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Two-Dimensional Electrophoretic Proteome Study of Serum Thermostable Fraction from Patients with Various Tumor Conditions

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Abstract—One of the problems of plasma proteomics is a presence of large major components. In this work, we use the thermostable fraction as a way to deplete these major proteins. The thermostable fraction of serum samples from patients with ovarian, uterus, and breast cancers and benign ovarian tumor was analyzed using two-dimensional electrophoresis combined with MALDI-TOF(-TOF)-mass spectrometry. Of them, α-1-acid glycoprotein and clusterin are expressly down-regulated in breast cancer, whereas transthyretin is decreased specifically in ovarian cancer. Apolipoprotein A-I forms have decreased spot volumes, while haptoglobin α1, in contrast, is elevated in several tumors. These data are partly consistent with previous art studies on cancer proteomics, which involve mass-spectrometry-based serum profiling techniques. Serum thermostable fraction may be recommended as a good tool for medium and small protein proteome investigation, in particular, by 2D-electrophoresis.

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Blood serum/plasma is an easily available and convenient specimen for diagnostic purposes. Plasma proteins originate from a variety of cells as a result of secretion or leakage from blood cells and cells of other tissues. Plasma exists in dynamic equilibrium, and any shift in this equilibrium may reflect changes in organism functioning.

The serum proteome can be analyzed by different methods, one of the most efficient being two-dimensional electrophoresis [1, 2]. Unfortunately, plasma analysis by the proteome approach is complicated by the presence of enormous amounts of major proteins. The dynamic range of plasma protein concentrations differs by at least nine orders of magnitude. The most abundant human plasma proteins are albumin and γ-globulins, which comprise about 80% of total protein content in human blood plasma. The presence of highly abundant components masks minor proteins and thus generally interferes with their detection and identification in proteome studies. Several methods, such as immunoaffinity chromatography, are commonly used for albumin and immunoglobulin depletion [2]. Using these methods, it is possible to decrease albumin and immunoglobulin concentrations 2-3-fold and to detect less abundant proteins. Thus, the application of depletion methods, which allow analysis of a different spectra of proteins, remains a very important goal [3].

In this study, we suggest the use of heat treatment of plasma to sufficiently deplete the serum proteome. The resultant subproteome, the so-called "thermostable fraction", has not been previously analyzed. To further illustrate the serum thermostable fraction approach, 2-DE maps of such fractions from patients with ovarian, uterus, and breast cancers and benign ovarian tumor were

Abbreviations: 2-DE) two-dimensional electrophoresis; MALDI-TOF) matrix-assisted laser desorption/ionization time of flight; MS) mass-spectrometry; SELDI) surface-enhanced laser desorption/ionization.

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obtained. These tumors were chosen for analysis because together they comprise 90% of all malignancies in women. Using our method we try to reveal cancer distinguishing proteins which would be further investigated as potential cancer biomarkers.

The recent proteome boom has changed the focus in plasma proteomics from 2-DE-based methods [4-7] to direct mass-spectrometry in the wide sense, which, inter alia, includes surface-enhanced laser desorption/ionization (SELDI) mass-spectrometry. Plasma or serum profiling on SELDI-MS chips became the most popular tool for disease biomarker discovery and new pattern-based diagnosis (reviewed in [8]). Initially being based on patterns, recent works in this art report the nature of detectable plasma proteome changes, such as certain identified products in the large-scale study of ovarian cancer sera by Zhang et al. [9]. Our study of thermostable fractions made it possible to compare cancer-related changes identified by 2-DE/MALDI-MS with plasma markers previously detected using the SELDI approach.

MATERIALS AND METHODS

Subjects. Patients of four disease groups were recruited as well as healthy women as controls. Blood samples from all cancer patients were taken preoperatively, and blood sera were obtained. The control group included 10 healthy women of age 29-47 years (average 36). The second group included 10 patients with ovarian cancer of age 30 to 66 years (average 48). Additionally, we used blood serum from 10 patients with uterus cancer of age 36-74 years (average 61), 10 patients with breast cancer of age 20-51 years (average 40), and 10 patients with ovarian benign tumor of age 25-71 years (average 53). Details on the subjects involved are listed in Table 1.

Serum thermostable fraction. Blood sera were mixed 1 : 1 (v/v) with buffer containing 20 mM EDTA, 0.2 M Tris-HCl (pH 9.0), and 7% (w/v) polyethylene glycol (PEG) 6000. The mixtures were incubated for 10 min at 98°C with shaking and left for 10 min at room temperature. The samples were then centrifuged at 10,000*g* for 15 min. Supernatants were collected and protein concentrations in samples were determined by bicinchonic acid assay [10].

Sample preparation and two-dimensional electrophoresis (2-DE). Isoelectrofocusing was carried out using IPG strips, pH 3-10 (Bio-Rad, USA). The strips were actively (50 V, 12 h) rehydrated in solution containing 70 μl of thermostable fraction (400-450 μg of protein per sample) and 330 μl of solubilization buffer (9 M urea, 4% CHAPS (w/v), 1.2% Nonidet P-40 (v/v), 100 mM dithiothreitol, 0.2% Ampholine pH 3-10 (w/v), and 0.1% Ampholine pH 8-10 (w/v) (Amersham-Pharmacia-Biotech, USA)). Isoelectrofocusing was initially carried out on a Protean IEF Cell (Bio-Rad) at 250 V for 15 min, then for 5 h using linear voltage increase up to 10,000 V, and then during 5 h at 10,000 V to a total of 70,000 V·h.

After isoelectrofocusing strips were incubated for 30 min in 0.125 M Tris-buffer, pH 6.8, containing 6 M urea, 30% glycerol, and 2% SDS. For the separation of proteins in the second dimension, 9-16% vertical gradient gels prepared using a standard protocol [11] were used. The size of gels used for all staining types was 0.15 × 20 × 20 cm. Silver staining with sodium thiosulfate and staining with Coomassie brilliant blue R-250 were carried out as described in [12] and [13], respectively.

Gel image analysis. 2-DE gels obtained have been scanned with resolution 300 dpi. Images were analyzed using Melanie III software (GeneBio, Switzerland). The conventional analysis involved (i) protein spot relative volume (%Vol) determination, which was expressed as the sum of pixel intensities in the certain spot divided to the sum of pixel intensities in all spots on the gel; (ii) gel alignment; and (iii) spot matching. Further, sets of %Vol values for every spot were processed by Kruskal–Wallis test, which is a non-parametric analog of Analysis of Variance (ANOVA), thereby testing whether there was a significant variation of the certain protein level between five specified patient groups. Because Kruskal–Wallis did not specify what group had deviating statistics, for all spots with validated variance ($p < 0.05$), all disease groups were compared with control group by the Wilcoxon paired test (see further Table 3).

Table 1. Subjects recruited in the study

Tumor type	Age	Morphology	Stage
Ovarian cancer*	47.6 ± 10.7	mucous GH^2, GL^3; serous GH^2, GL^3	$I\text{-}II^5$, $III\text{-}IV^5$
Uterus cancer	60.6 ± 14.1	adenocarcinoma^{10}	$I\text{-}II^7$, III^3
Breast cancer	40.8 ± 11.2	carcinoma^{10}	$I\text{-}II^8$, III^2
Ovarian benign tumor**	53.1 ± 14.7	serous^7, tecoma^2, mucous^1	

Note: Grade of differentiation: GL) low grade; GI) intermediate grade; GH) high grade. Superscript indicates number of patients.
* Adenocarcinoma.
** Cystadenoma.

Protein identification by MALDI-TOF(-TOF) mass-spectrometry. Protein spots were cut out (~3 mm^3) from 2-DE gels, destained, and in-gel digested with trypsin as described earlier [14]. Mass-spectrometry of trypsin digested serum proteins was performed using a Reflex III MALDI-TOF mass-spectrometer (Bruker, Germany). Peptide samples (0.2-1 μl) were mixed with an equal volume of 2.5-dihydroxybenzoic acid solution (20 mg/ml; Sigma, USA) in 20% acetonitrile and 0.1% trifluoroacetic acid, and the resulting droplets were dried in air. Mass-spectra were obtained for mass range from 800 to 4000 daltons in reflection mode and calibrated using internal standards (trypsin autolysis peaks, MH^+ 1046.54, 2212.10 daltons). Peptide peak lists were formed by the SNAP algorithm (XMass software, Bruker). Proteins were identified using the Mascot database search engine [15]. The search parameters were as follows: mass tolerance 100 ppm, NCBI protein sequence database, *Homo sapiens* taxon, one missed cleavage, variable modifications by propionamide for cysteines and oxidation for methionines.

In certain cases, when the peptide mass fingerprint-based identification was doubtful, the protein identity was confirmed by MS-MS peptide fragmentation. MS-MS spectra of the tryptic peptides were obtained using an Ultraflex MALDI-TOF-TOF mass-spectrometer (Bruker), equipped with 337 nm UV laser, in positive ion mode. Mass accuracy for fragment ions was about 0.03%.

RESULTS

Different methods of sample prefractionation for 2-DE were used to deplete serum of major proteins. We made several attempts to separate different serum fractions by barium salts, sulfosalicilic acid and PEG 6000 (for Ig sedimentation) (results not shown), and thermal treatment followed by protein composition analysis using 2-DE. Only the thermostable fraction gave proteomic maps suitable for our purpose. 2-DE gel images of whole serum and its thermostable fraction from the healthy subjects are shown in Fig. 1. The proteomic map of whole serum is comparable with the SWISS-2D PAGE reference plasma map (http://www.expasy.ch/ch2d/). As one can see from Fig. 1, some interesting differences have been revealed between serum (Fig. 1a) and thermostable fraction (Fig. 1b). The protein spots varying between whole serum and thermostable fraction were picked out and identified by the peptide mass fingerprint procedure, with some results being confirmed by tandem mass-spectrometry peptide sequencing. Results of protein identification are listed in Table 2. Application of fractions instead of whole sera leads to 2-3-fold decrease in albumin and immunoglobulin spot intensities (spots 2, 6, and 15 in Table 2, and the same spot numbers in Fig. 1) on the 2-DE proteomic map, this being comparable with the efficiency of affinity or immunoaffinity depletion [16, 17]. At the same time, this method of serum treatment

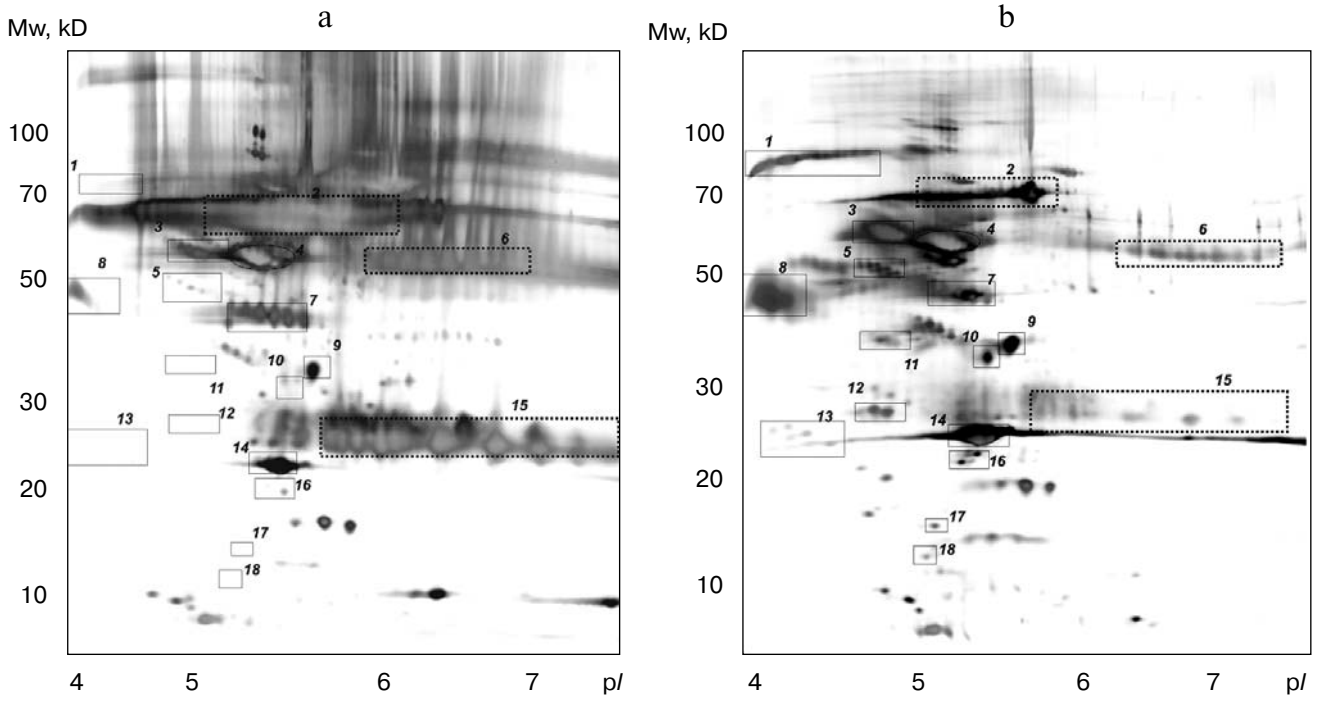

Fig. 1. 2-DE gel images of whole serum (a) and its thermostable fraction (b). Rectangles with dotted borders indicate decreased proteins in the serum thermostable fraction; rectangles with solid borders indicate increased proteins in the serum thermostable fraction; ovals indicate unchanged proteins. Protein identities are shown in Table 2.

Table 2. Identified protein differences between whole serum and its thermostable fraction

Spot number	Protein name	SwissProt AC	Mw (2-DE)/ Mw (SwissProt), kD	MALDI-MS sequence coverage, %
1	α-1-Acid glycoprotein	P02763	88.5/21.6	20*
2	Serum albumin	P02768	67.0/66.5	45
3	α-2-HS-glycoprotein	P02765	56.8/37.3	25*
4	α-1-Antitrypsin	P01009	54.3/44.3	44
5	Leucine-rich α-2-glycoprotein	P02750	48.1/34.4	31
7	Haptoglobin β	P00738	39.7/27.3	20*
6	Ig γ-2 chain C region	P01859	52.7/35.9	20*
8	α-1-Acid glycoprotein	P02763	42.3/21.6	36
9	Transthyretin	P02766	34.3/13.8	79
10	Transthyretin	P02766	32.3/13.8	41
11	Clusterin (ApoJ)	P10909	35.0/50.1	16*
12	Immunoglobulin G chain	P01591	25.6/15.6	43
13	Apolipoprotein A-I	P02647	23.1/28.1	18*
14	Apolipoprotein A-I	P02647	23.0/28.1	38
15	Ig κ chain C region	P01834	24.5/11.6	78
16	Apolipoprotein A-I and A-IV mixed fragment spots	P02647 P06727	20.1/28.1 20.1/43.4	53 31*
17	Haptoglobin α2	P00738	17.8/16.0	19*
18	Apolipoprotein A-I	P02647	14.6/28.1	32*
19	Transthyretin	P02766	13.8/13.8	85
20	Apolipoprotein A-I	P02647	11.8/28.1	36*
21	Haptoglobin α1	P00738	11.9/9.2	52*

Note: Mw (2-DE) represents the spot mass calculated from 2-DE image and Mw (SwissProt) represents the mass of mature protein with no account of post-translational modifications as defined in SwissProt.

* Identity was confirmed by tandem mass-spectrometry TOF-TOF peptide sequencing.

allows the observation of less abundant proteins with thermostable properties, such as apolipoproteins and glycoproteins (spots 1, 3, 8, 13, 16, 18, and 20 in Table 2, and the same spot numbers in Fig. 1). Resulting protein content in thermostable fractions was about 6 mg/ml, so protein depletion by this method was approximately 10-fold.

Further, we used this method for treatment of serum samples from healthy donors (control group, indicated as C) and patients with ovarian cancer (Ov), uterus cancer (Ut), breast cancer (Br), and benign ovarian tumor (Bov) followed by 2D-PAGE of the fractions. Each group consisted of samples from 10 patients. The Melanie III spot matching procedure was carried out for comparative analysis of 50 gels from patients groups. Protein differences were found in six gel areas containing the following protein spots: α-1-acid glycoprotein (area 1), clusterin (apo J) (area 2), transthyretin (area 3), apo A-I (area 4), apo A-I fragments (area 5), and haptoglobin α1 chain (area 6) as shown in Fig. 2. A summary of protein differences is presented in Table 3.

Table 3 shows that the α-1-acid glycoprotein spot (area 1, Fig. 2) and the clusterin spot (area 2) are significantly decreased in all disease states. At the same time,

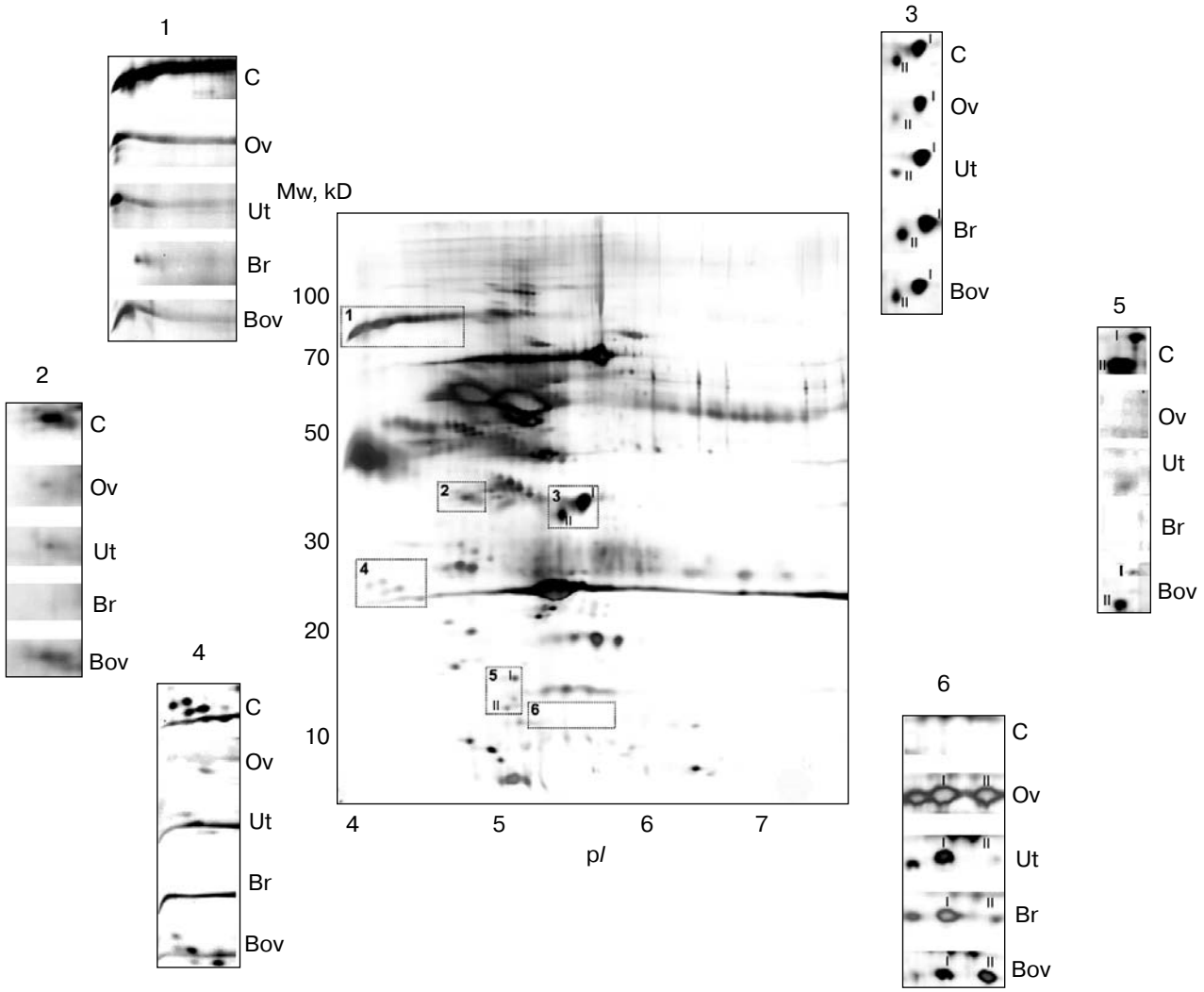

Fig. 2. Reference gel of serum thermostable fraction for control (C) and exemplary changing areas for ovarian cancer (Ov), uterus cancer (Ut), breast cancer (Br), and ovarian benign tumor (Bov). Area numbers correspond to proteins in Table 3.

for the breast cancer group, specific changes are observed in these two proteins, such that their spot volume is extremely low in this condition in comparison with other groups.

It is notable that significant decrease in a certain transthyretin spot (area 3) is observed which is specific for ovarian cancer sera, while in other conditions its volume remains similar to control.

It is well known that serum or plasma 2-DE map contains many spots that represent distinguishable forms of apolipoprotein A proteins, so-called isoforms (see, e.g., SWISS 2D-PAGE database, http://www.expasy.ch/ch2d). In our gels, such minor spots of apoA-I (areas 4 and 5, Fig. 2) disappear or are considerably decreased in all cancer groups. In this respect, benign ovarian condition has intermediate position, apoA-I spots being slightly and insignificantly down-regulated in the benign tumor. The haptoglobin α1 spot volume significantly increases in all cancers except uterus cancer. It should be noted that the latter protein may seemingly serve for distinguishing between benign and malignant ovarian tumors.

DISCUSSION

The first works on clinical proteomics reporting the use of proteome profiles to diagnose malignancies were met with enthusiasm because excellent diagnosis sensitivity and specificity of results were obtained [8]. These technologies mainly involved mass-spectrometry protein patterns. However, when the first identifications of discriminating MS peaks were made, they all occur to relate to acute-phase inflammatory protein, rather than to tumor cell-produced proteins. Clinical applicability of these proteins is now a subject of hot discussion [18]. Any

Table 3. Variation of protein spot volume between patient groups with ovarian (Ov), uterus (Ut), and breast cancers (Br) and ovarian benign tumor (Bov) and normal control (C)

Area number	Protein name	%Vol					
		Kruskal–Wallis test	Ov	C	Ut	Br	Bov
1	α-1-Acid glycoprotein (orosomucoid 1)	<0.001	1.04 ± 0.43**	3.26 ± 0.79	1.04 ± 0.68**	**0.33 ± 0.25****	1.73 ± 1.08**
2	Clusterin (apolipoprotein J)	<0.001	0.22 ± 0.20**	0.99 ± 0.79	0.21 ± 0.24**	**0.07 ± 0.04****	0.32 ± 0.25*
3	Transthyretin, spot I	<0.001	**0.93 ± 0.15****	1.38 ± 0.32	1.31 ± 0.31	1.63 ± 0.35	1.36 ± 0.21
3	Transthyretin, spot II	<0.001	**0.35 ± 0.15****	0.77 ± 0.21	0.56 ± 0.16	0.65 ± 0.13	0.66 ± 0.24
4	Apolipoprotein A-I (6 spots)	<0.001	0.11 ± 0.09**	1.21 ± 0.51	0.23 ± 0.19**	0.08 ± 0.06**	0.47 ± 0.43
5	Apolipoprotein A-I, spot I	<0.01	0.04 ± 0.02**	0.15 ± 0.09	0.04 ± 0.03**	0.02 ± 0.01**	0.06 ± 0.06*
5	Apolipoprotein A-I, spot II	<0.01	0.09 ± 0.08**	0.51 ± 0.59	0.08 ± 0.07**	0.08 ± 0.05**	0.21 ± 0.25
6	Haptoglobin α1, spot I	<0.025	1.06 ± 0.61**	0.40 ± 0.40	0.35 ± 0.49	0.87 ± 0.39**	0.62 ± 0.42
6	Haptoglobin α1, spot II	<0.01	0.81 ± 0.41**	0.24 ± 0.25	0.23 ± 0.35	0.58 ± 0.29*	0.30 ± 0.29

Note: In Kruskal–Wallis test column, probability values of null-hypothesis (no difference between samplings) were shown.
** $p < 0.01$; * $p < 0.05$; statistically significant difference calculated in Wilcoxon test against control group; group specific significant difference is bold typed.

way, it becomes clear that "biomarker" proteins discovered by proteomics should be tested by large-scale immunoassays to advance their clinical introduction.

With development of fast MS proteome profiling methods, such as SELDI-MS, the electrophoretic approaches for plasma-based diagnosis were given up due to their time-consuming character and relatively low reproducibility. Now we can compare the discriminating proteins obtained in this thermostable fraction 2-DE study with the extensive background on cancer diagnostics, especially, ovarian cancer, recently accumulated by SELDI experiments.

Historically, ovarian cancer was the first tumor characterized by diagnostics SELDI profiles [19]. This work was continued in some large-scale experiments, of which, in the paper by Zhang et al. [9], three cancer discriminating peaks were identified. Two of them represent truncated form of transthyretin and apolipoprotein A-I, which are significantly decreased in cancer. In our work, spots of these species are similarly decreased (Table 3, Fig. 2). The decrease in apoA level is a well-known cancer-associated phenomenon. More promising results concern transthyretin, which is decreased in our experiments specifically for ovarian cancer, although it is difficult to specify what certain form of this gene product constitutes the discriminating spot of Fig. 2 (area 3), probably corresponding to the transthyretin tetramer. Transthyretin is a known negative acute-phase transport protein, which previously was found to have significantly reduced levels in various acute liver diseases [20, 21].

Another large-scale SELDI-based study proposed haptoglobin α as a potential ovarian cancer biomarker [4]. Our thermostable fraction 2-DE results are consistent with the above work, where haptoglobin α1 spots demonstrated especially high volume in ovarian cancer. Surprisingly, the elevation of the allele form 2 of haptoglobin α was not detected in our study. This is probably due to the shift to allele 1 in the population studied where allele 2 may be simply absent in some subjects.

In this study, other specific group of protein spots with significant disease association includes clusterin (apolipoprotein J) and α-1-acid glycoprotein (oroso-

mucoid 1), which specifically outline breast cancer condition. Clusterin, a lipid-associated protein with unknown biological role, was not previously associated with cancer conditions. Orosomucoid, a member of the lipocalin family, is characterized in our experiments by abnormal spot position corresponding to doubled mass of the glycoprotein form, which is referred as 41-43 kD in mass [22]. It is that new spot which is down-regulated in disease, while some studies report the increase in plasma orosomucoid in malignancy [23]. Thus, plasma orosomucoid and clusterin behavior in cancer, such as, especially, breast cancer should be the subject for further investigation.

We used herein a thermostable fraction of serum as a way to enrich medium and small protein subproteome, and our results obtained for limited populations indeed resemble previous proteome-based cancer biomarker studies [4, 9] that recruited much larger subject sets. It should be therefore considered that serum thermostable fraction can be recommended as a good tool for medium and small protein proteome investigation in plasma or serum, in particular, by 2D electrophoresis.

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