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Regulation of survival and chemoresistance by HSP90AA1 in ovarian cancer SKOV3 cells

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Abstract Previous researches have showed that HSP90 AA is important in ovarian cancer, but the mechanism of HSP90AA is still unknown. This study aimed to investigate the role of the potential therapy target protein HSP90AA1 in ovarian cancer. The level of HSP90AA1 in ovarian cancer SKOV3 cell line was altered by RNAi and overexpression. Survival of these cell lines was investigated by tetrazolium-based assay and fluorescence-activated cell sorter (FACS). The chemosensitivity to cisplatin of the cell was also tested by FACS when HSP90AA1 was overexpressed. HSP90AA1 RNAi inhibited the proliferation of ovarian cancer SKOV3 cell line and increased the apoptosis. Furthermore, overexpression of HSP90AA1 decreased the chemosensitivity to cisplatin of SKOV3 cells and overexpression of HSP90AA1 could partially rescue the survival rate of SKOV3 cells which were treated with cisplatin. HSP90AA1 is required for the survival and proliferation of SKOV3 cells. High level of HSP90AA1 can increase chemoresistance to cisplatin of SKOV3 cells.

Keywords HSP90AA1 · Ovarian cancer · Apoptosis · Chemosensitivity · Chemoresistance

Background

Ovarian cancer has the highest mortality rate of all the gynecologic malignancies with most patients diagnosed at late stages [1]. Standard therapy for patients with advanced ovarian cancer includes primary cytoreductive surgery followed by combination chemotherapy [2]. Most of the patients are sensitive to the first-line chemotherapy, such as a single cisplatin (cis-diaminedichloroplatinum) treatment or in combination regimens. Cisplatin binds to and causes cross-linking of DNA, which ultimately triggers cancer cell apoptosis [3]. However, most of them will relapse within 2 years, which may be attributable to the following reasons: although systemic chemotherapy kills the majority of ovarian cancer cells leading to the clinical result of tumor shrinkage, the most important target, a small population of chemo-resistant cancer cells that possess tumorigenic capacity, is spared, thereby, allowing tumor regrowth [4].

Drug resistance is a multi-factor involved process that is not only associated with the unique genotype of tumors, but also mediated by cellular stress response to the microenvironment [5]. Heat shock proteins (HSPs) are a group of highly conserved chaperone proteins that are upregulated in a wide range of tumors by cell stress and are closely associated with a poor prognosis and resistance to therapy [6]. HSP90 is one of the most common heat-related proteins. HSP90 anti-apoptotic functions can largely be explained by its chaperone role in folding, stabilization, activation, and assembly of their "client" proteins such as Her2/ErbB2, Akt, Raf-1, Hif-1α, hormone receptors, survivin, mutant p53, and hTERT [7–9], which are oncogenic proteins. Thus HSP90 is a potential molecular target in cancer therapy [10]. Selective inhibitors of Hsp90, such as 17-allylamino, 17-demethoxygeldanamycin (17AAG) derived from geldanamycin can interact with the N domain ATP-binding pocket and stop the chaperone cycle, leading to the degradation of the client proteins and thereby promoting antitumor activity [11]. However, most of the clinical trials focus on HSP90 targeted inhibitors. The binding preference of HSP90 inhibitors to

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HSP90 sub family members [12] and the roles of HSP90 sub family members in ovarian cancer are not fully investigated.

In mammalian, HSP90 family is consisted of four major types, two cytosolic isoforms, the heat-inducible HSP90α encoded by gene HSP90AA1 and HSP90AA2 and the constitutively-expressed HSP90β encoded by HSP90AB1 [13]. Recent studies suggest that HSP90AA1 but not HSP90AA2 is expressed extracellularly and involved in cancer cell invasiveness [14]. Therefore, it would be interesting to determine the role of HSP90AA1 in ovarian cancer cells, with a particular focus on its putative effect in cell survival and its potential effects on the chemoresistance in the ovarian cancer cell.

Materials and methods

Antibodies, reagents and instruments

Antibodies were purchased from Cell Signaling. RPMI 1640 was purchased from Gibco and fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich. Annexin V-FITC/PI Apoptosis Detection Kit was obtained from Nanjing KeyGEN Biotech Co., Ltd. Enhanced chemiluminescence (ECL) kit and nonfat milk powder for immunoblotting were purchased from Guangzhou Maygene Co., Ltd. CO_2 incubator was purchased from Heraeus Ltd. Microplate absorbance Reader, fluorescence-activated cell sorter (FACS) Calibur, vertical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) systems and Gel imaging system were all obtained from Bio-Rad.

Cell culture

Human ovarian cancer cell line SKOV3 was purchased from Cell Bank of Chinese Academy of Science. Cells were cultured and passaged in RPMI 1640 supplemented with 10 % fetal bovine serum at 37 °C with 5 % CO_2.

siRNAs and plasmids

siRNAs corresponding to HSP90AA1 gene were designed online (http://sidirect2.rnai.jp/). The genebank ID of HSP90 AA1 is NM_001017963.2. Three pairs of sequences were designed (Sense sequence and antisense sequence in pairs): siRNA1: 5′-UUUUGUUGAGCUCUUCUUGAU-3′, 5′-CAA GAAGAGCUCAACAAAACA-3′; siRNA2: 5′-AUUACUAG CUCUGCUUU AGUG-3′, 5′-CUAAAGCAGAGCUAGUAA UGC -3′; siRNA3; 5′-AACAUGAAACUCAAAAAGCAU-3′, 5′-GCUUUUUGAGUUUCAUGUUGG-3′. siRNAs were synthesized by Shanghai GenePharma Co., Ltd. cDNA of HSP90AA1 was cloned by RT-PCR and inserted into pCDNA3.1(A+) (Invitrogen).

Cell transfection

siRNAs were dissolved in DEPC–H_2O in a concentration of 40 μM. pCDNA3.1-HSP90AA1 was purified as 1 μg/μl. Cells were plated into sixwell plates 24 h before transfection. Cells were transfected with siRNAs or plasmid using Lipofectamine 2000 (Invitrogen) at 70 % confluency based on manufacturer's recommendations.

MTT assays

Cells were trypsinized 24 h after transfection and seeded into 96-well plates in triplicate and incubated for 1–6 days, respectively. MTT of 50 μl (1 mg/ml) was added into each well and incubated for 4 h. The supernatant was discarded, DMSO of 150 μl was added to each well and shacked to completely dissolve. The absorbance of each well was determined using a microplate reader at a wavelength of 570 nm. The growth curve was created, using culture days for X-axis and absorbance average for Y-axis.

Cell apoptosis assays

Cells were trypsinized 48 h after transfection, centrifuged at 1,000 rpm for 5 min, then washed twice in PBS and the supernatant was discarded. Apoptosis was also evaluated using the Annexin V-FITC/PI Kit. Briefly, cells were re-suspended in 500 μl of binding buffer in the Kit, then treated with 5 μl of Annexin V-FITC and 5 μl Propidium Iodide for 5–15 min at room temperature (RT, 25 °C) in dark. The stained cells were analyzed in a flow cytometer within 1 h.

Western blotting assays

SKOV3 cells were trypsinized 48 h after transfection, then washed with chilled PBS three times and lysed in lysis buffer for 30 min on ice. Lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and protein concentrations were determined using BCA protein assay. Proteins of 20 μg were separated on a SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5 % nonfat milk for 2 h at RT. Anti-HSP90AA1 antibody was applied overnight at 4 °C, followed by a second antibody for 1 h at RT. ECL kit and X-ray film were used for chemiluminescence detection of the protein levels.

Statistical analyses

All results were given as mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical differences were compared using Student's *T* test by *SPSS13.0* software and statistical significance was accepted at $^{**}P < 0.01$.

Results

Effect of HSP90AA1 RNAi on SKOV3 cells

Analysis of HSP90AA1 knockdown efficiency in SKOV3 cells

In order to investigate the possible role of HSP90AA1 in ovarian cancer, three HSP90AA1 siRNAs were designed and transfected into SKOV3 cell line, which is an ovarian epithelial adenocarcinoma cell line resistant to cisplatin. Western blotting showed that the transfected siRNA3 remarkably decreased the HSP90AA1 protein level compared with the control among three siRNAs (Fig. 1). Thus, the following experiments were all performed using siRNA3.

HSP90AA1 is required for survival of SKOV3 cells

The cell survival was analyzed in siRNA transfected SKOV3 cells for 48 h. MTT assays were performed and the proliferating activity of the normal saline treated control was normalized to 100 %. The mock transfected cells had comparative activity as the control ones. However, HSP90AA1 RNAi significantly decreased the proliferative activity of SKOV3 cells (Fig. 2). This result indicates that HSP90AA1 is required for survival of SKOV3 cells.

HSP90AA1 RNAi induces apoptosis of SKOV3 cells

To further investigate the effects of HSP90AA1 RNAi, apoptosis was analyzed in siRNA transfected SKOV3 cells for 48 h. When HSP90AA1 siRNA was transfected, the

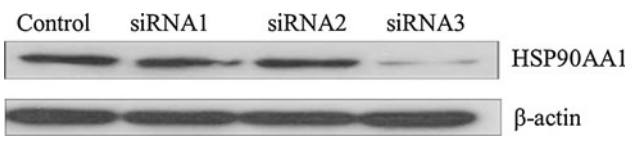

Fig. 1 Protein expression level of HSP90AA1 in control and HSP90AA1 RNAi SKOV3 cell lines. *Lane 1*: Scrambled siRNA *control*; *Lane 2*, *3*, *4*: *siRNA1*, *siRNA2*, *siRNA3* targeting *HSP90AA1*, respectively. The proteins were extracted and detected by indicated antibodies. *β-actin* was used as an internal control to ensure equal loading of each lane

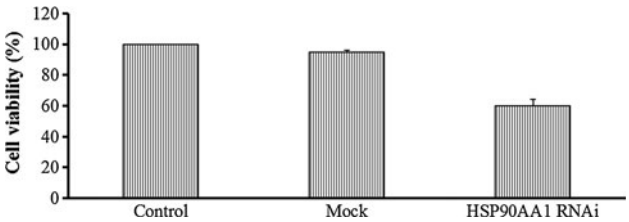

Fig. 2 HSP90AA1 is required for the survival of SKOV3 cells. MTT assay was performed to SKOV3 cells. *Control*: the normal saline treated cells; *Mock*: the mock transfected cells; Experiment group: *HSP90AA1 RNAi* by transfection. Three independent experiments were performed and *T* test was analyzed. ** indicated $P < 0.01$

Annexin V-FITC/PI dual staining showed that the ratio of the apoptosis cells increased to 20 ± 1.2 % (Fig. 3b), compared with the control rate of 3.8 ± 0.4 % (Fig. 3a). These FACS data suggest that loss of HSP90AA1 can induce apoptosis, which is consistent to the results of Fig. 2.

Effects of HSP90AA1 overexpression to SKOV3 cells

Analysis of HSP90AA1 overexpression level in SKOV3 cells

It is reported that HSP90 expression is upregulated in ovarian endometriosis [15], although the HSP90 expression has no prognostic relevance in epithelial ovarian carcinomas [16]. To further explore the role of HSP90 in ovarian cancer, it is necessary to perform the gain of function assay. Overexpression of HSPAA1 was performed by transfecting SKOV3 cells with recombination pcDNA3-HSP90AA1 plasmid. Western blotting analysis showed the level of HSP90AA1 protein was up-regulated in transfected cells, which was about two or three times more than the endogenous HSP90 protein level (Fig. 4).

HSP90AA1 overexpression induces chemoresistance of SKOV3 cells

Ovarian cancer is chemoresistant to many drugs, including cisplatin, which is a commonly used chemotherapy in clinical treatment. In our preliminary experiment, we have performed a series of experiment in order to determine the optimum doses of cisplatin to inhibit cell viability. The inhibition effect was gradually increased along with the increase in the cisplatin concentration. However, this effect was not significantly changed when the concentration exceeded 10 μg/ml. Therefore, we selected 10 μg/ml as our experiment concentration (Fig. 5). Compared with the control, the cell survival was inhibited significantly by cisplatin

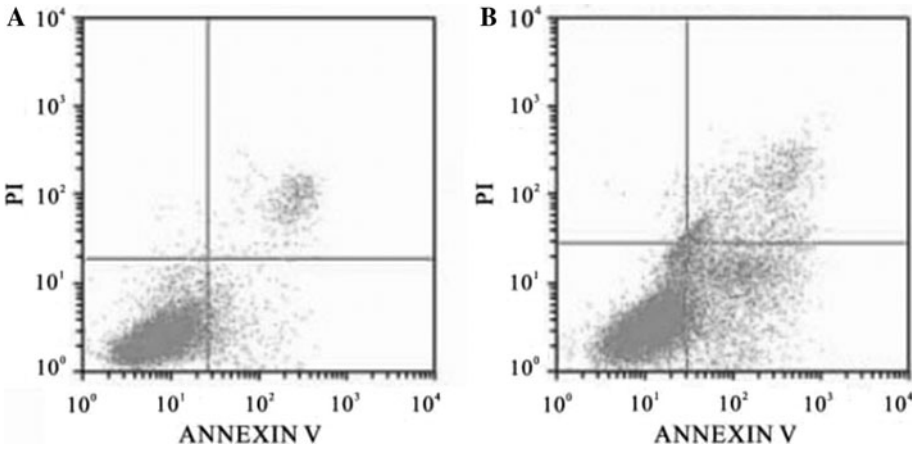

Fig. 3 HSP90AA1 RNAi induced apoptosis of SKOV3 cells. Cell apoptosis assay was performed using the Annexin V-FITC/PI kit and FACS analysis. **a** Control group: the normal saline treated cells, **b** Experiment group: HSP90AA1 RNAi. Annexin V-FITC positive cells are apoptosis cells while PI staining were negative to apoptosis cells, thus Annexin V-FITC (+)/PI (−) sections indicate apoptosis. The apoptosis percentage was 20 ± 1.2 % in RNAi cells while the control was only 3.8 ± 0.4 %

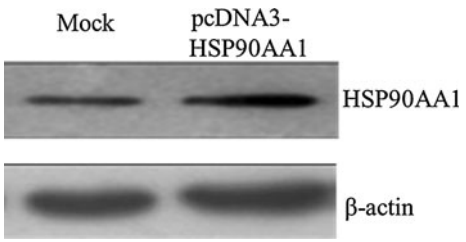

Fig. 4 Protein expression of HSP90AA1 in control and HSP90AA1 overexpression SKOV3 cell lines. *Lane 1*: *Mock* transfected control; *Lane 2*: *pcDNA3-HSP90AA1* transfected SKOV3 cells. The protein levels were detected by indicated antibodies. *β-actin* was used as an internal control to ensure equal loading of each lane

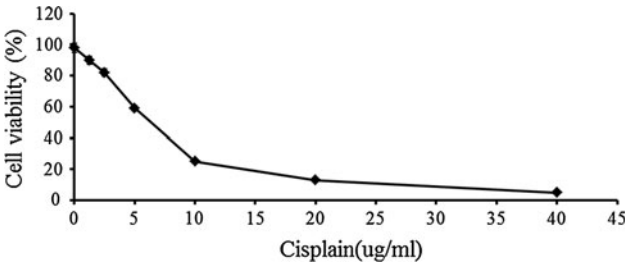

Fig. 5 A concentration response curve for cisplatin. *Cell viability* was gradually decreased along with the increase in the *cisplatin* concentration. However, the inhibition effect was not statistically significant when the concentration exceeded 10 μg/ml

at a concentration of 10 μg/ml (Fig. 6, lane 1, 2). HSP90AA1 recombinant plasmid was transfected to the cells, followed by cisplatin treatment for 24 h. Overexpressed HSP90AA1 dramaticlly recovered the cell survival in cisplatin treatment group, while the control group with no drug was unchanged (Fig. 6, lane 3, 4 compared with lane 1, 2).

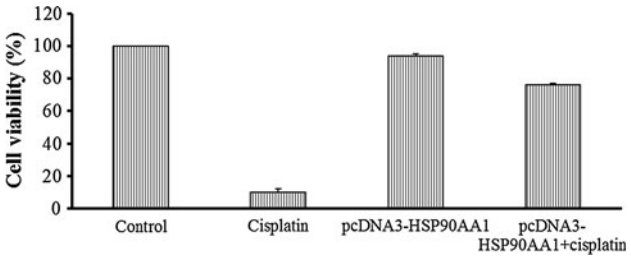

Fig. 6 HSP90AA1 overexpression reduced the cell death caused by cisplatin treatment. MTT assay was performed to SKOV3 cells. *Lane 1*: The mock transfected control cells; *Lane 2*: The mock transfected control cells treated by 10 μg/ml cisplatin. *Lane 3*: *pcDNA3-HSP90 AA1* transfected cells; *Lane 4*: *pcDNA3-HSP90AA1* transfected cells treated by 10 μg/ml cisplatin. Three independent experiments were performed and *T* test was analyzed. ** indicated $P < 0.01$

HSP90AA1 overexpression rescues the apoptosis of SKOV3 cells caused by cisplatin

In order to investigate the effect of overexpressed HSP90 AA1 to the chemoresistance of SKOV3 cells, Annexin V-FITC/PI dual staining followed by FACS assays were performed to analyze the apoptosis of the cell. Cisplatin (10 μg/ml) induced cell apoptosis to 80 ± 4.3 % while the apoptosis percentage of the untreated control cells was only 4.4 ± 0.2 % (Fig. 7a, b). When HSP90AA1 was overexpressed and followed by cisplatin treatment, the apoptosis percentage was decreased to 20 ± 1.1 % (Fig. 7c). Consistent to Fig. 6, HSP90AA1 overexpression reduced the apoptosis caused by cisplatin.

Discussion

HSP90 is a ubiquitously expressed molecular chaperone that is involved in the posttranslational folding and stability

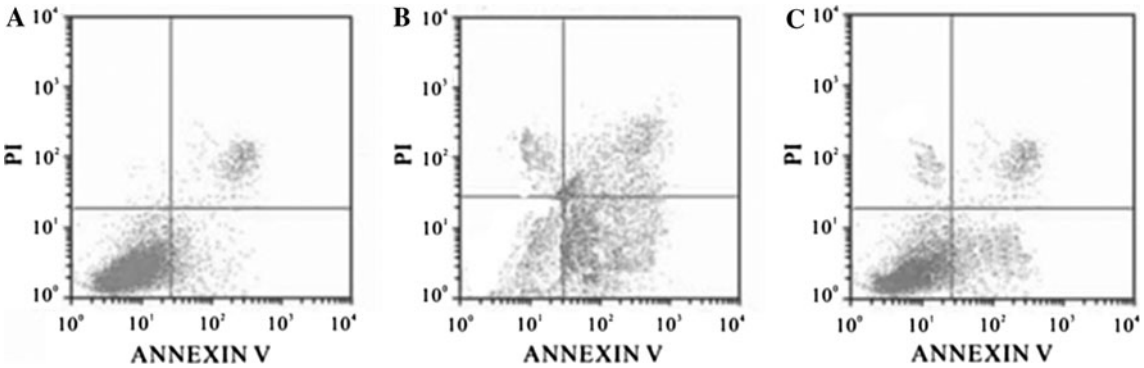

Fig. 7 HSP90AA1 overexpression decreased the percentage of apoptosis caused by cisplatin treatment. Annexin V-FITC/PI dual staining and FACS analysis were performed. **a** The mock transfected control cells: the apoptosis percentage was 4.4 ± 0.2 %, **b** The mock transfected control cells treated by 10 μg/ml cisplatin. The apoptosis percentage was 80 ± 4.3 %, **c** pcDNA3-HSP90AA1 transfected cells were treated by 10 μg/ml cisplatin. The apoptosis percentage was 20 ± 1.1 %

of multiple mutated, chimeric and over-expressed signaling proteins that promote the growth and/or survival of cancer cells [17]. Previous studies have shown that HSP90 is widely expressed in ovarian cancer and correlated with Federation International Of Gynecology And Obstetrics (FIGO) stage [18, 19]. Thus, it is conceivable that the specific inhibition of HSP90 sub-member, HSP90AA1 expression may affect the proliferation and apoptosis of cancer cells. By using siRNA approach, we first quantitatively evaluated the association between expression of HSP90AA1 and survival of ovarian cancer cell line SKOV3. As expected, the HSP90AA1 protein level in SKOV3 cell was significantly decreased after transfection with siRNA3, which result in lower proliferation activity and higher apoptosis rate compared with control.

It is a common phenomenon that tumor cells become resistant to chemotherapy. The cancer cells escape the caspase-mediated apoptotic process by overexpressing antiapoptotic proteins, or by inactivating proapoptotic factors [20]. Several lines of evidences have suggested the relationship of HSP proteins (one important anti-apoptotic protein family) and chemoresistance in ovarian cancer cells [21]. For example, Hsp70 is found highly expressed in cisplatin-resistant ovarian cancer cells and Hsp70 promotes chemoresistance, in part, by blocking Bax translocation to the mitochondria and mitochondrial protein release to cytosol [22]. Nuclear HSP90 expression is also significantly higher in post-chemotherapy compared to pre-chemotherapy effusions of advanced-stage ovarian carcinoma patients and cytoplasmic HSP90 expression is significantly associated with that of Bcl-2 in pre-chemotherapy effusions and marginally associated with cytoplasmic Survivin expression in post-chemotherapy effusions [23]. In this study, the HSP90 AA1 was also overexpressed by transfecting SKOV3 cells with recombination pcDNA3-HSP90AA1 plasmid. The cell survival in transfected SKOV3 cells was recovered to some extent, although undergoing cisplatin treatment, suggesting cisplatin chemoresistance. This may be attributable to persistent stabilization and activation of phosphatidyl inositol 3-kinase/AKT pathway by HSP90 [8, 24]. Interestingly, recent studies indicate cisplatin has high affinity for Hsp90 and binds to the C-terminal dimerization domain of Hsp90 [25, 26]. Cisplatin can inhibit Hsp90 function in vitro by preventing the rematuration of citrate synthase, a well-known Hsp90 client [27]. Thereby, the overexpressed HSP90AA1 may also bind and squelch cisplatin, ultimately decreasing the effective concentration of the chemotherapy and promoting cell survival. However, the exact mechanism still needed to be further explored.

Conclusion

In summary, this report investigated the role of HSP90AA1 in ovarian cancer cells. Our preliminary studies suggested that HSP90AA1 can function as factor in ovarian cancer cells and promotes chemoresistance to cisplatin. As the complexity of ovarian cancer and the wildly usage of HSP90 inhibitors, the inhibition of the HSP90 could potentially act as a promising adjuvant to chemotherapies to overcome drug resistance. Further studies would be informative to investigate the HSP90 inhibitors in combination with chemotherapy in clinic.

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