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Stage-specific analysis of plasma protein profiles in ovarian cancer: Difference in-gel electrophoresis analysis of pooled clinical samples

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Abstract

Introduction: Ovarian cancer is the leading cause of death from gynecological cancer. Non-specific symptoms early in disease and the lack of specific biomarkers hinder early diagnosis. Multi-marker blood screening tests have shown promise for improving identification of early stage disease; however, available tests lack sensitivity, and specificity. **Materials and Methods:** In this study, pooled deeply-depleted plasma from women with Stage 1, 2 or 3 ovarian cancer and healthy controls were used to compare the 2-dimensional gel electrophoresis (2-DE) protein profiles and identify potential novel markers of ovarian cancer progression. **Results/Discussion:** Stage-specific variation in biomarker expression was observed. For example, apolipoprotein A1 expression is relatively low in control and Stage 1, but shows a substantial increase in Stage 2 and 3, thus, potential of utility for disease confirmation rather than early detection. A better marker for early stage disease was tropomyosin 4 (TPM4). The expression of TPM4 increased by 2-fold in Stage 2 before returning to "normal" levels in Stage 3 disease. Multiple isoforms were also identified for some proteins and in some cases, displayed stage-specific expression. An interesting example was fibrinogen alpha, for which 8 isoforms were identified. Four displayed a moderate increase at Stage 1 and a substantial increase for Stages 2 and 3 while the other 4 showed only moderate increases. **Conclusion:** Herein is provided an improved summary of blood protein profiles for women with ovarian cancer stratified by stage.

Keywords: Difference in-gel electrophoresis, disease stage, protein depletion, ovarian cancer

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological cancer and the fifth leading cause of death from cancer in women[1] with risk increasing with age and decreasing with parity.[2,3] Initial symptoms are non-specific, thus, diagnosis at an early stage is challenging. Only 15% of cases are first diagnosed as localized primary cancer (i.e., Stage 1). While the 10-year survival rate after diagnosis at Stage 1 is 94%, at Stage 3 this declined to 28%.[1] Given the inverse relationship between prognosis and disease stage at diagnosis, early detection remains a major goal for clinicians to reduce long-term mortality. Due to the low prevalence of this disease (<40/100,000[4]) development of effective diagnostic assays or community-based screening tests remains a major challenge.[5]

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Currently, there is no early screening test available for ovarian cancer. Measurement of serum concentrations of cancer antigen (CA)-125 are used off-label as an aid in diagnosis. CA125 concentrations, however, are elevated in only 50% of patients with Stage 1 disease. Additionally, there are approximately 20% of patients with late stage disease who do not produce significantly elevated concentrations of CA125.[6] While CA125 is an extremely useful tool, accurate diagnosis cannot currently be determined by serological screening alone and requires more intensive/invasive/expensive examinations. Recently, multiple marker ovarian cancer diagnostics that include CA125 have been developed (OVA1™[7] and OvPlex™[8,9]) and display increased diagnostic efficiency when compared to CA125 alone, albeit with a substantially limited scope of use in the case OVA1™. Such assays establish proof-of-concept for the multi-marker assay approach and further support the search for additional markers that may improve diagnostic efficiency and the early detection of ovarian cancer.

Global analysis of plasma proteins (plasma proteomics) in relevant clinical samples is a key approach for the detection of molecules that may be differentially expressed with disease, and as such are of utility as bio-markers for the early detection of disease. The greatest challenge in plasma proteomics and bio-marker discovery is sample complexity. Plasma has an approximately 10 orders of magnitude difference[10] between most abundant (albumin) and least abundant proteins (e.g., some cytokines), an impossible dynamic range for detection and identification. For example, of thousands of proteins in plasma, the 22 most abundant proteins make up ~99% of the total protein mass.[11] Therefore, as abundant proteins are removed, the number of proteins in the sample that can be analyzed greatly increases. Removal of highly abundant proteins effectively enriches the low abundant protein fraction allowing the analysis of protein expression changes in disease/pathology that would otherwise be undetectable with current protein separation (e.g., chromatographic and electrophoretic) and identification (mass spectroscopy) techniques. Differential/clinical proteomics in plasma (and other similar biological fluids such as cervical-spinal fluid, bronchoalveolar lavage fluid, ascites etc.) relies on being able to detect and identify the less abundant proteins that are usually masked by the presence of more abundant species. As such, abundant protein depletion is essentially a prerequisite for the identification of novel disease-specific proteins in blood plasma.

In this study, we have utilised immuno-affinity depletion of abundant plasma proteins (leaving only ~1.5% of total initial protein mass) and fluorescence difference in-gel electrophoresis (DIGE) to analyse plasma protein signatures from normal and stage 1, 2, and 3 ovarian cancer patients. We present evidence for the differential regulation of a number of proteins, some of which decrease with stage, some that increase and some that seem to be relatively stage specific. These findings may prove useful in the hunt for complementary biomarkers for multi-marker diagnostics and also give insights into ovarian cancer biology.

MATERIALS AND METHODS

Cyanine dyes (CyDyes) Cy2, Cy3 and Cy5 DIGE Fluors (minimal dyes), Bind-Silane, Immobiline Dry-Strips, Dry Strip Cover Fluid, 3-[(3-cholamidopropyl) dimethylammonio]--1-propanesulfonate, Tris, 1,4-dithiothreitol, glycine, urea, thiourea, DeStreak solution, immobilized pH gel (IPG) strips 3-11 non-linear (NL) and Plus One™ ReadySol IEF 40% acrylamide, 3% bisacrylamide were from GE Healthcare (Buckinghamshire, UK). Dimethyl formamide (DMF) was from Sigma-Aldrich (Dorset, UK). All other chemicals were of the highest quality available.

Patients and plasma sample collection

This study was approved by the Alfred Hospital Human Research and Ethics Committee (HREC110/09) Melbourne, Australia. Plasma samples for this project were obtained from the Victorian Cancer Biobank under application 09004. Controls were age matched to samples from patients with Stage 1, 2 or 3 ovarian cancers. For each sample pool (control, Stage 1, Stage 2, and Stage 3), 100 µl aliquots from all available patients/controls plasma samples were combined to create four plasma pools [Table 1].

Sample preparation

Samples were pooled as follows: 100 µl from each sample in the Normal group (n = 14), Stage 1 (n = 6), Stage 2 (n = 5) and Stage 3 (n = 9). Pooled plasma was depleted of abundant proteins using the IgY14 LC-5 and SuperMix LC-2 Column Kits (Sigma, St Louis, MO), following the manufacturer's instructions. Briefly, 100 µl pooled plasma was diluted 1/5 in column dilution buffer and clarified with a 0.45 µm spin filter. Using a 2 ml injection loop coupled to an Agilent 1100 High Performance Liquid C hromatography system (HPLC, Agilent, Palo Alto, CA), the sample was introduced to the column and the flow-through fraction was collected. Bound material was eluted to waste with stripping buffer and the column regenerated. Depleted plasma samples were concentrated using Amicon Ultra-15 5 kDa (a lower molecular weight

Table 1: Sample details

Sample	*n*	Age	CA125 (*n*)
Controls	14	56.3±15.8	n/a
Stage 1	6	48.3±5.6	83.0±68 (3)
Stage 2	5	56.0±31.6	1295.0±2370.9 (5)
Stage 3	9	59.0±9.3	784.0±991.0 (6)

than required for 2-DE) molecular weight cut-off centrifugal devices according to manufacturer's instructions (Millipore Corporation). The solvent was exchanged by reconstituting the retentate to the original sample load volume using DIGE Labeling Buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris). This process was repeated twice. Conductivity was determined to be 250 μS/cm. Protein concentration was determined. The pH was adjusted to 8.5-8.7 with 100 mM HCl to optimise the CyDye labeling.

CyDye labelling

Depleted plasma samples were labeled using the fluorescent CyDyes (Cy3, Cy5) developed for DIGE following the manufacturer's instructions. Samples were paired (control v Stage 3 and Stage 1 v Stage 2) for CyDye labeling and 2D gel electrophoresis. A plasma sample pool (containing all four conditions) was also prepared for use as an internal and multi-gel standard.

Forty micrograms of protein were labeled with 200 pmol of amine reactive CyDyes (Control sample with Cy3, Stage 1-Cy5, Stage 2-Cy3, Stage 3-Cy5, internal standard-Cy2), freshly dissolved in anhydrous DMF. The labeling reaction was incubated at room temperature and was terminated by the addition of 10 nmol lysine. The labeled protein samples and the pooled internal standard were combined according to the experimental design. Equal volumes of 2 × lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 1% DTT and 1% IPG buffer) were added and if necessary samples were further diluted with a 1:1 mix of DIGE Labelling Buffer and 2 × lysis Buffer prior to cup loading.

2-DE

Isoelectric focusing was performed using rehydrated Immobiline™ Dry-Strips (13 cm, pH 3-11NL) for a total of 26,378 Vh at 20°C. Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the strips were equilibrated with 1% DTT followed by 2.5% iodoacetamide (both made up in 50 mM Tris pH 8.8, 30% glycerol, 6 M urea, 2% SDS). The strips were loaded onto 12.5% 13 cm (1 mm thick) hand cast polyacrylamide gels with low fluorescent glass treated with Bind-Silane (80% ethanol, 2% acetic acid, 0.01% Bind-Silane). The strips were overlaid with 0.5% agarose in SDS running buffer containing 0.02% bromophenol blue. The gels were run at 15 mA/gel for 60 min, 30 mA/gel for 120 min and then 45 mA/gel for 60 min at room temperature. A running buffer of 25 mM Tris, 192 mM glycine and 0.1% SDS was used.

Spot detection and analysis

CyDye DIGE Fluor labeled protein gels were scanned at 50 μm using a Typhoon Trio 9100 (GE Healthcare) and the scanning/capture specs outlined in Table 2. Gels were automatically aligned and spots detected using the Progenesis SameSpots v3.2.3107.24565 (nonlinear dynamics) workflow. Minimal spot editing followed. Normalization was performed using the software algorithm. Analysis was performed on spots with a greater than the 1.1-fold difference.

Spot-picking, tryptic digestion and matrix-assisted laser desorption ionisation (MALDI) target spotting

Gel spots that were up-or down-regulated by greater than 1.1 fold were selected for protein identification. Three hundred and fourteen gel plugs were excised by an Ettan Spot Handling workstation (GE Healthcare). However, 50 gel plugs were missing after robot malfunction. The gel plugs were first washed in 10 mM ammonium bicarbonate (Riedel-de Haen, Germany), followed by 100% acetonitrile (ACN, Lab-Scan Analytical Sciences, Ireland) and repeated once before plugs were allowed to dry at room temperature. Plugs were rehydrated on ice for 30 min with 20 μl sequencing grade trypsin (Promega, Madison, WI, 20 ng/μl in 20 mM ammonium bicarbonate). After rehydration, swollen plugs were covered with 20 μl of 20 mM ammonium bicarbonate and incubated @ 37°C overnight. Trypsin was de-activated by the addition of 2 μl formic acid (10%). Digest supernatant (3 μl) was applied to a Bruker Biosciences Anchorchip MALDI target, pre-prepared with α-Cyano-4-hydroxycinnamic acid (CHCA, see the thin layer affinity method in the Bruker Anchorchip manual). After 3 min, this solution was removed and the spot washed with 0.1% trifluoroacetic acid.

Mass spectrometry and data analysis: MALDI-mass spectrometry

MALDI-time-of-flight (TOF) tandem MS was performed on a Bruker Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). General instrument settings were as follows: Mode, positive, and reflector; pulsed ion extraction, 120 ns; laser intensity, 22-28%; laser frequency, 25 Hz; 600 laser shots were collected and summed for all MS data. The mass range (800-3,000 m/z) of the mass spectrometer was internally calibrated using the autolytic peaks of trypsin (842.510 and 2,211.1046 m/z). Matrix was suppressed using a high-gating factor. Signal suppression below 800 m/z was activated. Data acquisition was performed using the instrument-specific software, Flex-Control (Bruker Daltonics). Peaklists were generated using Flexanalysis (Bruker).

Table 2: DIGE CyDye parameters

Emission filters	PMT	Laser	Sensitivity
520 BP 40 Cy2	440	Blue (488)	Normal
580 BP 30 Cy3,	435	Green (532)	Normal
670 BP 30 Cy5	415	Red (633)	Normal

DIGE: Difference in-gel electrophoresis; PMT: Photomultiplier tube

Biotools software (Bruker) and the Mascot search engine were used to interrogate the SwissProt database (Release: 2010_04, 516081 sequences; 181677051 residues) and proteins were identified by peptide mass fingerprinting (PMF). Initial search parameters for PMF were: Taxonomy: Human; MS Tolerance: 100 ppm; Missing Cleavages: ≤1; Enzyme: Trypsin; Fixed Modifications: Carbamidomethylation; Variable Modifications: Oxidation (M). Identifications with Mascot expect probability values of <0.05 were then manually verified by examination of spectra and/or resubmission of peak lists to Mascot. We took a conservative approach to protein identification and based acceptance on a number of criteria other than these scores. These included theoretical and experimental Mr being in accordance, experimental peptide mass accuracy variation across the mass range and repeatability of identification across different gels. If multiple members of a protein family were identified those with the highest ranked hit were selected.

Mass spectrometry and data analysis: LC-MS

Digest supernatant was transferred to HPLC vials and placed in the autosampler tray of the HPLC system (Agilent 1100 Series). LC-ESI-MS/MS were performed using the 1100 Series HPLC coupled to an LC/MSD Trap XCT Plus Mass Spectrometer fitted with an HPLC Chip cube (Agilent, Palo Alto, CA). The HPLC Chip is comprised of a 40 nL enrichment column and a 75 μm × 43 mm separation column both packed with reversed phase resin (Zorbax 300SB-C18, 5 μm). Samples were loaded (8 μL) onto the enrichment column in ACN: Formic acid (4%:0.1%, v/v, 4 μL/min). A linear gradient (19 min, flow rate 0.5 μL/min, ACN: Formic acid, 4-50%:0.1%, v/v) was applied to the column to sequentially elute the bound peptides. A final gradient step was applied (19-20 min, ACN: Formic acid, 50-80%:0.1%, v/v) to strip the column of remaining proteins. All MS/MS spectra were collected using data dependent acquisition. Briefly, after the acquisition of a full MS scan (m/z 300-1800 at 8,100 m/z/sec) in the first scan event, the three most intense ions (precursor ions) present above a threshold intensity of 10,000 were subsequently selected for fragmentation (MS/MS scan m/z 100-2,000 at 26,000 m/z/sec). The collision energy for the MS/MS scan events was ramped from 30 to 200% of 1.3 V, for acquisition of the MS/MS scan, 3 spectra were averaged for each event. General instrument parameters were as follows; Capillary voltage: 2000 V, Skimmer: 40 V, Capillary Exit: 105.3V, Trap Drive: 77.8, Dry Gas: 5.0 L/min, Dry Temp: 350°C.

RESULTS AND DISCUSSION

In this study, we analyzed the protein profiles of pooled plasma from healthy women and those with ovarian cancer, stratified by disease stage [Table 1], in order to identify changes in proteins associated with this disease progression that could potentially be used to enhance early and accurate disease detection strategies. Currently, multivariate tests[7-9] are proving to be a promising improvement to the traditional univariate CA125 serological test and as such provide a proof of concept to continue seeking novel serological markers. Furthermore, by stratifying patients by disease stage we are able to better evaluate changes during disease progression. We took a proteomic approach, utilizing the IgY14/Supermix two column system (Sigma, St Louis MO) for depletion abundant proteins followed by DIGE[12] to determine differential regulation of protein spots and mass spectrometry methods to identify the proteins.

Depletion efficiency

Our depletion protocol removed an average of 98.6% (±0.50%, ~60 μg from 100 μl plasma/run, results not shown) of the total protein mass from the plasma. Due to the complexity of the plasma proteome and dynamic range of plasma protein concentrations,[10] depletion strategies are requisite for any in-depth profiling of protein species present in this tissue. There are numerous depletions methods available from dye affinity to remove serum albumin[13] to the more sophisticated antibody-based depletion columns preferred currently.[14] The aim of any depletion strategy is to remove high abundant proteins that may mask the analysis of lower abundant proteins where changes in quantity may be more indicative of attendant pathology and useful as a marker of disease. Compared to other depletion strategies, such as MARS 6 (Agilent) and the IgY14 column alone that remove ~80% and ~95% of plasma protein mass respectively,[14,15] the dual IgY14/Supermix column system depletes more effectively, only ~1% of the initial protein mass (also see[14]). This potentially allows lower abundant proteins to be detected with 20 times the sensitivity of a MARS 6 depletion method.

2D DIGE and mass spectrometric identification

Using three different fluorescent labels, Cy2, Cy3, and Cy5, DIGE allows the multiplexed, simultaneous analysis of up to three different samples in a single gel. The amine reactive Cy-dyes were specifically designed to ensure that proteins common to both samples have the same relative mobility irrespective of the label used.[16] After labeling, samples are pooled together prior to electrophoretic separation. Proteins labeled with the different dyes migrate together in both dimensions. In multi-gel experiments, the third sample, labeled with a third dye, usually consists of an internal standard prepared from a pool of all samples. After electrophoresis,

gels are scanned with the three different excitation/emission wavelengths [Table 2], allowing the separate analysis of each sample. Running multiple samples on the same gel allows the direct comparison of protein abundance in these samples, without the complication of inter-gel variation regularly seen in 2D electrophoresis. Furthermore, it also allows for cross-gel normalization where each gel is normalized to its own internal control before being compared to the other samples. Figure 1 shows an overlay image for a representative DIGE gel. After curating, SameSpots software detected and matched 557 spots across the four conditions (control and Stages 1, 2, and 3 ovarian cancers). Of these, 314 were up-or down-regulated by greater than 1.1 fold in at least one cancer stage compared to control. We successfully excised 264 of these spots for analysis. With a combination of MALDI-TOF PMF and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), proteins were identified with high confidence in 93 of these spots (35%, Table 3) varying in apparent mass from 10 kDa (the lower limit of gel resolution) to 150 kDa. This relatively low success rate for MS identification is due mainly to the sensitivity for detection of the DIGE Cy dyes being greater than the sensitivity of MS-based protein identification. Due to sample quantities we were limited to a 120 µg load of protein per gel in these studies. In our experience, the 13 cm IPG strips/gels used in this study have a loading capacity of ~300 µg. The detection sensitivity could, therefore, be potentially doubled by doubling protein load. Spot resolution, however, may be compromised if loading concentrations are increased too high (data not shown). Alternatively, if more samples could be obtained it is possible that the whole experiment could be up-scaled to 24 cm strips/gels.

Plasma protein depletion and identification

After analysis of the MS data, it was noted that a number of proteins (e.g., fibrinogens) anticipated to be removed by the depletion strategy were identified. This is common to most plasma protein depletion techniques.[14] The proteins targeted for depletion are very highly abundant compared to lower abundant species and anything less than 100% removal will leave traces in the depleted fraction. Indeed, fibrinogen is known to be not efficiently retained on the initial IgY14 column.[14] Also, depletion will depend on IgY antibody specificities. Post-translational modification (PTM) or protein truncation may change the avidity of binding or remove the binding epitope from a protein. In addition to incomplete removal of high abundant proteins, depletion kits likely remove some lower abundant proteins. All sample preparation methods will introduce their own unique sampling errors. In this study, we make the assumption that depletion of plasma proteins (of both high and low abundance) is consistent across sample pools. Only proteins that are present in the deconvoluted sample can be analyzed. These issues highlight the limitations of any plasma protein depletion strategy rather than invalidate the results of studies that utilize them.

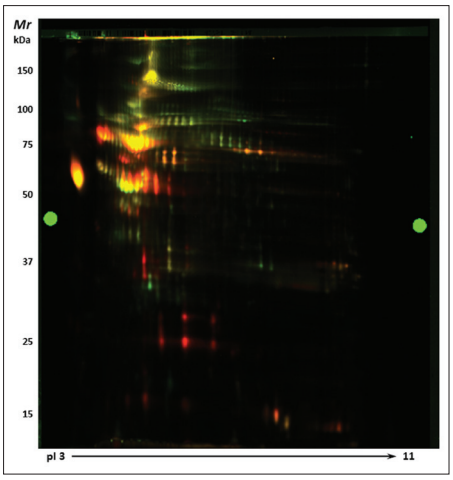

Figure 1: Typical difference in-gel electrophoresis gel image

Protein abundance changes

Normalized spot volumes (protein abundance) for each of the identified proteins are included in Table 3. Using the protein abundance values we found that 58 spots, with a single protein identification, were differentially up-or down-regulated by >1.5 fold in at least one cancer stage when compared to control (healthy patient pool) plasma. Figure 2 is a 2D gel reference map identifying the position of the differentially regulated protein spots while Figure 3 shows examples of the relative abundance (spot volume) for selected spots in each of the different plasma pools. To display these data in an easy to interpret format, relative protein abundance has been plotted as fold change compared to control (cancer stage spot volume/control spot volume) on a Log2 axis with the origin at one (no change, equal to control, Figure 4). In this way, we can easily identify trends across cancer stage, where bars to the right indicate an increase in expression and bars to the left a decrease when compared to the healthy patient pool (control) plasma.

Isoforms

From the 58 differentially regulated protein spots identified, 23 separate proteins are represented. In most cases, different protein isoforms were either similarly up-or down-regulated. For alpha-2-macroglobulin (A2MG) and hepatocyte growth factor-like protein (HGFL), however, both were identified at 12 different locations on the gel, some protein spots were up while others were down. Of the 12 spots containing HGFL, 9 were differentially regulated according to our criteria, and for 8, there was a consistent down regulation in ovarian cancer compared to control. For the ninth

Table 3: Identified protein spots and stage-specific, relative expression

Spot#	Average normalized volumes				Accession	Best score*	Coverage (%)	Peptides matched
	Control	Stage 1	Stage 2	Stage 3				
358	0.92	1.13	0.99	1.05	CERU_human	105	10	10
436	1.04	1.19	0.82	0.93	213 for mixture†			
					A2MG_human	132	17	21
					CERU_human	76	13	13
482	1.22	1.23	0.79	0.88	A2MG_human	110	13	17
567	0.97	1.07	1.04	1.32	A1AG1_human	58	28	5
580	1.08	1.18	1.00	0.64	HGFL_human	103	14	10
593	1.22	1.26	0.72	0.72	C1R_human	113	21	13
631	1.48	1.03	0.78	0.50	A2MG_human	73	15	17
634	1.50	0.79	0.94	0.52	FA12_human	58	9	7
636	1.25	1.14	0.86	0.72	HEP2_human	61	14	8
637	1.34	1.00	0.84	0.66	FA12_human	58	9	7
649	1.18	1.23	0.94	0.81	210 for mixture†			
					ALS_human	126	25	13
					CO9_human	68	18	11
658	0.88	1.00	0.86	0.82	HGFL_human	119	17	13
662	0.96	1.03	1.02	0.84	HGFL_human	85	13	8
674	1.02	1.11	0.86	1.08	IGHM_human	79	23	9
677	1.48	1.06	0.76	0.78	112 for mixture†			
					TRFE_human	47	13	9
					FA12_human	45	8	6
714	1.38	1.00	0.87	0.66	239 for mixture†			
					TRFE_human	176	30	22
					IGHM_human	49	17	8
716	1.06	0.94	0.83	1.08	213 for mixture†			
					ALS_human	83	25	13
					HEP2_human	60	23	13
					K2C7_human	58	23	11
717	1.05	1.02	0.86	1.14	176 for mixture†			
					HEP2_human	103	25	16
					ALS_human	58	11	11
718	1.55	0.91	0.69	0.66	TRFE_human	255	36	27
719	1.32	1.09	0.90	0.72	MUCB_human	62	14	6
738	1.05	1.14	0.86	1.02	HEP2_human	83	18	11
741	0.93	1.11	0.86	1.11	HEP2_human	57	14	8
748	1.35	1.13	0.84	0.73	HGFL_human	59	10	8
749	1.30	1.21	0.81	1.00	HEP2_human	125	30	17
751	1.43	1.09	0.75	0.79	HGFL_human	119	15	11
756	1.43	1.12	0.62	0.81	HGFL_human	61	14	10
764	1.57	1.07	0.65	0.87	FA11_human	84	15	9
798	0.32	0.69	1.77	1.59	FIBA_human	63	9	7
801	0.35	0.72	1.74	1.56	FIBA_human	118	16	11
806	0.36	0.69	1.57	1.71	FIBA_human	60	11	7
810	0.46	0.66	1.47	1.46	FIBA_human	77	14	10
825	0.57	0.86	1.02	1.88	AACT_human	121	33	15
836	0.72	0.92	1.03	1.64	AACT_human	114	30	12
842	1.13	1.18	0.87	0.89	A2MG_human	34	5	7
857	1.13	1.07	0.95	0.91	A2MG_human	60	7	9
858	0.88	1.13	0.93	1.18	FIBB_human	54	13	7
862	0.89	1.01	0.86	1.21	FIBB_human	94	23	11
869	1.25	1.12	0.72	0.83	A2MG_human	95	10	13
878	1.23	1.25	0.99	0.93	A2MG_human	67	11	16
882	0.57	0.69	1.59	1.51	A1AT_human	135	28	13
895	0.74	1.04	0.97	1.25	FIBB_human	120	29	14
899	0.89	1.12	1.02	1.22	FIBB_human	130	31	14
900	0.75	1.11	1.08	1.25	FIBB_human	174	38	19

Contd...

Table 3: Contd....

Spot#	Average normalized volumes				Accession	Best score*	Coverage (%)	Peptides matched
	Control	Stage 1	Stage 2	Stage 3				
907	0.67	1.02	1.25	1.26	146 for mixture†			
					FIBB_human	89	25	12
					FIBA_human	72	15	10
908	0.65	1.08	1.02	1.25	FIBG_human	57	18	6
909	0.60	1.01	1.24	1.27	175 for mixture†			
					FIBB_human	109	28	13
					FIBA_human	67	13	10
910	0.69	0.86	0.89	1.35	186 for mixture			
					FIBG_human	123	30	13
					CFAI_human	62	16	9
945	0.70	1.01	1.02	1.27	FIBG_human	119	30	11
954	0.45	0.48	1.49	2.08	A1AT_human	106	22	9
974	0.77	1.06	0.97	1.20	FIBG_human	115	27	10
982	0.79	1.06	0.99	1.28	FIBG_human	138	34	12
1005	1.12	1.06	0.85	0.82	CBPN_human	64	14	6
1067	0.88	0.90	0.97	0.99	ZA2G_human	157	37	14
1071	0.55	0.70	1.09	1.75	279 for mixture†			
					APOA4_human	157	46	19
					HPT_human	109	33	14
1084	1.08	1.09	0.87	0.75	ZA2G_human	147	42	14
1085	0.97	1.00	0.85	0.94	ZA2G_human	157	44	15
1227	2.00	1.07	0.36	0.88	HBA_human¥	99	14	2
1301	0.75	1.03	1.59	0.69	TPM4_human	118	29	11
1357	0.93	1.26	0.90	1.67	FIBA_human	53	8	7
1358	0.98	1.35	0.90	1.73	FIBA_human	58	10	8
1372	1.35	0.70	1.16	0.69	CAH1_human	68	22	5
1376	1.10	1.44	0.94	1.64	FIBA_human	72	11	9
1377	1.25	1.29	0.92	1.23	SAMP_human	55	20	4
1525	0.35	0.49	0.99	2.03	HPT_human¥	179	10	4
1552	0.39	0.47	1.02	2.01	HPT_human	56	11	5
1553	0.40	0.39	1.19	2.39	APOA1_human¥	39	10	2
1556	0.42	0.39	1.15	2.48	HPT_human¥	138	5	2
1573	0.47	0.84	2.83	1.38	AL3A1_human¥	39	7	2
1631	0.33	0.51	1.68	1.89	APOA1_human¥	41	8	2
1699	0.51	0.31	1.70	1.40	A2MG_human¥	78	2	2
1676	0.48	0.32	1.80	1.41	Mixture¥			
					A2MG_human¥	516	5	7
					IGHM_human¥	208	10	4
					FIBB_human¥	154	8	4
1701	0.44	0.43	1.52	2.09	A1AT_human	139	29	13
1708	0.48	0.73	1.04	2.07	AACT_human	96	23	9
1715	0.62	1.07	1.20	1.37	FIBA_human	52	10	6
1725	0.53	0.65	1.67	1.69	A1AT_human	125	30	13
1730	0.61	0.67	1.54	1.71	A1AT_human	132	30	14
1743	0.58	0.78	1.30	1.45	A1AG1_human	105	44	9
1746	1.81	1.26	1.09	0.71	PI16_human	59	13	8
1749	0.66	0.72	1.43	1.42	A2MG_human	92	13	15
1755	1.29	1.21	0.78	0.91	A2MG_human	101	10	11
1757	0.80	1.27	0.73	0.75	HGFL_human	110	15	11
1760	1.38	1.09	0.66	0.76	HGFL_human	64	11	8
1761	1.36	1.12	0.64	0.77	HGFL_human	38	5	4
1762	1.00	1.44	0.91	1.75	FIBA_human	55	9	8
1765	1.08	1.22	0.88	0.96	A2MG_human	115	13	17
1767	1.28	1.24	0.73	0.78	HGFL_human	77	11	8
1774	1.17	0.94	0.72	0.89	HGFL_human	118	17	12
1783	1.01	1.07	1.09	0.79	HGFL_human	86	13	9

Contd...

Table 3: Contd....

Spot#	Average normalized volumes				Accession	Best score*	Coverage (%)	Peptides matched
	Control	Stage 1	Stage 2	Stage 3				
1785	1.34	1.31	0.93	0.88	PROP_human	128	25	11
1787	1.15	1.10	0.85	1.06	HEP2_human	66	16	10
1788	1.18	1.13	0.88	1.13	119 for mixture†			
					HEP2_human	60	17	9
					FOLH1_human	59	9	8
1790	1.17	1.12	0.83	0.98	HEP2_human	99	18	10
1792	0.65	0.99	1.21	1.36	CO9_human	201	27	16

*Best score: The highest Mas./cot score for this spot number in all gels, †Mascot score for a mixture of proteins, ¥Identified by ESI-MS

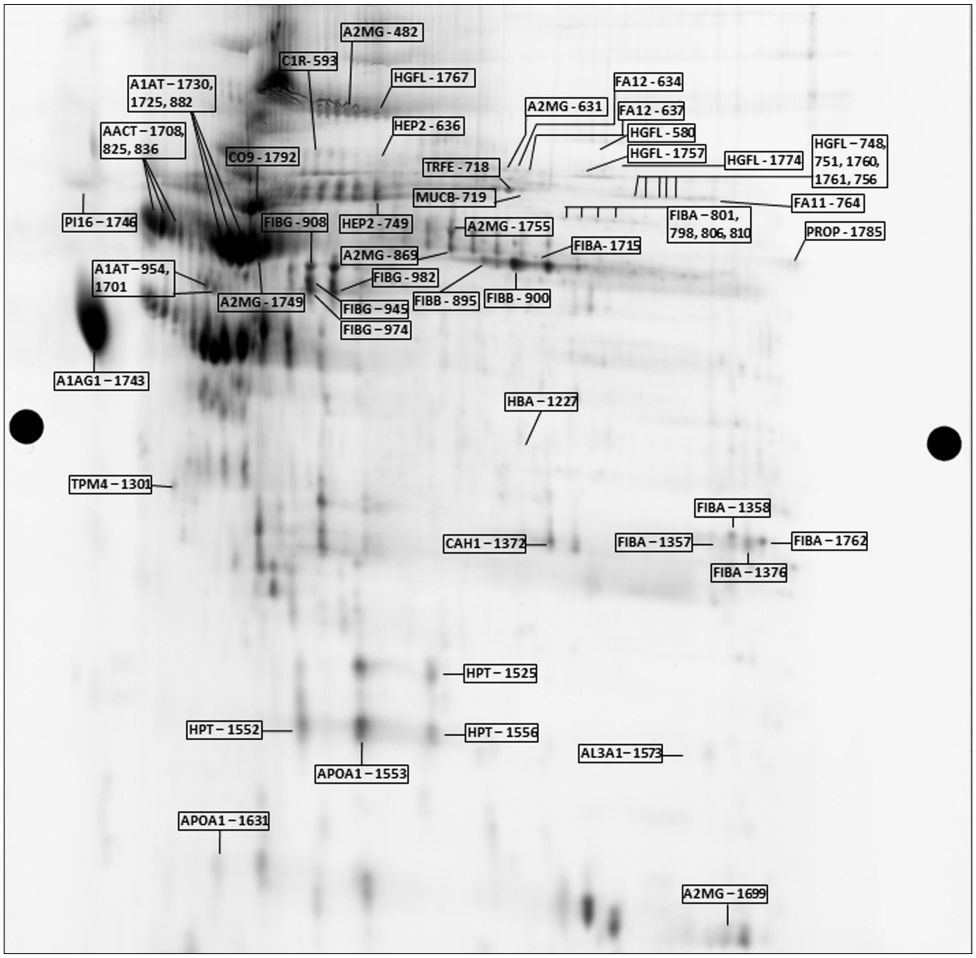

Figure 2: Annotated 2D reference map for proteins that are increased or decreased in any stage by >1.5 fold compared to control

protein spot (#1757), there was an increase observed for Stage 1 ovarian cancer [Figure 4c]. This spot is present at a slightly different MW and pI from the down-regulated isoforms [Figure 2]. Examination of the PMF data did not reveal any obvious sequence differences between these isoforms. Altered glycosylation, is known to be characteristic of cancerous cells (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco and part=A2667) and can radically change both the mass and (due to charged sugars such as sialic acid) charge of proteins, modifying their migration in both electrophoresis dimensions.

A2MG was similarly identified in 12 separate spots on the gel, including two that were identified in a mixture. Of the remaining ten, six were differentially regulated according to our criteria. Unlike HGFL, A2MG expression was more varied between isoforms, with four showing decreased expression in cancer and two increased [Figure 4a]. In this case, there are some discernable differences in the isoform sequences [Table 4] and MW and pI [Figure 2]. Several PTMs can change the physicochemical properties of the protein such that they run differently in 2-D gels. Both protein truncation and glycosylation (see above) have the potential to alter the

size and pI of proteins. A difference in the local or systemic availability of some proteases (in ovarian cancer) may be involved in enzyme truncation and therefore, responsible for some of the isoform differences we see. Interestingly, while fibrinogen alpha (FIBA) was uniformly increased (eleven spots identified, two mixtures and eight increased) there were also some noticeable differences in expression related to sequence of the protein fragments. Spots 798, 801, 806, and 810 are much more increased than the other FIBA spots [Figure 4b]. These spots are large N-terminal fragments while the lesser up-regulated spots (1357, 1358, 1715 and 1762) are lower MW, basic N-terminal fragments. It remains to be established why isoforms are expressed at different levels, however, there are a number of possibilities. These include known problems with resolution of basic proteins,[17,18] relative stability of different fragments in blood (smaller proteins and peptides are inherently more stable), and smaller fragments being homologous to sequences generated by different, homologous parent proteins. The specific changes in apparent abundance in this study may also represent changes in the extent of the glycosylation or proteolytic modification of isoforms with the disease progression. The data obtained in this study do not permit discrimination between these possibilities. Irrespective of the reasons, analysis of individual isoforms can prove beneficial in studying disease as some isoforms have a much stronger correlation to disease state and/or progression than their counterparts and has been previously shown in the literature for haptoglobin (HPT).[19]

Table 4: Up-and down-regulation in the ten A2MG protein spots, showing PMF sequence coverage

Spot	Fragment	~ αα	↑↓
482	N-terminal	135-945	↓
631	N-terminal	135-600	↓
842	C-terminal	1000-1420	NC
857	C-terminal	1000-1420	NC
869	C-terminal	1000-1420	↓
878	C-terminal	1000-1420	NC
1699	C-terminal	1004-1263	↑
1749	N-terminal	188-945	↑
1755	C-terminal	1000-1420	↓
1765	N-terminal	135-945	NC

PMF: Peptide mass fingerprinting; A2MG: Alpha-2-macroglobulin; NC: No change

Gene ontology–cellular component and biological process

Unsurprisingly, when curating the differentially expressed protein list for function [Figure 5] it was observed that the majority of the 23 differentially regulated proteins[18] were found in the secreted/extracellular space compartments [Figure 5a]. Of the remainder, three were cytoplasmic in origin and one each annotated to be from hemoglobin complex and membrane fraction. For biological process annotations, seven were involved in blood coagulation, three were acute phase proteins (APP), and four were involved in complement activation (one of which was a negative activator, see Figure 5b). Two more proteins were involved in immune or defense response and the remainder had several different annotations.

Potential biologies-up-regulated

Of the proteins we have identified as up-regulated in ovarian cancer, several have been linked to acute phase inflammatory pathways. alpha-1-acid glycoprotein-1 or Orosomucoid-1 (A1AG1), FIBA, FIBB, FIBG (fibrinogen alpha, beta and gamma respectively) and HPT are classified

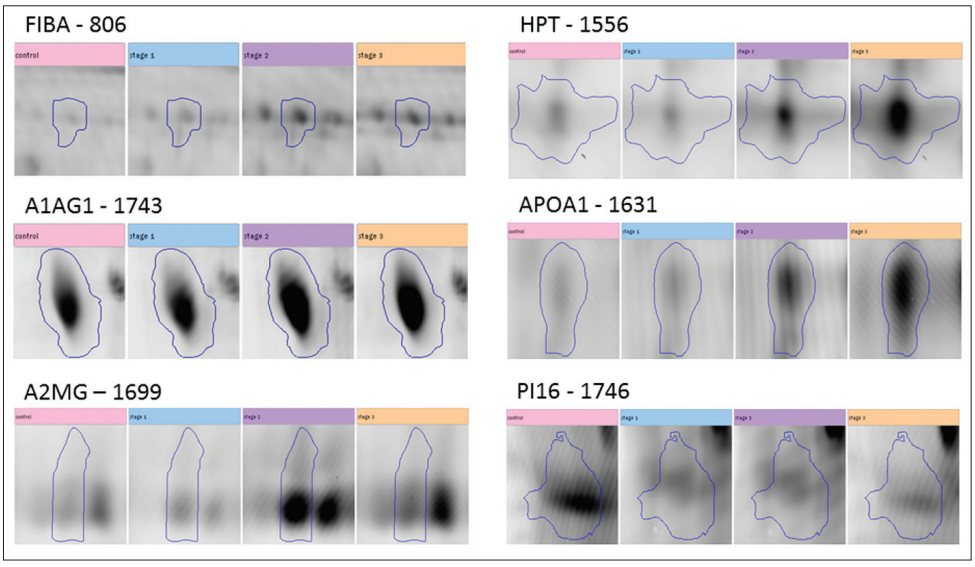

Figure 3: Some examples of protein abundance (spot volume) at different ovarian cancer stage. From left to right for each set; control, Stage 1, Stage 2, Stage 3

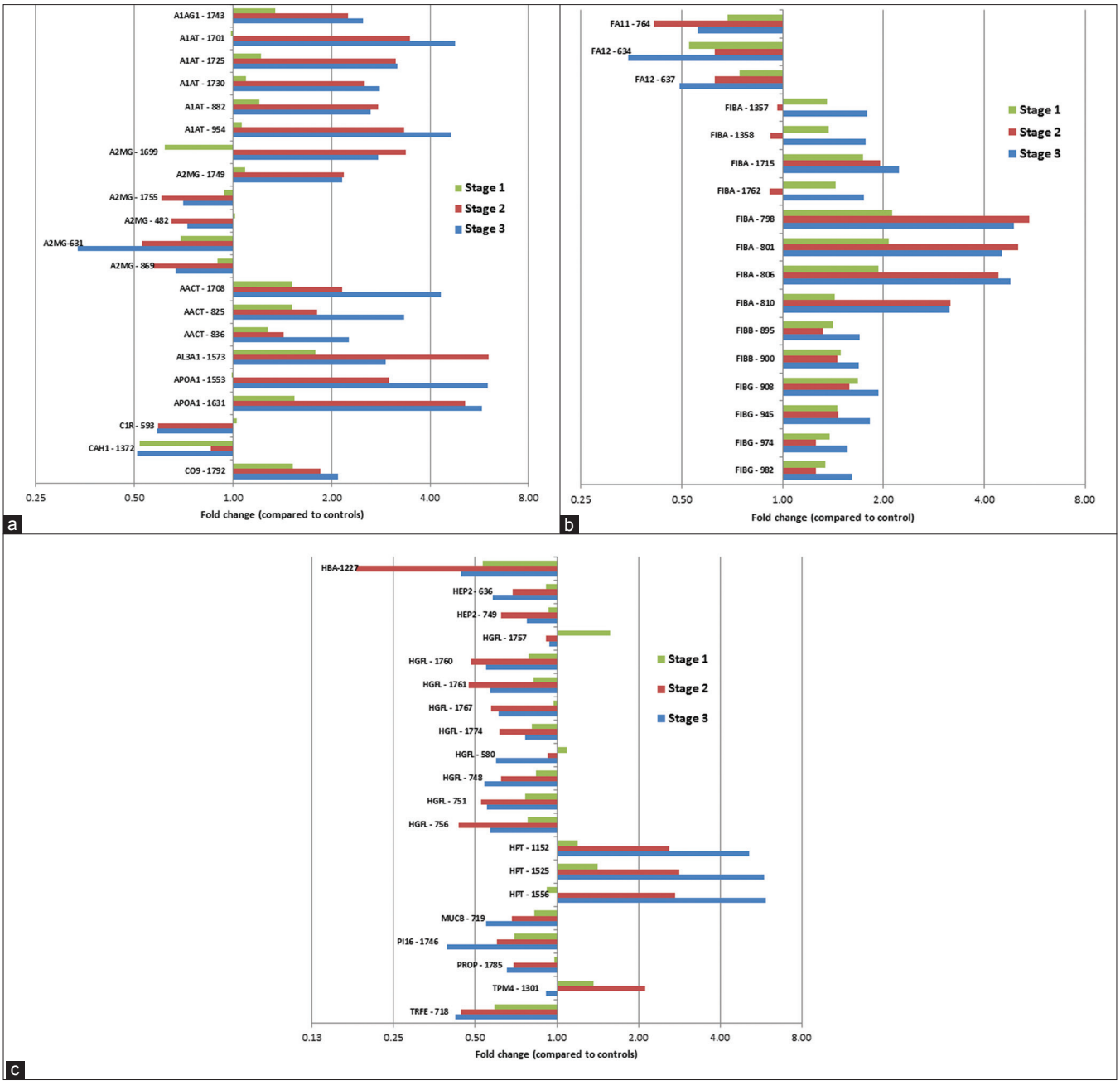

Figure 4: Protein abundance fold change for proteins that are increased or decreased in any stage by >1.5 fold compared to healthy sample pool (control = 1 on the x-axis) and are not part of a mixture as identified by MS. X-axis is log 2 to make fold increases and decreases relative, e.g., 2-fold increase (2) is the same magnitude change on x-axis as 2-fold decrease (0.5). For clarity, this figure is split into three alphabetically: (a) A-C; (b) F; (c) H-T

as APP.[20] Typically they are produced by the liver in response to stressful stimuli, including cancer, although other extra-hepatic cells, including granulocytes and epithelial cells, have been implicated in the expression of APPs.[20] HPT for example, is well-known to be increased in ovarian cancer.[19] Of the APPs identified A1AG1 is of particular interest because it has been previously identified as up-regulated in ovarian cancer serum and peritoneal fluid (ascites) compared to healthy controls.[21-23] In this study, we have been able to further stratify the observed increase in A1AG1 by stage of disease and show that levels increase progressively from Stage 1 (1.34-fold) to Stage 3 (2.5-fold) [Figures 3 and 4]. While A1AG1, as an APP, has been shown to be up-regulated in other conditions, it may still prove a useful adjunct in multiplexed diagnosis and disease monitoring. Another interesting identified up-regulated protein is aldehyde dehydrogenase 3A1 (AL3A1). Expression of AL3A1, in mice, is highest in lung and stomach tissue with the next highest from the 13 tissues tested being the ovaries.[24] AL3A1 is also of particular interest because

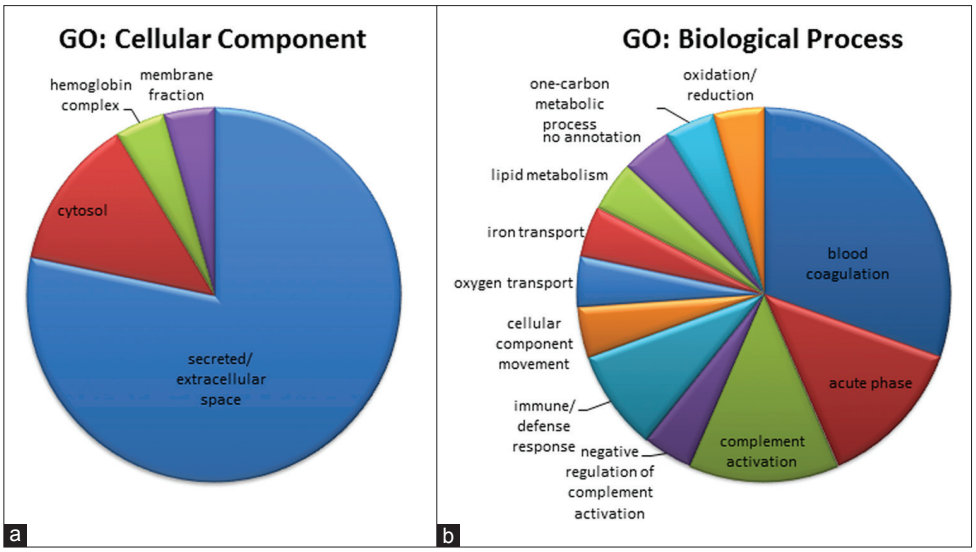

Figure 5: Swiss-Prot gene ontology annotations for (a) Biological process and (b) Cellular component for proteins found to be increased or decreased in any stage by >1.5 fold compared to control. One annotation was used for each protein

increased levels in breast, lung, and colon carcinoma cell lines have been linked with chemoresistance to cyclophosphamide and related agents.[25-27] Importantly, tissues with low-levels of expression, such as the liver have been shown to exhibit high-levels of expression in carcinomas arising from said tissue, with ~50% of liver cancers expressing high-levels of AL3A1.[28] While the specific expression in epithelial ovarian cancer tissue is unknown, our observation of an increase in circulating levels in this study combined with the fact that the ovaries are one of the few tissues with substantial expression, it may be expected that there would be a higher expression of AL3A1 in ovarian cancer tissue compared to normal ovary, similar to that seen with liver malignancies. If validated, circulating AL3A1 levels might be useful in diagnosis and prognosis.

Alpha-1-antitrypsin (A1AT), alpha-1-antichymotrypsin and apolipoprotein A1 are frequently up-regulated in cancer associated plasma,[15,29,30] particularly in the later stages of disease progression.[15,30] Complement component C9 (CO9) is known to be increased in acute leukemia, Hodgkin's disease and sarcoma.[31]

Potential biologies-down-regulated

Several proteins were found to down-regulated in ovarian cancer plasma pools compared to control. Of these, a number have known or purported roles in cancer. FA11 and FA12 (coagulation factors 11 and 12) and Heparin cofactor 2 (HEP2) are generally decreased across the board, trending down with a stage. It has been shown that coagulation factors (including HEP2) are significantly decreased in the prostate cancer patients prior to radical prostatectomy.[32] Serum levels of PI16, which is decreased more than two-fold in Stage 3 in our studies, also show a significant decrease in prostate cancer patients[33] and has been used as a prognostic marker for the recurrence of prostate cancer.[34] Similarly, properdin has long been known to be decreased in many cancer patients.[35] Hemoglobin (HBA) glycosylation has been linked to increased risk of cancer[36,37] and serum transferrin has been shown to be significantly decreased in cancer patients compared to healthy controls.[38] MUCB is the IgM heavy chain. It has been known for a long time that IgM may decrease in some cancers and has been shown to be decreased in ovarian cancer.[39,40] Inhibitors of carbonic anhydrase (CAH1) inhibit tumor cell growth,[41] suggesting that lower expression of CAH1 may support tumor development.

Potential biologies-variously-regulated

Two proteins, both of which were present in many different spots were found to have both up-and down-regulated spots. These proteins, HGFL and A2MG have been discussed above in regard to isoforms. HGFL, which is present as several protein spots (most of which are down-regulated) may be involved the migration of prostate cancer cells.[42] A2MG appeared in several spots, four of which were down-regulated and two that were up-regulated. It has been suggested that A2MG is cytotoxic for tumor cells;[43] a decrease therefore, may have potentiating effects on cancerous tissue. Decreased levels of A2MG have been found in prostate cancer patients with bone metastases (where it is inversely related to prostate-specific antigen, PSA, levels) and its measurement has been suggested for diagnosis and follow-up in these patients.[44,45]

Stage-specific differences

TPM4 showed a stage specific expression profile, with an

early increased observed at Stage 1 and Stage 2, followed by a return to normalcy on progression to Stage 3 [Table 3]. In a small study on cervical cancer, TPM4 showed a steady decline in expression from "normal" squamous cervical epithelium through cervical intraepithelial dysplasia to stage 1A2 squamous cell cervical carcinoma.[46] The study was very small, however, it may indicate that TPM4 is involved in early progression of ovarian cancer where the early increased plasma levels reflect an increase in the number of invasive cells and the decline observed after Stage 2 is a result of the specific down-regulation within said cell population. Alternatively, the increase observed from control to Stage 1 to Stage 2 could indicate the induction of an invasive phenotype early in disease progression that is no longer necessary during the late stage disease. A similar observation has been made of Protein S100-A9 in both ovarian and renal cell carcinomas.[15,30] Another tropomyosin, (TPM3) has been linked to epithelial-mesenchymal transition EMT in human hepatocellular carcinoma[47] and interestingly, like TPM4 expression in squamous cell cervical carcinoma and in our study, also decreases during the later stages in the human squamous cell lung carcinoma.[48]

Other proteins such as FIBB, A1AT, HPT, and others showed a steady increase from Stage 1 to Stage 3. This is fairly common, especially, among protein families that are involved in the inflammatory process, and understanding these changes will be important in developing more effective and accurate early screening blood tests.

CONCLUSIONS

In this study, we used pooled cancer plasma samples, efficient abundant protein depletion, DIGE and MS to identify proteins that are differentially regulated in the cancer. A number of these identified proteins have known or purported roles in ovarian or other cancers. By stratifying our analysis according to ovarian cancer stage, we were able to identify trends in protein change during disease progression, allowing a more robust screen of potential biomarkers. In particular, markers that were up-regulated in the early stages but not in later stages, proteins such as TPM4.

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