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Proteomic Analysis of Ovarian Cancer Tumor Fluid is a Rich Source of Potential Biomarkers

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Abstract

Proteomic analysis of body fluids can be used to identify proteins associated with malignant transformation. We investigated the potential of highthroughput proteomics to profile the proteins in ovarian cancer tumor fluid. Tumor fluid samples were obtained from 10 women with high-grade ovarian serous carcinoma and from 10 women with benign serous cystadenoma. The samples were subjected to immunodepletion of albumin and IgG, followed by in-depth proteomic profiling by LC-MS/MS. A total of 1135 proteins were identified, 358 (32%) were detected in both malignant and benign pools, 505 (44%) only in the malignant pool, and 272 (24%) only in the benign pool. The proteomic profile of tumor fluid was similar to human plasma and the secretome of ovarian cancer cells. Proteins related to cellular metabolism were predominantly present in tumor samples. TIMP1 was determined by ELISA, confirming the proteomic data and its significantly higher level in individual malignant tumor fluids when compared with benign tumor fluids. In the present study, proteomic analysis of tumor fluid permitted the identification of a large number of proteins expressed only in malignant tumors. Proteomic analysis of tumor fluid is a strategy in studies of biomarkers for ovarian cancer that can lead to significant advancements in the diagnosis and treatment of the disease.

Keywords: Ovarian cancer; Tumor fluid; Proteomic analysis; Biomarkers; ELISA immunoassay

Introduction

The proteome of an organism reflects cell function and metabolism. The same cell type can have different proteomes in response to drugs, pathogenic infections, pollution and various kinds of stress. Proteomics permits the study of the structure, function and control of biological systems by the analysis of the properties of proteins, such as identity, abundance, structure and activity, as well as modifications, interactions and translocations [1]. Like genomics, proteomics may contribute to the discovery of potential indicators of physiological state and changes that occur during cancer development and progression [2]. The expression of these biomarkers may reflect various events ongoing in tumor cells, such as hyperproliferation, altered patterns of gene expression, hyperplasia, genotoxicity, inflammation and enzymatic alterations related to tumor development, among others [3]. The study of the ovarian proteomic profiles represents a new frontier in ovarian cancer research, since this approach is able to identify thousands of proteins which could be coordinately altered and to simultaneously characterize a wide variety of post-translational events such as glycosylation, phosphorylation, proteolytic cleavages, etc. Taken together, all of these considerations indicate that comparative proteomics is an important approach to the elucidation of potential biomarkers for ovarian cancer detection, monitoring and treatment [4].

Blood is by far the most extensively studied body fluid in the search for biomarkers because proteins secreted by tumor cells are transported to the circulation by drainage via lymphatic or capillary systems, depending on their molecular weight [5,6]. Proteins related to cancer can be present in plasma at very low levels, about 1-10 pg/ml or less [7], and in the presence of very abundant physiological proteins it is difficult to identify their association with the disease using current methods. Most proteomic studies have used plasma, serum, tumor tissue and cultured cells as starting points [8-15]. Other proximal fluids such as ascites (accumulation of fluid in the peritoneal cavity)

represent an interesting alternative source for candidate discovery since they bathe non-adherent cancer cells and adjacent mesothelial cells and contain abundant information including growth, survival and metastasis signaling factors [16]. Additionally, tissue interstitial fluid (TIF) represents a rich sample source in terms of proteins because it hypothetically contains secreted, shed and/or effluxed proteins from the tumor and neighboring stroma [17]. Given that the concentration of disease biomarkers in the local tumor microenvironment is estimated to be 1000-1500 times higher than in blood [18], tumor fluid samples could be a rich source for the identification of ovarian cancer biomarkers.

In the present study we applied an in-depth proteomic profiling approach to characterize the intra-cystic tumor fluids (liquid accumulated inside the tumor cavity) of high grade serous ovarian cancer. The fluid is a potential resource for elucidating tumor-specific proteins and, therefore, a potential source of candidate biomarkers for ovarian cancer.

Methods

Patients and samples

Samples of tumor fluid were obtained from 10 women who had a diagnosis of high-grade ovarian serous carcinoma (mean age 51.2, range 36-72), who had not been treated with antineoplastic drugs or

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radiotherapy, and from 10 women who showed diagnosis of benign serous cystadenoma (mean age 49.7, range 37-71). All diagnosis were confirmed by histopathological analysis. The patients were recruited at the University Hospital (Ribeirão Preto, São Paulo, Brazil). The investigation was approved by the National Ethics Committee (CONEP: 1778/2010) and all patients gave written informed consent to participate.

Tumor fluid was collected from each tumor immediately after surgical removal and centrifuged at 20,000×g for 20 minutes. The resulting supernatant solution was stored at -80°C.

Protein quantification and analysis of sample protein profile by SDS-PAGE electrophoresis

Total protein quantification in each sample of tumor fluid was performed by the Bradford method using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Cat N° 500-0006 Lot. L9700067) according to manufacturer's instructions, and BSA as the standard. In order to obtain the electrophoretic profile of individual samples of tumor fluids, 2.5 µL of each sample was resuspended in electrophoresis buffer XT 4X (Bio-Rad, cat. 161-0791, Lot 1610791 Rev B) containing 5 mg/ml of dithiothreitol (DTT, GE, cat. 17-1318-02, Lot K42179653 133) and incubated at 100°C for 5 minutes. Electrophoresis was carried out with 8-16% Polyacrylamide Precise Protein Gels (Thermo Scientific, cat. #25243, LA2454983 Lot) at 70 V/gel for 1 hour and 40 minutes in a Mini-PROTEAN II Electrophoresis System (Bio-Rad, USA). All gels received 5 µL of a pre-stained protein standard (Thermo Scientific, Page Ruler Plus Prestained Protein Ladder, cat. #26619, Lot 00072585). Subsequently, the gels were stained with Coomassie Colloidal Concentrate (Sigma, Brilliant Blue G-Colloidal Concentrate, cat. #B2025-1EA, Lot 087K4364) according to the manufacturer's recommendations.

Sample pooling and albumin and IgG depletion

The high-grade ovarian serous carcinoma fluid pool (malignant pool) and benign serous cystadenoma fluid pool (benign pool) were obtained by combining a volume of each sample containing approximately 110 µg of total protein. Fluid from malignant and benign tumors, containing 1.2 mg and 1.1 mg of total protein, respectively, was submitted to albumin and IgG depletion using the Proteoprep Immunoaffinity Albumin and IgG Depletion Kit (Sigma, Code #PROTIA Lot 029K6025, Saint Louis, MO, USA). After immunodepletion, 160 µg and 130 µg of total protein were recovered from the malignant and benign pools, respectively. The recovery of proteins with this immunoaffinity chromatographic step was measured by the Bradford assay as described above.

Sample preparation for proteomic profiling

The proteins recovered from each pool in the immunodepletion step were denatured with urea to a final concentration of 8 M in 100 µM Tris-HCl buffer, pH 8.5. Samples were reduced with DTT at a 1:1 protein:DTT mass ratio for 2 hours at room temperature. Cysteine alkylation was carried out by adding a 1:7 protein:acrylamide mass ratio with incubation for 1 hour at room temperature. In order to remove urea and the reagents used for protein reduction and alkylation, malignant and benign fluid pools were subjected to dialysis and concentration in AMICON ULTRA 0.5 ml 10 K filter devices (Millipore #UFC501024 Lot Code R9EN02784). The fluid pools were centrifuged for 15 minutes at 14000×g, 20°C, and concentrated to a final volume of 20 µL each. The concentrated fractions were mixed with 40 µL of gel loading buffer diluted 2X (XT Sample Buffer 4X, Bio-Rad, 161-0791 Code #1610791

Lot B-Rev) and subjected to polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was used as the primary analytical tool for the characterization of proteins present in the sample. Precast Precise Protein Gels 8-16% (Thermo Scientific, Code # 25243 Lot LA2454983) were run in a Mini-PROTEAN II Electrophoresis System (Bio-Rad, USA). Electrophoresis was initially performed at 20 V for 40 minutes as a sample loading step and then at 70 V for 1 hour and 50 minutes until the end of the run by a marker dye. Five microliters of pre-stained protein standards (Thermo Scientific, Page Ruler Plus Prestained Protein Ladder, Code #26619 Lot 00072585) were also applied to one lane. To visualize the proteins, gels were stained with Coomassie Colloidal Concentrate (Brilliant Blue G-Colloidal Concentrate, Sigma, Code #B2025-1EA Lot 087K4364) according to manufacturer's recommendations. The gel was destained in water overnight and each lane of the gel was divided into 6 fractions.

For in situ tryptic digestion, each lane (malignant pool, benign pool) of the gel was cut into six fractions. The 12 resulting gel pieces were destained and SDS was removed by washing three times with 50 mM ammonium bicarbonate containing 50% acetonitrile and 40 µL trypsin (10 ng/µL, Promega, Code V511A Lot #30551310) diluted in 50 mM ammonium bicarbonate, pH>8.0. In-gel protein digestion was carried out at 37°C overnight. Peptides present in the gel bands were extracted twice with 150 µL of a 50% acetonitrile/2% formic acid solution for 2 hours. An additional extraction was carried out with a 200 µL solution of 100% acetonitrile/2% formic acid for 1 hour. The liquids collected from the extracts of the same sample were combined in a low-protein binding 0.5 ml tube. The 12 samples were dried in a Speed Vac (Thermo Scientific, Marietta, OH) and subjected to LC-MS/MS.

Protein identification and relative quantification by LC-MS/MS

Each of the 12 peptide extracts was dissolved in 5% acetonitrile and 0.1% formic acid and analyzed individually by LC-MS/MS in a nanoflow reversed-phase HPLC system connected to an LTQ Orbitrap mass spectrometer (Thermo Scientific). Chromatography was carried out with an in-house packed 75 mm inner diameter (New Objectives) 25 cm long column packed with Magic C₁₈ resin and eluted at 250 nL/min with 90 min linear gradients from 5% to 40% acetonitrile in 0.1% formic acid. MS/MS scans of the five most abundant doubly or triply charged peaks in the MS scan were recorded in data-dependent mode in the linear ion trap. Peptides and proteins were identified with the Labkey-Computational Proteomics Analysis System [18] using the X!Tandem search engine [19] and Peptide Prophet [20] and Protein Prophet [21] algorithms for the statistical validation of peptide data and protein grouping. MS data were searched against the human International Protein Index (IPI version 3.75). Search parameters for tryptic peptides included up to two missed cleavages, mass allowances of 0.5 Da for fragment ions, fixed cysteine modification with acrylamide (+71.0371) and variable methionine oxidation (+15.9949). Only peptides with a Peptide Prophet score above 0.90 and precursor ions with a delta mass less than 20 ppm were considered for protein identification. The list of proteins was generated with a Protein Prophet cut-off value of 0.9, representing an overall protein false discovery rate of 2% based on the Protein Prophet estimate and including proteins identified based on single peptide hits.

Data analysis and correlation

We used the Plasma Proteome Database [22] to determine the presence or absence of proteins in human plasma. Similarly, a

comparison of the proteins identified in the fluid with a comprehensive previous study of ovarian cancer cells [23] was carried out. The networks and functional analysis were generated by the use of Ingenuity Pathway Analysis (IPA, Ingenuity® Systems) and the gene ontology analysis was performed using Fatigo (<http://babelomics.bioinfo.cipf.es>). In addition, the Cluster 3.0 software (Stanford University) was used to compare the present results with those of a previous study [23] and to build a cluster.

TIMP1 ELISA immunoassay

TIMP1 (Metalloproteinase inhibitor 1) was determined using the HUMAN TIMP-1 ELISA KIT from GenWay Biothec, Inc. (San Diego, CA, USA) according to the manufacturer's recommendations. Individual samples of malignant and benign tumor fluid (1 µL diluted to 50 µL with kit's sample buffer) were analysed in duplicate. Data were analysed statistically by ANOVA and the Tukey Test.

Results

Protein quantification, sample protein profile and albumin and IgG depletion

First, in order to characterize individual cystic fluid samples, we measured the protein by the Bradford method. There was some variability among individual tumor fluid samples from each group (benign or malignant). Malignant fluid samples presented protein concentrations ranging from 33.9 to 109.3 mg/mL (average 59.03 mg/mL). Great heterogeneity was observed in the benign set of samples, which ranged from 1.3 to 104.5 µg/µL (average 41.61 mg/mL). Individual measurements are presented in Table 1. Similarly, the SDS-PAGE protein profile of individual samples indicated greater heterogeneity among samples of benign cystic fluid (Figure 1A). In addition, SDS-PAGE profiles showed a predominant concentration of proteins above 35 kDa and an intense diffuse band around 60-70 kDa, probably indicating the presence of large amounts of albumin. In fact, the SDS-PAGE profile of these cystic fluids resembled the profile of plasma/serum samples. Overall, all those profiles of benign and malignant samples for which protein concentrations were near the average (~50 mg/ml) were indistinguishable, indicating that major differences would represent low abundance proteins, thus supporting the need for in-depth proteomic profiling. For this reason, we chose to pool each set of malignant and benign samples based on their total protein concentration and to remove highly abundant proteins before proceeding with the proteomic analysis.

After immunoaffinity removal of albumin and immunoglobulins the recovery was 13% (0.80 mg/mL) and 12% (0.67 mg/mL) of the

initial amount of total proteins in the malignant and benign pools, respectively. As observed in Figure 1B, the SDS-PAGE profiles of immunodepleted pools were well distributed over the full range of molecular weights and the distribution of proteins indicated subtle differences between benign and malignant pools.

GEL-LC-MS/MS

We used SDS-PAGE to separate each cystic fluid pool into 6 fractions and GEL-LC-MS/MS to obtain a detailed proteomic profile. After approximately 200,000 peptide spectra were collected, 1135 proteins with individual IPI access numbers were identified in benign and malignant cystic fluid samples (the proteins are listed in Supplementary Table 1). These proteins correspond to 593 known genes. When comparing the profiles from benign and malignant cystic fluids, 358 common proteins were detected in both pools, 505 proteins were detected only in the malignant pool, and 272 proteins were detected only in the benign pool. Although some of these differences were due to highthroughput proteomic sampling considerations, we noted that the profile of malignant fluid was richer in terms of intracellular proteins. Interestingly, of the 593 known gene products detected, 425 (72%) are present in the human plasma proteome according to the Plasma Proteome Database [22], supporting our previous observation based only in the SDS-PAGE profile.

Data analysis and correlations

Using Ingenuity Pathways Analysis (IPA), we correlated the identified proteins with networks that indicate cellular functions, pathways or specific diseases. The proteins from the malignant fluid pool were associated with 15 networks, especially protein synthesis, protein trafficking and post-translational modifications, while the benign pool proteins were associated with 9 networks, mainly related to cellular movement and infectious diseases. The common proteins were associated with 14 networks, highlighting cell-to-cell signaling and interaction and cellular function and maintenance. Table 2 shows 3 sets of the most statistically significant networks (p-values<0.01): 4 for common proteins, 4 for proteins detected only in the benign pool and 5 for proteins detected only in the malignant pool.

Similarly, when we classified the proteins on the basis of molecular functions and biological processes, using the webtool FATIGO [24], we observed that proteins detected only in the malignant fluid pool are involved mainly in nucleotide/nucleic acid/nucleoside binding, metabolic processes and organelle organization, whereas proteins detected only in the benign fluid pool are involved in signal transducer/enzyme inhibitor/molecular adaptor activity, cell communication, development and growth (Figure 2A and 2B).

In order to extend the characterizations of malignant and benign cystic fluid proteins, we correlated our data with a previously published in-depth proteomic analysis of ovarian cancer cell lines and ovarian cancer tumor cells obtained from patient ascites [23]. We performed a cluster analysis in which protein abundance was estimated based on spectral counts, and observed that a total of 417 of the known genes identified in our study were also detected in the subset of secreted proteins from ovarian cancer cells. Most of these proteins (248) were enriched, especially in ovarian cancer cells from ascites. Furthermore, this analysis demonstrated that the pattern of proteins from the malignant pool was more similar to that of ovarian cancer cells from ascites than to the benign pool pattern or OVCAR3 and CaOV3 serous ovarian cancer-derived cell line patterns. The protein profile of the ES2 ovarian cancer cell line is significantly different from

Malignant individual tumor fluid		Benign individual tumor fluid	
Sample	mg/mL	Sample	mg/mL
M1	59.9	B1	21.1
M2	62.6	B2	53.9
M3	33.9	B3	66.4
M4	56.8	B4	73.9
M5	64.3	B5	1.3*
M6	40	B6	35.4
M7	74.7	B7	1.3*
M8	109.3	B8	104.5
M9	44	B9	51.3
M10	44.8	B10	7*
Average=59.03		Average=41.61	

Table 1: Protein quantification. Total protein quantification of malignant and benign individual tumor fluid samples was performed by the Bradford method (*): indicates samples with smaller amounts of protein.

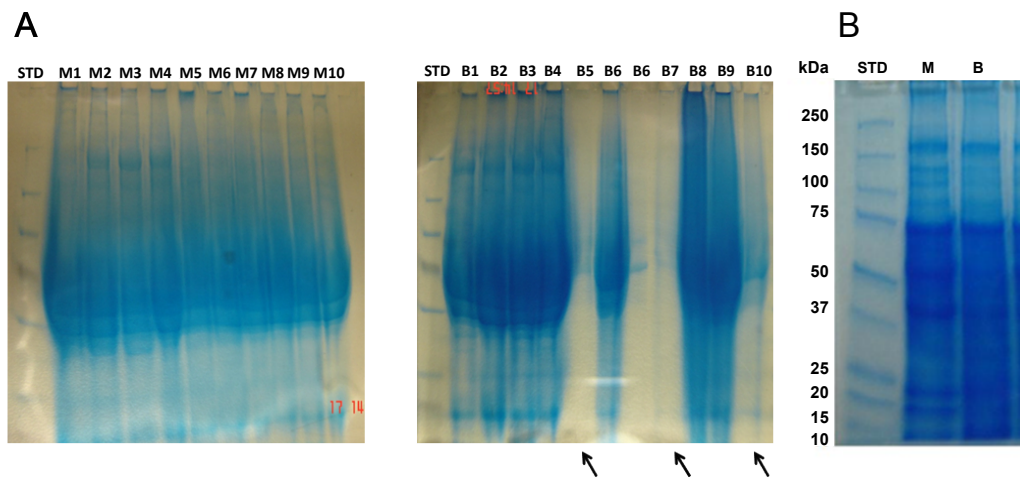


Figure 1: Protein profile of malignant and benign tumor fluids. A: 2.5 µl of each sample of malignant tumor fluids (left gel) and of benign tumor fluids (right gel) were applied to an SDS-PAGE gel (8-16%). Note that the benign fluid samples B5, B7 and B10, indicated by arrows, contain only a few proteins. B: SDS-PAGE gel of the malignant tumor fluid pool (M) and benign tumor fluid pool (B) after albumin and IgG depletion. STD: standard.

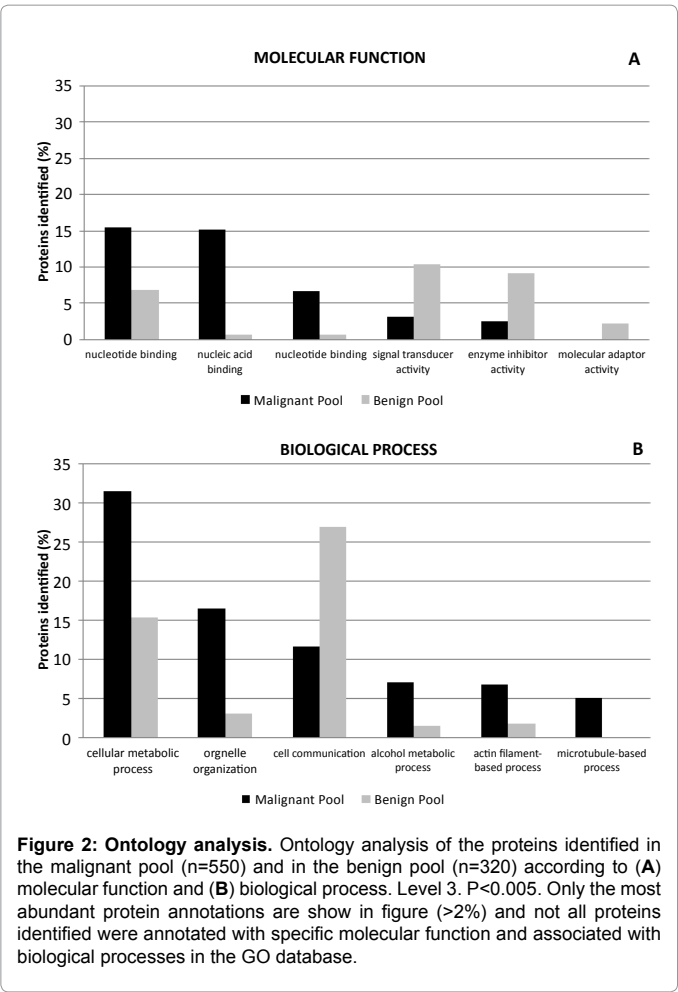


Figure 2: Ontology analysis. Ontology analysis of the proteins identified in the malignant pool (n=550) and in the benign pool (n=320) according to (A) molecular function and (B) biological process. Level 3. P<0.005. Only the most abundant protein annotations are show in figure (>2%) and not all proteins identified were annotated with specific molecular function and associated with biological processes in the GO database.

the other secretome profiles because ES2 cells are derived from clear ovarian cancer cell subtypes. Despite the similarities, we identified some proteins that were detected only in the malignant fluid pool or

only in the benign fluid pool, also indicating some differences between tumor fluids and ovarian cancer cell secretome (Figure 3) (count values are shown in Supplementary Table 2). Among the proteins detected only in the malignant fluid pool, proteins involved especially in blood coagulation and homeostasis (GP1BA, F13B, F10, C4BPB and PROC), response to wounding (GP1BA, F13B, F10, CRP, CFD, C4BPB, SAA2, PROC and MBL2), and acute inflammatory response (CRP, CFD, C4BPB, SAA2 and MBL2) were observed. Furthermore, proteins associated with ovarian cancer such as GPX3, CLIC1, SPAG9, SAA2 and MCAM were also detected only in the malignant fluid pool.

Determination of TIMP1 levels by ELISA Immunoassay

We further selected TIMP1, which was detected in malignant tumor fluid proteomic profile, for determination by ELISA immunoassay (Figure 4). TIMP1 concentrations in individual malignant tumor fluid samples (mean: 182.23 ± 92.99 ng/mL; range: 77.62 to 369.29 ng/mL) were significantly higher than in most of benign tumor fluid samples (mean: 78.69 ± 70.70 ng/mL; range: 0 to 194.78 ng/mL) (p<0.05).

Discussion

Some investigators have described weakness in proteomic analysis based on mass spectrometry to identify biomarkers in ovarian cancer, such as a low number of proteins identified, the lack of identification of low weight proteins, the low reproducibility of the results, and low sensitivity and specificity of discovered biomarkers in comparison with CA 125 [25]. With the improvement of mass spectrometry in terms of sensitivity, software used in searching and identification, and methods for peptide quantitation, these problems are expected to have been reduced. On the other hand, many publications have explored the potential use of individual proteins or collections of proteins as cancer biomarkers and have produced promising results. These proteins were selected for investigation as biomarkers because of their history as biomarkers for other diseases or because of their known function. Predictions from genomic analysis and gene-expression data have also been used to guide research. For most of these proteins, however, their role as a cancer biomarker has not been validated in the context of a defined clinical application. The advent of proteomic technologies

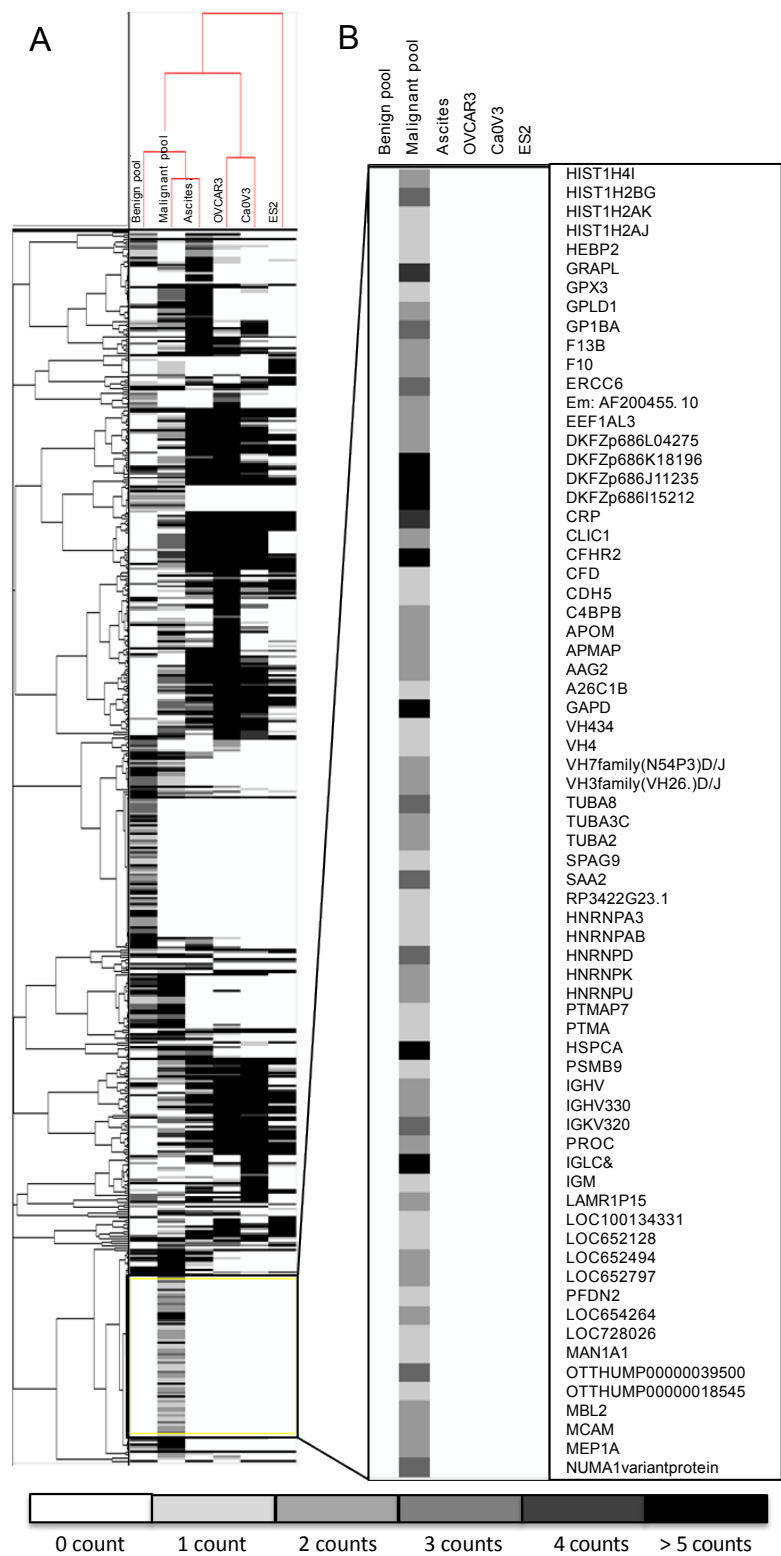


Figure 3: Cluster analysis. **A:** Cluster analysis of 593 genes detected in the benign pool and malignant pool in a comparative analysis with OVCAR3, CaOV3, ES2 and ovarian cancer cells from ascites, using spectral count data. Correlation coefficients (*R*): Malignant x Ascites (0.79); Malignant/Ascites x Benign (0.67); OVCAR3 x CaOV3 (0.70); Benign/Malignant/Ascites x OVCAR3/CaOV3 (0.34); Benign/Malignant/Ascites/OVCAR3/CaOV3 x ES2 (0.07). **B:** 69 genes detected only in the malignant pool.

	Associated Network Functions	Score	Focus Molecules	Molecules in Network
Proteins common to both pools	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function	47	29	26sProteasome,A2M,APOB,APOE,APOH,C3,C5,CFB,F2,F10,F11,HABP2,ITLN1,LCN2,LTF,MIF,MPO,MUC5AC/MUC5B,PLG,PON1,PRDX1,PRO C,PROS1,RBP4,SERPINA1,SERPINC1,SERPING1,SMPD2,TF
	Cellular Assembly and Organization, Cellular Function and Maintenance, Cellular Movement	40	28	ACTB,Actin,ACTN1,APOD,C2,C9,CAP1,CD55,CD59,CLU,EN01,Factin,FBLN1,FLNA,FN1,GAPDH,GSN,HGB1,HSP90AB1,Iit,ITI1,ITI2,LCP1,MDK,MUC4,NPC2,PGK1, VTN,YWHAZ
	Cancer	23	18	AHSG,ALB,APOA2,C1R,C1S,C4BPA,CD163,CFHR1,Fibrinogen,FOLR1,HPX,ITI1,ITI2,KLKB1,KNG1, MUC4,ORM1,SERPINA1,SERPINA3
	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function	18	15	AMBP,C1QA,C8A,CD163,CP,HRG,IGHA1,IGHA2,IGHG2,PGD,PGLYRP2,PIGR,PRDX2,TAP2,TUBB4B
Proteins detected only in the benign pool	Cellular Movement, Cell-To-Cell Signaling and Interaction, Tissue Development	43	25	ANXA1,C5,CD55,CD59,CDH13,CLU,DMBT1,DUOX2,EPS8L2,EZR,FBLN1,GNAI3,GNB2,GSN,MSN,MUC1,MUC4,RAC1,RAP1A,RAP1B,RDX,SDCBP,SPARC,SPP1,THY1
	Cell Death and Survival	15	12	ACE,ANXA4,BAIAP2,C7,CHI3L1,FBLN2,GNA13,IFITM2,PDCD6IP,PROM1,SPARCL1,UBA52
	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry	14	12	APOA2, C4B (includes others), CFB,COL1A2, CST3, GNAQ, HPX,LDL,MUC16,PIGR,QSOX1,TM4SF1
	Cellular Movement	14	11	ADAM9,ANXA2,CTSB,FN1, KRT19, OLFM4, S100A6, SLC9A3R1,SMPDL3B,SNX18,TTR
Proteins detected only in the malignant pool	Protein Synthesis, Molecular Transport, Protein Trafficking	43	30	ACTB,Actin,ACTR3,CAP1,ENO1,ERCC6,FASN,FGG,FLNA,GAPDH,GNB2L1,HNRNPA1,HNRNPK,HNRNPU,ILF3,KPNB1,MAP4,NCL,NPM1,NUMA1,NUTF2,OTUB1,PKM,PLEC,RPS8,RPS14,SSB,TAGLN2,XPO1,YBX1
	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Movement	41	29	ACTN4,ARHGDI1,C5,CALR,CAT,CDH5,CORO1A,EEF1A1,F10,Fibrinogen,FLNB,FN1,GP1BA,GPI,KNG1,LBP,LTF,MBL2,MCAM,MMP9,MPO,NA MPT,PROC,PSMB9,RHOA,RPS3A,SPTAN1,VCL,VCP
	Post-Translational Modification, Protein Folding	26	21	ANXA5,CAPZA1,CD44,CRP,CTTN,CYCS, FActin,HNRNPD,Hsp27,Hsp70,Hsp90,HSP90AB1,HSPA5,HSPA8,HSPB1,Igm, LCP1,LDL, NONO,PPIA,RPL6,SOD1,TIMP1,TLN1,TUBA1A,YWHAQ
	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	16	16	APOA4,APOM,ARPC2, DEFA1 (includes others),EEF1A2,GPLD1,HNRNPAB, Iit,ITI1,ITI2,LAP3, PSMB8,PSMB9,PSME2,PTGDS,SERPINA3
	Cell Morphology	14	14	APOE,ARHGEF2,C4BPB,CSE1L,EIF3A,FKBP4,HIST1H2BB,LDHB,PARK7,PLTP,RALY,TMSB10/TMSB4X,UCLH1,USP5

Score: the score is derived from a p-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1 in 100 chance that the Focus Genes are together in a network due to random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone.

Number of molecules: number of proteins detected in our study present in the network.

Table 2: Networks list. Principal networks of common proteins detected in both pools and proteins observed only in the benign fluid pool and only in the malignant fluid pool using Ingenuity Pathways Analysis (IPA).

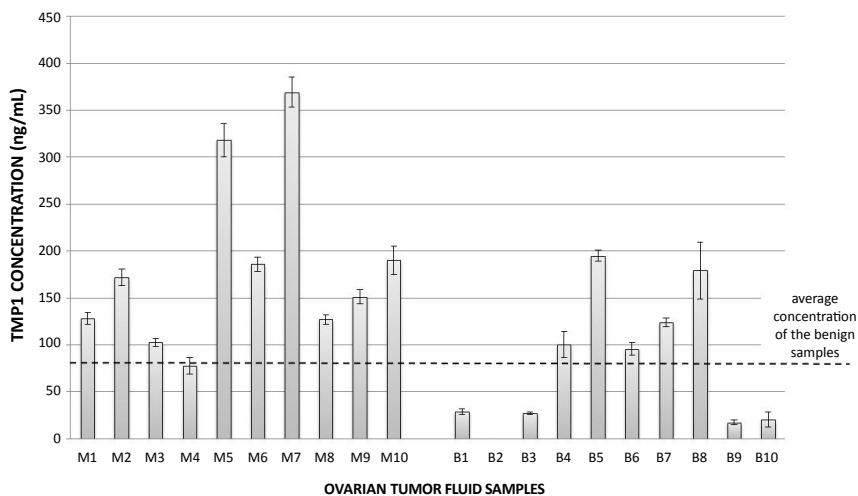


Figure 4: TIMP1 concentrations in tumor fluids. TIMP1 concentrations observed in individual malignant tumor fluid samples (M1- M10) and in individual benign tumor fluid samples (B1-B10) using ELISA immunoassay. Mean TIMP1 concentration was significantly higher in malignant tumor fluid samples (182.23 ± 92.99 ng/mL) than in benign tumor fluid samples (78.69 ± 70.70 ng/mL) (p<0.05). 90% of malignant tumor fluid samples have values above the average concentration of benign samples.

has allowed the systematic evaluation of complex proteins and the identification of differentially expressed proteins in cells, tissues and body fluids [26].

In an attempt to improve the results of proteomic studies, different body fluids have been used to characterize ovarian cancer, each one with their advantages and disadvantages. Most proteomic studies have used blood specimens as starting material. The reason is clear since a simple blood test would be ideal for a biomarker search. Furthermore, the sample may contain proteins or peptides reflecting organ -confined or small volume disease. Problematic, though, is the presence of a number of highly abundant proteins such as albumin, proteins from the complement and immunoglobulins, masking the less abundant and probably more interesting proteins or peptides [25].

The ovarian tumor fluid probably represents the local microenvironment that contains proteins secreted by tumor cells. Initial pathologic changes within that organ can therefore be reflected in proteomic patterns found in the ovarian tumor fluid before secretion into the bloodstream [27]. Studies using ovarian tumor fluid to identify diagnostic and prognostic markers have been reported [28-31]. Fluids from ovarian tumors, especially malignant ones, may exhibit higher concentrations of proteins also present in serum and urine but in a more diluted form.

In the present study, the total protein content of individual fluid samples showed a wide variation among tumors. On the other hand, many of these samples showed total protein concentrations similar to human plasma. The complexity of the tumor fluid samples, confirmed by protein quantification and pattern of proteins on SDS-PAGE gel, indicated the need for depletion of albumin and IgG. Without the overwhelming amounts of albumin, the probability of detecting relevant proteins increases.

The identification of the complete cell proteome is a complex challenge. Therefore, the combination of protein separation by SDS-PAGE followed by gel fractionation into various sections, combined with *in situ* enzyme digestion and MS/MS analysis may result in the identification of thousands (1000-4000) of different proteins that are present in micrograms amounts [32]. Here we considered the number of over 1000 identifiers (IPI) detected, corresponding to 593 genes, to be very representative due mainly to the complexity of the sample analyzed, but also due to the small amount of samples required for the experiment.

In a study published by Kristjansdottir et al. [27], mass spectrometry of ovarian cyst fluids, without depletion of high abundance proteins, was used to study 192 women (benign n=129, malignant n=63) with different histological types and grades of ovarian cancer tumors. The authors detected 1180 peaks (MALDI-TOF ions), 221 of which differed in abundance between benign and malignant ovarian tumors. However, they emphasized the importance of using careful and selective depletion of high abundance proteins from the ovarian cyst fluid which might increase access to other specific biomarkers. Other studies were performed using tumor fluid [28-31], but these investigations were directed at specific protein targets such as E-cadherin and calgranulin, also detected in our study.

More recently, a study [33] was conducted with ovarian cancer tissues (n=8, including three serous, three mucinous, and two endometrioid tumors) and normal ovarian epithelial tissues (n=8). Using the highthroughput proteomic technology of isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two-dimensional-liquid chromatography-tandem mass spectrometry, 1259

proteins were identified. Of those, 205 were potentially differentially expressed between ovarian cancer and normal ovarian tissues, and up-regulation of KRT8, PPA1, IDH2, and S100A11 was validated in ovarian tissue microarrays by immunohistochemistry [33]. In the present study, we also detected the PPA1 and S100A11 proteins.

Ontology analysis indicated that a significant portion of the malignant pool proteins participate in nuclear activity and metabolic processes. We observed a large number of proteins associated with nucleotide/nucleic acid/nucleoside binding. This is consistent with the fact that cancer cells have the ability to sustain chronic proliferation and therefore maintain a high rate of gene expression [34]. Our findings suggest that high-grade ovarian serous carcinomas exhibit heterogeneous metabolic alterations that extend beyond the Warburg effect [35]. At least some of the metabolic heterogeneity observed in our malignant sample is influenced by the tumor microenvironment [36].

Cancer cells must rewire cellular metabolism to support the demands of growth and proliferation, and these altered pathways represent attractive therapeutic targets [37,38]. Some target metabolic enzymes for cancer therapy described in the literature were also detected in our study, like FASN (fatty acid synthase) [39], PGAM (phosphoglycerate mutase) [40], PKM2 (Pyruvate kinase isozyme M2) [41], LDHA (Lactate dehydrogenase A) [42] and IDH1 (Isocitrate dehydrogenase 1) [43]. Recent advances in metabolite profiling methodologies associated with tumor fluid analysis have also contributed to the understanding of the metabolic processes of cancer cells. Furthermore, in the future, the tumor fluid can be used as an accessory method in tumor diagnosis and treatment, combined with a tissue biopsy and pathological methods, such as fine needle aspiration, which should have an impact on patient management.

Peptide counting provides abundance estimates that correlate reasonably well with those determined by other methods [44]. In our cluster analysis, we observed a good correlation between the malignant pool and ascites, followed by OVCAR3 and CaOV3, and then malignant pool/ascites and the benign pool. The similarity in protein profile observed between the malignant pool and ascites was expected, since cells from ascites were derived from a patient with serous ovarian adenocarcinoma. OVCAR3 and CaOV3 are derived from human serous ovarian adenocarcinoma [45,46] and ES2 is derived from clear cell carcinoma [47]. Differences in histology are paralleled by differences in protein profiles, as reflected in our cluster analysis [23]. This is evident from observations that the proteins of ES2 are significantly different from those of the cell lines analyzed [23] and are significantly different between malignant and benign pools.

Some differences were also observed between tumor fluids and cell lines, especially because some proteins were detected only in the malignant pool. These included GPX3 (glutathione peroxidase 3), CLIC1 (chloride intracellular channel 1), SPAG9 (sperm-associated antigen 9), SAA2 (acute phase serum amyloid A) and MCAM (melanoma cell adhesion molecule). Serum levels of GPX3, described as being reduced in various cancers including prostate, thyroid, colorectal, breast and gastric cancers, are decreased in women with papillary serous ovarian cancer [48]. Another case is CLIC1, detectable in ovarian cancer patient serum [49]. SPAG9 mRNA and protein expression was detected in 90% of epithelial ovarian cancer tissues and in three human ovarian cancer cell lines (A-10, SKOV-6 and CaOv-2). Specific SPAG9 antibodies were detected in 67% of epithelial ovarian cancer patients and not in serum from healthy individuals [50]. SAA2 has been shown to be extremely elevated in many cancer samples [51],

is relevant to ovarian cancer molecular pathogenesis and, at the same time, had the highest degree of up-regulation in disease conditions in the study described by Moshkovskii et al. [52]. MCAM is abnormally expressed in a variety of tumors and is closely associated with tumor metastasis, probably participating in the regulation of the Rho signaling pathway to protect ovarian cancer cells from apoptosis and to promote their malignant invasion and metastasis [53].

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) act together to control the operation of the extracellular matrix. TIMP expression is altered in benign and malignant tumors during invasion and metastasis, requiring the breakdown and removal of the extracellular matrix [54]. Studies have shown that TIMP1 is useful as a diagnostic biomarker for pancreatic adenocarcinoma when testing serum from patients compared with serum of healthy volunteers [55]. Moreover, TIMP1 in association with LCN2 (lipocalin) showed potential as a biomarker in serum for early detection of familial pancreatic cancer [56]. Likewise, TIMP1 levels were elevated more than 400-fold and 250-fold in ovarian tumor fluid and peritoneal ascites compared to murine plasma, respectively [57]. Furthermore, TIMP1 was detected in the secretome of OVCAR3, CaOV3, ES2 and ascites [23]. Here, we selected TIMP1 for further evaluation in individual tumor fluid samples since our proteomic profiling detected TIMP1 only in the malignant tumor fluid pool. ELISA immunoassay demonstrated that individual malignant tumor fluid samples showed significantly higher TIMP1 concentrations than most of the benign tumor fluid samples. This verification data agree with the aforementioned studies and support the results from our proteomic profiling of ovarian cancer tumor fluid.

The heterogeneity of samples commonly observed in other studies [27-31,58] may confound the interpretation of the results. Ovarian tumors can arise from different cell types, i.e., epithelial, germ, and sex cord stromal cells, with epithelial cells accounting for approximately 90% of all ovarian cancers [59]. Epithelial tumors are further grouped into different tumor types. Type I tumors include low-grade serous carcinoma, low-grade endometrioid carcinoma, mucinous carcinoma, and a subset of clear cell carcinomas, in most cases, borderline tumors [60-62]. Type I tumors are slow to develop, are generally confined to the ovary [63] and are also genetically stable, with each histologic subtype corresponding to a distinct genetic profile [61-63]. Differently, Type II tumors encompass high-grade serous carcinoma, undifferentiated carcinoma, malignant mixed mesodermal tumors (carcinosarcoma), and some clear cell carcinomas [60]. High-grade serous carcinomas are the most common Type II tumors and these tumors progress rapidly, harbor *TP53* mutations, and exhibit widespread changes in DNA copy number [60-64]. Because of these characteristics of ovarian tumors, analyzing separately each histologic type of tumor may represent the most significant advance in understanding the disease. Here, our analysis relied exclusively on high-grade ovarian serous carcinoma. We believe that a rigorous selection of the samples, using a single tumor type, can significantly contribute to the identification of relevant proteins in the study of cancer.

Conclusion

The proteomic analysis of tumor fluid permits the identification of a large number of proteins expressed in malignant tumors. This strategy is essential for understanding the mechanisms that regulate cell differentiation, leading to the development of different tumor behaviors in the same organ. On the basis of the results presented here, we suggest the use of tumor fluid in studies for therapeutic target

discovery and also as a possible material to assist with diagnosis and patient management.

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