research articles Journal of proteome research

Comprehensive Analysis of Conditioned Media from Ovarian Cancer Cell Lines Identifies Novel Candidate Markers of Epithelial Ovarian Cancer

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Received May 8, 2009

Ovarian cancer remains a deadly threat to women as the disease is often diagnosed in the late stages when the chance of survival is low. There are no good biomarkers available for early detection and only a few markers have shown clinical utility for prognosis, response to therapy and disease recurrence. We mined conditioned media of four ovarian cancer cell lines (HTB75, TOV-112D, TOV-21G and RMUG-S) by two-dimensional liquid chromatography−mass spectrometry. Each cell line represented one of the major histological types of epithelial ovarian cancer. We identified 2039 proteins from which 228 were extracellular and 192 were plasma membrane proteins. Within the latter list, we identified several known markers of ovarian cancer including three that are well established, namely, CA-125, HE4, and KLK6. The list of 420 extracellular and membrane proteins was cross-referenced with the proteome of ascites fluid to generate a shorter list of 51 potential biomarker candidates. According to Ingenuity Pathway Analysis, two of the top 10 diseases associated with the list of 51 proteins were cancer and reproductive diseases. We selected nine proteins for preliminary validation using 20 serum samples from healthy women and 10 from women with ovarian cancer. Of the nine proteins, clusterin (increase) and IGFBP6 (decrease) showed significant differences between women with or without ovarian cancer. We conclude that in-depth proteomic analysis of cell culture supernatants of ovarian cancer cell lines can identify potential ovarian cancer biomarkers that are worth further clinical validation.

Keywords: Epithelial ovarian cancer • biomarker • proteomics • mass spectrometry • cell lines • Oncomine • Human Protein Atlas

Introduction

Ovarian cancer (OvCa) kills more women than any other gynecological malignancy. For a cancer that accounts for only 3% of new cases, it is the fifth largest killer. The reason for the high case-to-fatality rate is that it is often diagnosed when the cancer has metastasized to other organs. The 5-year survival rate for patients with advanced disease (stage III and IV) is 10−30%.[1] In contrast, the 5-year survival rate for patients diagnosed with early stage disease can be as high as 94%.[1] These numbers clearly support the need for early diagnosis.

In general, ovarian malignancies arise in 3 major cell types. Epithelial ovarian cancer (EOC) accounts for more than 80% of the cases and is found on the surface epithelium. Stromal cell tumors arise in the connective tissue below the surface epithelium and account for 10% of cases. The third type arises from germ cells and accounts for less than 10% of cases. This study focuses on EOC, and in particular the serous, endometrioid, clear-cell and mucinous histological types.

The clinically accepted biomarker for EOC is CA-125.[2] Approximately 85% of clinically advanced ovarian carcinomas can be identified by measuring CA-125 levels.[3,4] However, this molecule is a poor marker for early detection due to frequent false positive and false negative results.[5] Other markers that have shown some clinical relevance in EOC are HE4,[6] osteopontin,[7] the carbohydrate antigens CA 15−3 and CA 19−9,[8] inhibin,[9] and several members of the kallikrein family (kallikreins 5, 6, 8,10, 11 and 14).[10−14] None of these proteins, however, have been effective early detection biomarkers nor have they reached the clinical efficacy of CA-125 for detecting recurrence and monitoring therapy.

Many strategies exist to uncover novel biomarkers for cancer, including gene expression profiling, protein microarrays, gene translocation/fusion analysis, peptidomics, and mass spectrometry (MS)-based profiling.[15] MS-based proteomic studies using EOC tissue,[16,17] ascites fluid,[18] and cancer cell lines[19,20] have contributed greatly to the list of potential protein markers.

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10.1021/pr900411g CCC: $40.75

Published on Web 08/10/2009

However, the selection of candidates and validation of these candidates have been major rate-limiting steps.

In this study, we used an MS-based profiling approach using cell culture supernatants as the source of proteins. Since many of the cancer markers studied to date are proteins that are secreted or shed into the circulation, our major focus was the set of extracellular and plasma membrane proteins. We performed comparisons of our data set with gene expression and protein expression data using the online search tools, Oncomine[21] and the Human Proteome Atlas (HPA),[22] respectively. In addition, we also compared our data with proteomic data of others to extend the already available resources. Our approach detected many well-known markers of ovarian cancer, such as CA-125, HE4, and KLK6, thus, confirming its effectiveness. In addition, several novel proteins were identified, whose role as biomarkers of EOC should be explored further.

Materials and Methods

Cell Lines. HTB-75, TOV-112D, and TOV-21G cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA. The RMUG-S cell line was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). HTB-75 cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS). TOV-112D and TOV 21G cell lines were grown in a 1:1 mixture of MCDB 105 medium and Medium 199, containing 10% FBS. RMUG-S cells were maintained in Ham's F12 medium containing 10% FBS. All media for cell culture was purchased from Invitrogen Canada, Inc. (Burlington, Ontario, Canada).

Cell Culture. Each cell type was seeded in T-175 cm^2 cell culture flasks and cultured to 80% confluency in normal growth medium (2 days). Eight flasks were grown per cell line and cells were washed 3 times with 30 mL of phosphate buffered saline (PBS). Following the washes, 30 mL of chemically defined serum-free CDCHO medium (Invitrogen) supplemented with 8 mM glutamine (Invitrogen) was added to each flask. HTB-75, TOV-112D, and TOV-21G cell lines were grown for 48 h, whereas RMUG-S was grown for 72 h in serum-free CDCHO medium. Following the growth in serum-free medium (SFM), the conditioned media (CM) were collected and centrifuged to remove cellular debris.

Sample Preparation. A total of 240 mL of CM was collected per cell-line. Each sample was centrifuged to remove cellular debris and then separated into four aliquots (60 mL) per cell line. Each aliquot represented a biological replicate, and thus, 4 replicates were available per cell line. In this study, we processed 3 replicates per cell line. Each replicate was dialyzed (3.5 kDa molecular mass cutoff) against 5 L of 1 mM ammonium bicarbonate with 2 buffer exchanges at 4 °C. Following dialysis, the replicates were lyophilized. Each lyophilized replicate was denatured using 8 M urea, reduced with 13 mM dithiothreitol (DTT, Sigma), and then alkylated using 500 mM iodoacetamide (Sigma). Following reduction and alkylation, the replicates were desalted using NAP5 columns (GE Healthcare). Each replicate was lyophilized and then trypsin-digested (Promega) overnight at 37 °C. Following trypsin digestion, each replicate was lyophilized once more.

Strong Cation Exchange Chromatography. The trypsin-digested and lyophilized replicate was resuspended in 120 μL of mobile phase A [0.26 M formic acid in 10% acetonitrile(ACN)]. The sample was injected into a PolySULFOETHYL A column with a 200-Å pore size and diameter of 5 μm (The Nest Group, Inc.) containing a hydrophilic, anionic polymer (poly-2-sulfethyl aspartamide). A 1-h separation was performed on an HPLC system (Agilent 1100) using a mobile phase B containing 0.26 M formic acid in 10% ACN and 1 M ammonium formate. The eluate was monitored at a wavelength of 280 nm. Fractions were collected every 5 min after the start of the run at a flow rate of 200 μL/min.

Mass Spectrometry. Fractions 6−11 obtained from strong cation exchange (SCX) chromatography were used for mass spectrometric analysis. Each fraction was loaded onto a ZipTip C18 pipet tip (Millipore; catalogue number ZTC18S096) and eluted in 4 μL of Buffer B [90% ACN, 0.1% formic acid, 10% water, 0.02% trifluoroacetic acid (TFA)]. The eluate was mixed with 80 μL of Buffer A, and 40 μL was injected via an autosampler into an Agilent 1100 series HPLC. The peptides were first injected onto a 2-cm C18 trap column (inner diameter, 200 μm), and then eluted from the trap column into a resolving 5-cm analytical C18 column (inner diameter, 75 μm) with an 8 μm tip (New Objective). The LC setup was coupled online with a 2-D linear ion trap (LTQ, Thermo, Inc.) mass spectrometer using a nano-ESI source in data-dependent mode. Each fraction was run on a 120 min gradient. The eluted peptides were subjected to MS/MS. DTAs were created using the Mascot Daemon (version 2.16) and extract_msn. We used the following parameters for DTA creation: 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. Mass spectra from each fraction were analyzed using Mascot (Matrix Science, London, U.K.; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2006.06.01) search engines on the nonredundant International Protein Index (IPI) human database version 3.27 (containing 67 528 entries). 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 Mascot and X!Tandem, with trypsin as the digestion enzyme. Six DAT files (Mascot) and six XML files (X!Tandem) were generated per replicate, per cell line. The DAT and XML files were uploaded and analyzed using Scaffold (v01_05_19, Proteome Software, Inc., Portland, OR). Peptide identifications and protein identifications were accepted if they could be established with greater than 95% probability using the PeptideProphet algorithm and greater than 80% probability using the ProteinProphet algorithm, respectively. The number of identified peptides was set to at least one. All biological samples were searched using the MudPIT (multidimensional protein identification technology) option. Sample reports were exported from Scaffold and the identified proteins were assigned a cellular localization based on information available from Swiss-Prot, Genome Ontology (GO), and other publicly available databases. To calculate the false positive error rate, each fraction was analyzed using a "sequence-reversed" decoy IPI human database version 3.27 by Mascot and X!Tandem and data analysis was performed as mentioned above.

Immunoassays. IGFBP5, IGFBP6, βIG-H3, and cystatin C ELISA kits were purchased from R&D Systems. The IGFBP4 kit was purchased from DSL, Inc., and the clusterin assay was purchased from ALPCO Laboratories. Immunoassays for IGFBP4, cystatin C, and clusterin were performed according to the manufacturer's instructions. Assays for IGFBP5, IGFBP6, and βIG-H3 were also performed according to the manufacturer's instructions but with a modification to the detection

step (see below). Some assays were not designed for use with serum and, therefore, required optimization (see Results).

Nonbiotinylated polyclonal and monoclonal antibodies to vasorin, EPCR, and IGFBP7 were purchased from R&D Systems, as were the recombinant proteins used as protein calibrators. Sandwich-type ELISAs were constructed in-house using a monoclonal or a polyclonal antibody for antigen capture and a biotinylated polyclonal antibody for detection. White polystyrene microtiter plates were coated with either 100 ng/100 μL (vasorin and IGFBP7) or 200 ng/100 μL (EPCR) of monoclonal or polyclonal antibody in coating buffer (50 mM Tris buffer, 0.05% sodium azide, pH 7.8) and stored at room temperature overnight. Fifty microliters of protein calibrators or samples and 50 μL of assay buffer [50 mM Tris, 6% BSA, 0.01% goat IgG, 0.1% bovine IgG (Sigma-Aldrich, Inc., St. Louis MO), 0.005% mouse IgG (Fortron Bio Science, Inc., Morrisville, NC), 0.05% sodium azide, pH 7.8] with 0.5 M KCl (vasorin and IGFBP7) or without KCl (EPCR) were added to wells and incubated for 90 min with shaking at room temperature. The plates were washed 6 times with washing buffer (5 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.8). Approximately 100 μL of biotinylated detection antibody (125 ng/mL in assay buffer containing 0.5 M KCl) 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.

Detection of IGFBP5, IGFBP6, and βIG-H3 was modified from the manufacturer's instructions and performed the same way as that for vasorin, IGFBP7, and EPCR. Approximately 100 μL (5 ng/well) of alkaline phosphatase-conjugated (ALP) streptavidin (Jackson ImmunoResearch) in sample buffer (6% BSA, 50 mM Tris, 0.06% sodium azide, pH 7.8) was added to each well and incubated for 15 min with shaking at room temperature. The plates were washed 6 times with the wash buffer, and then 100 μL of substrate buffer [0.1 mol/L Tris buffer, pH 9.1, containing 0.5 mmol/L diflunisal phosphate (DFP), 0.1 mol/L NaCl, and 1 mmol/L MgCl2] was added to each well and incubated for 10 min with shaking at room temperature. Approximately 100 μL of developing solution (1 mol/L Tris base, 0.15 mol/L NaOH, 2 mmol/L $TbCl_3$, 3 mmol/L EDTA) was added to each well and incubated for 1 min with shaking at room temperature. The fluorescence was measured with an EnVision 2103 time-resolved fluorometer (Perkin-Elmer).

Biotinylation of Detection Antibody. Biotinylated polyclonal antibodies to IGFBP5, IGFBP6 and βIG-H3 were provided with their kits. Biotinylated polyclonal antibodies to vasorin, and EPCR were purchased from R&D Systems. Approximately 50 ng of polyclonal anti-IGFBP7 antibody was incubated with 50 ng of biotin in 0.5 M $NaHC0_3$ for 1 h. This was used as the detection antibody for the IGFBP7 assay.

Clinical Specimens. Serum samples were collected from stage III−IV EOC patients and normal controls. Our protocols have been approved by the Institutional Review Board at the University Health Network, Toronto, Ontario, Canada.

Statistical Analysis. The Mann−Whitney test was used to determine statistical significance when comparing the concentrations of candidate biomarkers in normal and ovarian cancer sera.

Results

Optimization of Cell Culture Conditions. The optimization of culturing conditions (seeding density, type of serum-free media, culturing period, etc.) has been described previously.[23,24] Cell lines were grown first in regular growth medium containing 10% FBS (see Materials and Methods for culturing conditions of each cell line). To minimize contamination from proteins originating from FBS, each cell line was washed extensively with PBS, and with the exception of RMUG-S, grown in SFM for 48 h. The RMUG-S cell line was cultured for 72 h in SFM to increase the amount of protein in conditioned media. We measured KLK5 levels in the conditioned media by ELISA (Table 1) and used it as an internal control. Both HTB-75 (3 ng/mL) and RMUG-S (>10 ng/mL) expressed KLK5, whereas the other two cell lines did not.

Table 1. Cell Lines Used in This Study

cell line	histological origin	stage	number of proteins found	KLK5 levels (ng/mL)
HTB-75	Serous	III	1208	3 ng/mL
TOV-112D	Endometrioid	III	1252	not detected[a]
TOV-21G	Clear cell	III	885	not detected[a]
RMUG-S	Mucinous	unknown	467	>10 ng/mL

[a] KLK5 concentration was below detection limit of ELISA (<0.1 ng/mL) in cell culture supernatants.

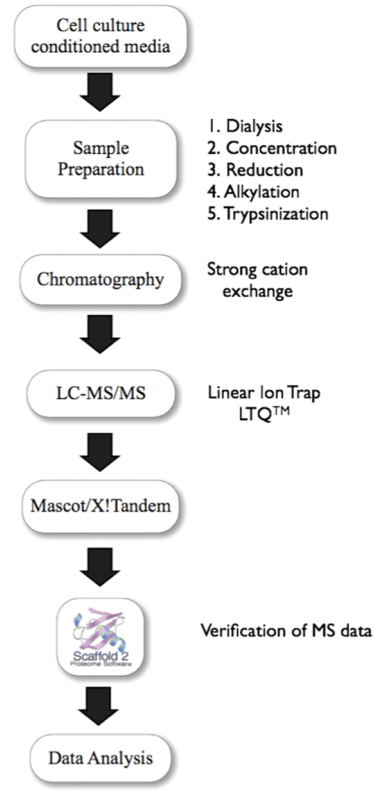

Figure 1. Outline of the experimental workflow.

Protein Identification by Mass Spectrometry. The workflow of this study is illustrated in Figure 1. In total, four ovarian cancer cell lines were used. Each cell line originates from an epithelial cancer of a particular histological type (serous, mucinous, endometrioid, and clear-cell). The details of each cell line used are listed in Table 1. Control samples were established in the same way as their corresponding experimental ones. Briefly, flasks without cells were incubated with regular growth medium for 48 h, washed extensively with PBS, and then incubated with SFM for the allotted time as their experimental counterpart. Approximately 29 proteins were found in the negative control for TOV-21G and TOV112D, 82 proteins for HTB-75, and 45 proteins for RMUG-S. These

Table 2. Breakdown of Proteins Identified in Each Cell Line Per Number of Unique Peptides Identified by MS

number of unique peptides	HTB75	TOV-112D	TOV-21G	RMUG-S
1	63	64	56	22
2	349	348	257	153
3	235	237	167	75
4	125	150	111	50
≥5	436	453	294	167

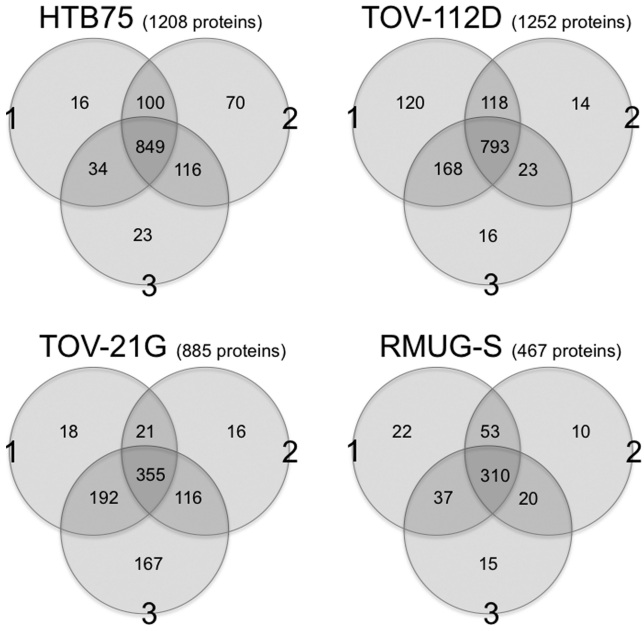

Figure 2. Overlap of proteins identified in the three replicates for each cell line. Three replicates per cell line (HTB75, TOV112D, TOV21G, and RMUG-S) were processed and analyzed. For each cell line, a majority of proteins identified were found in all three replicates.

proteins originated from FBS present in normal growth medium used to culture cells.

We found 1208 proteins in the conditioned medium of HTB-75, 1252 proteins for TOV-112D, 885 for TOV-21G, and 467 for RMUG-S. The breakdown of proteins based on the number of unique peptides identified by mass spectrometry is shown in Table 2. Supplementary Table 1A−D contain detailed information on all proteins identified in each cell line, including the number of unique peptides identified per protein, percentage sequence coverage per protein, sequence identified, the precursor m/z and charge state, and the score/E-value for each peptide. We examined the overlap of proteins identified in the three replicates analyzed per cell line. The overlap was 70% for HTB75, 63% for TOV-112D, 40% TOV-21G, and 66% for RMUG (Figure 2). Despite the reduced overlap for the TOV-21G line, 75% of the proteins in the TOV-21G conditioned media were found in at least two of the replicates. In total, we identified 2039 unique proteins (listed in Supplementary Table 2). We identified the internal standard, KLK5, in both HTB75 and RMUG-S, but, as expected, did not identify it in TOV21G and TOV112D. These results are consistent with the results of the ELISA testing (Table 1).

Subcellular Localization. Each protein was cross-referenced against the Gene Ontology database to determine the subcellular localization (Supplementary Table 2). A significant proportion of proteins were from intracellular locations such as the cytoplasm, Golgi, endoplasmic reticulum and the nucleus. This is most likely due to cell lysis, which is unavoidable with cultured cells. Figure 3 depicts the distribution of proteins based on subcellular location. Approximately 21% of the proteins identified were either extracellular or plasma membrane proteins. These proteins are listed in Supplementary Tables 3 and 4. Williams et al. recently published a comprehensive review, listing proteins that have been studied as biomarkers in serum or ascites fluid in EOC.[25] The list of extracellular and membrane proteins was compared with the aforementioned list and the common proteins are listed in Table 3. Known markers of ovarian cancer such as CA-125, CA 15−3, HE4, KLK6, and mesothelin were identified in this study.

Selection of Candidates. We used the following arbitrary criteria to pick candidates for study in serum of EOC cases and healthy individuals:

(1) The set of extracellular and membrane proteins was chosen as the starting point. It is reasonable to hypothesize that extracellular (secreted) and membrane proteins (ones that are shed) are more likely to enter the circulation. Comparing the list of the 228 extracellular and 192 plasma membrane proteins with that of the plasma proteome published by HUPO,[26] there was an overlap of 65 extracellular proteins and 29 plasma membrane proteins (Supplementary Tables 3 and 4). We eliminated known high-abundance proteins with concentrations greater than 5 μg/mL in plasma. Some proteins (serum levels >5 μg/mL), such as clusterin, were kept as candidates since their use as a biomarker in ovarian cancer has not been reported in the literature.

(2) Next, this set of extracellular and membrane proteins was compared with a list of 289 extracellular and membrane proteins of a separate study from our lab on the proteome of the ascites fluid.[27] Seventy-two proteins overlapped and these were selected for further investigation.

(3) We further eliminated proteins that have been reported previously as serological markers of ovarian cancer. By applying this criterion, 21 proteins were eliminated. The remaining 51 proteins are listed in Supplementary Table 5. The major biological functions and diseases associated with these proteins are illustrated in Figure 4.

(4) We searched for commercial ELISA kits for the 51 protein candidates. For candidates that did not have a commercial ELISA, we searched for monoclonal or polyclonal antibodies to construct in-house immunoassays.

(5) Proteins that did not have commercial ELISA kits or antibodies were not studied further. Of the remaining proteins that possessed an ELISA or antibodies, nine proteins were selected for preliminary validation with patient sera (Table 4).

Construction of Immunoassays. The immunoassays of the nine candidates were optimized before testing serum samples. We used the following stepwise approach to construct the immunoassays:

(1) The cystatin C and IGFBP4 ELISA kits were available in 96-well plate format with a precoated capture antibody. The clusterin ELISA kit was a competitive binding assay with precoated clusterin antigen. These assays had been already optimized for serum studies and therefore they were performed according to the manufacturer's instructions.

(2) For the other analytes, recombinant proteins of each candidate were used as standards, and assays were

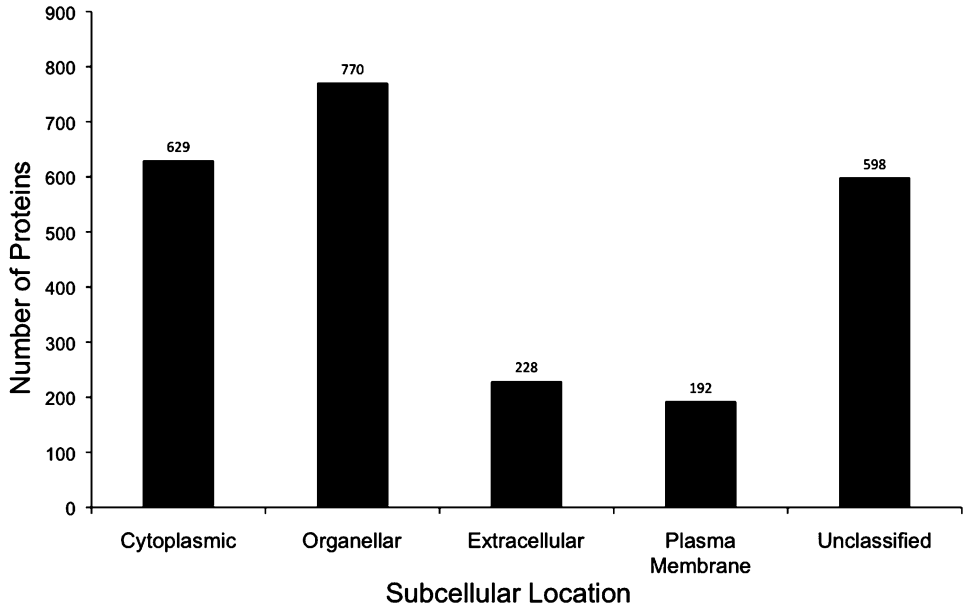

Figure 3. The number of proteins identified in each subcellular compartment. The 2039 proteins identified were cross-referenced with the Gene Ontology database to determine their subcellular location. The cytoplasmic proteins included those classified as cytoskeletal by Gene Ontology. The organellar designation includes proteins located in the mitochondria, endoplasmic reticulum, the Golgi, the nucleus, peroxisomes, and lysosomes. Unclassified proteins are those that either did not have a Gene Ontology classification or whose classifications were ambiguous and thus could not be placed in the other four categories. There is redundancy in this data as some proteins were placed in more than one compartment.

Table 3. Previously Studied Proteins in EOC That Were Identified in This Study

protein	subcellular location
Apolipoprotein A1	Extracellular
CA 125	Membrane
CA 15−3	Membrane
Cathepsin L	Extracellular
Epidermal Growth Factor Receptor	Membrane
Fibronectin	Extracellular
Fibulin	Extracellular
Human epididymal protein 4	Extracellular
Inhibin	Extracellular
Interleukin-6	Extracellular
Kallikrein-6	Extracellular
Macrophage-colony stimulating factor	Extracellular
Mesothelin	Extracellular
Osteopontin	Extracellular
α-1 antitrypsin	Extracellular

optimized to produce a linear standard curve. For some candidates, both a monoclonal and a polyclonal antibody were available. For these, sandwich-type immunoassays using both a monoclonal-polyclonal and polyclonal-polyclonal antibody configurations were constructed.

(3) Each ELISA was next tested for its efficacy in detecting endogenous protein. We used ascites fluid that was positive for each candidate as the test sample. Mass spectrometric analysis verified the presence of each candidate in the ascites fluid, as described elsewhere.[27]

(4) Last, we measured each analyte in serial dilutions of serum to examine the relationship between the signal measured and the corresponding dilution. All assays, except IGFBP5, produced linear dilution curves.

Preclinical Validation of Candidates. Since we were unable to establish a workable IGFBP5 immunoassay, we could not validate this candidate in this study. The eight remaining candidate proteins were evaluated using sera from EOC cases ($n = 10$) and normal healthy women ($n = 20$). For 6 of the candidates, there was no significant difference between groups. A significant difference was seen ($p = 0.0002$, Mann−Whitney U test) between the EOC cases and healthy controls for clusterin, with levels in EOC being higher (Figure 5). IGFBP6 was also significantly different ($p = 0.002$, Mann−Whitney U) between the EOC cases and healthy controls, with levels in EOC being lower than the controls.

To examine whether the difference between normals and EOC cases for clusterin and IGFBP6 is due to differences in gene expression, we searched the Oncomine gene expression database[21] for DNA microarray data on these two proteins in ovarian cancer and healthy tissue. Data showing clusterin mRNA expression in healthy and ovarian cancer tissue was not available. However, quantitative real-time PCR results presented by Hough et al. show that clusterin mRNA is overexpressed in ovarian cancer tissue.[28] Data for IGFBP6 showed that mRNA expression is lower in serous ovarian cancer compared to normal ovarian tissue. To further verify our findings, we conducted a search for immunohistochemistry data on both proteins using the Human Protein Atlas (HPA) database (www.proteinatlas.org). A detailed description of the HPA site is presented by Berglund et al.[22] We searched for tissue data using the following search parameters:

(1) Moderate to strong staining in at least 3 patients with ovarian cancer

(2) Negative staining in normal ovaries.

Clusterin showed staining in 5 out of 12 ovarian cancer tissue samples (data is not shown here but is available publicly at the HPA). Normal ovarian tissue showed no staining; however, data was available for the stromal and follicular regions only. IGFBP6 did not pass our search criteria as normal ovarian tissue (stromal tissue) showed weak staining (data available at the HPA).

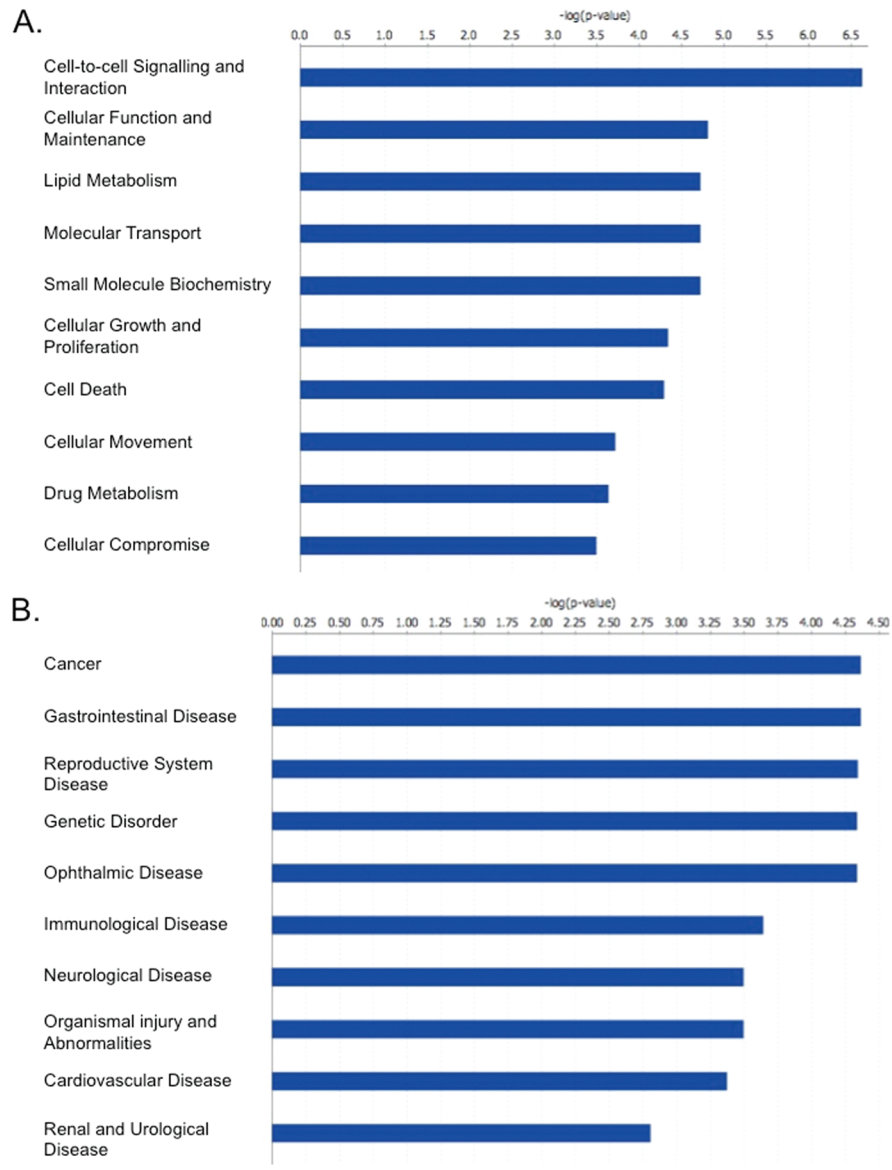

Figure 4. The major biological functions and diseases associated with the 51 candidate proteins. The list of 51 proteins (Supplementary Table 5) was analyzed using Ingenuity Pathway Analysis. The top 10 biological functions (A) and the top 10 diseases (B) associated with the 51 candidates are shown. The negative log of the *P*-value is shown on the *y*-axis. The greater the negative log of the *P*-value, the greater the number of proteins associated with a given function or disease.

Furthermore, in order to elucidate the global cellular functions of these two proteins, we examined clusterin and IGFBP6 using Ingenuity Pathway Analysis. The data showed clusterin to be involved in several biological functions pertinent to tumor pathology including cell development, growth and proliferation, and movement. In addition, the major diseases associated with clusterin were cancer, connective tissue disorders and endocrine disorders. The interactome of clusterin is shown in Supplementary Figure 1. The major biological functions for IGFBP6 were cell movement, growth and proliferation, and cell development. The major diseases associated with IGFBP6 were cancer, skeletal and muscular disorders, and respiratory disease. The interactome of IGFBP6 is shown in Supplementary Figure 2.

Discussion

Serum is a fruitful source of potential markers for ovarian cancer. It contains more than 100 000 protein forms with concentrations in the range of 10−12 orders of magnitude. The 20 most abundant proteins make up 99% of the total protein. The skewed protein distribution in serum is a major challenge when MS-based strategies are used in pursuit of low-abundance cancer biomarkers.[29,30] The main problem is that peptides from high-abundance proteins outcompete their low-abundance peptide counterparts for ionization. On the grounds of this difficulty and more, we opted to analyze conditioned medium from ovarian cancer cell lines, which is less complex than serum, yet is relevant to ovarian cancer pathobiology. Furthemore, cell lines are easy to maintain and propagate, and

Table 4. List of Candidates Tested and Details of Their Corresponding Immunoassay

protein candidate	company	assay type	antibody configuration (capture-detection)[a]
EPCR[b]	R&D Systems	in-house	mono-poly
Vasorin	R&D Systems	in-house	mono-poly
βIG-H3[c]	R&D Systems	Kit	mono-poly
Clusterin	ALPCO	Kit	Competitive ELISA[d]
Cystatin C	R&D Systems	Kit	mono-mono
IGFBP4[e]	DSL Inc.	Kit	poly-poly
IGFBP5	R&D Systems	Kit	mono-poly
IGFBP6	R&D Systems	Kit	mono-poly
IGFBP7	R&D Systems	in-house	mono-poly

[a] Mono for monoclonal antibody, poly for polyclonal antibody. Capture antibody is listed first. [b] EPCR, Endothelial cell protein C receptor (synonym: PROCR, protein C receptor). [c] βIG-H3, Transforming growth factor-beta-induced protein ig-h3. [d] ELISA plate was precoated with clusterin antigen. The assay was a competitive ELISA. [e] IGFBP, Insulin-like growth factor binding protein.

offer an inexhaustible source of mRNA and proteins. This material, in turn, can be rapidly processed for profiling experiments using technologies such as DNA micoarrays and mass spectrometry. In addition, the biological variation between samples from the same cell line is low, thus, allowing greater reproducibility compared to tissue and serum samples. The advantages of using cell culture supernatants for biomarker discovery are described in detail by Kulasingam et al.[31]

In this study, we analyzed the secretome of four cancer cell lines, each representing a histological type of epithelial ovarian cancer. The HTB-75, TOV-112D, TOV-21G, and RMUG-S cells lines are commonly used cell lines in ovarian cancer studies.[32−34] To represent serous carcinoma, we selected the HTB-75 cell line, the proteome of which is similar to tumor cells originating from serous carcinoma of the ovary.[19] The TOV-112D cell line represents the endometrioid histological type. The proteome of this cell line clusters closer to cell lines originating from endometrioid cancers of the ovary than to cell lines originating from other histological types of EOC.[35] The gene expression profile of TOV-21G, which represents clear-cell carcinoma, is different from cell lines originating from other histological types.[36] Thus, we believe that HTB-75, TOV-112D, and TOV-21G provide a distinct look at ovarian cancer. The choice of a mucinous carcinoma cell-line was RMUG-S. Studies comparing the gene or protein expression profile of RMUG-S with mucinous carcinoma of the ovary were not available in the literature. To our knowledge, this study is the first comparative proteomic study conducted using this particular cell line.

A total of 2039 proteins were identified from the four cell lines. Of these, 228 were extracellular and 192 were membrane proteins. The proportion of extracellular and membrane proteins (21%) relative to the total number proteins in this study is lower than for studies conducted on breast (34%)[23] and prostate cancer cell lines (39%);[24] however, this did not prevent us from identifying a large set of potential markers. Furthermore, a large proportion of proteins (29%) with unknown Gene Ontology annotations were identified and categorized as unclassified (Figure 3). Some of these proteins may indeed be extracellular or plasma membrane proteins.

Of the 420 extracellular and membrane proteins identified, 94 were found in plasma by HUPO[26] (see Supplementary Table 3 and Table 4). The small overlap may be due to several reasons. First, some extracellular and membrane proteins identified in this study have low abundance in plasma. With plasma being very complex, it is reasonable to assume that mass spectrometry is unable to identify these proteins. In addition, the elimination half-life for some proteins may be very short, meaning that they are either removed from the circulation rapidly, or are eliminated within their microenvironment before they can enter the circulation. Furthermore, some proteins may be localized to particular compartments or microenvironments in the body and thus never enter the circulation. Finally, some proteins are sensitive to sample handling and therefore are degraded during the experiment.

The Kislinger group[18] and the Hanash group[19] recently published two major proteomic studies using ascites fluid and ovarian cancer cell lines. Approximately, 44% of the proteins identified in our study overlapped with those if the Hanash study, whereas 29% of our proteins overlapped with those found in the Kislinger study (Figure 6). However, comparing just the extracellular and plasma membrane proteins, 75% of our proteins overlapped with those of the Hanash study. Taking all three studies together, a repository of 8256 proteins can be constructed, a valuable resource of proteins for further study in ovarian cancer. We have contributed an additional 1091 proteins that were not identified in the Kislinger and Hanash studies. A point to note is that only 555 proteins (7%) were common to all three studies. This is most likely due to the differences in experimental approach, sample types, the inherent variations in mass spectrometric analysis, and different bioinformatic platforms.

A key issue faced in this study was applying reasonable criteria to choose meaningful candidates. The criteria are dependent on the experimental questions being asked. In our analysis, we were interested in the extracellular and membrane proteins since these are likely to enter the circulation and have a higher chance of being measurable by a sensitive assay such as an ELISA. Therefore, our first criterion was to select extracellular and membrane proteins only. However, a drawback of using cancer cell lines is that they are no longer identical genetically or proteomically to the cancer from which they originate. Therefore, candidates chosen exclusively from a list of proteins secreted or shed by ovarian cancer cell lines may be biologically irrelevant to ovarian cancer. Ascites fluid bathes the ovarian tumor and it is reasonable to assume that some proteins found in ascites fluid originate from the tumor itself or its microenvironment. Therefore, by selecting proteins that are common to both cell lines and ascites fluid, the list can be narrowed to proteins that are biologically relevant to ovarian cancer. Indeed, by applying this criterion, many well-documented markers of ovarian cancer were found in our study including HE4[6,37] and KLK6.[11,38−40] CA-125 was also found in the conditioned media of cell lines, but was not identified in the ascites study to which we compared our list of proteins due to exclusion of proteins greater than 30 kDa.[27] Altogether, our study demonstrates the power of comparing the proteome of cell lines with that of a clinically relevant biological fluid to identify new markers. This strategy is transferable to other cancers such as lung, pancreatic, and liver.

Our final list of candidates included 51 proteins. Ingenuity pathway analysis revealed that two of the major disease types associated with these proteins were cancer and reproduction. This is encouraging given that our aim is to find biological markers of ovarian cancer. In addition, some of the major molecular functions associated with these proteins include cell-to-cell interaction, cellular function and maintenance, and cell

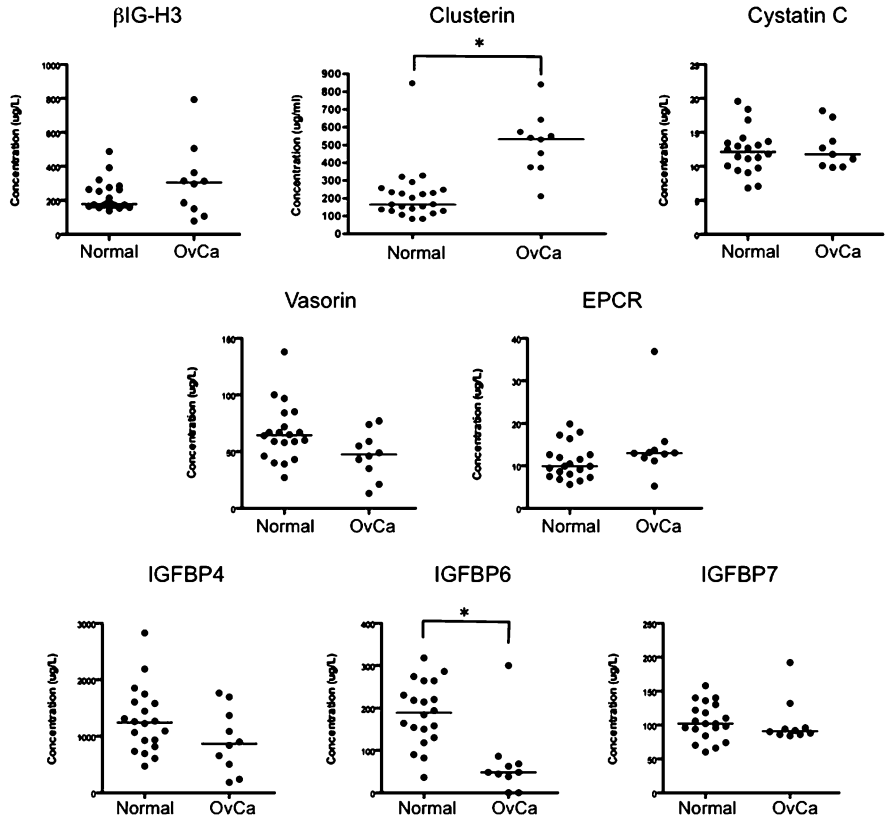

Figure 5. Initial screening results of the 8 candidates tested in serum of EOC patients and healthy individuals. *Normal* designates women without ovarian cancer. *OvCa* designates individual serum samples from EOC cases. Mann−Whitney test was used to calculate *P*-values and comparisons that are significantly different from each other ($p < 0.01$) are indicated with an asterisk (*). Horizontal bar through each data set shows the median.

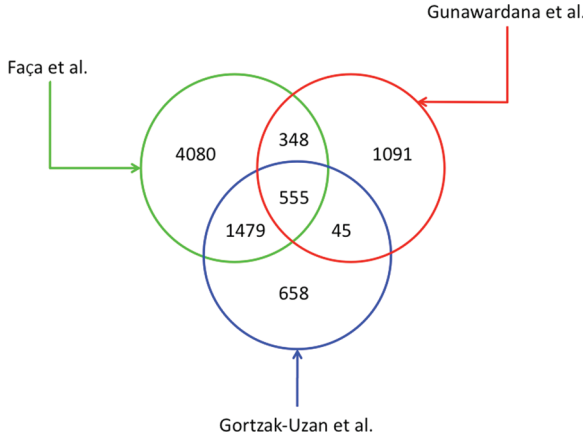

Figure 6. Comparing the proteins identified in this study with those found in other proteomic profiling studies for ovarian cancer. The lists generated by Faca et al. and Gortzak-Uzan et al. were compared with our list. A repository of 8256 proteins can be generated.

growth and proliferation. These functions are known to be important for tumor growth and metastasis.[41]

A major bottleneck in identifying markers using a proteomic approach is candidate validation. It is imperative that good antibodies and immunological assays are developed to evaluate the numerous potential markers identified in studies so far. In our study, some promising candidates could not be studied due to the lack of immunological reagents. We analyzed 8 proteins that had ELISA kits or antibodies available. From this panel, both IGFBP6 and clusterin showed significant differences between EOC cases and healthy individuals.

Current standards imply that a good biomarker is one that is preferably elevated in tissues or biological fluids; clusterin showed such promise as a potential marker. To our knowledge, clusterin levels in the serum of EOC cases have not been reported previously. Regarding the candidates that did not show promise in serum, they cannot be dismissed since their role in the pathogenesis of ovarian cancer needs to be determined. Although clusterin showed promise, its effectiveness as a marker for early detection still remains an open question. Our initial screen did not use serum from early stage EOC, and therefore, the use of clusterin to detect early stage EOC cannot be ascertained. In addition, the sample size ($n = 30$) in this study is relatively low. Therefore, further studies to test clusterin need a large cohort ($n > 100$) with a substantial number of early stage cancer patients.

Immunohistochemistry data available from the HPA show that clusterin is expressed in greater amounts in ovarian cancer tissue relative to healthy tissue. The immunohistochemistry data is supported by the gene expression data published by Hough et al.[28] showing upregulated clusterin mRNA in ovarian cancer. The results of our ELISA are concordant with the studies mentioned above. These results raise the question as to why clusterin is upregulated in ovarian cancer. Clusterin is important in several cellular functions including apoptosis,[42,43] cell migration,[44,45] and cell development.[46] Recent evidence suggest that clusterin may be involved in ovarian cancer-related processes. Findings by Park et al.[47] showed that high clusterin expressing ovarian cancer cells are resistant to Paclitaxel and

that high clusterin expression correlated with poor survival. Further studies are ongoing to understand the pathobiological role of clusterin in ovarian cancer.

Abbreviations: ACN, acetonitrile; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EOC, epithelial ovarian cancer; GO, gene ontology; HUPO, Human Proteome Organization; HPA, Human Protein Atlas; IPI, international protein index; KLK5, kallikrein 5; MS, mass spectrometry; SCX, strong cation exchange chromatography; SFM, serum-free media; TFA, trifluoroacetic acid.

Acknowledgment. This work was supported by a University-Industry grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Proteomic Methods, Inc., Toronto, Canada. We thank Dr. Vathany Kulasingam and Dr. Girish Sardana for their advice with the design of this project. We also thank Ioannis Prassas and Uros Kuzmanov for helpful discussions.

Supporting Information Available: Single peptide identifications mass spectra; figures of proteins that interact with clusterin and with IGFBP6. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR900411G