Proteomic Analysis to Differentiate Malignant from Benign Ovarian Tumors

Li Li*, Yi Xu, Chun-Xia Yu

Abstract

Clinically, elevated cancer antigen 125 (CA-125) in blood predicts tumor burden in a woman’s body, especially in the ovary. However, determining whether a tumor is benign or malignant is difficult. Differentially expressed proteins in the serum of patients with OC and benign ovarian tumor, both having elevated CA-125, may be involved in pathways that modulate malignant and benign tumors (BT). Therefore, they are logical candidates as markers to identify whether an ovarian tumor is malignant or benign. Mass spectrometry-based quantitative proteomics, such as isobaric tags for relative and absolute quantitation (iTRAQ) combined with 2D-nano-liquid chromatography-OrbiTrap tandem mass spectrometer, allow global identification and quantification of proteins in complex samples and are well suited for discovering potential biomarkers for human diseases (DeSouza et al., 2005; Wu et al., 2006).

In the present paper, we report a quantitative proteomic analysis of serum from women with elevated CA-125, which helps identify proteins to differentiate malignant from benign ovarian tumors. Two important differential proteins were further validated using western blot technology in serum samples.

Materials and Methods

Sample collection and preparation

Fasted blood samples were obtained from 21 OC patients, 16 BT patients, and 20 healthy women from the Cancer Hospital, Xinjiang Medical University (Urumqi, China).
China). The OC and BT patients all had elevated CA-125 (> 35 units/mL). The OC patients were categorized in terms of histopathological features and stages according to the tumor-node-metastasis classification of tumors. The clinical diagnoses and pathological reports of all the patients were obtained from the hospital. Clinical data such as age, clinical assay value of CA-125, and histological staging are shown in Table 1. The protocol for the present study was approved by the Cancer Hospital Institutional Review Board, and all participants gave informed consent before they were involved in the study.

Data were analyzed anonymously

Serum from OC patients, BT patients, and healthy controls (HC) were used in our proteomic discovery and validation experiments. Serum collection and processing were according to our SOP. In brief, serum from women with OC and BT were collected prior to surgery and chemotherapy. Age-matched HC controls were recruited through an OC screening program. All serum samples, regardless of the collection source, were processed using the same protocol. Blood was collected in 10 cc Vacutainer Serum Separator Tubes and allowed to sit for 30 min at room temperature. The tubes were centrifuged at 3000 rps (1000 × g) for 5 min, split into multiple 1 mL aliquots of serum, and then stored at -80 °C until analysis.

The serum proteomic discovery experiments were performed in pooled sample from each group. The serum from the OC and BT patients was compared with that from the HC group. The pooled samples were analyzed in triplicates; each pooled sample had a total of 10 cases. The validation experiments for serum samples were performed case by case, including all the serum samples previously recruited, which are 57 in total.

Immunological interaction

We used a commercial immunoaffinity depletion method prior to proteomic analysis, multiple affinity removal system (MARS) columns (Agilent Technologies, USA) performed in Prominence LC system (Shimadzu, Kyoto, Japan), to collect flow-through fraction (low-abundant proteins). This procedure was done to reduce the dynamic range of proteins in the serum and increase the likelihood of identifying medium and low-abundance serum proteins via mass spectrometry.

After removing the majorly abundant proteins, protein concentrations were determined using the Bradford Reagent (Bio-Rad, Hemel Hempstead, UK) assay in a test tube format with albumin as a reference standard (1 μg/μL to 10 μg/μL).

Peptide labeling using the iTRAQ® chemical reagent

Depleted sera were labeled with isobaric tags for relative and absolute quantification (iTRAQ, Applied Biosystems, California, USA) of the medium and low abundance proteins recovered from the MARS column. This procedure was done to identify differentially expressed proteins in the sera. Three samples were labeled with the iTRAQ tags as follows: Sample A (OC-pooled sample), 118.1 tag; Sample B (BT-pooled sample), 119.1 tag; and Sample C (HC-pooled sample), 121.1 tag.

The labeled peptides from the three samples were combined into one tube and dried in a vacuum concentrator. A SepPac™ C18 cartridge (Waters Corporation, Milford, MA) was used to exchange the buffer and remove trypsin and hydrolyzed unbound iTRAQ reagents from the labeled peptides.

Peptide fractionation

The concentrated iTRAQ-labeled sample was added to 2 mL of Diluent buffer [10 mM potassium phosphate and 25% acetonitrile (ACN)]. The pH of the buffer was adjusted below 3 using phosphoric acid. The samples were subjected to cation exchange chromatography using a PolySULFOETHYL A HPLC column [(2.1 × 100 mm)i.d., PolyLC Inc, Columbia, MD, USA]. A gradient flow of 0.2 mL/min was used, and twenty-four 0.4 mL fractions were collected in 1.5 mL microfuge tubes. The pH values of buffers A (10 mM potassium phosphate with 25% ACN) and B (high-salt buffer containing 10 mM potassium phosphate, 500 mM potassium chloride, and 25% ACN) were adjusted to pH < 3 using phosphoric acid. The following gradient was applied: 5 min 100% buffer A; 5 min to 45 min increasing to 30% buffer B; 45 min to 50 min increasing to 80% buffer B, maintained for 5 min; 56 min 100% buffer A, maintained for 15 min. Fractions were collected starting at 6 min and at 2 min intervals.

Reversed phase LC-MS/MS analysis

The peptides were resuspended with 20 μL solvent A (A: water with 0.1% formic acid; B: ACN with 0.1% formic acid) and separated using a Nano Aquity UPLC system (Waters Corporation, Milford, USA) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with an online nanoelectrospray ion source (Michrom Biosources, Auburn, USA). An 18 μL peptide sample was loaded onto the Captrapp Peptide column (Michrom Biosources, Auburn, USA), with a flow of 20 μL/min for 5 min. Subsequently, the sample was eluted with a three-step linear gradient as follows: starting from 5% A to 45% B for 70 min, increased to 80% B for 1 min, and then sustained on 80% B for 4 min. The column was re-equilibrated at initial conditions for 15 min. The flow rate and temperature of the column were maintained at 500 nL/min and 35 °C, respectively. The electrospray voltage of 1.9 kV versus the inlet of the mass spectrometer was used.

LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 300-1600) were acquired in the Orbitrap with a mass resolution of 60,000 at m/z 400, followed by four sequential HCD-MS/MS scans. The automatic gain control was set to 500,000 ions to prevent over-filling of the ion trap. The minimum MS signal for triggering MS/MS was set to 10,000. In all cases, one microscan was recorded. The lock mass option was enabled, and the polydimethyllysineolsoxane ion signal (protonated (Si(CH$_3$)$_2$O)$_n$; m/z 445.120025) was used for internal calibration of the mass spectra.

MS/MS scans were acquired in the Orbitrap with a mass resolution of 7,500. For MS/MS, precursor ions were
activated using 45% normalized collision energy and an activation time of 30 ms.

**Peptide sequencing and data interpretation**

All MS/MS spectra were identified using SEQUEST [v.28 (revision 12), Thermo Electron Corp.] against the human Swiss-Prot database (Release 2010_04). The searching parameters were set as follows: partial trypsin cleavage with two missed cleavage was considered; fixed modification of cysteines by methyl methanethiosulfonate; iTRAQ modification of peptide N termini, methionine oxidation, and lysine residues was set as variable modification; peptide mass tolerance was 15 ppm; and fragment ion tolerance was 0.1 Da. The Trans Proteomic Pipeline software (revision 4.2) (Institute of Systems Biology, Seattle, WA) was used to identify the proteins and calculate protein ratios. The peptide results were filtered using Peptide Prophet1, with a p-value over 0.90, and a Protein Prophet2 probability of 0.95 was used for the protein identification results. The false positive rate was < 1% for the experiment.

**Western blot**

Differentially expressed serum protein markers determined via iTRAQ were validated using western blot. The total protein (30 mg) was separated through 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Amersham Bioscience). The membranes were blocked with 5% bovine serum albumin in TBST buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Tween-20) and then incubated with primary antibody for 1 h at room temperature. Subsequently, the membranes were washed and incubated with corresponding horseradish peroxidase-conjugated secondary antibody. The washed membranes were developed with enhanced chemiluminescence reagent (Amersham Bioscience). The images were captured with LAS-3000 instrument (Fuji, Japan) and calculated using Grey analysis.

**Results**

We analyzed in triplicates the iTRAQ-labeled pooled serum samples from the three groups (OC, BT, and HC). The peptide results were filtered using Peptide Prophet1 (Keller et al., 2002) with a p-value over 0.90, and a Protein Prophet2 probability of 0.95 was used for the protein identification results. The false positive rate was < 1% for the experiment.

We obtained quantitative data on 326 proteins. Proteins with expression ratios of over 1.2-fold in increase or at least 1.5-fold in decrease were considered differentially expressed.

We identified 9 overexpressed and 75 underexpressed proteins in OC compared with HC (Table 1 in Supplemental Digital Content). We identified 32 overexpressed and 103 underexpressed proteins in BT compared with HC (Table 2 in Supplemental Digital Content). In addition, we identified 77 overexpressed and 43 underexpressed proteins (Table 2) in OC compared with BT.

**Gene ontology analysis**

Gene ontology analysis was operated according to the method in nature protocol (Huang et al., 2009) using DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov). The gene list was submitted to DAVID. We accessed DAVID analytic modules through the tools menu page, ran “Gene Name Batch Viewer,” and finally explored the results. Table 3 lists the top 10 terms that are significantly enriched (p < 0.004) by molecular function.

**Protein validation**

Two differentially expressed proteins between OC and BT sera were validated via western blot: apolipoprotein A-4 (APOA4) and natural resistance-associated macrophage 1 (NREM1). The expression of APOA4 and NREM1 were significantly increased in the OC serum samples compared with the BT and HC sera.

**Table 1. Clinical Data for the Serum Samples in This Study**

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**Figure 1. The Western Blotting Result for APOA4**

Protein (A for ovarian cancer group; B for ovarian benign tumor group; and C for healthy group; digitals after the A, B, or C for the number of each serum sample). The value below each gel was the grey level.

**Figure 2. The Western Blotting Result for NREM1**

Protein (A for ovarian cancer group; B for ovarian benign tumor group; and C for healthy group; digitals after the A, B, or C for the number of each serum sample). The value below each gel was the grey level.
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Table 3. Gene Ontology Analysis of the Differentially Expressed Proteins: the 32 Terms that are Significantly Enriched (p<0.05) by Molecular Function

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</table>

(NRAM1).

The western blot result of APOA4 verified the quantitative proteomic results. APOA4 protein exhibited a good stability for individuals in each group. In the proteomic profiling, APOA4 protein showed 0.71 and 1.18 in OC and BT sera, respectively, compared with the value of 1 in HC. The western blot result for the protein was consistent with the profiling result. The mean grey level values of the OC, BT, and HC groups were 0.54, 0.83, and 0.61, respectively (Figure 1).

Based on the western blot result, NRAM1 protein was robustly consistent with the profiling result but was not very acceptable at the stability in individual. It fluctuated for each serum sample in each group (Figure 2).

Discussion

Recently, proteomic profiling of serum or plasma has been widely performed to discover cancer biomarkers (Amon et al., 2010; Gao et al., 2011; Qiu et al., 2011). In OC proteomic research, Amon et al. found 54 quantified serum proteins and 358 peripheral extracellular fluid proteins, in which 17 proteins are identified in both materials and 14 are extracellular (DeSouza et al., 2005; Wu et al., 2006; Amon et al., 2010). Pan et al. demonstrated that extracellular matrix proteins, catenins, and P53 binding protein 1 are important for chemotherapy response in OCs (Pan et al., 2009).

One successful aspect of Amon's study is the design of the serum experiment, which included three different types of subjects: OC patients, BT patients, and healthy volunteers. In the aforementioned research, proteins up-regulated in cancer relative to the healthy but not relative to the benign were eliminated from consideration. The same was true for proteins up-regulated relative to the benign but not to the healthy. Only proteins in the cancer versus the benign comparison became the key point (Amon et al., 2010). In the present study, we used a similar strategy to focus on differentially expressed proteins in the comparison between OC and BT, both having elevated CA-125. Our goal was to identify proteins to differentiate malignant from benign tumors in serum with elevated CA-125. We obtained 80 overexpressed and 44 underexpressed proteins in the comparison (Table 2) between OC and BT for further validation, we selected two important proteins among the differential proteins: APOA4 and NRAM1. APOA4 is a member of the APOA1/C3/A4/A5 gene cluster located on the long arm of the human chromosome 11 (Lai et al., 2005; Dieplinger et al., 2009). Members of this cluster are all involved in lipid and lipoprotein metabolism and thus in many ways associated with cardiovascular disease. APOA4 is a 46 kDa glycoprotein that is almost exclusively produced in intestinal enterocytes and secreted into the lymph. APOA4 was first identified as a component of chylomicrons and high-density lipoproteins (Betsiegil and Utermann, 1979). Dieplinger found that APOA4 has a decreasing tendency from the sera in HC to those in BT and OC (Dieplinger et al., 2009). In the present study, we observed a decreased APOA4 in the OC serum both in the results of proteomic profiling and western blot. However, we observed an elevated APOA4 (1.18 in the BT serum in the proteomic profiling) in the BT serum along with elevated CA-125. This result is consistent with the data of Dieplinger and other previous studies. The protein validation of APOA4 in the OC serum was first performed by a Western blot and confirmed by immunohistochemistry (De Souza et al., 2005; Wu et al., 2006; Amon et al., 2010). Pan et al. demonstrated that extracellular matrix proteins, catenins, and P53 binding protein 1 are important for chemotherapy resistance in OCs (Pan et al., 2009).
against phagocytosed microorganisms (Goswami et al., 2001). However, prolonged accumulation of toxic radicals can have detrimental effects, causing cell or tissue damage and contributing to the development or progression of numerous diseases, including cancer and autoimmunity. Toxic radicals generated due to macrophage activation in response to stimuli are reactive oxygen (generically referred to as oxidants: superoxide, hydrogen peroxide, hypochlorous acid, singlet oxygen, and hydroxyl radical) and nitrogen (nitric oxide by the inducible nitric oxide synthase) intermediates (Schumacker, 2006). In humans, there are two predominant NRAMP1 (GT) n Z-DNA promoter alleles: allele 2 and allele 3. These alleles have been reported to account for opposing levels of NRAMP1 gene expression; that is, allele 3 drives high gene expression, whereas allele 2 drives low gene expression (Searle and Blackwell, 1999). Allele 3 is associated with autoimmunity and cancer but protects against infectious diseases, whereas allele 2 is associated with infections, such as tuberculosis, but protects against autoimmunity and cancer (Searle and Blackwell, 1999). These observations suggest that chronic hyperactivation of macrophages associated with allele 3 is functionally linked to cancer and autoimmune disease susceptibility, whereas the poor level of NRAMP1 expression promoted by allele 2 contributes to infectious disease susceptibility (Searle and Blackwell, 1999).

In the present proteomic profiling, NRAM1 protein was elevated in OC (1.27) and decreased in BT (0.67). This result is consistent with previous studies. The western blot verification on the protein in the sera showed that the protein was roughly consistent with the profiling result but was unstable on the value in individuals and fluctuated for each individual serum sample in each group. The reason for this phenomenon needs further investigation.

In summary, the quantitative proteomic profiling found on the serum of women with elevated CA-125 helped identify proteins that can differentiate malignant from benign ovarian tumors. A novel protein, APOA4, has the potential to be a marker for malignant and benign tumor differentiation in serum with elevated CA-125.

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**References**


