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Identification of candidate circulating cisplatin-resistant biomarkers from epithelial ovarian carcinoma cell secretomes

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Background: The majority of patients diagnosed with advanced epithelial ovarian carcinoma (EOC) relapse with resistant disease, and there are no biomarkers that possess clinical utility to identify or monitor these patients. This study aimed to identify secreted proteins ('secretome') collected from human EOC cell lines that differ in their inherent platinum sensitivity.

Methods: Secreted proteins collected from conditioned medium from ovarian cancer cell lines that vary in their sensitivity to cisplatin were digested with trypsin and analysed by liquid chromatography-tandem mass spectrometry for peptide identification.

Results: Of the 1688 proteins identified, 16 possessed significant differential abundances ($P < 0.05$) between the platinum-resistant and -sensitive cell lines. A number of these were verified by immunoblot, including COL11A1, which was also found to be associated with worse progression-free survival (PFS; $N = 723$) and overall survival (OS; $N = 1183$) as assessed from publicly available transcript expression data from ovarian cancer tumour specimens.

Conclusion: Secretome proteomics of EOC cells resulted in the identification of a novel candidate biomarker, COL11A1. The expression level of COL11A1 correlates to worse PFS and OS, and is predicted to reside in peripheral circulation making this an attractive candidate for validation in sera as a biomarker of cisplatin resistance and poor outcome.

Epithelial ovarian carcinoma (EOC) is one of the top 10 most frequently occurring cancers in women with an estimated 22 240 new cases in the United States in 2013, 14 030 of whom are predicted to succumb to their disease (Siegel *et al*, 2013). Epithelial ovarian carcinoma is the most lethal gynaecological malignancy largely attributable to its typical late stage (stage III or IV) diagnosis (Heintz *et al*, 2006). Advanced-stage EOCs are characterised by extensive tumour heterogeneity, genomic instability and defects in p53 signalling and homologous recombination (The Cancer Genome Atlas Research Network, 2011). Current front-line therapies include cytoreductive surgery followed by adjuvant platinum- and taxane-based chemotherapy. Despite the

high initial response to primary therapy, a high proportion of patients relapse with platinum-resistant disease, which represents a major hurdle in successful management of ovarian cancer patients.

Platinum-based drugs are among the most active and commonly used chemotherapy agents for solid tumors including ovarian cancer, lung cancer and colorectal cancer. Platinum drugs, such as cisplatin and carboplatin, are alkylating-like agents that adduct DNA resulting in intra- and inter-strand crosslinks that accumulate to produce overwhelming levels of DNA damage, and ultimately apoptosis. The mechanisms underlying platinum resistance are manifold and poorly understood, but likely involve reduced uptake of and increased inactivation of the drug, increased

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DNA repair capacity and modulation of apoptosis evasion mechanisms (Siddik, 2003; Galluzzi *et al*, 2012). Identification and clinical validation of surrogate biomarkers of platinum resistance would represent a major advance in ovarian cancer patient care; however, no such biomarkers have yet been identified.

Unfortunately, serum-based proteomic biomarker discovery efforts have been met with serious limitations due to the high dynamic range of serum proteins (Anderson *et al*, 2004; Hanash *et al*, 2008). These limitations prompted discovery strategies from alternative sample sources, such as conditioned media or proximal fluids (Faca *et al*, 2008; Gortzak-Uzan *et al*, 2008; Gunawardana *et al*, 2009; Kuk *et al*, 2009). This rationale is supported by the frequent observation that secreted and/or shed proteins from the tumour microenvironment, such as CA-125, are often present at higher relative concentrations in proximity to the tumour site than in peripheral circulation (Sedlacek *et al*, 2002). Therefore, characterisation of secreted proteins from tumour cells may provide an ideal source to identify candidate protein biomarkers for validation in serological samples.

In this study, conditioned media was harvested from three papillary serous ovarian cancer cell lines (CaOV3, OVCAR3 and OV90) along with that from an undifferentiated EOC cell line A2780 and its isogenic, yet cisplatin-resistant daughter cell line A2780-CP20. Proteins harvested from the conditioned media from these cell lines were digested and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the relative abundances of the identified proteins were determined by spectral counting, ultimately resulting in 16 proteins that possessed significant differential abundances (*Z*-statistic test, $P < 0.05$) between the platinum-resistant and -sensitive cell lines. Proteins that were elevated in the conditioned medium from platinum-resistant EOC cells were further evaluated for potential clinical utility by determining the relationship between the level of candidate biomarkers and measures of clinical outcome including progression-free survival (PFS) and overall survival (OS) in ovarian cancer patients using publicly available transcript expression data with corresponding clinical information (Ganzfried *et al*, 2013). Of the candidates identified from the secretome analysis, higher than median expression of collagen, type XI, alpha 1 chain (COL11A1) was associated with worse PFS and OS.

MATERIALS AND METHODS

Cell lines and tissue culture conditions. The human EOC cell lines A2780 and A2780-CP20 were purchased from the European Collection of Cell Cultures (Salisbury, UK). A2780 and A2780-CP20 cells were maintained in RPMI-1640 media supplemented with 10% foetal bovine serum albumin (FBS; ATCC, Manassas, VA, USA). The human epithelial OVCAR3 cell line, established from malignant ascites of a patient with progressive ovarian adenocarcinoma and resistant to clinically relevant concentrations of adriamycin, melphalan and cisplatin, was obtained from ATCC. OVCAR3 was maintained in RPMI-1640 media with 20% FBS and 0.01 mg ml⁻¹ human insulin (Sigma-Aldrich, St Louis, MO, USA). CaOV3 and OV90 were also obtained from ATCC and were cultured in DMEM media containing 10% FBS. All cells were cultured in monolayer using 75 cm² tissue culture flasks and maintained at 37 °C supplemented with 5% CO₂. The identities of all cell lines used were verified by short tandem-repeat testing and were confirmed to be mycoplasma-free (Universal Mycoplasma Detection Kit; ATCC, Manassas, VA, USA).

Cell proliferation assay. Cells were trypsinised with 0.25% trypsin-EDTA (ATCC) and counted with 0.4% trypan blue using a TC10 automated cell counter (Bio-Rad, Hercules, CA, USA). Cells were seeded in 96-well plates with 5000–10 000 cells per well.

Once cells were attached (~24 h post seeding), media was removed and replaced with fresh media containing cisplatin (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM). An MTS cell proliferation assay was performed 72 h post treatment using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 20 μl of the CellTiter 96 Aqueous One Solution reagent was added to each well and incubated at 37 °C for 2 h. Absorbance at 490 nm was measured using the xMark microplate spectrophotometer (Bio-Rad), and the CompuSyn software (ver 3.0.1; ComboSyn, Inc., Paramus, NJ, USA) was used to obtain the half maximal inhibitory (IC₅₀) values for each cell line.

Secretome collection and sample preparation. Ovarian cancer cells were cultured in conditions as appropriate until they were ~70% confluent (15 × 10⁶ cells per 75 cm²) and washed three times with 10 ml of serum- and phenol red-free RPMI-1640 media to remove residual FBS. To collect the secretome, cells were incubated with 10 ml serum- and phenol red-free RPMI-1640 media for 24 h at 37 °C supplemented with 5% CO₂. For each cell secretome sample, cultured media (secretome) was pooled from three 75 cm² tissue culture flasks and centrifuged at 500 g at 4 °C to remove any floating debris. A protease and phosphatase inhibitor cocktail (Halt, Thermo Fisher Scientific Inc., Rockford, IL, USA) and 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) were added to the secretome samples. The secretome samples were buffer-exchanged with 25 mM ammonium bicarbonate (AMB; Thermo Fisher Scientific Inc., Fair Lawn, NY, USA) and concentrated to 500 μl using 3 K MWCO Ultra Centrifugal Filters (Amicon Millipore, Billerica, MA, USA). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., San Jose, CA, USA). Secretome samples were maintained at -80 °C before sample preparation for proteomic analysis or immunoblotting.

Ten micrograms of proteins harvested from conditioned medium from A2780 and A2780-CP20 cells were electrophoresed briefly into the stacking portion of a one-dimensional gel (4–15% Mini-PROTEAN TGX gel, Bio-Rad). The gel was stained for 10 min with Coomassie (SimplyBlue SafeStain; Invitrogen, Carlsbad, CA, USA) and the stained gel bands containing the entire 'secreted proteome' were excised and destained in 50% acetonitrile (ACN; Sigma-Aldrich) and 25 mM AMB at ambient temperature on a vortex platform for 1 h. Gel bands were dehydrated in 100% ACN and incubated in 25 mM AMB, 10 mM dithiothreitol (Sigma-Aldrich) at 56 °C for 30 min to reduce disulphide bonds of reactive cysteine residues, followed by alkylation with 25 mM AMB and 45 mM iodoacetamide (Sigma-Aldrich) in darkness for 45 min at ambient temperature. Gel slices were dehydrated in 100% ACN and rehydrated with 20 ng μl⁻¹ sequencing-grade modified trypsin (Promega), and 25 mM AMB on ice for 30 min. Excess trypsin was removed, gel bands were washed with 500 μl of 25 mM AMB and in-gel digestion was performed at 37 °C for 16 h. Tryptic peptides were extracted with 70% ACN and 5% formic acid (Sigma-Aldrich), lyophilised by vacuum centrifugation and resuspended in 25 mM AMB. Samples were centrifuged at 16 000 g to remove particulates, and supernatant was stored at -80 °C.

Liquid chromatography-tandem mass spectrometry. Seven replicate injections (1 μg each) of each secretome digest were analysed by nanoflow LC (Easy-nLC; Thermo Fisher Scientific Inc., San Jose, CA, USA) coupled online with an LTQ-Orbitrap Velos MS (Thermo Fisher Scientific Inc., San Jose, CA, USA). Following system equilibration, each sample was loaded onto a 2-cm reversed-phase (C18) vented pre-column (Thermo Fisher Scientific Inc., San Jose, CA, USA) at 3 μl min⁻¹ for 6 min with mobile phase A (0.1% formic acid in water). Peptides were resolved on a 100 μm inner diameter × 360 μm outer diameter × 200-mm long

capillary column (Polymicro Technologies, Phoenix, AZ, USA) slurry packed in house with 5 μ m diameter, 100 Å pore size reversed phase (Magic C18 AQ, Bruker Michrom Bioresources, Auburn, CA, USA) at a constant flow rate of 200 nl min⁻¹ by development of a linear gradient of 0.33% mobile phase B (0.1% formic acid in ACN) per min for 120 min. The column was washed for 15 min at 95% mobile phase B and then equilibrated to 100% mobile phase A before the next sample injection. The LTQ-Orbitrap Velos MS was configured to collect high resolution ($R = 60\,000$ at m/z 400) broadband mass spectra (m/z 375–1800) using the lock mass feature for the polydimethylcyclsiloxane ion generated in the electrospray process (m/z 445.12002) from which the 20 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS. MS conditions were set as follows: electrospray voltage, 1.9 kV; no sheath and auxiliary gas flow; capillary temperature, 220 °C; and S-Lens RF level, 69%. The ion selection threshold for the Orbitrap (MS) was set at 1.0×10^6 with a maximum ion accumulation time of 500 ms. Tandem MS were collected in the high-pressure linear ion trap (MS/MS) with the following settings: ion threshold, 5000; minimum intensity, 3000; maximum ion accumulation time, 25 ms; and activation time, 10 ms. Dynamic exclusion (60 s) was used to minimise redundant selection of peptides for MS/MS.

Peptide identification and spectral counting. Tandem mass spectra were searched against the UniProt human protein database (downloaded on 25/10/2012, 68 109 sequences) from the European Bioinformatics Institute (<http://www.ebi.ac.uk/integr8/>) using the Mascot Daemon (Matrix Science Inc., Boston, MA, USA). The data were searched with a precursor mass tolerance of 10 p.p.m. and a fragment ion tolerance of 0.6 Da. Cysteine carbamidomethylation (m/z 57.021464) was set as a fixed modification and methionine oxidation (m/z 15.99492) was set as a dynamic modification, and a maximum of two missed tryptic cleavages were allowed. Identified peptides were filtered using an ion score cutoff of 33 resulting in a false peptide discovery rate of <1% for all peptides identified (determined from an automatic decoy database search). Peptides whose sequence mapped to multiple protein isoforms were grouped as per the principle of parsimony (Marengo *et al*, 2010). Total peptide spectral matches (spectral counts) were derived by summing the results for each UniProt accession from each of the seven replicate LC-MS/MS analyses for each secretome sample, and normalisation was performed using the lowest total peptide spectral matches value across all samples in the comparison group. A value of 0.5 was added to each spectral count value before log₂ transformation to enable ratio values to be calculated for proteins identified in one cell line but not another (McDonald, 2009).

Statistical analyses for proteomics data. Statistical analyses for the enrichment of specific proteins among differentially expressed proteins were performed using a Z-statistic test on the log₂-transformed spectral count ratios. The Z-statistic test was used to calculate P-values with the minimum significance level set to $P < 0.05$. Primary accessions, with isoform designations removed, were used when comparing EOC secretome protein identifications to other published data sets. Secretome proteins identified from the cisplatin-resistant A2780-CP20 cell line were also compared with those identified from cisplatin-sensitive A2780, CaOV3 and OVCAR3 cell lines to derive mutually significant differentially abundant proteins between the sensitive and resistant phenotypes.

Immunoblot analyses. Thirty micrograms of secretome were resolved by 4–15% mini-PROTEIN TGX gels (Bio-Rad) and transferred to PVDF membranes using Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's protocol. Membranes were blocked for 1 h at ambient temperature in 5% blocking grade non-fat dry milk (Bio-Rad), 0.1% Tween-20, and $1 \times$ tris-buffered saline. Membranes were incubated with primary

antibody overnight at 4 °C with gentle rocking, washed three times for 15 min in 0.1% Tween-20, $1 \times$ tris-buffered saline and incubated with secondary antibody for 1 h at ambient temperature with gentle rocking. Primary antibodies used were as follows: anti-matrix-degrading metalloproteinases (MMP)1 (1:1000, clone H00004312-B01P; Abnova, Taipei, Taiwan), anti-MMP10 (1:1000, clone H00004319-B01P; Abnova), anti-CTH (1:1000, clone HPA023300; Sigma-Aldrich) and anti-Col11A1 (1:500, clone NBP1-55803; Novus Biologicals, Littleton, CO, USA). The secondary antibodies were goat anti-rabbit IgG HRP-linked (1:2000, clone 7074S, Cell Signaling, Beverly, MA, USA) and goat anti-mouse IgG (H+L)-HRP (1:2000, clone 31430, Thermo Fisher Scientific Inc., San Jose, CA, USA). Membranes were washed three times for 15 min in 0.1% Tween-20, $1 \times$ tris-buffered saline and incubated with SuperSignal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific, San Jose, CA, USA) for 5 min. Images were acquired using a ChemiDoc XRS+ system (Bio-Rad). Membranes were stained with Ponceau S (Sigma-Aldrich) to verify loading equivalency.

Statistical analysis of public gene expression and patient outcome data. CuratedOvarianData, a manually curated data collection by Ganzfried *et al* (2013), were downloaded on 23 April 2013 from the web site (<http://bcf.dfc.harvard.edu/ovariancancer>) using the statistical computing language 'R' with the Bioconductor package. It included 23 data sets from 11 platforms and provided curated gene expression and clinical data for a total of 2970 samples. In this collection, there were 15 data sets from Affymetrix platforms. From a total of 2970 cases with public gene expression data, 1183 cases meet the eligibility criteria for this candidate gene analysis. Log₂-transformed gene expression data must be available from frozen primary tumors using one of three platforms (Affymetrix HG-U133A, Affymetrix HG-U133 Plus 2.0 and Affymetrix HG-HT U133A) from women with a diagnosis of invasive serous adenocarcinoma of the ovary and survival data. Data from cell lines, normal human surface epithelium, breast cancer metastasis, unknown samples, borderline tumors, non-serous ovarian cancers and patients who died within 2 weeks of diagnosis were excluded from the analysis. Data from Agilent platforms or from formalin-fixed and paraffin-embedded tumors were also excluded. Disease site, cell type, tumour stage and survival data including survival status and survival time in months from diagnosis were available on all of the eligible patients ($N = 1\,183$). PFS data was available in a subset ($N = 723$). A total of 22 277 probe sets (representing 13 435 unique genes) had expression data across the eligible 1183 samples in data sets from six sources (GSE14764, GSE18520, GSE26712, GSE9891, PMID17290060 and TCGA) (Dressman *et al*, 2007; Bonome *et al*, 2008; Tothill *et al*, 2008; Denkert *et al*, 2009; Mok *et al*, 2009; The Cancer Genome Atlas Research Network, 2011). The combat method was implemented in the Surrogate Variable Analysis Bioconductor package (R package version 3.6.0; www.bioconductor.org). SVA was used to combine log₂-transformed gene expression data from the six data sets to adjust for the between-experiments batch effects.

Statistical analysis of the clinical relevance of the candidate biomarkers was performed using R and SPSS v. 20 (IBM Corp., Pittsburgh, PA, USA). Cox regression analysis was performed to evaluate the relationship between expression of candidate genes of interest and PFS or OS with unadjusted hazard ratios (HRs) and 95% confidence intervals (CIs) estimated for each unit increase in average gene expression for each of the candidate biomarkers. Cox regression modelling was also performed with expression levels categorised at the median without and with adjustments for tumour stage. Kaplan–Meier method was used to estimate PFS and OS probabilities for average gene expression data categorised at the median as low (\leq median) vs high ($>$ median). Differences in PFS

and OS distributions between groups were assessed using the log-rank test. The Mantel–Haenszel common odds ratio and 95% CI were estimated to examine the relationship between candidate genes categorised at the median and tumour stage. The association between categorised expression of an individual gene and stage was examined using Fisher’s exact test. All tests were two-sided.

RESULTS

Secretome collection. The half maximal inhibitory (IC₅₀) values for each EOC cell line were determined before the collection and secretome proteomic analysis as follows (listed from the most platinum sensitive to most platinum resistant): OVCAR3, IC₅₀ = 3.9 μM; A2780 IC₅₀ = 4.2 μM; CaOV3, IC₅₀ = 5.0 μM; OV90, IC₅₀ = 11.2 μM; and A2780-CP20 IC₅₀ = 23.3 μM. Secretome samples were harvested at 24 h after incubation in serum-free media when all cell lines displayed healthy morphologies and none of the cultures had evidence of free, floating or dead cells. Thirty millilitres of conditioned medium yielded ~ 500 μg of total protein for each cell line.

Protein identification from ovarian carcinoma cell line secretomes. The LC-MS/MS analyses yielded a total of 160 411 peptide identifications representing 1983 aggregate proteins from the five EOC cell line (A2780, A2780-CP20, CaOV3, OVCAR3 and OV90) secretome samples (Supplementary Tables 1 and 4). The number of peptides and proteins identified from each cell line is shown in Table 1. There were a total of 1688 proteins identified with two or more peptide sequences in any of the five EOC secretome samples, among which 269 were identified in common across the secretome samples from these five cell lines (Figure 1). The spectral count values were used to identify statistically significant alterations in protein abundances from the secretome of the cisplatin-sensitive A2780 cell line compared with the isogenic cisplatin-resistant daughter cell line (A2780-CP20), where 205 proteins were found to be differentially abundant (*P* < 0.05, Supplementary Table 2, Supporting Information), of which 67 proteins were elevated in the secretome from A2780-CP20 as compared with A2780. The proteins identified in the secretome from A2780-CP20 were also compared with those from OVCAR3 and CaOV3 (both having similar cisplatin IC₅₀ values as the A2780 cells). Sixteen proteins were identified as differentially abundant in platinum-sensitive cells (A2780, CaOV3 and OVCAR3) compared with platinum-resistant cells (A2780-CP20) (Figure 2, Table 2 and Supplementary Table 3).

Evidence of biological and translational potential. Public proteomic and gene expression data were used to further

interrogate the candidate biomarkers from our secretome analyses to determine whether the secretome from human ovarian cancer cell lines represents a rich source for candidate secreted or shed proteins with biological and translational potential as candidate circulating biomarkers. The *in vitro* EOC secretome data were compared with the human plasma proteome (Farrah *et al*, 2011) and the human-derived protein entries in the secreted protein database (Figure 3A and Table 2) (Chen *et al*, 2005). Overall ~60% of the secretome proteins identified in this investigation were also identified in the human plasma proteome and/or were annotated in the secreted protein database, strongly suggesting that the secretome from *in vitro* cultures are viable and valuable sources

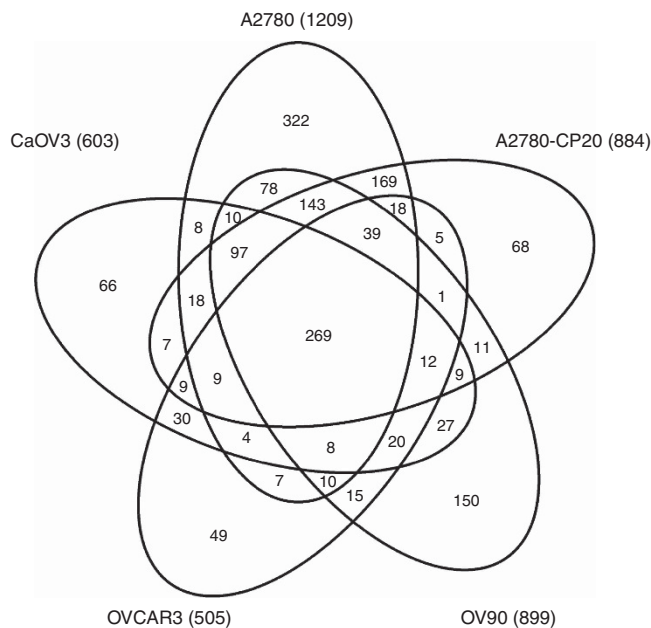


Figure 1. Venn diagram of 1688 secretome proteins identified with two or more spectral counts from five EOC cell lines.

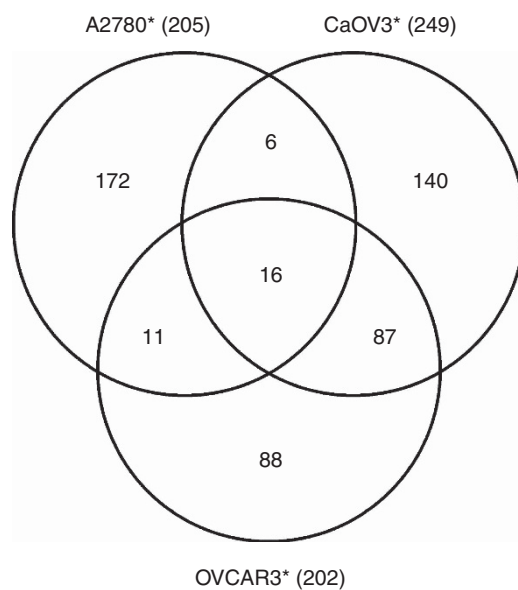


Figure 2. Venn diagram of significant differential proteins between cisplatin-resistant A2780-CP20 and cisplatin-sensitive A2780, OVCAR3 and CaOV3, where 16 mutually differential proteins were identified. *Significant differential proteins compared with A2780-CP20.

Table 1. Numbers of peptides and proteins identified from the EOC secretome

Secretome sample	Total peptide sequences identified	Unique peptide sequences identified	Total protein accessions identified	Protein accessions identified with ≥ two unique peptides
A2780	46689	6802	1396	1209
A2780-CP20	32840	4738	1046	884
CaOV3	20643	2955	712	603
OV90	38558	5654	1060	899
OVCAR3	21681	2564	607	505

Abbreviation: EOC = epithelial ovarian carcinoma.

Table 2. Sixteen secretome proteins identified with significant differential abundances ($P < 0.05$) between cisplatin-sensitive (A2780) and cisplatin-resistant A2780-CP20) cells compared with publicly available proteomic data sets and secreted/plasma databases

Differentially abundant proteins in cisplatin-resistant (A2780-CP20) relative to cisplatin-sensitive (A2780) ovarian cancer cells	Uniprot accession	Secretome analysis						Human plasma proteome ^b	Human secreted proteome database ^c	Hoskins et al (2011)		Faca et al (2008)		
		OVCAR3	A2780	CaOV3	OV90	A2780-CP20	TIF			Ascites	OVCAR3	CaOV3	Ascites	
Increased														
3-ketoacyl-CoA thiolase (ACAA2)	P42765	0 ^a	0	0	0	22	X		0	0	0	0	15	
Cerebellin-2 (CBLN2)	Q8IU88	0	8	0	0	58			0	0	0	0	0	
Collagen alpha-1(II) chain (COL2A1)	P02458	0	0	0	0	35		X	0	0	1	5	0	
Collagen alpha-1(XI) chain (COL11A1)	P12107	0	0	0	0	212		X	0	0	0	0	0	
Cystathionine gamma-lyase (CTH)	P32929	0	3	0	7	40			0	0	0	1	0	
Inhibin beta A chain (INHBA)	P08476	0	0	0	0	64			0	0	0	0	6	
Interstitial collagenase (MMP1)	P03956	0	5	0	48	189		X	0	0	0	0	2	
Stromelysin-1 (MMP3)	P08254	0	60	0	0	944		X	0	0	0	0	0	
Stromelysin-2 (MMP10)	P09238	0	109	0	16	740		X	0	0	0	0	0	
Pappalysin-1 (PAPPA)	Q13219	0	0	0	0	23			0	0	0	0	0	
Protocadherin-10 (PCDH10)	Q9P2E7	0	0	0	0	31			0	0	0	0	0	
Semaphorin-3D (SEMA3D)	O95025	0	0	0	0	63			0	0	0	0	0	
Torsin-1B (TOR1B)	O14657	0	2	0	2	19		X	0	0	0	0	0	
Decreased														
Aggrin (AGRN)	O00468	465	24	108	304	1	X	X	15	2	277	60	109	
D-3-phosphoglycerate dehydrogenase (PHGDH)	O43175	39	144	12	76	0	X		58	0	50	8	11	
Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1)	P09936	17	35	36	0	0	X		38	0	14	27	2	

Abbreviation: TIF = tissue interstitial fluid.
^aSpectral count.
^bFarrah et al, 2011.
^cChen et al, 2005.

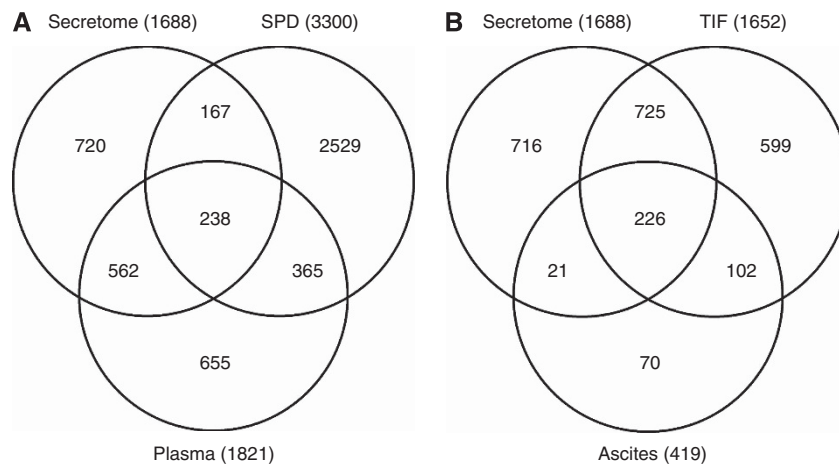


Figure 3. Comparison of proteins identified from the current ovarian cancer cell secretome analyses to publicly available databases/data sets, including (A) the secreted protein database (SPD) and the Human Proteome Organization (HUPO) plasma proteome; and (B) TIF and ascites from ovarian cancer patients.

of candidate circulating biomarkers in humans. Of the 16 most differential proteins in our secretome analysis of cisplatin-sensitive compared with cisplatin-resistant cells, four were also found in the human plasma proteome (ACAA2, Agrin (AGRN), D-3-phosphoglycerate dehydrogenase (PHGDH) and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1)) and eight were also detected in the secreted protein database (COL2A1, COL11A1, MMP1, MMP3, MMP10, TOR1B and AGRN). AGRN, an extracellular matrix (ECM) protein that was observed to be lower in abundance in the cisplatin-resistant A2780-CP20 secretome, was present in both the human plasma proteome and the secreted proteome databases (Table 2). Matrix-degrading metalloproteinases have important roles in ECM remodelling and tumour microenvironment dynamics (Sole *et al*, 2004). Of the three MMPs elevated in the platinum-resistant A2780-CP20, MMP3 was also detected in the human secreted proteome database and has been shown to degrade AGRN.

We then compared our EOC secretome results with previously published data from proximal fluid studies, including those derived from proteomic analyses of ascites and tissue interstitial fluid (TIF) collected from papillary serous EOC patients (Figure 3B and Table 2) (Hoskins *et al*, 2011). Ascites is fluid in the peritoneal cavity that commonly accumulates in ovarian cancer patients and represents a complex milieu of detached ovarian cancer cells and soluble protein (Gortzak-Uzan *et al*, 2008; Kuk *et al*, 2009; Hoskins *et al*, 2011), whereas TIF is harvested by *ex vivo* incubation of finely diced solid tumors in buffered solutions (Celis *et al*, 2005; Teng *et al*, 2010; Teng *et al*, 2011). Approximately 60% of the secretome proteins identified from EOC cells in this study have been previously reported from proteomic studies of ascites and/or *ex vivo* TIF. When comparing the 16 differentially abundant secretome proteins from platinum-sensitive vs platinum-resistant EOC cells to those observed in TIF and ascites, AGRN was observed in both ascites and TIF, whereas UCHL1 and PHGDH were observed only in TIF. UCHL1 and PHGDH were more abundant in the secretome from platinum-sensitive EOC cells. UCHL1, a deubiquitinating enzyme, has been suggested to be a tumour suppressor in multiple cancers, including ovarian cancer (Okochi-Takada *et al*, 2006; Yu *et al*, 2008; Seliger *et al*, 2009), that

is silenced by promoter methylation (Okochi-Takada *et al*, 2006; Yu *et al*, 2008; Seliger *et al*, 2009). In addition, UCHL1, which induces G₀/G₁ cell cycle arrest and apoptosis by stabilising p53,

Table 3. Clinical characteristics for invasive serous ovarian cancer patients with progression-free survival and/or overall survival data

	Progression-free survival data		Overall survival data	
	Cases	Percent	Cases	Percent
Age at diagnosis (years)				
<50	139	19.2	185	15.6
50–59	249	34.4	292	24.7
60–69	173	23.9	242	20.5
70–79	138	19.1	190	16.1
80+	24	3.3	34	2.9
Missing			240	20.3
Site				
Ovarian	723	100	1183	100
Stage				
I	31	4.3	34	2.9
II	37	5.1	41	3.5
III	564	78.0	951	80.4
IV	91	12.6	154	13.0
Advanced (III or IV)			3	0.3
Cell type				
Serous adenocarcinoma	723	100	1183	100
Grade				
1	20	2.8	26	2.2
2	134	18.5	219	18.5
3	556	76.9	921	77.9
Missing	13	1.8	17	1.4
Debulking status				
Optimal	470	65.0	689	58.2
Suboptimal	179	24.8	348	29.4
Missing	74	10.2	146	12.3
Data source				
GSE14764 (Affy U133A) ^a			68	5.7
GSE18520 (Affy U133 Plus 2.0) ^b			53	4.5
GSE26712 (Affy U133A) ^c			185	15.6
GSE9891 (Affy U133 Plus 2.0) ^d	213	29.5	216	18.3
PMID17290060 (Affy U133A) ^e			116	9.8
TCGA (Affy HT U133A) ^f	510	70.5	545	46.1
Events				
Alive NED	232	32.1	299	25.3
Alive with progression	138	19.1	190	16.1
Alive with unknown progression status			63	5.3
Deaths	353	48.8	631	53.3

^aDenkert *et al*, 2009.
^bMok *et al*, 2009.
^cBonome *et al*, 2008.
^dTothill *et al*, 2008.
^eDressman *et al*, 2007.
^fThe Cancer Genome Atlas Research Network, 2011.



Figure 4. Immunoblotting verification of selected target secretome proteins. MMP1, MMP10, CTH and COL11A1 were selected for validation by western blotting in EOC secretome.

was shown to be expressed in normal breast cell lines and tissues but downregulated or silenced in breast cancer cell lines and tissues (Xiang *et al*, 2012). PHGDH, amplified in melanoma and breast cancer, was demonstrated to contribute to carcinogenesis by metabolic transformation specifically through diversion of glycolytic flux (Locasale *et al*, 2011). Liu *et al* (2013) showed that PHGDH was over expressed in aggressive glial brain tumors and provided evidence of a non-metabolic role in promoting tumorigenesis. Our results showed a decrease of PHGDH in the cisplatin-resistant ovarian cancer cells compared with the sensitive cells.

Finally, we compared our *in vitro* secretome results in the cisplatin-sensitive CaOV3 and OVCAR3 ovarian cancer cells with those published by Faca *et al* (2008) in the same cell lines and found an overlap of 61% and 70% of the proteins, respectively. When we focused on the 16 candidate biomarkers, AGRN, PHGDH and UCHL1 were the most abundant proteins observed in secretome samples from CaOV3 and OVCAR3 in our study and that reported by Faca *et al* (2008) (Table 2). In addition, both studies provide confirmation that the cisplatin-sensitive CaOV3 and OVCAR3 did not secrete or shed detectable levels of 11 biomarkers and only low levels of two others (Faca *et al*, 2008). Of note, COL11A1 was only identified in this study from the secretome of the cisplatin-resistant cell line, A2780-CP20. Faca *et al* (2008) also studied the secretome harvested from ovarian cancer ascites cells and detected three candidate biomarkers associated with cisplatin sensitivity (ACAA1, INHBA and MMP1) and three associated with cisplatin resistance (AGRN, PHGDH and UCHL1), suggesting that the patient may have had a heterogeneous cancer made up of clonally distinct populations of sensitive and resistant tumour cells (Table 2).

Immunoblot verification. From the panel of 16 differentially abundant secretome proteins from platinum-sensitive compared with platinum-resistant EOC cells (Table 2), MMP1, MMP10, CTH and COL11A1 were selected for verification by immunoblot in independent biological replicate samples based on known mechanisms of action and relevance in cancer. These secretome immunoblots unequivocally verify the spectral count-based proteomic findings from all five EOC cell lines (Figure 4).

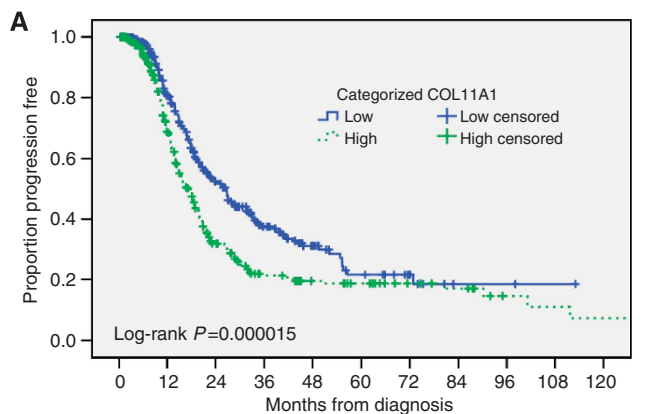
Evidence of clinical relevance. Public gene expression data was used to further interrogate the four candidate biomarkers from our *in vitro* secretome analysis that passed immunoblot verification to evaluate the clinical relevance of some of the candidate biomarkers. Table 3 provides the clinical characteristics for the eligible 1183 women with invasive serous ovarian cancer, batch corrected Affymetrix gene expression data from primary tumors with PFS ($N=723$) and survival data ($N=1183$) from six sources (Dressman *et al*, 2007; Bonome *et al*, 2008; Tothill *et al*, 2008; Denkert *et al*, 2009; Mok *et al*, 2009; The Cancer Genome Atlas Research Network, 2011). Cox regression analysis was initially performed to evaluate the relationship between expression of the four candidate biomarkers of interest (*MMP1*, *MMP10*, *CTH* and *COL11A1*) and risk of disease progression and death for each unit increase in average gene expression across a probe set (Table 4). Among the candidates evaluated, *COL11A1* was strongly associated with both PFS and OS. Higher levels of *COL11A1* expression were associated with an increased risk of disease progression (unadjusted HR = 1.078, 95% CI = 1.035–1.124, $P=0.0003$) and death (unadjusted HR = 1.070, 95% CI = 1.035–1.106, $P=7 \times 10^{-5}$). The associations between higher expression of *COL11A1* and worse PFS (adjusted HR = 1.053, 95% CI = 1.010–1.097, $P=0.015$) and OS (adjusted HR = 1.059, 95% CI = 1.024–1.094, $P=0.001$) held after adjusting for tumour stage.

Analyses were then performed with expression levels categorised at the median. Women with primary tumors with high (>6.8722) relative to low (≤ 6.8722) transcript expression values of *COL11A1* had worse PFS (Figure 5A, log-rank $P=1.5 \times 10^{-5}$) and OS (Figure 5B, log-rank $P=0.002$). High *COL11A1* expression was also associated with an increased risk of disease progression (unadjusted HR = 1.534, 95% CI = 1.261–1.866, $P=2 \times 10^{-5}$) and death (unadjusted HR = 1.287, 95% CI = 1.1–1.505, $P=0.002$). The association between categorised expression of *COL11A1* and PFS or OS remained statistically significant even after adjusting for tumour stage (Table 5, adjusted HR = 1.237, 95% CI = 1.058–1.448, $P=0.008$ or adjusted HR = 1.373, 95% CI = 1.126–1.674, $P=0.002$, respectively). Women with high *COL11A1* expression were also shown to have an increased chance of having advanced-stage disease (odds ratio = 3.978, 95% CI = 2.261–6.998, $P=1.7 \times 10^{-6}$).

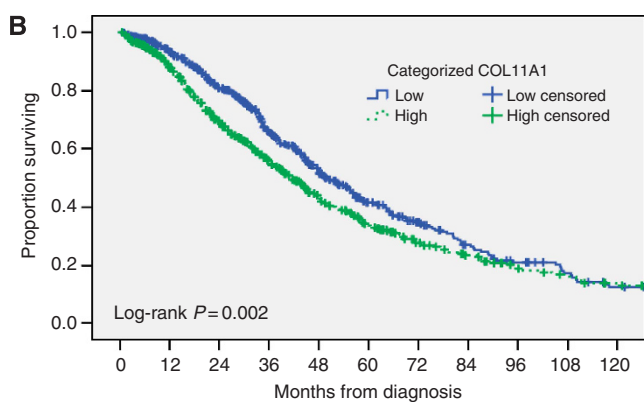
Table 4. Relationship between the expression of candidate genes and progression-free survival or overall survival in women with invasive serous ovarian cancer and public Affymetrix gene expression data

Gene (Affymetrix probe set identifier)	Progression-free survival (N = 723)						Overall survival (N = 1183)					
	Unadjusted modelling			Adjusted modelling ^b			Unadjusted modelling			Adjusted modelling ^b		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Continuous variables^a												
<i>MMP1</i> (204475_at)	1.001	0.944–1.062	0.964	0.995	0.938–1.056	0.875	0.998	0.950–1.048	0.923	0.997	0.948–1.047	0.893
<i>MMP10</i> (205828_at)	1.151	0.945–1.402	0.163	1.185	0.964–1.458	0.108	1.066	0.881–1.291	0.509	1.080	0.889–1.312	0.438
<i>CTH</i> (206085_s_at and 217127_at)	0.893	0.714–1.116	0.320	0.898	0.717–1.124	0.347	0.860	0.709–1.043	0.126	0.871	0.718–1.057	0.162
<i>COL11A1</i> (204320_at and 37892_at)	1.078	1.035–1.124	0.0003	1.053	1.010–1.097	0.015	1.070	1.035–1.106	0.00007	1.059	1.024–1.094	0.001
Categorised <i>COL11A1</i>^c												
Low	1.000			1.000			1.000			1.000		
High	1.534	1.261–1.866	0.000018	1.237	1.058–1.448	0.008	1.287	1.100–1.505	0.002	1.373	1.126–1.674	0.002

Abbreviations: CI = confidence interval; HR, hazard ratio; MMP = matrix-degrading metalloproteinases.
^aHR and 95% CI estimated for each unit increase in expression of each gene.
^bAdjusted for tumour stage categorised as early vs advanced.
^cCategorised at the median as low (≤ 6.8722) or high (> 6.8722).
Significant P -values < 0.05 were bolded.



Categorized COL11A1	Total	No event	Event	Median ± SE PFS time
Low ≤ median	374	184	190	26.6 ± 1.8 months
High > median	349	132	217	17.3 ± 1.1 months



Categorized COL11A1	Total	Alive	Dead	Median ± SE survival time
Low ≤ median	594	310	284	49.6 ± 2.7 months
High > median	591	243	348	41.6 ± 2.0 months

Figure 5. Kaplan-Meier plots for PFS (A) and OS (B) for women with low (≤ median) or high (> median) tumour expression of COL11A1. There were 723 women with 138 events (recurrences) included in the PFS analysis and 1183 women included in the OS analysis with 631 deaths.

DISCUSSION

Development of drug resistance represents a major obstacle in treating ovarian cancer patients and ultimately results in disease progression and death. This obstacle underscores the clinical need for biomarkers to identify and monitor patients that develop drug resistance to more effectively triage patients to therapies that reverse resistance and/or have distinct mechanisms of action. We have conducted a LC-MS/MS analysis of secreted proteins harvested from human EOC cell lines, including A2780, A2780-CP20, CaOV3, OVCAR3 and OV90, that identified 16 significantly differentially abundant proteins from platinum-sensitive vs platinum-resistant cells. Thirteen proteins were elevated and three proteins were reduced in platinum-resistant compared with platinum-sensitive EOC cells. Among these, MMP1, MMP10, CTH and COL11A1 were observed to be elevated in the secretome from platinum-resistant compared with platinum-sensitive EOC cells, and verified by immunoblot analysis.

Matrix-degrading metalloproteinases have major roles in cancer biology including proteolysis of ECM-releasing growth factors and generation of angiogenesis inhibitors, as well as processing and

Table 5. Relationship between the categorised COL11A1 expression and tumour stage^{a,b}

Tumour stage	Categorised COL11A1	
	Low ≤ 6.8722	High > 6.8722
Early stage (I + II)	59 (78.7%)	16 (21.3%)
Advanced stage (III + IV)	533 (48.1%)	575 (51.9%)

^aFisher's exact test $P = 2.67 \times 10^{-7}$.
^bMantel-Haenszel common odds ratio = 3.978, 95% confidence interval = 2.261–6.998, $P = 1.7 \times 10^{-6}$.

activation of signalling molecules to control tumour cell growth, survival, invasion and metastasis (Bauvois, 2012). Urinary MMPs have been shown to predict ovarian cancer in patients with normal CA-125 levels (Coticchia *et al*, 2011). In addition, several MMP transcript expression levels have been indicated to be associated to platinum-resistant head and neck cancer cell lines (Ansell *et al*, 2009). Matrix-degrading metalloproteinase-1 is known to activate protease-activated receptor-1, a G-protein-coupled receptor that promotes angiogenesis and metastasis in peritoneal mouse models of ovarian cancer (Agarwal *et al*, 2008). Salivary MMP1 levels have been shown to be elevated in oral squamous cell carcinomas (Stott-Miller *et al*, 2011). Matrix-degrading metalloproteinase-10 is involved in tumour initiation, cancer cell migration, cancer stem cell maintenance and expansion (Regala *et al*, 2011; Justilien *et al*, 2012). Matrix-degrading metalloproteinase-10 gene expression has been shown to be highly expressed in platinum-resistant (A2780-CP70 and A2780-CP200) when compared with platinum-sensitive cells (A2780) (Solar and Sytkowski, 2011). CTH, an enzyme that catalyses the last step in the trans-sulfuration pathway from methionine to cysteine, is regulated by the PI3K/Akt pathway (Yin *et al*, 2012). The trans-sulfuration pathway links methionine metabolism to the production of redox-modulating proteins and has been suggested to have a role in tumour development by altered redox equilibrium (Rosado *et al*, 2007). A patent has been filed by Abnova and the Japan National Cancer Center on CTH as an *in vitro* biomarker distinguishing ovarian cancer patients with poor prognosis (Tesshi Yamada and Wilber, 2012). Further, CTH has been suggested to be a provocative drug target where preliminary data demonstrates increased sensitisation of CTH-overexpressing cells to platinum when CTH is inhibited (Tesshi Yamada and Wilber, 2012). Elevated levels of gene and protein expression of CTH have also been reported in a multi-drug-resistant oesophageal squamous carcinoma cell line (EC109/CDDP) when compared with its parent line EC109 (Wen *et al*, 2010). COL11A1 overexpression has been shown to be associated with multiple advanced-stage cancers including ovarian carcinoma (Kim *et al*, 2010). COL11A1, along with other ECM proteins, could contribute to ECM remoulding and tumorigenesis in ovarian cancer promoting tumour cell survival and migration. Together, these observations suggest that this subset of 16 proteins, including MMP1, MMP10, CTH, and COL11A1, may hold potential as circulating biomarkers of platinum resistance, and warrant further verification in clinically relevant serum samples from recurrent/resistant and non-recurrent EOC patients.

Finally, high COL11A1 was associated with advanced stage, worse PFS and worse OS in women with invasive serous ovarian cancer as assessed from publicly available transcript expression data with clinical outcomes. The secretome from human ovarian cancer cell lines represents a rich source of candidate secreted or shed proteins with translational potential and clinical relevance as candidate circulating biomarkers. Well annotated tumour tissues,

sera and/or plasma from EOC patients are being assembled to further interrogate the clinical relevance of protein levels of COL11A1 and the other candidate biomarkers from our *in vitro* secretome analysis as markers of cisplatin resistance and poor outcome. The ultimate goal will be to develop a panel of circulating biomarkers that can be deployed to identify/monitor platinum resistance, and triage-resistant patients to new standards of care that abate resistance and enhance outcome.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, Department of the Air Force or the Department of Defense.

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