Regular Article

Ovarian cancer marker of 11.7 kDa detected by proteomics is a serum amyloid A1

Sergei A. Moshkovskii[1], Marina V. Serebryakova[1], Konstantin B. Kuteykin-Teplyakov[1], Olga V. Tikhonova[1], Eugene I. Goufman[1], Victor G. Zgoda[1], Irina N. Taranets[2], Oleg V. Makarov[2] and Alexander I. Archakov[1]

[1] Institute of Biomedical Chemistry, Moscow, Russia
[2] Russian State Medical University, Moscow, Russia

In this study, to reduce the number of major plasma components, we examined thermostable plasma fractions to search for a biomarker of ovarian cancer. An apparent cancer biomarker of 11.7 kDa was detected in these fractions using ProteinChip SELDI-TOF mass spectrometry system. This peak invariably appeared with another close peak of about 11.5 kDa, suggesting that it is a derivative of a larger mass molecule. Of 27 cancer plasma specimens, 15 (55.6%) demonstrated this peak pair, whereas only 2 of 34 controls specimens (5.8%) were shown to express it with low intensity. Using a method involving cysteine modification by 4-vinylpyridine (4-VP), 2-DE and HPLC, these peaks were identified by mass spectrometry as serum amyloid A1 (11.68 kDa) and its *N*-terminal arginine-truncated form (11.52 kDa).

Received: March 22, 2004
Revised: December 25, 2004
Accepted: December 27, 2004

Keywords:
Biomarker / Ovarian cancer / SELDI / Serum amyloid A

1 Introduction

In recent years, proteomics has become an extensively developing technique in the field of biotechnology. A primary goal of proteomics is biomarker discovery for various human disease conditions. Plasma and serum are considered to be the source of choice in molecular diagnostics [1]. However, although readily accessible from patients, plasma proteome analysis is hampered by the number of major component present, such as serum albumin, immunoglobulins, *etc*., the predominance of which raises the problem of detection of the less abundant marker species [2].

At present, particular attention is being paid to cancer biomarker proteomics. Several researches have tried to provide early cancer detection *via* sophisticated software processing of serum/plasma mass spectrometry profiles [3–5], where the result is typically based on a pre-trained artificial intelligence system decision. On the other hand, many reports relate to the precise identification of cancer proteomics marker [6, 7] to develop affinity-based diagnostics tests. These approaches are promising, but the results obtained are still preliminary and are being extensively discussed by skilled artisans [8].

To deplete the number of major plasma components in serum samples in this study, we used thermostable plasma fractions in the search for a ovarian cancer biomarker. An apparent cancer biomarker of 11.7 kDa was detected in these fractions using the ProteinChip™ SELDI-MS system. Previous reports have described a biomarker of similar molecular mass that is elevated in ovarian cancer [6] and lung cancer [7, 9]. Ye *et al.* [6] indirectly identified this protein as a glycosylated haptoglobin alpha 1 (HA1) subunit, while another report [7] suggested the same protein to be serum amyloid A1 (SAA1). Moreover, Wagner *et al.* [9] associated this serum protein to a protein expressed in lung cancer cells, predicting that the 11.7 kDa MS peak is a protein kinase C inhibitor (calculated mass 11.5 kDa).

Correspondence: Dr. Sergei A. Moshkovskii, Center of Proteomics, Institute of Biomedical Chemistry, 10 Pogodinskaya Str., Moscow, 119121, Russia
E-mail: sergei.moshkovskii@ibmc.msk.ru
Fax: +7-095-245-0857

Abbreviations: 4-VP, 4-vinylpyridine; **HA**, haptoglobin alpha; **SAA**, serum amyloid A; **SAX**, strong anion exchanger

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Thus, in view of the intricate situation surrounding this marker, it is necessary to identify it unambiguously by proteomics techniques.

2 Materials and methods

2.1 Subjects

All patient-related specimens were collected following the recommendations of the Ethics Committee of the Ministry of Health of the Russian Federation. Disease blood specimens were collected preoperatively from 27 women with epithelial ovarian cancer (aged 25–55, average 43 years). As controls, specimens from 34 healthy women (aged 18–55, average 41 years) were used.

2.2 Thermostable plasma fraction

Plasma samples containing 20 mM EDTA were mixed 1:1 with 0.2 M Tris-HCl, pH 9.0, containing 7% w/v polyethylene glycol (PEG) 6000. Mixtures were incubated for 10 min at 98°C with shaking, and maintained for 10 min at room temperature. The samples were then centrifuged at 10 000 × *g* for 15 min. Thermostable supernatants were collected and protein concentration was determined by Bradford assay [10].

2.3 SELDI-TOF MS

All solvents for mass spectrometry were of HPLC grade (Merck, Germany). For the strong anion exchanger (SAX) ProteinChip assay (Ciphergen Biosystems, USA), the thermostable fraction (20 μL) was mixed with 80 μL binding/washing buffer (0.1 M Tris-HCl, pH 8.5, 0.01% Triton X-100), and the resultant 100 μL was applied to the spot of a prepared SAX2 chip and processed using the manufacturer's protocol in a 96-well Bioprocessor (Ciphergen Biosystems).

For the metal affinity Ni-IMAC3 ProteinChip assay (Ciphergen Biosystems), the thermostable fraction (20 μL) was mixed with 80 μL binding/washing buffer (Dulbecco's phosphate-buffered saline, pH 7.2; Fluka, USA) and applied to the spot of a prepared Ni-IMAC3 chip and processed using the manufacturer's protocol in a 96-well Bioprocessor.

After incubation and washing, spots, regardless of the chip surface type, were coated by two 0.5 μL additions of saturated sinapinic acid matrix (Ciphergen Biosystems) solution in 0.5% trifluoroacetic acid (TFA), 50% acetonitrile. Chips were inserted into the SELDI-TOF Protein Biology System II (Ciphergen Biosystems), and spectra were collected automatically in the 6000–100 000 Da range with laser intensity 230, detector sensitivity 9 and 90 laser shots *per* spot. Spectra were calibrated using external calibration with a Peptide Standard kit (Ciphergen Biosystems), equine cytochrome *c* (12 361 Da), whale sperm myoglobin (17 200 Da) and bovine serum albumin (66 431 Da).

2.4 Modification of protein cysteine residues by 4-VP

The thermostable plasma fraction (20 μL was acidified with TFA (Merck; 1 μL of 5% TFA) to pH 7.5, and DTT was added to a final concentration of 10 mM (Aldrich, USA; 1 μL of 0.2 M DTT in acetonitrile). The reaction was maintained for 30 min at 58°C. The reduced cysteine residues of the sample were modified by adding 4-VP (Sigma, USA; 3 μL of 0.2 M in acetonitrile) to a final concentration of 24 mM, and incubating for 15 min at 58°C.

2.5 MALDI-TOF MS

The 4-vinylpyridine-modified and starting thermostable fractions, 10 μL of each, were desalted with ZipTip C18 (Millipore, USA) following the manufacturer's protocol. Polypeptides of interest were eluted from ZipTip using 60% acetonitrile solution containing 0.1% TFA. Of each resultant sample, 1 μL was mixed on the MALDI target with 1 μL 2,5-dihydroxybenzoic acid matrix solution (Sigma, 50 mM in 0.1% TFA) and air dried.

Mass spectra of whole proteins and tryptic digests were obtained using the MALDI-TOF-mass spectrometer Reflex III (Bruker Daltonics, Germany) equipped with a 337 nm UV laser, with positive ions being registered. Mass accuracy obtained by external calibration in the linear mode for proteins was within about 0.05%. Mass spectra of digests were obtained in reflectron mode; additional calibration using the peaks of trypsin autolysis was performed, and the resulting mass accuracy was less than 0.01%.

MS-MS spectra of the tryptic peptides were obtained using the MALDI-TOF-TOF-mass spectrometer Ultraflex (Bruker Daltonics, Germany), equipped with a 337 nm UV laser, in positive ion mode. Mass accuracy for fragment ions was about 0.03%.

2.6 2-DE

All reagents were from Bio-Rad (USA) except where specified otherwise. Samples were prepared as follows. Solubilization buffer comprised 7 M urea, 2 M thiourea, 65 mM DTT, 5% Ampholine 3–10 (Amersham Biosciences, USA), 4% CHAPS (Amersham Biosciences), 1.5% NP-40. (1) Mini-gels: 22 μL of human blood serum thermostable fraction (90 μg total protein) was added to 66 μL solubilization buffer and mixed (mini-gels). In parallel, 3 μL human blood plasma (180 μg total protein) was added to 27 μL solubilization buffer and mixed; (2) Large gels: 30 μL of human blood serum thermostable fraction (120 μg total protein) was added to 90 μL solubilization buffer and mixed.

Gel composition for IEF contained 7 M urea, 4% acrylamide/bis-acrylamide (32:1), 2% Ampholine 3–10, 3% Ampholine 5–8 (both from Amersham Biosciences). 0.02% APS and 0.1% TEMED were added as polymerization initiators. Samples were run in tubes: (1) with 1.0 mm diameter and 66 mm length on a Mini-PROTEAN 3 IEF Cell (Bio-Rad); volt-

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

age profile: 100 V for 1 h, 200 V for 1 h, 300 V for 1 h, 400 V for 1 h, 500 V for 12 h, 600 V for 1 h (room temperature), or (2) with 1.5 mm diameter and 140 mm length on a PROTEAN II xi 2D Cell (Bio-Rad); voltage profile: 100 V for 1 h, 200 V for 1 h, 300 V for 1 h, 400 V for 1 h, 500 V for 1 h, 600 V for 1 h, 700 V for 10 h, 900 V for 0.5 h (room temperature).

SDS-PAGE as a 2-D separation was performed at room temperature on gradient polyacrylamide gels of 9–16%: (1) gels 85 × 70 × 1.0 mm, Mini-PROTEAN 3 DodecaCell, at 16 mA *per* gel for 2 h, or (2) gels 160 × 180 × 1.5 mm, PROTEAN II xi Multi-Cell, 40 mA *per* gel for 4.5 h.

Finally, gels were washed and stained using the silver/thiosulfate procedure [11]. Gels were scanned and image analyzed using the Melanie IV software (Genebio, Switzerland). The vol% parameter of gel spots was determined as the integration of optical density over the area of the spot divided by the total integration of optical density over the whole image.

2.7 HPLC separation of thermostable plasma

To remove salts and large proteins from the 50 μL thermostable plasma fraction, a 0.1 mL self-made cartridge with Lichrosorb C_{18} 10 μm particles, pore size 300 (Merck) was used. The elution of the proteins of interest was performed by the following steps: (1) 1 mL 0.1% TFA in water, (2) 0.2 mL 30% acetonitrile with 0.1% TFA in water, (3) 0.2 mL 70% acetonitrile with 0.1% TFA in water. This fraction was evaporated and redissolved in 50 μL 10% acetonitrile with 0.1% TFA in water for HPLC.

HPLC analyses were performed on a high-performance liquid chromatography system MilliChrom A-02 (Chromatography Institute ECONOVA, Russia). A narrow-bore 75 × 2 mm column packed with 5 μm particles of Nucleasil C_{18}, pore size 300 (Macherey-Nagel, Germany), was used. Separations were performed at 35°C; a dual wavelength (220 and 280 nm) detector was employed. The elution gradient profile was as follows: (1) elution solvents: (A) 0.1% TFA in water (pH 2.2), (B) acetonitrile with 0.1% TFA; (2) linear gradient: 0–100% B for 20 min; flow rate 100 μL/min. Fractions of approximately 20–40 μL were collected for subsequent analysis manually. Fraction compositions were monitored by SELDI-MS; 2 μL of fraction was applied on NP20 chip (Ciphergen) and processed with α-cyanohydroxicinnamic acid matrix according to the manufacturer's user manual.

2.8 Protein identification by PMF

Spots of interest from 2-D gels were manually excised and proteins were digested by modified porcine trypsin (Promega, USA) as generally described [12]. The HPLC fraction (20 μL) was dried by evaporation on a Vacuum Concentrator 5301 (Eppendorf, Germany), products were then redissolved in 5 μL 100 mM ammonium bicarbonate solution containing 10 mg/mL trypsin (Promega) and the reaction was incubated for 1 h at 58°C. Digest MALDI-TOF mass spectra were obtained substantially as described above. Proteins were identified by PMF with MASCOT software (Matrix Science, USA) using the NCBI database with following parameters: trypsin enzyme, 1–2 missed cleavages, oxidation (M) and propionamide (C) variable modifications, mass tolerance 150 ppm. TOF-TOF spectra of peptide fragmentation were analyzed using the BioTools software version 2.1 (Bruker Daltonics, Germany).

3 Results

3.1 Proteomics of thermostable plasma fraction

Even in the presence of a significant amount of PEG 6000, the thermostable plasma fraction with a protein concentration of about 4 mg/mL was compatible with the ProteinChip™ affinity surfaces. The optimal surface was the SAX due to the conditions used for fraction preparation (basic pH and Tris buffer). SAX protein profile of the fraction in the range 6–150 kDa was highly reproducible between different samples and differed both qualitatively and quantitatively from the profile of whole plasma obtained in similar fashion. Figure 1 illustrates the successful serum albumin depletion in a course of obtaining the thermostable fraction.

3.2 Ovarian cancer marker of 11.7 kDa

Visual examination of thermostable fraction SAX profile yielded an evident biomarker peak of approximately 11 680 Da specific for cancer plasma. More particularly, this peak invariably appeared with another close peak of about 11 520 Da with a quite similar intensity (Fig. 2), thereby suggesting that it is a derivative of a larger mass molecule. Of the 27 cancer plasma specimens, 15 (55.6%) showed this peak pair, whereas only 2 of 34 controls specimens (5.8%) expressed it, and with low intensity. The statistical evaluation

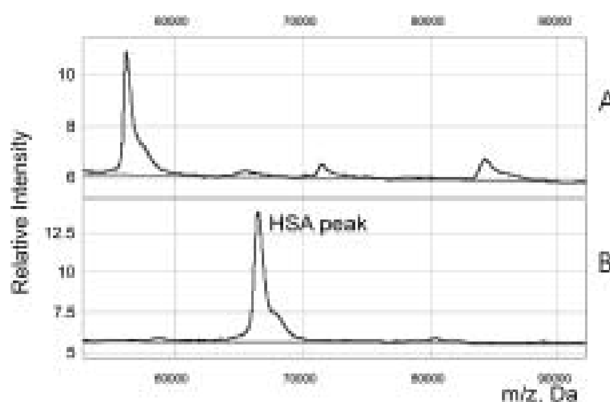

Figure 1. Serum albumin depletion by thermostable fractioning. SELDI-MS of the thermostable fraction (A) and whole plasma (B) both retained on an SAX surface. HSA is a human serum albumin peak.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

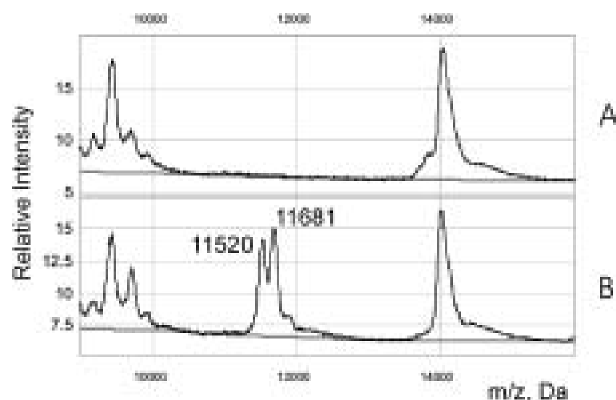

Figure 2. The 11.5/11.7 kDa ovarian cancer biomarker. SELDI-MS of exemplary thermostable fractions retained on an SAX surface. (A) A control plasma, no. 35; (B) a cancer plasma, no. 1, with marker masses indicated.

of the intensity value for both peaks proved their disease marker properties when the cancer and control groups were tested by a sample-independent *t*-test (Fig. 3). The two-tailed *t*-test probability values of the difference between control and cancer peaks were 0.002 for both the 11.52 and 11.68 kDa proteins.

3.3 Indirect identification of 11.5/11.7 kDa marker

To reveal whether the above-mentioned peaks relate to SAA1 with an exactly matched average mass of 11 682 Da, or to HA1 [6], or to another product, we applied the method of cysteine modification, which has been previously used for small protein identification by MS [13]. Cysteine modification by the 105 Da 4-VP moiety would yield a mass shift that would correspond to the number of cysteines in sequence, if any. Since the HA1 sequence contains three cysteine residues, whereas the SAA1 sequence lacks cysteines, distinguishing between these two proteins may be elegantly performed using this approach.

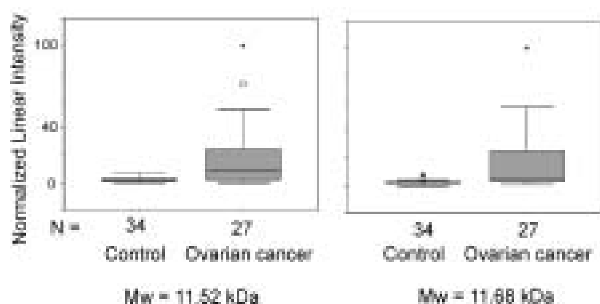

Figure 3. Statistical difference between intensity of 11.5/11.7 kDa SELDI-MS peaks for ovarian cancer and control groups. The normalized linear MS signal intensity data calculated by Ciphergen's Biomarker Wizard™ software are shown as 'box and whiskers' diagram.

The acidified and 4-VP-modified thermostable plasma fractions have fortunately shown high compatibility with the surface of the SAX ProteinChip™ (data not shown) and, after Zip-Tip™ purification, with conventional MALDI process. Figure 4 shows that the described peak dyad of about 11 530/11 684 Da average molecular mass does not shifted after 4-VP modification. At the same time, the 13 766-Da peak shifts to 13 869 Da, *i.e.*, to one modified cysteine; this may represent human transthyretin which has an average mass of 13 760 Da and one cysteine residue in its sequence. Similarly, the shift of the peak at 15 916 Da to 16 549 Da may suggest that this peak is an HA2 protein (database mass 15 938 Da and six cysteine residues in its sequence). The lack of a shift for the 11 530/11 684 peaks after modification leads to conclusion that these peaks correspond to a protein without cysteine.

Since in previous report [6] the 11.7 kDa peak was detected on a Ni-IMAC metal affinity surface, we applied the starting and modified samples to a Ni-IMAC protein chip. This surface has been shown to be not completely suitable for the protein inventory of the fractions. However, peaks of 11.7 and 15.9 kDa were detected in mass region of interest (Fig. 5). The first of these peaks has no shift, while the second one shifts to indicate six cysteines, suggesting that 11.7 kDa product may be SAA1 and 15.9 kDa product may be HA2.

The second argument indicating that the marker of interest is SAA1 was found in the literature. Interestingly, the difference between the twin peaks of 11.5/11.7 kDa matches the mass of one arginine moiety, *i.e.*, about 157 Da (Figs. 2, 4). Kiernan *et al.* [14] have recently shown that the two main SAA forms in human serum are whole SAA1 alpha (1–104 residues, 11 683 Da) and its fragment lacking the *N*-terminal arginine residue (2–104 residues, 11 526 Da).

Taken together, these data establish the identity of the above marker peaks as SAA1 alpha and its *N*-terminally truncated form.

3.4 Comparison of SELDI data and 2-DE maps in respect of SAA

The SELDI data described above should be confirmed by parallel proteomics techniques. Therefore, the thermostable fractions of selected plasma samples were subjected to a 2-DE procedure. Two spots on these gels, which were located below the 14 kDa marker were confidently identified by peptide mass fingerprinting as SAA (NCBI ID gi:225986) with the following MASCOT results: score 171–280; expectation $9.3 \times 10^{12} - 1.2 \times 10^{23}$; sequence coverage 77–94%. Moreover, a deduced sequence for selected peptides from digest mass spectra was confirmed by TOF-TOF fragmentation; see Fig. 6 for the product of 2177.9 kDa.

To examine the correlation between SELDI peaks and SAA spots obtained on gels, a series of 2-DE experiments was performed, which involved a cancer sample with highly intensive SELDI peaks of 11.5/11.7 Da (sample 1), a cancer

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

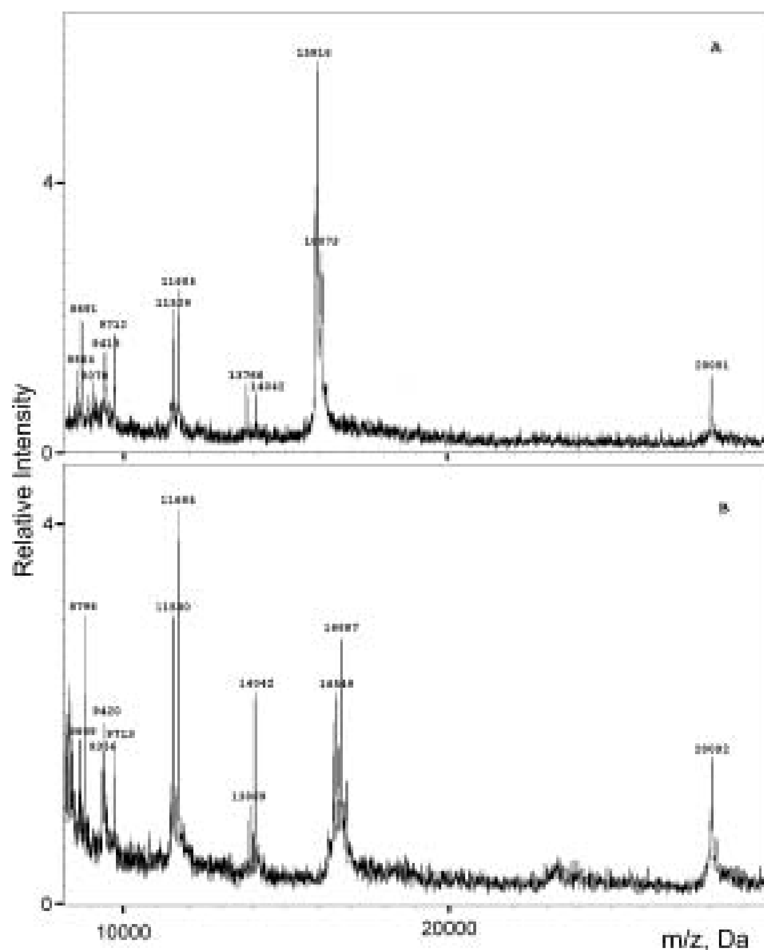

Figure 4. MALDI-TOF mass spectra of exemplary ovarian cancer plasma thermostable fraction before (A) and after 4-VP modification of protein cysteine residues (B).

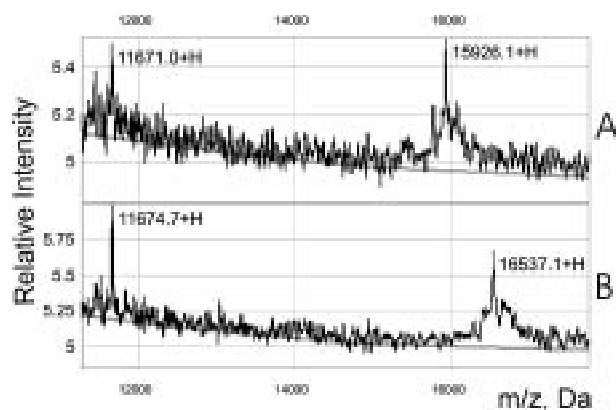

Figure 5. SELDI-TOF mass spectra of exemplary ovarian cancer thermostable plasma fraction before (A) and after 4-VP modification of protein cysteine residues (B) retained on a Ni-IMAC metal affinity surface.

sample with less intensive marker peaks (no. 10), and a control sample (no. 29) without marker peaks detected. As shown in Fig. 7, there is a correlation between the SELDI peak intensity and the SAA spot volume. Of the two gel spots, the basic one is deduced to be the complete form of SAA of 11.7 kDa, whereas the acidic one is apparently the *N*-terminal arginine-truncated form. The ratio between these two spot volumes (spots 1 and 2, Fig. 7) on gels of samples 1 and 10 corresponds to the ratio between the 11.7 and 11.5 kDa peaks on the SELDI spectra of the samples (Table 1).

Interestingly, the 2-D gels of these samples contain spots of HA1 identified by PMF (spots 4 and 5, Fig. 7). The volume of these two spots do not relate to the 11.5/11.7 kDa peak intensities. In particular, sample 1, with largest 11.5/11.7 kDa peaks, has no HA1 spots on the 2-D gel (Fig. 7).

The SAA spots were clearly detected on the 2-D gels of whole plasma (sample 1, Fig. 8), *i.e.*, plasma samples could probably be used for this marker detection without prefractionation. Thus, the conclusion on marker peak identity as SAA was confirmed by parallel 2-DE analysis.

3.5 Isolation of the 11.7 kDa product by liquid chromatography and its direct identification

To identify the 11.7 kDa component of interest directly, the latter was isolated from the thermostable fraction by liquid chromatography. The sample was prefractioned on a self-

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

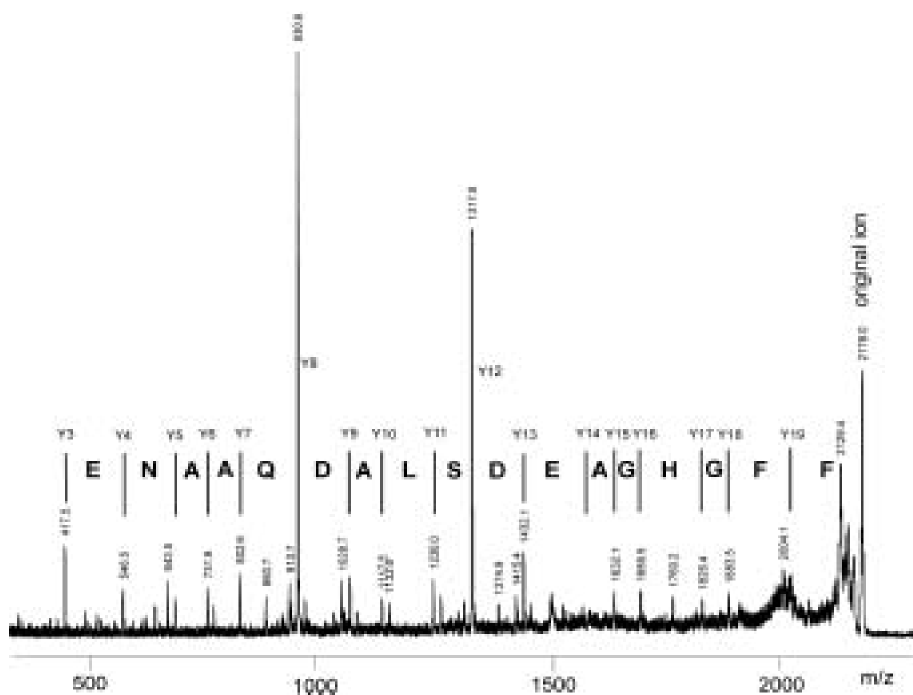

Figure 6. MALDI-TOF-TOF mass spectrum of the peptide with 2177.9 kDa monoisotopic mass from digest of the 2-D gel spot 1 (plasma no. 1, ovarian cancer), which was identified by PMF as SAA peptide 68–87 (FFGHGAEDS LADQAANEWGR). The deduced sequence is confirmed by MS/MS: Y-ions are shown by bars.

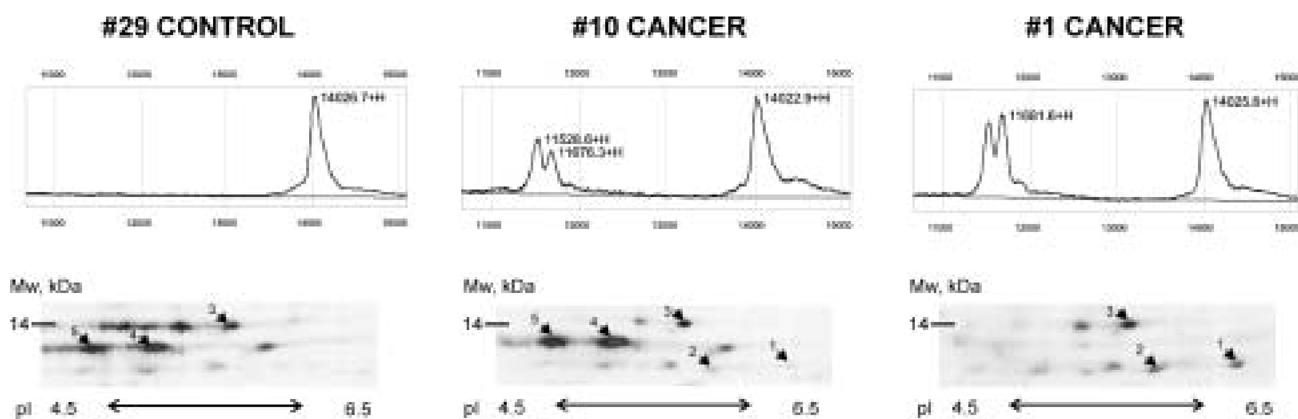

Figure 7. Comparison of SELDI-MS (SAX surface) and 2-DE data for thermostable fractions of three exemplary plasma samples (control no. 29; ovarian cancer nos. 1 and 10). SELDI-MS: 11.7 kDa peak, putative full-length form of SAA1; 11.5 kDa peak, putative *N*-terminal arginine-truncated form. 2-DE: 1, SAA1 (NCBI ID gi:225986) as determined by PMF and TOF-TOF fragmentation, putative full-length form; 2, SAA1 as determined by PMF, putative *N*-terminal arginine-truncated form; 3, transthyretin (gi:4507725) as determined by PMF; 4 and 5, HA1 (gi:67586, residues 19–101) as determined by PMF. Gels used were 160 × 180 × 1.5 mm.

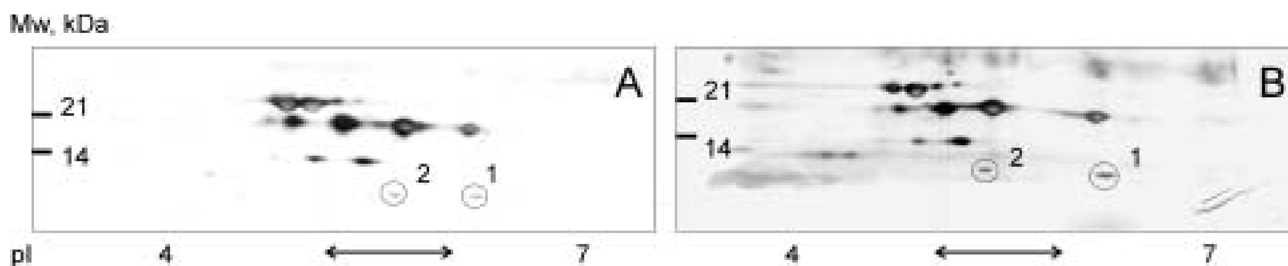

Figure 8. 2-DE gel fragments of exemplary ovarian cancer plasma (B) and its thermostable fraction (A). Circles show SAA1 species identified by peptide mass fingerprinting. 1, putative full-length form; 2, putative *N*-terminal arginine-truncated form. Mini-gels used were 85 × 70 × 1.0 mm.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Comparison between SELDI and 2-DE data for three exemplary plasma samples (data shown in Fig. 7), which confirm the SAA identity

	control no. 24	cancer no. 10	cancer no. 1
SELDI peak of 11.68 kDa; AUC[a)]	0	337	1371
SAA, basic 2-DE spot; %Vol[b)]	0	0.10	0.66
SELDI peak of 11.52 kDa; AUC	0	436	1035
SAA, acidic 2-DE spot; %Vol	0	0.26	0.53
Total AUC of 11.52/11.68-kDa peaks	**0**	**773**	**2406**
Total %Vol of SAA spots	**0**	**0.36**	**1.19**

a) AUC, area under the curve as determined by Ciphergen ProteinChip® Software for spectra normalized by total ion current
b) %Vol, relative spot volume as determined by Melanie IV Software (GeneBio, Switzerland)

made reverse-phase cartridge, where the fraction eluted by 70% acetonitrile and 0.1% TFA appeared to be enriched in the 11.5/11.7 kDa peaks of interest (see Fig. 4) as shown by MALDI and SELDI-MS (spectra not shown).

This preparation was then resolved by HPLC. Three close fractions (nos. 5–7) with elution time of 12.5–13.5 min (approximately 50% acetonitrile) were shown to have the sought MS peaks (Fig. 9). Fraction 7 comprised the 11.7 kDa product in a substantially isolated form, with minor admixtures of 11.5 and 8.6 kDa products, and thus could be used for ultimate identification of the sought product. After trypsin digestion, this fraction yielded an excellent SAA1 peptide mass fingerprint having 89% sequence coverage (Fig. 10).

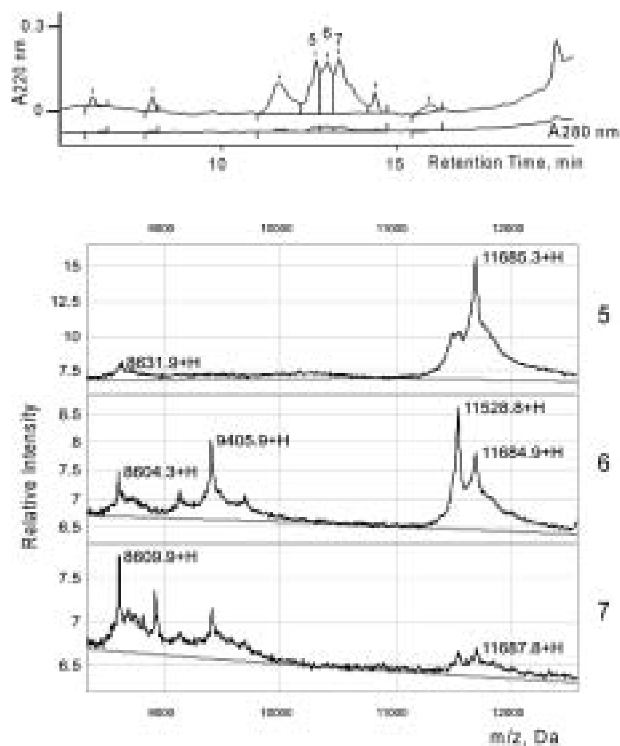

Figure 9. SELDI-TOF analysis of HPLC fractions 5–7 containing the SAA peaks of 11.5/11.7 kDa. Top: partial chromatogram with fractions 5–7 indicated. Bottom: mass spectra of three fractions.

All major peaks of Fig. 10 relate to SAA1, the deduced sequences for two of them being confirmed by TOF-TOF fragmentation (spectra not shown). Thus, the direct identification of a marker for ovarian cancer with a molecular mass of 11.7 kDa was achieved.

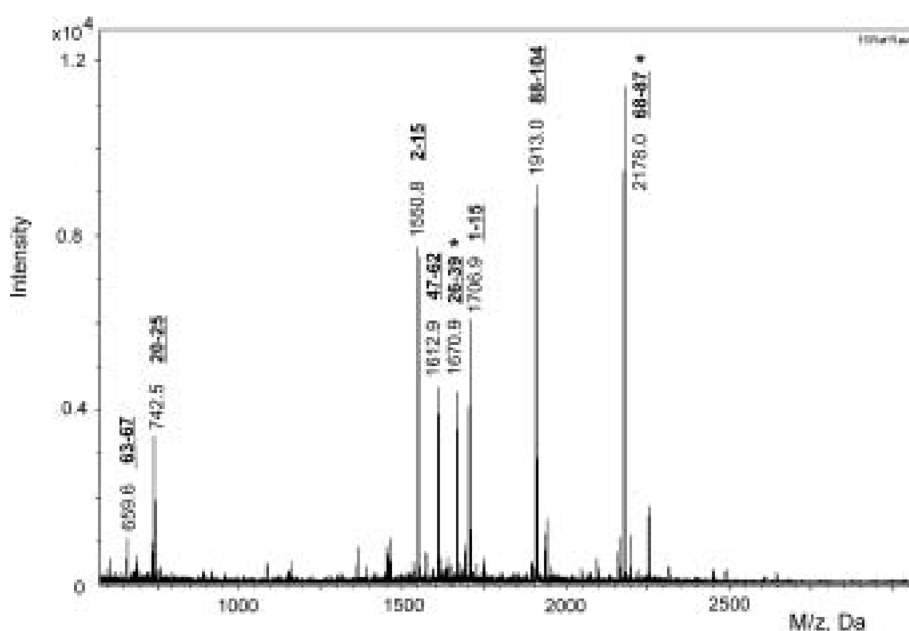

Figure 10. MALDI-TOF mass spectrum of HPLC fraction 5 digested by trypsin. The peaks of SAA1 peptides are designated by position of certain peptide in SAA1 sequence. Two peaks with sequence confirmed by TOF-TOF fragmentation are indicated by asterisks.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

4 Discussion

Serum amyloid A is a well-known acute-phase reactant [15]. The plasma level of this apolipoprotein increases in a wide variety of different disease conditions. Among others, there are reports of elevated SAA levels in lung cancer [7,16] and in renal cell carcinoma [17].

Previously, it has been shown that HA1 is an evident ovarian cancer serum biomarker [6]. We supplement these data with information that the plasma level of SAA1 is also elevated in this cancer type. At first sight, the marker properties shown for these inflammation proteins are of limited clinical application value due to their low specificity *a priori*. However, since there is a lack of sufficiently specific markers for most cancer types, data on the disease behavior of many plasma species, such as apolipoproteins, may be clinically useful. Attempts to use such a protein level estimation for cancer prognosis have already been made [16]. We believe that summarizing proteomics data can result in the development of diagnostic plasma protein arrays, which may be useful in a non-invasive determination of cancer stage, extent of malignancy, prognosis, *etc*.

On the other hand, the present work is promising in the context of plasma SAA complexity in cancer. The proteomics of serum amyloids has been extensively studied in relation to amyloidosis and chronic inflammation [14], and, similarly, attention should be paid to the arrangement of plasma SAA forms in different stage and type of tumors.

The authors thank Dr. Dmitry D. Petrunin and Prof. Vadim M. Govorun (Institute of Physico-Chemical Medicine, Moscow, Russia) for valuable technique advice. This work was supported by 'Proteomics for medicine' program of Ministry of Industry, Science and Technology of Russian Federation and by the National Medicine Assistance Fund (Russia). The work of S. Moshkovskii is individually supported in part by the National Medicine Assistance Fund (Russia).

5 References

[1] Liotta, L. A., Ferrari, M., Petricoin, E., *Nature* 2003, *425*, 905.

[2] Anderson, N. L., Anderson, N. G., *Mol. Cell. Proteomics* 2002, *1*, 845–867.

[3] Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B. *et al.*, *Lancet* 2002, *359*, 572–577.

[4] Hilario, M., Kalousis, A., Muller, M., Pellegrini, C., *Proteomics* 2003, *3*, 1716–1719.

[5] Kozak, K. R., Amneus, M. W., Pusey, S. M., Su, F., Luong, M. N., Luong, S. A., Reddy, S. T., Farias-Eisner, R., *Proc. Natl. Acad. Sci. USA* 2003, *100*, 12343–12348.

[6] Ye, B., Cramer, D. W., Skates, S. J., Gygi, S. P., Pratomo, V., Fu, L., Horick, N. K. *et al.*, *Clin. Cancer Res.* 2003, *9*, 2904–2911.

[7] Howard, B. A., Wang, M. Z., Campa, M. J., Corro, C., Fitzgerald, M. C., Patz, E. F. Jr., *Proteomics* 2003, *3*, 1720–1724.

[8] Pearl, D. C., *Lancet* 2002, *360*, 169–170.

[9] Wagner, M., Naik, D., Pothen, A., *Proteomics* 2003, *3*, 1692–1698.

[10] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248–254.

[11] Blum, H., Beier, H., Gross, H. G., *Electrophoresis* 1987, *9*, 93–99.

[12] Havlis, J., Thomas, H., Sebela, M., Shevchenko, A., *Anal. Chem.* 2003. *75*, 1300–1306.

[13] Zhang, L., Yu, W., He, T., Yu, J., Caffrey, R. E., Dalmasso, E. A., Fu, S. *et al.*, *Science* 2002, *298*, 995–1000.

[14] Kiernan, U. A., Tubbs, K. A., Nedelkov, D., Niederkofler, E. E., Nelson, R. W., *FEBS Lett.* 2003, *537*, 166–170.

[15] Uhlar, C. M., Whitehead, A. S., *Eur. J. Biochem.* 1999, *265*, 501–523.

[16] Benson, M. D., Eyanson, S., Fineberg, N. S., *Cancer* 1986, *57*, 1783–1787.

[17] Kimura, M., Tomita, Y., Imai, T., Saito, T., Katagiri, A., Ohara-Mikami, Y., Matsudo, T., Takahashi, K., *Cancer* 2001, *92*, 2072–2075.

© 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim