Journal of Molecular Biomarkers & Diagnosis

Wang, et al., J Mol Biomark Diagn 2014, 5:4
DOI: 10.4172/2155-9929.1000184

Research Article Open Access

Putative Surrogate Biomarkers to Predict Patients with Acquired Platinum Resistance in Ovarian Cancer

Mu Wang*, **Dawn P G Brown**, **Jinsam You and Kerry G Bemis**

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

***Corresponding author**: Mu Wang, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS 4053, Indianapolis, USA, Tel: 317- 278-0296; Fax: 317- 274-4686; E-mail: muwang@iu.edu

Rec date: May 07, 2014; **Acc date:** May 30, 2014; **Pub date:** Jun 03, 2014

Abstract

Over 15,000 women die from ovarian cancer and there are approximately 23,000 new cases diagnosed each year. Platinum-based chemotherapy is still the primary treatment for ovarian cancer. Most patients with the disease are initially responsive to chemotherapeutic treatment. However, a majority of ovarian cancer patients eventually relapse and become refractory to additional treatment. This drug-resistance is a major impediment to the successful treatment of ovarian cancer. To date the mechanisms of drug-resistance remain poorly understood. Previous studies have suggested that many proteins, such as BRCA1, BRCA2, MDR1, MRP1, MDM2, hMLH1, HSP27, and HSP70, are differentially expressed in drug-resistant ovarian tumor cells by mRNA differential display analysis. However, biomarkers that can be used to differentiate chemotherapy responders from non-responders have not yet been developed. With recent developments in proteomic technologies, differential protein expression in complex biological samples can be analyzed. In this cell model based study, we applied a label-free protein quantification technology to discover potential protein biomarker candidates that can differentiate chemo-drug responders from non-responders. This experimental approach could also serve as a model tool for further clinical validation and biomarker development for other diseases.

Keywords: Ovarian cancer; Biomarker; Platinum resistance

Abbreviations:

AUC: Area-Under-the-Curve; CV: Coefficient-of-Variation; IPI: International protein index; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; LDA: Linear Discriminant Analysis; MRM: Multiple-Reaction-Monitoring; OC: Ovarian Cancer; XIC: Extracted Ion Chromatogram

Introduction

Ovarian cancer (OC) ranks second among gynecological cancers in the number of new cases and first among gynecological cancers in the number of deaths each year [1]. Epithelial ovarian carcinomas (EOCs) account for 85 to 90 percent of all cancers of the ovaries. About 65% of women with epithelial OC will die within five years of their diagnosis [1,2]. Despite improvements in aggressive debulking surgery and the initial good response of patients to platinum-based chemotherapies (primarily paclitaxel & carboplatin), there has been little improvement in the overall survival rates for advanced epithelial OC patients in over three decades [2]. Two major factors are thought to contribute to the drawback of OC patient care: 1) disease stage. Most patients are diagnosed with advanced disease; and 2) chemodrug resistance. The majority of patients (70~80%) initially respond well to platinum-based chemotherapy but later become refractory to additional treatment over time [3,4]. Furthermore, there remains intense debate about the cellular origins, precursor lesions, and histological classification of the disease. With so many unknowns, it is not surprising that progress in reducing mortality in women diagnosed with OC has been so limited.

However, the most recent scientific advances and discoveries have given continued hope and potential that can improve OC patient care [5]. For some OC types, the molecular characterization of tumors has led to better strategies not only for predicting patient-care outcomes, so that treatment can be targeted more effectively, but also for the development of new therapies [5]. While some molecular mechanisms of platinum resistance are beginning to be deciphered, effective predictive biomarkers or molecular signatures of resistance remain largely unknown. Identification of such predictive biomarkers would significantly improve our current understanding of the disease, allow risk stratification of patients prior to treatment and potential allocation of patients likely to be resistant to platinum to more aggressive treatment regime (i.e., drugs now in development or in clinical trials. Good examples are ABT-888 and Topotecan Hydrochloride treatment regime for chemo-resistant tumors, Farletuzumab, Doxorubicin, and Bevacizumab for combined chemotherapies).

So far, many proteins have been suggested as defining factors associated with drug resistance [3-7], including BRCA1, DNA-PK, ERCC1, MDR1, HSP27, HSP70, ATP7A, ATP7B, and CTR1 [8-17]. Unfortunately, variations in protein expression in clinical samples have complicated the development of readily applicable platinum resistance biomarkers mainly due to sample heterogeneity. We contend that the use of a panel of predictive biomarkers would yield higher predictive power than the use of a single or small number of biomarkers. Successful identification of such a specific panel for platinum resistance could directly impact clinical applications, as patients who are predicted to not derive sustained benefit from platinum-based chemotherapy could be directed towards more aggressive therapies incorporating novel agents targeting mechanism(s) of drug resistance.

Proteomic technologies offer the promise of a comprehensive understanding of cancer and its therapeutic responsiveness [18-20]. During the last decade, these technologies have played a key role in characterizing proteins from the discovery of predictive biomarkers and drug targets to the validation of biomarker candidates [21-23]. Despite many advances in technology and bioinformatics, today almost all the disease indicative biomarkers only consist of a single or small number of biomarkers, and they are often not sensitive, with high false discovery rate and poor accuracy. Thus there is an urgent need to develop more sensitive, more specific, and clinically useful biomarkers to precisely characterize the disease not only at the diagnostic and prognostic levels, but to monitor disease progression [24]. In addition, with the sensitivity and accuracy of today's state-of-the-art technologies, the challenges remain huge in identifying plasma/serum protein biomarker candidates due to its heterogeneity and wide dynamic range [25]. However, compared to identifying meaningful disease related protein biomarker candidates in plasma or serum, the challenges of identifying them in cells and tissues are significantly reduced due to the significantly lower protein dynamic range [26,27]. A key benefit of cell or tissue model samples is the fact that the differential protein expression can be directly investigated at the origin of the disease. Therefore, disease indicating protein expression differences are expected to be more pronounced in suitable cell or tissue samples compared to the blood stream where the relevant tissue derived proteins are expected to be detected after significant dilution [25]. Both prostate specific antigen (PSA) and human epidermal growth factor receptor 2 (HER2) are good examples for cell-derived biomarkers that are subsequently validated for clinical use [28-31]. In this study, we apply a mass spectrometry-based unbiased proteomic approach and cell-based models to identify potential biomarkers that are capable of predicting the drug response of the OC patient to chemotherapeutic treatment and therefore have a potential to guide clinicians in choosing the best treatment option in OC patient care.

Materials and methods

Chemicals and reagents

The ammonium bicarbonate, dithiothreitol (DTT) and iodoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and mass spec grade water were purchased from Honeywell Burdick & Jackson (Morristown, NJ, USA). Modified trypsin was purchased from Promega (Madison, WI, USA). Fetal Bovine Serum was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). RPMI-1640 media was purchased from Cambrex Bio Science (Walkersville, MD, USA).

Cell lines

Two pairs of well-characterized human ovarian cancer cell lines were used for this study: A2780 (cisplatin-sensitive) & A2780-CP (cisplatin-resistant) and 2008 (cisplatin-sensitive) & 2008-C13*5.25 (cisplatin-resistant) [32,33]. The cell lines were obtained from Dr. Stephen B. Howell of University of California at San Diego (La Jolla, California, USA). All cell lines were handled under identical conditions and maintained at 37°C in a humidified incubator containing 5% CO_2 in RPMI-1640 supplemented with 15% fetal bovine serum. In this particular study, the sensitive cell lines were grown in the absence of cisplatin, whereas the resistant cell lines were grown in the absence and presence of cisplatin, respectively.

Cell line characterization

The Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche Diagnostics, Indianapolis, IN, USA) was used to determine cisplatin cytotoxicity. Cells were seeded at ~2,000 cells per well in a 96-well plate with 100 µL of cell suspension solution and placed in an incubator at 37°C, 5% CO2, and allowed to attach overnight. They were then treated with various concentrations of cisplatin (0, 0.1, 0.2, 1, 2, 5, 10, 20, and 30 µM, respectively) and the plates were incubated for an additional 48 hrs. Cell proliferation was quantified based on BrdU incorporation during DNA synthesis in proliferating cells, according to the manufacturer's instructions. IC50 values were determined from dose-response graphs.

Cell culture preparation for proteomic analysis

The two pairs of cell lines described above were grown in forty-eight 100-mm tissue culture plates with RPMI-1640 media containing 15% fetal bovine serum at 37°C in a 5% CO2 incubator. Six plates for each condition (see Table 1 for details). Two resistant cell lines were treated with 10 and 20 µM of cisplatin, respectively, for 24 hrs before the fresh media were used to replace the media containing cisplatin. The rationale for choosing these two concentrations was based on the observation that the majority of the cisplatin-sensitive cells would not survive at 20 µM cisplatin concentration, while at least 50% of the cisplatin-resistant cells would survive (Figure 1). Upon 95% confluency, cells were detached from the plates by trypsin, washed three times with 5 mL of ice-cold phosphate-buffered saline (PBS) and stored at -80°C until use.

Protein sample preparation

Cells were homogenized in the hypotonic lysis buffer (100 µL of freshly made 8 M Urea, 10 mM DTT solution). Resulting cell lysates were reduced and alkylated by triethylphosphine and iodoethanol, and then digested by trypsin according to a previously published procedure [34]. This procedure allows all steps to be carried out in one tube without washing or filtering steps. Tryptic peptide concentration was determined by the Bradford Protein Assay [35]. The same lysis buffer was used as the background reference for protein assay and the buffer for protein standards (BSA).

Mass spectrometric analysis

Peptides were prepared and subjected to LC/MS analysis as previously described [36]. There were eight groups in this study: two pairs of cell lines each including a sensitive cell line and a resistant cell line with no drug treatment (Table 1, Groups 1&2 and 5&6), the resistant cell lines (A2780-CP and 2008-C13*5.25) with 10 µM and 20 µM of cisplatin treatment, respectively (Table 1, Group 3&4 and 7&8). There were 6 samples per group, yielding a total of 96 randomized HPLC injections. Samples were run on a Surveyor HPLC system (Thermo-Fisher) with a C18 microbore column (Zorbax 300SB-C18, 1mm x 5cm). All tryptic peptides (20 µg) were injected onto the column in random order. Peptides were eluted with a linear gradient from 5 to 45% acetonitrile developed over 120 min at a flow rate of 50 µL/min, and effluent was electro-sprayed into the LTQ mass spectrometer (Thermo-Fisher). The data was collected in the "Triple-Play" (MS scan, Zoom scan, and MS/MS scan) mode. The acquired data were processed by a proprietary algorithm developed by Higgs, et al. [36]. Database searches against the IPI (International Protein Index) human database and the NCBI Non-Redundant-homo sapiens

database were carried out using both the X!Tandem and SEQUEST algorithms. Protein quantification was carried out using the same proprietary protein quantification algorithm [36]. All extracted ion chromatograms (XIC) were aligned by retention time within a one-minute window. Each aligned peak must match parent ion, charge state, daughter ions (MS/MS data) and retention time (within a one-minute window) to pass through the "filter". After alignment, the area-under-the-curve (AUC) for each individually aligned peak from each sample was measured, normalized, and these were compared for relative abundance. All peak intensities were transformed to a log2 scale before quantile normalization [37].

Linear discriminant analysis

Linear discriminant analysis (LDA) was carried out in SAS (version 9.2 for Windows, SAS Institute Inc., Cary, NC, USA). The relative abundances of the selected product ions of proteins were tested for significance using a one-factorial multivariate analysis of variance (ANOVA). Then, to determine which peaks can discriminate between groups, a step-wise discriminant analysis was carried out to calculate the canonical discriminant functions. The first two canonical discrimination functions normally can represent over 98% of the separation of different proteins. The classification functions were then obtained to predict the group to which the unknown samples belong.

Results and discussion

Characterization of cisplatin-resistance

Cisplatin resistance in these cells was determined using the Cell Proliferation ELISA, BrdU Assay. The results from these cell proliferation assays are shown in Figure 1. There was an 11.8-fold resistance to cisplatin in the A2780/CP as compared to its sensitive counterpart, A2780, based on IC50 values. Likewise, the 2008/C13*5.25 subline showed a 34.8-fold greater resistance to cisplatin than the 2008 cell line. Phenotype of the resistant cell lines was stable even in the absence of cisplatin [15].

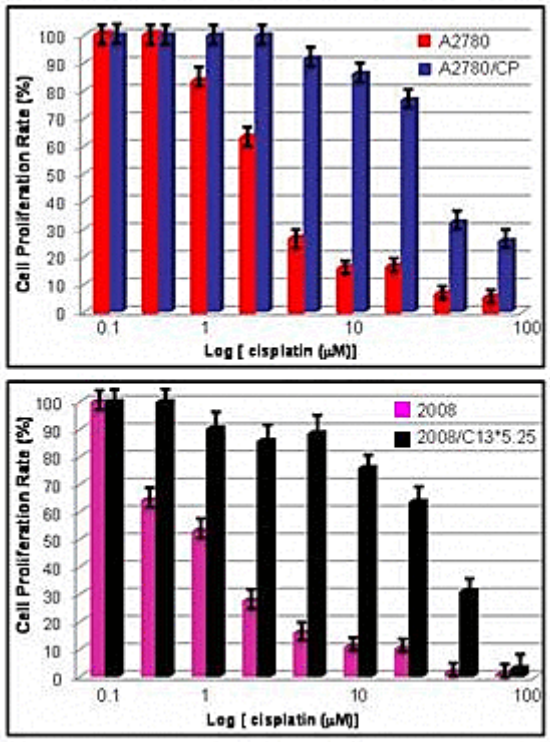

Figure 1: Dose-dependent cell proliferation assay of A2780/A2780-CP (cisplatin-sensitive/cisplatin-resistant) and 2008/2008-C13*5.25 (cisplatin-sensitive/cisplatin-resistant) ovarian cancer cells. Three replicates were performed. $P<0.05$. X-axis is in logarithmic scale

Group Number	Group Name	Group Abbreviation	Cell Type	Cisplatin Treatment (μM)	Number of Samples Per Group	Total Number of Replicate Injections Per Group
1	A2780	C1S	Sensitive	0	6	12
2	A2780CP	C1R00	Resistant	0	6	12
3	A2780CP-10	C1R10	Resistant	10	6	12
4	A2780CP-20	C1R20	Resistant	20	6	12
5	2008	C2S	Sensitive	0	6	12
6	2008C13	C2R00	Resistant	0	6	12
7	2008C13-10	C2R10	Resistant	10	6	12
8	2008C13-20	C2R20	Resistant	20	6	12

Table 1: Experimental Design

Protein Identification

In this study, a total of 2,117 proteins were identified and quantitatively compared. A summary of the data is shown in Table 2. Proteins were categorized into priority groups based on the quality of the peptide identification. The Peptide ID Confidence assigns a protein to a 'HIGH' or 'INTERMEDIATE' classification based on the peptide with the highest peptide ID Confidence (the best peptide) among all the peptides identified for a particular protein.

Proteins which have at least one identified peptide with a confidence level ≥90% are assigned to the 'HIGH' category regardless of whether there are other peptides having low confidence or not. Proteins which have no peptide with a confidence level ≥90% but at least one peptide with a confidence between 75-89% are assigned to the 'INTERMEDIATE' category. All peptides with confidence less than 75% were filtered out for further analysis. SEQUEST and X! Tandem database search algorithms were used for peptide sequence identification. Each algorithm compares the observed peptide MS/MS spectrum and a theoretically derived spectra from the database to assign quality scores (*XCorr* in SEQUEST and *E-Score* in X!Tandem). These quality scores and other important predictors are combined in a proprietary algorithm that assigns an overall score, %ID Confidence, to each peptide. The assignment is based on a random forest recursive partition supervised learning algorithm [36,38].

Protein Priority	Peptide ID Confidence	Multiple Peptide Sequences	Number of Proteins Identified	Number of Significant Changes	Median Overall %CV
1	HIGH (≥90%)	YES	855	760	12.87
2	HIGH (≥90%)	NO	583	390	23.65
3	INTERMEDIATE (75~89%)	YES	27	15	24.77
4	INTERMEDIATE (75~89%)	NO	652	404	31.95
Overall			2,117	1,569	19.55

Table 2: Summary of the Study Using a LC/MS-based Label-free Protein Quantification Method

The confidence in protein identification increases with the number of unique amino acid sequences identified. Therefore we also categorized proteins depending on whether they have only one or multiple sequences of the required confidence. A protein is classified as 'YES' in the 'Multiple Sequences' column if it has at least two distinct amino acid sequences with the required ID confidence; otherwise it is classified as 'NO'. Priority assignments reflect our level of confidence in the protein identification. Priority 1 proteins would have the highest likelihood of correct identification and Priority 4 proteins the lowest. This priority system is based on the quality of the amino acid sequence identification (Peptide ID Confidence) and whether one or more sequences were identified (Multiple Sequences). Many would view any protein identification outside of priority 1 as questionable [39]. All data processing was carried out on a Linux cluster using highly parallel processing and data qualification and filtering software.

Table 2 shows a summary of the results from this study. A total of 5,282 distinct amino acid sequences corresponding to 2,117 different proteins were identified in these samples. Among them, 855 proteins were in the Priority 1 group and 760 of them showed significant changes. The significance threshold is set to control the False Discovery Rate (FDR) at less than 5%. A False Discovery is a protein declared to have a significant change when it does not. The replicate median % Coefficient of Variation (%CV) (technical variation) for the Priority 1 proteins was 11.71% and the combined replicate plus sample median %CV was 12.87% (technical plus biological variations). The %CV is the Standard Deviation divided by the Mean on a % scale. There were also 809 proteins that had significant changes among the 1,262 proteins that were less confidently identified (Priorities 2-4).

Protein quantification

For protein quantification, every peptide quantified has an intensity measurement for every sample. The intensity measurement is a relative quantity, given by the area-under-the-curve (AUC) from the extracted ion chromatogram (XIC) after background noise removal. The AUC is measured at the same retention time for each sample after the sample chromatograms have been aligned [36]. The intensities are then transformed to the log scale where base 2 has become customary. The log transformation serves two purposes. First, relative changes in protein expression are best described by ratios. However, ratios are difficult to statistically model and the log transformation converts a ratio to a difference which is easier to model. Second, as is frequently the case in biology, the data better approximate the normal distribution on a log scale [40]. This is important because normality is an assumption of the ANOVA models used to analyze this data. The base of the log transform is arbitrary with base 2 the most common with genomic data. Base 2 is popular because a two-fold change (or doubling, or 100% increase) yielding an expression ratio of 2 is transformed to 1 on a log base 2 scale (i.e., a two-fold change is a unit change on the log base 2 scale). After log transformation, the data are then quantile normalized [37]. Quantile normalization is a method of normalization that essentially ensures that every sample has a peptide intensity histogram of the same scale, location and shape. This normalization removes trends introduced by sample handling, sample preparation, total protein differences as well as changes in instrument sensitivity while running multiple samples.

If multiple peptides have the same protein identification then their quantile normalized log base 2 intensities are averaged to obtain log base 2 protein intensities. The average of the normalized log peptide

intensities is a weighted average. A peptide is weighted proportionally to the peptide ID Confidence for its protein category and receives a weight of zero if it is outside that category. For example, a 'HIGH' category protein gives zero weight to peptides with < 90% Confidence. The log base 2 protein intensity is the final quantity that is fit by a separate ANOVA statistical model for each protein. The ANOVA is a statistical model that separates the variation due to groups, samples and replicates and constructs the appropriate statistical model for discovering group differences.

Quality assurance and quality control

In this study, all injections were performed using the same C18 microbore column. To assess the stability of the column and instrument, chicken lysozyme was spiked into every sample at a constant amount before tryptic digestion. After tryptic digestion, 9 chicken lysozyme peptides were quantified (all peptides with ID confidence > 75%). The overall mean for each group is displayed by the line across the plot. This plot presents a visual quality control assessment of the ability of the instrument to measure a constant amount of protein over sequential injections. Since a constant amount of chicken lysozyme was spiked into all the samples, it should show no significant change between groups. If there is a significant group effect (i.e. if q-value < 0.05), then one should interpret significant changes in other proteins with smaller fold changes as possibly due to spurious effects. In this experiment, the largest absolute fold change for chicken lysozyme was 1.16 with a q-value of 0.0667. Even though this is not a significant change based on the q-value threshold of <0.05, any significant fold changes of absolute magnitude less than 1.16 in this study should be interpreted with caution.

Statistical analysis

The number of significant changes between groups, the maximum absolute fold changes and the exhibited variability (Coefficient of Variation) for each Priority level are displayed in Table 2. It gives the number of proteins with significant changes for each Priority level. The threshold for significance is set to control the False Discovery Rate (FDR) for each two group comparison at 5% [41].

Dealing with high-dimensional data has always been a major problem in pattern recognition, group classification, and machine learning. Linear discriminant analysis (LDA) is one of the most popular methods for dimension reduction. It projects high-dimensional data onto a lower dimensional space by maximizing the separation of data points from different classes and minimizing the dispersion of data from the same class simultaneously, thus achieving maximum class discrimination in the dimensionality-reduced space. In this study, LDA was performed to evaluate the beneficial effects of multiplexing. As shown in Figure 2, when any combination of 5 markers from Table 3 was chosen, classification accuracy for sensitive vs. resistant reached optimal level, demonstrating that a combination of surrogate biomarkers could separate chemoresponders from non-responders fairly well. Again, it should be mentioned that this small proof-of-principle profiling experiment has some shortcomings concerning the limited number of cell lines and sample size. These initial results must be validated thoroughly and independently to substantiate the relation between over-expression of these biomarkers and chemodrug resistance.

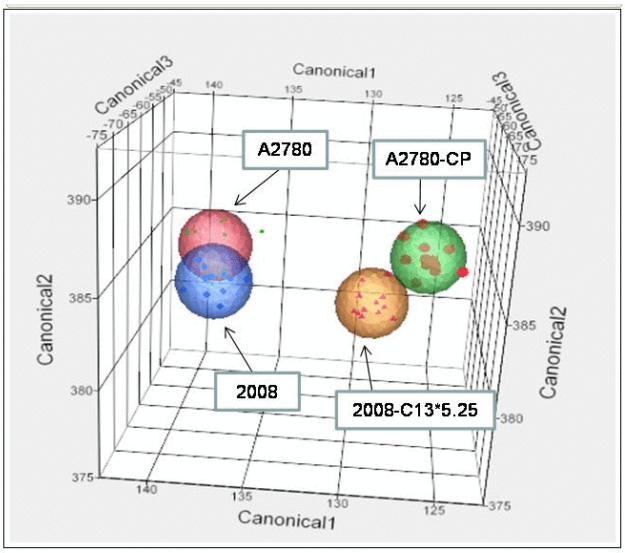

Figure 2: Linear Discriminant Analysis. Red: A2780 (sensitive); Blue: 2008 (sensitive); Green: A2780-CP (resistant); Gold: 2008-C13*5.25 (resistant)

Protein Accession Number	Protein Annotation	% ID Confidence	Fold-Change
IPI00455527.1	Heat-shock 70kD protein binding protein	>99	4.4
IPI00306960.2	Asparaginyl-tRNA synthetase	>99	4
IPI00025512.2	Heat-shock protein beta-1	>99	3
IPI00455315.2	Annexin A2	>99	2.6
IPI00026052.1	Heat-shock factor binding protein 1	>99	2.4
IPI00024057.1	Transgelin-2	>99	2.4
IPI00017448.1	40S ribosomal protein S21	>99	2.4
IPI00218914.1	Aldehyde dehydrogenase 1A1	>99	2.3
IPI00419134.1	Epiplakin	>99	2.3

IPI00106642.4	Dihydropyrimidinase-like 2	>99	2
IPI00105407.1	Aldo-keto reductase family 1 member B10	>99	2
IPI00414320.1	Annexin A11	>99	2

Table 3: Cisplatin Resistance Biomarker Candidates

Putative surrogate biomarkers

In this study to discover platinum-based chemotherapy response predictive biomarkers, we applied a label-free LC/MS-based protein quantification proteomic method to examine the global protein expression of two pairs of epithelial OC cell lines: A2780/A2780-CP (cisplatin-sensitive/cisplatin-resistant) and 2008/2008-C13*5.25 (cisplatin-sensitive/cisplatin-resistant). The rationale for choosing these cell lines was based on the fact that these resistant sub-cell lines were derived by continuous treatment of the parent sensitive cell lines with increasing concentrations of cisplatin in the media [32,33,42,43], eliminating other potential factors, such as cell type and buffer conditions, that can complicate data interpretation when sensitive vs. resistant phenotypes are compared. Thus, a proteomic comparison of these well-defined cell lines under identical conditions may provide an opportunity to discover a specific panel of biomarkers for cisplatin resistance.

Table 3 lists a panel of candidate biomarkers for chemotherapy response prediction. These candidates were selected based on three criteria: 1) they were identified with high confidence - multiple peptides with peptide identification confidence level >99% and greater than two-fold expression changes; 2) LDA results show that these chosen proteins are capable of discriminating sensitive (A2780 & 2008) and resistant (A2780-CP & 2008-C13*5.25) phenotypes at ~100% specificity (Figure 2) they all have relatively high abundance in the human proteome, and more importantly, they all have been previously detected and quantified by mass spectrometry-based proteomic studies in abundant protein depleted human plasma samples (internal confidential data generated from a study supported by the NCI's Clinical Proteomic Technologies for Cancer program). Since plasma and serum are easily accessible biospecimens, future validation in these samples will no doubt provide more valuable data for future clinical applications. Furthermore, most of the candidates we identified in this study such as annexin A11, transgelin-2, asparaginyl-tRNA synthetase, heat-shock protein beta-1, aldehyde dehydrogenase 1A1, epiplakin, have been previously identified by others to be involved in cancer chemodrug resistance at either gene or protein level [44-49].

In this biomarker discovery study, we quantitatively identified over 2,000 proteins, and more than 700 of them showed significant changes in protein expression level across all comparison groups. When only the sensitive and resistant groups were compared, however, only 95 proteins showed significant changes in protein expression levels, suggesting that the other 605 detected proteins might be cell line specific. After data sorting and bioinformatics analysis for these 95 proteins-of-interest, a panel of proteins which could be used as biomarkers capable of distinguishing platinum-based chemotherapy responders from non-responders is determined. However, because only a couple pairs of cell lines are used for this study, we must not over-interpret the data. When other epithelial ovarian cancer cell lines such as TOV-81D, TOV-112D, and OVCAR-3 are used, we expect to see an even smaller number of overlap among differentially expressed proteins. Thus future validation study needs to be carried out before they can be used for clinical validation and clinical applications.

There is compelling evidence that high and medium abundance proteins in a given tissue or plasma sample have value as clinical biomarkers; therefore, there may be applications for specific assays even without antibody enrichment such as mass spectrometry-based multiple reaction-monitoring (MRM) multiplexed assays [50-52]. If the MS-based measurement strategy proves to be robust, it could enable routine and relatively inexpensive measurements of clinically specific biomarkers. A combination of biomarker discovery and candidate-based biomarker validation in large sample sets would provide an effective staged pipeline for generation of valid protein biomarkers of disease, risk, and therapeutic response [53].

Biomarkers are measurable and quantifiable biological parameters that can be used as a basis for health and physiology related assessments, such as disease risk and drug efficacy [54,55]. Alterations in the expression levels of proteins used as biomarkers have been proven to be indicative of causes of illnesses [56]. The limitations of non-proteomic classical approaches prevent large-scale analysis of proteins. Using a newly developed robust high-throughput LC/MS-based protein quantification technology [36], global protein expression profiles of cisplatin-sensitive and cisplatin-resistant OC cells can be obtained. Since this study was performed with established cell lines, small variation would be expected among the samples we compared. Conversely, it is expected that a large variance will be observed when plasma or serum samples from patients are analyzed. Hence, there is a critical need to use clinically relevant samples and a large sample set to obtain statistically valid data for future clinical biomarker assay development. In addition, since individual proteins discovered could be too variable to give confident results from patient plasma or serum samples, the potential protein biomarker candidates must be chosen carefully to ensure that they are truly independent of one another and must be validated by other method(s) before moving forward to the next step of the biomarker development pipeline (clinical applications). It is important to note that many of our putative surrogate biomarker candidates have been previously reported to be involved in the development of chemoresistance [44-49], suggesting that the differential protein expression we observed from the chemosensitive and chemoresistant cells are most likely specific to chemotherapeutic treatment and therefore possible to serve as surrogate biomarkers for prediction of treatment response. Mass spectrometry-based methods allow more efficient ruling-in and ruling-out of candidate biomarkers than comparable reagent-based approaches, and it can save up to 70% on development time [57]. Current study not only provides critical data for biomarker development for OC patient-care, but could also have a potential societal impact. For example, patients who will not benefit from platinum-based chemotherapy will not have to go through this 'standard' patient-care procedure, preventing them suffering from

adverse side effects. In addition, it could also save millions of dollars in Medicare/Medicaid for ineffective treatments.

Conclusion

Cancer remains one of the leading causes of death worldwide. Advances in proteomic technologies have significantly improved biomarker discovery and validation. However, the use of biomarker(s) for personalized medicine is still in its infant stage. Even though there is a crucial need for information about biomarkers for drug resistance in OC, currently no sensitive and specific biomarkers to predict a patient's response to chemotherapy are available. Thus, identification and validation of potential biomarker candidates of cisplatin resistance become clinically valuable for diagnostics, prognostics, patient stratification, and treatment evaluation/guidance. Mass spectrometry-based technologies have advantages in accuracy, specificity, throughput, assay development time (normally 3-6 months), and cost. The innovative approach of ruling-in and ruling-out candidate biomarkers using this method is more efficient than reagent-based methods. Utilizing and monitoring a panel of biomarkers (instead of single markers) can also make diagnostics more accurate. In addition, the work described in this paper can be used as a model system for the development of other disease biomarkers and for understanding biological pathways.

Despite the great advances in the application of mass spectrometry in biomarker discovery and validation, several challenges remain. Low abundant proteins, small expression changes, and post-translational modifications are still difficult to be detected. In addition, accurate selection of samples for study, standardization of sample collection and storage conditions, utilization of other methodologies to reduce sample complexity, and refined bioinformatic and statistical analysis to process data are necessary elements to improve the biomarker development process. Our future plan for this particular research is to develop an MRM based assay to validate these biomarkers in plasma samples using both a "training set" and a "test set". Our ultimate goal is to develop a clinically useful assay (either MRM-based or ELISA) for the assessment of cisplatin resistance in OC patients. In addition, longitudinal studies involving plasma samples from OC patients before and after chemotherapeutic treatment to differentiate acquired drug resistance biomarkers from intrinsic resistance biomarkers may also be very helpful in OC clinical biomarker development.

Acknowledgements

Mass spectrometric analysis was carried out at the Indiana University School of Medicine Proteomics Core with support in part from the Indiana Genomics Initiative (INGEN) and the Indiana 21st Century Research and Technology Fund.

References

1. Landis SH, Murray T, Bolden S, Wingo PA (1999) Cancer statistics, 1999. CA: A Cancer Journal for Clinicians 49: 8-31.
2. Levi F, Lucchini F, Negri E, Boyle P, La Vecchia C (2004) Cancer mortality in Europe, 1995-1999, and an overview of trends since 1960. Int J Cancer 110: 155-169.
3. Yamamoto K, Okamoto A, Isonishi S, Ochiai K, Ohtake Y (2001) Heat shock protein 27 was up-regulated in cisplatin resistant human ovarian tumor cell line and associated with the cisplatin resistance. Cancer Lett 168: 173-181.
4. Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC (1998) The biology of ovarian cancer. Semin Oncol 25: 281-304.
5. Lawrenson K, Gayther SA (2009) Ovarian cancer: a clinical challenge that needs some basic answers. PLoS Med 6: e25.
6. Zamble DB, Lippard SJ (1995) Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem Sci 20: 435-439.
7. Johnson SW, Laub PB, Beesley JS, Ozols RF, Hamilton TC (1997) Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. Cancer Research 57: 850-856.
8. Zhou C, Smith JL, Liu J (2003) Role of BRCA1 in cellular resistance to paclitaxel and ionizing radiation in an ovarian cancer cell line carrying a defective BRCA1. Oncogene 22: 2396-2404.
9. Kim CH, Park SJ, Lee SH (2002) A targeted inhibition of DNA-dependent protein kinase sensitizes breast cancer cells following ionizing radiation. J Pharmacol Exp Ther 303: 753-759.
10. Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, et al. (2002) Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell 109: 459-472.
11. Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al. (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. Science 297: 606-609.
12. Deeley RG, Cole SP (1997) Function, evolution and structure of multidrug resistance protein (MRP). Semin Cancer Biol 8: 193-204.
13. Oguri T, Fujiwara Y, Isobe T, Katoh O, Watanabe H, et al. (1998) Expression of gamma-glutamylcysteine synthetase (gamma-GCS) and multidrug resistance-associated protein (MRP), but not human canalicular multispecific organic anion transporter (cMOAT), genes correlates with exposure of human lung cancers to platinum drugs. Br J Cancer 77: 1089-1096.
14. Mi RR, Ni H (2003) MDM2 sensitizes a human ovarian cancer cell line. Gynecol Oncol 90: 238-244.
15. Katano K, Kondo A, Safaei R, Holzer A, Samimi G, et al. (2002) Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. Cancer Res 62: 6559-6565.
16. Samimi G, Katano K, Holzer AK, Safaei R, Howell SB (2004) Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B. Mol Pharmacol 66: 25-32.
17. Holzer AK, Samimi G, Katano K, Naerdemann W, Lin X, et al. (2004) The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. Mol Pharmacol 66: 817-823.
18. Pietrowska M, Marczak L, Polanska J, Behrendt K, Nowicka E, et al. (2009) Mass spectrometry-based serum proteome pattern analysis in molecular diagnostics of early stage breast cancer. J Transl Med 7: 60.
19. Taylor AD, Hancock WS, Hincapie M, Taniguchi N, Hanash SM (2009) Towards an integrated proteomic and glycomic approach to finding cancer biomarkers. Genome Med 1: 57.
20. Wang P, Whiteaker JR, Paulovich AG (2009) The evolving role of mass spectrometry in cancer biomarker discovery. Cancer Biol Ther 8: 1083-1094.
21. Schiffer E, Vlahou A, Petrolekas A, Stravodimos K, Tauber R, et al. (2009) Prediction of muscle-invasive bladder cancer using urinary proteomics. Clin Cancer Res 15: 4935-4943.
22. Buhimschi CS, Bhandari V, Han YW, Dulay AT, Baumbusch MA, et al. (2009) Using proteomics in perinatal and neonatal sepsis: hopes and challenges for the future. Curr Opin Infect Dis 22: 235-243.
23. Morrison CJ, Butler GS, Rodríguez D, Overall CM (2009) Matrix metalloproteinase proteomics: substrates, targets, and therapy. Curr Opin Cell Biol 21: 645-653.
24. Foster CS, Cooper CS (2009) Urgent need to develop independent biomarkers for functional, diagnostic and prognostic application in oncology research. Biomark Med 3: 329-333.
25. Schiess R, Wollscheid B, Aebersold R (2009) Targeted proteomic strategy for clinical biomarker discovery. Mol Oncol 3: 33-44.
26. Eriksson J, Fenyö D (2007) Improving the success rate of proteome analysis by modeling protein-abundance distributions and experimental designs. Nat Biotechnol 25: 651-655.

27. Tyers M, Mann M (2003) From genomics to proteomics. Nature 422: 193-197.
28. Zaviacic M (1997) Prostate-specific antigen and history of its discovery. Bratisl Lek Listy 98: 659-662.
29. Polascik TJ, Oesterling JE, Partin AW (1999) Prostate specific antigen: a decade of discovery--what we have learned and where we are going. J Urol 162: 293-306.
30. Kharaziha P, Ceder S, Li Q, Panaretakis T (2012) Tumor cell-derived exosomes: a message in a bottle. Biochim Biophys Acta 1826: 103-111.
31. Shah S, Chen B (2010) Testing for HER2 in Breast Cancer: A Continuing Evolution. Patholog Res Int 2011: 903202.
32. Hamilton TC, Winker MA, Louie KG (1985) Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol 34: 2583-2586.
33. DiSaia PJ, Sinkovics JG, Rutledge FN, Smith JP (1972) Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. Am J Obstet Gynecol 114: 979-989.
34. Hale JE, Butler JP, Gelfanova V, You JS, Knierman MD (2004) A simplified procedure for the reduction and alkylation of cysteine residues in proteins prior to proteolytic digestion and mass spectral analysis. Anal Biochem 333: 174-181.
35. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
36. Higgs RE, Knierman MD, Gelfanova V, Butler JP, Hale JE (2005) Comprehensive label-free method for the relative quantification of proteins from biological samples. J Proteome Res 4: 1442-1450.
37. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193.
38. Higgs RE, Knierman MD, Freeman AB, Gelbert LM, Patil ST, et al. (2007) Estimating the statistical significance of peptide identifications from shotgun proteomics experiments. J Proteome Res 6: 1758-1767.
39. Carr S, Aebersold R, Baldwin M, Burlingame A, Clauser K, et al. (2004) The need for guidelines in publication of peptide and protein identification data: Working Group on Publication Guidelines for Peptide and Protein Identification Data. Mol Cell Proteomics 3: 531-533.
40. Limpert E, Stahel WA, Abbt M (2001) Log-normal Distributions across the Sciences: Keys and Clues. BioScience 51: 341-352.
41. Reiner A, Yekutieli D, Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics 19: 368-375.
42. Holzer AK, Katano K, Klomp LW, Howell SB (2004) Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. Clin Cancer Res 10: 6744-6749.
43. Yang X, Xing H, Gao Q, Chen G, Lu Y, et al. (2005) Regulation of HtrA2/Omi by X-linked inhibitor of apoptosis protein in chemoresistance in human ovarian cancer cells. Gynecol Oncol 97: 413-421.
44. Song J, Shih IeM, Chan DW, Zhang Z (2009) Suppression of annexin A11 in ovarian cancer: implications in chemoresistance. Neoplasia 11: 605-614.
45. Cai J, Chen S, Zhang W, Hu S, Lu J, et al. (2014) Paeonol reverses paclitaxel resistance in human breast cancer cells by regulating the expression of transgelin 2. Phytomedicine 21: 984-991.
46. Kim M, Park YJ, Kim OJ, Lee GY, Chung EJ, et al. (2003) Gene expression profiles related with overcoming cisplatin resistance in human cancer cell lines. Cancer Therapy 1: 21-29.
47. Smith L, Welham KJ, Watson MB, Drew PJ, Lind MJ, et al. (2007) The proteomic analysis of cisplatin resistance in breast cancer cells. Oncol Res 16: 497-506.
48. Duong HQ, Hwang JS, Kim HJ, Kang HJ, Seong YS, et al. (2012) Aldehyde dehydrogenase 1A1 confers intrinsic and acquired resistance to gemcitabine in human pancreatic adenocarcinoma MIA PaCa-2 cells. Int J Oncol 41: 855-861.
49. Dressman HK, Berchuck A, Chan G, Zhai J, Bild A, et al. (2007) An integrated genomic-based approach to individualized treatment of patients with advanced-stage ovarian cancer. J Clin Oncol 25: 517-525.
50. Seegmiller JC, Barnidge DR, Burns BE, Larson TS, Lieske JC, et al. (2009) Quantification of urinary albumin by using protein cleavage and LC-MS/MS. Clin Chem 55: 1100-1107.
51. Kuhn E, Addona T, Keshishian H, Burgess M, Mani DR, et al. (2009) Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. Clin Chem 55: 1108-1117.
52. Fortin T, Salvador A, Charrier JP, Lenz C, Lacoux X, et al. (2009) Clinical Quantitation of Prostate-specific Antigen Biomarker in the Low Nanogram/Milliliter Range by Conventional Bore Liquid Chromatography-Tandem Mass Spectrometry (Multiple Reaction Monitoring) Coupling and Correlation with ELISA Tests. Mol Cell Proteomics 8: 1006-1015.
53. Anderson L, Hunter CL (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 5: 573-588.
54. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, et al. (2009) The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. Oncologist 14: 320-368.
55. Halperin I, Morelli M, Korczyn AD, Youdim MB, Mandel SA (2009) Biomarkers for evaluation of clinical efficacy of multipotential neuroprotective drugs for Alzheimer's and Parkinson's diseases. Neurotherapeutics 6: 128-140.
56. Ullah MF, Aatif M (2009) The footprints of cancer development: Cancer biomarkers. Cancer Treat Rev 35: 193-200.
57. Hoofnagle AN, Wener MH (2009) The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. J Immunol Methods 347: 3-11.