RESEARCH ARTICLE

Identification of Cisplatin-Resistance Associated Genes through Proteomic Analysis of Human Ovarian Cancer Cells and a Cisplatin-resistant Subline

Jing Zhou[1], Yue-Hua Wei[2], Mei-Yan Liao[2]*, Yan Xiong[1], Jie-Lan Li[2], Hong-Bing Cai[1]

Abstract

Chemoresistance to cancer therapy is a major obstacle to the effective treatment of human cancers with cisplatin (DDP), but the mechanisms of cisplatin-resistance are not clear. In this study, we established a cisplatin-resistant human ovarian cancer cell line (COC1/DDP) and identified differentially expressed proteins related to cisplatin resistance. The proteomic expression profiles in COC1 before and after DDP treatment were examined using 2-dimensional electrophoresis technology. Differentially expressed proteins were identified using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and high performance liquid chromatography-electrospray tandem MS (NanoUPLC-ESI-MS/MS). 5 protein spots, for cytokeratin 9, keratin 1, deoxyuridine triphosphatase (dUTPase), aarF domain containing kinase 4 (ADCK 4) and cofilin1, were identified to be significantly changed in COC1/DDP compared with its parental cells. The expression of these five proteins was further validated by quantitative PCR and Western blotting, confirming the results of proteomic analysis. Further research on these proteins may help to identify novel resistant biomarkers or reveal the mechanism of cisplatin-resistance in human ovarian cancers.

Keywords: Cisplatin resistance - ovarian cancer - proteomics - MALDI-TOF-MS - electrospray tandem MS

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Introduction

Ovarian cancer is the fifth most common cause of cancer deaths in women and accounts for the highest tumor-related mortality of gynecologic malignancies (Jemal et al., 2009). Cisplatin and its analogues are the key compounds of chemotherapy for human ovarian cancers, but chemoresistance is a major obstacle hindering the successful treatment of ovarian cancer patients (Ozols et al., 1985; Yuan et al., 2003). The optimal cytoreductive surgery followed by a first-line platinum-based chemotherapy is an effective strategy, however, the 5-year survival rate for stage III and IV disease is about 20%-30%.

A number of genes, such as GST-pi, LRP, MDR1, XIAP, HER2/neu, hMLH 2, and hMSH1, BRCA 2, mdrl, BCL-2 and BCL-XL (Hamada et al.,1994; Veneroni et al., 1994; Aebi et al., 1996; Marth et al., 1997; Sasaki et al., 2000; Rudin et al., 2003; Williams et al., 2005 Yuan et al., 2011), have been linked to drug resistance in ovarian cancer, whereas general consensus for biomarkers has not been established to detect tumor resistance to special chemotherapy. Because the gene transcription may be inconsistent to protein level due to modification during a post-translational process, it is very necessary to perform high-throughput studies at protein level, besides the mRNA level (Yan et al., 2007). In our study, cisplatin-resistance associated proteins were discovered by comparing cell line of resistant cell and its parental cell, using two-dimensional gel electrophoresis (2-DE), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and high performance liquid chromatography-electrospray tandem MS (NanoUPLC–ESI- MS/MS). Based on our previous study, we have further confirmed and identified 5 proteins to be down-regulated in resistant lines. They may be involved in the mechanisms of cisplatin resistance in ovarian cancer.

Materials and Methods

Materials

Cisplatin was puchased from QiLu drug manufacturer (ShanDong, China). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-dipherytetradium bromide was purchased from Sigma (St.Louis, MO). Protease inhibitor cocktail was purchased by Roche (Mannheim, Germany). DyNAmo PCR Master Mix was purchased from Finnzymes (Espoo, Finland).

[1]*Department of Gynecology Oncology,* [2]*Medical Imaging Central, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China*
**For correspondence: Meiyanl2002@Yahoo.com.cn*

Rabbit polyclonal anti-ADCK 4 antibody, mouse monoclonal anti-cofilin 1 antibody, mouse monoclonal anti-cytokeratin 9, mouse monoclonal anti-keratin 1, mouse monoclonal anti-dUTPase, mouse monoclonal anti-cofilin1 and mouse anti-actin antibody were all purchased from Santa Cruz Biotechnology, Inc(Europe, USA).

Cell Lines and Culture Conditions

Human ovarian cancer cells (COC1) and its cisplatin-resistant sublines (COC1/DDP) were provided by Wuhan University Type Culture Collection and the COC1/DDP cells were of 6.5-fold resistance to DDP(Zhou et al., 1996). Two cell lines were maintained in DMEM containing 10% fetal calf serum. Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cell lines grew in a monolayer and were passaged when cultures were 70%-80% confluent.

2-DE

Cell samples were lysed in solubilization buffer (100 μL per 10^7 cells) containing 40 mM Tris, 8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 60 mM dithiothereitol (DTT), 1 mM ethylene diamine tetracetic acid, 1 × protease inhibitor cocktail, 0.1 g/L RNase A, and 0.1 g/L DNase I with sonication (5 s/cycle, 5 cycle; 0 °C). After centrifugation at 14 000×g for 30 min at 4 °C, the supernatant was collected. Protein concentration was determined by Bradford protein assay. The protein samples were stored at - 80 °C in aliquots until use.

2-DE was performed as described (Cecconi et al., 2005). Proteins (1.2 mg) were diluted to 350 μL with rehydration solution (8 M urea, 4% CHAPS, 20 mM DTT, 0.5% immobilized pH gradient buffer, trace of bromphenol blue) and loaded into 18 cm (pH 3-10) nonlinear immobilized pH gradient DryStrip (Amersham Biosciences, Little Chalfont, UK). The IPGphor system (Amersham Biosciences) was used for the first dimension isoelectric focusing, for a total running time of 80 000 Vh. Prior to the second dimension separation, the strip was equilibrated for 15 min with equilibration solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS, 0.3% DTT, and trace of bromphenol blue. A second equilibration step was also carried out for 15 min in the same solution except for DTT, which was replaced by 2.5% iodoacetamide. Separation in the second dimensional electrophoresis was carried out in the PROTEAN II xi Cell(Bio-Rad, Hercules, CA) with a 13% SDS-polyacrylamide gel at a constant current of 20 mA/gel for the initial 40 min and 30 mA/gel thereafter until the bromphenol blue dye marker reached the bottom of the gel. The experiments were repeated twice to determine the variability.

2-DE Image Analysis

Protein patterns were directly visualized by Coomassie Brilliant Blue R-350 staining. The two dimensional electrophoresis patterns were captured with the ImageScanner (Amersham Biosciences). Spot detection, quantification, and matching analysis were performed with the ImageMaster 2D Platinum 5.0 software (Amersham Biosciences). Spot intensity was expressed as percentage of the spot volume in the total sum of all spot volumes on the gel. Image analysis was performed by comparing the quantity of matched spots between resistant sublines and the parental (sensitive) cell lines.

In-Gel Digestion and Peptide Mass Fingerprinting by MALDI-TOF-MS and NanoUPLC-ESI-MS/MS

After matching the gel image with the analytical software, in-gel digestion was performed with a previously published protocol (Yang et al., 2005).Target proteins were prepared as indicated by the instruments' manufacturer. A saturated solution of α-cyano-4- hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. One microliter of matrix solution and sample solution (1:1) was mixed and applied onto the target plate. 3 spots mass spectra of MALDI-TOF-MS were obtained on a Bruker REFLEX III MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) and the other 2 spots were obtained on a NanoUPLC-ESI-MS/MS (nanoACQUITY UPLC, Waters, USA). NanoUPLC-ESI -MS/MS was performed as described (Liu et al., 2010). The peptide mass fingerprints obtained were used to search through the SwissProt and NCBInr database by the Mascot software. There was no limitation in protein mass and fixed modification. Monoisotopic peptide masses and MH+ mass values were used to search the database. A peptide mass tolerance of 100 ppm and one missed cleavage was allowed. Six matching peptides were the minimal requirement for an identity assignment. Variable modifications such as oxidation of methionine and carbamidomethyl modification of cysteine were considered.

Quantitative PCR

Total RNA was isolated with TRIzol Reagent according to the supplier's instructions. The isolated RNA was used for the preparation of first-strand cDNA by reverse transcription. The RNA samples were incubated in 25 μL of reaction buffer at 42 °C for 60 min. For the determination of the five target cDNA contents, reactions containing 0.25 mM of each primer, 1 μL of template cDNA, and 5 μL DyNAmo PCR Master Mix in a total of 10 μL was performed in a Chromo 4 thermocycler (MJ Research, Waltham, MA). β-actin cDNA fragments were amplified as internal positive controls. The PCR conditions included an initial denaturation of 10 min at 95 °C, followed by 40 cycles consisting of 30 s of denaturation at 95 °C, 30 s of primer annealing at 56 °C, and 25 s of elongation at 72 °C. Data were analyzed using Opticon Monitor 3.0 software. The amplification of the target fragments from cisplatin-resistant cells, relative to their amplification in the corresponding parental cells, was determined by quantitative PCR using the ΔΔCT method (Livak et al., 2001). Quantitative PCR assays were conducted in triplicate for each sample, and mean value was used for calculation. The sequence of each primer and product length are shown in Table 1.

Western Blot Analysis

Cells were lysed in Laemmli Sample Buffer (Bio-

Table 1. DNA Sequence of the Forward and Reverse Primers, Length of Products, and Annealing Temperature for Quantitative PCR

primer designation	sequence	product length (bp)	Annealing temperature (°C)
dUTPase	GCTTGGCTGCAAAACACTTT		
	TCCAGTGGAACCAAAACCTC	227	56
keratin 1	ATCAACTACCAGCGCAGGAC		
	AAAGCCACTACCACGTCCAC	198	56
cytokeratin 9	CTCCTGGCAAAGATCTCACC		
	GCAGCTCAATCTCCAACTCC	213	56
ADCK4	TTCCGATTCATGCAGACTGA		
	CTGGACTTCTGCAGGACACA	182	56
cofilin1	ATGCCCTCTATGATGCAACC		
	TTCATGCTTGATCCCTGTCA	158	56
β-acting	CACGATGGAGGGGCCGGACTCATC		
	TAAAGACCTCTATGCCAACACAGT	240	56

Rad) on ice. The lysates were heated to 100 °C for 5 min and centrifuged (12 000 × g for 5min at 4 °C) to remove insoluble material. Following electrophoresis, proteins were electrophoretically transferred to polyvinylidene difluoride membrane at a constant current 200 mA for 1 h in ice-cooled transfer buffer. Membranes were blocked in 5% nonfat milk for 1 h at room temperature and then incubated overnight at 4 °C in the relevant primary antibody. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, peroxidase activity was visualized with the chemiluminescence kit according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were carried out by One-way ANOVA and Student's t test. When necessary, data were logarithmically converted into normal distribution of variable to remove heterogeneity of variance before analysis. $P<0.05$ was regarded as statistically significant.

Results

2-DE Maps of All Cell Lines

The protein lysates and 2-DE gels were processed in parallel. 2-DE was carried out on the protein samples of the two cell lines and was repeated at least three times from different samples. On average, 1581 protein spots were detected in COC1 profile compared to 1518 protein spots which were found in the COC1/DDP cell line after Coomassie Brilliant Blue R-350 staining by the auto-detect spots menu of analysis software and manual cleanup.

All the maps showed great similarity between the resistant subline and its parental cell line in which the matching rate reached 93.6%. In the matched spot, a 2.5-fold or higher difference in spot intensity was considered significant. With the image analysis software, we found that 97 protein spots were expressed at significantly different levels in the COC1 cell line to the COC1/DDP (41 were up-regulated, 56 were down-regulated). The pI of the differentially expressed spots ranged between 4 and 9, and the molecular weight was about 14-80 kDa (see Figure 1).

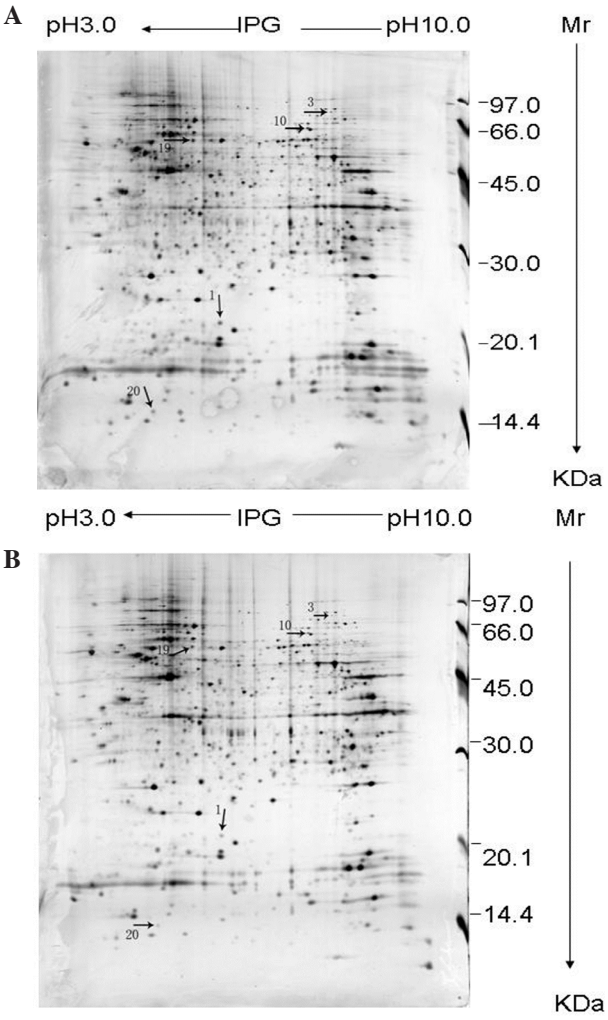

Figure 1. A. Representative picture of Coomassie Blue-stained 2-DE map of the sensitive cell line COC1. B. representative picture of Coomassie Blue-stained 2-DE map of the resistant cell line COC1/DDP. The representative differentially expressed proteins between resistant subline and its parental cell were marked (arrow) on the map

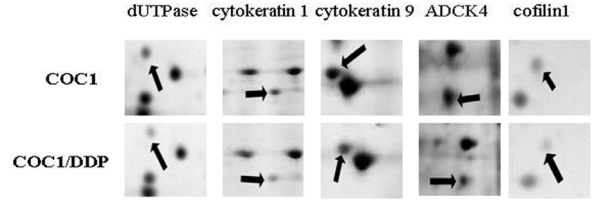

Figure 2. Representative Picture of Differentially Expressed Proteins Between COC1 and COC1/DDP after 2D Electrophoresis in Triplicate gGels. The spots with arrow indicated the target protein

Protein Identification by MALDI-TOF-MS and NanoUPLC-ESI-MS/MS

97 protein spots in all samples were found to be significantly different in spot intensity by statistical analysis ($P < 0.05$). In this study, we choose 3 spots to perform MALDI-TOF-MS (Figure 1, Table 2) and 2 spots to perform NanoUPLC-ESI-MS/MS (Figure 1, Table 3). Protein identification was repeated at least twice with spots from different gels for guaranteeing the reliability. The result showed that the matched spots from different gels were the same protein, and all the proteins

Table 2. Differentially Expressed Proteins in COC1/DDP Identified by MALDI-TOF-MS Compared with COC1

Spot	Protein name	NCBInr ID[a]	theoretical pI/Mr (Da)	Queries matches	score[b]	Expression
3	keratin 1	gi/11935049	7.82/66027	15	319	Lower
10	cytokeratin 9	gi/435476	7.37/ 62092	20	729	Lower
20	cofilin1	gi/5031635	4.96/18491	5	141	Lower

[a]ID, identification; [b]A score of more than 66 is significant (P<0.05)

Table 3. Differentially Expressed Proteins in COC1/DDP Identified by NanoUPLC-ESI- MS/ MS Compared with COC1

Spot	Protein name	NCBInr ID[a]	theoretical pI/Mr (Da)	Queries matches	score[b]	Expression
1	dUTPase	gi/4503423	6.05/17737	8	100	Lower
19	ADCK4 9	gi/217416386	5.43/55817	11	71	Lower

[a]ID, identification; [b]A score of more than 66 is significant (P<0.05)

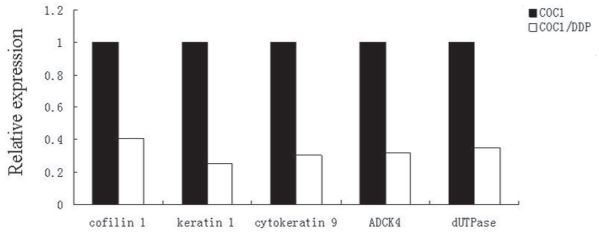

Figure 3. Relative Quantification of dUTPase, Keratin 1, Cytokeratin 9, ADCK 4, Cofilin 1 Related to Cisplatin Resistance Using the Comparative Method, β-actin was Used as Internal Standards. *P< 0.01 by the t test, when comparing its parental cell for each target mRNA. Relative gene expression of COC1 and its cisplatin-resistant subline for target mRNA. Results represent mean±SD of three separate experiments

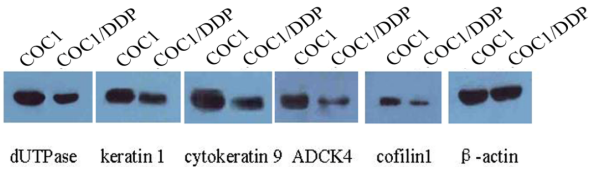

Figure 4. Western Blot Characterization of dUTPase, Keratin 1, Cytokeratin 9, ADCK 4, Cofilin1 in COC1 andCOC1/DDP. Forty micrograms of total proteins were run in SDS-PAGE. The protein bands of of dUTPase, keratin 1, cytokeratin 9, ADCK 4, cofilin 1 and β-acting are located at 23, 67, 55, 62, 18 and 42 kDa, respectively

spots showed the same expression trends in COC1/DDP cells. Interestingly, the top five proteins that were significantly changed were all downregulated in COC1/DDP cells, compared with its parental cells (Figure 2). Spot 1 (dUTPase), spot 3 (keratin 1) and spot 20(cofilin 1) decreased about 2.5-fold in COC1/DDP group, spot 10 (cytokeratin 9) decreased about 3-fold and spot 19 (ADCK 4) decreased around 5-fold.

Validation of Five Differential Proteins by Quantitative PCR and Western Blot

To validate the expression of 5 genes identified by 2-DE, we measured their mRNA level by quantitative PCR. As shown in Figure 3, in COC1/DDP cells, the mRNA expression levels of dUTPase, keratin 1, cofilin 1, cytokeratin 9 and ADCK 4 are greatly reduced to 35%, 25%, 49%, 30% and 30% of the control cells. Moreover, results of western blot showed that the protein expression levels of dUTPase, keratin 1, cofilin 1, cytokeratin 9 and ADCK 4 are greatly reduced to 40%, 40%, 40%, 33% and 20% in COC1/DDP cells compared with in COC1 cell (Figure 4). Consistent with the 2-DE result, both mRNA and protein level were significantly decreased in the cisplatin-resistant subline.

Discussion

For DNA/RNA sequence information provides only a static and limited snapshot, there is an increasing demand in proteomic techniques, which provide a new biological discipline contributing greatly to our understanding of gene function in the post-genomic era, protein expression and protein-protein interactions via a global, high-resolution capacity, and high-throughput study (Yan et al., 2007). Development of drug resistance can be attributed to various factors that include altered drug metabolism or uptake, avoidance of apoptotic cell death, increased repair of drug induced damage, altered gene expression and drug target (Kartalou et al., 2005). Through proteomic analysis, we found five proteins including dUTPase, keratin 1, cytokeratin 9, ADCK 4, cofilin 1, which were significantly downregulated in cisplatin-resistant human ovarian cancer cell line (COC1/DDP), and may play some roles in cisplatin resistance.

In COC1/DDP cell line, the expression of dUTPase, keratin 1, cytokeratin 9, ADCK 4, cofilin 1 showed a consistent down-regulated expression in both mRNA and protein level, by quantitative PCR and western blot (Figure 3 and 4). In neuroblastoma, dUTPase displayed a down-regulation in the etoposide-resistant clone (Andrea et al., 2005). However, in colon cancer cells, induced expression of dUTPase was reported to confer resistance to fluorodeoxyuridine (FUdR) (Canman et al., 1994). These suggest that the role of dUTPase in chemotherapy is according to the cell context. In chemoresistant stage IIIc primary serous epithelial ovarian cancer tissues, keratin 1 was under-expressed and significantly correlated with poor overall survival (Kim et al., 2011). Cytokeratin 9 was down-regulated in a colorectal cancer (CRC) cell line SW480 after 5-Fu treatment, which suggest that expression of cytokeratin 9 may be closely associated with the response to chemotherapy (Wong et al., 2008). The gene product of cofilin 1 (CFL1) is responsible for severing actin filaments and regulating actin polymerization and depolymerization during cell migration (DesMarais et al., 2005). This protein was reported to be related to apoptotic cell death, cancer invasion, metastasis, and chemoresistance (Wang et al.,

2004; Zhu et al., 2006; Wang et al., 2006; Yan et al., 2007). Yan et al. (2007) also reported that CFL1 was involved in the platinum-resistance in ovarian cancer cell lines, lines using proteomics-based approaches. They speculated that CFL1 may exert platinum-resistant action through modulating the actin cytoskeleton and, thereby, further inhibit apoptotic cell death in response to chemotherapeutic agents. The activity of CFL may be related to chemoresistance and cause a poor prognosis. Chua et al. (2003) reported that the active form of cofilin is targeted to mitochondria after initiation of apoptosis and induces cytochrome c leakage from mitochondria. The functions of ADCK4 remain poorly understood. According to our current knowledge, the gene of ADCK 4 encodes a protein with two copies of a domain found in protein kinases. The encoded protein has a complete protein kinase catalytic domain, and a truncated domain that contains only the active and binding sites of the protein kinase domain, however, it is not known whether the protein has any kinase activity. Multiple transcript variants encoding different isoforms have been found for this gene (provided by RefSeq, Sep 2011).

Taken together, our findings support that dUTPase, keratin 1, cytokeratin 9, ADCK 4, cofilin 1 probably play important roles in the development of cisplatin resistance. Clearly, further studies will be required to clarify these mechanisms. The distinct function roles for them in regulation of cisplatin resistance courage us to pursue that the use of marker proteins as clinical utility for early detecting drug resistance and preventing poor prognosis.

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