

Decreased concentrations of retinol-binding protein 4 in sera of epithelial ovarian cancer patients: A potential biomarker identified by proteomics

LUCIE LORKOVA¹, JANA POSPISILOVA¹, JAN LACHETA², SERGIU LEAHOMSCHI¹,
JAROSLAV ZIVNY², DAVID CIBULA², JAN ZIVNY¹ and JIRI PETRAK^{1,3}

¹Institute of Pathological Physiology, and ²Department of Gynecology and Obstetrics, First Faculty of Medicine, Charles University, Prague; ³Institute of Hematology and Blood Transfusion, Prague, Czech Republic

Received August 29, 2011; Accepted September 30, 2011

DOI: 10.3892/or.2011.1513

Abstract. Ovarian cancer is the fifth leading cause of cancer death in women. Absence of a reliable biomarker precludes early diagnosis of the disease. To identify new proteins with potential diagnostic or prognostic value for the therapy of ovarian cancer we performed comparative proteomic analysis of sera from ovarian cancer patients and healthy women. We analyzed serum samples from 10 patients diagnosed with epithelial ovarian cancer and 10 age-matched healthy women. To decrease the extremely wide dynamic range of protein concentrations in serum we used combinatorial hexapeptide libraries. Serum samples were then subjected to proteomic 2-DE analysis. Three proteins with differential abundance were found and identified by mass spectrometry: α -1-antitrypsin, apolipoprotein A-IV and retinol-binding protein 4. Identification of α -1-antitrypsin and apolipoprotein A-IV confirms previous studies but the identification of significantly decreased levels of RBP4 in ovarian cancer patients represents a novel observation. We verified the decrease of RBP4 levels in ovarian cancer patient sera by two independent methods and determined absolute RBP4 concentrations in patients and healthy women. We excluded possible non-cancer factors that could be responsible for the observed RBP4 decrease. We propose a connection of RBP4 with epithelial ovarian cancer and advocate the potential of RBP4 as a candidate diagnostic or prognostic biomarker.

Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women. While overall 5-year survival is only 16-40%, in patients with early stage (FIGO I) ovarian cancer survival rate is 95% (1,2). Unfortunately, vast majority of the patients is diagnosed in advanced stage disease (FIGO III/IV), mostly because of absence of specific symptoms and by lack of reliable serum markers for early disease. Effective screening tests are yet to be developed.

The most widely used serum marker for ovarian cancer, mucin protein CA125, has low sensitivity, its serum concentration is increased in less than half of early stage patients. Furthermore, its specificity is also insufficient, increased levels have been reported also in patients with benign gynecological diseases, endometriosis, cirrhosis and heart disease (3). Enormous effort has therefore been developed to identify and implement new serum biomarkers with sufficient sensitivity and specificity for detection and monitoring of epithelial ovarian cancer. Several candidate molecules have been discovered (4,5). Unfortunately, none of the identified proteins or peptides proved to be of sufficient sensitivity and specificity as a clinically applicable diagnostic marker.

Comparative proteomic analyses of serum or plasma are, among other obstacles, hindered by extremely high dynamic range of individual protein concentration in serum exceeding 10 orders of magnitude (6). Effective methods to decrease the concentration range of serum proteins are based on either immunodepletion of the most abundant serum/plasma proteins or, more recently, on equalization of protein concentrations by interaction with combinatorial hexapeptide library coupled to beads (7,8). The latter method has gained attention under commercial name ProteoMinerTM and was used in our current study. Equalized samples were then subjected to 2-DE differential proteomic analysis. We identified three proteins that were present in serum of EOC patients in concentrations significantly different than in sera of age-matched healthy women. Two of the proteins have been identified in EOC patients previously. Identification of retinol binding protein 4 (decreased in sera of EOC patients) is a novel observation; therefore its altered concentrations were further tested, verified and quantified in individual serum samples.

Correspondence to: Dr Jiri Petrak, Institute of Pathological Physiology, First Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 120 00 Prague, Czech Republic
E-mail: jpetr@lf1.cuni.cz

Abbreviations: EOC, epithelial ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics; IUCC, International Union against Cancer; ELISA, enzyme-linked immunosorbent assay; BMI, body-mass index; RBP4, retinol-binding protein 4; TTR, transthyretin; PBS-T, phosphate-buffered saline with 0.1% Tween-20

Key words: ovarian carcinoma, proteomics, combinatorial hexapeptide libraries, biomarkers, retinol-binding protein, cachexia

Materials and methods

All chemicals were from Sigma-Aldrich, unless stated otherwise. The study was approved by the Ethics Committee of the Charles University in Prague, First Faculty of Medicine (IRB approval IGA MZ CR 1.LF UK 19/05).

EOC patient selection. Serum samples were collected at the Department of Gynecology and Obstetrics of the First Faculty of Medicine and General Teaching Hospital after informed consent from both patients and healthy age-matched women after overnight fasting. Patient samples were collected at the time of preliminary diagnosis before surgery and chemotherapy. The diagnosis was confirmed histologically after the surgery and only the samples from patients with confirmed EOC were included in the study. Tumor typing and staging were performed by the Department of Pathology according to the criteria of the International Federation of Gynecologists and Obstetricians (FIGO) and the International Union against Cancer (IUCC).

Serum and plasma collection. Blood was collected into BD Vacutainer tubes (BD, USA) with sodium heparin (plasma) and without additives (serum). The tubes were kept at room temperature for 5 min and centrifuged at 1500 x g for 5 min. Collected plasma was then re-centrifuged at 15000 x g for 20 min to remove remaining platelets. The serum and plasma were aliquoted into 2 ml screw cap tubes (Axygen, USA) and stored at -80°C.

Serum equalization - hexapeptide ligand library treatment. Concentration of the most abundant serum proteins was reduced using the ProteoMiner Enrichment Kit (Bio-Rad Laboratories, CA, USA). Pooled sera from control healthy women (controls, n=10) and EOC patients (patients, n=10) were used as a starting material (10 ml each pool). To obtain sufficient amount of equalized sera, 10 depletions were performed from each pooled sample, each depletion with a fresh ProteoMiner column and 1 ml of pooled sera. The procedure was carried out according to the manufacturer's instructions. Serum samples relatively enriched in medium- and low-abundant proteins were eluted and pooled. The combined equalized samples (2.7 ml), were precipitated by 40 ml of cold acetone at -20°C overnight.

Two-dimensional electrophoresis. Precipitated equalized serum protein pellets were dissolved each in 2.8 ml of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 1% ampholytes (GE, USA) and 0.002% bromophenol blue). Protein concentration was determined and adjusted to 4.4 mg/ml. IPG strips (24 cm, pH 4-7, GE, USA) were rehydrated overnight in 450 µl of the sample, representing 2 mg protein per strip. Six technical replicates were run for each sample.

Isoelectric focusing was performed with a Bio-Rad Protean IEF cell for 80 kVh, with maximum voltage not exceeding 5 kV, current limited to 50 µA per strip and temperature set to 20°C. Strips were equilibrated and reduced in equilibration buffer A (6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 2% SDS and 450 mg DTT per 50 ml of the buffer) for 15 min and then alkylated in equilibration buffer B (6 M urea, 50 mM Tris pH 8.8, 30% glycerol, 2% SDS and 1.125 mg iodoacetamide per 50 ml). Equilibrated strips were then placed on the top of 11% SDS-PAGE and secured in place by molten agarose.

Electrophoresis was performed in a Tris-glycine-SDS system using a 12 gel Protean Plus Dodeca Cell apparatus (Bio-Rad) with buffer circulation and external cooling (20°C). Gels were run at constant voltage of 200 V for 6 h. Following electrophoresis gels were washed 3 times for 15 min in deionized water to remove SDS. Washed gels were stained in CBB (Simply Blue SafeStain, Invitrogen, Carlsbad, USA) overnight and then destained in deionized water.

Gel image analysis. Stained gels were scanned with a GS 800 calibrated densitometer (Bio-Rad). Image analysis was performed with Progenesis PG200/PG220 (Nonlinear Dynamics, UK) in semi-manual mode with six gel replicates for each group. Normalization of gel images was based on total spot density, and integrated spot density values (spot volumes) were calculated after background subtraction. Average spot volume values (averages from the all 6 gels in the group) for each spot were compared between the groups. Protein spots were considered differentially expressed if they met both of the following criteria: average normalized spot volume difference >2-fold and statistical significance ($p < 0.05$) of the change determined by the t-test.

MALDI mass spectrometry, protein identification. Differentially expressed proteins were excised from gels, cut into small pieces and washed four times with 25 mM ammonium bicarbonate in 50% acetonitrile. The supernatant was removed and the gel was partially dried in a SpeedVac concentrator. Gel pieces were then reconstituted in a cleavage buffer containing 25 mM ammonium bicarbonate and sequencing grade trypsin (5 ng/ml; Promega, WI, USA). After overnight digestion, the resulting peptides were extracted with 50% ACN/0.1% TFA. Extracted peptide mixture (0.5 µl) was deposited on a steel MALDI target and allowed to air-dry at room temperature. After complete evaporation, 0.5 µl of the matrix solution [α -cyano-4-hydroxycinnamic acid in aqueous 50% ACN/0.1% TFA (5 mg/ml)] was added. MALDI mass spectra were measured on Autoflex II instrument (Bruker Daltonics, Bremen, Germany).

Spectra were acquired in the mass range between ~700 and 3200 Da and calibrated internally using the monoisotopic $[M+H]^+$ ions of Peptide calibration standard II (Bruker Daltonics, Bremen, Germany). Peak lists in XML data format were created using the flexAnalysis 3.1 program with the SNAP peak detection algorithm. No smoothing was applied, and the maximal number of assigned peaks was set to 50. After peak labeling, all known contaminant signals were manually removed. The peak lists were searched using the MASCOT search engine against the SwissProt 2009_11 database subset of human proteins with the following search settings: peptide tolerance 50 ppm, 1 missed cleavage, fixed carbamidomethylation of cysteine, variable acetylation of protein N-term and oxidation of methionine. No restrictions on protein molecular weight or pI value were applied. Proteins with a Mascot score over the threshold 55 for $p < 0.05$ calculated for the used settings were considered as identified.

Western blotting. Individual serum samples (10 µg of proteins) were combined with SDS loading buffer containing DTT, boiled 5 min and separated on 10% SDS-PAGE minigels in Tris-glycine-SDS buffer. Electrophoresis was performed using

Table I. Details on patients included in the proteomic analysis study.

| Patients | Age | BMI ^a | Tumor size | Node involvement | Metastases status | Grade | Histology | Stage | CA125 (U/ml) | RBP4 (μg/ml) |
|-----------|----------|------------------|------------|------------------|-------------------|-------|------------|-------|--------------|--------------|
| P1 | 44 | 17.2 | pT3b | pNx | M0 | G3 | Serous | III | 917 | 32.3 |
| P2 | 46 | 29.7 | pT3c | pNx | M0 | G3 | Serous | III | 4100 | 8.1 |
| P3 | 49 | 24.4 | pT3b | N0 | M0 | G2 | Serous | III | 149 | 27.8 |
| P4 | 54 | 21.5 | pT1c | pNx | M0 | G1 | Mucinous | I | 350 | 32.1 |
| P5 | 59 | 25.7 | pT1c | pN0 | M0 | G3 | Serous | I | 19 | 45.4 |
| P6 | 55 | 21.3 | pT1c | pN1 | M0 | G3 | Clear cell | III | 318 | 31.5 |
| P7 | 56 | 24.0 | pT3c | pN1 | M0 | G3 | Serous | III | 1685 | 35.7 |
| P8 | 56 | 27.7 | pT3c | pNx | M0 | G3 | Mixed | III | 2500 | 27.2 |
| P9 | 58 | 19.9 | pT3c | pN1 | M0 | G1 | Serous | III | 592 | 35.9 |
| P10 | 59 | 25.4 | pT3c | pN1 | M1 | G2-3 | Serous | IV | 364 | 23.2 |
| Mean ± SD | 53.6±5.3 | 23.7±3.8 | | | | | | | | |

^aBMI, body mass index.

Table II. Details on healthy controls included in the proteomic analysis study.

| Healthy controls | Age | BMI ^a | RBP4 (μg/ml) |
|------------------|----------|------------------|--------------|
| C1 | 44 | 24.8 | 51.3 |
| C2 | 44 | 24.7 | 48.8 |
| C3 | 45 | 21.7 | 50.1 |
| C4 | 46 | 34.6 | 45.1 |
| C5 | 50 | 22.7 | 47.7 |
| C6 | 55 | 24.7 | 54.2 |
| C7 | 56 | 25.4 | 40.8 |
| C8 | 57 | 31.6 | 49.5 |
| C9 | 58 | 24.7 | 45.5 |
| C10 | 58 | 22.7 | 43.1 |
| Mean ± SD | 51.4±5.8 | 25.8±4.1 | |

^aBMI, body mass index.

Mini-Protean Tetra Cell (Bio-Rad Laboratories) at constant voltage for 30 min at 45 V, and then at 90 V until the dye front reached the gel bottom. Proteins were then transferred to 0.45 μm PVDF membranes (Millipore, MA) in semi-dry blotter (Hoeffer, Canada) at 0.8 mA/cm². Membranes were incubated with PBS-T (phosphate-buffered saline with 0.1% Tween-20) for 2 h. As a primary antibody, mouse anti-RBP4 (Sc-69795, Santa Cruz Biotechnology, CA, USA) diluted 1:500 in PBS-T or rabbit anti-transferrin (Sigma) diluted 1:1000 was added for 1 h. After thorough washing with PBS-T, secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibody or goat anti-rabbit IgG antibody (both Santa Cruz Biotechnology) diluted 1:10,000 was added for 1 h. Membranes were thoroughly washed in PBS-T and then in PBS. Signal was developed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and membranes were exposed to X-ray film

(Kodak, CR), developed and scanned. For detection of human IgG as an internal loading control, the membrane were stripped and re-probed with a swine anti-IgG antibody (Sevapharma a.s., Czech Republic) conjugated with HRP diluted 1:10,000.

RBP4 ELISA. The quantitative determination of human retinol-binding protein 4 (RBP4), α-1-antitrypsin and apolipoprotein A4 concentrations in patient and control sera was performed in triplicates using the Quantikine Human RBP4 immunoassay (R&D Systems, MN, USA), human apolipoprotein AIV ELISA Kit (Millipore, MA, USA) and α-1-antitrypsin Clearance ELISA (Immuno Diagnostik AG, Germany) according to the manufacturer's instructions using ELISA Reader Sunrise (Tecan, Austria).

Measurement of plasma vitamin A. Vitamin A serum levels were determined by HPLC method using ClinRep Complete Kit for vitamins A and E in Plasma (Iris technologies international, Germany) on ECOM HPLC according to the manufacturer's instructions.

Statistical analysis. Statistical significances of normalized optical spot density, values from ELISA tests and retinol measurements by HPLC was determined by Student's t-test. Correlation between variables was determined by Correlation Pearson's coefficient using software Statistica (StatSoft Inc., USA).

Results

Patients and controls. The women enrolled in the study were patients diagnosed with EOC (histologically confirmed) and age-matched healthy women. Average age was 53.6±5.3 for patients and 51.4±5.8 for healthy controls. Details are provided in the Tables I and II.

Serum equalization and proteomics. To eliminate potential inter-individual variability, our analysis was performed with

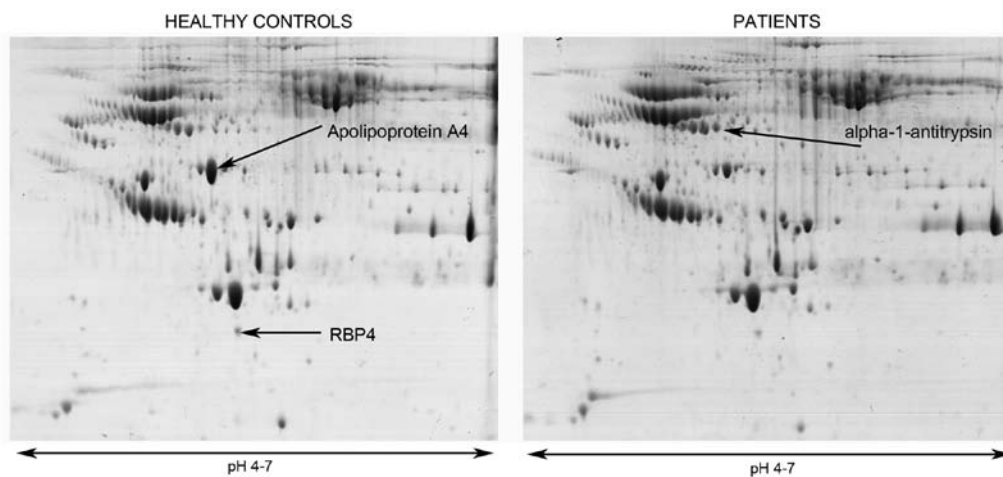


Figure 1. 2-DE analysis of sera after hexapeptide ligand library treatment. 2-DE proteomic analysis of pooled sera from healthy women and EOC patients after ProteoMiner equalization. Twelve 20.5x25-cm gels were analyzed, 6 replicates with pooled patient samples and 6 gels with pooled healthy control samples. Gels were stained, scanned and analyzed by Progenesis PG200/PG220 image analysis software. Three spots of significantly different density between the groups were found and the three proteins were identified by MALDI mass spectrometry using peptide mass fingerprinting (Table III).

Table III. Peptide mass fingerprint identification of proteins with differential concentrations (difference at least 2-fold and statistical significance ($p < 0.01$) in sera of EOC patients.

| Sequence coverage ^a (%) | Matched peptides | Mascot score ^b | Swiss-Prot access no. ^c | Protein name | Fold-change |
|------------------------------------|------------------|---------------------------|------------------------------------|---------------------------|-------------|
| Down-regulated in EOC patients | | | | | |
| 58 | 28 | 335 | P06727 | Apolipoprotein A-IV | -2.3 |
| 57 | 11 | 153 | P02753 | Retinol-binding protein 4 | -2.2 |
| Up-regulated in EOC patients | | | | | |
| 42 | 17 | 115 | P01009 | α -1-antitrypsin | 2.0 |

^aSequence coverage is the number of amino acids spanned by the assigned peptides divided by the sequence length. ^bMASCOT score helps to estimate correctness of the individual hit. It is expressed as $-10 \times \log(P)$ where P is the probability that the observed match is a random event.

^cSwissProt access no. is the code under which the identified protein is deposited in the SwissProt database.

pooled serum samples from 10 patients diagnosed with EOC (patients) and 10 healthy age-matched women (controls). Later verifications by Western blotting and ELISA were done with individual serum samples.

The dynamic range of individual protein concentrations in serum was reduced or equalized by interaction of serum proteins with of random hexapeptide library immobilized on beads (ProteoMiner) (8). Using the equalized pooled sera we performed classical 2-DE differential proteomic analysis. Equalization of serum samples with hexapeptide ligand library beads was effective, increasing the number of spots detected in 2-DE gels ~1.5 fold compared to untreated sera in a pilot experiment (data not shown).

Twelve 2-DE gels were analyzed (six replicates with pooled patient samples and 6 gels with pooled healthy control samples). On average we detected 410 protein spots per gel upon colloidal Coomassie staining. Quantitative analysis of normalized spot density using Progenesis PG200/PG220 software revealed statistically significant difference [normalized spot volume difference at least 2-fold and statistical significance ($p < 0.01$)] between the control and patient groups in 3 spots (Fig. 1). All three proteins present in the spots of differential density were identified by

MALDI-TOF mass spectrometry using peptide fingerprint method (Table III). The only up-regulated protein found in sera of ovarian cancer patients has been identified as α -1-antitrypsin. Two proteins with concentrations decreased in patient sera were apolipoprotein A-IV and retinol-binding protein 4 (RBP4).

Retinol binding protein 4 is decreased in EOC patients. α -1-antitrypsin and apolipoprotein A-IV belong among the 40 most abundant plasma proteins, with concentrations 18-40 $\mu\text{mol/l}$ and 3-6 $\mu\text{mol/l}$, respectively (9). We verified the altered concentrations in these two proteins by ELISA in all individual patient and control serum samples. α -1-antitrypsin was significantly ($p=0.015$) upregulated in patients while apolipoprotein A-IV was markedly downregulated ($p=0.0001$) (Fig. 2). Both proteins were identified by proteomic serum analyses as potential ovarian cancer markers previously (10,11) and we therefore focused our attention to retinol binding protein 4 (RBP4) as the novel observation and the potentially more interesting candidate biomarker (concentration in patient serum decreased 2.2-fold, $p < 0.001$).

Since our proteomic analysis was performed with pooled and equalized serum samples, it was necessary to confirm

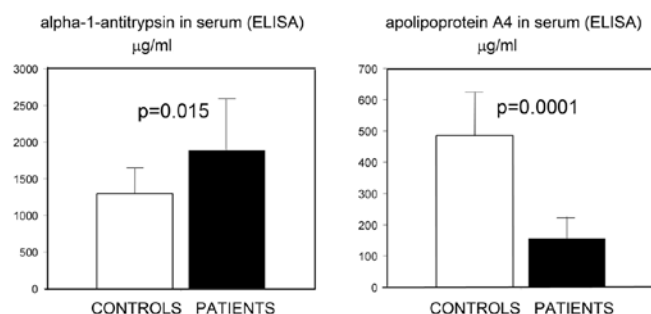


Figure 2. Verification of altered concentrations of α -1-antitrypsin and apolipoprotein A-IV in patient and healthy women sera. Serum concentrations of the proteins were determined in all 10 patient and 10 control serum samples using ELISA kits. α -1-antitrypsin was confirmed to be significantly up-regulated in patients and apolipoprotein A4 was significantly downregulated. Mean values, and statistical significances are shown.

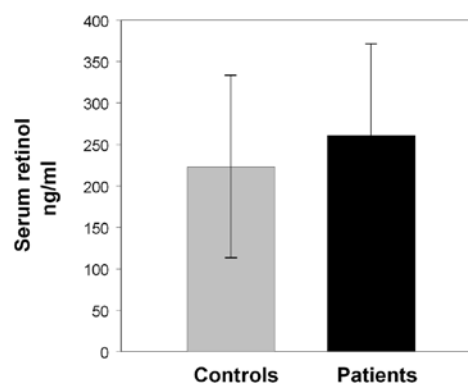


Figure 4. Serum retinol levels in EOC patients and age-matched healthy women. Levels of serum vitamin A (retinol) regulate RBP4 secretion by liver. Decreased retinol levels could affect RBP4 levels in our study. We therefore determined serum retinol levels in all 10 patients and controls by HPLC. Mean values of serum retinol concentrations are shown.

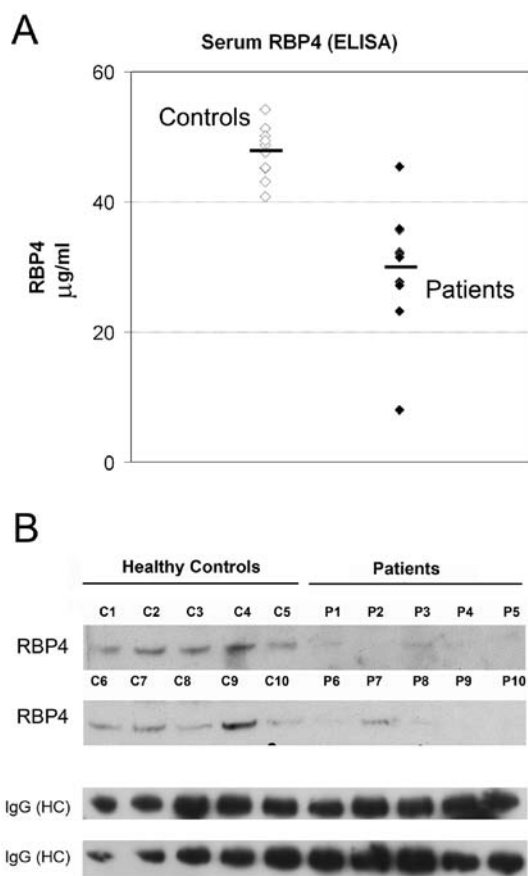


Figure 3. Verification of altered RBP4 concentration. (A) Absolute RBP4 concentrations in serum samples were determined by ELISA. Each measurement represents average value from 3 replicates. Individual serum RBP4 concentrations can be found in Tables I and II. (B) Western blot analysis of RBP4 concentration in individual serum samples was performed. To enable relative comparison between healthy controls and patients five control and five patient samples (10 µg protein each) were run on each gel and processed simultaneously. Membranes were re-probed with anti-human IgG (HC) as an internal standard.

the altered RBP4 abundance in the individual, crude serum samples before the ProteoMiner equalization. To determine also absolute concentrations of RBP4 we used an ELISA

test to measure RBP4 in the original set of the all individual non-equalized serum samples from 10 EOC patients and 10 controls. As seen in Fig. 3A, ELISA results confirmed decreased serum concentration of RBP4 in the EOC patients. The average RBP4 concentration in patient serum samples (29.9 µg/ml) was 1.6-fold decreased compared to the control samples (47.7 µg/ml, $p < 0.007$). Distribution of the RBP4 concentrations suggests that a threshold exists at 38-40 µg/ml distinguishing healthy age-matched women from most ovarian cancer patients in our cohort. For the individual RBP4 levels see Tables I and II. Serum RBP4 concentrations negatively correlate with ovarian cancer marker CA125 levels in the patient group ($r = -0.715$, $p = 0.015$).

To provide additional verification (using different antibodies, than the one used for ELISA) we performed also RBP4 immunodetection using Western blotting with non-equalized sera from all 10 individual patients in the group and the 10 healthy controls (Fig. 3B). The results also confirmed that RBP4 was significantly decreased in patient samples compared to sera from healthy women. Equal sample loading (10 µg per lane) was ensured by careful and repeated determination of protein concentration in samples. Internal standard [total human IgG (HC)] is shown only for rough loading control. Due to high individual variability of serum protein levels, there is currently no reliable and generally accepted internal standard for serum samples (similar to β -actin, GAPDH or tubulin used for tissues).

Serum retinol-binding protein 4 is a 21-kDa lipocalin produced by liver, adipocytes, macrophages and some epithelial cells. It is the principal transport protein for retinol (vitamin A). RBP4 levels in blood are normally maintained within narrow limits with one exception. RBP4 secretion by its main producer, liver, is tightly regulated by availability of retinol levels. In vitamin A deficiency is RBP4 retained in liver, upon retinol repletion RBP4 associates with the vitamin and is secreted into blood (12,13). We therefore tested, whether the decreased concentration RBP4 identified in EOC patients could be attributed to decreased retinol levels. We measured retinol levels in individual serum samples of all 10 individual patients and the 10 healthy controls in our study. As seen in Fig. 4, average serum retinol concentration is comparable

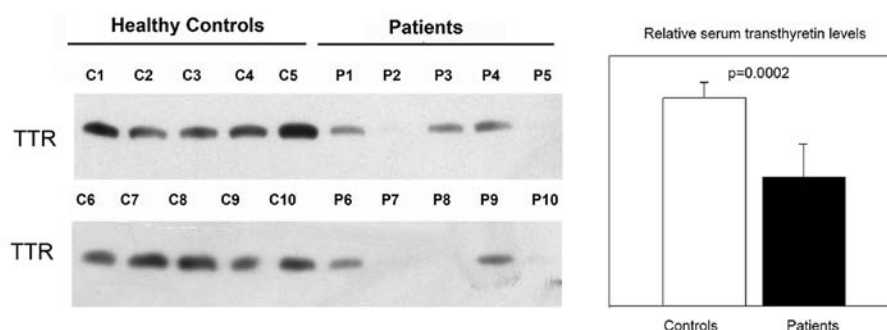


Figure 5. Relative serum transthyretin (TTR) levels in patients and controls. (A) Western blot analysis of TTR concentration in individual serum samples was performed. To enable relative comparison between healthy controls and patients, five control and five patient samples (10 μ g protein each) were run on each gel. (B) Densitometric analysis of the TTR bands from the Western blot analysis.

between the patients and healthy women. Hence, we concluded that the decrease in RBP4 concentration in EOC patients is independent on serum vitamin A levels.

RBP4 is a relatively small protein and to avoid glomerular filtration it associates with transthyretin (TTR) (12). Interestingly, transthyretin has also been identified as down-regulated in blood of ovarian cancer patients and considered as a potential biomarker (14-16). We determined relative serum transthyretin levels in our group of patients and control women and confirmed its down-regulation in our EOC patients (1.6-fold change, $p=0.002$) (Fig. 5). There was no marked correlation of TTR levels with RBP4.

Discussion

RBP4 is secreted by liver, adipose tissue and some epithelia including ovarian and serves as an important transporter of retinol. Retinoids play an important role in fundamental aspects of human physiology such as hematopoiesis, reproduction and cell proliferation. Anti-cancer effect of retinoids was reported long ago (17). Alterations of vitamin A and retinoid homeostasis are found in many tumors. Defects in expression of retinol metabolism genes, namely its crucial components cellular retinol-binding protein 1 (CRBP1) and RBP4, have been previously reported in ovarian cancer and connected with the oncogenic process in a rat model of ovarian cancer (18,19). Moreover, defective conversion of retinol to retinoic acid has been demonstrated in ovarian carcinoma cell lines (20).

The observed decrease in concentration of RBP4 in sera of EOC patients identified here may be theoretically attributed to decreased RBP4 production by ovary. Regrettably, we do not know how much RBP4 ovary contributes to the total circulating RBP4 pool. Considering the fact, that liver and adipose tissue are believed to be the main producers of the circulating RBP4, it remains to be determined whether decreased production of RBP4 in ovary may be reflected in total circulating RBP4 levels in EOC patients.

Alternative hypothesis which considers a systemic process and the liver and/or adipose tissue as a source of the altered RBP4 levels can also be proposed. We demonstrated that levels of retinol are comparable between patients and controls. Decreased levels of RBP4 in patients therefore can not be explained by different retinol availability between the groups. In addition to its role as a vitamin A transporter RBP4 attracted

wide attention as a molecule involved in insulin resistance in mice (21) and as a protein elevated in serum of patients with impaired glucose tolerance and type 2 diabetes (insulin resistance) (22). These observations were followed by many other reports, in the wider metabolic area related to insulin and glucose and fat metabolism. RBP4 has thus been shown to be elevated in obese patients with polycystic ovary syndrome (disease associated with insulin resistance) (23) and in patients with renal dysfunction and cardiac disease in type 2 diabetes patients (24). To exclude a potential influence of such a cancer-unrelated factor we verified anamnesis of our healthy controls and patients. None of the women involved in our study had history, or evidence of type 2 diabetes.

What could then be the connection between RBP4 energy metabolism and ovarian cancer? Is it a tumor-specific response or rather a reflection of general metabolic changes taking place in cancer patients? Controversy exists, whether there is a general correlation between RBP4 levels and body-mass index in otherwise healthy women. Whereas some investigators showed lower serum RBP4 levels in healthy lean women compared to obese (22), others demonstrated that there is no such a correlation (25). However, since levels of RBP4 have been reported to decrease in morbidly obese patients after weight loss due to gastric banding surgery (26), we tested a hypothesis that the decreased levels of RBP4 observed in our patient group could be attributed to cancer-induced cachexia. However, comparison of body-mass indexes (BMI) between our patients and healthy controls showed, that there is only a marginal and statistically insignificant ($p=0.126$) BMI decrease in our group of EOC patients and there is no correlation of serum RBP4 concentration with BMI ($r=-0.001$, $p=0.99$). This observation therefore does not support the hypothesis that the reduced RBP4 level in blood of EOC patients is caused by cancer-related decrease in BMI. However, we are aware, that the decrease in serum RBP4 in EOC patients may be an early sign of cancer-triggered nutritional changes before they become apparent by weight loss. Such a marker would be of wider clinical interest because cancer-induced cachexia indeed complicates therapy and has been implicated in up to 20% of cancer-related deaths (27,28).

We identified significantly decreased RBP4 concentrations in sera of EOC patients. We excluded influence of diabetes, serum retinoid levels and BMI as potential causes of the decreased RBP4 levels and we therefore believe that the phenomenon is cancer related. We are fully aware that the low

number of patients enrolled in our proteomic discovery phase of the study must be compensated for in future verification process using large cohorts of patients and controls stratified by stage/grade and other factors.

The decreased RBP4 concentration in sera of EOC patients is either directly connected with altered retinoid metabolism and RBP4 production in ovary or it is a reflection of a more general process involving energy metabolism or other systemic changes. In both cases, the cancer-related information represented by RBP4 serum levels, is of clinical interest and should be evaluated as a potential biomarker.

So far, none of the candidate molecules identified by proteomic analyses of ovarian cancer (10, 11, 14 and many others) have been implemented into clinical practice as a single diagnostic biomarker. However, as demonstrated by recent development, combined informative power of several weak biomarkers can be valuable in clinical decision-making in assessment of ovarian tumors (29). We believe that RBP4 can increase diagnostic performance of such a multivariate biomarker panel in future.

Acknowledgements

This study was supported by grants from the Ministry of Health CR, (IGA MZCR NT12248-5 to J.Z. and NS10300-3 and UHKT No. 023736 to J.P.), from the Ministry of Education, Youth and Sports, CR (VZ 0021620806 to J.Z., LC06044 to J.P. and SVV-2011-262507 to L.L.), from the Grant agency of the Czech Republic (305/09/1390 to J.P.) and Grant agency of the Charles University (GAUK 251180 111210 to Jana Pospislova). Authors would like to thank Milada Kostirova for plasma vitamin A measurements and Ondrej Vit for his assistance with western blotting. Special thanks to Mrtva Ryba.

References

- Heintz APM, Odicino F, Maisonneuve P, *et al*: Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynaecological Cancer. *Int J Gynaecol Obstet* 95: S161-S192, 2006.
- Skirnisdottir I and Sorbe B: Prognostic factor for surgical outcome and survival in 447 women treated for advanced (FIGO-stages III-IV) epithelial ovarian carcinoma. *Int J Oncol* 30: 727-734, 2007.
- Moore RG, MacLaughlan S and Bast RC Jr: Current state of biomarker development for clinical application in epithelial ovarian cancer. *Gynecol Oncol* 116: 240-245, 2010.
- Husseinizadeh N: Status of tumor markers in epithelial ovarian cancer has there been any progress? A review. *Gynecol Oncol* 120: 152-157, 2011.
- Narasimhan K, Changqing Z and Choolani M: Ovarian cancer proteomics: Many technologies one goal. *Proteomics Clin Appl* 2: 195-218, 2008.
- Anderson NL and Anderson NG: The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 1: 845-867, 2002.
- Pernemalm M, Lewensohn R and Lehtio J: Affinity prefractionation for MS-based plasma proteomics. *Proteomics* 9: 1420-1427, 2009.
- Boschetti E and Righetti PG: The ProteoMiner in the proteomic arena: a non-depleting tool for discovering low-abundance species. *J Proteomics* 71: 255-264, 2008.
- Hortin GL, Sviridov D and Anderson NL: High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin Chem* 54: 1608-1616, 2008.
- Chen Y, Lim BK, Peh SC, Abdul-Rahman PS and Hashim OH: Profiling of serum and tissue high abundance acute-phase proteins of patients with epithelial and germ line ovarian carcinoma. *Proteome Sci* 6: 20, 2008.
- Dieplinger H, Ankerst DP, Burges A, Lenhard M, *et al*: Afamin and apolipoprotein A-IV: novel protein markers for ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 18: 1127-1133, 2009.
- Muto Y, Smith JE, Milch PO and Goodman DS: Regulation of retinol-binding protein metabolism by vitamin A status in the rat. *J Biol Chem* 247: 2542-2550, 1972.
- Peterson PA, Rask L, Ostberg L, Anderson L, Kamwendo F and Pertoft H: Studies on the transport and cellular distribution of vitamin A in normal and vitamin A-deficient rats with special reference to the vitamin A-binding plasma protein. *J Biol Chem* 248: 4009-4022, 1973.
- Zhang Z, Bast RC Jr, Yu Y, *et al*: Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 64: 5882-5890, 2004.
- Kozak KR, Su F, Whitelegge JP, Faull K, Reddy S and Farias-Eisner R: Characterization of serum biomarkers for detection of early stage ovarian cancer. *Proteomics* 5: 4589-4596, 2005.
- Mählck CG and Grankvist K: Plasma prealbumin in women with epithelial ovarian carcinoma. *Gynecol Obstet Invest* 37: 135-140, 1994.
- Clarke N, Germain P, Altucci L and Gronemeyer H: Retinoids: potential in cancer prevention and therapy. *Expert Rev Mol Med* 6: 1-23, 2004.
- Roberts D, Williams SJ, Cvetkovic D, Weinstein JK, Godwin AK, Johnson SW and Hamilton TC: Decreased expression of retinol-binding proteins is associated with malignant transformation of the ovarian surface epithelium. *DNA Cell Biol* 21: 11-19, 2002.
- Cvetković D, Williams SJ and Hamilton TC: Loss of cellular retinol-binding protein 1 gene expression in microdissected human ovarian cancer. *Clin Cancer Res* 9: 1013-1020, 2003.
- Williams SJ, Cvetkovic D and Hamilton TC: Vitamin A metabolism is impaired in human ovarian cancer. *Gynecol Oncol* 112: 637-645, 2009.
- Yang Q, Graham TE, Mody N, *et al*: Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436: 356-362, 2005.
- Graham TE, Yang Q, Blüher M, *et al*: Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 354: 2552-2563, 2006.
- Tan BK, Chen J, Lehnert H, Kennedy R and Randeva HS: Raised serum, adipocyte, and adipose tissue retinol-binding protein 4 in overweight women with polycystic ovary syndrome: effects of gonadal and adrenal steroids. *J Clin Endocrinol Metab* 92: 2764-2772, 2007.
- Cabré A, Lázaro I, Girona J, *et al*: Retinol-binding protein 4 as a plasma biomarker of renal dysfunction and cardiovascular disease in type 2 diabetes. *J Intern Med* 262: 496-503, 2007.
- Kowalska I, Straczkowski M, Adamska A, Nikolajuk A, Karczewska-Kupczewska M, Oziomek E and Gorska M: Serum retinol binding protein 4 is related to insulin resistance and nonoxidative glucose metabolism in lean and obese women with normal glucose tolerance. *J Clin Endocrinol Metab* 93: 2786-2789, 2008.
- Haider DG, Schindler K, Prager G, Bohdjalian A, Luger A, Wolzt M and Ludvik B: Serum retinol-binding protein 4 is reduced after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab* 92: 1168-1171, 2007.
- Inui A: Cancer anorexia-cachexia syndrome: current issues in research and management. *CA Cancer J Clin* 52: 72-91, 2002.
- MacDonald N, Easson AM, Mazurak VC, Dunn GP and Baracos VE: Understanding and managing cancer cachexia. *J Am Coll Surg* 197: 143-161, 2003.
- Ueland FR, Desimone CP, Seamon LG, *et al*: Effectiveness of a multivariate index assay in the preoperative assessment of ovarian tumors. *Obstet Gynecol* 117: 1289-1297, 2011.