Serum Levels of an Isoform of Apolipoprotein A-II as a Potential Marker for Prostate Cancer

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ABSTRACT

Purpose: We recently showed that protein expression profiling of serum using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has potential as a diagnostic approach for detection of prostate cancer. As a parallel effort, we have been pursuing the identification of the protein(s) comprising the individual discriminatory “peaks” and evaluating their utility as potential biomarkers for prostate disease.

Experimental Design: We employed liquid chromatography, gel electrophoresis and tandem mass spectrometry to isolate and identify a protein that correlates with observed SELDI-TOF MS mass/charge (m/z) values. Immunodepletion, immunoassay, and Western analysis were used to verify that the identified protein generated the observed SELDI peak. Subsequent immunohistochemistry was used to examine the expression of the proteins in prostate tumors.

Results: An 8,946 m/z SELDI-TOF MS peak was found to retain discriminatory value in each of two separate data sets with an increased expression in the diseased state. Sequence identification by liquid chromatography-MS/MS and subsequent immunoassays verified that an isoform of apolipoprotein A-II (ApoA-II) is the observed 8,946 m/z SELDI peak. Immunohistochemistry revealed that ApoA-II is overexpressed in prostate tumors. SELDI-based immunoassay revealed that an 8.9-kDa isoform of ApoA-II is specifically overexpressed in serum from individuals with prostate cancer. ApoA-II was also overexpressed in the serum of individuals with prostate cancer who have normal prostate-specific antigen (0.4-4.0 ng/mL).

Conclusions: We have identified an isoform of ApoA-II giving rise to an 8.9K m/z SELDI “peak” that is specifically overexpressed in prostate disease. The ability of ApoA-II to detect disease in patients with normal prostate-specific antigen suggests potential utility of the marker in identifying indolent disease.

INTRODUCTION

Due to the high prevalence of prostate cancer, there has been a large-scale search for potential biomarkers useful in the early detection and prognosis of prostate cancer. The “gold standard” diagnostic marker for prostate cancer is prostate-specific antigen (PSA) and the rapid incorporation of aggressive PSA testing has resulted in a dramatic reduction in the identification of advanced stages of prostate cancer as well as deaths secondary to prostate cancer (1, 2). However, the significantly high false-positive rate of PSA combined with its widespread clinical application has lead to a tremendous increase in the number of unnecessary biopsies of the prostate (3). In addition, recent reports, such as those from the Prostate Cancer Prevention Trial (4), highlight the inability of PSA to separate aggressive prostate cancer from clinically indolent disease (5). Such reports support the concept that no single marker will accurately reflect the complex phenotypic changes associated with development of cancer. There has been, therefore, an increasing emphasis on the need to determine multiple protein biomarkers for use in the diagnosis/prognosis of prostate cancer. The development of high-throughput methods that are able to analyze large segments of the proteome promise to facilitate the identification of multiple protein panels for cancer diagnostics.

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a useful tool for integrating separation and analysis of complex mixtures of proteins. The protein profiles are generated using specific surface chemistry to affinity capture proteins from complex biological mixtures. Captured proteins are then analyzed by TOF-MS, generating a spectral map depicting approximations of the molecular weight (mass/charge or m/z) and relative concentration (intensity) of each protein (ion). The technique is a convenient, high-throughput tool to segregate proteins from complex bodily fluids like serum and generate comparative protein profiles. SELDI technology has now been widely used for diagnosis of cancer and other diseases in a large number of studies (reviewed by refs. 6–9).

SELDI ProteinChip technology has proven to be highly promising in cancer diagnostics (10). Our group first showed that using the spectral peaks of SELDI protein profiles significantly increases the sensitivity for detecting transitional cell carcinoma of the bladder (11). The application of a pattern recognition algorithm to the data from the protein profiles was reported to be successful for the identification of ovarian cancer (12).
Identification of a Serum Marker of Prostate Cancer

We subsequently showed the utility of protein expression profiling using an automated decision tree algorithm as an accurate assay for the detection of prostate cancer (13, 14). Indeed, following these initial publications there have been numerous reports of the successful application of this approach to cancer diagnostics (15–29). All of these findings strongly support the potential usefulness of profiling of protein expression, coupled with decision algorithms, for improving the early detection/diagnosis of prostate cancer. Our efforts in profiling of protein expression in prostate cancer are paralleled with approaches to identify the biomarkers represented by the subset of m/z peaks we foresee as comprising a potential group of proteins that will form a diagnostic profile of prostate cancer. Specifically, we targeted m/z peaks for further identification that showed significant area under the curve for discriminating between any two groups (>0.7 for disease versus nondisease) and were observed as clearly delineated well-expressed m/z peak events. Identification of these cancer biomarkers will assist in successful implementation of profiling-based diagnostics, as well as facilitate the development of more traditional multiprotein antibody array or multiplex immunoassays for the early detection of prostate cancer. It may also potentially help elucidate important steps in the prostate cancer disease process.

MATERIALS AND METHODS

Serum Samples. Serum samples were obtained from the Virginia Prostate Center Tissue and Body Fluid Bank. Blood samples collected under the same protocols from properly consented patients diagnosed with either biopsy proven prostate cancer or benign prostate hyperplasia (BPH; PSA 4-10 ng/mL with multiple negative biopsies) were obtained from the Department of Urology, Eastern Virginia Medical School, and the samples of healthy men cohort (normal; PSA < 4.0 ng/mL with no evidence of prostate disease) were obtained from free serum samples collected under the same protocols from properly consented patients with cancers of the head and neck, colon, and lung or benign prostate hyperplasia (BPH; PSA 4-10 ng/mL with multiple negative biopsies) were obtained from the samples of healthy men cohort (normal; PSA < 4.0 ng/mL with no evidence of prostate disease). All samples were processed based on manufacturer’s directions. The material was dried in a speed-vac, rehydrated in a 12.5 ng/μL modified sequencing grade trypsin solution (Promega, Madison, WI) and incubated in an ice bath for 40 to 45 minutes. The excess trypsin solution was then removed with SilverQuest destaining solution following manufacturer’s instructions. The gel was silver stained, the stain was first removed with SilverQuest silver staining kit (Invitrogen Life Technologies). All the gels were stained using a SilverQuest silver staining kit (Invitrogen Life Technologies).

Protein Purification. The accurate mass and chemical affinity of the protein of interest was established based on the SELDI profile and the ProteinChip type and this information formed the basis for designing the two-dimensional purification process. Whole unfraccionated serum samples from verified overexpressing and underexpressing samples were subjected to affinity and reverse-phase chromatography in parallel. Isolated fractions were then separated by mass through single-dimension SDS-PAGE and silver stained for visualization of protein bands.

Metal Affinity Chromatography. To complement the chip chemistry used for SELDI profiling (IMAC3-Cu2+), whole serum was subjected to immobilized metal affinity capture (IMAC) HyperCel filtration (Ciphergen Biosystems, Inc., Fremont, CA) as per the manufacturer’s instructions on a Biomek 2000 Automated Laboratory Workstation (Beckman Coulter, Inc., Fullerton, CA). Briefly, IMAC resin was regenerated with high-performance liquid chromatography (HPLC) water for 30 minutes. After washing the resin thoroughly with water, the resin was charged with 0.1 mol/L CuSO4 for 30 minutes. Resin was then washed thoroughly with HPLC water followed by PBS. Twenty microliters of serum were processed in urea as described previously (14) and incubated with the resin for 40 minutes. After collecting the unbound proteins in the flow through and washing the resin thoroughly with PBS, bound proteins were eluted off the resin in buffer(s) containing 50 to 250 mmol/L imidazole in PBS. Fractions were analyzed using one-dimensional SDS-PAGE and IMAC3-Cu2+ ProteinChips.

Reverse-Phase Chromatography. An Agilent 1100 series HPLC system (Agilent Technologies, Inc., Palo Alto, CA) was used for all chromatographic steps. Serum (20 μL) was processed in a buffer containing urea (final volume, 0.75 mL) and then diluted 1:1 in buffer A (0.5% acetonitrile and 0.1% trifluoroacetic acid); 0.7 mL was loaded onto a Zorbax Eclipse XDB C-8 column (150 × 4.6 mm) packed with rapid resolution 3.5 μm C-8 beads (Agilent Technologies) maintained at 25°C at a flow rate of 1 mL/min. Proteins were eluted in a 60 minutes, 0% to 85% linear gradient of buffer B (100% acetonitrile and 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min; 0.5 mL fractions were collected in an automated time based mode. Effluent was monitored at 210, 214, and 280 nm. The column was thoroughly washed in buffer B and then reequilibrated in buffer A between successive runs. Statistical processing and reporting of the data used ChemStation for LC 3D (Rev. A.09.01; Agilent Technologies). Eluted fractions were dried and subjected to one-dimensional SDS-PAGE.

SDS-PAGE. Individual fractions collected by chromatography were separated using the NuPAGE 4% to 12% Bis-Tris gels electrophoresed with the XCell SureLock mini-vertical gel electrophoresis system (Invitrogen Life Technologies, Carlsbad, CA). All the samples were processed based on manufacturer’s instructions. All the gels were stained using a SilverQuest silver staining kit (Invitrogen Life Technologies).

Liquid Chromatography Mass Spectrometry Analysis. Protein bands were excised from one-dimensional polyacrylamide gels. Gel slices were cut into 1 to 2 mm cubes; washed thrice with 500 μL Ultrapure water and incubated in 100% acetonitrile for 45 minutes. If the gel was silver stained, the stain was first removed with SilverQuest destaining solution following manufacturer’s directions. The material was dried in a speed-vac, rehydrated in a 12.5 ng/μL modified sequencing grade trypsin solution (Promega, Madison, WI) and incubated in an ice bath for 40 to 45 minutes. The excess trypsin solution was then removed and replaced with 40 to 50 μL of 50 mmol/L ammonium bicarbonate (pH 8.0) and the mixture was incubated overnight at 37°C. Peptides were extracted twice with 25 μL.
50% acetonitrile, 5% formic acid and dried in a speed-vac. Digests were resuspended in 20 μL. Buffer A (5% acetonitrile, 0.1% formic acid, and 0.005% heptafluorobutyric acid) and 3 to 6 μL were loaded onto a 12 cm × 0.075 mm fused silica capillary column packed with 5 μmol/L diameter C-18 beads (The Nest Group, Southboro, MA) using a N2 pressure vessel at 1,100 p.s.i. Peptides were eluted over 55 minutes, by applying a 0% to 80% linear gradient of Buffer B (95% acetonitrile, 0.1% formic acid, and 0.005% HFBA) at a flow rate of 130 μL/min with a precolumn flow splitter resulting in a final flow rate of ~200 nL/min directly into the source. The Finnigan LCQ Deca XP (ThermoFinnigan, San Jose, CA) was run in an automated collection mode with an instrument method composed of a single segment and four data-dependent scan events with a full MS scan followed by three MS/MS scans of the highest intensity ions. Normalized collision energy was set at 30, activation Q was 0.250 with minimum full scan signal intensity at 5 × 10^5 and a minimum MS^2 intensity at 1 × 10^4. Dynamic exclusion was turned on using a 3-minute repeat count of 2 with the mass width set at 1.50 Da. Sequence analysis was done with SEQUEST (TurboSequest obtained from ThermoFinnigan) using an indexed human subset database of the nonredundant protein database from National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).

**Western Blotting.** The samples were analyzed by SDS-PAGE and electrotransferred onto nitrocellulose membrane at 100 V for 1 hour at 4°C in transfer buffer [30 mmol/L Tris, 150 mmol/L Glycine (pH 8.0) + 20% methanol]. Nonspecific sites were blocked by 5% nonfat milk in TBS for 1 hour. The membrane was then incubated with either polyclonal goat anti-apolipoprotein A-II (ApoA-II) antibodies (Rockland Immunocytencies, Inc., Gilbertsville, PA) or mouse monoclonal anti-ApoA-II antibodies (Biodesign International, Saco, ME) in the blocker (1:3,000) followed by incubation with 1:5,000 dilution of anti-goat immunoglobulin G (IgG) horseradish peroxidase conjugate (Rockland Immunocytencies) or anti-mouse IgG horseradish peroxidase conjugate (Biodesign International), respectively, in TBST (0.1% Tween 20 in TBS). The blot was washed thoroughly with TBST (0.1% Tween 20 in TBS) between successive incubations. Immunoreactive protein bands were detected using an Enhanced Chemiluminescence Western blotting kit (Amersham Biosciences Co., Piscataway, NJ). Blots overlaid with detection reagents were exposed to the Enhanced Chemiluminescence Hyperfilm for 1 to 30 seconds and then developed.

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were removed from the serum using protein A-agarose beads and saved. Immunodepleted serum was analyzed by SDS-PAGE, Western blotting, and SELDI.

**SELDI-Immuoassay.** Protein G–coated PS20 Protein-Chips were incubated with 0.25 mg/mL mouse monoclonal anti-ApoA-II antibodies (Biodisgn International) for 2 hours at 25°C. Anti-PSA (Biodisgn International) was used as a control. Fifty microliters of each serum sample were diluted 1:1 in 0.5% Triton X-100 in PBS and incubated on the chips overnight at 4°C. Chips were washed in PBS buffers containing 1% to 0.1% Triton X-100 between successive incubations. For a quantitative immunoassay, 0 to 1,000 μg/mL of ApoA-II purified from human plasma (Biodisgn International) was added to the chips in place of serum to make a titration curve.

**Immunohistochemistry.** The method used for performing immunohistochemistry has been described extensively elsewhere (30, 31). The conditions of the secondary detection method were kept constant, and then the concentrations and conditions of incubation of the primary antibodies were varied. Optimal concentrations were also tested with various antigen recovery techniques. A set of dilutions were done using either the polyclonal (Rockland Immunochromicals) or the monoclonal antibodies (Biodisgn International) to ApoA-II. These dilutions indicated that the optimal dilution of the polyclonal antibody was 1 to 7,000 and for the monoclonal antibody 1:2,000. Citric acid (pH 6.0) with boiling in a pressure cooker for 5 minutes was elected to use as the antigen recovery method. The immunohistochemical approach is briefly described as follows: 5-μm sections were cut from formalin fixed paraffin embedded tissues. The tissue sections were attached to slides by heating at 60 degrees for 2 hours, deparaffinized, and rehydrated with three baths of xylene followed by graded alcohols to 70% and then Tris buffer. The slides were then boiled in a pressure cooker for 5 minutes in coplin jars filled with 0.01 mol/L citric acid (pH 6.0) for antigen retrieval. Endogenous peroxidases were quenched with five minutes of 3% peroxide.

The protein block and the secondary antibody were specific for the primary antibody used. Anti-apoipoprotein A-II from Biodisgn is a mouse monoclonal, thus 3% goat serum was used for 20 minutes as a protein block, the primary antibody at a dilution of 1:2,000 was incubated for one hour and biotinylated goat anti-mouse from Richard-Allan Scientific (Kalamazoo, MI) was used as the secondary detection system. Anti-apoipoprotein A-II from Rockland Immunochromicals was a goat polyclonal and therefore, 3% horse serum was used for 20 minutes as a protein block. After 1 hour incubation with the primary polyclonal antibody at a dilution of 1:7,000, a biotinylated mouse anti-goat (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:1,000 was applied as secondary antibody for 20 minutes. Streptavidin peroxidase (Richard-Allan Scientific) incubated for 20 minutes was used as the label for both antibodies. The 3,3′-diaminobenzidine chromagen was from a BioGenex kit (BioGenex, San Ramon, CA). The sections were lightly counter stained with hematoxylin, dehydrated through graded alcohols to xylene and the coverslips mounted with Permount.

**RESULTS**

**A Peak at 8.943 m/z Has Significant Cross-Study Predictive Power.** We examined data from our previously published protein expression profiling analysis of serum for the detection of prostate cancer (13, 14) and a more recent study involving a separate population of 186 prostate cancer, 142 BPH, and 219 normal samples using the same methods.5 We found several SELDI-TOF MS peaks that retained discriminatory value in each of the two data sets. One of these peaks was a ~8.9K m/z peak with highly significant P value in both populations. The 8.9K m/z peak was one of the 24 diagnostic SELDI peaks (13, 14) that displayed a differential expression in the diseased versus healthy state (Fig. 1). The intensity values for the 8.9K m/z peak were similar in the two studies suggesting that the corresponding protein is consistently overexpressed in serum from individuals with disease of the prostate. Overall, the peak was most intense in the prostatic hyperplasia (BPH) and prostate cancer. Although the greatest discriminatory value was between normal and diseased, there was some separation using the 8.9K m/z peak between BPH and prostate cancer (Table 1). The peak retained a significant area under the curve (>|0.62) for most of the paired tests and performed comparably in the two independent serum studies that were run 2 years apart indicating its consistently high significance in separating the diseased state from normals.

**Isolation and Purification of a Protein that Corresonds to the Observed 8.9K m/z Peak.** To identify the protein that gives rise to the 8.9K m/z peak from prostate cancer specimens, whole sera were fractionated using IMAC-Cu²⁺ affinity fast protein liquid chromatography or C-8 hydrophobic HPLC in parallel (Fig. 2). We chose the two purification schemes and processed multiple sample pairs as separate mutually confirming approaches. Pairs of normal and control sera were either fractionated on IMAC HyperCel resin or C-8 reverse-phase HPLC (see MATERIALS AND METHODS) and the resulting fractions analyzed by SELDI-TOF MS to identify the fraction(s)

Table 1  Prostate serum study analysis of 8.9K m/z SELDI protein peak

<table>
<thead>
<tr>
<th>Group</th>
<th>P</th>
<th>Area under the curve</th>
<th>*Positive group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Study (year 2000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal versus (BPH + prostate cancer)</td>
<td>&lt;0.000000001</td>
<td>0.7820861678</td>
<td>BPH + prostate cancer</td>
</tr>
<tr>
<td>BPH versus prostate cancer</td>
<td>0.0000341830</td>
<td>0.6561108552</td>
<td>BPH</td>
</tr>
<tr>
<td>Serum Study (year 2002)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal versus (BPH + prostate cancer)</td>
<td>&lt;0.000000001</td>
<td>0.8023516455</td>
<td>BPH + prostate cancer</td>
</tr>
<tr>
<td>BPH versus prostate cancer</td>
<td>0.0002874212</td>
<td>0.6337090389</td>
<td>BPH</td>
</tr>
</tbody>
</table>

*Group with the 8.9K m/z intensity value greater than or equal to the cutoff value is considered the “positive group” to compute the true positive ratio (sensitivity) and the false positive ratio (1-specificity) for each cutoff value of the peak.

5 Unpublished data.
containing the 8.9K m/z peak. The selected fractions were then subjected to SDS-PAGE and silver staining to visualize the proteins. In each case, a protein band, with an apparent molecular weight between 6 and 10 kDa on the SDS-PAGE, was observed to be overexpressed in the prostate cancer samples and was excised from the gels for analysis.

**Identification of Apolipoprotein A-II as the Protein Corresponding to the Observed 8.9K m/z Peak.** The differentially expressed protein bands isolated from each of the SDS-PAGE gels were subjected to tandem mass spectrometry as described. Three major tryptic fragments were obtained of m/z 1,200.9 (VKSEPQLAEAK), 1,157.2 (SKEQLTPLIK), and 972.6 (SPELQAEAK) with two of them overlapping in the same region. A SEQUEST search of the peptides in the indexed human subset of the nonredundant protein database from National Center for Biotechnology Information identified the excised protein as apolipoprotein A-II (ApoA-II).

![Fig. 2](image)

**Verification that Serum Apolipoprotein A-II Gives Rise to the Observed 8.9K m/z Peak.** Although the identification of ApoA-II as the overexpressed protein that consistently arose out of the isolation process was associated with high confidence, we needed to confirm that serum ApoA-II comprises the 8.9K m/z peak observed in SELDI-TOF MS. To achieve this assurance, we first verified that a polyclonal antibody specific to ApoA-II recognizes the differentially expressed band by Western analysis and each of the peptide ions observed is shown (see Supplementary Fig. 1). The expected tryptic fragments of the apolipoprotein sequence (Id) suggest these peptides as the major feasible ions to be captured by the tandem mass spectrometer (range 400-2,000 m/z). It is important to note that the same peptide ions and resulting protein identification was achieved for each of the parallel purification schemes. The same protein purification and identification procedures were repeated on multiple normal and cancer serum samples, and each time the excised protein band was identified as ApoA-II.
of the serum fractions derived from the isolation/purification steps. All of the fractions examined showed a differential expression of ApoA-II that coincided with the differential expression of a prominent silver stained band at the same molecular weight. In Fig. 3, we show an example of this comparison using a fraction from HPLC separation of a paired prostate cancer and healthy male (normal). Thus, we showed that the polyclonal anti-ApoA-II antibodies recognize a serum protein that is overexpressed in prostate cancer and migrates at the same Rf as the originally targeted protein.

Because we had verified that polyclonal anti-ApoA-II antibodies were effective at recognizing a protein of the expected size, we were able to use this reagent to selectively immunodeplete ApoA-II from serum. This result was also confirmed using mouse monoclonal antibodies against ApoA-II (Fig. 4). The selective removal of ApoA-II from serum should result in a loss of the 8.9K m/z peak in the SELDI-TOF MS spectra of prostate cancer serum. The immunodepletion studies were conducted using three separate sets of samples consisting of "precleared" sera from normal, BPH, and prostate cancer as described in MATERIALS AND METHODS. In each case, we compared the intensity of the 8.9K m/z peak in the crude serum, precleared serum, ApoA-II immunodepleted serum, anti-ApoA-II-bound, and IgG-bound proteins. In all cases, the immunodepletion significantly reduced the 8.9K m/z peak 5-fold. Immunodepletion with a control IgG antibody did not result in any reduction of the 8.9K m/z peak. We further examined the same fractions by separation on SDS PAGE and immunoblotting with monoclonal anti-ApoA-II antibodies (Fig. 4C). In this analysis, the steady-state levels of serum ApoA-II, as identified by anti-ApoA-II, in each of the steps of the immunodepletion were consistent with the relative amounts of the observed 8.9K m/z peaks. Furthermore, the depleted protein bands migrated with purified ApoA-II on SDS-PAGE. The combined analysis using immunodepletion and Western blotting confirmed that the 8.9K m/z peak observed with SELDI-TOF MS is most likely a form of ApoA-II.

Apolipoprotein A-II Is Expressed in Prostate Cancer, Prostatic Intraepithelial Neoplasia, and Prostate Basal Cells. Immunohistochemistry using either the monoclonal antibody or the polyclonal antibody against ApoA-II showed the same general patterns of staining. Prostatic adenocarcinoma stained stronger than other benign elements in the prostate. The pattern of staining of cancer cells was variable, primarily cytoplasmic and membranous and with rare nuclear staining (Fig. 5). Staining of the cancer cells was accentuated on the advancing edge of the tumor. Histologically normal appearing prostate glands showed...
little staining of luminal cells, whereas basal cells were stained with minimal to moderate levels. The luminal and basal cells of both low-grade and high-grade prostatic intraepithelial neoplasia also stained variably. Fluid in endothelial and interstitial spaces also stained variably.

Serum Levels of an Isoform of Apolipoprotein A-II Are Specifically Overexpressed in Prostate Cancer. One of the outcomes of the immunodepletion study was the determination that the anti-ApoA-II antibodies were efficient at the immunocapture of ApoA-II from serum. Thus, using this antibody, we developed a SELDI-based immunoassay for detection of serum ApoA-II. We incubated whole serum that was prepared for SELDI-TOF MS analysis on PS20 ProteinChip coated with anti-ApoA-II antibodies as described in MATERIALS AND METHODS. In an initial analysis of the 8.9K m/z peak in triplicate samples of normal, BPH, prostate cancer, the relative intensities of the 8.9K m/z peak as detected in the SELDI-based immunoassay were comparable to the relative intensities observed for the same samples examined by standard SELDI-TOF MS (see Supplementary Fig. 2).

We next established a titration curve for the 8.9K m/z peak value using purified ApoA-II analyzed in the SELDI-based immunoassay platform. Using the values obtained in the titration of purified ApoA-II, we then screened a group of 50 normal, 51 BPH, and 48 prostate cancer serum samples that were part of our earlier serum profiling studies. The resulting relative amounts of ApoA-II in serum as determined using the immunoassay was comparable to the relative differences observed using the SELDI-TOF MS 8.9K m/z trace (Fig. 6). Thus, the immunoassay reveals that the SELDI-TOF MS profile of the 8.9K m/z peak accurately represents the actual concentration of ApoA-II in serum. With this technique, we have also shown a successful immunoassay approach for detection of ApoA-II in serum.

During the establishment of the titration curve, we noted that the ApoA-II purified from human plasma (Biodesign International) consists of three major isoforms based upon mass differences (Fig. 6A). Although, the reported mass for ApoA-II is 8.7 kDa (32, 33), we observed that, in the purified mixture, the 8.7K m/z peak is dominated by an 8.8K m/z peak. We also report that the 8.9K m/z peak comprises a third form of ApoA-II, also seen in the purified protein. We extended the immunoassay analysis to include cancer and control serum samples from head and neck squamous cell carcinoma, colon, and lung. In these samples, the 8.9K m/z peak was not overexpressed either in the immunoassay or in the standard SELDI-TOF MS analyses (Fig. 7). The values for head and neck squamous cell carcinoma were elevated but still significantly lower than those for either prostate cancer or BPH. Thus, the 8.9-kDa isoform of ApoA-II is specific for prostate cancer when compared with other common

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Fig. 5 A, prostate cancer infiltrating under uninvolved prostate glands (double-headed arrow) which shows minimal staining even in basal cells. The pattern of staining, which is cytoplasmic, membranous and nuclear in cancer, is accentuated slightly on the advancing edge and is variable (weak to strong). Inset, prostate cancer demonstrating scattered nuclear (single-headed arrows) staining. B, weak to strong staining in the luminal cells of prostatic intraepithelial neoplasia and prostate cancer (single-headed arrows) present above uninvolved prostate glands with little staining (double-headed arrow). C, basal cells of uninvolved prostate glands demonstrating moderate staining (arrows). Endothelial spaces also show some staining. D, peri-neural prostate cancer with membranous and cytoplasmic staining (arrows). Original magnification, ×400 (A and B) and ×600 (C and D).
cancer groups. Interestingly, when we examined the 8.7- and 8.8-kDa forms of ApoA-II in these sera, the values detected were extremely low and the correlation with either disease states was lost (Fig. 8). Contrary to the published results of the molecular weight of ApoA-II in serum, we see an 8.9-kDa form as the predominant species in sera of prostate cancer and controls and it is this form that holds a diagnostic potential in prostate cancer.

**Apolipoprotein A-II Is Overexpressed in Serum from Prostate Cancer Cases in which Prostate-Specific Antigen Fails to Detect.** The utility of a biomarker such as ApoA-II should be examined in relationship to PSA. In the present analysis, ApoA-II would likely present as a biomarker with a relatively low specificity with respect to the combined noncancer group. The difficulty in distinguishing prostate cancer from benign disease is also the case with PSA and thus ApoA-II might be expected to have similar false-positive rates. However, we also examined the ability of ApoA-II to discriminate cancer from controls when the PSA values are <4.0 ng/mL. In this range, the ApoA-II may prove a useful biomarker for contributing to the PSA test by complementing this marker in the range where PSA fails to detect cancer. Using 4.0 ng/mL as a clinical cutoff value, we analyzed the intensity of the 8.9K m/z peak in 40 cancers and 154 controls (see Supplementary Fig. 3). When the distribution of intensity of the 8.9K m/z peak was compared in these normal and prostate cancer samples (PSA, 0-4 ng/mL), most of the normal samples had an average intensity between 0 and 20, whereas a substantial proportion of the prostate cancer samples had an average intensity >20 (Fig. 9). Thus, the relative intensity value of the 8.9K m/z peak was a strong discriminator even when PSA failed to detect cancer.

**DISCUSSION**

The development of multiparametric diagnostic assays (e.g., those that measure multiple proteins) holds tremendous promise for fulfilling clinical demands for accurate molecular analyses in modern medicine. The incorporation of expression differences of serum proteins into such a diagnostic platform may prove to be an important variable in the realization of difficult diagnostic objectives. The identification of the individual differentially expressed proteins that comprise the diagnostic expression profile is essential to facilitating real progress in the development of a robust accurate diagnostic platform, because classic measurements of serum levels of proteins that comprise the profiles will help to stabilize/normalize the profile from patient to patient. In addition, if the proteins are identified and specific high affinity antibodies are generated to them, then more direct and potentially less expensive methods for analysis can be developed.

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**Fig. 6** Detection of ApoA-II levels in serum. A group of 50 NO (healthy), 51 BPH (benign), and 48 PCa (prostate cancer) samples were screened for ApoA-II levels using the SELDI-based immunoassay on mouse monoclonal antibody anti-ApoA-II-coated PS20 ProteinChips. A, spectra obtained from pure ApoA-II protein on the SELDI immunoassay. The spectra are displayed in an overlayed layout, color coded for each concentration (0-1,000 μg/mL). B, data obtained from pure ApoA-II spectra was used to establish a titration curve. C, 8.9K m/z area observed in the serum samples was used to calculate the serum levels of ApoA-II based on the titration curve. About 4-fold increase in the protein amount in PCa samples is observed as compared with NO. D, relative normalized intensity observed in the SELDI-TOF-MS 8.9K m/z trace on IMAC-Cu²⁺ ProteinChips.
In fulfillment of these goals, we have identified an 8.9-kDa isoform of ApoA-II as a component of a protein expression profile that detects prostate disease. ApoA-II is the second most abundant protein in high-density lipoproteins (25% of protein mass) and is primarily synthesized by liver (34). The expression of the protein is regulated both at transcriptional and translational levels. In humans, ApoA-II consists of two identical 77-residue polypeptide chains linked by a disulfide bridge between Cys-6 residues with a monomer mass of 8,707.9 Da (33). When we examined ApoA-II purified from human plasma (Biodesign International), we observed three species separated by mass; specifically, we observed an 8,706.5 \text{ m/z} and 8,808.4 \text{ m/z} and an 8,936.9 \text{ m/z} species with 8,808.4 \text{ m/z} being the predominant form in the purified protein. However, in the sera from healthy, BPH and prostate cancer cases in our study, the predominant species is the 8,936.9 \text{ m/z} isoform and this is the only form specifically able to detect prostate cancer. The differences in the observed mass of the predominant isoform of purified ApoA-II and the discriminatory immunoassay captured serum isoform may be due to degradation of ApoA-II during purification or selective isolation of the smaller species. ApoA-II is initially synthesized as a preprotein composed of 100 amino acid residues, 23 residues longer than the mature protein (32, 35). There is an 18 amino acid signal peptide and a five amino acid prosegment that is cleaved upon secretion of the protein into the medium (36). With respect to the expected mass of serum ApoA-II, several studies have determined that a range of predicted and observed sizes are expected due to alternative oxidation patterns, cysteinylation, sialylation, and the deletion of COOH-terminal glutamine (37–40).

Although ApoA-II is the second most abundant protein in high-density lipoproteins, its function is largely unknown. Both in mice and humans, ApoA-II influences, by an unknown mechanism, plasma levels of free fatty acids (41). In vitro studies have shown that ApoA-II can displace ApoA-I from high-density lipoprotein particles (42), stimulate or inhibit hepatic lipase (43), inhibit lecithin cholesterol acyl transferase (44), and inhibit the actions of cholesterol ester transfer protein (reviewed by ref. 45). The ApoA-II-deficient state has also been associated with decreased levels of plasma free fatty acid, glucose, and insulin suggesting a metabolic disturbance compatible with hypersensitivity to insulin (46). In most studies, a constant steady-state level of ApoA-II is essential for maintaining homeostatic regulation of function. In addition, there is an
increasing number of reports suggesting a relationship between cancer susceptibility and proteins of the lipid metabolic pathway, including apolipoproteins (47–52). For example, members of the apolipoprotein family, such as apolipoprotein D (ApoD), have been shown to be overexpressed in prostate cancer (47, 52). Interestingly, a large amount of ApoD occurs primarily as a disulfide linked heterodimer of ApoA-II in human plasma (53). Although the role that these proteins play in cancer is unknown, there is some evidence that they may be involved in functions of cell proliferation/apoptosis (51, 54). In fact, the staining pattern, which we report here for ApoA-II in normal and tumoral prostatic epithelial cells, is consistent with this suggested role.

The normal prostatic epithelium is composed of two distinct compartments, the basal cell layer and the luminal secretory cell layer. A third compartment of transiently proliferating cells have been recently described (55). The basal cells are the stem cells of the prostatic epithelium that proliferate actively and are refractory to undergo apoptosis after castration. Consistent with this function, they express the nuclear proliferation antigen proliferating cell nuclear antigen, overexpress the apoptosis inhibitor Bcl-2, lack expression of cell cycle inhibitor p27, and lack or express low levels of androgen receptor (55). In contrast, secretory luminal cells are nonproliferating terminally differentiated cells, which strongly express p27, lack expression of proliferating cell nuclear antigen and do not overexpress Bcl-2. In addition to being the source of PSA production, benign secretory cells express high levels of the androgen receptor and undergo apoptosis after castration. Benign prostate hyperplastic glands show a multilayer papillary epithelium with a prominent compartment of transiently proliferating cells in which p27 is down-regulated when compared with luminal cells. The majority of the benign prostatic glands that we evaluated here for ApoA-II expression by immunohistochemistry showed a selective or preferential expression of this protein in basal cells, variable expression pattern in multilayer hyperplastic glands and weak to no expression in secretory luminal cells. This staining pattern correlates with those of cell proliferation markers such as proliferating cell nuclear antigen (51, 54). In fact, the staining pattern, which we report here for ApoA-II in normal and tumoral prostatic epithelial cells, is consistent with this suggested role.

We report that by using a simple value cutoff for ApoA-II, patients with prostate cancer and BPH can be distinguished from healthy men as defined as randomly selected asymptomatic men.
with PSA values < 4.0 ng/mL. Although more studies are needed to evaluate this, it seems as though the separation between patients with prostate cancer versus BPH is marginal. Thus, the expected potential false-positive rate of ApoA-II may not improve the existing capabilities of PSA. However, ApoA-II retains the discrimination between disease and nondisease when PSA levels are <4.0 ng/mL or in other words, in cases of prostate cancer in which PSA would have failed to detect the disease. Thus, the use of ApoA-II in combination with PSA may extend the utility of this test. We are now examining samples that have PSA levels <4.0 ng/mL with no clinical symptoms and a normal prostate volume, to determine if ApoA-II is able to detect prostate cancer in this population. This is especially important in light of the recent results from the Prostate Cancer Prevention Trial showing that a significant number of advanced cancers go undetected in patients with PSA values of <4.0 ng/mL (4).

In addition to a potentially increased range of prostate cancer detection of ApoA-II compared with PSA, our results also address some of the questions being raised with respect to the eventual utility of protein expression profiling. We were able to show that the relative intensities of a m/z peak detected via mass spectrometry were comparable to the actual serum levels when the protein was measured by immunoassay. This helps to address the question of whether the platform can reflect relative quantities of some specific protein in a mixture as complex as serum. Furthermore, we provide evidence that there are m/z peaks that can be linked by conventional approaches to biological disease and show that this protein was consistently detected in two separate studies by both SELDI-TOF MS and immunoassay. In addition, the overexpression of ApoA-II was specific for prostate cancer and increased expression of ApoA-II was not observed in cancers of head and neck, colon, or lung. Thus, the expression pattern of ApoA-II could be specific for prostate disease and ApoA-II is neither a general cancer marker nor an artifact of mass spectrometry.

These results identify a protein peak detected by SELDI-TOF MS that is diagnostic for prostate cancer and BPH. The identified protein is an apparent 8.9-kDa species of the serum protein ApoA-II, and ApoA-II is specifically overexpressed in prostate cancer when compared with cancers of the head and neck, colon, or lung. The correlation was observed using mass spectrometry protein expression profiling as well as mass spectrometry–based immunoassay. We propose that the incorporation of an immune-based assay for ApoA-II in conjunction with protein expression profiling may provide for a more robust assay platform than mass spectrometry alone. In addition, ApoA-II may provide an important contribution to a multiplexed immunoassay or antibody array. Because overexpression of ApoA-II was also observed in patients with prostate cancer having PSA < 4.0 ng, we propose that analysis of ApoA-II in serum may extend the utility of current blood testing for prostate cancer.

REFERENCES


Serum Levels of an Isoform of Apolipoprotein A-II as a Potential Marker for Prostate Cancer


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