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Research Article

Protein expression patterns associated with advanced stage ovarian cancer

This is a comparative proteomic study on biopsies from patients with ovarian cancer to identify potential diagnostic/prognostic biomarkers in both healthy and tumor tissue, interstitial fluid (normal interstitial fluid and tumoral interstitial fluid and peritoneal effusion. Protein expression/identification was evaluated by 2-DE and MS analysis: six proteins showed differential expression in tumoral interstitial fluid and tumor tissue compared to normal interstitial fluid and healthy tissue: five were found to be down-regulated and identified as galectin 3, glutathione *S*-transferase A-2, retinol binding protein 1, phosphatidylethanolamine-binding protein and annexin 5, while the calgranulin, was significantly upregulated in all pathological samples, including the ascitic fluid. Validation of S100A8 overexpression in carcinoma tissue was obtained by immunohistochemistry. To our knowledge, this is the first study to report an over-expression of calgranulin by 2-DE associated with MS/MS analysis on surgical biopsy. The reduced expression of galectin 3 and retinol binding protein 1 in cystic fluid and serum of patients with early stage disease is confirmed in this study. The results highlight alterations in proteins that control cell-cycle progression and apoptosis, as well as factors that modulate the activity of signal transduction pathways. Moreover, this study suggests that calgranulin expression may be used as a diagnostic and/or prognostic biomarker.

Keywords:
2-DE / Biomarkers / Calgranulin / Ovarian cancer DOI 10.1002/elps.201000654

1 Introduction

Ovarian cancer is the leading cause of mortality among all types of female reproductive system cancers and one of the most aggressive and lethal epithelial cancers in women [1]. Cancer statistics from the National Cancer Institute in Bethesda (available at http://seer.cancer.gov/statfacts/html/ovary.html) show that only 19% of ovary cancer cases are diagnosed at a localized stage, while 74% are discovered after the cancer has spread to regional lymph nodes or has already metastasized. Considering that the 5-year relative survival rate of ovarian cancer at the localized stage is 92.7%, the need for improving early detection is essential.

There are several difficulties in early diagnosis. Early stage ovarian cancer is usually asymptomatic and a simple physical examination is commonly unsatisfactory. Further, the common diagnostic tool, the trans-vaginal ultrasonography, often fails to distinguish between benign and malignant lesions.

These difficulties in early diagnosis and the poor prognosis associated with advanced stage disease have generated the need for research focusing on a proteomic marker for diagnostic purposes. Currently, the serum glycoprotein CA-125 is the commonly used biomarker, which lacks optimum specificity and sensitivity, because clinically significant elevations in CA-125 occur in a variety of other conditions, both benign diseases and malignant tumors [2]. To date, CA-125 is only approved for the use in monitoring disease recurrence [3].

Several reports have led to the belief that no single marker is likely to be a sufficient predictor for the diagnosis of ovarian cancer, emphasizing the need for development of panel of multiple diagnostic/prognostic markers. This seems to be necessary because of the high heterogeneity demonstrated by most cancers. It has been suggested by several studies that different histological types of ovarian cancers represent distinct disease entities [4–6]; in fact, it appears that endometrioid ovarian cancer

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Abbreviations: GST A-2, glutathione *S*-transferase A-2**; IAA,** iodoacetamide**; NIF,** normal interstitial fluid**; PEBP,** phosphatidylethanolamine-binding protein 1**; RBP-1,** retinol binding protein 1**; TIF,** tumoral interstitial fluid

arises probably from the metaplastic Mullerian epithelium rather than the ovarian surface celomatic sheet, which is more likely in the case of serous carcinoma. This reflects the fact that gene expression profiles are different in the two histological types regardless of the stage and grade of the disease [7].

Several studies investigated the possibility of using biomarkers combination strategies that consist of CA-125 and other similar serum glycoprotein for increasing sensitivity, but they reveal an insufficient predictability of disease in screening tests of the general population for early stage cancer [8, 9]. The U.S. Food and Drug Administration recently cleared the OVA1 test, which measures the levels of five proteins in blood samples in patients with ovarian cancer, in order to select the best surgical option. The OVA1 test promises to significantly improve public health; however, it is not intended for ovarian cancer screening or for a definitive diagnosis of ovarian cancer. Consequently, there is an urgent need to develop detection methods to improve the sensitivity and specificity not only in early ovarian cancer detection but also in monitoring treatment, another important factor in improving ovarian cancer outcome. The current optimal follow-up/monitoring strategy for an asymptomatic patient with advanced ovarian cancer following initial treatment is undefined.

The identification of proteins differentially expressed in ovarian cancer with respect to the same subjects' healthy ovarian tissue may provide markers of early stage ovarian cancer and/or give indications of a molecular subtype that could help in defining the treatment.

Several studies have analyzed the proteomic profiles of ovarian tumor tissue [10, 11], cell lines [12–14], ascites fluid [15] and blood samples from ovarian cancer patients [16, 17]. This study's aim is to investigate and define protein expression patterns associated with advanced stage ovarian cancer, to enable a better understanding of the cancer-associated molecular events. This understanding will in turn lead to the definition of a panel of diagnostic and/or prognostic markers. We also investigated proteins secreted by the cancer cell into the interstitial fluid, as it has also been proven that cancer growth and progression also depends on stromal factors present in the tumor microenvironment [18, 19]. Moreover, protein secreted into interstitial fluids could be present in blood serum, representing potential biomarkers of the disease. Studies regarding interstitial fluids in breast cancer suggest that protein patterns predictive of response to chemotherapy may be present in the interstitial fluid [20].

Proteomic profiling of differentially expressed proteins in cancer ovarian tissue, tumoral interstitial fluid (TIF) and ascitic fluid, compared with healthy tissue sample and normal interstitial fluid (NIF), allowed the identification of protein spots consistently differentially expressed between normal and cancer samples in this study. Data obtained by 2-DE and MS analysis were confirmed by immunohistochemistry.

2 Materials and methods

2.1 Patients and samples collection

Six consecutive patients with endometrioid or serous ovarian carcinoma subtypes who presented at the Department of Obstetrics and Gynecology at the S. Maria Nuova Hospital in Reggio Emilia (Italy), and who had not been previously treated with chemotherapy prior to surgical resection, were enrolled in this study (Table 1). Previous treatment with chemotherapy was the only excluding prerequisite for this study. Patients were not selected according to any other prerequisite.

Patient mean age was 69.8 years (range 58–79 years). Due to the rarity of early stage diagnosis and the difficulty in obtaining controlateral healthy ovary samples from young patients for fertility reasons, all patients enrolled were advanced in age and at advanced stages of the disease. Informed consent was obtained from the patients and biological sample handling was performed according to the local ethics committee guidelines.

Paired samples of healthy and tumor tissue and peritoneal effusions were collected at the time of primary surgery. Formalin-fixed paraffin-embedded blocks were made from each tissue sample, stained with hematoxylin and eosin, and examined by light microscopy for histopathological characterization. Ovarian cancer was classified and graded (grading I–III) according to the World Health Organisation (WHO) guidelines; distribution of the tumor was defined according to the International Federation of Gynecology and Obstetrics (FIGO) stages. The majority of ovarian tumor samples were classified as stage III, grade 2 tumors. Overall, the tumor specimens all contained over 90% tumor cells with necrosis. No tumor cells were detected in healthy tissue samples.

Specimens for proteomic analysis were processed immediately after collection, stocked and frozen at $-80^{\circ}C$.

2.2 TIF and NIF

All tissues (about 0.25 g) were minced into 1- to 2-mm^3 pieces, carefully rinsed twice in phosphate-buffered saline (PBS) and placed in a conical plastic tube for processing as described in recent studies [20, 21] to obtain TIF and NIF. The final recovered supernatant was precipitated o/n at $-20^{\circ}C$ by adding 14 V of a mix composed by 12 V acetone, 1 V methanol and 1 V tri-butyl-phosphate. Thereafter, the samples were centrifuged at $2000 \times g$ for 15 min and the protein pellets resuspended in 200–400 μL rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 25 mM DTT, 0.2% ampholytes and a trace of bromophenol blue).

2.3 Proteins tissue extraction

After TIF and NIF recovery, tumor tissue pellets were crushed to frozen powder by using a pestle under cooling in

Table 1. Patients characteristics

Sample ID	Histological type	Age at diagnosis	Tumor grade	Stage at diagnosis[a)]
1F	Endometrioid	78	G2	III C
2M	Endometrioid	58	G3	III C
3M	Endometrioid	70	G2	II C
4L	Serous-papillary	58	G2	III C
5G	Serous-papillary	79	G2	III C
6C	Serous-papillary	76	G3	III C

a) Referred to the International Federation of Gynecology and Obstetrics (FIGO) staging system.

liquid nitrogen. The frozen powder was incubated for 1 h at room temperature in 1.2 mL of extraction buffer (7 M urea, 2 M thiourea, 3% CHAPS, 40 mM Tris, pH 8.3, 1% ampholytes) containing a protease inhibitors cocktail (Roche Complete EDTA-free, Okano). Samples were sonicated at 4°C in rehydration buffer (10 s/cycle, 2 cycles) for improving cell lysis. The lysates were centrifuged at 13 000 × *g* for 15 min at 4°C and supernatants collected and precipitated o/n at −20°C with 12 V of ice-cold acetone. The protein pellet was recovered by centrifugation at 10 000 × *g* for 15 min at 4°C and resuspended in 300–500 μL of rehydration buffer.

2.4 Peritoneal fluid

After centrifugation of peritoneal fluid at 2000 × *g* for 15 min, 250 μL of the supernatant was precipitated at −20°C o/n with a precipitation mix (8 V acetone, 1 V methanol) and then centrifuged at 10 000 × *g* for 20 min at 4°C. The protein pellet was finally solubilized in 300 μL of rehydration buffer.

2.5 2-DE

To assure the reliability of data and reduce experimental variation, this analysis was performed in duplicate for each sample type.

Before performing the 2-DE separation, protein concentrations of all samples were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) according to the Neno Drop instructions.

For IEF, 120 μg of proteins were mixed with up to 300 μL of the rehydration buffer. This mixture was used to rehydrate 17 cm, pH 3–10 nonlinear ReadyStrip™ IPG Strips (Bio-Rad) for 12 h at 20°C, with a constant voltage (50 V) applied across the gel strips, which were placed in the Protean IEF Cell focusing tray (Bio-Rad). The rehydrated gels were electrophoresed at 250 V for 15 min, subjected to a linear voltage ramp to 10 000 for 3 h, and then focused up to 75 000 V/h. Temperature was maintained at 20°C. After IEF, the IPG strips were equilibrated in SDS-PAGE equilibration buffer, containing 1% w/v DTT, by gentle shaking for 15 min. The procedure was repeated with SDS-PAGE equilibration buffer, containing 2.5% w/v iodoacetamide (IAA). Then, the IPG gel was transferred onto 13 and 10% polyacrylamide gels and SDS-PAGE was performed in a Protean II xi Cell (Bio-Rad) in TGS running buffer (25 mM Tris, 1.92 mM glycine, 1% SDS, pH 8.3). Gels were run at a constant temperature (10°C), 20 mA/gel for the initial 30 min and 500 V/gel thereafter, until bromophenol blue dye marker settled to the bottom of the gel.

Proteins were visualized with a silver staining protocol compatible with protein digestion and MS analysis as described by Shevchenko et al. [22], modified according to our previous published study [23].

2.6 Image analysis

Gel images were acquired with a laser scanner (EPSON Perfection 4490). Spot detection, matching and quantification analysis were carried out with the PDQuest 7.3.1, 2-D Image Analysis software program (Bio-Rad). The quantity of protein in each spot was normalized by the total valid spot intensity according to the manufacturer's instruction. Only the spots that seemed to show a greater than twofold change in expression compared to controls were selected.

2.7 Tryptic in-gel digestion of proteins and MS analysis

Protein spots were excided from gels with an end-removed pipette tip and transferred into a microcentrifuge tube (0.5 mL). Briefly, protein pieces were destained by incubation with 200 L of 1:1 v/v solution 30 mM potassium hexacyano-ferrate (III) and 100 mM sodium thiosulfate, washed twice with 100 L of water for 15 min and shrunk with 100% acetonitrile until the gels turned white. Proteins were then reduced adding 50 μL of a DTT solution (10 mM DTT in 50 mM ammonium bicarbonate) and sequentially alkylated with an IAA solution (55 mM IAA in 50 mM ammonium bicarbonate). The gels were dried for 15 min in a speed vac (Savant Speed-Vac concentrator). A volume of 30 μL trypsin (Promega, Madison, WI, USA) solution (12.5 ng/μL in 25 mM ammonium bicarbonate) was then added, and the gel pieces were incubated at 4°C for 30 min. After digestion, trypsin solution was removed and the samples were incubated at 37°C o/n in the same solution

without trypsin. Resulting supernatants, representing peptide solution, were recovered and concentrated in a speed vac.

After resuspension in 5% formic acid, MS analysis of the peptides was performed on an ESI-Q-TOF-MS/MS Ultima Global (Waters), equipped with nano-electrospray (nano-ESI) and coupled with HPLC CapLC (Waters).

2.8 MS data processing

MS data were automatically registered in PKL file format, analyzed and searched with a mammalian public protein/genome database using MASCOT MS/MS ion search program version 2.2.06 (Matrix Science, http://www.matrixscience.com). Search parameters were set as follows: species, *Homo sapiens*; enzyme trypsin; allowance of one missed cleavage site; carbamidomethylation as fixed modification; peptide tolerance $\pm$0.8 Da; MS/MS tolerance error of $\pm$0.4 Da; monoisotopic mass values and protein mass unrestricted. The mowse score cut-off for 95% protein identification was set to 37. Protein identification was repeated at least once using spots from different gels. The highest score hits among MASCOT search results were selected. The Micromass software (MassLynx^{TM}; version 4.1–2005) allows for the automated selection of peptides for fragmentation (and therefore primary structure determination) when peptide ions above a certain detection level are recorded. Since ESI normally produces multiply charged peptide ions, parameters were chosen so that only multiply charged ions were selected for sequencing by MS/MS. The database searched was the Swiss-Prot 57.8 8 October 2009 (509 019 sequences; 178 948 533 residues).

2.9 Immunohistochemical staining

Biopsy specimens were fixed in 19% buffered formalin and subsequently embedded in paraffin. Five-millimeter sections were stained with hematoxylin/eosin for histopathologic evaluation. Detailed immunohistophenotypic analysis was performed on routinely fixed, paraffin-embedded tissue sections, according to a standard immunoperoxidase technique, using the anti-CalgranulinA (FL-83) rabbit polyclonal antibody (Santa Cruz Biotechnology, CA) at a dilution of 1:100. The analysis was performed on both the tumors and the controlateral ovarian tissue. Additionally, ten paraffin blocks of healthy ovarian tissue, obtained from women with uterine leiomyomata, were used as controls. Staining for S100-A8 protein was scored into four groups according to a semi-quantitative method: (−) no positive cells observed, (+) $<$20% positive cells, (++), 20%$<$50% positive cells, (+++)50% positive cells.

Immunophenotypic sections were photographed with a Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan) using a Plan Fluor X lens with the Nikon digital sight DS-L1 camera system.

2.10 Statistical analysis

Student's *t*-test (two-tail) was used for statistical analyses. The level of significance was $p < 0.05$.

3 Results

Tissue samples and interstitial and peritoneal fluids from women with advanced ovarian carcinoma were analyzed by 2-DE and MS analysis. The clinical characteristics of the patients and the histopathological data are listed in Table 1 and indicate that the endometrioid and the serous-papillary histological types were equally represented. The mean patients age was 69.8 years $\pm$ 9.7, the tumor grades were G2 (four patients) or G3 (two patients), while the stage at diagnosis was mostly IIIC (five patients), according to the International Federation of Gynecology and Obstetrics.

The proteomic analysis was performed to assess molecular differences between ovarian cancer tissue/ TIF and healthy ovarian tissue/NIF: the healthy samples were obtained from paired controlateral ovarian samples, taken during surgery. To document reproducible repeats of detected protein patterns, each sample was analyzed twice by 2-DE.

Healthy ovarian tissue for each patient was analyzed first, and protein spots were separated on each gel, with a mean number of 520 spots (Fig. 1). From these visualized protein spots, 137 spots were excised and subjected to ESI-Q-TOF-MS/MS analysis. A total of 118 proteins were identified in ovary proteome map as representing 58 unique proteins (Fig. 1, arrows). Positions of the identified proteins on 2-DE gel were in the expected range of their theoretical molecular weights (mw) and p*I*. Most protein spots were clustered between 8 and 60 kDa, and between p*I* 4 and 9. The overall patterns of protein expression in all the 2-DE gels were similar.

All proteins identified are described in Table 2. In Supporting Information Table 1S, the amino acid sequences of peptides identified by mass spectroscopy for protein spots derived from 2-DE gels of ovarian tissue samples is reported. The heat shock protein B6 (HSPB6) was identified by a single peptide. For this protein, the sequence identified, the precursor m/z and charge observed, along with the score for the peptide are reported in Supporting Information Table 2S. The MS/MS spectrum is reported in Supporting Information Fig. 1S. The comparison between healthy and tumor ovarian tissues (as illustrated in Fig. 2, where the representative 2-DE gels of a single patient are reported) showed different protein patterns, especially in the low molecular range (10–20 kDa).

The 2-DE analysis of TIF and NIF showed a similar protein expression profile (see Fig. 3). Down- or upregulated proteins in pathological samples were evaluated using PD Quest 2-D software. Both in tissue and interstitial fluid, the expression profiles of six proteins (indicated by arrows in Figs. 2 and 3) were significantly changed in ovarian

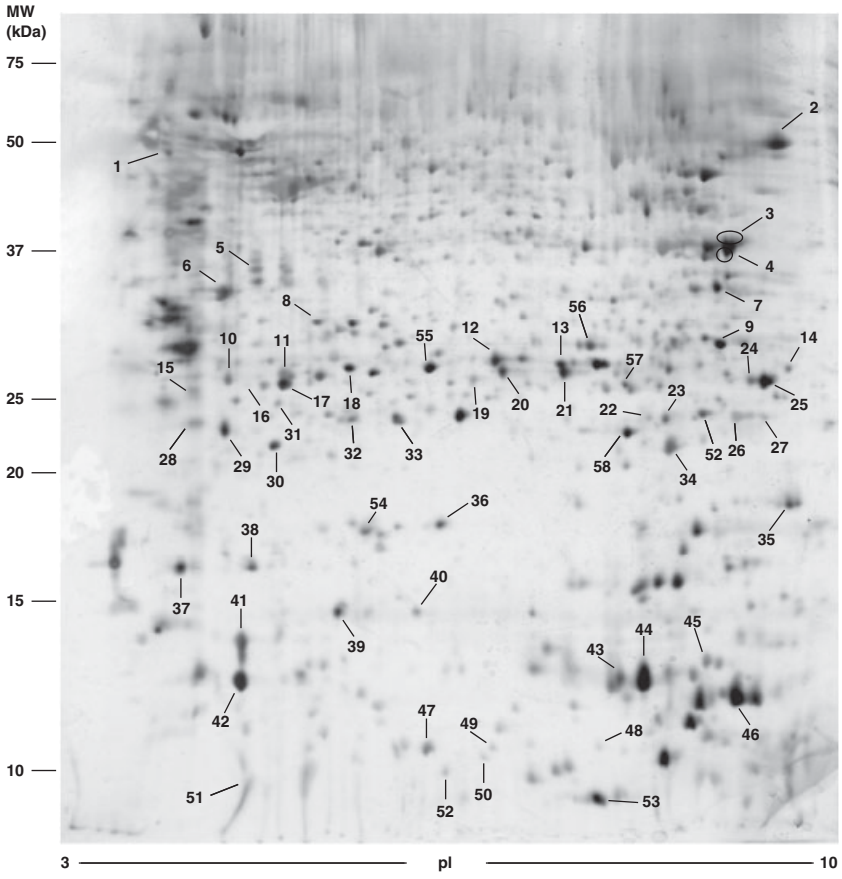

Figure 1. Map of the reference profile of protein spots separated in normal ovarian tissue by 2-DE. Total protein (120 μg) was loaded onto an IGP strip (pH 3–10 NL), separated in the second dimension on a 13% polyacrylamide gel and visualized by silver staining. Spot numbers in the gel image reflect the numbers listed for the proteins identified in Table 2.

carcinoma with respect to healthy ovary tissue. The S100 calcium binding protein A8–calgranulin-A were upregulated in TIF and ovarian cancer tissue with respect to NIF and healthy ovary tissue: a 3.67- and 3.58-fold change, respectively. Conversely, five proteins were downregulated in all the types of cancer samples in comparison to healthy samples: these were annexin 5 (ANXA5), galectin 3 (Leg3), glutathione *S*-transferase A-2 (GST A-2), cellular retinol binding protein (RBP-1) and phosphatidylethanolamine-binding protein (PEBP-1). In Table 3, the changes in expression profile of the six differentially expressed proteins are reported. Each gel was quantified by PDQuest, which resulted to be statistically significant by applying the Student's *t*-test to duplicate samples of all six patients.

The proteomic analysis of the peritoneal fluids obtained from the study patients demonstrated the presence of several proteins (Fig. 4), which were identified by mass spectroscopy (Table 4). The amino acid sequence of the identified peptides is reported in Supporting Information Table 2S. Two spots (11 and 12) were identified by a single peptide as haptoglobin-related protein (HPTR). For these single-peptide-based protein identifications, further information is presented in the supporting information (Supporting Information Table 2S and Supporting Information Fig. 2S). It should be noted that protein S100A8 was always present in the peritoneal fluids.

The upregulation of S100A8 in pathological tissue samples and in cells obtained from peritoneal fluid was also confirmed by immunohistochemistry (Fig. 5). In all the cases, a strong and diffuse (+++) immunoreactivity was present in the neoplastic cells, either with a cytoplasmic or a nuclear pattern of positivity; in two cases there was also a moderate (++) positivity in the ovarian stroma intermingled within the neoplastic epithelial cells. Benign epithelia, e.g. tubaric one, were consistently negative. The benign surface ovarian epithelium was slightly positive (+) in the cytoplasm in focalized zones. Results from healthy ovarian tissue of women with uterine leiomyomata and from healthy controlateral ovary of the patients were absolutely superimposable. In Fig. 5, the neoplastic cells of a serous papillary carcinoma were found strongly positive (+++) with S100A8. An immunohistochemical analysis was performed on a histological section (Fig. 5, panel A) and on a cytological peritoneal smear (Fig. 5, panel B). Controlateral non-neoplastic ovary was found to express S100A8 protein in surface epithelium with few myeloid positive cells in the stroma (Fig. 5, panel C).

4 Discussion

Most ovarian tumors consist of serous and endometrioid subtypes: serous carcinomas account for around 50% of all

Table 2. Ovarian cancer tissue proteins separated by 2-DE electrophoresis and identified by MS

Spot #	Identification (SWISS-PROT)	Gene	Sequence coverage (%)	Spot mw (kDa)	Spot p*I*	Swiss-Prot access #
1	Retinoblastoma-binding protein 4 (RBBP4)	RBBP4	23	47.8	4.74	Q09028
2	Elongation factor 1-α 1 (EF1A1)	EEF1A1	4	50.4	9.1	P68104
3	Malate dehydrogenase mitochondrial (MDHM)	MDH2	37	35.9	8.92	P40926
4	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	GAPDH	26	36.1	8.58	P04406
5	Osteoglycin (MIME)	OGN	11	34.2	5.46	P20774
6	Annexin 5 (ANXA5)	ANXA5	41	35.9	4.94	P08758
7	Hydroxyacyl-coenzyme A dehydrogenase mitochondrial (HCDH)	HADH	7	34.3	8.88	Q16836
8	Proteasome activator complex subunit 2 (PSME2)	PSME2	22	27.5	5.44	Q9UL46
9	Galectin-3 (LEG3)	LGALS3	31	26.1	8.61	P17931
10	Calcium-activated neutral proteinase small subunit 1 (CPNS1)	CAPNS1	6	28.4	5.05	P04632
11	Glutathione-S-transferase Mu-3 (GSTM3)	GSTM3	40	26.9	5.37	P21266
12	Peroxiredoxin-6 (PRDX6)	PRDX6	34	25.1	6.02	P30041
13	Triosephosphate isomerase (TPIS)	TPI1	52	26.8	6.51	P60174
14	Adenylate kinase 3 (KAD3)	AK3	31	25.5	9.15	Q9UIJ7
15	Tumor protein D52 (TPD52)	TPD52	37	24.3	4.94	P55327
16	Rho GDP dissociation inibitor 1 (GDIR1)	ARHGDIA	15	23.2	5.03	P52565
17	Apolipoprotein A-I (APOA1)	APOA1	43	30.7	5.56	P02647
18	Serum amyloid P-component (SAMP)	APCS	27	25.5	6.1	P02743
19	Glutathione-S-transferase Mu2 (GSTM2)	GSTM2	46	25.8	6.02	P28161
20	Glutathione-S-transferase Mu1 (GSTM1)	GSTM1	35	25.9	6.24	P09488
21	Glutathione-S-transferase Mu1 (GSTM1)	GSTM1	35	25.9	6.24	P09488
22	Flavin reductase (BLVRB)	BLVRB	31	22.2	7.31	P30043
23	Peroxiredoxin-1 (PRDX1)	PRDX1	45	22.3	8.27	Q06830
24	Cleavage and polyadenilation specificity factor 5 subunit (CPSF5)	NUDT21	17	26.3	8.85	O43809
25	Glutathione S-transferase A2 (GSTA2)	GSTA2	25	25.5	8.51	P09210
26	Adenylate kinase isoenzyme 1 (KAD1)	AK1	23	21.7	8.73	P00568
27	Transgelin (TAGL)	TAGLN	40	22.6	8.88	Q01995
28	Translationally-controlled tumor protein (TCTP)	TPT1	14	19.6	4.84	P13693
29	Lactoylglutathione lyase (LGUL)	GLO1	21	20.8	5.25	Q04760
30	Synthase subunit d, mitochondrial (ATP5H)	ATP5H	18	18.5	5.21	O75947
31	Ubiquitin-conjugatin enzyme E2 K (UBE2K)	UBE2K	18	22.5	5.33	P61086
32	Glutathione-S-transferase P1 (GSTP1)	GSTP1	48	23.5	5.43	P09211
33	Abhydrolase domin-containing protein 14B (ABHEB)	ABHD14B	20	22.4	5.94	Q96IU4
34	Phosphatidylethanolamine-binding protein 1 (PEBP1)	PEBP1	56	21	7.01	P30086
35	Peptidyl-prolyl cis-trans isomerase B (PPIB)	PPIB	41	23.7	9.33	P23284
36	Heat shock protein β 6 (HSPB6)	HSPB6	8	17.2	5.95	O14558
37	Cytochrome b5 (CYB5)	CYB5A	42	15.3	4.88	P00167
38	Eukariotic translation initiation factor 5A-1 (IF5A1)	EIF5A	21	17	5.08	P63241
39	Transthyretin (TTHY)	TTR	64	15.9	5.52	P02766
40	Ubiquitin-conjugatin enzyme E2 N (UBE2N)	UBE2N	30	17.1	6.13	P61088
41	Retinol binding protein (RET1)	RBP1	41	15.9	4.99	P09455
42	Galectin-1 (LEG1)	LGALS1	22	14.9	5.34	P09382
43	Hemoglobin subunit β (HBB)	HBB	93	15.9	6.81	P68871
44	Hemoglobin subunit β (HBB)	HBB	93	15.9	6.81	P68871
45	Profilin 1 (PROF1)	PFN1	45	15.2	8.48	P07737
46	Hemoglobin subunit α (HBA)	HBA1-HBA2	63	15.2	8.73	P69905
47	Protein S 100-A8-calgranulin A (S100A8)	S100A8	25	11.8	6.56	P05109
48	Protein S 100-A8-calgranulin A (S100A8)	S100A8	44	10.9	6.51	P05109
49	β-2 microglobulin (B2MG)	B2M	29	13.8	6.06	P61769
50	Histone H4 (H4)	HIST1H4A	29	11.2	11.36	P62805
51	Protein S 100-A6 (S10A6)	S100A6	17	10.2	5.33	P06703
52	Peroxiredoxin-1 (PRDX1)	PRDX1	45	22.3	8.27	Q06830
53	Ubiquitin	RPS27A	69	8.5	6.56	Q91888
54	Superoxide dismutase [CuZn] (SODC)	SOD1	16	16	5.7	P00441
55	Heat shock protein β 1 (HSPB1)	HSPB1	22	22.8	5.98	P04792
56	Abhydrolase domain containing protein 11 (ABHDB)	ABHD11	25	34.7	9.5	Q8NFV4
57	GTP-binding nuclear protein Ran (RAN)	RAN	20	24.5	7.01	P62826
58	Superoxide dismutase (Mn) mitochondrial (SODM)	SOD2	32	24.8	8.35	P04179

The first column denotes the 2-DE gel image spot number as illustrated in Fig. 1, associated with a commonly used protein name (column 2). Column 3 indicates the known gene symbol; column 4 shows the sequence coverage, which is the percentage of amino acids sequenced for the identified protein; column 5 represents the theoretical mw of the unprocessed protein; column 6 indicates the p*I* of the protein; column 7 specifies the SWISS-PROT primary protein accession numbers as identified by MS.

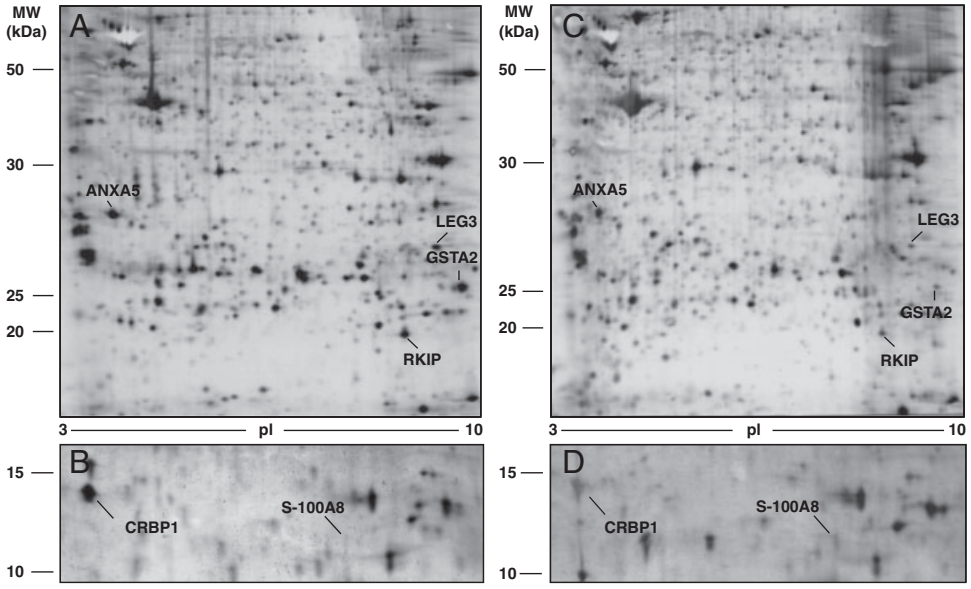

Figure 2. Comparison between proteome of normal ovarian tissue and the corresponding tumor tissue. Representative gels derived from normal ovarian tissue (A and B) and ovarian cancer tissue (C and D) of a single patient. To obtain a better separation of protein spots, the samples were run on 10% (A and C) and 13% (B and D) polyacrylamide gels. A total of seven differentially expressed protein spots in tumor tissue are annotated and identified by MS analysis, as reported in Table 2.

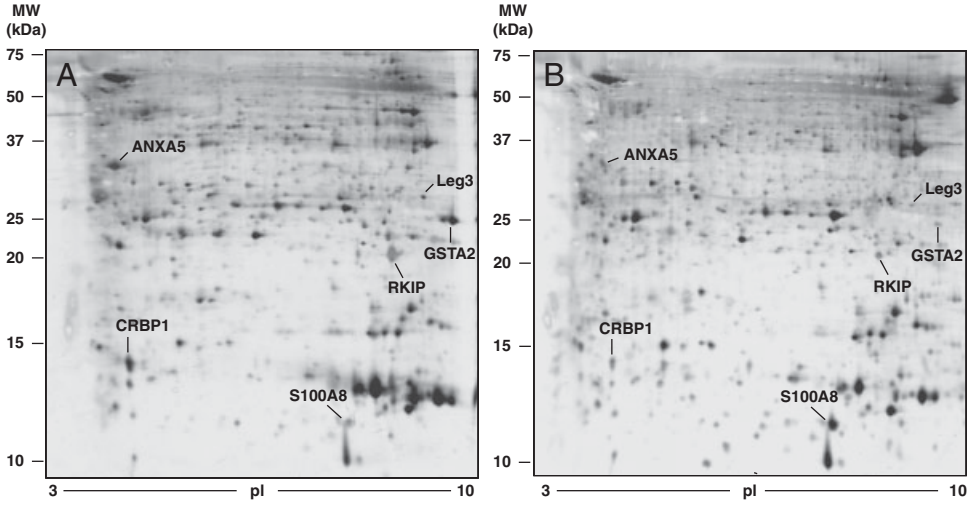

Figure 3. Comparison between proteins secreted by ovarian normal interstitial fluid (NIF) and by ovarian tumoral interstitial fluid (TIF). 2-DE images of protein in NIF (panel A), obtained from healthy ovarian tissue biopsies, and in TIF (panel B), obtained from ovarian cancer biopsies. Molecular weight (kDa) and isoelectric values (p*I*) are shown on the image. Arrows indicate proteins differentially expressed and identified by MS analysis (as reported in Table 2).

epithelial ovarian cancers, while endometrioid carcinomas account for around 25% [24]. Despite the development of new therapeutic approaches, relapse occurs in the majority of advanced stage patients despite a complete response to initial treatments. At least 70–90% of these patients die with drug (therapy)-resistant cancer.

Due to possible analysis of thousands of proteins that could be simultaneously altered, comparative proteomics is a promising mode of possible biomarker discovery for cancer detection and monitoring. Therefore, for this study, proteomic strategies were used to study protein expression profiles of six patients' metastatic ovarian tumors compared with paired normal ovarian tissues, focusing on discriminating proteins in low molecular weight proteome, as their role in ovarian cancer is mostly unknown. Among the ovarian proteins identified by MS analysis, we found that six discriminating proteins showed large differences in the expression profile between normal and pathological samples. It is already known that these six proteins are differentially expressed in tumor samples. Our finding that the same altered pattern of expression is present in interstitial fluids confirms that these proteins are secreted into the extracellular microenvironment by the cancer cells and may therefore be detected in peritoneal fluid and eventually in blood.

This study's findings include the downregulation of the expression of ANXA5 from the normal ovarian tissue to the metastatic tumor tissue. This protein has previously been identified as an anticoagulant protein belonging to the annexins, a multigene family of phospholipids binding proteins [25]. Several other biological properties have since been described for the ANXA5 protein, including Ca^{2+}-channel activity, immune modulation and collagene receptor [26, 27]. The function of ANXA5 as masking phosphatidylserine on the outer cell membrane, protecting apoptotic cells from phagocytosis, has also previously been

Table 3. Modification in protein expression in tumoral tissue and interstitial fluid

Protein name	Fold change tumoral versus normal tissue	Fold change TIF versus NIF
ANXA5	$-1.88 \pm -0.48^{*}$	$-5.605 \pm -3.29^{***}$
PEBP	$-4.21 \pm -2.90^{***}$	$-2.82 \pm -0.69^{*}$
GSTA2	$-4.67 \pm -1.88^{*}$	$-27.39 \pm -21.24^{***}$
LEG3	$-2.19 \pm -0.69^{*}$	$-5.10 \pm -4.42^{****}$
S100A8	$3.67 \pm 1.50^{**}$	$3.58 \pm 1.11^{*}$
RET1	$-6.33 \pm -3.30^{**}$	$-5.01 \pm -4.28^{****}$

The fold change indicates the direction and the magnitude of the change in expression level. Data are expressed as mean ± standard deviation; p value is expressed as $^{*}p<0.0001$, $^{**}p<0.001$, $^{***}p<0.01$ and $^{****}p<0.05$.

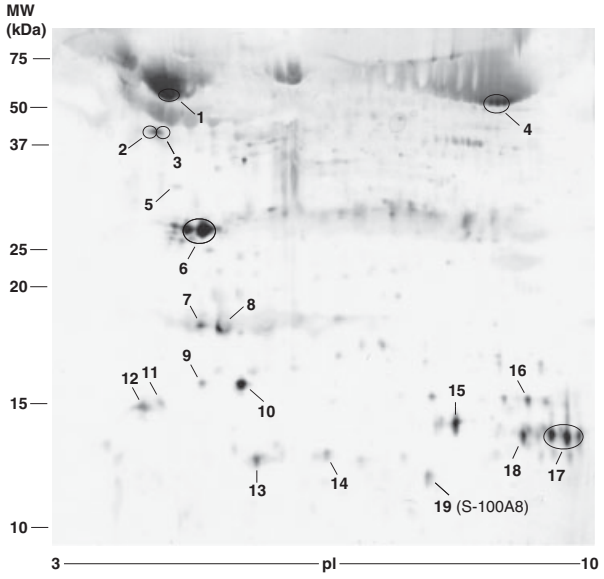

Figure 4. Protein pattern from a representative peritoneal fluid of ovarian tumor. The peritoneal fluid was collected at surgery and prepared for protein 2-D as described in Section 2. Spot numbers in the gel reflect the numbers listed for the proteins in Table 3. The presence of protein S-100A8 in peritoneal fluid is indicated.

outlined [28, 29]. However, to our knowledge, this is the first study to highlight ANXA5 as a possible ovarian cancer biomarker. In contrast with this study's results, an upregulation of ANXA5 has been reported in other cancer types, for example, squamous cervical cancer [30, 31], head and neck squamous carcinoma [30, 31], where it is suggested to be potentially connected to proliferation and/or metastatic propagation, even though its physiological significance is still not clearly defined.

A decreased expression of galectin 3 protein in cancer tissue and interstitial fluid was also found in all six study tissue samples. Galectin 3 is a β-galactoside-binding phosphoprotein expressed widely in epithelial and immune cells and is an intracellular and extracellular lectin, which interacts with intracellular glycoproteins, cell surface molecules and extracellular matrix proteins [32]. It is involved in a variety of biological functions, including tumor cell adhesion, proliferation, differentiation, angiogenesis, cancer progression and metastasis [33, 34]. The expression of galectin 3 has been proven to be associated with tumor invasion and metastatic potential in human head and neck [35], thyroid [36] and gastric cancers [37]. In contrast, for some tumors such as breast [33], prostate [38] and endometrial cancer [34], the expression of galectin 3 is inversely correlated with the metastatic potential. In conjunction with other study findings, this study suggests that the expression of galectin-3 may depend on tumor or tissue-specific factors that in turn may modulate its levels.

Cellular retinol binding protein 1 (RBP-1) is one of three cellular-retinol-binding proteins described in humans that regulates the uptake and subsequent esterification of retinol and its bioavailability [39]. Retinoids play an important role in fundamental physiological processes such as vision, reproduction, hematopoiesis and differentiation of epithelial tissues. They have been shown to inhibit the growth of human cancer cells in vitro [40], and to be effective chemopreventive and chemotherapeutic agents in ovarian and in other human epithelial and hemotopoietic tumors [41]. A reduction of RPB-1 gene expression has already been reported in breast [42], endometrial [43] and ovarian carcinoma [44], suggesting that a reduction of RBP-1 expression may significantly contribute to the oncogenic process, through altering the vitamin A metabolism. While the reduction of RBP-1 expression is reported in less differentiated endometrial carcinomas, it has been demonstrated that in breast [45] and serous ovarian tumors [44], its reduction appears to be associated with early stage carcinogenesis process: a correlation with tumor stage and grade has been demonstrated.

PEBP-1 is a member of the PEBP family [46], characterized by the ability to block the activation of several signaling pathways including mitogen-activated protein kinase (MEK), G-proteins and NFkappa B, thereby abrogating the antiapoptotic properties of these signaling pathways and their ongoing survival [47, 48]. It has been demonstrated that PEBP is a metastasis suppressor gene [49]; its loss enhances angiogenesis and vascular invasion, and inhibits apoptosis in many types of cancer [50]. Independent studies have demonstrated that in primary colorectal [51] and prostate tumors [49], the downregulation of PEBP-1 can predict the risk of early cancers falling into relapse, while the level its expression is significantly and inversely associated with progression to metastatic disease. The role of PEBP-1 in ovarian cancer so far has not been described. We hypothesize that the lower expression of PEBP-1 in ovarian cancer tissue may indicate a higher risk of relapse and should be investigated in future studies.

Glutathione *S*-transferase (GST) comprises a family of enzymes involved in DNA protection from electrophilic metabolites of carcinogens and reactive oxygen species through the conjugation to reduced glutathione [52]. Selective

Table 4. Proteins obtained from peritoneal fluid and identified by ESI-Q-TOF-MS/MS

Spot #	Identification (Swiss-Prot)	Gene	Protein sequ. cover. (%)	mw (KDa)	p*I*	Swiss-Prot acc. #
1	α-1-Antitripsin (A1AT)	SERPINA1	26	46.8	5.37	P01009
2	Complement C3 (CO3)	C3	7	18.8	6.02	P01024
3	Clusterin (CLUS)	CLU	13	53.0	5.89	P10909
4	Ig gamma-1-chain C region (IGHG1)	IGHG1	43	36.5	8.46	P01857
5	Aminopeptidase B (AMPB)	RNPEP	7	39.8	5.95	Q9H4A4
6	Apolipoprotein A-1 (APOA1)	APOA1	59	30.7	5.56	P02647
7	Transthyretin (TTHY)	TTR	34	15.9	5.52	P02766
8	Haptoglobin (HPT)	HP	13	45.8	6.13	P00738
9	Transthyretin (TTHY)	TTR	33	15.9	5.52	P02766
10	Transthyretin (TTHY)	TTR	64	15.9	5.52	P02766
11	Haptoglobin-related protein (HPTR)	HPR	6	39.4	6.42	P00739
12	Haptoglobin-related protein (HPTR)	HPR	6	39.4	6.42	P00739
13	Serum amyloid protein A (SAA)	SAA1	52	13.5	6.28	P02735
14	Serum amyloid protein A (SAA)	SAA1	52	13.5	6.28	P02735
15	Hemoglobin subunit β (HBB)	HBB	78	16.1	6.75	P68871
16	Fibrinogen α chain (FIBA)	FGA	9	95.6	5.70	P02671
17	Fibrinogen α chain (FIBA)	FGA	9	95.6	5.70	P69905
18	Hemoglobin subunit α (HBA)	HBA1; HBA2	19	15.3	8.72	P69905
19	Protein S100-A8-calgranulin A (S100A8)	S100A8	31	10.8	6.51	P05109

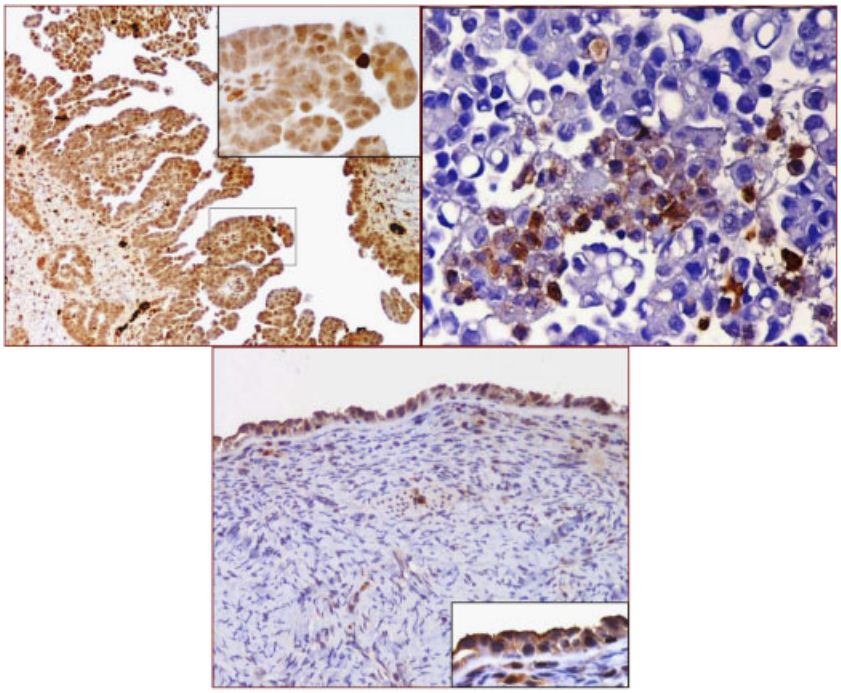

Figure 5. Calgranulin expression in ovarian tissues and in peritoneal fluids of ovarian cancer patients. Immunohistochemical staining of S100A8 (calgranulin A) in ovarian tumor and peritoneal fluid. In A, the S100A8 expression in the neoplastic cells can be noted. In B, cells obtained from peritoneal fluids also express S100A8. In C, non-neoplastic ovary express S100A8 protein in surface epithelium with few myeloid positive cells in the stroma.

downregulation of the GST A-2 protein has been proven in neoplastic kidney cells [53] and a decreased expression is also present during the progression of prostatic neoplasia from benign epithelium to high-grade intra-epithelial neoplasia and carcinoma [54]. In the present study, the downregulation of both GST A-2 and PEBP-1 may indicate a poor prognosis for patients in terms of survival.

S100 proteins incorporates a family of at least 24 different members, which are characterized by high homology, low molecular weight and two Ca^{2+}-binding EF-hands [55]. These proteins are involved in numerous cellular functions, such as protein phosphorylation, enzyme activation, interaction with cytoskeletal components and calcium homeostasis and are regulators of many cellular processes such as cell growth, cell cycle progression, differentiation, transcription and secretion [56]. The current study observed an upregulation of protein S100A8 in analyzed tumor specimens, confirmed by immunohis-

tochemistry. The presence of S100A8 protein was also detected in the peritoneal fluid in all six analyzed cases. Ott et al. [57] were first to identify the S100A8 protein in cystic fluid and serum of women with ovarian carcinomas. Moreover, they demonstrated that borderline or early-stage ovarian carcinomas express significantly less calgranulin with respect to well-advanced carcinomas. On the basis of these combined results, the authors suggest that calgranulin might serve as a diagnostic biomarker to distinguish between malignant and benign ovary tumors and that the amount of detectable calgranulin probably correlates with tumor progression.

Cancer research for various cancer types has proposed that the formation of homo- and hetero-dimers, the binding of Ca^{2+} and the interaction with effector molecules are essential for cancer development and progression, as seems to be the case in ovarian cancer. Several studies have suggested that S100 proteins promote cancer progression and metastasis through cell survival and apoptosis pathways [58] and significant alterations in the expression of the S100 members have been found in different tumor types [59]. Therefore, changes in the expression and/or function of S100 proteins might represent a key step during cancer development and progression, and enhanced S100A8 levels in pathological conditions of chronic inflammation as well as in cancer, suggest a possible role in inflammation-associated carcinogenesis.

Furthermore, a strong correlation of S100A8 upregulation has been found in skin [60], breast [61], lung [62], gastric [63], colorectal [64], pancreatic [65] and prostate [66] cancer. In human bladder cancer, overexpression of S100A8 is associated with stage progression, invasion, metastasis and poor survival [67]. There is at least one report showing a calgranulin decreased expression in esophageal squamous cell carcinoma [68].

It can be stated that a better estimation of the biological importance of certain proteins with regard to the progression from preneoplastic tissue alterations to malignant tumors, as well as the prediction of the metastasis-forming potential by biomarkers, will be a necessary prerequisites for providing a more detailed insight and understanding of tumor progression.

The six proteins identified in the study samples cannot be considered specific biomarkers for ovarian carcinoma, as they have also been detected in other cancer types. However, as the identified proteins are unregulated in advanced state disease, correlated with aggressive disease behavior, and are only identified in malignant tumor, it may suggest that the panel of proteins could be a sensitive indicator for the spread and eventual relapse of the tumor following primary therapy (surgery and/or chemotherapy). Further, calgranulin alone may suffice as a diagnostic and/or prognostic biomarker, distinguishing between malignant and benign ovary tumors, and the amount detected may indicate tumor progression. Although this pilot study is limited by a small patient sample size of advanced stage disease only, the preliminary results are promising and encourage further, larger scale trial. The authors suggest that further studies should include comparison between serous and endometrioid cancers, in order to demonstrate proteomic expression differences within these two histotypes, regardless of the grade and stage. Moreover, the presence in the tumoral cell of a strong positivity at the nuclear level, respect to the normal epithelial cell's weak positivity in the cytoplasm, may reflect the existence of S100A8 phosphorylation, and merits further investigation.

A combined ''second look'' laparotomy could also be very important in establishing factors affecting ovarian carcinoma progression.

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