Proteins Associated with Cisplatin Resistance in Ovarian Cancer Cells Identified by Quantitative Proteomic Technology and Integrated with mRNA Expression Levels* [S]

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Nearly all women diagnosed with ovarian cancer receive combination chemotherapy including cis- or carboplatin. Despite high initial response rates, resistance to cisplatin develops in roughly one-third of women during primary treatment and in all women treated for recurrent disease. ICAT coupled with tandem MS is a quantitative proteomic technique for high throughput protein expression profiling of complex protein mixtures. Using ICAT/MS/MS we profiled the nuclear, cytosolic, and microsomal fractions obtained from IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) ovarian cancer cell lines. The proteomes of cisplatin-sensitive and -resistant ovarian cancer cells were compared, and protein expression was correlated with mRNA expression profiles. A total of 1117 proteins were identified and quantified. The relative expression of 121 of these varied between the two cell lines. Sixty-three proteins were overexpressed in cisplatin-sensitive, and 58 were overexpressed in cisplatin-resistant cells. Examples of proteins at least 5-fold overexpressed in resistant cells and with biological relevance to cancer include cell recognition molecule CASPR3 (13.3-fold), S100 protein family members (8.7-fold), junction adhesion molecule Claudin 4 (7.2-fold), and CDC42-binding protein kinase β (5.4-fold). Examples of cancer-related proteins at least 5-fold overexpressed in sensitive cells include hepatocyte growth factor inhibitor 1B (13.3-fold) and programmed cell death 6-interacting protein (12.7-fold). The direction of changes in expression levels between proteins and mRNAs were not always in the same direction, possibly reflecting post-transcriptional control of protein expression. We identified proteins whose expression profiles correlate with cisplatin resistance in ovarian cancer cells. Several proteins may be involved in modulating response to cisplatin and have potential as markers of treatment response or treatment targets. Molecular & Cellular Proteomics 5:433–443, 2006.

Ovarian cancer ranks fourth in cancer mortality among women in the United States accounting for more than 16,000 deaths annually (American Cancer Society Statistics for 2005). Most cases are advanced at diagnosis because ovarian cancer typically does not cause symptoms until having spread outside the ovary. Platinum compounds, given as a combination of cis- and carboplatin, are the most active ovarian cancer chemotherapy and standard treatment for nearly all woman diagnosed with ovarian cancer. Although most patients initially respond to this treatment, few are cured (1). Resistance to chemotherapy is the major cause of treatment failure. Resistance to cisplatin occurs in roughly one-third of women during primary treatment and in all patients treated for recurrent disease (2). Currently resistance can only be determined retrospectively after patients have experienced the burden and toxicity of ineffective therapy. Outcomes for women with ovarian cancer could be improved by the identification of biomarkers capable of identifying resistant tumors and better therapies for treating them.

Proteins that are differentially expressed in cisplatin-resistant and -sensitive ovarian cancer cells are likely to be involved in pathways that modulate the sensitivity of ovarian cancer to cisplatin and are logical candidates for treatment response biomarkers and therapeutic targets. ICAT/MS/MS is a novel proteomic technology that allows for the identification and quantification of the relative abundance of proteins in a complex mixture. The approach involves labeling of protein lysates prior to LC-MS/MS with ICATs specific to sulfhydryl groups that greatly reduces sample complexity, allows detection of low abundance proteins, and aids in quantification of relative protein abundance between samples (3). We used ICAT/MS/MS to compare the proteome of IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) cell lines and to identify differentially expressed proteins. Identified proteins were categorized based on functional characteristics and cellular localization. We also correlated the protein expression of 121 proteins we identified with the mRNA expression profiles generated using massively parallel signature sequencing.
(MPSS)\(^1\) (4).\(^2\) MPSS is a highly sensitive differential expression profiling technology for mRNAs as it can sequence over 1 million signatures (tags) from a cDNA library and allows direct comparison of different cDNA libraries by counting the number of the same signatures (tags) that are sequenced. We determined concordant and discordant expression at mRNA and protein levels in a subset of 92 highly differentially expressed proteins: 37 are concordant, i.e., expression level changes in the same direction, and 55 are discordant, possibly reflecting posttranscriptional control of protein expression.

**EXPERIMENTAL PROCEDURES**

**Ovarian Cancer Cell Lines and Extraction Conditions**—IGROV-1/CP cisplatin-resistant ovarian cancer cells are derived experimentally from IGROV-1, the cisplatin-sensitive parental line. IGROV-1 cells were exposed to an initial dose of cisplatin and allowed to recover for several weeks at which time cisplatin exposure was reiteratated with escalating doses. During this process a few surviving cells proliferated and recolonized the cultures, yielding a cisplatin-resistant strain (5).

Nuclear, cytoplasmic, and membrane fractions were prepared from IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) cell lines by differential centrifugation (6). Cell lines were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C. Approximately 1.0 × 10\(^7\) cells were harvested in ice-cold PBS and centrifuged at 900 × g for 10 min at 4 °C. The resulting cell pellet was washed twice with ice-cold PBS and then resuspended in lysis buffer (10 mM Hepes, pH 7.4, 30 mM NaCl, 1% SDS, and protease inhibitors). The suspension was mixed for 15 min at 4 °C, homogenized using 10 strokes with a Dounce homogenizer, and centrifuged at 900 × g for 20 min at 4 °C. The resulting pellet (nuclear fraction) was resuspended in lysis buffer. The supernatant was centrifuged at 100,000 × g for 2 h. The resulting supernatant was used as the cytoplasmic fraction. The final pellet (microsomal fraction) was resuspended in lysis buffer.

**ICAT Labeling and Microcapillary LC-MS/MS Analysis**—For ICAT labeling, 1 mg of either cytoplasmic or microsomal proteins and 0.5 mg of nuclear proteins from IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) cell lines were denatured with 6 M urea and 0.05% SDS and immediately reduced with 5 M dithiothreitol. Each of the three samples was labeled with second generation ICAT reagents (acid-cleavable) either in light (\(^{13}\)C for cisplatin-sensitive) or in heavy (\(^{15}\)C for cisplatin-resistant) isotopes (Applied Biosystems, Foster City, CA). Equal amounts of the two labeled samples were combined and digested into peptides by trypsin (Promega, Madison, WI). ICAT-labeled peptides were subsequently purified by cation exchange chromatography and avidin affinity chromatography. Peptide mixtures were analyzed by microcapillary HPLC-ESI-MS/MS using an ion trap mass spectrometer (LCQ-DecaXP, ThermoFinnigan) as described previously (7).

**Data Analysis and Software Programs**—We used SEQUEST to match peptide tandem mass spectra to sequences in the human International Protein Index (IPI) database (Version 2.31) (8). A copy of the SEQUEST parameter is provided in the supplemental documents. We used the Peptide Prophet and the Protein Prophet programs to measure the quality of peptide and protein identification (9, 10). Relative protein expression ratios were quantified using the ASAPRatio software tool (Institute for Systems Biology) (11) and confirmed manually. To classify the identified proteins into functional categories, Gene Ontology (GO) analysis was performed using GoMiner (12) (discover.ncbi.nih.gov/gominer). For the analysis, Human Genome Organization IDs were retrieved using MatchMiner (discover.ncbi.nih.gov/matchminer), and redundant IDs were removed before GoMiner analysis. All proteomic data were stored and systematically analyzed at the Systems Biology Experiment Analytical Management System at the Institute for Systems Biology (www.peptideatlas.org/repository/).

**MPSS**—Total RNA was isolated from cisplatin-sensitive and cisplatin-resistant cell lines using TRIzol (Invitrogen). cDNA libraries comprising 21 bases adjacent to the poly(A) proximal DpnII site were prepared according to the Megaceclone protocol (Lynx Therapeutics, Hayward, CA). The resulting libraries were amplified and loaded onto microbeads. Approximately 1.6 × 10\(^6\) microbeads were loaded into each flow cell, and signature sequences were determined by serial enzymatic reactions. An average of 7 × 10\(^6\) sequences were obtained from each sequencing run, and 16 runs were preformed for each library. A comprehensive reconstruction of human transcripts based on genome data was undertaken by mapping the MPSS signatures to the current human reference transcript sequence collections from the National Center for Biotechnology Information (Refseq NM and XM sections) and ENSEMBL Version 8.30.

**Immunoblot Analysis and Antibodies**—Whole cell lysates of IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) cell lines were electrophoresed on a 4–20% gradient SDS-PAGE gel (Bio-Rad). Briefly 20 μg of protein was mixed with SDS-PAGE loading buffer (0.5  μ Tris, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromphenol blue, 0.05% DTT), serially diluted 2-fold, and electrophoresed at 110 V. The proteins were transferred to a low autofluorescence PVDF membrane (Immobilon-FL, Millipore Corp., Bedford, MA) for 120 min at 200 mA. Membranes were washed twice in TBS (0.05  μ Tris, pH 7.4, 0.15  μ NaCl)-Tween 20 (TBS-T) and blocked overnight at 4 °C in the Qdot Western kit blocking buffer (SEA BLOCK, Pierce). Monoclonal mouse anti-spectrin β (Calbiochem), monoclonal mouse anti-annexin IV (BD Biosciences), and polyclonal rabbit anti-integrin α5 (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:1000 in Qdot blocking buffer. Membranes were incubated with primary antibodies for 60 min at room temperature with rocking. Washing was carried out for 30 min using three changes of TBS-T. The blots were incubated further for 60 min at room temperature with secondary antibodies, either Qdot 565 goat anti-mouse IgG or Qdot 655 goat anti-rabbit IgG (Quantum Dot Corp., Hayward, CA) diluted 1:10,000 in Qdot blocking buffer followed by washing three times in TBS-T for 5 min each. Finally the membrane was rinsed twice with TBS, and the protein bands were detected using the Bio-Rad GelDoc imaging system (Bio-Rad) using UV transillumination equipped with a wide bypass filter centered at 415 nm. Protein quantification was performed with ImageQuant software (Amersham Biosciences).

**RESULTS**

Identification of Proteins Differentially Expressed between Cisplatin-resistant and -sensitive Ovarian Cancer Cells—ICAT/MS/MS is a quantitative proteomic approach that enables rapid and comprehensive analysis of the proteomes of two comparable samples (3). Here we used this approach to examine differential levels of protein expression prepared from the cytoplasmic, nuclear, and microsomal fractions of cisplatin-sensitive IGROV-1 and cisplatin-resistant derivative
IGROV-1/CP cell lines. Using Peptide Prophet score \( p > 0.5 \) as a selection criterion and further excluding those peptides with bad spectrum quality by visual inspection, 1117 proteins were quantified (Supplemental Table S1). A differential protein expression ratio of 1.75 was selected as significant based on a hypothesis test using an empirical probability density function (obtained by kernel density estimation) for the ratios of all 1117 proteins identified. A -fold change of 1.75 corresponded to a \( p \) value of 0.05. Of these 1117 proteins, 63 were increased, 58 were decreased, and 995 were not changed comparing the cisplatin-sensitive with the -resistant cells. The median ratio and standard deviation of the 1117 dataset was 0.947 and 0.246, respectively. Table I lists 121 differentially expressed proteins with their respective fractions: nuclear, microsomal, and cytoplasmic. Four of the 121 differentially expressed proteins (Table I, Footnote e) were identified from different cellular fractions either due to cross-contaminations or due to their true distribution in different fractions. The expression ratios obtained from different independent fractions (therefore different MS/MS runs) are very close to each other with an average coefficient of variance (CV) of 20.5%. In the 1117 protein dataset, 263 proteins were identified in multiple fractions, and the average CV is 28.15% and median CV is 19.95% (data not shown). Most of the variance probably comes from technical replicates of different MS/MS runs. However, it is possible that some of the variance may reflect true differential enrichment of proteins in different cellular fractions between cisplatin-resistant and -sensitive cells. We therefore chose not to combine proteins identified from different fractions but to keep them as different entries. We also decided not to combine and average protein expression ratios from different cellular fractions for the same protein. In Supplemental Table S1 we provide the complete list of 1117 proteins with their expression ratios and errors analyzed by the ASAPRatio program (11). 363 proteins have two or more peptide identifications, including 263 proteins with peptides from different fractions (and additionally some have multiple peptide identifications from each fraction) and 100 with more than one peptide identifications from the same fraction. 408 proteins have single peptide identifications. The total number of unique proteins identified and quantified is 771.

In Table I, Footnote d denotes proteins shown in previous studies to be dysregulated in human cancer. Some of the proteins overexpressed in cisplatin-resistant cells have been previously associated with chemoresistance. These include junction adhesion molecule Claudin 4 (7.5-fold) (13), CDC42-binding protein kinase \( \beta \) (5.4-fold) (14), and mitogen-activated protein kinase kinase kinase 6 (3.7-fold) (15, 16). A large number of proteins not previously known to be altered in chemoresistance were identified. Examples include similar to cell recognition protein CASPR3 isoform 1 (13.3-fold), putative S100 calcium-binding protein (6.9-fold), p37 AUF1 (5.1-fold), mitochondrial carrier homolog 2 (4.6-fold), peroxiredoxin (3.3-fold), and numerous ribosomal proteins (Table I).

We identified a number of “uncharacterized proteins” as differentially expressed between these two cell lines. These include unnamed proteins, hypothetical proteins, and a range of proteins that have distinctive prefixes in their titles. KIAA, FLJ, and other proteins with similar names are submitted to protein databases based on translations from nucleotide sequences and possess distinguishing features (i.e. all KIAA proteins theoretically have large molecular weights). Furthermore several proteins were identified and named based on their sequence sharing high percentages of similarity with well characterized proteins. Uncharacterized proteins that were increased in cisplatin resistance included hypothetical protein FLJ34068 (2.9-fold) and several proteins with “no match” to existing databases. Uncharacterized proteins included hypothetical protein KIAA1636 (3.5-fold), similar to RIKEN cDNA 4732495G21 gene (2.7-fold), and similar to RIKEN cDNA 2900070E19 gene (2.1-fold) (Table I).

The overall amounts of spectrin \( \beta \), integrin \( \alpha 5 \), and annexin IV were measured in whole cell lysates by probing Western blots of one-dimensional PAGE-separated proteins with specific antibodies. Comparing the signal of IGROV-1 lysate with the corresponding lane for the IGROV-1/CP lysate using ImageQuant software (Amersham Biosciences) showed a 4-fold increase for integrin \( \alpha 5 \) and a 2-fold increase for annexin IV protein expression in the chemosensitive cell line. Conversely the Western blot showed a 6-fold increase in spectrin \( \beta \) in the chemoresistant cell line. These results showed that the Western blot analysis confirmed the differential expression of these three proteins between IGROV-1 and IGROV-1/CP cell lines originally measured in the ICAT analysis (Fig. 1).

Identification of Enriched GO Categories and Pathways Differentially Expressed between Cisplatin-resistant and -sensitive Ovarian Cancer Cells—To gain an overall picture of the cellular changes during the transition of ovarian cancer cells from cisplatin-sensitive to -resistant phenotype, we sought to see whether any GO categories were enriched in the differentially expressed proteins we identified. Using all 1117 proteins (taking unique entries) we identified as the background list, we used GoMiner (12) to test whether there were any enrichments of under- or overexpressed proteins. The results (Table II) indicated that there were significant changes in GO categories between cisplatin-resistant and -sensitive cells. Enriched GO molecular function terms in cisplatin-resistant cells include RNA and nucleic acid binding and hydrolase activities, whereas those enriched GO terms in cisplatin-sensitive cells include heat shock protein binding and kinase activity. Enriched GO biological process terms in cisplatin-resistant cells include DNA, RNA, and protein biosyntheses; processing and metabolism; and MAPKKK cascade, whereas those enriched in cisplatin-sensitive cells include secretion, regulation of body fluids, and coenzyme biosynthesis (Table II).

We also used the Panther pathway analysis tool (panther.appliedbiosysems.com) to analyze pathways that were sig-
Proteome Changes in Cisplatin-resistant Ovarian Cancer Cells

| IPI no.       | Cellular fractions | No. of peptides identified | Normalized chemo s/ chemo r (ICAT ratio) | Normalized errors<sup>a</sup> | MPSS tpm<sup>b</sup> | MPSS p value (Z test) | Protein descriptions                                           |
|--------------|--------------------|-----------------------------|------------------------------------------|-------------------------------|---------------------|----------------------|---------------------------------------------------------------|
| IPI00005589  | Nuclear            | 1                           | 0.007                                    | 0.001                         | 1010                | 1411                 | 0                                                                 |
| IPI00005589  | Microsomal         | 4                           | 0.232                                    | 0.223                         | 347                 | 71                   | 0.0024                                                        |
| IPI00005589  | Cytosolic          | 3                           | 0.102                                    | 0.111                         | 1082                | 1119                 | 0.54                                                           |
| IPI00005589  | Nuclear            | 1                           | 0.075                                    | 0.076                         | 959                 | 762                  | 0.000004                                                      |
| IPI00005589  | Nuclear            | 1                           | 0.114                                    | 0.073                         | 78                  | 53                   | 0.0013                                                        |

Proteins that displayed altered expression between chemosensitive and chemoresistant cells

snRNP, small nuclear ribonucleoprotein; IGF, insulin-like growth factor; ABC, ATP-binding cassette; E3, ubiquitin-protein isopeptide ligase; chemo s, chemosensitive; chemo r, chemoresistant.
| IPI no.    | Cellular fractions | No. of peptides identified | Normalized chemo s/chemo r (ICAT ratio) | Normalized errors | MPSS tpm<sup>a</sup> | MPSS p value (Z test) | Protein descriptions                                      |
|-----------|--------------------|-----------------------------|----------------------------------------|-------------------|----------------------|----------------------|----------------------------------------------------------|
| IPI00024911 | Cytosolic          | 1                           | 0.531                                  | 0.469             | 333                  | 230                  | Endoplasmic reticulum protein ERp29 precursor             |
| IPI00385701 | Nuclear            | 1                           | 0.533                                  | 0.076             | —                    | —                    | Hypothetical protein                                     |
| IPI00222228 | Microsomal         | 1                           | 0.534                                  | 0.088             | —                    | —                    | Vigilin                                                  |
| IPI00328753 | Nuclear            | 3                           | 0.537                                  | 0.47              | 138                  | 115                  | Splice isoform 1 of Q86UP2                  |
| IPI00333592 | Cytosolic          | 9                           | 0.538                                  | 0.811             | 2756                 | 3598                 | Hypothetical protein                                     |
| IPI00011528 | Nuclear            | 1                           | 0.54                                  | 0.227             | 0                    | 0                    | Cleavage stimulation factor, 50-kDa subunit             |
| IPI00003399 | Microsomal         | 8                           | 0.553                                  | 0.857             | 2756                 | 3598                 | Hypothetical protein                                     |
| IPI00016786 | Microsomal         | 1                           | 0.555                                  | 0.543             | 120                  | 244                  | Splice isoform 2 of P21181 cell division control protein |
| IPI00385025 | Cytosolic          | 3                           | 0.556                                  | 0.274             | 15                   | 0                    | Isocitrate dehydrogenase                                 |
| IPI00076042 | Cytosolic          | 1                           | 0.559                                  | 0.057             | —                    | —                    | Short heat shock protein 60                              |
| IPI00167147 | Nuclear            | 3                           | 0.561                                  | 0.561             | 240                  | 184                  | Similar to E1B 55 kDa-associated protein 5              |
| IPI00374970 | Microsomal         | 3                           | 0.567                                  | 0.147             | 61                   | 33                   | Septin 10                                                |
| IPI0013396 | Microsomal         | 1                           | 0.569                                  | 0.277             | 13                   | 44                   | U1 small nuclear ribonucleoprotein C                     |
| IPI00027107 | Cytosolic          | 1                           | 0.569                                  | 0.28              | 76                   | 39                   | Tu translation elongation factor, mitochondrial         |
| IPI00288941 | Nuclear            | 1                           | 1.757                                  | 0.394             | 3                    | 13                   | Splice isoform 2 of Q96AE4 Far upstream element-binding |
| IPI00163782 | Cytosolic          | 1                           | 1.77                                   | 0.205             | 2                    | 1                    | Splice isoform 2 of Q96AE4 Far upstream element-binding |
| IPI00170436 | Microsomal         | 1                           | 1.771                                  | 0.251             | 75                   | 73                   | Hypothetical protein                                     |
| IPI00171473 | Nuclear            | 1                           | 1.782                                  | 1.326             | 39                   | 21                   | Hypothetical protein                                     |
| IPI00328762 | Nuclear            | 1                           | 1.788                                  | 0.199             | —                    | —                    | ABC A13                                                  |
| IPI00064239 | Microsomal         | 1                           | 1.795                                  | 0.349             | 6                    | 3                    | Hypothetical protein                                     |
| IPI00186884 | Cytosolic          | 1                           | 1.796                                  | 0.383             | —                    | —                    | Similar to CG1383 gene product                           |
| IPI00382858 | Cytosolic          | 1                           | 1.8                                    | 2.204             | 7                    | 0                    | Similar to t-3-methyllysine hydroxylase                  |
| IPI00291922 | Cytosolic          | 1                           | 1.819                                  | 0.545             | 164                  | 109                  | Proteasome subunit α type 5<sup>a</sup>                 |
| IPI00027718 | Microsomal         | 2                           | 1.82                                   | 0.234             | 2                    | 4                    | Ellis-van Creveld syndrome protein                       |
| IPI00026185 | Cytosolic          | 1                           | 1.834                                  | 0.458             | 209                  | 568                  | Splice isoform 1 of P47756 F-actin capping protein β subunit<sup>b</sup> |
| IPI00013230 | Cytosolic          | 1                           | 1.848                                  | 1.156             | 31                   | 49                   | Glycyl-TRNA synthetase                                   |
| IPI00185326 | Nuclear            | 1                           | 1.867                                  | 0.24              | 0                    | 0                    | F-box and leucine-rich repeat protein 10                  |
| IPI00165467 | Microsomal         | 1                           | 1.897                                  | 0.399             | 19                   | 0                    | IGF-II mRNA-binding protein 3                           |
| IPI00376825 | Cytosolic          | 1                           | 1.899                                  | 1.039             | —                    | —                    | Similar to DG34A16.1 (similar to PGAM)                   |
| IPI00220644 | Microsomal         | 1                           | 1.909                                  | 0.366             | 1390                 | 1135                 | Pyruvate kinase 3 isoform 2                              |
| IPI00013944 | Nuclear            | 1                           | 1.925                                  | 1.447             | 216                  | 332                  | Splice isoform of heterogeneous nuclear ribonucleoproteins |
| IPI00013651 | Nuclear            | 1                           | 1.944                                  | 0.214             | 0                    | 7                    | Hypothetical protein                                     |
| IPI0030243 | Cytosolic          | 1                           | 1.948                                  | 0.631             | 0                    | 8                    | Splice isoform 1 of Q12920 proteasome activator complex subunit 3 |
| IPI00007812 | Microsomal         | 1                           | 1.968                                  | 0.573             | 0                    | 10                   | Vacular ATP synthase subunit b, brain isofrom            |
| IPI00400924 | Nuclear            | 1                           | 2.028                                  | 0.852             | —                    | —                    | Similar to ribosomal protein L13α<sup>d</sup>             |
| IPI00333763 | Nuclear            | 1                           | 2.061                                  | 1.468             | 4                    | 100                  | Similar to RIKEN cDNA 2900007E19 gene                     |
| IPI00026185 | Nuclear            | 2                           | 2.066                                  | 0.526             | 209                  | 568                  | Splice isoform 1 of P47756 F-actin capping protein β subunit<sup>d</sup> |
| IPI00008248 | Nuclear            | 1                           | 2.075                                  | 0.509             | 28                   | 38                   | Anaphase-promoting complex subunit 7<sup>d</sup>          |
| IPI00017625 | Cytosolic          | 1                           | 2.096                                  | 3.118             | 0                    | 4                    | Organic cation transporter (SLC22A2)                     |
| IPI00012462 | Nuclear            | 1                           | 2.163                                  | 1.343             | 11                   | 38                   | CDA02                                                   |
Proteome Changes in Cisplatin-resistant Ovarian Cancer Cells

Table I—continued

| IPI no.       | Cellular fractions | No. of peptides identified | Normalized chemo s/ chemo r (ICAT ratio) | Normalized errors<sup>a</sup> | MPSS tpm<sup>b</sup> | MPSS p value (Z test) | Protein descriptions |
|--------------|--------------------|-----------------------------|------------------------------------------|-------------------------------|----------------------|----------------------|----------------------|
| IPI00180776  | Cytosolic          | 1                           | 2.174                                    | 0.277                         | 105                  | 14                   | 0                   | 29-kDa protein       |
| IPI00184512  | Nuclear            | 1                           | 2.186                                    | 0.483                         | 105                  | 14                   | 0                   | Hypothetical protein |
| IPI00219674  | Nuclear            | 1                           | 2.439                                    | 0.6                           | 34                   | 130                  | 0                   | Cysteine- and glycine-rich protein 1 |
| IPI00005159  | Microsomal         | 1                           | 2.487                                    | 2.523                         | 13                   | 12                   | 0.91                | Actin-like protein 2 |
| IPI00221225  | Nuclear            | 2                           | 2.539                                    | 0.965                         | 332                  | 93                   | 0                   | Annexin IV<sup>d</sup> |
| IPI00293443  | Microsomal         | 1                           | 2.575                                    | 0.892                         | 7                    | 0                    | 0.04                | Signal recognition particle 14-kDa protein |
| IPI00295857  | Nuclear            | 1                           | 2.657                                    | 0.805                         | 79                   | 35                   | 0.000563            | Coatomer α subunit   |
| IPI00180983  | Microsomal         | 1                           | 2.674                                    | 0.438                         | 197                  | 109                  | 0.000213            | Hepatocellular carcinoma autoantigen<sup>d</sup> |
| IPI00003269  | Cytosolic          | 1                           | 2.694                                    | 0.96                          | —                    | —                    | —                   | Similar to RIKEN cDNA |
| IPI00152578  | Microsomal         | 1                           | 2.697                                    | 0.693                         | —                    | —                    | —                   | Splice isoform 1 of Q8TE04 |
| IPI00396967  | Nuclear            | 1                           | 2.705                                    | 0.252                         | 7                    | 0                    | 0.04                | Four and a half LIM domains 2 isoform 1 |
| IPI00012066  | Nuclear            | 2                           | 2.809                                    | 4.594                         | 0                    | 6                    | 0.02                | Poly(C)-binding protein 2 isoform b |
| IPI00062210  | Cytosolic          | 1                           | 2.83                                     | 0.436                         | —                    | —                    | —                   | Amiloride-sensitive cation channel 4 isoform 1 (ACCN4)<sup>d</sup> |
| IPI00329389  | Microsomal         | 1                           | 2.91                                     | 0.196                         | 1540                 | 2576                 | 0                   | 60 S ribosomal protein L6<sup>d</sup> |
| IPI00219568  | Cytosolic          | 1                           | 3.126                                    | 0.305                         | —                    | —                    | —                   | Phosphoglycerate kinase 2 |
| IPI00197577  | Microsomal         | 1                           | 3.159                                    | 0.64                          | 2546                 | 3267                 | 0                   | Glutathione S-transferase Pi<sup>d</sup> |
| IPI00029623  | Microsomal         | 1                           | 3.288                                    | 0.956                         | 341                  | 278                  | 0.06                | Proteosome subunit α type 6<sup>d</sup> |
| IPI00030466  | Cytosolic          | 1                           | 3.536                                    | 1.059                         | 0                    | 0                    | 1                   | Hypothetical protein KIAA1636 |
| IPI00030847  | Nuclear            | 1                           | 3.645                                    | 0.769                         | 37                   | 4                    | 0.000015            | Transmembrane 9 superfamily protein member 3 precursor |
| IPI00386128  | Cytosolic          | 1                           | 3.737                                    | 7.804                         | 21                   | 130                  | 0                   | HDJ2 protein (DNAJ1) |
| IPI00032406  | Cytosolic          | 1                           | 3.773                                    | 0.506                         | 0                    | 20                   | 0.000911            | DnaJ homolog subfamily a member 2 (DNAJ2)<sup>d</sup> |
| IPI00028740  | Cytosolic          | 1                           | 4.002                                    | 0.6                           | 0                    | 0                    | 1                   | Transforming protein p21/H-ras-1<sup>d</sup> |
| IPI00002773  | Cytosolic          | 1                           | 4.029                                    | 3.161                         | 0                    | 14                   | 0.00372             | Splice isoform 2 of P52333 |
| IPI00009747  | Nuclear            | 1                           | 4.043                                    | 0.416                         | 153                  | 63                   | 0.000007            | Tyrosine-protein kinase JAK3<sup>d</sup> |
| IPI00006097  | Nuclear            | 1                           | 4.383                                    | 1.065                         | 146                  | 172                  | 0.22                | Tumor necrosis factor receptor superfamily member 3 precursor<sup>d</sup> |
| IPI00306604  | Microsomal         | 2                           | 4.452                                    | 4.679                         | 134                  | 341                  | 0                   | Integrin α5 precursor<sup>d</sup> |
| IPI0000873  | Microsomal         | 1                           | 4.692                                    | 1.503                         | 0                    | 0                    | 1                   | Integrin α3 precursor<sup>d</sup> |
| IPI00173349  | Cytosolic          | 1                           | 5.869                                    | 6.203                         | —                    | —                    | —                   | Hypothetical protein |
| IPI00000873  | Microsomal         | 1                           | 5.973                                    | 1.024                         | 155                  | 53                   | 0                   | Valyl-tRNA synthetase 2 |
| IPI00011302  | Nuclear            | 1                           | 6.387                                    | 6.412                         | 0                    | 137                  | 0                   | CD59 glycoprotein precursor |
| IPI00306604  | Nuclear            | 1                           | 6.756                                    | 1.543                         | 134                  | 56                   | 0                   | Integrin α6 precursor<sup>d</sup> |
| IPI00258904  | Nuclear            | 1                           | 8.186                                    | 1.228                         | —                    | —                    | —                   | Similar to peptidyl-prolyl cis-trans isomerase |
| IPI00246058  | Nuclear            | 1                           | 12.7                                     | 0.156                         | 32                   | 49                   | 0.09                | Programmed cell death 6-interacting protein<sup>d</sup> |
| IPI00376403  | Nuclear            | 1                           | 13.328                                   | 1.889                         | 66                   | 129                  | 0.0001             | Hepatocyte growth factor activator inhibitor 1B<sup>d</sup> |
| IPI00103422  | Microsomal         | 1                           | 29.152                                   | 47.247                        | 0                    | 6                    | 0.07                | Coxackievirus-adenovirus receptor isofrom CAR/47<sup>d</sup> |
| IPI00003348  | Nuclear            | 1                           | 38.692                                   | 78.489                        | 273                  | 261                  | 0.65                | Guanine nucleotide-binding protein G/G<sub>y</sub>/G<sub>y</sub> subunit 2 |
| IPI00008433  | Nuclear            | 1                           | 45.411                                   | 46.759                        | 369                  | 447                  | 0.02                | 40 S ribosomal protein S5<sup>d</sup> |

<sup>a</sup> Calculated by the ASAPRatio program.

<sup>b</sup> mRNA expression levels represented by transcript per million (tpm) analyzed by MPSS.

<sup>c</sup> —, no data available.

<sup>d</sup> Indicates proteins previously shown to change expression in cancer.

Some proteins were identified in more than one fractions and are listed more than once.

Significantly changed in the differentially expressed proteins. In the Panther database, there are 61 pathways listed. Using all the proteins we identified as the reference list and using the binomial test (17), we identified three pathways (glycolysis, interleukin signaling pathway, and PI 3-kinase pathway) that were significantly (p < 0.05) up-regulated in cisplatin-sensitive cells and one pathway (fibroblast growth factor signaling pathway) that was marginally (p = 0.056) up-regulated in cisplatin-resistant cells (Table III). These four pathway maps with the changed proteins colored by their increased or decreased expression.
In addition to the proteomic data, we generated expression profiles of IGROV-1 and IGROV-1/CP cells using MPSS. We were therefore able to correlate the protein and mRNA expression between IGROV-1 and IGROV-1/CP. We converted the protein IDs and MPSS signatures to Unigene IDs to compare the MPSS data with the ICAT/MS/MS data. We limited this comparison to 63 proteins that have reliable MPSS measurement (p value <0.05 by Z test) and ICAT data (1.75-fold, p < 0.05). There was concordance in the direction of differential expression between cisplatin-resistant and -sensitive cells at the protein and transcript level in 28 of 63 (44.44%) proteins. Examples of cancer-related genes overexpressed at both the protein and transcript levels in resistant cells include mitogen-activated protein kinase kinase kinase 6, protein disulfide isomerase, NHP2-like protein 1, and DNA replication licensing factor MCM3. Examples of cancer-related genes overexpressed at both the protein and transcript levels in cisplatin-sensitive cells include lanosterol synthase, transmembrane 9 superfamily protein member 3, hepatocellular carcinoma autoantigen, and annexin IV. However, in 35 of 63 (55.56%) proteins, the expression levels were discordant between the mRNAs and the proteins, i.e. high protein level but lower transcript level or vice versa. This finding suggests that posttranscriptional regulation of protein expression is an important factor in controlling the final protein concentration in the cell.

**DISCUSSION**

Cisplatin (cis-diaminodichloroplatinum) contains a platinum central atom surrounded by two chloride and two NH₃ groups in the cis position. Mechanisms explaining cisplatin resistance include the reduction of cisplatin accumulation inside cancer cells, the ability to detoxify cisplatin, faster repair of cisplatin-DNA adducts, modulation of apoptotic pathways, up-regulation in transcription factors, and a higher concentration of glutathione and metallothioneins (18). Our goal was to use high throughput proteomic technologies to help us understand cisplatin resistance and to identify candidate biomarkers of cisplatin treatment response or novel therapeutic targets. In this study we applied high throughput ICAT/MS/MS proteomic technology to compare the proteomes of an isogenic pair of cisplatin-sensitive and -resistant ovarian cancer cell lines. In the end, we quantified the expression of 1117 proteins and identified 121 proteins as differentially expressed between cisplatin-resistant and -sensitive cells. 37 proteins have been reported as being cancer-related as determined by gene array or other techniques (Table I). However, we viewed our ICAT experiment as a global profiling approach to identify putative candidates that we can evaluate individually. We have not determined the reproducibility or the uncertainty levels in our measurements by repeating the experiments and mass spectrometry analyses multiple times (ideally three biological replicate experiments with three technological replicate MS runs, i.e. a total of nine ICAT/MS/MS runs). At this point in time it would be more cost effective to evaluate the biological variance at individual protein levels using much cheaper technology such as ELISA and quantitative Western blot analysis as we have done for a few examples. We considered running nine ICAT/MS/MS too costly at this point in time.

Analysis of differentially expressed genes or proteins by GO categories offers a global view of the biological meaning of a global genomic or proteomic dataset. In our analysis, we identified enriched GO categories in both cisplatin-sensitive and -resistant cells. By GO molecular function, heat shock protein binding GO term is overrepresented in cisplatin-sensitive cells (Table II). We showed that DNAJ1 and DNAJ2, both DNAJ (Hsp40) homologs, were expressed at higher levels in cisplatin-sensitive cells compared with -resistant cells, suggesting loss of DNAJ family proteins may play an important role in conferring the resistant phenotype in ovarian cells. Recently Shridhar et al. (19) found that loss of expression of a new member of the DNAJ protein family that belongs to the heat shock protein binding GO term conferred resistance to cisplatin. In contrast, many GO categories including those with hydrolase activities, pyrophosphatase activity, ATP-dependent RNA helicase activity, and nucleoside triphosphatase activity were overrepresented in cisplatin-resistant cells. These GO categories have metabolic activities and may be involved in active cisplatin metabolism, resulting in fast cisplatin turnover or degradation and thus conferring a resistant phenotype.

By GO biological process, many processes involved in RNA splicing, processing, and DNA replication were overrepresented in cisplatin-resistant cells. Increased activities in these biological processes may allow faster repairs of cisplatin-induced DNA damages such as cisplatin-DNA adducts, thus resulting in a resistant phenotype. Interestingly the MAPKKK cascade, a cascade of at least three protein kinase activities culminating in the phosphorylation and activation of a mitogen-activated protein kinase, was overrepresented in cisplatin-resistant ovarian cancer cells. The biological variance at individual protein levels using much cheaper technology such as ELISA and quantitative Western blot analysis as we have done for a few examples. We considered running nine ICAT/MS/MS too costly at this point in time.
## TABLE II

*Enriched GO terms in cisplatin-resistant and -sensitive cells*

| GO ID | Total | Changed | p value (changed) | Term |
|-------|-------|---------|------------------|------|
|       |       |         |                  |      |
| **By molecular function (out of 632 GO terms)** |       |         |                  |      |
|       |       |         |                  |      |
| **Up in chemoresistant cells** |       |         |                  |      |
| 3723  | 67    | 10      | 0.0083           | RNA binding                               |
| 3676  | 117   | 14      | 0.0117           | Nucleic acid binding                      |
| 4004  | 3     | 2       | 0.0129           | ATP-dependent RNA helicase activity       |
| 8186  | 4     | 2       | 0.0247           | RNA-dependent ATPase activity             |
| 16462 | 39    | 6       | 0.039            | Pyrophosphatase activity                  |
| 16818 | 39    | 6       | 0.039            | Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides |
|       |       |         |                  |      |
| **Up in chemosensitive cells** |       |         |                  |      |
| 31072 | 3     | 2       | 0.0173           | Heat shock protein binding                 |
| 16301 | 17    | 4       | 0.0371           | Kinase activity                           |
|       |       |         |                  |      |
| **By biological process (out of 842 GO terms)** |       |         |                  |      |
|       |       |         |                  |      |
| **Up in chemoresistant cells** |       |         |                  |      |
| 6396  | 39    | 9       | 0.0005           | RNA processing                            |
| 8380  | 22    | 6       | 0.0021           | RNA splicing                              |
| 16070 | 52    | 9       | 0.0046           | RNA metabolism                            |
| 44237 | 271   | 25      | 0.0087           | Cellular metabolism                       |
| 6445  | 8     | 3       | 0.0127           | Regulation of translation                  |
| 6417  | 8     | 3       | 0.0127           | Regulation of protein biosynthesis        |
| 6414  | 8     | 3       | 0.0127           | Translational elongation                   |
| 51246 | 8     | 3       | 0.0127           | Regulation of protein metabolism           |
| 398   | 15    | 4       | 0.0141           | Nuclear mRNA splicing, via spliceosome     |
| 377   | 15    | 4       | 0.0141           | RNA splicing, via transesterification reactions with bulged adenosine as nucleophile |
| 375   | 15    | 4       | 0.0141           | RNA splicing, via transesterification reactions |
| 43283 | 97    | 12      | 0.0168           | Biopolymer metabolism                     |
| 9889  | 9     | 3       | 0.0181           | Regulation of biosynthesis                |
| 8152  | 285   | 25      | 0.0212           | Metabolism                                |
| 6397  | 26    | 5       | 0.0247           | mRNA processing                           |
| 165   | 4     | 2       | 0.0247           | MAPK cascade                              |
| 43037 | 26    | 5       | 0.0247           | Translation                               |
| 50875 | 342   | 28      | 0.0267           | Cellular physiological process            |
| 6468  | 11    | 3       | 0.0324           | Protein amino acid phosphorylation        |
| 16071 | 28    | 5       | 0.0334           | mRNA metabolism                           |
| 6412  | 49    | 7       | 0.0372           | Protein biosynthesis                      |
| 6270  | 5     | 2       | 0.0394           | DNA replication initiation                |
| 30154 | 5     | 2       | 0.0394           | Cell differentiation                      |
| 9059  | 51    | 7       | 0.0454           | Macromolecule biosynthesis                |
|       |       |         |                  |      |
| **Up in chemosensitive cells** |       |         |                  |      |
| 46903 | 7     | 3       | 0.0127           | Secretion                                |
| 50878 | 3     | 2       | 0.0173           | Regulation of body fluids                 |
| 9108  | 4     | 2       | 0.0329           | Coenzyme biosynthesis                     |
|       |       |         |                  |      |
| **By cellular component (out 205 GO terms)** |       |         |                  |      |
|       |       |         |                  |      |
| **Up in chemoresistant cells** |       |         |                  |      |
| 5681  | 9     | 4       | 0.0017           | Spliceosome complex                       |
| 30529 | 48    | 9       | 0.0025           | Ribonucleoprotein complex                 |
| 43234 | 101   | 13      | 0.0084           | Protein complex                           |
| 43229 | 269   | 24      | 0.0218           | Intracellular organelle                   |
| 43226 | 269   | 24      | 0.0218           | Organelle                                |
| 43231 | 218   | 20      | 0.0404           | Intracellular membrane-bound organelle    |
| 43227 | 218   | 20      | 0.0404           | Membrane-bound organelle                  |
|       |       |         |                  |      |
| **Up in chemosensitive cells** |       |         |                  |      |
| 5829  | 30    | 6       | 0.0231           | Cytosol                                 |

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TABLE III

| Table III: Changed pathways in cisplatin-sensitive and -resistant cells |
|---------------------------------------------------------------|
| **Pathway** | **No. of hits in the reference set** | **No. of hits in the comparison set** | **Expected no. of hits** | **p value** |
|---------------|-------------------------------------|-------------------------------|------------------------|-----------|
| **Up in cisplatin-sensitive pathways** | | | | |
| Glycolysis | 7 | 3 | 0.58 | 0.020 |
| Interleukin signaling pathway | 3 | 2 | 0.25 | 0.026 |
| PI 3-kinase pathway | 8 | 3 | 0.66 | 0.028 |
| **Up in cisplatin-resistant pathways** | | | | |
| FGF signaling pathway | 12 | 3 | 0.87 | 0.056 |

*Using binomial test.*

**Fibroblast growth factor.**

Cisplatin-resistant cells. MAPKKK cascades lie downstream of many signaling pathways and have been shown to play important roles in chemoresistance (for a review, see Ref. 20). We identified three pathways (glycolysis, interleukin signaling pathway, and PI 3-kinase pathway) in the Panther database (panther.appliedbiosystems.com) that were significantly \((p < 0.05)\) up-regulated in cisplatin-sensitive cells. Ras, Jak, and G\(\beta\)\(\gamma\) protein in the PI 3-kinase pathway were overexpressed in cisplatin-sensitive cells (Supplemental Fig. 1). Overexpression of these proteins can result in activation of PI 3-kinase (catalytic subunit P110 and regulatory subunits P101 or P85) that catalyze phosphorylation of PI(4,5)P\(_2\) to PI(3,4,5)P\(_3\) (phosphatidylinositol 3,4,5-trisphosphate). PI(3,4,5)P\(_3\) can activate kinase 3-phosphoinositide-dependent protein kinase-1, which in turn can activate kinase AKT (also known as protein kinase B), resulting in phosphorylation of BAD (BCL2 antagonist of cell death) and caspase-9, two proteins important for apoptosis. Caspase-9 is involved in the activation caspase cascade responsible for apoptosis execution. The function of the proapoptotic molecule BAD is regulated by phosphorylation of three serine sites (serines 112, 136, and 155). Phosphorylation of BAD results in loss of the ability of BAD to heterodimerize with the survival proteins BCL-XL or BCL-2, therefore promoting apoptosis.

The major mechanism of cisplatin-induced tumor killing is via induction of apoptosis (21). We also identified other genes related to apoptosis (Table I). We identified hepatocyte growth factor activator inhibitor 1B as overexpressed in cisplatin-sensitive cells. Hepatocyte growth factor is a cellular protein that offers protection from apoptosis (22). Overexpression of hepatocyte growth factor activator inhibitor 1B can reduce the expression of hepatocyte growth factor and its apoptosis protection efforts, thus increases apoptosis of cells, and results in sensitive phenotype. We also identified programmed cell death 6-interacting protein (PDCD6IP; alias, Alix) as overexpressed in cisplatin-sensitive cells. PDCD6IP plays a role in modulating apoptosis as it interacts with apoptosis-related proteins such as PDCD6 and endophilins (23, 24). However, the role of PDCD6IP in regulating chemotherapy response is still not clear.

Cisplatin is very reactive against SH-containing molecules and thiols molecules in cells. We identified two SH- and thiol-containing molecules as overexpressed in cisplatin-sensitive cells: cysteine- and glycine-rich protein 1 and glutathione S-transferase Pi. It was proposed previously that these SH- or thiol-containing proteins can detoxify cisplatin (25), but it is still possible that binding to these proteins can cause cellular damage and cell death.

In addition to confirmation of previously proposed mechanisms of cisplatin resistance in ovarian cancer cells, our study identified many new differentially expressed proteins, pathways, and enriched GO terms that may confer novel insights into the mechanisms of cisplatin resistance and allow us to generate novel hypotheses for testing. We identified many cation transport proteins as overexpressed in cisplatin-sensitive cells such as proton ion transport protein H\(^+\)-transporting ATPase, sodium ion transport protein ACCN4 (amiloride-sensitive cation channel 4), and organic cation transporter SLC22A2 (solute carrier family 22, member 2). One can hypothesize that overexpression of these proteins allows easier transport of cisplatin into cells and thus increases the intracellular concentration of cisplatin and results in a more sensitive phenotype to cisplatin. How cisplatin is transported into cells remains unknown. It should be noted that the parent cisplatin PtCl\(_2\)(NH\(_3\))\(_2\) generated cisplatin-aguivated derivatives including PtCl\((\text{H}_2\text{O})(\text{NH}_3)\)\(_2\) and Pt\((\text{H}_2\text{O})_2\)(\text{NH}_3)\(_2\) and hydroxido derivatives PtCl(\text{OH})(\text{NH}_3)\(_2\) and Pt(\text{OH})\(_2\)(\text{NH}_3)\(_2\) in plasma and aqueous environment (26). These different forms of cisplatin derivatives may require different transporters.

We also identified annexin IV as overexpressed in cisplatin-sensitive cells. Annexins are a family of structurally related water-soluble proteins possessing a hydrophilic surface that can bind to phospholipids in a calcium-dependent manner and form voltage-dependent calcium channels within planar lipid bilayers. Despite their structural similarities, annexins have diverse functions including cell division, apoptosis, calcium signaling, growth regulation, and secretory function involving both exocytotic and endocytotic pathways. Annexin IV has a role in carcinogenesis as it was found to be overexpressed at both the RNA and protein levels in renal cell carcinoma cells (27, 28). The role of annexin IV in ovarian carcinogenesis has not been studied. However, annexin IV was found to be overexpressed in a paclitaxel-resistant lung cancer cell line (H460/T800), and transfection of annexin IV...
cDNA into 293T cells results in a 3-fold increase in paclitaxel resistance (29). Combination therapy (cisplatin or carboplatin with paclitaxel) is common therapy for ovarian cancers, but cisplatin and paclitaxel may have opposite effects on the expression of certain proteins. The role of annexin IV in the chemotherapy response of ovarian cancer warrants further investigation.

We identified Claudin 4 (CLDN4) as overexpressed in cisplatin-resistant cells. CLDN4 is an integral membrane protein and component of the tight junction of cells. The tight junction provides the barrier to the passage of ions and molecules through the paracellular pathway (movement of molecules through the intercellular spaces between epithelial cells) and to the movement of proteins and lipids between the apical and the basolateral domains of the plasma membrane (30). Disruption of tight junctions is often associated with cancer development (31, 32). For example, CLDN4 mRNA has been identified as overexpressed relative to normal epithelial in several types of cancer including ovarian (33, 34) and prostate cancer (35). However, CLDN4 protein expression measured using immunohistochemical staining is reduced in invasive breast and advanced gastric adenocarcinoma relative to normal or precancerous tissues (36, 37). The relationship between increased CLDN4 protein expression and cisplatin resistance is unclear at present. Indeed to our knowledge, no studies on the relationship between tight junction and chemotherapy response have been carried out. We hypothesize that the balance of cellular uptake and export of cisplatin can change the intracellular levels of cisplatin accumulation. The proteins involved in tight junctions (e.g. Claudin 4) and ion transport and ion channels (e.g. cation transport proteins and annexin IV) may all play roles in regulating this balance and result in a sensitive or resistant phenotype.

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