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Original Article

Vimentin and PRG4 as candidate biomarkers of ovarian cancer using serum proteomic analysis of 2D-DIGE

Bo Bi, Lei Fang, Ruiping Han, Jiemin Gan, Huimin Zhou, Wenrong Jiang, Hu Zhao

Department of Clinical Laboratory Medicine of Huadong Hospital Affiliated to Fudan University, Shanghai 200040, China

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Abstract: Epithelial ovarian cancer (OC) is the most lethal of gynecologic malignancies, largely due to the advanced stage at diagnosis in most patients. It is therefore an urgent task to screen out reliable biomarkers for the early diagnosis and prognostic prediction of OC. In this study, we used pooled sera depleted of the most highly abundant proteins to reduce the dynamic range of proteins, and thereby enhance the identification of serum biomarkers using the quantitative proteomic method of 2-dimensional difference gel electrophoresis (2D-DIGE), and further validate the protein expression by ELISA and immunohistochemistry. OC specimens were first investigated by 2D-DIEG, of which 24 were confirmed by ELISA and IHC. It was found that vimentin (VIM) and proteoglycan 4 (PRG4) were significantly up-regulated in the sera from OC patients, suggesting that the two proteins may prove to be promising biomarkers of OC.

Keywords: 2D-DIGE, ovarian cancer, proteomics, biomarker, vimentin, proteoglycan 4

Introduction

Ovarian cancer (OC) ranks the 5th leading cause of cancer death in women, causing 140,000 deaths yearly in China [1]. More than 80% OC cases were metastatic at the time of diagnosis, which is the main reason for the high mortality of the disease. Indeed, the survival rate of OC patients could increase to more than 90% if it could be detected at an early stage [2], and therefore it is primarily important to find sensitive and specific biomarkers for early diagnosis of OC.

CA125 is the most commonly used biomarker of OC, knowing that it is an antigen protein normally expressed in coelomic epithelial cells, and elevated in about 90% of late-stage OC patients [3, 4]. However, CA125 expression is normal in about 50% stage I OC patients, indicating that it is not sensitive enough for screening of early OC. The incidence of OC is relatively low, accounting for about 40 out of 10,000 persons. As the specific biomarkers currently available in clinical practice cannot meet the requirement of screening of early OC [5], it is necessary to find more sensitive and specific biomarkers for early OC screening.

Quantitative proteomics is an important tool for screening molecular biomarkers for disease diagnosis and prediction of the therapeutic effect, in which two-dimension difference gel electrophoresis (2D-DIGE) is a common technique [6]. Albrethsen et al [7] used 2D-DIGE to assess the difference between cisplatin-sensitive and cisplatin-resistant OC cell strains. Krockenberger et al [8] used 2D-DIGE to assess the protein difference between high-invasive and low-invasive OC strains.

The aim of the present study was to use proteomics (**Figure 1**) to determine serum protein biomarkers of OC by a combination of serum protein fractionation by immunoaffinity substraction of high-abundant proteins, 2D-DIGE and mass spectrometry (MS), and finally confirm the serum protein levels by ELISA and immunohistochemistry (IHC).

Materials and methods

Subjects

Serum samples were obtained from 12 patients with serous OC and 12 non-cancer females as controls between January 2009 and March

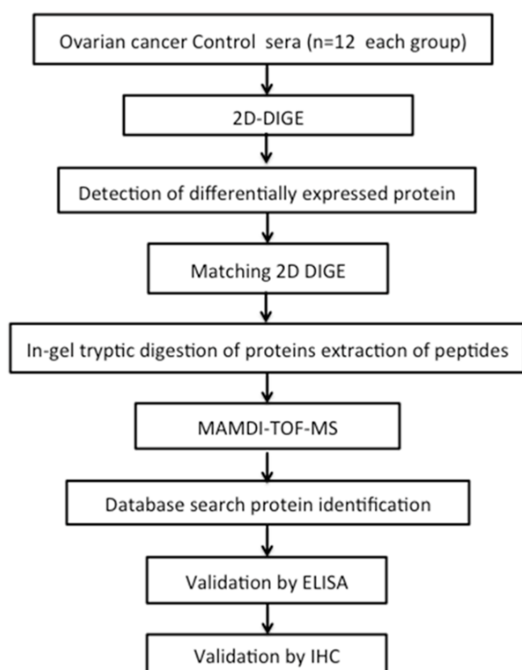

Figure 1. Workflow of the serum proteome analysis for identification of differentially expressed proteins in two groups. Twelve sera from OC group and 12 sera from normal control group were first subjected to a reduction of serum complexity by removing the abundant proteins by immunodepletion columns. The immunoaffinity-processed sera were parallelly subjected to 2D-DIGE, and differentially expressed proteins identified through DIGE image analysis were matched with the preparative gel. The protein spots were excised as gel plugs, prepared for tryptic digestion and identified by MS. The conformation and pilot validation of identified proteins were performed by ELISA and IHC.

2014 in the Departments of Gynecology and Obstetrics of Huadong Hospital and Zhongshang Hospital (Shanghai, China). Of the 12 serum OC samples, 7 were from patients with stage III OC tumors, 4 from stage IV OC tumors, and one from a stage I and II OC tumor. The mean age of the 12 OC patients was 60.5 ± 7.3 (range 24-65) years. The samples were immediately centrifuged at 3000 g for 10-15 min at 5°C. The supernatant (serum) was collected and kept in aliquots of 50 μl. The control samples were obtained from 12 voluntary women without cancer who aged between 15 and 56 years with a mean of 53.2 ± 8.2 years. Informed consent was obtained from all patients and volunteers, and the research protocol was approved by the Ethical Committee of Fudan University (Shanghai, China) according to the ICH GCP guideline and the Declaration of Helsinki.

Removal of high-abundant proteins from serum samples

The serum samples were processed using a 4.6 × 50 mm^2 Multiple Affinity Removal Column (Agilent Technologies, Palo Alto, CA) to selectively remove albumin and IgG. The column was attached to an EZChrome Elite HPLC (Agilent Technologies, Palo Alto, CA, USA), which can process 25 μl human serum per sample run. The samples were processed according to the manufacturer's instructions. For each sample, a low-abundant fraction was collected and buffer exchanged into 15 mmol/l Tris-HCl pH 7.4 using a 5000-Da molecular weight cutoff spin concentrator (Agilent Technologies, Palo Alto, CA, USA). Protein quantification was performed by mixing the sample with a Coomassie protein assay reagent (Abcam, Cambridge, UK) and measuring the absorbance at 595 nm based on the Bradford method. Bovine serum albumin (BSA) was used as a protein standard. Approximately 92% total serum protein was removed using the described method.

2D-DIGE

2D-DIGE was performed as previously described [9]. Briefly, 10 μl unfractionated whole human serum sample was subjected to isoelectric focusing (IEF) in 11 cm rehydrated precast immobilized dry strips pH 4-7 (GE Healthcare Bio-Sciences, CA, USA). For the second dimension, focused samples in the strips were subjected to electrophoresis using the 8-18% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). All samples were analyzed in duplicate.

Coomassie Blue and silver staining

The 2D-DIGE gels were developed by silver staining as described previously [12] and stained with Coomassie Blue for MS analysis according to the modified method of Shevchenko et al [13]. Coomassie Blue was used in this case as the higher number of peptides was recovered from the in-gel digestion of Coomassie stained gel plugs compared to the plugs from silver-stained gels.

Mass spectrometry

Highly resolved spot clusters of proteins of interest were preliminarily identified by visual

comparison with the SWISS ExPASy standard plasma protein reference [14] before being subjected to precise identification by MS. In-gel trypsin digestion was performed according to the method of Shevchenko et al [13]. MS analysis was performed by mixing peptide solutions with matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 0.5% trifluoroacetic acid (TFA) and 50% acetonitrile (ACN) in a ratio of 1:1. The mixture (0.5 μl) was then applied onto the sample slide. The spectrum was calibrated using peptides included in the matrix solution, approximately 3 pmol Ile7Ang III and hACTH 18-39 with the expected m/z at 897.523 [(M + H)+, monoisotopic] and 2465.191 [(M + H)+, monoisotopic], respectively. Protein spots that were not positively identified were subsequently sent for MS/MS instrument analysis (Applied Biosystem 4700 Proteomic Analyzer) at Fudan University School of Biological Sciences (Shanghai, China). Gel plugs were kept in fresh Eppendorf tubes containing small volumes of milliQ water to ensure that they remained hydrated prior to analysis. MS/MS instrument-related parameters included the reflection mode, nitrogen laser (337 nnq, 3 ns pulse width, 3 Hz repetition rate), delayed ion extraction for 100 ns, grid voltage 68%, vacuum 40-008, accumulation of 150 single scans of mass spectrometric signals, and detection of the positive ion spectrum. Pancreatin self-decomposition peaks 842.51 and 2211.1046 were used as internal reference.

Database searching

Peaklist data obtained from PMF and MS/MS analyses were generated using the Ettan MS/MS software (version 2.0) and 4000 Series Explorer software (version 4), respectively. The data were exported to the MASCOT search engine (Matrix Science Ltd., London, UK; version 2.3). The procedures are as follows: (i) Clicking Peptide Mass Fingerprint for entry; (ii) selecting NCBInr database and setting the search indexes using three possible decomrations: Carbamidomethyl (C), Oxidation (M) and Pryro-glu (N-tern E); the maximum allowable peptide mass error was set as 0.1 Da, and each peptide was allowed to have one incomplete cleavage site; (iii) determine the type of protein obtained; and (iv) display the matched segment and amino acid sequence of the obtained peptide.

Image analysis

The Image scanner (Version 6) was used to capture and store 2D-DIGE gel images, evaluate the protein profiles, and present information obtained from the 2D-DIGE gels. To detect proteins that were differentially expressed in the sera, the percentage of volume contribution (% vol), which refers to the volume percentage of a protein taken against the total spot volume of all proteins including the unresolved peptides in each gel, was calculated. Data obtained in such expression are independent of variations attributing to protein loading and staining.

ELISA

Competitive ELISA was performed as previously reported [9-11] in a single-blind manner, in which the operator was not aware of the source of the serum samples and clinical information of the patients. Serum vimentin (VIM) and proteoglycan 4 (PRG4) concentrations were reflected by their ability to inhibit the specific binding of antisera. The primary antiserum (IgG fraction) comprised mouse anti-human VIM and PRG4 (Abcam, Cambridge, UK). Peroxidase conjugated donkey anti-mouse IgGs (Abcam, Cambridge, UK) were used as the secondary antiserum. Assay was not performed for LRG because of antiserum to the protein was not commercially available. Enzyme activity was revealed by addition of 3, 3', 5, 5' tetramethylbenzidine (Abcam, Cambridge, UK). Protein levels in the test sera were proportional to the percentage of inhibition of substrate hydrolysis. Serum protein concentrations were interpolated from kit-specific standard curves generated in GraphPad Prism 6.0 (GraphPad Software).

Immunohistochemical staining

The 24 tissue specimens were paraffin embedded, sliced, routinely de-waxed with dimethylbenzene, dehydrated with gradient alcohol to inactive endogenous peroxidase, cultured in 3% H_2O_2 37°C for 10 min, washed with PBS for 5 min × 3, mounted with normal goat serum working solution for 10 min at 37°C, added with 1:200 anti-VIM (Abcam, Cambridge, UK) and 1:200 anti-PRG4 (Abcam, Cambridge, UK), incubated at 4°C overnight, washed with PBS for 5 min × 3, added with biotin-labeled secondary antibody, incubated at 37°C for 30 min,

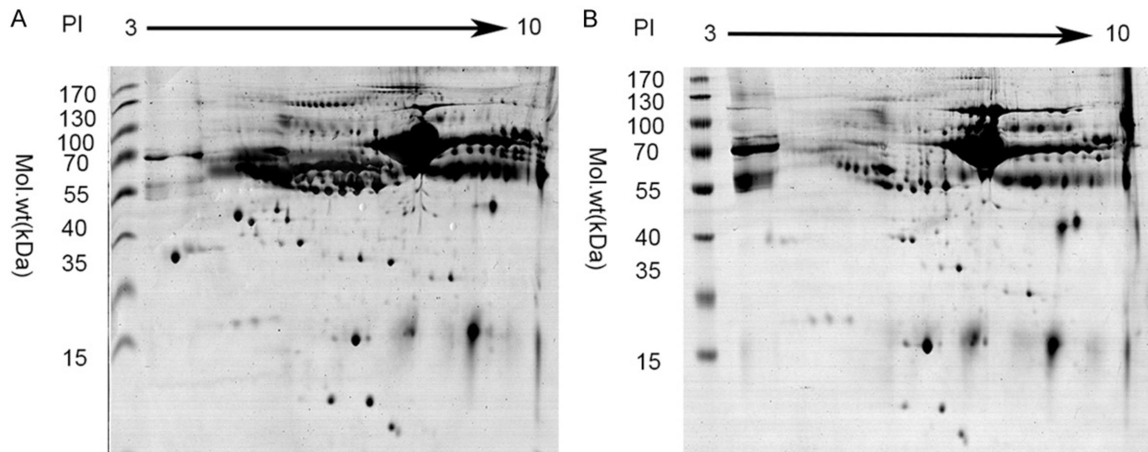

Figure 2. 2D-DIGE comparison of different methods used for depletion of high-abundant proteins. A. An untreated serum sample. B. Depletion of two high-abundant proteins from the serum using the ProteoExtract Albumin/IgG Removal Kit.

washed with PBS for 5 min × 3, added with horse radish peroxidase-labeled working solution, incubated at 37°C for 30 min, washed with PBS for 5 min × 3, stained with DAB/H_2O_2, thoroughly washed with running water, restained with hematoxylin, dehydrated routinely, and finally mounted.

Any brown cytoplasmic or cytomembrane staining of cells was considered positive for VIM and PRG4. The tissue sections were screened at a high power magnification (200 ×), and 5 areas with the most intense expression were selected. A section with a mean percentage of positive cells < 10% was considered to have low expression, and a section ≥ 10% was considered as having high expression.

Statistical analysis

The statistic software SPSS 13.0 for Windows (SPSS, Chicago, IL) was used to calculate the significance according to Student t-test. *P*-values of less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

Removal of high-abundant proteins using MARS

To detect low-abundant proteins, two most abundant proteins in the sera were removed using multiple affinity removal system (MARS) columns, which allowed for clear visualization of several other proteins. These depleted samples were further concentrated and desalted using ultracentrifuge filters to exclude proteins with a molecular mass < 5 kDa. Using this method, we successfully depleted the samples of six high-abundant proteins with no substantial loss of other proteins in the samples (**Figure 2**).

Several proteins expressed differently in serum from different groups

Using Lab Scan image scanner software analysis, 768 ± 58 protein spot features were identified in the immunoaffinity-depleted sera. DIGE was then performed on the depleted samples from the two groups, including the pooled sera from the OC patients and those from normal controls. T test analysis showed that nine of the protein spots with more than two-fold significant difference underwent significant changes, of which six protein spots were up-regulated and the remaining three protein spots were down-regulated, all showing a two-fold difference in expression ($P < 0.01$) (**Figure 3B** and **Table 1**).

Two differentially expression protein up-regulated in the sera of OC patients

Protein spot cutting, in-gel trypsin digestion and MS/MS analysis were performed on protein spots that underwent statistically significant changes in the D2-DIGE protein profiles, and the original MS data thus obtained were analyzed in MASCOT database to finally define

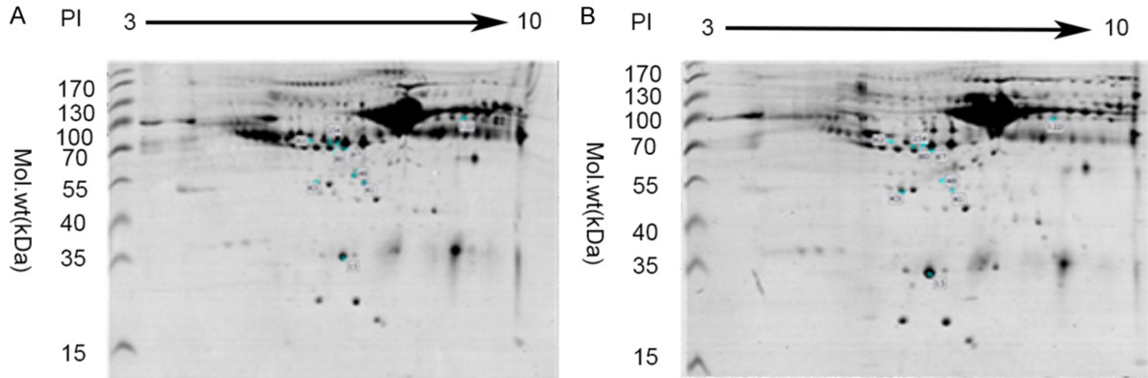

Figure 3. A map of differentially expressed protein spots between the two groups. A. Normal control group. B. OC group.

Table 1. 2D-DIGE analysis of different protein spots between group A and B

Master number[a]	T-test[b]	Mean ratio[c]
15	0.0012	-2.38
41	0.0025	2.12
43	0.0035	-2.32
48	0.0018	2.16
67	0.0026	-2.23
80	0.0026	5.76
82	0.0038	6.35
122	0.0037	2.29
254	0.0028	2.15

[a]Master number indicates the number of different positions for the different protein spots on the gels. [b]Paired-sample *t*-test. [c]Ratio of differential expression. Group B/A indicates the OC group *vs.* the normal control group. A positive value indicates that the expression level of the latter is higher than the former. A negative value indicates that the expression level of the latter is lower than the former.

the names of the differentially expressed proteins in serum. In the present study, we finally identified two differentially expression protein molecules that were up-regulated in the sera of OC patients (**Table 2**).

VIM and PRG4 mainly expressed in the cytoplasm and plasma membrane of OC cells

IHC staining showed that VIM and PRG4 were mainly expressed in the cytoplasm and plasma membrane of OC cells. The expression of VIM and PRG4 in the OC tissue was 73% and 67% *vs.* 33% and 27% in the normal control group, respectively ($P < 0.05$) (**Figure 4**; **Table 3**).

VIM level and PRG4 in serum expressed higher in OC group

To confirm the aberrantly expressed proteins in the sera of the normal control and OC groups, competitive ELISA was carried out using antisera against VIM and PRG4. The results are demonstrated in **Figure 5**. The serum VIM level and the expression of PRG4 in OC group were significantly higher than those in the normal control group ($P < 0.001$).

The ROC curves based on the ELISA data

ROC curves based on the ELISA data for the two were obtained, demonstrating there is a statistically significant difference in protein concentration between the Ovarian Cancer patients and controls ($P < 0.001$). The area under the curve values are VIM = 0.91, and PRG4 = 0.95 (**Figure 6**).

Discussion

Early diagnosis is one of the essential factors in improving the 5-year survival rate of OC patients. Most OC patients lost their best time of treatment when they noticed the clinical symptoms because the disease had progressed to the late stage. The clinical symptoms and signs of early OC are usually not obvious and not specific, making the early diagnosis especially difficult and causing a high death rate. It is therefore especially important to screen highly sensitive and specific serum biomarkers to improve the early diagnosis of OC.

Recently, some researchers [14] used 2D-DIGE to screen candidate serum biomarkers by com-

Table 2. MS identification of protein spot clusters from 2D-DIGE serum protein profiles

Master number[a]	Protein	Official Full Name	Accession number[b]	Sensitivity	Tolerance	*P* Value
80	VIM	Vimentin	P08670	0.44656	0.219355	0.014767
82	PRG4	Proteoglycan 4	Q92954	0.086953	0.045551	0.010232

Mass spectrometry was used to analyze the protein spot cluster. [a]Master number are as in **Figure 3**. [b]Accession numbers are from the MASCOT database http://www.matrixscience.com.

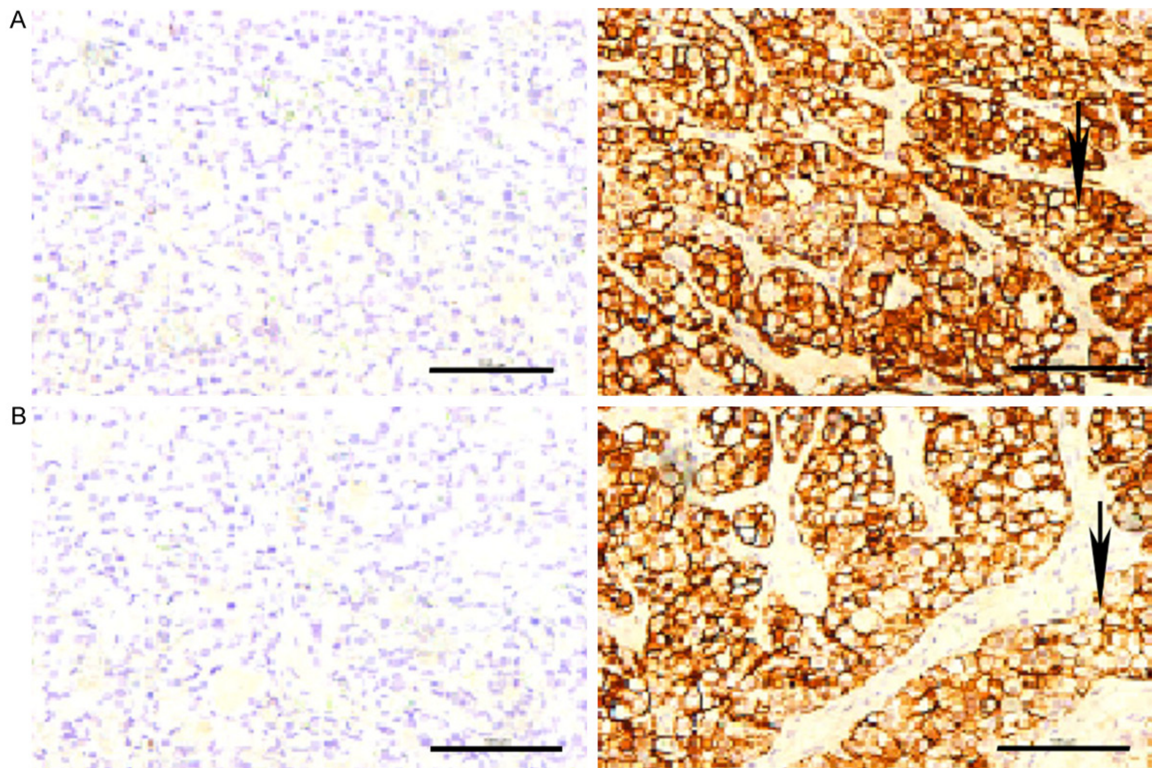

Figure 4. IHC analysis of protein expression in the ovarian tissue of normal controls and OC patients. IHC staining was performed using antisera to (A) VIM and (B) PRG4. (A and B) Demonstrate strong positive VIM and PRG4 expressions (arrow) in the OC tissue *vs.* negative expression in the normal ovarian tissue.

Table 3. VIM and PRG4 expressions in the OC and normal tissues

	Cases	Positive	Negative	*P* Value
Ovarian cancer tissues				
VIM	12	11	1	0.00167
PRG4	12	10	2	0.00184
Normal controls				
VIM	12	0	12	0
PRG4	12	0	12	0

parison between OC patients and normal controls, computerized the differentially expressed proteins, analyzed them by MS/MS to screen out tumor-related serum biomarkers, and finally used ELISA and IHC to compare these differentially expressed proteins between OC and normal ovarian tissues. In their MS/MS analysis and 2-DE, Ebert et al [15] found that thrombin light chain A may be a serum biomarker of OC, and serum amyloid A may be a useful biomarker of gastric cancer, and their results were later confirmed by IHC [16]. Unfortunately, these moleculobiological markers finally failed to prove effective for the screening and differential diagnosis of OC.

In the present study, we obtained two proteins (VIM and PRG4) and found that they were closely associated with OC and could be used for differential diagnosis and prognostic prediction of OC. To the best of our knowledge, this is the

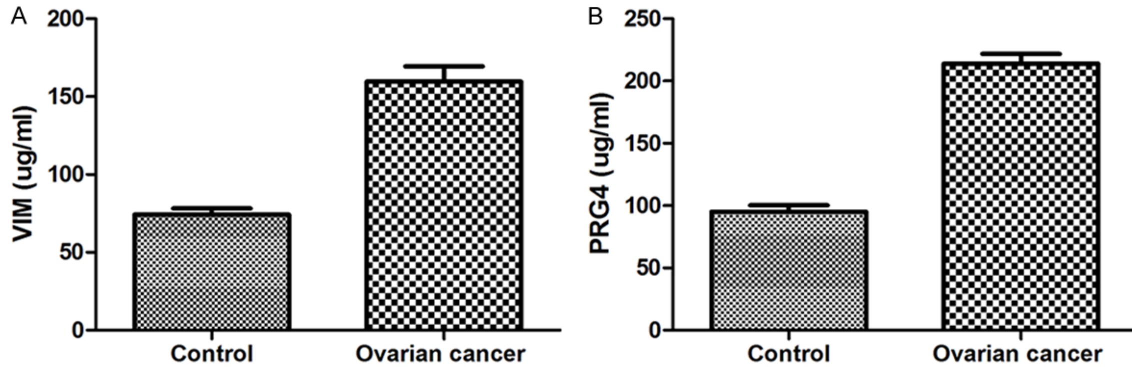

Figure 5. Analysis of serum protein expression by competitive ELISA. Competitive ELISA was performed using mouse anti-human VIM and PRG4 as primary antibodies. Analyses were performed in triplicate. Statistical analysis was performed with GraphPad Prism5.5 applying Mann Whitney test (two tailed). Values are shown as a mean with SEM.

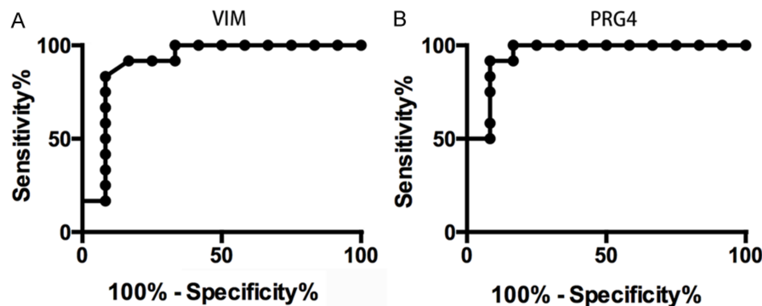

Figure 6. ROC curves based on the ELISA data. The result indicated there is a statistically significant difference in protein concentration between the Ovarian Cancer patients and controls. The area under the curve (AUC) values are VIM = 0.91, and PRG4 = 0.95.

first study reporting the correlation between VIM and OC, and also the first research on the role of VIM in the development and progression of OC.

The result of ELISA in our study showed that VIM was highly expressed in the serum of OC patients, and the result of IHC suggested that VIM was also highly expressed in the OC tissue. VIM is a type III intermediate filament protein and expressed in mesenchymal cells and some ectodermal cells [17]. Abnormal expression of VIM can alter the composition of cytoskeleton protein, thus turning the cubic shape of epithelial cells to the spindle shape of fiber-like cells and making them susceptible to migration and translocation. VIM is also reported to be correlated with the degree of malignancy of tumor cells [18]. VIM has been found to be expressed in various epithelial carcinomas including breast cancer, hempatocellular carcinoma (HCC), clear cell renal cell carcinoma (CCRCC), prostate cancer, gastric cancer and lung cancer, and its high expression means poor differentiation, strong invasiveness and metastatic susceptibility of cancer cells [19-25]. However, there is also clinical evidence that the expression of VIM in the tumor tissue of breast, kidney and prostate cancers had nothing to do with the degree of tumor malignancy [26]. The specific expression of VIM intermediate filaments in histocytes, its structure and related functions have tempted researchers to link VIM with tumor occurrence, differentiation, local invasion, metastasis and prognosis. It is generally believed that VIM is the prerequisite of invasion and metastasis of tumor tissues [22].

Recent studies using 2D-DIGE and MS/MS showed that the OC-to-control ratio of PRG4 was about 2. The result of ELISA suggested that the expression of PRG4 was elevated in the serum of OC patients, and the result of IHC showed that the expression of PRG4 was also up-regulated in the OC tissue. PRG4 is a large-molecular-weight viscous glycoprotein, whose binding with urinary enzyme-like plasmin receptor activators and inhibitors would affect the activity of plasmin and cell adhesiveness. In addition, heme protein fragments could regulate the metabolism of extracellular matrix proteins such as collagen and fibronectin metabolism [27]. PRG4 participates in boundary

lubrication by forming a strong repulsive force on the articular surface due to the large amounts of amino polysaccharides attached to the middle segment of PRG4, which promote protein glycosylation, increase the molecular mass and enrich negative charges [28].

Using 2D-DIGE and MS/MS technique, we found that the VIM and PRG4 levels in the sera of OC patients were significantly higher than those in the non-cancer sera, suggesting that VIM and PRG4 may be biomarkers of OC. Studies currently available have shown that high-abundant serum proteins have higher tumor specificity than low-abundant proteins. However, the discovery of OC markers is only the first step in our work. As the sample size of the present study is relatively small, more clinical studies and larger clinical samples are needed to detect and confirm the expression of VIM and PRG4 in early OC patients.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

HSPA9, Heat shock protein 9; FGA, Fibrinogen alpha chain; UBP1, Upstream-binding protein 1; VIM, Vimentin; PRG4, Proteoglycan 4; FLNB, Filamin-B; HSPA5, Heat shock protein 5; IHC, immunohistochemistry.

Address correspondence to: Hu Zhao, Department of Clinical Laboratory Medicine of Huadong Hospital Affiliated to Fudan University, Shanghai 200040, China. E-mail: zhaohuhuadong@126.com

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