Advances in Brief

Haptoglobin-α Subunit As Potential Serum Biomarker in Ovarian Cancer: Identification and Characterization Using Proteomic Profiling and Mass Spectrometry

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Abstract

Purpose: The objective of this study was to identify and characterize new serum biomarkers in ovarian cancer patients using mass spectrometric protein profiling and specific immunological assays.

Experimental Design: Serum samples from 80 cancer patients and 91 healthy women were analyzed by surface enhanced laser desorption and ionization-mass spectrometry (MS) profiling. A candidate biomarker was purified by affinity chromatography, and its sequence was determined by liquid chromatography-tandem MS. An antibody was generated from the synthesized peptide for quantitative validation in the cases and controls. CA125 was determined and compared with matched normal serum as a control.

Results: Using surface enhanced laser desorption and ionization, we found a serum biomarker at ~11700 Da, which had peak intensity significantly higher in cases (1.366) compared with controls (0.208, \( P = 0.002 \)), and subsequently identified this as the α chain of haptoglobin. ELISA indicated that Hp-α was ≈2-fold higher in cancer serum compared with normal, benign tumor, and other gynecological cancers (\( P < 0.05 \)) and had 64% sensitivity at 90% specificity alone and 91% sensitivity and 95% specificity if combined with CA125.

Conclusions: Haptoglobin-derived α subunit is a potential marker for ovarian cancer that is complementary to CA125. MS-based protein profiling is a valuable tool for screening protein markers and useful to detect post-translational modification of tumor-associated proteins or abnormal metabolic products. However, confirmation of protein identity with specific antibodies is crucial for clinical application and functional studies.

Introduction

The identification of cancer biomarkers advances the possibility for early detection, better monitoring of tumor progression, and even targeting therapy. Such markers are especially needed for ovarian cancer, which is associated with advanced stage at presentation and poor survival (1, 2). Classical approaches for cancer biomarker identification used tumor cells to immunize animals and screen for antibodies that could efficiently recognize the antigen (3). This approach is limited by its high cost and labor intensity but has produced the best known marker, CA125, approved for ovarian cancer monitoring. More recently, tumor mRNA has been compared with normal tissue mRNA to identify up-regulated genes in cancer tissue using cDNA micro-arrays to identify a variety of markers, including prostasin, osteopontin, and He4 (4, 5). A limitation of the cDNA micro-array approach is that transcriptional activity in the tumor does not necessarily reflect the protein observed peripherally, because various protein–protein interactions and post-translational modifications may alter the protein patterns found in circulation.

Time of flight mass spectrometry technology offers a powerful and sensitive tool to study post-translational protein profiles. SELDI-MS3 provides a proteomic high throughput approach that profiles the mass/charge of potential biomarkers. There has been considerable interest in analyzing the SELDI-MS spectral "proteomic pattern" as a marker for disease detection (6, 7). Near perfect sensitivity and specificity have been achieved in separating cancer from control specimens, but identification of the specific proteins constituting the SELDI

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3 The abbreviations used are: SELDI-MS, surface enhanced laser desorption and ionization mass spectrometry; IMAC, immobilized metal affinity capture; TBST, [10 mM Tris-HCl, 100 mM NaCl, and 0.1% (volume for volume) Tween 20 (pH 7.5)]; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; Hb, hemoglobin; biotin-Hp, biotin-labeled haptoglobin; Hp, haptoglobin.

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discriminatory patterns has not been attempted in these studies. Here, we report our progress in using SELDI-MS protein chip technology combined with LC-MS/MS, and traditional immunological approaches to identify and validate a potential ovarian cancer biomarker suggested from SELDI profiling.

Materials and Methods

Biological Specimens. All patient-related biological specimens were collected and archived under protocols approved by the Human Subjects Committees of the Partners HealthCare System (Boston, MA) and Institutional Review Board for The University of Texas, Southwestern Medical Center at Dallas. Serum was collected preoperatively from women requiring surgery for a “pelvic mass,” at Brigham and Women’s Hospital, Massachusetts General Hospital, and The University of Texas, Southwestern Medical Center at Dallas. Current analysis is based on serum specimens from 175 women collected before surgery; 80 of them proved to have epithelial ovarian cancer, 44 had benign gynecologic tumor, and 51 had other types of gynecologic cancer. A total of 91 serum specimens was also available from normal women selected from the general population and collected as part of a population-based case control study of ovarian cancer (8). Specimens were generally processed within 4 hours of collection, aliquoted, and stored at store at −80°C.

SELDI-MS Profiling of Serum Protein. Unfractionated serum samples were thawed and mixed with equal volume of PBS buffer (5 µl) containing 1% CHAPS and 8 m urea. The mixture was spun for 30 s before using for protein chip binding. The protein chip IMAC3 was activated by 50 mM Cu²⁺ for 10 min and followed by two washes with high-performance liquid chromatography grade water. Ten µl of serum, mixed as above, were added to the surface spots of the IMAC3 array and incubated with 40 µl of binding buffer (0.1 M sodium phosphate, 0.5 M sodium chloride, and 10 mM imidazole) for 30 min. After two washes with binding buffer and water, the air-dried array was then treated with saturated sinapinic acid in 0.5% trifluoroacetic acid and 50% acetonitrile before applied on SELDI-MS (Protein Biology System II; Ciphergen, Biosystems, Freemont, CA). Mass resolution and accuracy were assessed by routine calibration with 5733.58 and 12230.92 Da polypeptides. The chip was read and analyzed under the following settings: (a) laser intensity 250; (b) detection sensitivity 10; (c) 50 shots per sample; and (d) auto-identify peaks from 3000 to 50,000 Da. We have achieved a mass accuracy of 0.1% for protein and polypeptide of 3,000–30,000 mass/charge (m/z) in this system.

Protein Purification and Identification. A serum sample of 0.5 ml from a cancer patient with the protein pattern of interest was mixed with equal volume of PBS buffer containing 1% CHAPS and 8 m urea. The sample was then applied on the Sephadex G-25 column for desalting and removing insoluble fractions from the serum. The metal-chelating column (HiTrap; Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used for purification according to the provided protocol. Elution buffers containing 20 mM sodium phosphate, 0.5 M NaCl (pH 4.5–6.5), were applied. The eluted fractions of interest ~11,700 Da were first applied on the SELDI-MS to confirm the polypeptide peak and then separated by 10–20% gradient SDS-PAGE. The separated proteins were visualized by Coomassie staining. The band of interest was excised from the gel and subjected to in-gel digestion with trypsin (9). The resultant polypeptides were further separated by liquid chromatography with online sequence analysis by LC-MS/MS (10, 11). The fragmentation ladders (the b and y ion series) from the lowest to highest mass were used to identify the amino acid residues of the peptides.

Antibody Generation and Western Blotting. The amino acid sequence, NKKQWINKAVGDKLPEC, from the identified Hp-α fragment was selected for peptide synthesis based on predicted antigenicity and used for generating a rabbit polyclonal antibody (BioSource International, Hopkinton, MA). The affinity-purified antibody developed was then used for the Western blot and ELISA. A total of 0.5 µg of human Hp protein (98–100% purity; Sigma) was incubated with 10 µl of Hp cleavage solution (50% urea and 2% β-mercaptoethanol; Ref. 12) to generate both α and β chains as positive controls. Aliquot sera (0.5 µg) samples were mixed with PBS buffer containing 1% CHAPS and 8 m urea before loading or on the 15% SDS-PAGE. After the proteins transferred to polyvinylidene difluoride membrane, 5% of fat-free milk in TBST was used for blocking. A monoclonal antibody (1:5000) against human Hp (Sigma) was also applied for blocking the cross-reaction between Hp-α and intact Hp protein. The purified primary antibody against Hp-α was used at 1:5000 diluted in TBST with 5% milk (w/v) for 2 h. The membrane was then washed three times with TBST for 10 min/wash. The serum Hp-α peptides were detected by the second antibody conjugated to horseradish peroxidase and visualized by the enhanced chemiluminescence detection system (Pierce).

Quantitative Validation by Enzyme-linked Immunosorbent Assay. The total Hp-α level was quantified by using direct ELISA with the purified polyclonal antibody against Hp-α and the monoclonal antibody against intact Hp protein (Sigma). Individual serum samples (1 µl) were mixed with 2 µl of PBS buffer containing 1% CHAPS and 8 m urea. The mixture was then diluted (1:1000) in coating buffer [0.1 M carbonate (pH 9.8)]. The diluted mixture of 200 µl was added onto a 96-well plate and incubated overnight at 4°C for antigen coating. The synthetic Hp-α peptide fragment was used as the antigen for standard calibration in each assay. After six washes with buffer (5 mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween 20), the antigen was blocked by incubation at 37°C for 2 h, with 1% BSA and 2-3 µg (1:5000) of monoclonal antibody against human Hp protein, in the buffer containing 50 mM Tris-HCl (pH 7.5) and 0.05% NaN₃. After three washes, the polyclonal Hp-α antibody (1:3000) in 50 mM Tris-HCl (pH 7.5), with 6% BSA was added and incubated for 1 h at 37°C. After seven washes, the second antibody conjugated with horseradish peroxidase was diluted (1:5000) in 50 mM Tris-HCl (pH 7.5), with 6% BSA, and applied for incubation at 37°C for 30 min. After nine washes, the antigen concentration was recovered using the Turbo-TMB (Pierce) initiated chemiluminescence reaction and measured at A₄50nm, according to the protocol.

CA125 Assay. CA125 levels in serum were measured by an electrochemiluminescence immunoassay (CA125 II) performed on automatic immune analyzer (Elecsys2010; Roche Diagnostics, Indianapolis, IN). The working range of the immune assay is 0.6–5000 units/ml. The upper 95th percentile
limit for CA125 levels in healthy women is 35 units/ml in this system.

**Biotin-labeling Hp and Serum Enzymatic Reaction.** Intact Hp protein (mixture of 1-1, 2-1 forms; Sigma) was labeled with NHS-LC-Biotin (Pierce) as described previously (13). A total of 200 μg of Hp in 200 μl of PBS buffer was added with 20 times mol excess of fresh prepared NHS-LC-Biotin and incubated for 1 h at room temperature. The unlabeled free NHS-LC-Biotin was removed by microcentrifugation (Millipore, Bedford, MA). Biotin-Hp (0.4 μg) was incubated with serum samples (2 μl) at room temperature for 30 min to 6 h. The reaction samples were analyzed by Western blot under nonreducing conditions. Biotin-Hp-α and -β chains were detected by enhanced chemiluminescence. The total amount of biotin-Hp-α on the membrane relative to controls was quantified by densitometry and normalized with the same control sample in each blot.

**Statistical Analysis.** For comparison of the difference between the means of the protein marker peak intensity from SELDI-MS profiles between cancer and normal serum samples, Student’s t test was applied on the logarithmic scale. The total Hp-α concentration in serum quantitatively measured by ELISA and biotin Hp-α, transformed to the logarithmic scale, was analyzed by ANOVA to compare the difference among populations of ovarian cancer, benign tumor, other gynecological cancers, and normal controls. To assess the impact on sensitivity and specificity when both CA125 and Hp-α are used to predict cancer status, a logistic regression model, including terms for
both measurements (log transformed), was fit in cases and normal controls. This model included menopausal status to account for differences in the proportion of postmenopausal subjects between cases and controls. To validate the sensitivity and specificity predicted by the model without using a separate validation data set, we used the "leave-one-out" method of cross-validation. This technique iteratively omits one observation from the dataset to obtain parameter estimates and then uses these estimates to classify the omitted subject as a case or control (14).

Results

Serum Protein Profiling. The SELDI-MS profile pattern of the metal-binding polypeptides was displayed according to their mass:charge ratio (m/z) and analyzed with the biomarker software (Ciphergen) that distinguishes differences in polypeptide peaks between subject groups based on their mass intensity. One candidate marker was identifiable both by visually comparing mass spectrum profiles and using the protein peak discriminator software (Fig. 1A). From the initial SELDI serum protein profiling of 80 cancer cases and 91 normal healthy controls, a polypeptide at ~11,700 Da was frequently found in cancer patients at high intensity but less so in controls. The average (±SE) mean peak intensity in cases was 1.366 (±0.33) compared with 0.123 (±0.03) in controls (P = 0.002).

Purification and Identification of the Polypeptide Biomarker. To purify the polypeptide peak that appeared at 11,700 Da shown in the IMAC3 profiles, one sample of cancer serum was applied to a Cu²⁺-activated chelating column (Hi-Trap). The eluted protein fraction from the column was separated on a 10–20% gradient SDS-PAGE, and the protein of interest was identified as shown in Fig. 1B (see arrow of left panel). The purified candidate protein from the cancer case was confirmed by SELDI-MS, which corresponded with the peaks at ~11,400 and 11,700 Da. The corresponding polypeptides were unable to be purified from normal control serum (Fig. 1B, right panel).

The precise amino acid sequence of the isolated polypeptide was next determined by liquid chromatography with online sequence analysis by LC-MS/MS. The sequence data showed...
that five different polypeptides from pro-Hp-1 were detected (Fig. 2, A and B). Two of these peptides were partially tryptic peptides, which corresponded to the NH₂ and COOH terminus of the polypeptide biomarker. The fragmentation ladders of first polypeptide (964.5 MH⁺/H₁₁₀₀₁, the b and y ion series) are displayed as an example for identification of the amino acid residues of PKNPANPVQ peptide (Fig. 2C). Considering all peptides detected, the amino acid sequence of the biomarker corresponded to Hp-₁/₉₂₅₁ polypeptide.

Elevation of Hp-₁ in Cancer Serum. To further explore and validate Hp-₁ as an ovarian cancer biomarker, we performed Western blot analysis using a specific polyclonal antibody against the epitope peptide to detect the Hp-₁ in serum samples. Because the peptide sequence of Hp-₁ is derived from gene duplication of Hp-₁ (15), the polyclonal antibody reacted with both Hp-₁ and Hp-₂ but not with β chain as shown in Fig. 3A (Lanes 2 and 3). Western blotting revealed that Hp-₁ is elevated in cancer sera but not in controls. The level of Hp-₂ is also increased in cancer sera (Fig. 3A).

To quantify the total Hp-₁ amount in case and control serum samples, the ELISA we developed was applied to a population of 80 patients with ovarian cancers, 44 with benign ovarian tumor, 51 with other gynecological cancers, and 91 normal controls. The log-transformed mean of total Hp-₁ unit (µg/ml) in sera of ovarian cancer patients was 63 and significantly different from 37, 42, and 50 for the normal controls (P < 0.0001), benign ovarian tumor (P < 0.05), and other gynecological cancers (P < 0.05), respectively (Fig. 3B).
Table 1 Mean value of preoperative Hp-α and CA125 in the selected subtypes of ovarian cancer and control subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>CA125 level, units/ml</th>
<th>Mean ± SD</th>
</tr>
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<tr>
<td>Normal controls</td>
<td>91</td>
<td>37 (1.5)</td>
<td>&lt;0.00001</td>
<td>15 (2.0)</td>
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<tr>
<td>Benign conditions</td>
<td>44</td>
<td>42 (2.1)</td>
<td>&lt;0.05</td>
<td>21 (2.5)</td>
</tr>
<tr>
<td>Other GYN cancer</td>
<td>51</td>
<td>50 (1.6)</td>
<td>&lt;0.00001</td>
<td>22 (2.9)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>77</td>
<td>63 (1.8)</td>
<td>229 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>10</td>
<td>46 (1.8)</td>
<td>31 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Nonmucinous</td>
<td>67</td>
<td>66 (1.8)</td>
<td>422 (4.6)</td>
<td></td>
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<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>53 (1.6)</td>
<td>58 (5.3)</td>
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<tr>
<td>II</td>
<td>3</td>
<td>100 (1.5)</td>
<td>151 (6.3)</td>
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<tr>
<td>III</td>
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<td>469 (4.0)</td>
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</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>55 (1.5)</td>
<td>966 (5.7)</td>
<td></td>
</tr>
</tbody>
</table>

**Footnote:**

a ANOVA was used to compare mean log-transformed Hp-α/CA125 levels in normal controls, benign conditions, and other gynecologic cancers with mean log-transformed Hp-α/CA125 levels in ovarian cancers.

Three cases are missing menopausal status, and two are missing cancer stage information.

compared with the normal controls (78% of cases versus 51% of controls). Adjustment for menopausal status was necessary in the model because CA125 levels were significantly lower in premenopausal compared with postmenopausal cases (data not shown). The combined use of Hp-α and CA125 yielded an estimated sensitivity of 79 at 96% specificity in premenopausal women (Fig. 3C) and 94% sensitivity and 93% specificity in postmenopausal women (Fig. 3D). The model demonstrated 91% sensitivity and 95% specificity in the combined two groups. The logistic model was validated using a leave-one-out cross-validation procedure, and the cross-validated estimate of sensitivity was 88 at 95% specificity, demonstrating no substantial loss in sensitivity upon cross-validation.

**Hp-α Elevation and Protein Interactions in Cancer Serum.** To test whether the elevated level of Hp-α in the sera of ovarian cancer patients resulted from overexpression by tumor cells, quantitative reverse transcription-PCR with three sets of DNA primers and Western blot analysis was performed using four normal and seven malignant ovarian epithelial cell lines. Neither the mRNA coding for Hp-α nor the peptide was detected (data not shown). To test the hypothesis that dissociation of Hp into Hp-α and β subunits may be caused by the presence of specific protein–protein interaction, such as proteases in cancer serum, the biotin-Hp was incubated with sera from cancer patients and controls. Western blotting showed that biotin-labeled α₁, α₂, and β fragments were detected in biotin-Hp samples incubated with cancer sera, and the intensity of the Hp-α increased with the time of incubation. However, only a trace of Hp-β fragment was detected when biotin-Hp was incubated with the normal serum (Fig. 4A). The individual serum from different case and control groups was incubated with the same amount of biotin-Hp. The cleaved Hp-α fragments were 3-2-fold higher in sera from ovarian cancer patients than in sera from normal, benign ovarian tumors, or other gynecological cancers (Fig. 4B). This is consistent with the ELISA data that total Hp-α is about two times higher than the controls. In addition, pretreatment of cancer sera by boiling for 10 min eliminated detectable Hp-α or β subunits (Fig. 4C). This suggests that Hp-α subunit elevation is likely caused by a specific enzymatic cleavage or specific protein–protein interactions.

**Discussion**

Time of flight mass spectrometry technology offers a powerful and sensitive tool to study post-translational protein profiles in serum obtained from cancer and normal subjects. Recently, there has been considerable interest in analyzing the SELDI-MS spectral “proteomic pattern” (6, 16). The high dimensional array, created by the spectrum of thousands of peptides and their intensities, provides discriminatory power for separating any given set of case and control specimens. However, validation of the methodology requires demonstration that the discriminatory algorithm is reproducible among different laboratories and different sets of case control specimens. Furthermore, it seems desirable to know the identity of the biomarkers in the pattern to understand their significance in disease pathogenesis, as has been reported with the use of SELDI-MS to identify amyloid-β peptide as a diagnostic marker for Alzheimer’s disease (17, 18) and α-defensin 1, 2, 3 as a favorable prognostic marker in acquired immune deficiency and contributed to anti-HIV-1 activity (19). In this report, we have used...
SELDI-MS and LC-MS/MS technologies and identified Hp-α as a potentially useful polypeptide biomarker for ovarian cancer.

The full scope of biological functions of Hp in a variety of diseases is not well understood (20, 21). Intact Hp is a glycoprotein mainly secreted by the liver cells in response to a variety of stimuli (22, 23) and functions as a Hb scavenger by binding free Hb and recycling the iron. Hp is made up of Hp-α and Hp-β subunits, which linked to the β chain via disulfide bonds to form different Hp biotypes (Fig. 2D). Cells and tissues other than liver, including cancer cells (24), intestinal, seminiferous, and endometriotic epithelium, may also produce Hp (25). Besides its scavenging and inflammatory response functions, Hp has been shown to be involved in the regulation of epidermal cell transformation (26), immune suppression in cancer (27, 28), and angiogenesis (29).

Interestingly, other studies have identified increased intact Hp in sera from ovarian cancer patients, specifically of glycosylated forms produced by fucosylation (30, 31). But none of these studies has described any intrinsic changes in the content of Hp-α. Hence, we provide evidence that the Hp-α subunit is specifically elevated in sera of ovarian cancer patients. Although the cause and consequence of this elevation in ovarian cancer is not clear yet, we speculate that the dissociation of α and β subunits from intact Hp is a key source. Furthermore, it is possible that the dissociated fragments may cause abnormal physiological function, i.e., Hp-α may interfere with the immune system because it has structural and functional homology with human 7S immunoglobulins (32). Thus, Hp-α might affect the immune cellular response (28), as a potent immunosuppressant (27), or abolish the native Hp-Hb complex (33) in cancer patients. These tumor-related functions of Hp-α and -β subunits and the protein–protein interaction with circulating antigens in ovarian cancer will require future study.

At the specificity of 90%, Hp-α has 64% sensitivity and is not superior to the 87% observed for CA125. However, additional validation studies may improve the performance by using a more specific antibody. Additional testing should be done on sets of samples with a larger size of early stage disease and serum from other cancers or medical conditions, such as liver diseases. Despite the poor sensitivity by itself, it appears Hp-α is complementary to CA125, resulting in 91% sensitivity and 95% specificity in the combined logistic model.

In conclusion, we have demonstrated that MS-based protein chip technology combined with liquid chromatography and LC-MS/MS has identified Hp-α as a novel ovarian cancer serum biomarker. The elevated Hp-α in ovarian cancer sera is most likely attributable to abnormal protein–protein interaction in the circulation rather than overproduction from tumor cells, providing evidence that cancer biomarkers may not always need to be derived directly from cancer cells. Serum protein fragments or peptides derived indirectly from cancer antigens could also be valuable cancer markers. These post-translational, modification, and metabolic peptide biomarkers would not otherwise be identified using either the classical approach or newer ones with cDNA microarrays.

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References


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