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Original Research Article

Identification of Proteins Participating in the Cisplatin Resistance Following Treatment with Cisplatin in A2780 and A2780CP Ovarian Cell Lines

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HIGHLIGHTS

- The major problem during treatment with cisplatin is the appearance of acquired resistance in cancer cells.
- The cisplatin- resistant and sensitive ovarian cell lines of A2780 were evaluated in this study.
- Proteomics analysis was run using 2D gel electrophoresis after cisplatin treatment.
- Three proteins were determined as the key players of resistance against cisplatin in A2780 cell line.

ABSTRACT

Ovarian cancer is the most fatal gynecological cancer and the 8th most prevalent type of cancer in Iran. Chemotherapy regimen for the treatment of this type of cancer is mostly based on platinum agents and paclitaxel. The major problem during treatment with cisplatin is the appearance of acquired resistance in cancer cells in the first 6 months of therapy. Owing to inefficacy of second line regimens, it seems necessary to find out the molecular mechanisms of cisplatin resistance and find an efficient strategy against the resistant cancer cells. In this study, two ovarian cancer cell lines, A2780-sensitive and A2780CP (resistant to cisplatin) were evaluated. To acquire the protein expression profile, a culture of each line containing 1.5×10^7 cells was divided into the two groups of control and treatment cells. The treatment group cells were treated with cisplatin at pre-determined IC_{50} concentration for 6 hours. Then, the total proteins of 7×10^6 cells were extracted. The proteome of each group (20 µg) was used for subsequent separation of proteins by two-dimensional gel electrophoresis using 7 cm IPG strips. The results of protein pattern changes were analyzed by one-way ANOVA. At least 230 proteins were detected in each gel, from which, about 45 proteins were differentially expressed in each model of comparison. However, by considering the results of all models of analysis on the protein expression profile of two cell lines, three proteins were determined as the key players of resistance against cisplatin. Our results can be considered in the therapeutic regimen of ovarian cancers with cisplatin as important knowledge for defending against resistant cancer cells.

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Introduction

Ovarian cancer with the prevalence of yearly at least 240000 cases causing 150000 deaths annually, is the 8th

most frequent cancer among women (Webb and Jordan, 2017). According to the most recent guidelines on the treatment of epithelial type of this cancer, alongside surgical procedures, platinum-based agents such as carboplatin and cisplatin plus paclitaxel are the main choices of chemotherapy regimens prescribed by oncologists (Ledermann et al., 2013; Armstrong et al., 2019). Despite hopeful response of patients to platinum agents, there are a group of patients, referred to "Platinum resistant", suffering from the relapse of cancer in the first 6 months of therapy. This group has shown to have the survival of less than a year, and low response to further therapy with platinum agents which is a major implication of using these drugs (Davis et al., 2014). Having a deep insight into the underlying mechanisms behind the resistance is of great importance.

Cisplatin, an agent with the mechanism of DNA adduct formation, was firstly approved by the Food and Drug Administration (FDA) to be used in testicular, ovarian, head and neck cancers in 1979 (Muggia, 2009). DNA-adduct formation and as a result, cell cycle arrest at G2 phase together with oxidative stress induction are the major mechanisms of cisplatin-driven apoptosis (Sorenson et al., 1990; Dasari and Tchounwou, 2014). With regards to the mechanisms of resistance to platinum-based drugs, Stewart has divided the factors involved in the resistant mechanism into two categories. First, "Classical factors" of resistance, the most important mechanisms of this group, are DNA repair mechanisms which increase drug efflux and drug detoxification. Second, the "Newer Factors" group of resistance consists of heat shock proteins, cell signaling proteins and cell cycle related proteins (Stewart, 2007). Glutathione as a major member of anti-oxidant defense system of the cells has been studied extensively as one of the key players of resistance (Jamali et al., 2015).

Whatever the underlying molecules or mechanisms are involved in resistance, proteins are the functional correspondent of cells phenotypes, characteristics and activities. Thus, studying the whole proteome of the sensitive and resistant cells would give a complete, precise and comprehensive attitude towards cisplatin resistance. In this study, the whole protein expression profiles of sensitive (A2780S) and resistant (A2780CP) ovarian carcinoma cell lines with and without treatment by cisplatin were studied. Three-model comparison of proteome of cells provides more supplemented information on resistance, compared to the last studies. In the first model, the proteome profiles of sensitive and resistant cells were compared. In the second model, the protein expression profiles of sensitive and resistance cell lines were compared following treatment. Then, in the third model, alterations in protein expression profiles were investigated after the cell treatment with cisplatin to elucidate the proteins expressed differentially in cells and the changes in their expression following treatment with cisplatin.

Materials and Methods

Cells and culture

A2780S and A2780CP cell lines (Pasteur institute, Tehran, Iran) were cultured in T-75 flasks (Jet Biofil, China) in RPMI 1640 medium (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA) and 1% Penicillin/Streptomycin (Gibco®, USA) and sub-cultured three times before running the experiments. Growth condition was fixed for all cultures in a humid CO_2 (5%) incubator at 37 °C. On the day of experiment, flasks were divided into the two groups of control and treatment, which in the treatment group, cisplatin was added to the media to reach the concentration equal to IC_{50} (6 h) of cisplatin on the mentioned cell lines. The cells were then kept in the incubator for a period of six hours. Attached cells were washed 3 times with washing buffer (Isotonic sucrose-Tris pH 7, Sigma Aldrich, Germany) and harvested using a cell scraper (Jet Biofil, China).

Protein extraction

After counting, 7×10^6 cells were collected and washed again 3 times by washing buffer and centrifuged. Lysis buffer (500 µL, containing 7 M Urea, 2 M Thiourea, 4% Chaps, 40 mM Tris, 0.2% Biolyte, 50 mM DTT, protease cocktail inhibitor, pH 8.8) was added on the cells, pipetted and then, frozen for 20 minutes in -20°C freezer. To complete cell lysis, a modified procedure of cell lysis of the published work was conducted (Wu et al., 2009). Briefly, exploiting probe homogenizer, the lysis solution was sonicated 2 s with the power of 200 W followed by 5 s break for a total of one minute while held in the ice. Then, the microtubes containing lysis solution were undergone gentle vortexing as described in the reference. Subsequently, centrifugation was done for 15 minutes at 15 °C with the power of 20000 g. Following that, protein-rich supernatant was collected and to which, then, DNase and RNase were added and stored at room temperature for one hour. To separate un-solubilized DNA and RNA, again the same centrifugation procedure was done. After that, by adding 8 volumes of cold acetone-methanol (8-1, Merck, Germany) to the supernatant, it was stored at -20°C overnight to precipitate proteins.

2D gel electrophoresis of extracted proteome

Precipitated proteins were dried off acetone-methanol and solubilized in 250 µL of rehydration buffer (7 M Urea, 2 M Thiourea, 4% Chaps, Bromophenol Blue-Tris, 0.2% Biolyte, and 50 mM DTT, pH 8.8). To assure

complete solubilization, the solution was kept in room temperature for 4 hours alongside frequent vortexing. Micro-scale Bradford assay (Takara, Japan) was utilized for determination of protein concentration. In order to eliminate the interferences of the components of the rehydration buffer with Bradford reagent, the absorption of rehydration buffer was measured to eliminate background absorption. Then, 20 µg of protein was loaded on 7 cm immobilized pH gradient (IPG) strip pH 3-10 (Bio-Rad, USA) and strips were stored overnight to rehydrate them, passively. Trays were filled with mineral oil (Titran, Iran) to prevent strip drying.

Prior to run the isoelectric focusing (IEF) as the first dimension of 2D electrophoresis, a paper soaked in a 15 mM DTT solution was placed between the connection point of the strip (the pH=10 end) and the cathode. Isoelectric focusing was performed using π-focus IEF strip system (Elettrofor scientific instruments, Italy) by applying a total of 12000 V.H. energy to strips with the following program: 200 V for 1 hour, gradual increase to 1500 V in 1 hour, 2000 V for 30 minutes, gradual increase to 3000 V in 1 hour, Hold on 4000 V till the end of program. Quickly after IEF, strips were equilibrated in equilibration buffer I (6 M Urea, 0.375 M Tris, 20% Glycerol, 2% SDS, 2% DTT) followed by the second equilibration in equilibration buffer II (6 M Urea, 0.375 M Tris, 20% Glycerol, 2% SDS, 2.5% Iodoacetamide, bromophenol blue), each step lasting 20 minutes.

To avoid protein loss, swiftly after equilibration, strips were located on the top of 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and embedded with 0.5% fresh agarose. The second-dimension separation was run under 70 V constant voltage for 30 minutes followed by 120 V constant voltage.

Silver staining of SDS-PAGE gels

For the staining of the gels, a modified protocol of fast silver nitrate staining was done (Chevallet et al., 2006). In brief, the gels were fixed overnight and then, washed by 20% alcohol solution and deionized water. Then, the gels were sensitized in sodium thiosulfate solution (Merck, Germany) for 1 minute followed by being incubated in staining solution for 30 minutes. After a 10s wash by deionized water, the gels were soaked in sodium carbonate solution 6% (Titran, Iran) for 8 minutes. The staining reaction was stopped by stopping solution (50% methanol, 6% Acetic acid glacial) and the gels were kept in 2% acetic acid solution in deionized water till image acquisition.

Gel image analysis

Images of the gels were captured and scanned using GS-800 densitometer (Bio-Rad, USA). For gel analysis, which was done by Samespot® software, one gel image was selected as the reference, and after a precise manual and automatic alignment, 3 models of analysis were done. In the first model, A2780CP treated gel was compared with A2780S treated gel. In the second model, the same comparison as model 1 was done with the control group. Then, in the third model, the control and treated gels of each cell lines were compared with each other. Identified spots were evaluated in facets of relative expression changes and one-way ANOVA for p-value determination of each spot was employed. Spots with more than 2-fold alteration in expression and the p-value below 0.05 were selected as candidates for having a role in cisplatin resistance. Using 2D gel databases, spots identities were predicted based on their pI and molecular weight in defined 2D gel maps of HepG2, epithelial colorectal and Hela cancer cells at https://world-2dpage.expasy.org.

Results and Discussion

Protein extraction

The results of Bradford assay for determining the concentration of soluble proteome of cell lysates (6×10^7 cells) are shown in Table 1. The correlation coefficients of all Bradford assays were higher than 0.97. The data of Table 1 depicts a highly efficient protein extraction. An average of 160 pg of protein has been extracted from each cell. The high efficiency of protein extraction might be due to the usage of a combination of different methods for cell lysis. In our study, sonication, freezing, gentle vortexing and a lysis buffer containing surfactant, urea and thiourea with high capacity of protein solubilization were used.

Table 1. Results of protein extraction from different cell lines

Cell line (group)	A2780S (Control)	A2780S (Treatment)	A2780CP (Control)	A2780CP (Treatment)
Protein concentration (mg/mL)	3.63 (±0.16)	4.42 (±0.18)	3.42 (±0.19)	4.56 (±0.23)
Total amount of protein extracted (mg)	0.91 (±0.04)	1.11 (±0.5)	0.86 (± 0.05)	1.14 (±0.06)

Proteome analysis

Figure 1a illustrates the 2D map of the total proteome of A2780S-control. A total of 237 spots has been identified that each represented a protein differentially expressed in cell lines applying the third model of analysis (Figure 1b). As an example, Figure 2 shows the analysis of one spot compared in the 2D image of A2780S-control and A2780CP-control.

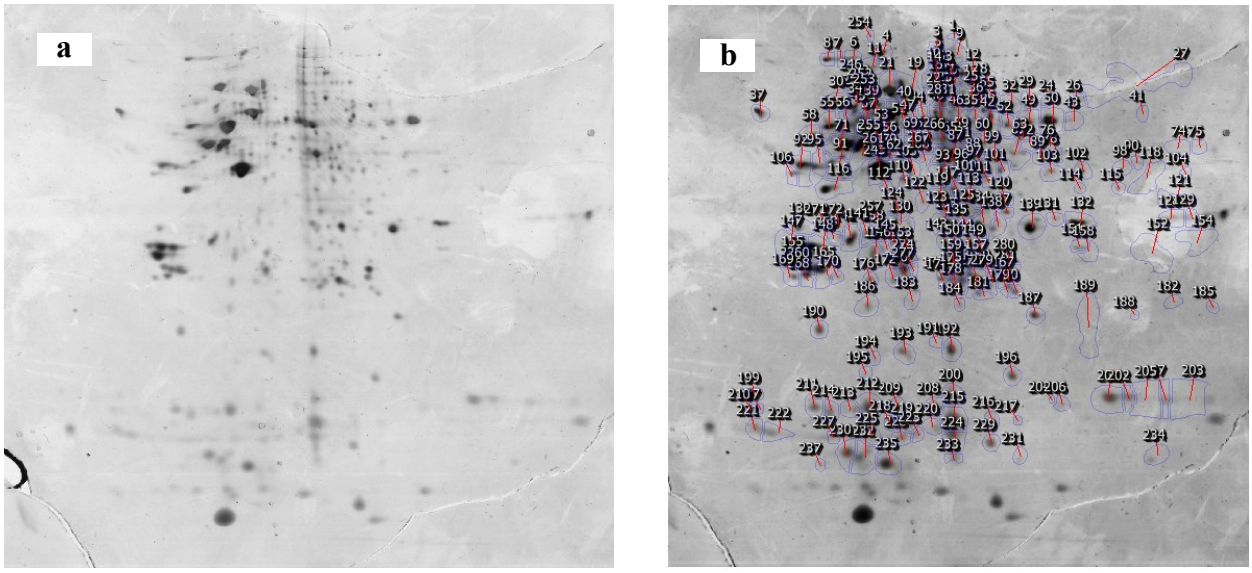

Figure 1. Image of 2D electrophoresis gel of the total proteome of A2780S-control staining with silver nitrate (a), The mapping image of 237 dots on 2D electrophoresis gel image of the total proteome of A2780S-control staining with silver nitrate (b).

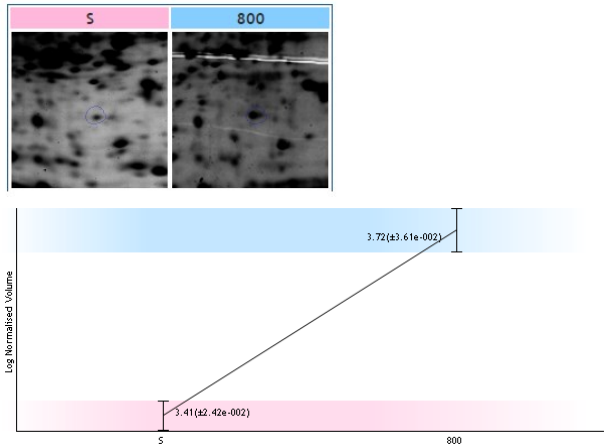

Figure 2. The diagram of the comparison of a spot in the gel image of A2780S-control and A2780CP-control.

Proteins with altered expression in all models of analysis

At least 45 proteins had significant alterations in their relative expression in each model of comparison, but the relative expression of only a slight number of proteins was altered harmoniously. Table 2 shows the spots which were differentially expressed in both models of analysis.

Spot 226 (predicted pI 5.6, MW: 15.6 kDa): The amount of this protein has been increased in all models of analysis significantly. The predicted proteins for this spot are fatty acid binding protein-1 and superoxide dismutase. It has been shown that Fatty acid binding protein-1 expressions are increased in response to cisplatin nephrotoxicity (Huang et al., 2001; Will et al., 2008). Knocking down the Superoxide dismutase as one of the members of ROS scavenging system in cells is shown to sensitize the resistant ovarian cancer cells to cisplatin (Kim et al., 2010). Thus, this protein could be one of the main players of cisplatin resistance. Moreover, Superoxide dismutase overexpression certainly has a role in cisplatin resistance.

Spot 229 (Predicted pI 7.1, MW: 14. kDa): The expression of this protein has risen in all models of analysis. The predicted protein, Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A), has been mentioned by another study for its contribution in resistance to cisplatin in the resistant subline of ovarian cancer A2780^{CisR} (Huq et al., 2014).

Table 1. Results of protein extraction from different cell lines

Spot number	Fold change 1st model (p. value)	Fold change 2nd model (p. value)	Fold change 3rd model (p. value)	pI	MW (kDa)	Predicted Protein(s)
226	1.8↑	3.9↑	2↑	5.2-5.4	10-15	- FABP-1 -Superoxide dismutase
229	2.5↑	4.9↑	1.6↑	6.9-7.1	10-15	- Peptidyl-prolyl cis-trans isomerase A
214	3.3↓	2.2↓	Not detected	4.1-4.3	15-20	-Calmodulin

Spot 214 (Predicted pI 4.1, MW: 16.7 kDa): The amount of this protein has decreased in the first and second model of analysis. Calmodulin is the predicted spot of this protein which is down-expressed in resistant cell lines and after treatment with cisplatin. Calmodulin has been previously reported to have a role in resistance to cisplatin. It has 4 binding sites for calcium ion which is an important second messenger (Castagna et al., 2004).

A2780CP cell line firstly was established in the lab in 1990 by induction of resistance by stepwise elevation of cisplatin during a long-time exposure to cisplatin rather than a selection of resistance cell populations by a short exposure to the high concentration of cisplatin. It was also mentioned in the paper that the level of resistance could be increased by induction with higher concentrations of cisplatin (Schilder et al., 1990). It can be concluded that cisplatin resistance in this cell line is mostly induced in the presence of cisplatin, although some leading correspondents of cisplatin resistance may be active without the existence of drug in the medium. In last studies regarding identification of key proteins of cisplatin resistance using 2D gel electrophoresis-mass spectrometry, treatment with cisplatin had not been considered as an independent variable, and claimed proteins were the results of the only comparison of sensitive and resistant cell lines without treatment with cisplatin (Lincet et al., 2012; Zhou et al., 2012). It is our hypothesis that key players of cisplatin resistance are activated or included in resistance in the presence of cisplatin in the medium and adding the results of cisplatin treatment to the results of the first and second mode of comparison will reduce the complexity of results. Indeed, the number of proteins that are shown to be comprised in resistance will decrease remarkably. The results of Castgna and colleagues also confirm our hypothesis. In their study, they had considered the treatment with cisplatin and compared the gels in 4 ways. There were only 7 proteins which were identified in more than one way of analysis of cisplatin resistance in A431 sensitive and resistant cell lines. These proteins were Calmodulin, Voltage-dependent anion-selective channel (VDAC-1), Microtubule-associated protein RP/EB, Heat-shock cognate 71 kDa protein, Stathmin, 14-3-3 and Peroxiredoxin (isoforms 1, 2 and 6) (Castagna et al., 2004).

Conclusion

Resistance to cisplatin is the most causing complication of the treatment of ovarian cancer with cisplatin. 2D gel electrophoresis of the whole proteome of sensitive and resistant ovarian cell lines has been studied and some proteins have been reported that may have an important role in the resistance against cisplatin. In this study, in addition to cell subline (sensitive or resistant), treatment with cisplatin has been taken into account for gel analysis and protein identification. By merging the results of 3 modes of analysis and detecting the proteins that their expressions have been changed in a similar pattern, three proteins were reported to be mostly included in the generation of resistance. Considering the results of cisplatin treatment with other models of comparison concurrently, led to the identification of main changes in proteins expression, which in following step, were applied to predict the main proteins involved in the resistance progress against cisplatin in ovarian cancer cell line.

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Competing Interests

The authors declared that there is no conflict of interest.

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