondrial isolation buffer was used to resuspend the pallet. Sirt3 activity was assessed using a Sirt3 Deacetylase Fluorometric Assay Kit (Cyclex, Japan) according to the manufacturer's instructions.

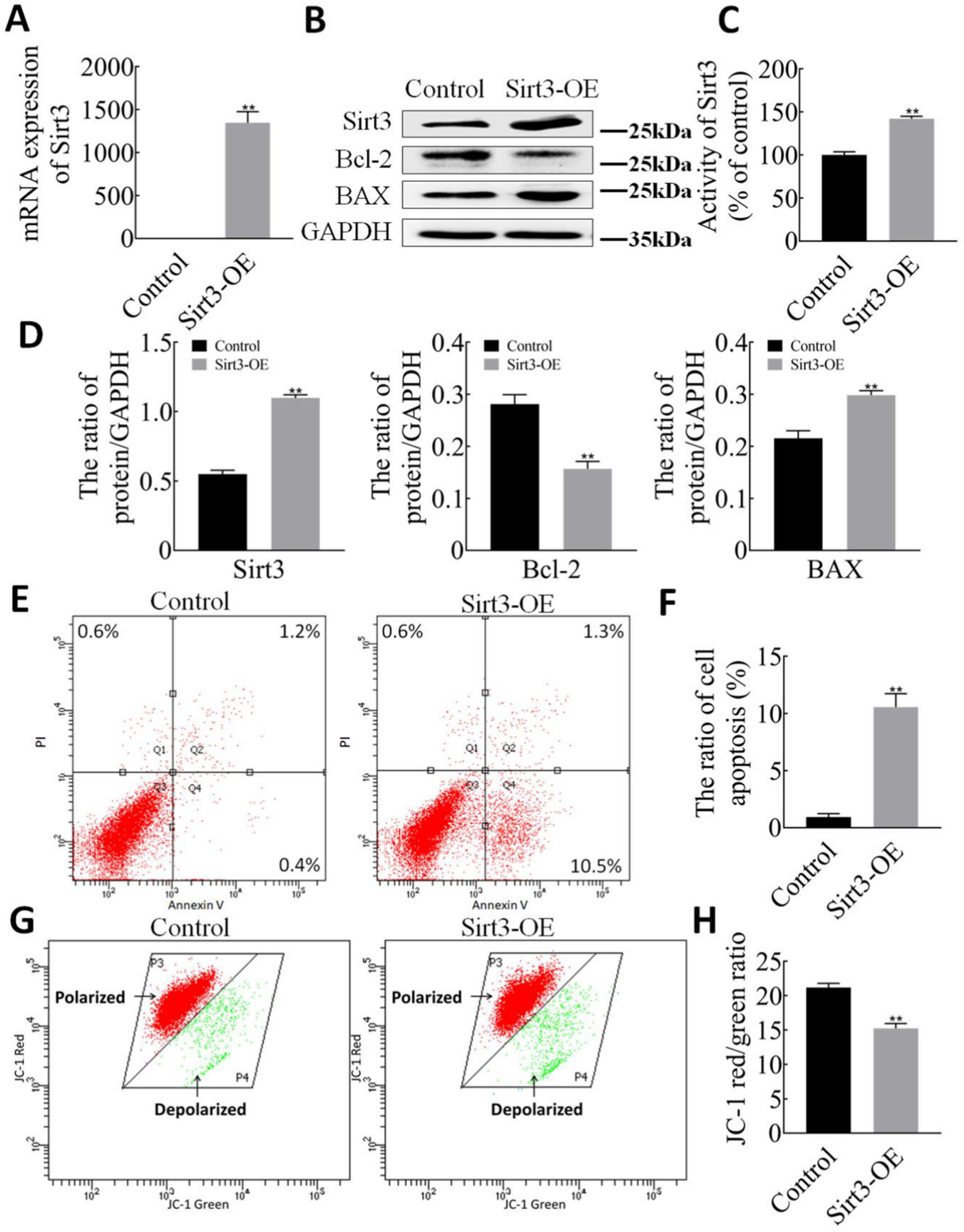


Fig. 3. Activity of Sirt3 was involved in apoptosis and mitochondrial membrane potential.
(A) High level of Sirt3 mRNA was measured by qRT-PCR. The gene was normalized to GAPDH. $^{**}p < 0.01$ vs control cells. (B) SKOV3 cells were transfected with Sirt3 overexpression plasmid. Western blot analysis was performed with Sirt3, BAX, Bcl-2, and GAPDH antibodies. GAPDH was used as the loading control. (C) After transfected with Sirt3 overexpression plasmid, mitochondria were collected by Mitochondria Isolation Kit. The activity of Sirt3 was assessed using a Sirt3 Deacetylase Fluorometric Assay Kit. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (D) Quantitation of Sirt3, BAX, and Bcl-2. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (E) Apoptosis was analyzed by flow cytometry after stained with Annexin V/PI. (F) Apoptosis data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (G) JC-1 was used to assess MMP. The data were obtained by flow cytometry. (H) The MMP data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control.

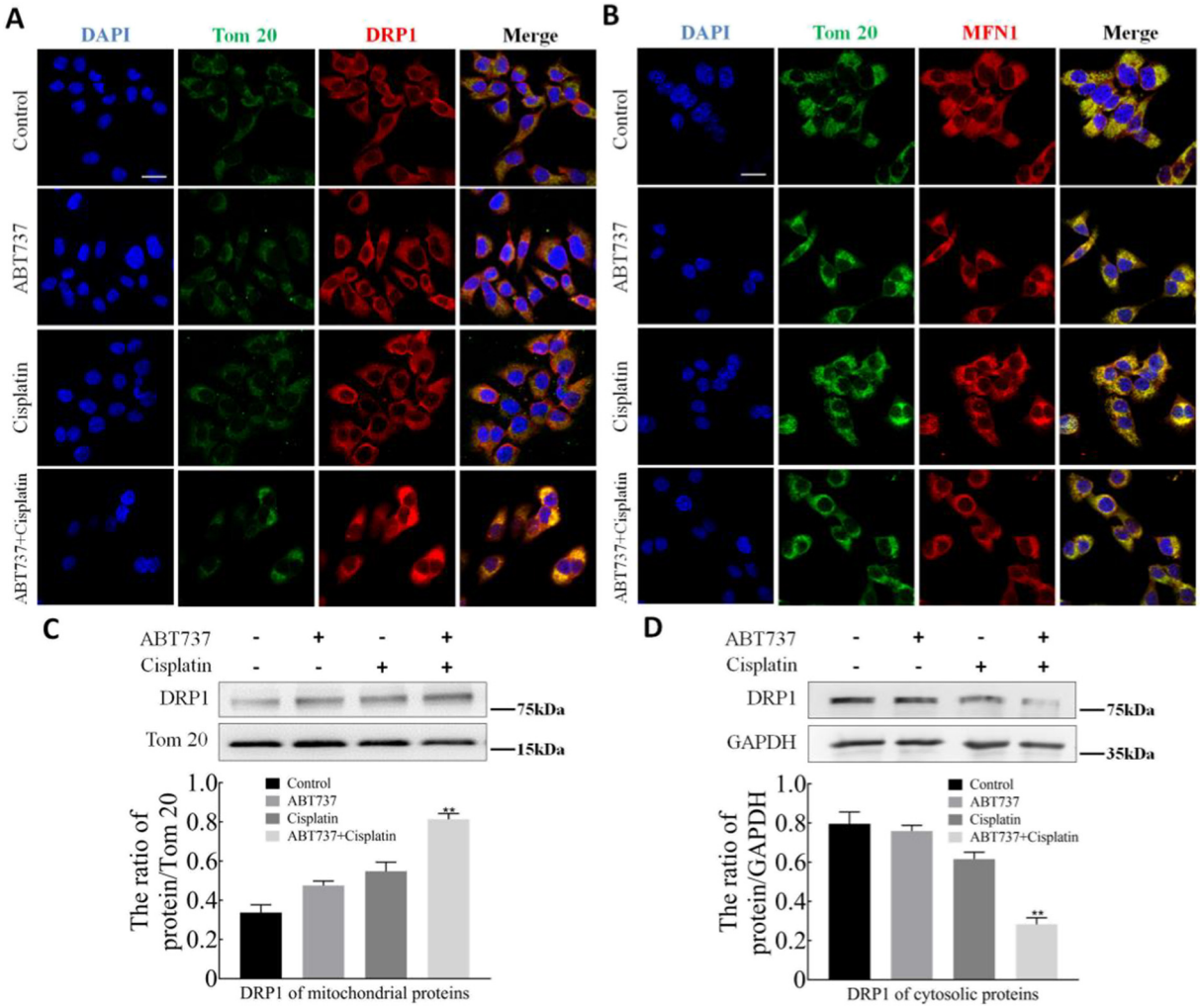


Fig. 4. Cisplatin accompanied with ABT737 promoted mitochondrial fission in SKOV3 cells.
(A) Immunofluorescence of Tom 20 (green) and DRP1 (red) in SKOV3 cells after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). The DRP1 co-localization with mitochondria was observed by confocal microscopy (bar, 20 μm). (B) Immunofluorescence of Tom 20 (green) and MFN1 (red) in SKOV3 cells after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). The MFN1 co-localization with mitochondria was observed by confocal microscopy (bar, 20 μm). (C, D) Mitochondrial fractions and cytosolic fractions were prepared after 12 h treatment with cisplatin (2.5 μg/mL) and/or ABT737 (20 μM). Western blot analysis was performed with DRP1, Tom 20, and GAPDH antibodies. Tom 20/GAPDH was used as the loading control. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. RNA isolation and quantitative real-time PCR

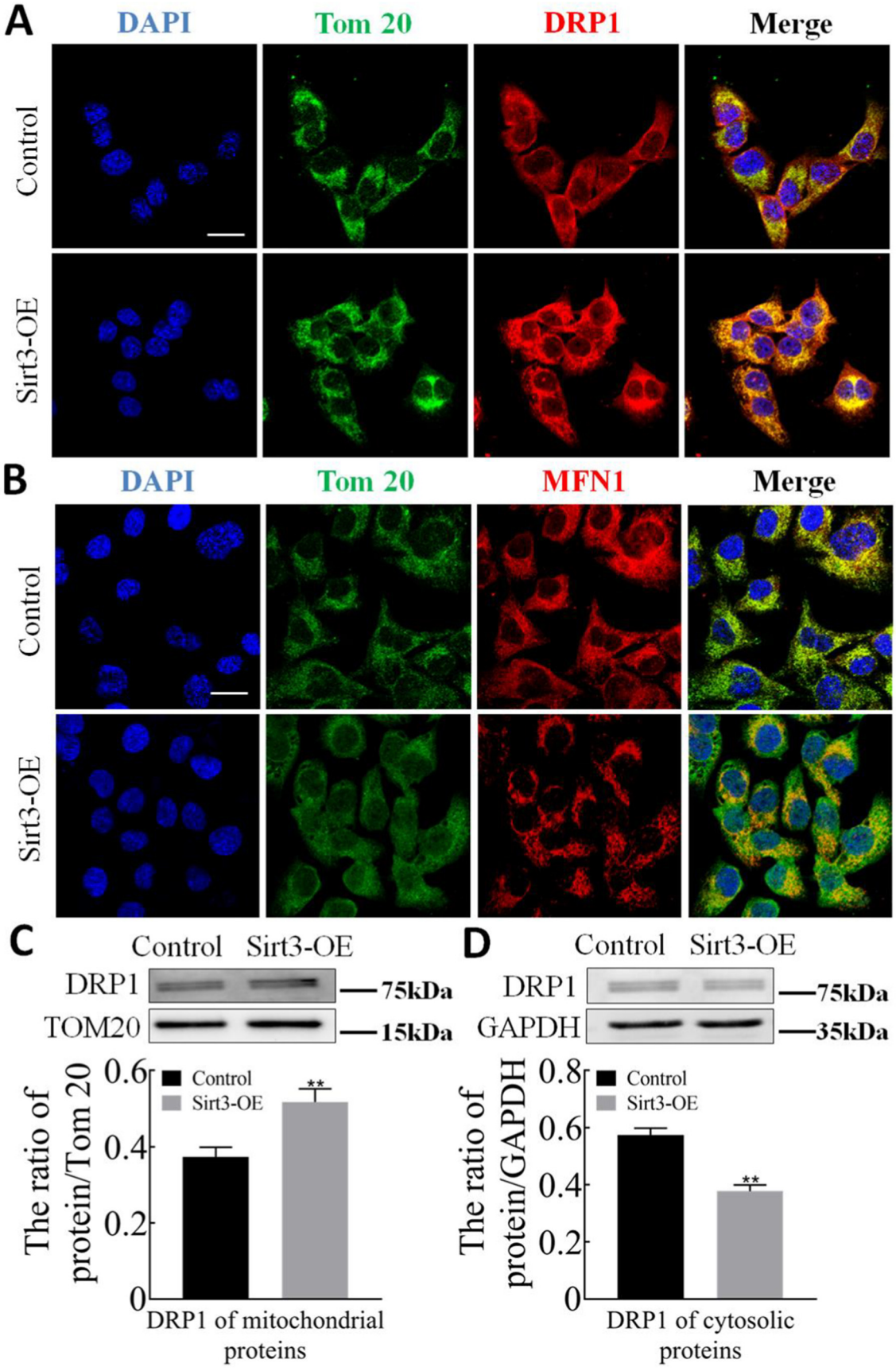
RNA fraction was isolated from ovarian cancer cells using Trizol reagent (Invitrogen, USA). cDNA was obtained by reverse transcription with PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). For quantitative PCR analysis, cDNA were amplified using SYBR Green PCR Master Mix (Bio-Rad, USA) on 7500 RealTime PCR Instrument (Applied Biosystems). Each sample was tested in triplicate. ΔΔCT method was employed to determine the fold change in gene expression level. Melting curves were used to verify specificity of real-time PCR. The expression of Sirt3 gene and the control GAPDH gene was determined by quantitative real-time PCR. All reactions were run in triplicate. The PCR primers were as follows: Sirt3 F: 5′-ACCCAGTGGCATTCCAGAC-3′ and R: 5′-GGCTTGGGGTTGTGAAA GAAG-3′; GAPDH F: 5′-CAATGAC CCCTTCATTGACC-3′ and R: 5′-GACAAGC TTCCCGTTCTCAG-3′.

2.10. Immunofluorescence staining and confocal laser microscopy

The expression of DRP1, MFN1 and Tom 20 was tested by Immunofluorescence staining. SKOV3 cells seeded on chamber slides in six-well plate were incubated for 12 h, and we treated the cells with cisplatin and/or ABT737 for 12 h. Cells were fixed with 4% paraformaldehyde at 4 °C overnight, followed by washing three times with PBS. 0.5% Triton X-100 was adopted to permeate cells for 10 min. Cells were blocked with 5% bovine serum albumin (BSA) in PBS at room temperature for 30 min, then incubated with the primary antibody for 1 h followed by three times washing with PBS. Samples were incubated with another primary antibody for 1 h, then washed for three times with PBS and incubated in secondary antibody in the dark for 2 h followed by stained with DAPI (0.5 μg/mL) for 10 min at room temperature. Finally, cells were observed and the fluorescent images were captured by a Nikon laser scanning confocal microscope.

2.11. siRNA targeting Sirt3 knock-down

Knock-down Sirt3 expression in SKOV3 cell lines was performed siRNA targeting Sirt3. SKOV3 cells were transfected with 50 nM Sirt3 siRNA (Si-Sirt3: 5′-CCAGT GGCATTCCAGACTT-3′) (Shanghai GenePharma Co., Ltd., China) or control siRNA (Shanghai GenePharma Co., Ltd., China) using Lipofectamine 2000 (Life Technologies).



(caption on next page)

Fig. 5. Activity of Sirt3 promoted mitochondrial fission in SKOV3 cells.
(A) Immunofluorescence of Tom 20 (green) and DRP1 (red) in Sirt3 overexpressed cells and SKOV3 control cells. The DRP1 co-localization with mitochondria was observed by confocal microscopy (bar, 20 μm). (B) Immunofluorescence of Tom 20 (green) and MFN1 (red) in Sirt3 overexpressed cells and SKOV3 control cells. The MFN1 co-localization with mitochondria was observed by confocal microscopy (bar, 20 μm). (C, D) Mitochondrial fractions and cytosolic fractions were prepared after transfected with Sirt3 overexpression plasmid for 24 h. Western blot analysis was performed with DRP1, Tom 20, and GAPDH antibodies. Tom 20/GAPDH was used as the loading control. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.12. Statistical analysis

The results were represented as mean values ± standard deviation (S.D) and data were evaluated separately for at least three independent experiments. Student's *t*-test was used for statistical analyses. Statistical analysis was performed with SPSS 22.0 (SPSS Inc., Chicago, IL). The $^{*}p$ value < 0.05 was considered significant and $^{**}p < 0.01$ was considered extremely significant.

3. Results

3.1. ABT737 sensitized SKOV3 cells to cisplatin

To detect the antitumor effect of cisplatin accompanied with ABT737 in SKOV3 cells, MTT assay and colony formation assay were conducted. MTT assay results showed that cisplatin or ABT737 alone inhibited the viability of ovarian cancer cells. There was a dramatic decrease of viability when the cells were treated with cisplatin and ABT737 together (Fig. 1A). The colony formation assay showed that the combination of two drugs had no colony in SKOV3 cells (Fig. 1B).

We then investigated whether ABT737 combined with cisplatin induced apoptosis in SKOV3 cells. The flow cytometry analysis revealed that cells treated with cisplatin and ABT737 resulted in a profound increase in apoptosis (Fig. 1C and D). In addition, western blot for Bcl-2, BAX, and Cleaved caspase 3 confirmed the increase of mitochondrial pathway apoptosis in SKOV3 cells when these two drugs were used together (Fig. 1E and F). These results demonstrated that cisplatin accompanied with ABT737 inhibited viability and induced mitochondrial pathway apoptosis in SKOV3 cells.

3.2. Cisplatin accompanied with ABT737 up-regulated Sirt3 activity and affect mitochondrial membrane potential

Sirt3, a mitochondrial protein, is required for apoptosis induced by silencing Bcl-2 [17]. We then investigated whether cisplatin combined with ABT737 was able to influence the expression of Sirt3. There was no significant difference in the protein expression of Sirt3 (Fig. 2A), but the expression of Sirt3 was increased in mitochondria (Fig. 2B). GAPDH was not detected, which proved that mitochondrial fractions were isolated successfully (Fig. 2B). We further detected the NAD^+-dependent histone deacetylases activity of Sirt3 in mitochondria. The enzyme activity of Sirt3 increased significantly after being treated with cisplatin and ABT737 (Fig. 2C).

ABT737 as an inhibitor of Bcl-2/Bcl-xl can induce BAX/BAK activation and eventually result in changes in the permeability of the mitochondrial outer membrane as well as activation of caspase pathway and apoptosis [18]. We next investigated the influence on the mitochondrial membrane under the treatment of these two drugs. Cisplatin or ABT737 alone had an impact on mitochondrial membrane potential (MMP), while ABT737 combined with cisplatin had an extremely significant decline of MMP (Fig. 2D and E). These data indicated that cisplatin accompanied with ABT737 increased the expression and enzyme activity of Sirt3 in mitochondria and depolarized the mitochondrial membrane.

3.3. Activity of Sirt3 was involved in apoptosis and mitochondrial membrane potential

To further determine the function of activated Sirt3 in cell death and mitochondrial dysfunction, we evaluated the effect of Sirt3 by overexpressing Sirt3 in SKOV3 cells. The mRNA and protein expression of Sirt3 had a significant increase in transfected cells (Fig. 3A, B and D). Meanwhile, the enzyme activity of Sirt3 was increased in Sirt3 overexpression cells (Fig. 3C).

Significant upregulation in apoptosis could be seen in Sirt3 overexpression cells (Fig. 3E and F). Less Bcl-2 and more BAX protein expression were observed in Sirt3 overexpression cells (Fig. 3B and D). These results indicated that excessive activation of Sirt3 promoted mitochondrial pathway apoptosis, which might be a feedback effect on the expression of Bcl-2 and BAX. Moreover, excessive activation of Sirt3 could also decrease the mitochondrial membrane potential (MMP) (Fig. 3G and H).

3.4. Cisplatin accompanied with ABT737 promoted mitochondrial fission in SKOV3 cells

Recently, studies have shown that cancer biology can be regulated by mitochondrial fission/fusion dynamics, which can be associated with Bcl-2 family proteins and Sirt3. To investigate mitochondrial dynamics in SKOV3 cells, we used MFN1 and DRP1 to detect the mitochondrial fusion and fission respectively. The translocase of the outer mitochondrial membrane 20 (Tom 20) was used to locate the outer membrane of mitochondria.

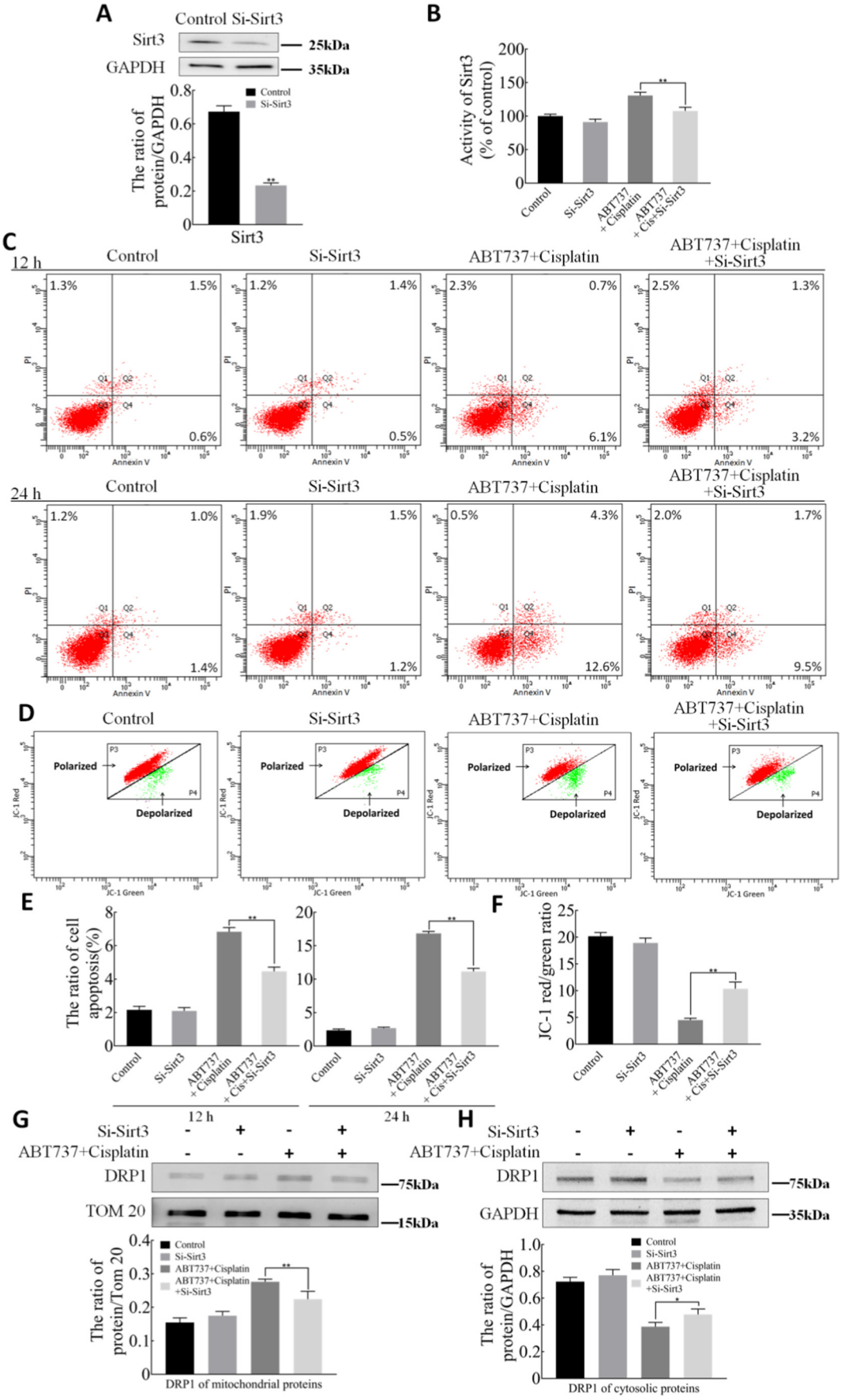
Immunofluorescence analysis showed that the expression of DRP1 markedly increased and no significant change on MFN1 in mitochondria when SKOV3 cells were treated with cisplatin and ABT737 together (Fig. 4A and B). Moreover, most DRP1 was co-localized with mitochondria. The mitochondrial fission was also examined by western blot. DRP1 expression increased in mitochondria and decreased in cytoplasm after the combination of these two drugs (Fig. 4C and D).

3.5. Activity of Sirt3 promoted mitochondrial fission in SKOV3 cells

Furthermore, we investigated whether the activation of Sirt3 could also influence the mitochondrial fission and fusion. Immunofluorescence analysis showed that the activation of Sirt3 increased the expression of DRP1 and that most DRP1 was co-localized with mitochondria, which meant excessive Sirt3 caused the fission mediated by DPR1 (Fig. 5A). There was no significant difference on MFN1 expression and co-location with mitochondria between Sirt3 overexpressed cells and control cells (Fig. 5B). The mitochondrial fission was also examined by western blot. DRP1 expression increased in mitochondria and decreased in cytoplasm after overexpression of Sirt3 in SKOV3 cells (Fig. 5C and D).

3.6. Knock-down Sirt3 expression could partially reverse the antitumor effect of cisplatin accompanied with ABT737

To verify that ABT737 enhanced ovarian cancer cells sensitivity to cisplatin through the activation of Sirt3, we knocked down Sirt3 in SKOV3 cells (Fig. 6A). We found that Si-Sirt3 prevented the increase of enzyme activity of Sirt3 when induced by cisplatin and ABT737



(caption on next page)

Fig. 6. Knock-down Sirt3 expression could partially reverse the antitumor effect of cisplatin accompanied with ABT737.
(A) SKOV3 cells were transfected with Si-Sirt3 (50 μM) or control siRNA (50 μM) using Lipofectamine 2000. Western blot analysis was performed with Sirt3 antibodies. GAPDH was used as the loading control. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. control. (B) After transfected with Si-Sirt3 (50 μM) for 24 h, SKOV3 cells were treated with cisplatin (2.5 μg/mL) and ABT737 (20 μM) for 12 h. Mitochondria were collected by Mitochondria Isolation Kit. The activity of Sirt3 was assessed using a Sirt3 Deacetylase Fluorometric Assay Kit. The data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. ABT737 + Cisplatin. (C) After transfected with Si-Sirt3 (50 μM) for 24 h, SKOV3 cells were followed by treatment with cisplatin (2.5 μg/mL) and ABT737 (20 μM) for 12 h or 24 h. Apoptosis was analyzed by flow cytometry after stained with Annexin V/PI. (D) After transfected with Si-Sirt3 (50 μM) for 24 h, SKOV3 cells were followed by treatment with cisplatin (2.5 μg/mL) and ABT737 (20 μM) for 12 h. JC-1 was used to assess mitochondrial membrane potential. The data were obtained by flow cytometry. (E) Apoptosis data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. ABT737 + Cisplatin. (F) The MMP data are expressed as mean ± SEM of three independent experiments; $^{**}p < 0.01$ vs. ABT737 + Cisplatin. (G, H) After transfected with Si-Sirt3 (50 μM) for 24 h, SKOV3 cells were followed by treatment with cisplatin (2.5 μg/mL) and ABT737 (20 μM) for 12 h. Mitochondrial fractions and cytosolic fractions were prepared and subjected to western blot analysis using antibodies against DRP1, Tom 20, and GAPDH. The data are expressed as mean ± SEM of three independent experiments; $^{*}p < 0.05$ vs. ABT737 + Cisplatin. $^{**}p < 0.01$ vs. ABT737 + Cisplatin.

(Fig. 6B).

The flow cytometry analysis revealed that Si-Sirt3 inhibited the apoptosis of ovarian cancer cells induced by cisplatin and ABT737 (Fig. 6C and E). Moreover, the down-regulation of mitochondrial membrane potential (MMP) was partially reversed by Si-Sirt3 (Fig. 6D and F). In addition, the knock-down of Sirt3 reduced the expression of DRP1 in mitochondria induced by cisplatin and ABT737, the opposite effect could be observed in cytoplasm (Fig. 6G and H).

All these results indicated that ABT737 enhanced ovarian cancer cells' sensitivity to cisplatin by increasing the activation of Sirt3, which induced the mitochondrial fission dynamics and increased the mitochondrial pathway apoptosis.

4. Discussion

Ovarian cancer is one of the malignant tumors in the female reproductive system with a poor prognosis [19]. Platinum-based chemotherapy, a standard treatment for ovarian cancer, can be used at all stages of the disease [20]. As platinum's analogue, cisplatin has been employed for decades to treat ovarian cancer. However, the resistance of cisplatin is the main obstacle to its treatment.

Overexpression of Bcl-2 can enhance resistance to cisplatin in cancer cells. Therefore, Bcl-2 is regarded as a target of cancer treatment [21]. Several small molecule antagonists of Bcl-2 have been identified and synthesized these days, such as GX15–070, AT 101, Gossypol, Isoxazolidine, ABT-737 and ABT-263 [22]. It has been reported that the anti-tumor effect of cisplatin can be significantly improved by accompanying it with ABT737 in ovarian cancer [4,23]. Our results showed that compared with ABT737 or cisplatin alone, the combination significantly inhibited the cell viability and increased the mitochondrial pathway apoptosis (Fig. 1). They further suggested that mitochondrial dysfunction induced by ABT737 increased the sensitivity of cisplatin to SKOV3 cells.

Reports have shown that Bcl-2 family proteins can regulate the link between cancer and mitochondrial dynamics [24]. Mitofusin 1/2 (MFN1/2) proteins are involved in the fusion process of outer membranes, whereas OPA1 is responsible for inner membranes [10]. Mitochondria dynamin-related protein 1 (DRP1) plays an essential role in regulating mitochondrial fission by recruitment onto mitochondria, which promotes mitochondrial membrane constriction and subsequent mitochondrial fragmentation [25]. Our results showed that cisplatin, when accompanied with ABT737, induced the fission of mitochondria.

Sirt3 is a NAD^+-dependent protein deacetylase related to mitochondrial biogenesis and mitochondrial dynamics [26,27]. In our experiments, there was no significant difference in the protein expression of Sirt3 under the treatment of cisplatin and ABT737 (Fig. 2A). But the expression and enzyme activity of Sirt3 in mitochondria were increased (Fig. 2B and C).

To further illustrate whether the activity of Sirt3 is associated with cell death and mitochondrial dysfunction, Sirt3 overexpression plasmid was transfected into SKOV3 cells. The mRNA expression, protein expression, and the enzyme activity of Sirt3 were increased in Sirt3 overexpression cells (Fig. 3A-D). Results showed that increasing activation of Sirt3 induced apoptosis, decreased mitochondrial membrane potential, and promoted the fission process of mitochondria, which was similar to our findings in the combination of cisplatin and ABT737. However, the effect of Sirt3 overexpression on mitochondrial function alone was weaker than that of cisplatin and ABT737, indicating combination treatment was partly achieved by activating Sirt3 to regulate the mitochondrial fission.

We knocked down Sirt3 expression in SKOV3 cells (Fig. 6A) revealing that the loss of Sirt3 partially reversed apoptosis and the down-regulation of mitochondrial membrane potential (MMP) caused by cisplatin and ABT737 (Fig. 6B-F). In addition, the knock-down of Sirt3 reduced the activity of Sirt3 when induced by the combination of two drugs showing that the activation of Sirt3 is one of the key pathways for the treatment of cisplatin and ABT737.

Human Sirt3 exists in two forms, a full-length (FL) protein of 44 kDa peptide and a processed form [28]. Sirt3 localizes to the mitochondria and the nucleus under normal cell growth conditions. The degradation of FL Sirt3 is mediated by the ubiquitin-proteasome pathway through the ubiquitin protein ligase SKP2 activity [29]. Studies show that Sirt3 is transferred from the nucleus to the mitochondria upon cellular stress as well as overexpression of Sirt3 itself [30]. In this study, we found that the change of Sirt3 activity was caused when Sirt3 was transferred into the mitochondria under the treatment of cisplatin and ABT737, which means that Sirt3 can be a key factor in this process.

In summary, our research shows that the activation of Sirt3 is an important factor in increasing the sensitivity of ovarian cancer cells to cisplatin when induced by Bcl-2 inhibitor ABT737. This promotes mitochondrial fission and increases the mitochondrial pathway apoptosis. Meanwhile, our findings suggest that the activation of Sirt3 is an attractive therapeutic target for ovarian cancer.

Acknowledgements

This study was funded by the 13th Five-year Science and Technology Project of Jilin Provincial Education Department (JJKH20180190KJ), Research Foundation of Jilin Provincial Department of Science & Technology (20170414038GH, 20180101102JC, and 20180414037GH), and the Youth Foundation of Health and Family Planning Commission of Jilin Province (2017Q025 and 2017Q003).

References

[1] I.J. Jacobs, U. Menon, A. Ryan, A. Gentry-Maharaj, M. Burnell, J.K. Kalsi, N.N. Amso, S. Apostolidou, E. Benjamin, D. Cruickshank, D.N. Crump, S.K. Davies, A. Dawnay, S. Dobbs, G. Fletcher, J. Ford, K. Godfrey, R. Gunu, M. Habib, R. Hallett, J. Herod, H. Jenkins, C. Karpinskyj, S. Leeson, S.J. Lewis, W.R. Liston, A. Lopes, T. Mould, J. Murdoch, D. Oram, D.J. Rabideau, K. Reynolds, I. Scott, M.W. Seif, A. Sharma, N. Singh, J. Taylor, F. Warburton, M. Widschwendter, K. Williamson, R. Woolas, L. Fallowfield, A.J. McGuire, S. Campbell, M. Parmar, S.J. Skates, Ovarian cancer screening and mortality in the UK collaborative trial of ovarian Cancer screening (UKCTOCS): a randomised controlled trial, Lancet 387 (10022) (2016) 945–956.

[2] U.A. Matulonis, A.K. Sood, L. Fallowfield, B.E. Howitt, J. Sehouli, B.Y. Karlan, Ovarian cancer, Nat Rev Dis Primers 2 (2016) 16061.
[3] C. Holohan, S. Van Schaeybroeck, D.B. Longley, P.G. Johnston, Cancer drug resistance: an evolving paradigm, Nat. Rev. Cancer 13 (10) (2013) 714–726.
[4] Q. Xie, J. Su, B. Jiao, L. Shen, L. Ma, X. Qu, C. Yu, X. Jiang, Y. Xu, L. Sun, ABT737 reverses cisplatin resistance by regulating ER-mitochondria Ca2+ signal transduction in human ovarian cancer cells, Int. J. Oncol. 49 (6) (2016) 2507–2519.
[5] R.C. Taylor, S.P. Cullen, S.J. Martin, Apoptosis: controlled demolition at the cellular level, Nat. Rev. Mol. Cell Biol. 9 (3) (2008) 231–241.
[6] Y. Soini, P. Paakko, V.P. Lehto, Histopathological evaluation of apoptosis in cancer, Am. J. Pathol. 153 (4) (1998) 1041–1053.
[7] L. Galluzzi, L. Senovilla, I. Vitale, J. Michels, I. Martins, O. Kepp, M. Castedo, G. Kroemer, Molecular mechanisms of cisplatin resistance, Oncogene 31 (15) (2012) 1869–1883.
[8] G. Radha, S.C. Raghavan, BCL2: a promising cancer therapeutic target, Biochim Biophys Acta Rev Cancer 1868 (1) (2017) 309–314.
[9] T. Oltersdorf, S.W. Elmore, A.R. Shoemaker, R.C. Armstrong, D.J. Augeri, B.A. Belli, M. Bruncko, T.L. Deckwerth, J. Dinges, P.J. Hajduk, M.K. Joseph, S. Kitada, S.J. Korsmeyer, A.R. Kunzer, A. Letai, C. Li, M.J. Mitten, D.G. Nettesheim, S. Ng, P.M. Nimmer, J.M. O'Connor, A. Oleksijew, A.M. Petros, J.C. Reed, W. Shen, S.K. Tahir, C.B. Thompson, K.J. Tomaselli, B. Wang, M.D. Wendt, H. Zhang, S.W. Fesik, S.H. Rosenberg, An inhibitor of Bcl-2 family proteins induces regression of solid tumours, Nature 435 (7042) (2005) 677–681.
[10] A. Autret, S.J. Martin, Bcl-2 family proteins and mitochondrial fission/fusion dynamics, Cellular and molecular life sciences: CMLS 67 (10) (2010) 1599–1606.
[11] A.P. Trotta, J.E. Chipuk, Mitochondrial dynamics as regulators of cancer biology, Cellular and molecular life sciences: CMLS 74 (11) (2017) 1999–2017.
[12] C. Sheridan, S.J. Martin, Mitochondrial fission/fusion dynamics and apoptosis, Mitochondrion 10 (6) (2010) 640–648.
[13] C. Sheridan, P. Delivani, S.P. Cullen, S.J. Martin, Bax- or Bak-induced mitochondrial fission can be uncoupled from cytochrome C release, Mol. Cell 31 (4) (2008) 570–585.
[14] J.A. Hall, J.E. Dominy, Y. Lee, P. Puigserver, The sirtuin family's role in aging and age-associated pathologies, J. Clin. Invest. 123 (3) (2013) 973–979.
[15] H.S. Kim, K. Patel, K. Muldoon-Jacobs, K.S. Bisht, N. Aykin-Burns, J.D. Pennington, R. van der Meer, P. Nguyen, J. Savage, K.M. Owens, A. Vassilopoulos, O. Ozden, S.H. Park, K.K. Singh, S.A. Abdulkadir, D.R. Spitz, C.X. Deng, D. Gius, SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress, Cancer Cell 17 (1) (2010) 41–52.
[16] G. Marfe, M. Tafani, M. Indelicato, P. Sinibaldi-Salimei, V. Reali, B. Pucci, M. Fini, M.A. Russo, Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction, J. Cell. Biochem. 106 (4) (2009) 643–650.
[17] S.J. Allison, J. Milner, SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways, Cell Cycle 6 (21) (2007) 2669–2677.
[18] A. Gross, BCL-2 family proteins as regulators of mitochondria metabolism, Biochim. Biophys. Acta 1857 (8) (2016) 1243–1246.
[19] A. Halama, B.S. Guerrouahen, J. Pasquier, N.J. Satheesh, K. Suhre, A. Rafii, Nesting of colon and ovarian cancer cells in the endothelial niche is associated with alterations in glycan and lipid metabolism, Sci. Rep. 7 (2017) 39999.
[20] J. Li, J. Wang, R. Chen, Y. Bai, X. Lu, The prognostic value of tumor-infiltrating T lymphocytes in ovarian cancer, Oncotarget 8 (9) (2017) 15621–15631.
[21] P.E. Czabotar, G. Lessene, A. Strasser, J.M. Adams, Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy, Nat. Rev. Mol. Cell Biol. 15 (1) (2014) 49–63.
[22] D. Zhai, C. Jin, A.C. Satterthwait, J.C. Reed, Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins, Cell Death Differ. 13 (8) (2006) 1419–1421.
[23] Y. Xu, W. Gao, Y. Zhang, S. Wu, Y. Liu, X. Deng, L. Xie, J. Yang, H. Yu, J. Su, L. Sun, ABT737 reverses cisplatin resistance by targeting glucose metabolism of human ovarian cancer cells, Int. J. Oncol. 53 (3) (2018) 1055–1068.
[24] E.A. Tanner, T.A. Blute, C.B. Brachmann, K. McCall, Bcl-2 proteins and autophagy regulate mitochondrial dynamics during programmed cell death in the Drosophila ovary, Development 138 (2) (2011) 327–338.
[25] C. Hu, Y. Huang, L. Li, Drp1-dependent mitochondrial fission plays critical roles in physiological and pathological progresses in mammals, Int. J. Mol. Sci. 18 (1) (2017).
[26] T. Shi, F. Wang, E. Stieren, Q. Tong, SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes, J. Biol. Chem. 280 (14) (2005) 13560–13567.
[27] Y. Wang, X. Sun, K. Ji, L. Du, C. Xu, N. He, J. Wang, Y. Liu, Q. Liu, Sirt3-mediated mitochondrial fission regulates the colorectal cancer stress response by modulating the Akt/PTEN signalling pathway, Biomed. Pharmacother. 105 (2018) 1172–1182.
[28] B. Schwer, B.J. North, R.A. Frye, M. Ott, E. Verdin, The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase, J. Cell Biol. 158 (4) (2002) 647–657.
[29] T. Iwahara, R. Bonasio, V. Narendra, D. Reinberg, SIRT3 functions in the nucleus in the control of stress-related gene expression, Mol. Cell. Biol. 32 (24) (2012) 5022–5034.
[30] M.B. Scher, A. Vaquero, D. Reinberg, SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress, Genes Dev. 21 (8) (2007) 920–928.