Mining the Ovarian Cancer Ascites Proteome for Potential Ovarian Cancer Biomarkers*⑤

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Current ovarian cancer biomarkers are inadequate because of their relatively low diagnostic sensitivity and specificity. There is a need to discover and validate novel ovarian cancer biomarkers that are suitable for early diagnosis, monitoring, and prediction of therapeutic response. We performed an in-depth proteomics analysis of ovarian cancer ascites fluid. Size exclusion chromatography and ultrafiltration were used to remove high abundance proteins with molecular mass ≥30 kDa. After trypsin digestion, the subproteome (≤30 kDa) of ascites fluid was determined by two-dimensional liquid chromatography-tandem mass spectrometry. Filtering criteria were used to select potential ovarian cancer biomarker candidates. By combining data from different size exclusion and ultrafiltration fractionation protocols, we identified 445 proteins from the soluble ascites fraction using a two-dimensional linear ion trap mass spectrometer. Among these were 25 proteins previously identified as ovarian cancer biomarkers. After applying a set of filtering criteria to reduce the number of potential biomarker candidates, we identified 52 proteins for which further clinical validation is warranted. Our proteomics approach for discovering novel ovarian cancer biomarkers appears to be highly efficient because it was able to identify 25 known biomarkers and 52 new candidate biomarkers that warrant further validation. *Molecular & Cellular Proteomics 8:661–669, 2009.*

Accounting for ~3% of all new cancer cases in 2008 (1) with a 1 in 59 (1.7%) lifetime probability of developing the disease, ovarian cancer is the most lethal gynecological malignancy deeming 5–6% of all cancer deaths (1). Hidden deep within the pelvis, ovarian cancer is relatively asymptomatic in early stages, and because of the lack of adequate screening, ovarian cancer has resulted in the majority of cases being presented with late stage disease in association with a low 5-year survival rate of 25–40%. When presented at an early stage, the 5-year survival rate exceeds 90% and most patients are cured by surgery alone (2). Although the most widely used serum marker for ovarian cancer is carbohydrate antigen 125 (CA125),[1] its utility as a screening marker is limited because of its high false positive rates and elevation in other malignancies such as uterine, fallopian, colon, and gastric cancer (3, 4) as well as in non-malignant conditions such as pregnancy and endometriosis (5). These reasons alone demonstrate the need and immediate benefit in using biomarkers with increased sensitivity and specificity for early diagnosis, prognosis, or monitoring of ovarian cancer.

Many advanced stage ovarian cancer patients exhibit rapid growth of intraperitoneal tumors along with abdominal distention as a result of accumulation of ascites fluid in the peritoneal cavity. Mechanistically ascites formation occurs as malignant cells secrete proteins, growth factors, and cytokines that cause neovascularization, angiogenesis, increased fluid filtration, and/or lymphatic obstruction (6–8) resulting in the buildup of serum-like fluid within the abdomen. This local microenvironment of secreted and shed proteins by the ovarian tumor cells is an excellent reservoir for the identification of useful ovarian cancer biomarkers (9).

Body fluids have been shown to be excellent media for biomarker discovery (10). Because ascites fluid contains many cells of tumor origin in addition to other soluble growth factors that have been associated with invasion and metastasis (11, 12), this fluid contains the secretome of ovarian cancer cells while reflecting other microenvironmental factors of the malignancy. Thus, applying the ever advancing technique of proteomics analysis on ascites, it may be possible to discover novel biomarkers that are more sensitive and specific than those currently available.

Mass spectrometry has been widely used to identify the proteome of fluids (13–16), and specifically Gortzak-Uzan *et al.* (17) have recently attempted to identify the proteome of ascites, both cellular and fluid fractions. As biomarkers may be present at low concentrations, (18) and ascites, like serum, contains many high abundance proteins (with a protein concentration range spanning at least 9 orders of magnitude (19)), extensive sample fractionation is necessary if biomarkers are to be found successfully using mass spectrometry.

[1] The abbreviations used are: CA125, carbohydrate antigen 125; KLK6, kallikrein 6; IPI, International Protein Index.

In this study, we present an in-depth subproteome analysis of ascites fluid based on multiple separation and fractionation techniques followed by mass spectrometry analysis. Although Kislinger and co-workers (17) previously identified over 2500 proteins within ascites, only 229 proteins were identified in the fluid fraction. Here we report the most extensive ascites fluid subproteome consisting of 445 unique proteins, many of which overlap with previous data (17) including proteins proposed as candidate serological ovarian cancer biomarkers. After applying multiple data mining criteria to our list of proteins, we assembled a group of 52 proteins that represent good candidates for future investigation as ovarian cancer biomarkers.

<div align="center">MATERIALS AND METHODS</div>

Patients and Specimens—Ascites fluid was obtained, with informed consent and Institutional Review Board approval, from women with advanced stage ovarian cancer undergoing paracentesis. These patients had stage IV serous ovarian carcinoma, and they had been treated previously with surgery plus carboplatin/paclitaxel chemotherapy.

Sample Collection and Preparation—Ascites fluids were aliquoted in 1-ml portions and centrifuged at 16,000 × g for 30 min at 4 °C three times to separate the fluid from lipids and cellular components.

Gel Filtration—Gel filtration was performed using a 0.75 × 60-cm TSK-Gel G3000SW column (Tosoh Bioscience) attached to an Agilent 1100 HPLC system. The column was equilibrated with either (i) phosphate/sulfate buffer (10 mM NaH_2PO_4, 10 mM Na_2SO_4, pH 6.8) or (ii) 100 mM ammonium bicarbonate buffer, pH 7.8. 500 μl of ascites were loaded onto the system at a flow rate of 0.5 ml/min for 1 h. Forty successive injections were performed, collecting eluted fractions at 1-min intervals, starting at 20 min (column void volume). A total of 39 fractions, containing 20 ml each, was collected for each buffer type. Gel filtration experiments were performed in duplicate for each buffer. Each fraction was then analyzed for kallikrein 6 (KLK6) and total protein followed by lyophilization to dryness.

Centrifugal Ultrafiltration—15 ml of ascites were added to a pre-rinsed 50- or 100-kDa nominal molecular mass limit cutoff Amicon Ultra-15 centrifugal filter device (Millipore). After 5 min of centrifugation at 4000 × g in a swinging bucket rotor, unfiltered ascites was topped to 15 ml with water. This process was repeated until 15 ml of filtered ascites were obtained. The filtered ascites was then lyophilized to dryness and underwent trypsin digestion (see below). The 50-kDa-filtered ascites was analyzed directly by LC-MS/MS, whereas the 100-kDa-filtered ascites underwent strong cation exchange liquid chromatography (see below) prior to LC-MS/MS analysis with a reverse-phase C_{18} column.

KLK6 ELISA—The concentration of KLK6 in each eluted gel filtration fraction was measured by a sandwich-type immunoassay (20). In brief, a KLK6-specific monoclonal antibody (clone 27-4; developed in house) was first immobilized in a 96-well white polystyrene plate by incubating 250 ng/100 μl/well in a coating buffer (50 mmol/liter Tris, 0.05% sodium azide, pH 7.8) overnight. After washing three times with washing buffer (5 mmol/liter Tris, 150 mmol/liter NaCl, 0.05% Tween 20, pH 7.8), 50 μl of each pooled gel filtration fraction diluted 1:3 in 6% BSA or 50 μl of KLK6 standards were pipetted into each well in addition to 50 μl of assay buffer (50 mmol/liter Tris, 6% BSA, 0.01% goat IgG, 0.005% mouse IgG, 0.1% bovine IgG, 0.5 mol/liter KCl, 0.05% sodium azide, pH 7.8) and incubated for 1 h with shaking at room temperature. The plates were washed six times with the washing buffer after which biotinylated detection antibody solution (100 μl; 15 ng of anti-KLK6 (E24) monoclonal antibody in assay buffer)

was added to each well and incubated for 1 h at room temperature with shaking. The plates were then washed six times with the washing buffer. Subsequently alkaline phosphatase-conjugated streptavidin solution (5 ng/well; Jackson ImmunoResearch Laboratories, Westgrove, PA) in 6% BSA buffer (in 50 mmol/liter Tris, 0.05% sodium azide, pH 7.8) was added to each well and incubated for 15 min with shaking at room temperature. The plates were washed six times with the washing buffer, and substrate buffer (100 μl; 0.1 mol/liter Tris buffer, pH 9.1) containing 1 mmol/liter substrate diflunisal phosphate, 0.1 mol/liter NaCl, and 1 mmol/liter $MgCl_2$ was added to each well and incubated for 10 min with shaking at room temperature. After adding 50 μl of developing solution containing Tb^{3+}-EDTA complex, the fluorescence of each well was measured with a Wallac Envision 2103 multilabel reader. More details are given elsewhere (20).

Total Protein Assay—Total protein of each ascites fraction was quantified using a Coomassie (Bradford) protein assay reagent (Pierce). 5 μl of each pooled gel filtration fraction and 5 μl of water were loaded in duplicate in a microtiter plate along with the reagent, and protein concentrations were estimated by reference to absorbance obtained for a series of bovine albumin standard protein dilutions.

Trypsin Digestion—Each lyophilized sample was denatured using 8 M urea and reduced with DTT (final concentration, 13 mM; Sigma) at 50 °C followed by alkylation with 500 mM iodoacetamide (Sigma) with shaking at room temperature in the dark. The samples were then desalted using a NAP5 column (GE Healthcare). Samples were lyophilized and resuspended in trypsin buffer (1:50 ratio of trypsin (Promega, sequencing grade modified porcine trypsin):protein concentration; 120 μl of 50 mM ammonium bicarbonate, 100 μl of methanol, 150 μl of water) overnight in a 37 °C waterbath and then lyophilized to dryness.

Strong Cation Exchange Liquid Chromatography—Trypsin-digested lyophilized sample was reconstituted in 120 μl of mobile phase A (0.26 M formic acid in 10% acetonitrile). The samples were directly loaded onto a PolySULFOETHYL A™ column (The Nest Group, Inc.) containing a hydrophilic, anionic polymer (poly-2-sulfoethyl aspartamide) with a pore size of 200 Å and a diameter of 5 μm. A 1-h fractionation run was performed using HPLC with an Agilent 1100 system at a flow rate of 200 μl/min. A linear gradient of mobile phase B (0.26 M formic acid in 10% acetonitrile and 1 M ammonium formate) was added as the elution buffer. The eluate was monitored at a wavelength of 280 nm. Forty fractions, 200 μl each, were collected every minute after the start of the elution gradient. These 40 fractions were pooled into eight combined fractions (each pool consisting of five fractions) and concentrated to ~200 μl using a SpeedVac system preceding mass spectrometry analysis. Prior to each run, a protein cation exchange standard (Bio-Rad) was applied to evaluate column performance.

Mass Spectrometry—The samples from each pooled fraction of each individual separation experiment were desalted using a ZipTip C_{18} pipette tip (Millipore) and eluted in 4 μl of Buffer B (90% acetonitrile, 0.1% formic acid, 10% water, 0.02% trifluoroacetic acid). 80 μl of Buffer A (95% water, 0.1% formic acid, 5% acetonitrile, 0.02% trifluoroacetic acid) were added to each sample, and 40 μl were loaded on an Agilent 1100 HPLC system by the autosampler and injected onto a 2-cm C_{18} trap column (inner diameter, 200 μm). Peptides were eluted from the trap column onto a resolving 5-cm analytical C_{18} column (inner diameter, 75 μm) with an 8-μm tip (New Objective). This liquid chromatography setup was coupled on line to a two-dimensional linear ion trap (LTQ, Thermo Inc.) mass spectrometer using a nano-ESI source in data-dependent mode. Each fraction underwent a 120-min gradient, and eluted peptides were subjected to MS/MS. Data files were created using the Mascot Daemon (version 2.2) and extract_msn. The parameters for data file creation were:

minimum mass, 300 Da; maximum mass, 4000 Da; automatic precursor charge selection; minimum peaks, 10 per MS/MS scan for acquisition; and minimum scans per group, 1.

Data Analysis—The resulting mass spectra from each fraction were analyzed using Mascot (Matrix Science, London, UK; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2006.06.01) search engines on the non-redundant International Protein Index (IPI) human database (version 3.27) that included the forward and reversed sequences for calculating false positive error of each protein. Up to one missed cleavage was allowed, and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues. A fragment tolerance of 0.4 Da and a parent tolerance of 3.0 Da were used for both search engines with trypsin as the digestion enzyme. The resulting files were all loaded into Scaffold (version 2.0; Proteome Software Inc., Portland, OR), which validated each MS/MS-based peptide and protein identification. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the PeptideProphet algorithm (21). Protein identifications were accepted if they could be established at greater than 95% probability and contained at least one identified peptide. Protein probabilities were assigned by the ProteinProphet algorithm (22). The DAT and XML files for each fraction were inputted into Scaffold to cross-validate Mascot and X!Tandem data files. All biological samples were searched with multidimensional protein identification technology option clicked. Sample reports were exported from Scaffold, and each protein identification was assigned a cellular localization based on information available from Swiss-Prot, Gene Ontology, Euk-mPLoc (23), and other publicly available databases.

RESULTS

Complex biological fluids such as serum and ascites fluid contain thousands of proteins with a concentration range spanning at least 9 orders of magnitude (19). The major challenge preventing in-depth analysis of these proteomes by mass spectrometry is the presence of abundant proteins such as albumin and immunoglobulins, which make up 65–97% of serum proteins. These abundant proteins limit the ionization efficiency during mass spectrometric analysis, preventing the identification of low abundance proteins. Although various techniques have been used for albumin and immunoglobulin depletion (24, 25), we chose to perform size exclusion chromatography and centrifugal ultrafiltration to fractionate ascites fluid on the basis of molecular mass. Because the top 20 most abundant serum proteins have molecular masses greater than 30 kDa, we arbitrarily chose 30 kDa as the approximate molecular mass cutoff for the identification of the ascites fluid subproteome.

Identification of Proteins by Mass Spectrometry-Gel Filtration—20 ml of ascites fluid from one patient with disseminated ovarian cancer were used, and size exclusion chromatography was performed in duplicate using two different mobile phase buffer solutions: phosphate/sulfate and ammonium bicarbonate. After performing KLK6 ELISA and total protein assay on the eluate, fractions containing KLK6 (~30 kDa) and lower molecular mass proteins were selected for further fractionation or trypsin digestion and mass spectrometry (Figs. 1 and 2). Although with this method the majority of albumin and immunoglobulins were removed, some early
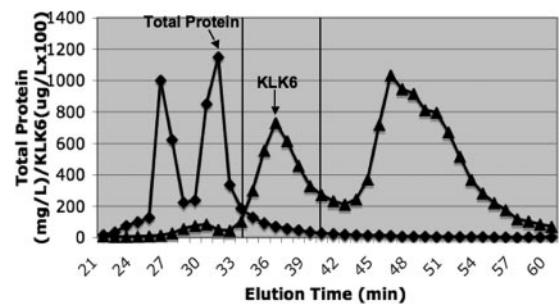
FIG. 1. **Elution profile of total protein (♦) and KLK6 (■) during one gel filtration.** Fractions with molecular mass of ≤30 kDa (*first vertical line*) were collected and analyzed by LC-MS/MS. Fractions *between* the *two vertical lines* were rechromatographed to remove additional high abundance proteins. For more details, see the text. Monomeric KLK6 (approximate molecular mass, 30 kDa) elutes at fractions 37–38; the second peak likely represents fragmented KLK6.
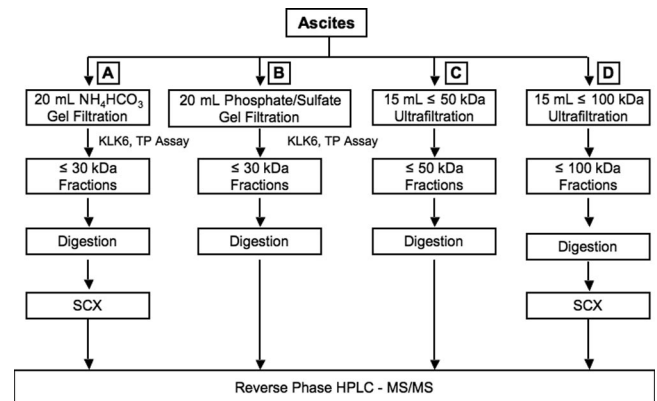
FIG. 2. **Ascites fluid fractionation protocol prior to LC-MS/MS analysis.** For more details, see the text. *A*, NH₄HCO₃ buffer gel filtration, strong cation exchange (*SCX*)-LC-MS/MS. *B*, phosphate/sulfate buffer gel filtration, LC-MS/MS. *C*, 50-kDa ultrafiltration, LC-MS/MS. *D*, 100-kDa ultrafiltration, strong cation exchange-LC-MS/MS. Digestion was with trypsin. *TP*, total protein.

fractions still contained a significant amount of total protein (Fig. 1). Hence 10 fractions starting from the KLK6 peak were collected and refractionated with gel filtration to remove additional amounts of high abundance proteins. Four hundred and four proteins were identified with the ammonium bicarbonate buffer, and 231 proteins were identified using the phosphate/sulfate buffer system (duplicate analysis with both systems). There was a 46% overlap between the two buffer systems; when data were combined, a total of 434 unique proteins were identified (supplemental Fig. 1). Only 30 additional proteins were identified with the phosphate/sulfate buffer system.

Identification of Proteins by Mass Spectrometry-Ultrafiltration—15 ml of ascites from two different patients with late stage ovarian cancer underwent ultrafiltration using Millipore centrifugal ultrafiltration devices with a nominal molecular mass cutoff of 50 and 100 kDa. These cutoffs were chosen based on the guidelines provided by the manufacturer regarding yields of proteins in the eluates. To obtain a good yield of
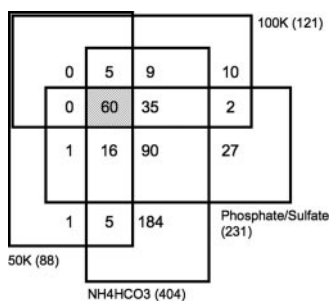
FIG. 3. **Number of proteins identified with each fractionation method.** In total, 445 proteins were identified. *50K*, 50 kDa; *100K*, 100 kDa.
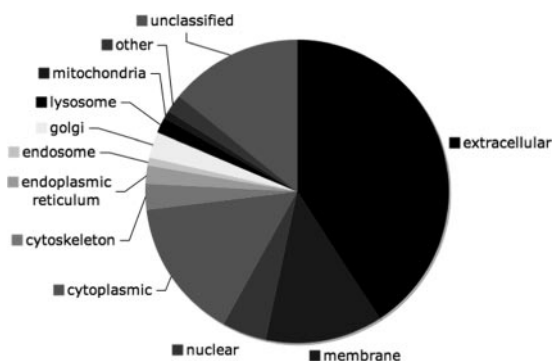

FIG. 4. **Classification of 445 ascites proteins by subcellular localization.**

FIG. 5. **Selection of 52 candidate ovarian cancer biomarkers based on the criteria shown above and as described in text.**

filtrated proteins with molecular mass of ≤30 kDa, it was suggested to use ultrafiltration devices with a nominal molecular mass cutoff 2–4 times higher than the desired protein mass. Eighty-eight and 121 proteins were identified from the 50- and 100-kDa filtrates, respectively. There was a 45% overlap between the 50- and 100-kDa filtrates; when data were combined, a total of 144 unique proteins were identified (supplemental Fig. 2). When all data were combined (gel filtration and ultrafiltration) the number of unique proteins identified was 445 (Fig. 3).

A complete list of proteins identified along with their number of unique peptides in each experiment is presented in supplemental Table 1. Redundancies and false positive proteins were removed from the list. Supplemental Table 2 contains detailed information on all proteins identified for each experiment, including number of unique peptides identified per protein, peptide sequences, precursor ion mass, and charge states. A total of 445 unique proteins were identified from all six individual experiments; 215 more proteins were identified in the soluble ascites fraction than the previously published proteome of ascites fluid by Gortzak-Uzan *et al.* (17). Our false positive rate was 2.4%.

Cellular Localization of Identified Proteins—Each unique protein identified was classified according to its cellular localization based on information available from Swiss-Prot, Gene Ontology, and other publicly available databases. Fig. 4 shows the cellular distribution of the 445 proteins with known
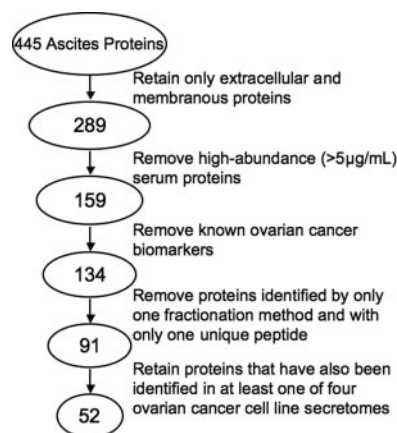
localizations. When one protein is localized in more than one cellular compartment, all of the categories were accounted for non-exclusively. This resulted in a total percentage greater than 100%. Of the proteins, 14% were not classified as none of the sources were informative. The majority of the classified proteins were extracellular (40%) and membrane-bound (12%). One hundred and fifty-seven of the 445 identified ascites fluid proteins were also identified in the Plasma Proteome Database (supplemental Table 1). This does not mean that the remaining 288 proteins are exclusive to ascites fluid as the plasma proteome is incomplete and still requires more in-depth analysis. Our data suggest that many of the proteins identified are secreted by the tumor cells or the tumor microenvironment.

Identification of Candidate Biomarkers—To identify biomarker candidates, we applied a set of filtering criteria to our list of unique proteins (Fig. 5). 1) We removed proteins that are not extracellular or membranous: from our list of 445 unique proteins, we eliminated 148 proteins, resulting in a shortened list of 289 extracellular and membranous proteins (supplemental Table 3). We chose to focus on extracellular and membranous proteins as these proteins have the highest potential of being found in the circulation and hence can be detected by non-invasive serum-based tests. 2) We removed all known high abundance serum proteins (concentration >5 μg/ml) such as albumin, immunoglobulins, and complement-related proteins: of the 289 extracellular and membranous proteins, 130 were classified as high abundance serum proteins and were removed, leaving a list of 159 proteins. 3) We removed proteins previously studied in the serum of ovarian cancer patients: the 159 remaining proteins were individually searched in PubMed. Twenty-five of these proteins were examined in the past as candidate ovarian cancer biomarkers (Table I). Five of those belong to the kallikrein family of biomarkers as described previously by our group (40–45). 4) We removed proteins found in only one fractionation protocol and with a single unique peptide: 43 proteins were removed

TABLE I
Identified proteins previously examined as ovarian cancer biomarkers

Protein name[a]	Molecular mass	Refs.
	Da	
AFM, afamin precursor	69,052	36
CHI3L1, chitinase-3-like protein 1 precursor (YKL-40)	42,609	37
CLEC3B, hypothetical protein DKFZp686H17246 (tetranectin)	17,776	38, 39
KLK10, kallikrein-10 precursor	30,120	40, 41
KLK11, isoform 1 of kallikrein-11 precursor	27,448	42
KLK6, kallikrein-6 precursor	26,838	43
KLK7, isoform 1 of kallikrein-7 precursor	27,507	44
KLK9; KLK8, isoform 1 of neuropsin precursor	28,029	45
LCN2, neutrophil gelatinase-associated lipocalin precursor	22,571	46
LGALS1, galectin-1	14,698	47, 48
MMP2, 72-kDa type IV collagenase precursor	73,867	49–51
PEBP1, phosphatidylethanolamine-binding protein 1	21,039	52
PLAUR, isoform 1 of urokinase plasminogen activator surface receptor precursor	36,959	38, 53, 54
RBP4, plasma retinol-binding protein precursor	22,992	55
SERPINA3, isoform 1 of α_1-antichymotrypsin precursor	50,583	34
SERPINE1, plasminogen activator inhibitor 1 precursor	45,042	56
SERPINF2, α_2-antiplasmin precursor	55,047	57
SPP1, isoform A of osteopontin precursor	35,405	58–60
TF, serotransferrin precursor	77,032	61, 62
THBS1, thrombospondin-1 precursor	129,364	63, 64
TIMP1, metalloproteinase inhibitor 1 precursor	23,153	65
TIMP2, metalloproteinase inhibitor 2 precursor	24,382	51
TMEM110; ITIH4, isoform 1 of inter-α-trypsin inhibitor heavy chain H4 precursor	103,308	65
TTR, transthyretin precursor	15,869	35, 55, 56, 62
WFDC2, isoform 1 of whey acidic protein four-disulfide core domain protein 2 precursor	12,974	59, 66, 67

[a] For protein IPI accession numbers, see supplemental Table 1.

with 91 proteins remaining for further selection. 5) We have previously completed analysis of the proteome and secretome (secreted and membrane-bound proteins) of four ovarian cancer cell lines (HTB75, serous; TOV112D, endometrioid; TOV21G, clear cell; and RMUG-S, mucinous) and identified a total of 1689 proteins.[2] One hundred and fifty-four proteins overlapped with our list of 445 ascites proteins, whereas 73 of these proteins overlapped with our list of 289 extracellular and membranous ascites fluid proteins (supplemental Table 4). We used the results of the cell line analysis to further confirm the proteins identified within our ascites samples. Of our 91 remaining proteins, 52 proteins were identified as extracellular or membranous proteins in the supernatant of at least one of the four ovarian cancer cell lines studied. These remaining 52 proteins, which passed all of our selection criteria, represent our panel of candidate ovarian cancer biomarkers (Table II).

DISCUSSION

One of the main obstacles in proteomics analysis of biological fluids is the presence of high abundance proteins (26), mainly albumin and immunoglobulins. In human serum the top 10 most abundant proteins comprise over 95% of all proteins present in this fluid (27), and the top 20 are greater than 30 kDa in molecular mass. These abundant proteins, especially

human serum albumin, generate massive amounts of ions that often result in the inaccurate representation and identification of ions from the low abundance proteins because of the limited number of ions passed on for tandem mass spectrometry analysis. Hence these high abundance proteins must be depleted or removed to efficiently identify the proteins of low molecular mass and low abundance by mass spectrometry. Various methods have been used previously for the removal of albumin or immunoglobulins such as dye affinity resins or protein A/G beads (28, 29), yet these approaches are limited as albumin and other high abundance proteins often act as transport proteins by binding (and thereby concentrating) many low abundance proteins and peptides. Thus, removal of serum albumin and other abundant proteins may inadvertently remove many small proteins and peptides of interest (30).

Alternative approaches for biomarker discovery without the problems associated with high abundance molecules include analysis of tissue culture supernatants of cancer cell lines grown in serum-free media (31). Also recently Faça *et al.* (32) characterized the cell surface proteome and the proteins released into the extracellular milieu of three ovarian cancer cell lines and identified over 6000 proteins as candidate biomarkers and therapeutic targets.

By utilizing different separation methods in combination with mass spectrometry, we identified 445 proteins within the

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[2] C. G. Gunawardana, C. Kuk, C. R. Smith, I. Batruch, A. Soosaipillai, and E. P. Diamandis, manuscript in preparation.

TABLE II

Panel of 52 candidate ovarian cancer biomarkers

COMP, cartilage oligomeric matrix protein; EGF, epidermal growth factor; GM2, GalNAcβ1→4(NeuAcα2→ 3)Galβ1→4Glcβ1-1′Cer.

Protein name[a]	Molecular mass
	Da
AGRN, agrin precursor	214,820
BCAM, Lutheran blood group glycoprotein precursor	61,042
C14orf141; LTBP2, latent transforming growth factor β-binding protein 2 precursor	195,039
CD248, isoform 1 of endosialin precursor	80,840
CD59, CD59 glycoprotein precursor	14,159
CLU, clusterin precursor	52,477
COMP 80-kDa protein	79,676
CPA4, carboxypeptidase A4 precursor	47,334
CST3, cystatin-C precursor	15,781
CST6, cystatin-M precursor	16,493
CTGF, isoform 1 of connective tissue growth factor precursor	38,073
DAG1, dystroglycan precursor	97,563
DKK3, Dickkopf-related protein 3 precursor	38,272
DSC2, isoform 2A of desmocollin-2 precursor	99,945
DSG2, desmoglein 2 preproprotein	122,276
ECM1, extracellular matrix protein 1 precursor	60,655
EFEMP1, isoform 1 of EGF-containing fibulin-like extracellular matrix protein 1 precursor	54,621
FAM3C, protein FAM3C precursor	24,663
FBLN1, isoform C of fibulin-1 precursor	74,442
FOLR1, folate receptor α precursor	29,801
FSTL1, follistatin-related protein 1 precursor	34,967
GLOD4, uncharacterized protein C17orf25	54,995
GM2A, ganglioside GM2 activator precursor	20,805
GPX3, glutathione peroxidase 3 precursor	25,488
HSPG2, basement membrane-specific heparan sulfate proteoglycan core protein precursor	468,788
HTRA1, serine protease HTRA1 precursor	51,269
IGFBP2, insulin-like growth factor-binding protein 2 precursor	35,119
IGFBP3, insulin-like growth factor-binding protein 3 precursor	31,656
IGFBP4, insulin-like growth factor-binding protein 4 precursor	27,916
IGFBP5, insulin-like growth factor-binding protein 5 precursor	30,552
IGFBP6, insulin-like growth factor-binding protein 6 precursor	25,304
IGFBP7, insulin-like growth factor-binding protein 7 precursor	29,112
LRG1, leucine-rich α_2-glycoprotein precursor	38,162
MST1, hepatocyte growth factor-like protein precursor	80,360
MXRA5, matrix-remodeling-associated protein 5 precursor (adlican)	312,263
NID2, nidogen-2 precursor	151,377
NPC2, epididymal secretory protein E1 precursor	16,552
NUCB1, nucleobindin-1 precursor	53,862
PCOLCE, procollagen C-endopeptidase enhancer 1 precursor	47,955
PLEC1, isoform 1 of plectin-1	531,708
PLTP, isoform 1 of phospholipid transfer protein precursor	54,723
PROCR, endothelial protein C receptor precursor	30,697
PROS1, vitamin K-dependent protein S precursor	75,105
PSAP, isoform Sap-mu-0 of proactivator polypeptide precursor	58,094
QSCN6, isoform 1 of sulfhydryl oxidase 1 precursor	82,561
SECTM1, secreted and transmembrane protein 1 precursor	27,021
SERPINA6, corticosteroid-binding globulin precursor	45,124
SOD1 16-kDa protein (superoxide dismutase 1)	16,104
SVEP1, polydom (Sel-Ob)	390,478
TAGLN2; CCDC19, transgelin-2	22,374
TGFBI, transforming growth factor-β-induced protein ig-h3 precursor	74,665
VASN, vasorin precursor	71,696

[a] For protein IPI accession numbers, see supplemental Table 1.

soluble fraction of ascites fluid. Recently the proteome of ascites fluid was reported by Gortzak-Uzan *et al.* (17). Although they identified over 2200 proteins, only 229 were found in the soluble fraction of ascites. Because the serum proteome contains thousands of proteins, the list of 229 proteins is unlikely to represent the full proteome of soluble

ascites fluid. As serum protein concentrations range over 9 orders of magnitude, we aimed to identify a more extensive proteome of ascites fluid, focusing on low molecular mass (≤30 kDa).

Using different mobile phase systems (ammonium bicarbonate and phosphate/sulfate) and size exclusion chromatography, we identified 404 and 231 proteins, respectively. Combining these proteins with the 88 and 121 proteins identified from the 50- and 100-kDa centrifugal ultrafiltration experiments, we identified a total of 445 unique proteins within ascites fluid; this is more than any other published proteome of soluble ascites and almost doubles the proteins reported earlier (17). Our results indicate that by combining different sample fractionation methods a greater coverage of the ascites fluid proteome can be obtained, thus allowing for better biomarker selection. The overlap between our own soluble ascites fluid proteome (445 proteins) and that of Gortzak-Uzan (17) (220 proteins) was 28% (supplemental Table 3).

Although ascites fluid is the buildup of peritoneal fluid accumulated from infiltrated circulating serum, its composition may be different due to the presence of the burdening ovarian tumor. By comparing our identified ovarian ascites fluid subproteome with the human plasma proteome database, only 34% of our proteins were common. Even taking into account that the plasma proteome is not complete and that our focus was on low molecular mass proteins, our data suggest that ascites fluid has a significantly different composition than plasma, and its proteins reflect the contribution of the tumor microenvironment.

Classification of the identified proteins by Swiss-Prot, Gene Ontology as well as other publicly available databases indicated that 52% of the proteins within ascites fluid are extracellular or membranous (Fig. 4) as would be expected for an extracellular biological fluid. With over half of the proteins defined as extracellular or membranous, it is highly plausible that many of these proteins are shed by the tumor cells, allowing for an efficient selection of candidate ovarian cancer biomarkers.

The proteins identified within ascites fluid reflect the pathobiological state of ovarian cancer. Because ascites accumulation is often linked to advanced ovarian cancer, it is likely that many of these identified proteins represent promising new biomarkers. On the other hand, not all proteins in ascites represent tumor-associated antigens. By applying an arbitrary set of selection criteria, we were able to minimize the list of candidate biomarker proteins to a more manageable number (~50) for further selection and validation. From the list of extracellular and membranous proteins, we eliminated high abundance proteins, proteins previously studied as serological biomarkers for ovarian cancer, proteins identified with a single unique peptide from only one fractionation protocol, and proteins that were not identified in at least one supernatant of four different ovarian cancer cell lines. The identification of 25 known secreted or membrane-bound ovarian can-

cer biomarkers (Table I) supports our view that the outlined approach can identify novel biomarkers.

From our panel of 52 candidate biomarkers (Table II), 31 proteins were also identified within the ascites fluid proteome by Gortzak-Uzan *et al.* (17) (supplemental Table 5). However, these authors did not select any of these proteins for further investigation as they had applied a different set of criteria for biomarker selection. This underlines the fact that despite successful identification of proteins in fluids by mass spectrometry the criteria for narrowing down the list of candidates are also of paramount importance.

Although many of our filtering criteria were somewhat subjective, our discovery strategy appears to be efficient as 25 of our 289 identified extracellular or membranous proteins were previously studied as potential serum ovarian cancer biomarkers (Table I). It is very likely that our list of 52 candidates also includes novel ovarian cancer biomarkers. Although the most widely studied biomarker for ovarian cancer, CA125, was not identified in any of our experiments, this can be explained by the fact that CA125 is highly glycosylated with a molecular mass ranging from 190 to 2700 kDa (33) and was therefore excluded during sample preparation. We acknowledge that other glycosylated proteins of molecular mass of ≥30 kDa may have not been identified. Additionally many of our candidates (Table II) have molecular masses ≥30 kDa, implying that they are likely fragmented in ascites fluid. This has also been reported by others who observed truncated forms of transthyretin and cleavage fragment of inter-α-trypsin inhibitor heavy chain H4 (35).

The major challenge in biomarker discovery using proteomics is the validation phase. In the future, we intend to validate some, or all, of our 52 candidate ovarian cancer biomarkers by using ELISAs or other quantitative techniques and serum as the fluid of choice. Such analysis is currently problematic because of the lack of immunological reagents or assays that have the capability of measuring low levels of these antigens in serum.

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