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Quantitative Proteome Analysis of Multidrug Resistance in Human Ovarian Cancer Cell Line

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ABSTRACT

In order to understand the molecular mechanisms of multidrug resistance (MDR) in ovarian cancer, we employed the proteomic approach of isobaric tags for relative and absolute quantification (iTRAQ), followed by LC-MS/MS, using the cisplatin-resistant COC1/DDP cell line and its parental COC1 cell line as a model. A total number of 28 proteins differentially expressed were identified, and then the differential expression levels of partially identified proteins were confirmed by Western blot analysis and/or real-time RT-PCR. Furthermore, the association of PKM2 and HSPD1, two differentially expressed proteins, with MDR were analyzed, and the results showed that they could contribute considerably to the cisplatin resistance in ovarian cancer cell. The differential expression proteins could be classified into eight categories based on their functions, that is, calcium-binding proteins, chaperones, extracellular matrix, proteins involved in drug detoxification or repair of DNA damage, metabolic enzymes, transcription factor, proteins related to cellular structure and proteins relative to signal transduction. These data will be valuable for further study of the mechanisms of MDR in the ovarian cancer. J. Cell. Biochem. 109: 625–633, 2010.

KEY WORDS: MULTIDRUG RESISTANCE; OVARIAN CANCER; iTRAQ; MASS SPECTROMETRY

Multidrug resistance (MDR) describes a phenomenon of cross-resistance of tumor cells to several structurally unrelated chemotherapeutic agents after exposure to a single cytotoxic drug. The phenomenon of MDR is a multifactor event in which several mechanisms act simultaneously. Firstly, tumor cells could greatly reduce the intracellular concentration of cytotoxic drugs by the adenosine triphosphate-driven efflux pump functions of P-gp, MRP, and BCRP [Lee et al., 2004]. Secondly, anticancer drugs could not arrive at their targets on account of transportation of the intracellular cytotoxic drugs to other subcellular structures by LRP [Yeh et al., 2004]. Finally, structural alterations in the drug target enzymes and proteins greatly increased their detoxification, and alterations in cellular metabolism enhanced the ability of tumor cells to repair DNA damage and resist to apoptosis [Stein et al., 2003; Depeille et al., 2005]. Although these pathogenesis studies on MDR of tumors have been undertaken successfully, the mechanisms of MDR are intricate and have not been fully elucidated yet [Yang et al., 2007].

Ovarian cancer is one of the most common malignancies tumors in adult females in the world with a very high mortality. However, resistance to anticancer drugs is a major obstacle towards a successful treatment of ovarian cancer. Drug resistance mechanisms in ovarian cancer cells have been broadly explored, but they are still unclear. Cisplatin-resistant COC1/DDP, which is derived from its parental ovarian cancer cell line COC1 by stepwise selection in vitro using cisplatin, and can also display cross-resistance to other anticancer drugs such as adriamycin, mitomycin C, and 5-fluorouracil, is a useful cell model for investigating the mechanisms underlying MDR in ovarian cancer [Zhang et al., 2005]. Meanwhile the quantitative proteomics approach offers us a new tool to study the pathogenesis of MDR of cancer cells. Isobaric tags for relative and absolute quantification (iTRAQ) analysis is a gel

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free mass spectrometry technique that uses isobaric amine specific tags to compare the peptide intensities between samples and infer quantitative values for corresponding proteins [Zhang et al., 2008]. Using this approach, we identified 28 differentially expressed proteins, and further functional studies suggested PKM2 and HSPD1 could be related to MDR, which may lead to a better characterization of the COC1/DDP cell line, and therefore a better understanding of its multidrug resistant phenotype.

MATERIALS AND METHOD

REAGENTS

The iTRAQ kits were purchased from Applied Biosystems (Foster City, CA). Sequence grade modified trypsin was purchased from Promega (Madison, WI). Bromophenol blue, Bis, TEMED, low molecular weight marker, Tris-base, SDS, glycine, PVDF membrane, and ECL kit were purchased from Amersham Biosciences (Uppsala, Sweden). Monoclonal or polyclonal antibodies against Talin, PKM2, TOP1, EEF2, HSPD1, and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PKM2 siRNA and HSPD1 siRNA kits were also from Santa Cruz Biotechnology. All other reagents were from Sigma (St. Louis, MO).

CELL LINES

Cisplatin-resistant human ovarian cell line COC1/DDP and its parental cell line COC1 were obtained from Chinese Culture Collection. Cisplatin-sensitive human ovarian cell line A2780 was originated from the American Type Culture Collection. These cells were cultured in RPMI1640 medium containing 10% fetal calf serum.

PROTEIN SAMPLE PREPARATION AND iTRAQ LABELING

Cells were lysed in lysis buffer (7 M urea, 1 mg/ml DNase I, 1 mM Na_3VO_4, and 1 mM PMSF) and prior subjected to centrifugal at 15,000 rpm for 30 min at 4°C. The supernatant was collected and the concentration of the total proteins was determined using 2D Quantification kit (Amersham Biosciences). For each sample, a total of 100 μg of protein was precipitated by the addition of four volumes of cold acetone and stored in −20°C for 2 h. The precipitated protein was then dissolved in solution buffer, denatured, and cysteines blocked according to the manufacturer (Applied Biosystems). Each sample was then digested with 20 μl of 0.25 μg/μl trypsin (Promega) solution at 37°C overnight and labeled with the iTRAQ tags as follows: (i) parental cell line COC1 − 115 tag and (ii) cisplatin-resistant cell line COC1/DDP − 117 tag. The labeled samples were pooled prior to further analysis.

STRONG CATION EXCHANGE CHROMATOGRAPHY

To reduce sample's complexity during LC-MS/MS analysis, the pooled samples were diluted 10-fold with loading buffer (10 mM KH_2PO_4 in 25% acetonitrile at pH 3.0) and subjected to an ICAT Cartridge-Cation Exchange column (Applied Biosystems). The column is washed with 1 ml loading buffer before sequential peptide elution with 0.5 ml aliquots of 10 mM KH_2PO_4 at pH 3.0 in 25%acetonitrile containing 100, 200, and 350 mM KCl, respectively. Eluted peptide was desalted using a C18 Sep-Pak (Waters). Cleaned peptide fractions were dried and subjected to LC-MS/MS analysis.

ESI-Q-TOF-MS ANALYSIS

Mass spectrometric analysis was performed using a nano-LC coupled online to QStarXL mass spectrometer (Applied Biosystems). Peptides were loaded on a 75 cm × 10 cm, 3 mm fused silica C18 capillary column, followed by mobile phase elution: buffer (A) 0.1% formic acid in 2% acetonitrile/98% Milli-Q water and buffer (B): 0.1% formic acid in 98% acetonitrile/2% Milli-Q water. The peptides were eluted from 2% buffer B to 100% buffer B over 60 min at a flow rate 300 nl/min. The LC eluent was directed to ESI source for Q-TOF-MS analysis. The mass spectrometer was set to perform information-dependent acquisition (IDA) in the positive ion mode, with a selected mass range of 300–2,000 m/z. Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to 3 s. The two most abundantly charged peptides above a 10 count threshold were selected for MS/MS and dynamically excluded for 60 s with ±50 mmu mass tolerance.

DATABASE ANALYSIS

Peptide identification and quantification were performed using ProteinPilot software packages (Applied Biosystems). Each MS/MS spectrum was searched against the IPI human protein database v3.49 and protein identification was accepted based on ProteinPilot confidence scores. Relative quantification of proteins, in the case of iTRAQ, is performed on the MS/MS scans and is given by the peak areas ratio at m/z 115 and 117 Da. Error factor (EF) and P-value are calculated using ProteinPilot software which gave an indication of the deviation and significance in the protein quantification. For the selection of differentially expressed proteins we considered the following situation: (1) the protein must contain at least two unique peptides with high score and confidence (2) the protein is considered as differentially expressed if its ratio ≥ 2 or ≤ 0.5 with P-value ≤ 0.05 [Lu et al., 2008].

REAL-TIME QUANTITATIVE RT-PCR ANALYSIS

Quantitative RT-PCR was performed on the Roche LightCycler system (Roche Diagnostics, Mannheim, Germany) using SYBR Green I dye, which binds preferentially to double-strand DNA. The specific primers for PKM2 were 5′-AAATCACGCTGGATAACGC-3′ (sense) and 5′-CATCAAACCTCCGAACCC-3′ (antisense), for VDAC1 were 5′-AATGACGGGACAGAGTTT-3′ (sense) and 5′-CCTATCAGGCTG-GAGTTG-3′ (antisense), for PDX1 were 5′-TATGCCAGATGGT-CAGTT-3′ (sense) and 5′-CAGGGAGGTCATTTACAG-3′ (antisense), and for β-Actin were 5′-CTTAGTTGCGTTACACCCTTTC-3′ (sense) and 5′-ACCTTCACCGTTCCAGTTTT-3′ (antisense), respectively. After denaturation at 95°C for 10 s, amplification was performed for 40–45 cycles at 95°C for 5 s, 52°C for 10 s, 72°C for 10 s with a single fluorescence measurement, after which a melting curve program was employed. The fluorescence data were acquired after the extension step in PCR reactions. Thereafter, PCR products were analyzed by generating melting curve. Since the melting curve of a product is sequence specific, it can be used to distinguish between non-specific and specific PCR products. To verify the melting curve

results, the PCR products were sequenced using 377 Prism automated DNA sequencer (ABI). The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001]. Real-time quantitative RT-PCR analysis was repeated at least three times.

WESTERN BLOT ANALYSIS

The cells were lysed at 4°C for 30 min in a lysis buffer (50 mM Tris, pH 7.4, 100 mM $NaCl_2$, 1 mM $MgCl_2$, 2.5 mM Na_3VO_4, 1 mM PMSF, 2.5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 5 μg/ml of aprotinin, pepstatin A, and leupeptin). The cell lysates were centrifuged at 15,000 rpm for 15 min at 4°C. Protein concentration was determined using 2D Quantification kit (Amersham Biosciences). The protein samples (about 20 μg) were separated using SDS–PAGE. After SDS–PAGE electrophoresis, proteins were transferred to PVDF membranes. The membranes were blocked overnight at 4°C with 5% non-fat dry milk in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM $NaCl_2$, 0.5% Tween-20), followed by 3 h of incubation with the primary antibody (1:1,500–1:2,000 dilution) in TBS-T buffer containing 5% non-fat dry milk at room temperature. After washing three times with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, or rabbit anti-goat IgG as a secondary antibody (1:3,000 dilution) for 1 h at room temperature. The membranes were then washed three times in TBS-T buffer and the reactions were visualized with ECL detection system. All of the Western blot analyses were repeated at least three times.

ADMINISTRATION OF PKM2 siRNA AND HSPD1 siRNA TO CELLS

The cells were transfected with PKM2 siRNA (sc-62820), or HSPD1 siRNA (sc-29351) (Santa Cruz Biotechnology) according to the siRNA transfection protocol provided by the manufacturer. Briefly, COC1, A2780, and COC1/DDP cells were plated into 6-well plates and 96-well plates at the density of 10^5 cells/ml medium, respectively. When the cells were 60–80% confluent, they were transfected with 10 nmol/L of specific siRNA, and control siRNA after a pre-incubation for 20 min with siRNA transfection reagent in siRNA transfection medium (Santa Cruz Biotechnology). After 4 h of transfection, the medium was replaced with RPMI1640 medium containing 10% fetal calf serum, and continued to culture the cells for additional 44 h. The cells were then incubated with different concentrations of cisplatin. After 24-h incubation, PKM2 or HSPD1 expression level was determined by Western blot analysis described above, and the cell viability of COC1, A2780, and COC1/DDP was examined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay or crystal violet assay described previously [Flick and Gifford, 1984; Plumb et al., 1989].

TRANSFECTION OF THE FULL-LENGTH PKM2 GENE TO CELLS

The full-length PKM2 gene was amplified from cDNA synthesized by RT-PCR using the total RNA extracted from cells COC1 as the template. The following primers were used for PCR: forward: 5-cgggatccatgtcgaagccccatagtga-3 (*Bam*HI site underlined); reverse: 5-ccggaattctcacggcacaggaacaacac-3 (*Eco*RI site underlined). The purified PCR products were digested with *Bam*HI and *Eco*RI, and then cloned into the pcDNA3.1 vector (Invitrogen Life Technologies, Grand Island, NY) followed by sequence confirmation by DNA sequencing analysis. COC1/DDP cells were transfected with pcDNA3.1/PKM2 or pcDNA3.1/control vector using lipofectamine 2000 following the manufacturer's protocol (Invitrogen Life Technologies). Briefly, COC1/DDP cells were seeded into 6-well plates, and when the cells were 90% confluent, they were transfected with 4 μg pcDNA3.1/PKM2 or pcDNA3.1/control vector. After 48 h incubation, the transfected COC1/DDP cells were seeded in each well of 96-well plates and exposed to different concentrations of cisplatin for additional 24 h to investigate their sensitivity to cisplatin. The PKM2 expression level in COC1/DDP transfectants was determined by Western blot analysis, and the cell viability was examined using MTT assay or crystal violet assay described above.

STATISTICAL ANALYSIS

The data were expressed as mean ± SE, and analyzed with the Student's *t*-test between two groups. It was considered statistically significant if *P*-value was less than 0.05.

RESULTS

iTRAQ ANALYSIS OF DIFFERENTIALLY EXPRESSED PROTEINS

Our goal was to identify differentially expressed proteins related to MDR in ovarian cancer and to subsequently validate a subset of these proteins. We employed the cisplatin-resistant COC1/DDP, and its parental ovarian cancer cell line COC1 for this study. Proteins from these cell lines were labeled with iTRAQ reagents as parental cell line COC1, 115 tag and cisplatin-resistant cell line COC1/DDP, 117 tag. Thus the ratio of 117:115 would indicate the relative abundance of MDR related proteins. To increase the coverage of protein identification and/or the confidence of the data generated, two separate preparations were made, and each was analyzed by LC/MS/MS.

The annotation of 28 differential expression proteins is summarized in Table I. In COC1/DDP, 11 proteins were found to be down-regulated and 17 proteins were up-regulated as compared with cell line COC1. These 28 proteins, which were differentially expressed between the COC1 and COC1/DDP, could be classified into eight functional categories, that is, calcium-binding proteins, chaperones, extracellular matrix, proteins involved in drug detoxification or repair of DNA damage, metabolic enzymes, transcription factor, proteins related to cellular structure, and proteins relative to signal transduction, based on the Swiss-prot database (Fig. 1). Figure 2 illustrates the representative MS/MS of PKM2, HSPD1, and Actin respectively, showing their differential expression levels. The expression levels of PKM2 were significantly decreased (Fig. 2A) and HSPD1 were significantly increased (Fig. 2B) compared to cell line COC1 (labeled with 115 tag), whereas Actin expressions remains unchanged (Fig. 2C).

VALIDATION OF DIFFERENTIAL EXPRESSION PROTEINS

The differential expression levels of the proteins identified by iTRAQ approach were validated using Western blot and/or real-time quantitative RT-PCR. Figure 3A shows relative mRNA expression levels of PKM2, VDAC1, and PDX1 normalized against Actin. The mRNA levels of PDX1 and VDAC1 are up-regulated in

TABLE I. iTRAQ Analysis of Differentially Expressed Proteins Between Cisplatin-Resistant COC1/DDP (ITRAQ 117) and COC1 (ITRAQ 115)

Number	Accession	Gene	Protein	117:115	PVal 117:115	Function
1	IPI00026663.2	ALDH1A3	Aldehyde dehydrogenase 1A3	0.09	3.51E − 09	Drug detoxification or repair of DNA damage
2	IPI00329573.9	COL12A1	Isoform 1 of collagen alpha-1 (XII) chain precursor	0.12	4.54E − 05	Extracellular matrix
3	IPI00296099.6	THBS1	Thrombospondin-1 precursor	0.12	2.51E − 06	Extracellular matrix
4	IPI00022774.3	VCP	Transitional endoplasmic reticulum ATPase	0.25	9.79E − 12	Signal transduction
5	IPI00298994.6	TLN1	Talin-1	0.34	9.28E − 08	Cellular structure
6	IPI00294739.1	SAMHD1	SAM domain and HD domain-containing protein 1	0.35	0.000424114	Signal transduction
7	IPI00402184.4	SYNCRIP	Isoform 4 of heterogeneous nuclear ribonucleoprotein Q	0.36	0.007829933	Cellular structure
8	IPI00877938.1	IARS	Isoleucyl-tRNA synthetase	0.38	0.02896693	Metabolic enzymes
9	IPI00872028.1	NUMA1	NUMA1 variant protein	0.40	7.28E − 07	Cellular structure
10	IPI00479186.5	PKM2	Isoform M2 of Pyruvate kinase isozymes M1/M2	0.43	2.40E − 32	Metabolic enzymes
11	IPI00021812.2	AHNAK	Neuroblast differentiation-associated protein AHNAK	0.49	1.56E − 19	Signal transduction
12	IPI00419258.4	HMGB1	High mobility group protein B1	2.08	2.23E − 07	Cellular structure
13	IPI00303476.1	ATP5B	ATP synthase subunit beta, mitochondrial precursor	2.13	6.25E − 06	Metabolic enzymes
14	IPI00000874.1	PRDX1	Peroxiredoxin-1	2.18	0.002378281	Drug detoxification or repair of DNA damage
15	IPI00010796.1	P4HB	Protein disulfide-isomerase precursor	2.20	0.009726817	Chaperone
16	IPI00000877.1	HYOU1	Hypoxia up-regulated protein 1 precursor	2.23	4.33E − 05	Chaperone
17	IPI00658000.2	IGF2BP3	Isoform 1 of insulin-like growth factor 2 mRNA-binding protein 3	2.25	1.53E − 05	Signal transduction
18	IPI00000105.4	MVP	Major vault protein	2.40	0.000669224	Cellular structure
19	IPI00438229.2	TRIM28	Isoform 1 of transcription intermediary factor 1-beta	2.59	3.79E − 05	Transcription factor
20	IPI00784154.1	HSPD1	60 kDa heat shock protein, mitochondrial precursor	2.61	6.23E − 09	Chaperone
21	IPI00020984.1	CANX	Calnexin precursor	2.90	2.55E − 07	Calcium-binding proteins
22	IPI00004358.4	PYGB	Glycogen phosphorylase, brain form	3.34	0.003972082	Metabolic enzymes
23	IPI00413611.1	TOP1	DNA topoisomerase 1	3.42	0.001343002	Drug detoxification or repair of DNA damage
24	IPI00216308.5	VDAC1	Voltage-dependent anion-selective channel protein 1	4.04	2.11E − 08	Signal transduction
25	IPI00299000.5	PA2G4	Proliferation-associated protein 2G4	4.19	2.68E − 08	Signal transduction
26	IPI00186290.6	EEF2	Elongation factor 2	4.29	2.15E − 37	Transcription factor
27	IPI00291006.1	MDH2	Malate dehydrogenase, mitochondrial precursor	4.34	6.36E − 05	Drug detoxification or repair of DNA damage
28	IPI00646304.4	PPIB	Peptidylprolyl isomerase B precursor	4.57	4.28E − 07	Metabolic enzymes

cisplatin-resistant COC1/DDP, whereas the mRNA level of PKM2 is down-regulated, as compared to COC1. This trend is similar to their protein expression level obtained in iTRAQ approach. Figure 3B shows a representative Western blot analysis result of Talin, PKM2, TOP1, and EEF2 expression in the two cell lines. Compared with COC1, cisplatin-resistant COC1/DDP had an obvious up-regulation of TOP1 and EEF2, and a marked down-regulation of Talin and PKM2.

THE ASSOCIATION OF PKM2 AND HSPD1 WITH MDR

To study the functional role of PKM2 down-regulation in COC1/DDP, COC1 was transfected with PKM2 siRNA. Firstly, siRNA-induced inhibition of PKM2 expression was determined by Western blot analysis. As shown in Figure 4A, transfection of COC1 cells with PKM2 siRNA significantly reduced PKM2 protein levels, whereas PKM2 protein expressions were not significantly suppressed by control siRNA. We next evaluated the effect of PKM2 siRNA transfection on cisplatin resistance in COC1. COC1 cells were treated with PKM2 siRNA for 2 days, and for additional 24 h incubation with different concentrations of cisplatin. The cell viability of COC1 was examined using MTT and crystal violet assay. As shown in Figure 4A, transfected COC1 cells with PKM2 siRNA resulted in cisplatin resistance, with significant increase in cell viability. Another cisplatin-sensitive ovarian cell line A2780 also was induced of cisplatin resistance by PKM2 siRNA demonstrated by MTT and crystal violet method (data not shown). To further determine if the decreased expression of PKM2 potentially contributes to drug resistance, we also transfected the full-length PKM2 gene to COC1/

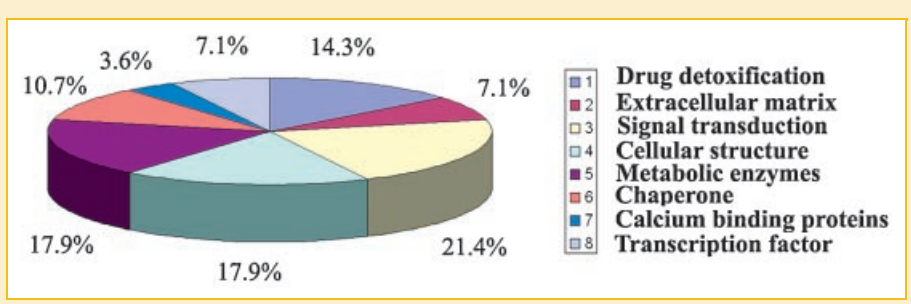

Fig. 1. Pie chart showing the various functional categories as a percentage of the 28 differentially expressed proteins based on the Swiss-prot database.

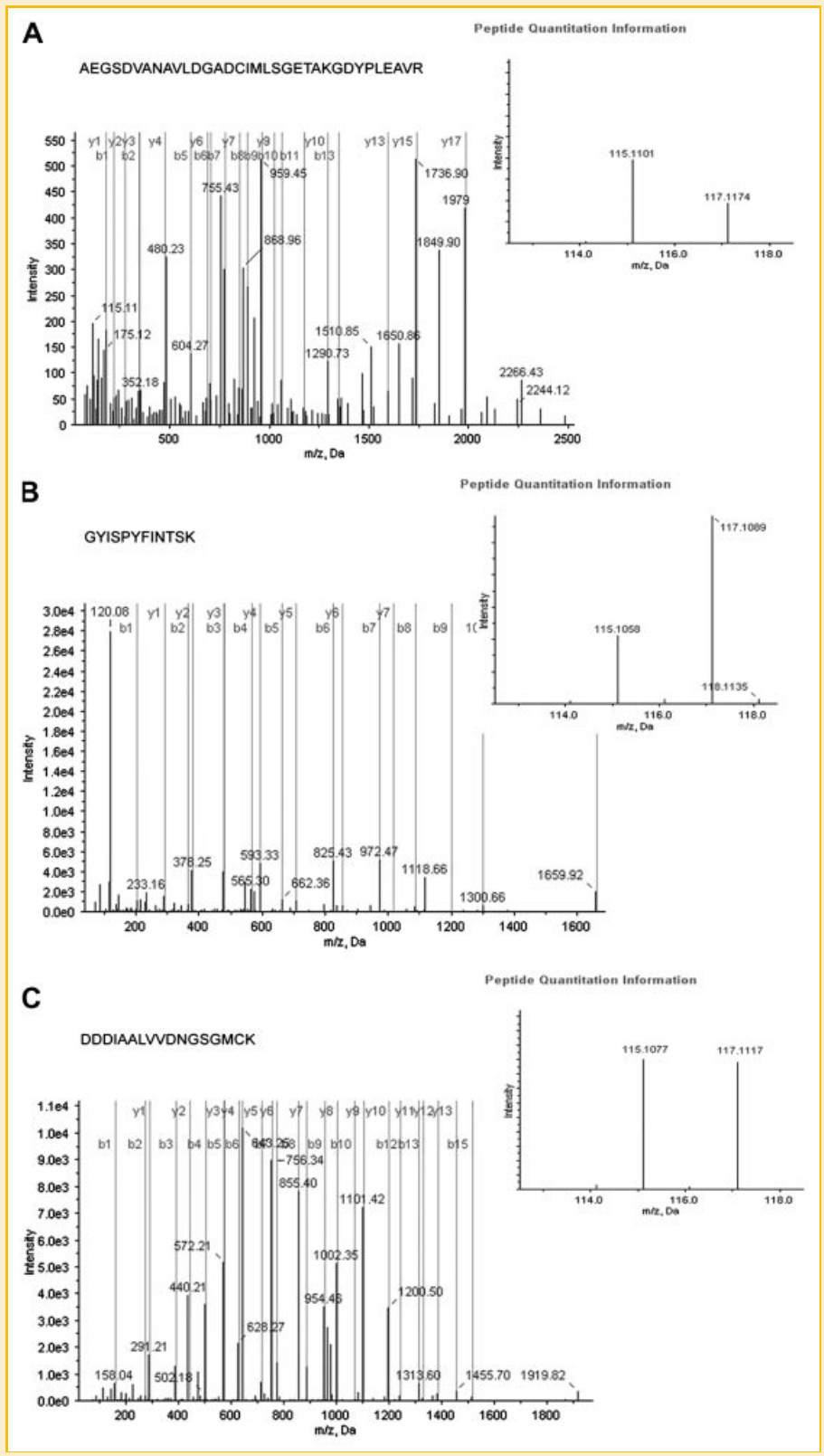

Fig. 2. A representative MS/MS spectrum showing the peptides from PKM2 (A), HSPD1 (B), and Actin (C). Compared with cell line COC1 labeled with 115, expression levels of PKM2 were significantly decrease (A) and HSPD1 were significantly increase (B), whereas Actin expressions was not significantly changes (C) in cisplatin-resistant COC1/DDP labeled with 117.

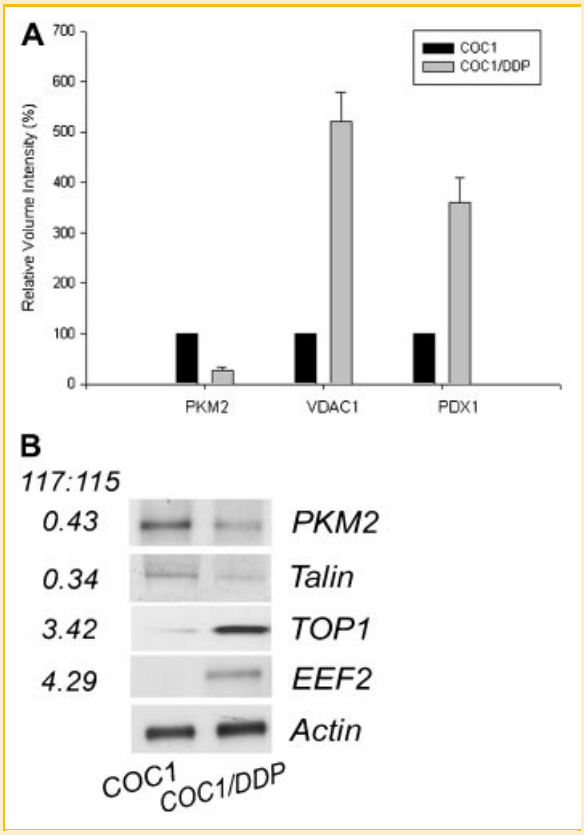

Fig. 3. A: Relative mRNA expression levels of PKM2, VDAC1, and PDX1 after normalization with Actin mRNA levels as determined by real-time RT-PCR. Compared with COC1, cisplatin-resistant COC1/DDP had an obvious up-regulation of PDX1 and VDAC1 and down-regulation of PKM2, which were identical with the protein level changes in iTRAQ analysis. B: A representative Western blot analysis result of Talin, PKM2, TOP1, and EEF2 expression in the two cell lines. Compared with COC1, cisplatin-resistant COC1/DDP had an obvious up-regulation of TOP1 and EEF2, and a marked down-regulation of Talin and PKM2.

DDP cells. As shown in Figure 4B, after 48 h transfection, the PKM2 protein level increased in the pcDNA3.1/PKM2 cells compared with the control cells. It was obvious that the over-expression of PKM2 in COC1/DDP could enhance cisplatin chemosensitivity, with the significant decrease of the cell viability. Finally, the role of HSPD1 in the development of MDR in COC1/DDP was also verified. HSPD1 siRNA transfection could significantly decrease the cell viability of COC1/DDP (about 12.5%) after incubated with 20 μM cisplatin for 24 h, compared with control siRNA. All these clearly demonstrated that PKM2 and HSPD1 involved in MDR in ovarian cancer.

DISCUSSION

In this study, we used iTRAQ proteomic approach to identify proteins with differential expression between the cisplatin-resistant ovarian cancer cell line COC1/DDP and its parental cell line COC1 cells. We found 28 proteins with significant alterations in expression between the two cell lines. Among them, six of the proteins, that is, PKM2, VDAC1, Talin, TOP1, EEF2, and PDX1 were validated using real-time RT-PCR analysis and/or Western blot analysis. These findings illustrate that multiple mechanisms may cause drug resistance in ovarian cancer cells, and they may contribute partially to chemotherapeutic resistance in ovarian cancer treatment. We discuss some of the key proteins discovered in this work in the following text.

Among identified metabolic enzymes, the levels of PKM2 markedly decreased in COC1/DDP. The mRNA level and protein level of PKM2 were also lower in COC1/DDP compared with COC1. Pyruvate kinase has four isoforms (L, R, M1, and M2), and the type M2 is expressed in proliferating cells, such as embryonic stem cells, embryonic carcinoma cells, as well as cancer cells [Mazurek, 2007]. At this stage, a rational correlation of PKM2 with MDR remains unknown. As we know that cisplatin is inactivated by covalent linking to GSH. Low PKM2 activity could increase NADPH production, which was necessary for the reduction of oxidized GSH [Mazurek and Eigenbrodt, 2003]. In additional, inhibition of PKM2 activity can decelerate tumor cell proliferation, but without affecting apoptotic cell death [Spoden et al., 2008]. To further determine the biological role of PKM2 in the cell, Christofk et al. [2008] applied RNAi to knock down PKM2 expression in H1299 lung cancer cells resulting in reduced glycolysis and decreased cell proliferation. Moreover, we observed that, compared with COC1, COC1/DDP cells grew slowly in RPMI1640. It is accepted if tumor cells did not divide or grew slowly, they could escape injury of chemotherapeutic drugs such as cisplatin. Yoo et al. [2004] used a proteomics approach to study MDR of gastric cancer, had also reported a lower expression and activity of PKM2 in cisplatin-resistant gastric cancer cells, and the suppression of PKM2 expression by ASO increased the cell resistance to cisplatin. In this study, PKM2 was observed to be under-expressed in COC1/DDP. We demonstrated that the suppression of PKM2 expression in parental cell line COC1 and another cisplatin-sensitive ovarian cell line A2780 can confer resistance to cisplatin. Furthermore, transfection of full-length PKM2 gene to COC1/DDP cells can increase the sensitivity to cisplatin. This is obvious that the under-expression of PKM2 was involved in the MDR phenotype of COC1/DDP. Thus it shows that metabolic enzymes can contribute to the drug resistance.

The expressions of two chaperone proteins obviously changed in COC1/DDP. HSPD1 (HSP60) is primarily a mitochondrial protein and though to function as folding key proteins after import into the mitochondria [Bukau and Horwich, 1998]. Cellular expression of HSP60 was shown to increase in response to thermal, toxic, ischemic and other types of injury [Lindquist, 1986]. Kirchhoff et al. [2002] demonstrated that Hsp60 can inhibit apoptosis by sequestering Bax and preventing its translocation to the mitochondrial membrane. Although it has been reported HSP60 was over-expression in three different cell model systems including cisplatin-resistant ovarian cancer, oxaliplatin-resistant ovarian cancer, and cisplatin-resistant bladder cancer [Abu-Hadid et al., 1997]. However, whether increased HSP60 is mechanistically related to MDR, remains unclear. In our case, we also observed increased expression of HSP60 in cisplatin-resistant cell line COC1/DDP. To study the functional role of HSP60 up-regulation in COC1/DDP, COC1/DDP cells were transfected with HSP60 siRNA to evaluate the effect on

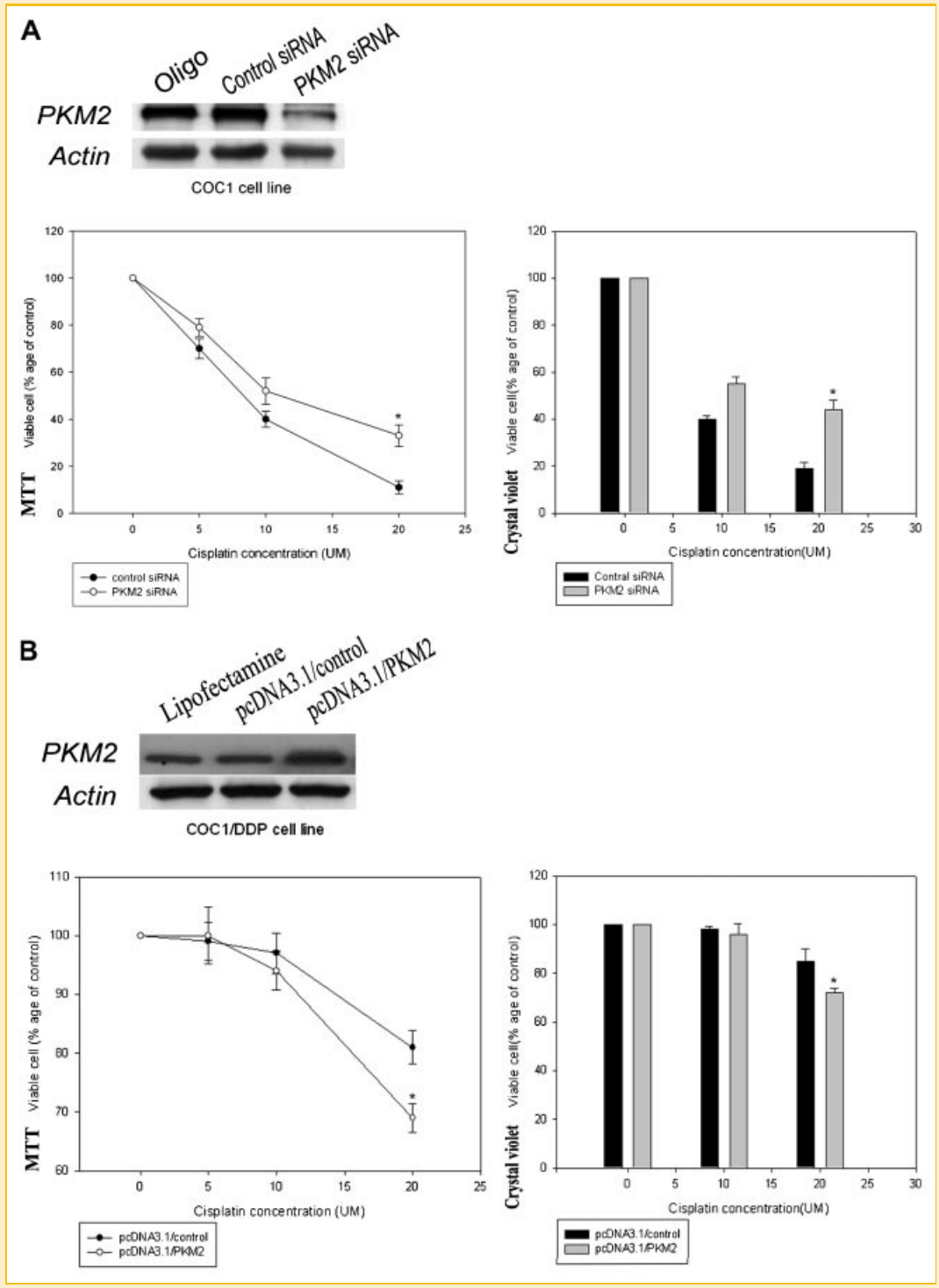

Fig. 4. A: Western blot analysis showed that transfection of COC1 cells with PKM2 siRNA significantly reduced PKM2 protein levels, whereas PKM2 protein expressions were not significantly suppressed by control siRNA and oligofectamine. Oligofectamine (a cationic lipid, Invitrogen, Carlsbad, CA), cells treated with oligofectamine only. Furthermore, MTT and crystal violet assay showed that transfection of COC1 cells with PKM2 siRNA could result in cisplatin resistance, with the significant increase of the cell viability. B: After 48 h transfection, Western blot analysis showed that transfection of COC1/DDP cells with pcDNA3.1/PKM2 significantly increased PKM2 protein levels, compared with the pcDNA3.1/control and lipofectamine. Subsequently, MTT and crystal violet assay showed that pcDNA3.1/PKM2 transfection could increase the cisplatin sensitivity of COC1/DDP. The experiment was repeated in triplicate. Points, mean viable cells (% of control) from three experiments; bars, SE. $^{*}P \leq 0.05$ differ from control by *t*-test.

the cisplatin chemosensitivity. Our results showed that HSP60 siRNA transfection could significantly decrease the cell viability of COC1/DDP cells incubated with cisplatin, and supported that the increased expression of HSPD1 was correlated with the MDR of cancer cells possibly by inhibiting cisplatin-induced cell apoptosis. HYOU1, also known as 150-kDa oxygen-regulated protein, has a protective role of which under hypoxic conditions was shown to be partly due to the suppression of apoptosis [Ozawa et al., 1999]. Anticancer treatment for prostate cancer by using an antisense HYOU1 expression vector, which resulted in the reduction of tumorigenicity [Miyagi et al., 2002]. Also up-regulation of HYOU1 in cancer cells can inhibit celecoxib-induced apoptosis [Namba et al., 2007], thereby no doubt HYOU1 correlated with chemotherapeutic resistance.

It has been proposed that extracellular effectors such as matrix components, which is well-known process tumor growth, metastasis and progression of atherosclerosis, may provide sanctuary to cancer cells by preventing stress-induced cell death [Rintoul and Sethi, 2001]. Down-regulated extracellular matrix structural protein, COL12A1 (isoform 1 of collagen XII alpha-1 chain), was discovered in this study. Type XII collagen interacts with type I collagen, which has previously been shown to regulate expression of E-cadherin at cell–cell junctions [Koenig et al., 2006]. In recent years, E-cadherin has been correlated with MDR. An anti-E-cadherin antibody can increase the chemosensitivity of MCF-7 cells, which express a high level of E-cadherin [Nakamura et al., 2003]. Işeri et al. [2009] also demonstrated alterations in gene expression levels of type IV collagen, type VI collagen, type XII collagen, and type XVI collagen in drug-resistant MCF-7 cells. The correlation between Type XII collagen and MDR in COC1/DDP needs to further study.

We showed that calnexin, a calcium-binding protein, was over-expressed in cisplatin-resistant ovarian cancer cell line COC1/DDP. Calnexin is a unique endoplasmic reticulum (ER) chaperon that promotes proper folding and prevents aggregation of nascent protein chains [Zapun et al., 1999]. Recently, it has been shown that calnexin is involved in resistance to apoptosis [Takizawa et al., 2004]. Another data also reported that the depletion of calnexin sensitized cells to tunicamycin-induced apoptosis [Delom et al., 2007]. Ng and Shore [1998] hypothesized that calnexin and Bap31 form a protein complex to trigger apoptosis during ER stress. In light of the function of calnexin above, it is conceivable that over-expression of calnexin in cisplatin-resistant COC1/DDP may be important for cells to survive environmental stresses, such as drug attack.

One of the major differentially expressed proteins groups between COC1/DDP and COC1 is proteins related to cellular structure. Such as HMGB1, in the nucleus, it bends and plasticizes DNA. It interacts with a number of proteins, including p53, general and specific transcription factors, nuclear factor-κB, etc. [Bianchi and Agresti, 2005]. Mammalian cells lacking HMGB1 are hypersensitive to DNA damage induced by psoralen plus UVA irradiation or UVC radiation [Lange et al., 2008]. It seemed that HMGB1 is a potential modulator of anticancer therapy targeted against DNA. Major vault protein (MVP), also called lung resistance-related protein, which is widely distributed in normal tissues, and over-expressed in multidrug-resistant cancer cells, mediates drug resistance via a transport process [Steiner et al., 2006]. Treatment of colon cancer cell lines SW620 with sodium butyrate-induced MVP expression, which resulted in an increase of the resistance to doxorubicin. Moreover anti-MVP polyclonal antibody reversed this drug resistance [Kitazono et al., 2001]. In our iTRAQ analysis, MVP was also observed to be over-expressed in COC1/DDP, and no doubt major vault protein was related to MDR.

Antioxidants and some proteins related to repair of DNA damage were closely correlated with the development of MDR. Inside cells, GSH and thioredoxin represented the major reducing agents, detoxification of which was commonly recognized as one of the major mechanisms of MDR. Finally, there are proteins including transcription factor, and those relative to signal transduction, which were found to be differentially expressed between COC1/DDP and COC1, but have not been associated with MDR previously. The correlation between these proteins and MDR in COC1/DDP will be subjected to future study.

Resistance to anti-cancer drugs is one of the major problems faced during chemotherapy of ovarian cancer, but the mechanism of its MDR is still unclear. In this study, we focused our attention on those proteins that changed in expression levels in cisplatin-resistant cell line COC1/DDP. As a result, 28 differential expressional proteins possibly associated with MDR of COC1/DDP were identified, and association of PKM2 and HSP60 with MDR was verified. These data were valuable for further to study the mechanism of MDR in human ovarian cancer, and also provide some new clues for investigating other tumors MDR.

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