Normal, Benign, Preneoplastic, and Malignant Prostate Cells Have Distinct Protein Expression Profiles Resolved by Surface Enhanced Laser Desorption/Ionization Mass Spectrometry

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ABSTRACT

Purpose: The objective of this study was to discover protein biomarkers that differentiate malignant from nonmalignant cell populations, especially early protein alterations that signal the initiation of a developing cancer. We hypothesized that Surface Enhanced Laser Desorption/Ionization-time of flight-mass spectrometry-assisted protein profiling could detect these protein alterations.

Experimental Design: Epithelial cell populations [benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN), and prostate cancer (PCA)] were procured from nine prostatectomy specimens using laser capture microdissection. Surface Enhanced Laser Desorption/Ionization-time of flight-mass spectrometry analysis was performed on cell lysates, and the relative intensity levels of each protein or peptide in the mass spectra was calculated and compared for each cell type.

Results: Several small molecular mass peptides or proteins (3000–5000 Da) were found in greater abundance in PIN and PCA cell lysates. Another peak, with an average mass of 5666 Da, was observed to be up-regulated in 86% of the BPH cell lysates. Higher levels of this same peak were found in only 22% of the PIN lysates and none of the PCA lysates. Expression differences were also found for intracellular levels of prostate-specific antigen, which were reduced in PIN and PCA cells when compared with matched normals. Although no single protein alteration was observed in all PIN/PCA samples, combining two or more of the markers was effective in distinguishing the benign cell types (normal/BPH) from diseased cell types (PIN/PCA). Logistic regression analysis using seven differentially expressed proteins resulted in a predictive equation that correctly distinguished the diseased lysates with a sensitivity and specificity of 93.3 and 93.8%, respectively.

Conclusions: We have shown that the protein profiles from prostate cells with different disease states have discriminating differences. These differentially regulated proteins are potential markers for early detection and/or risk factors for development of prostate cancer. Studies are under way to identify these protein/peptides, with the goal of developing a diagnostic test for the early detection of prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer and, second to lung cancer, causes the greatest number of cancer deaths in American males (1). The PSA3 serum test has contributed to earlier detection, however, 65–75% of moderately elevated PSA levels are attributed to BPH, often resulting in unnecessary biopsies (2). Several approaches have been undertaken to improve the PSA test such as measuring PSA velocity (3), PSA density (4), and assessing ratios between free, complexed, and total PSA serum values with various degrees of success (5). Combinations of markers such as free PSA, IGF-I, and IGFBP-3 have resulted in improved diagnostic discrimination between BPH and prostate cancer (6). It is becoming increasingly clear that because of the inherent molecular heterogeneity and multifocal nature of prostate cancer (7), additional improvement in early detection, diagnosis, and prognosis will likely require the measurement of a panel of biomarkers.

The proteome is the full complement of proteins that regulate the physiological and pathophysiological phenotype of a cell. Because proteins initiate all cell functions and pathways, identifying differentially expressed proteins between normal and pathological states can lead to a better understanding of the

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3 The abbreviations used are: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; IGF, insulin-like growth factor; LCM, laser capture microdissection; MS, mass spectrometry; TOF, time of flight; SELDI, Surface Enhanced Laser Desorption/Ionization; PIN, prostate intraepithelial neoplasia, PCA, prostate cancer; IMAC, Immobilized Metal Affinity Capture; N, normal.
cellular mechanisms involved in disease. Some proteins are
down-regulated and others are up-regulated with the onset of
disease, depending on a protein’s specific function, whereas
others undergo disease-specific posttranslational modifications
(8–10). The identification of changes in protein expression and
modification that occur in the early stages of a developing
cancer could lead to the discovery of protein biomarkers
and novel strategies for the improvement of early detection,
diagnosis, and therapy of cancer. Therefore, examining the
proteome of a cell holds great potential for the discovery of new
biomarkers.

As a result of the microheterogeneity of organ-based can-
cers, studies of pure cell populations are required to achieve
precision in the search for disease-associated biomarkers. LCM
microscopes have been used successfully for the procurement of
pure populations of cells for genetic analysis (11, 12), protein
expression changes in cancer cells using two-dimensional elec-
trophoresis (13), and MS (14–16). Advances in MS have lead to
the evolution of several proteomic applications: from the map-
ping of peptide digests of proteins isolated from two-dimen-
sional electrophoresis to direct and rapid proteome profiling of
cells and body fluids. For example, matrix-assisted desorption
ionization-TOF-MS has been used to look for protein changes in
breast cancer cell lines (17) and in the serum of cutaneous melanoma patients (18). In addition, tandem MS systems are
capable of extracting peptide sequence information for sequence
tagging and protein identification (19). Another innovative MS
technology, SELDI, has been used to compare the patterns of protein expression in two physiological states of Yersinia pestis
(20) and in the profiling of amyloid β peptide variants (21). Our
laboratory has successfully applied SELDI to the identification
of specific protein changes in the urine of bladder cancer pa-
cients (22) and the detection of prostate cancer-associated bi-
omarkers, PSA, prostate-specific membrane antigen, prostate
acid phosphatase, and prostate secretory protein in cell lysates,
sperm, and seminal plasma (16). Using the various affinity
surfaces of Proteinchip arrays, SELDI can reduce complex protein mixtures to sets of proteins with common properties.

The advantage of the SELDI protein profiling method is the ability to simultaneously detect multiple protein changes with a
high degree of sensitivity (pmol to amol; Ref. 23) in a rapid high
throughput process. Clear spectra are obtained with predomi-
nately singly charged ions and mass deviations of <0.02% for
internally calibrated spectra (24, 25). This precision makes it
possible to delineate very small proteins and peptides, as well as
differential posttranslation modifications such as phosphoryla-
tion and glycosylation (26). Recently, SELDI protein profiling
has been shown to provide reproducible and specific protein
patterns of LCM cell lysates harvested from different cancer
types and grades (15, 27).

This report describes the combinatorial use of LCM and
SELDI technologies to detect protein differences in cell lysates of
pure populations of normal, benign (BPH), premalignant
(PIN), and malignant prostate (PCA) cells. The objectives of
this study were to discover potential biomarkers that could be
used to differentiate malignant from the nonmalignant cell pop-
ulations, especially early protein alterations that signal the ini-
tiation of a developing cancer. The latter would be especially
useful as potential markers for early detection and/or as risk

factors for development of prostate cancer. Differential expres-
sion of several individual protein peaks was observed for BPH,
PIN, and PCA epithelial cells with respect to the expression
levels found in matched normal epithelial cells. Combinations
of these signature or differentially regulated proteins/peptides
could distinguish PCA and PIN from normal and BPH. How-
ever, in most cases, it was difficult to differentiate PCA from
high-grade PIN. Thus, these protein alterations could represent
early signals of a developing malignant lesion and may be useful
as markers of early detection.

MATERIALS AND METHODS

Patient Specimens.

Prostate tissues were procured from consenting patients undergoing radical prostatectomy. The age of the patients ranged from 44 to 68 years and consisted of five
Caucasians and four African Americans. The tissues were pro-
cessed immediately and stored in the Virginia Prostate Center’s
Bio-repository. Tissue pieces harvested for LCM were immedi-
ately embedded in optimal cutting temperature compound and
stored at −80°C. One cryosection was H&E stained and exam-
ined by a pathologist to identify cells of interest for microdis-
section. Mirrored-stained sections, fixed in formalin and paraf-
fin embedded, were also used to further aid in the identification
of specific cell types. Additional serial frozen sections at 8 μm
were used for microdissection.

LCM. Pure populations of normal luminal epithelia,
BPH, PIN, and PCA epithelial cells were microdissected from
frozen tissue sections using the PixCell II Laser Capture Micro-
dissection Microscope (Arcturus Engineering, Inc., Mountain
View, CA) essentially as described by Emmert-Buck et al. (28).
The procedure for staining frozen sections for LCM was fol-
lowed with slight modifications: the hematoxylin step was omit-
ted and protease inhibitors (Complete; Roche Biomedical Indi-
apolis, IN) were added to the staining baths. A total of 1000
laser shots totaling 3000–6000 cells was procured for each cell
type. Matched benign and diseased epithelial cell types were
harvested from each prostate sample. In some cases, stroma
cells were also procured from the same section directly adjacent
to the cells of interest. Each cell population was estimated to be
>98% homogeneous based on careful examination of captured
cells by the pathologist. Samples were standardized by total
number of laser shots, and duplicate samples were captured from
the same areas of each serial section to check reproduc-

Cell Lysates and SELDI Proteinchip Array Binding.

Cell lysates were immediately prepared after microdissection by
adding 4 μl of a lysis buffer containing 20 mM HEPES (pH 8.0)
with 1% Triton X-100 directly on the LCM cap. Each lysate was
diluted 1:10 in PBS buffer, giving a total volume of 40 μl.
Lysates were vortexed for 10 min at 4°C and centrifuged briefly
to remove cellular debris. The supernatant was added to an
IMAC3 Proteinchip Array (Ciphergen Biosystems, Inc., Fre-
mont, CA), pretreated with 100 mM CuSO4, following the manu-
facturer’s instructions. This surface was chosen because it
produced the most robust spectra of the LCM lysates and for its
ability to bind phosphorylated proteins. A bioprocessor (Ciphe-
gen Biosystems, Inc.) was fitted on top of the chip arrays to
permit the addition of the 40-μl sample. To control for variation,
cell lysates harvested from each prostate tissue were analyzed on a single biochip. The array was then incubated with the diluted lysate overnight at room temperature on an orbital shaker. After removal of the lysate, each spot was washed twice with PBS, followed by a final water rinse.

**SELDI Analysis.** The arrays were allowed to air dry, and a saturated solution of sinapinic acid (Ciphergen Biosystems, Inc.) in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was added to each spot. TOF mass spectra were generated in a Ciphergen Protein Biology System II by averaging 120 laser shots collected in the positive mode at laser settings of 225 and 280. Data were calibrated externally using purified peptide and protein standards.

**Protein Profile Evaluation and Peak Expression Scoring.** Spectra were analyzed with the Ciphergen Peaks 2.1 software and relative abundance for each peak were calculated as follows. The relative abundance of the proteins was subdivided into three classes: low (+), 1–30% of spectral scale; medium (+ +), 31–60%; and high (+ + +), 60–100% and analyzed for each matched set of cell types. Numerical values were then assigned to the abundance levels (i.e., (+), 33; (+ +), 66; and (+ + +), 100) and averaged for each cell type to represent the protein expression between prostate cell types in graphical form.

**Statistical Analysis.** Sensitivity is defined as the percentage of diseased (BPH/PIN/PCA) cell types for which the biomarker of interest is present (true positive/total number of diseased lysates × 100). Specificity is defined as the percentage of cell types for which the biomarker of interest is not positive (true negative/total number of lysates without disease × 100). The statistical significance of the differences in peak expression scores between all possible pairs among the four cell types was calculated using the Wilcoxon signed rank test. A logistic regression analysis was then performed using the most significant differentially expressed proteins.

**RESULTS**

**Sample Harvesting and SELDI Profile Evaluation.** Pure populations of organ-matched benign (normal or BPH), PIN (high grade), and PCA epithelial cells were obtained from nine prostatectomy specimens examined. In four of the prostate specimens, all four cell types were identified and harvested, and PIN cells were obtained for all nine prostate tissues. The total number of cell types for each group was as follows: eight normal, seven BPH, nine PIN, and seven PCA. An average of 5000 cells was microdissected in duplicate for each cell type, resulting in 62 cell lysates analyzed by SELDI-TOF-MS. In two samples, we were able to microdissect two different foci of PIN and PCA. Additionally, adjacent stroma cells were microdissected from a selected subset of tissues to compare with the epithelial cell profiles.

**Visual Analysis of SELDI Data Revealed Differential Protein Profiles.** Processing the lysates on an immobilized metal affinity capture surface pretreated with CuSO4 resolved between 50 and 90 protein or peptide peaks in the mass range of 3 to 100 kDa. Fig. 1 is a representative spectrum of the protein profile of a PCA cell lysate. The advantages of the SELDI technology over two-dimensional electrophoresis in resolving molecular mass protein or peptide species below m/z 10,000 (10 kDa) is evident. The protein profiles of each set of matched lysates were compared visually to identify differences. Expression profiling of the samples revealed several protein pattern differences, indicating up- and down-regulation or possible altered protein processing between the prostate cell types. Fig. 2A is the SELDI spectra and gel-view, showing a differentially expressed group of proteins between 4000–6000 Da in epithelial cells obtained from the same prostate tissue specimen. Three peaks (4030, 4358, and 4753 Da) are present or up-regulated in the PIN and PCA lysates. Fig. 2B shows multiple SELDI gel-view of matched cell types obtained from three different prostatectomy specimens exhibiting increased expression of a peak at an average mass of 4749 Da in the PIN and PCA samples. Duplicate samples exhibited a high degree of reproducibility. In contrast, regions of heterogeneity were present in the protein profiles derived from two different foci of PCA in patient 2 and two foci of PIN in patient 3. However, overexpression of the 4749 Da protein is still observed. Profile differences were also found in the BPH cell lysates. Fig. 2C is an example of a peak at 5666 Da, which appears to be up-regulated in the BPH epithelial cells when compared with the spectra of the other matched cell types.

**Differential Expression of Intracellular PSA Was Observed between Prostate Cell Types.** To assess the potential of the SELDI technology, initial studies in our laboratory focused on the detection of known prostate cancer markers (16). Prostate-specific membrane antigen, prostate acid phosphatase, prostate secretory protein, and PSA were detected from cell lysates and body fluids. In this study, a peak of average mass 28.4 kDa was detectable in the protein profiles of epithelial cells using the IMAC surface pretreated with CuSO4. This mass is consistent with the molecular mass of free PSA (29) and has been confirmed as such in our laboratory from LCM prostate cells lysates using a SELDI immunoassay (data not shown).

To determine how the expression of intracellular PSA differed between each cell type, the PSA peaks were compared in the protein profiles of organ-matched sets of lysates. Differential PSA expression between the organ-matched cell types was observed. In 5 of 9 of the PIN samples and 4 of 7 of the PCA samples, the PSA peak was reduced when compared with matched normal epithelia. An example of this decrease in intracellular PSA levels can be seen in Fig. 3. A large PSA peak at 28,393 Da was present in the normal prostate epithelia but, as expected, is absent from adjacent stroma cells. The PIN and PCA cells had a greatly reduced PSA peak. Normal epithelial cells procured directly adjacent to the PCA foci, however, exhibited a large amount of intracellular PSA. Serum PSA values for patients donating tissue for this study were between 5.5 and 26.2 ng/ml. Unfortunately, no correlation could be made between the relative intracellular PSA levels in PCA cells observed in the SELDI profiles and serum PSA values.

**Identification of Differentially Expressed Peaks of Diagnostic Value.** A visual comparative analyses of the SELDI profiles suggested that several proteins exhibited differential regulation between the four cell types. However, to more precisely select proteins with high biomarker potential, it was necessary to standardize peak levels throughout the matched populations. The relative abundance of the peaks was subdi-
vided into three classes: low (+), 1–33% of spectral scale; medium (++) , 33–66%; and high (+++), 66–100%. Each individual spectrum was expanded in specific mass ranges to assist in determining the relative levels of each observed peak. An in-house computer program was used to cluster the peaks to obtain an average mass for each peak within a mass error range of 0.15%. Of an average of 70 peaks commonly observed, 14 (21%) of these peaks displayed some expression differences (up- or down-regulation or altered processing) between the epithelial cell profiles (see Table 1). Most species in this mass range were present in epithelial cell types only with the exception of peaks at 3448 Da and the 4361 Da peak, which were also present in adjacent stroma cells. The scores in Table 1 were converted to numerical values and plotted in a histogram showing the average differences in expression levels between cell types subtracted from the expression level in normal epithelial (see Fig. 4). Thus, overexpression and underexpression for each peak listed was normalized and evaluated for BPH, PIN, and PCA cells.

**Proteins Overexpressed in BPH, PIN, and PCA Identified from SELDI Profiles.** Generally, the PCA and PIN levels of expression profiles were similar. As seen in Fig. 4, there were higher levels of a group of small molecular mass peptides/proteins between 3000 and 5000 Da in the PIN and PCA profiles. The first of these, 3448 Da, was found at increased levels in the BPH, PIN, and PCA samples. Three peaks (4036, 4361, and 4749 Da) were overexpressed in PIN and PCA with the highest level of abundance in PCA lysates. A summary of

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**Fig. 1** Representative SELDI spectra of one LCM sample from PCA epithelial cells in m/z ratios of 3000–10000, 10000–20000, and 20000–80000.
Fig. 2  A, representative spectra and gel-view of matched prostate epithelial cell lysates (normal BPH, PIN, and PCA) showing protein alterations in the range of 4000–5000 Da (m/z). (Arrows indicate overexpressed peaks in PIN and PCA). *Gray scale display of the raw spectra called gel-view because it looks like a stained one-dimensional electrophoresis gel. B, SELDI gel-view protein profiles of lysates prepared from cells procured from different prostatectomy specimens. The box identifies a peak with an average mass of 4749 Da that appears to be overexpressed in PIN and PCA epithelial cells. Replicate samples(*) show good reproducibility of the protein patterns. The different protein patterns observed for two different PCA foci in patient 2 and the two PIN foci in patient 3 may be the result of genetic heterogeneity. C, representative spectra and gel-views of matched prostate epithelial cell lysates showing increased expression of a peak at m/z of 5666 in the BPH sample. N, normal.
the percentage of each cell type exhibiting those most significantly increased or decreased proteins is presented in Table 2. The peaks at 4036 and 4361 Da were overexpressed in 71% of the PCA lysates and 44% of the PIN samples. Additionally, two peaks (4639 and 2418 Da) were specific to PCA cell lysates but were seen in only 43% of PCA samples tested. The peak at 4749 Da was up-regulated in 67 and 57% of PIN and PCA lysates, respectively. Furthermore, this up-regulation of PSA was significant for N versus PIN and approaches significance for BPH versus PIN. The 5666 Da protein was significantly up-regulated when comparing N versus BPH and, interestingly, is significantly down-regulated in BPH versus PIN and BPH versus PCA. No correlation could be made between age or race of the patients and the appearance of differentially expressed or processed peaks.

Proteins Underexpressed in BPH, PIN, and PCA Profiles. As seen in Fig. 4, a few proteins were underexpressed in BPH, PIN, and PCA cells when compared with the expression levels in normal epithelial cells. Expression of the 5666-Da peak, which was overexpressed in BPH profiles, was reduced in 22% of PIN and 71% of PCA cell lysates at significant levels (see Tables 2 and 3). In addition, decreased expression of an 11,744-Da protein and PSA (28,442 Da) was found in the BPH, PIN, and PCA cell lysates. The reduced expression of intracellular PSA was found in 56 and 57% of PIN and PCA cell lysates, respectively. Furthermore, this underexpression of PSA was significant in N versus PIN and approaches significance (P = 0.066) for N versus PCA (Table 3).

Biomarker Combination of Identified Peaks Improved Prediction of Cell Lysate Disease State. Because no single peptide or protein was discovered to be differentially expressed in all of the PIN or PCA profiles, various combinations of selected proteins were evaluated to identify a panel of markers (expression levels) that could improve disease classification of each specific cell type (Table 4). A biomarker combination was classified as positive if any marker in the combination was present in a sample and negative if none of the markers were detected in a specimen. Because most of these markers were overexpressed in both PCA and PIN, the combinations did not improve the specificity for each of the diseased cell types when evaluated individually.

Better discrimination was achieved by combining the benign cell types (normal, BPH) and comparing them to diseased cell types (PIN or PCA). The sensitivity and specificity of each marker for benign versus disease were then calculated both individually and in additive combinations. By combining markers 1 (p4036) and 3 (p4639), there was an improvement in the sensitivity for PCA from 71 to 86% while maintaining a specificity of 100% for PIN or PCA combined. This combination of markers also identified 44% of the PIN samples. Combining marker 2 (p4361) and marker 3 (p4639) increased the sensitivity for PCA to 100% while the specificity decreased to 87% for PIN and PCA. By combining markers 2 and 4 (p4749), 100% of the PCA and 89% of the PIN samples could be correctly identified with a slight decrease in specificity. Combinations involving three or more of the markers did not improve the overall specificity and sensitivity. The 5666-Da protein (marker 5, Table 4) had a sensitivity of 86% and specificity of 88% for detecting BPH. This marker also provided some discrimination between BPH and PIN lysates having 22% sensitivity and 68% specificity for PIN epithelia.
Table 1  Comparison of the expression levels* of proteins in lysates of LCM-procured prostate cells (epithelial and stromal)

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Molecular mass (Da)</th>
<th>CA3491</th>
<th>CA3495</th>
<th>CA3501</th>
<th>CA3502</th>
<th>CA3512</th>
<th>CA3517</th>
<th>CA3511</th>
<th>CA3513</th>
<th>CA3539</th>
<th>CA3540</th>
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<th>CA3546</th>
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<td>+</td>
<td>+</td>
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<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
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<td>+</td>
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<td>PCA</td>
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<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>CA3495 N*</td>
<td>4,413</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
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* Relative expression levels of proteins: +, 1–33% of SELDI spectral scale; ++, 33–66%; and ++++, 66–100%, 0, peak not detected.

Multivariable analysis of the peak expression data was evaluated to determine whether the simultaneous overexpression or underexpression of several proteins in combination could be used to predict benign versus diseased cell lysates. Logistic regression analysis performed with the seven most significant differentially expressed peaks [4,036, 4,361, 4,413, 4,639, 4,729, 5,666, and 28,422 Da (PSA)]. As seen in Table 5, a predictive equation based on diseased (PIN/PCA) versus benign (normal/BPH) cell lysates resulted in 93.3% specificity and 93.8% sensitivity for PIN or PCA cell lysates. Therefore, these biomarkers in combination may have clinical value, especially if detectable in biopsy or body fluid samples.

DISCUSSION

The development of cancer is a multistep process encompassing multiple events involving oncogenic and tumor suppressor gene products. These events can occur pre- or posttranslationally and will be reflected in differential changes in a myriad of proteins. Analyzing and interpreting the proteomic changes that occur in prostate disease progression is a daunting task made even more difficult by the biological heterogeneity of the disease. Advances in TOF-MS, resulting in the SELDI technology, have provided an approach for the sensitive and direct analysis of proteins in complex biological samples. Previous studies in this and other laboratories have demonstrated the successful application of this technology to profile a few microdissected prostate cell specimens (15, 16, 30). In this study, we combined LCM with SELDI to generate protein profiles from 62 prostate cell lysates derived from nine prostatectomy specimens. Our results demonstrate that the protein profiles of normal prostate epithelia, BPH, PIN, and PCA display discriminating differences.
Protein extracts prepared from prostate cell types were analyzed using an IMAC3 protein biochip pretreated with CuSO₄. On average, 70 protein peaks were detected from the cell lysates using this surface. Overall, there were a relatively large number of common peaks in the benign and diseased epithelial cell profiles and very few peaks that would, based on presence or absence, be candidate biomarkers for the disease progression and/or diagnosis of prostate cancer. It was therefore determined that calculating expression levels of peaks would enhance the significance of the results and identify possible expression differences between the samples. Peak abundance levels were calculated for diseased cell profiles as compared with levels found in matched benign cell types. Of the common 70 peaks observed, 15 (21%) of these peaks displayed dysregulation in the diseased profiles.

Several small molecular mass peptides or proteins (3000–5000 Da) had increased expression levels in the PIN and PCA cell extracts. Although, the clusters of peaks in this range could originate from proteolysis and cleavage products of larger proteins, they nonetheless were consistently detected in common cell types and were considered part of the general profile. The chymotrypsin-like activity of PSA has been shown to facilitate the proteolysis of semenogelin from seminal plasma (31), and IGF-binding protein 3 (32). Such stable cleavage products of proteins may be indicative of changes occurring in the prostate disease cycle. Furthermore, because the IMAC surface can bind phosphorylated peptides or proteins, it is feasible that some of the changes observed are attributable to differential phosphorylation of the proteins in the diseased cell types. Of interest in this study, two peaks were identified with an average mass of 4827 Da.

### Table 2 Percentage of samples (BPH/PIN/PCA) displaying differential expression of selected proteins when compared with matched normal samples

<table>
<thead>
<tr>
<th>Molecular mass (Da)</th>
<th>BPH % of samples</th>
<th>PIN % of samples</th>
<th>PCA % of samples</th>
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<tbody>
<tr>
<td></td>
<td>Overexpression</td>
<td>Underexpression</td>
<td>Overexpression</td>
</tr>
<tr>
<td>3,448</td>
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<td>67</td>
</tr>
<tr>
<td>4,036</td>
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</tr>
<tr>
<td>4,361</td>
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<td>14</td>
<td>44</td>
</tr>
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<td>4,413</td>
<td>43</td>
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<td>33</td>
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<td>4,639</td>
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<td>0</td>
</tr>
<tr>
<td>4,749</td>
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<td>0</td>
<td>67</td>
</tr>
<tr>
<td>5,666</td>
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<td>0</td>
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<tr>
<td>8,445</td>
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<td>0</td>
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</tr>
<tr>
<td>11,744</td>
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<td>43</td>
<td>11</td>
</tr>
<tr>
<td>14,696</td>
<td>14</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>24,184</td>
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<td>28,422</td>
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<tr>
<td>48,308</td>
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</tr>
<tr>
<td>53,830</td>
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<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 4** Expression levels of peptide/proteins compared with the expression levels in matched normal epithelial cells. The average expression in normal cell lysates was subtracted from the average expression levels found in the other cell types. Bars above 0, overexpression; bars below 0, underexpression.
These two peaks may represent a dephosphorylation event occurring in transition from benign to PIN/PCA. The average mass shift between these proteins (78 Da) is close to the calculated mass shift of 79 Da for a phosphorylation event. Prominent examples of aberrant phosphorylation of proteins found in cancer studies include extracellular signal-regulated kinase 1/2 in breast cancer (33) and androgen receptor in prostate cancer (34).

It is also quite possible that these small molecules could be intact functional proteins or peptides, examples of which include prohormones, growth factors, amidated peptides, and defensins. In a recent study by Rocchi et al. (35), PC-3 and Du145 prostate cancer cell lines were found to produce and secrete a multifunctional amidated peptide (adrenomedullin, molecular mass 6 kDa). In the same study, increased levels of adrenomedullin immunostaining were found in PCA epithelia when compared with normal epithelia. The activity of the enzyme peptidylglycine-amidating monooxygenase was also demonstrated in prostate cancer cell lines. This enzyme produces N-amidated bioactive peptides from their inactive glycine-extended precursors. The importance of the role of these small molecular mass proteins may have previously been overlooked as a result of the difficulty in detection using two-dimensional analysis.

Previous studies have shown a cytogenetic link between high-grade PIN and prostate cancer strengthening its role as a precursor lesion (36). In addition, 50% of patients with high-grade PIN present with cancer detected in a subsequent biopsy (37). Therefore, the identification of proteins specifically associated with PIN would have tremendous impact as markers for the early detection of prostate cancer. In our study, PIN and...

<table>
<thead>
<tr>
<th>Peak (Da)</th>
<th>N° vs. BPH</th>
<th>N vs. PIN</th>
<th>N vs. PCA</th>
<th>BPH vs. PIN</th>
<th>BPH vs. PCA</th>
<th>PIN vs. PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,036</td>
<td>0.317</td>
<td>0.083</td>
<td>0.059</td>
<td>0.025(^a)</td>
<td>0.039(^a)</td>
<td>0.102</td>
</tr>
<tr>
<td>4,361</td>
<td>0.564</td>
<td>0.059</td>
<td>0.038(^b)</td>
<td>0.046(^b)</td>
<td>0.102</td>
<td>0.083</td>
</tr>
<tr>
<td>4,413</td>
<td>0.083</td>
<td>0.157</td>
<td>0.317</td>
<td>0.655</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td>4,639</td>
<td>1.000</td>
<td>1.000</td>
<td>0.083</td>
<td>1.000</td>
<td>0.157</td>
<td>0.083</td>
</tr>
<tr>
<td>4,749</td>
<td>0.317</td>
<td>0.034(^b)</td>
<td>0.102</td>
<td>0.059</td>
<td>0.109</td>
<td>0.564</td>
</tr>
<tr>
<td>5,666</td>
<td>0.038(^b)</td>
<td>1.000</td>
<td>0.059</td>
<td>0.024(^a)</td>
<td>0.039(^a)</td>
<td>0.408</td>
</tr>
<tr>
<td>28,422 (PSA)</td>
<td>0.083</td>
<td>0.041(^c)</td>
<td>0.066</td>
<td>0.680</td>
<td>0.180</td>
<td>0.564</td>
</tr>
</tbody>
</table>

\(^a\) N, normal.
\(^b\) Significant \(P_s(\leq0.05)\) based on overexpression as compared with N or BPH.
\(^c\) Significant \(P_s(\leq0.05)\) based on underexpression as compared with N or BPH.

### Table 4

Sensitivity and specificity of single and combinations of markers for the detection of diseased cells

<table>
<thead>
<tr>
<th>(Marker) expression</th>
<th>Sensitivity</th>
<th>Specitivity</th>
<th>Sensitivity/Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA</td>
<td>PIN</td>
<td>PCA</td>
</tr>
<tr>
<td>(1) increase of m/z 4036</td>
<td>71%</td>
<td>44%</td>
<td>83%</td>
</tr>
<tr>
<td>(2) increase of m/z of 4361</td>
<td>71%</td>
<td>44%</td>
<td>75%</td>
</tr>
<tr>
<td>(3) presence of m/z 4639</td>
<td>43%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>(4) increase of m/z of 4749</td>
<td>57%</td>
<td>67%</td>
<td>71%</td>
</tr>
</tbody>
</table>

Combination

| 1 + 2 | 86% | 67% | 67% | 64% | 75%/87% |
| 1 + 3 | 86% | 44% | 83% | 77% | 63%/100% |
| 1 + 4 | 86% | 78% | 67% | 68% | 81%/94% |
| 2 + 4 | 100% | 89% | 54% | 55% | 94%/80% |
| 3 + 4 | 86% | 67% | 71% | 68% | 75%/94% |
| 1 + 2 + 3 | 86% | 67% | 67% | 64% | 81%/94% |
| 1 + 2 + 4 | 100% | 89% | 54% | 59% | 94%/80% |

<table>
<thead>
<tr>
<th>(Marker) expression</th>
<th>Sensitivity</th>
<th>Specitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BPH</td>
<td>PIN</td>
</tr>
<tr>
<td>(5)(^b) increase of m/z 5666</td>
<td>86%</td>
<td>22%</td>
</tr>
</tbody>
</table>

\(^b\) The increase in abundance of each marker was determined from matched normal or BPH epithelial cell lysates.

Table 5

Classification table based on logistic regression analysis using expression levels of seven proteins: [p4036, p4361, p4413, p4639, p4749, p5666, and p28422 (PSA)]

<table>
<thead>
<tr>
<th>Observed</th>
<th>Predicted (PIN/PCA)</th>
<th>% correct</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIN/PCA</td>
<td>N°</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>93.3 (specificity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93.8 (sensitivity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93.5 (overall)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) N, no; Y, yes.

Da ± 26.5 (common in the benign cell types) and 4749 Da ± 26.1 (higher abundance in PIN/PCA; Fig. 2A). These two peaks may represent a dephosphorylation event occurring in transition from benign to PIN/PCA. The average mass shift between these proteins (78 Da) is close to the calculated mass shift of 79 Da for a phosphorylation event. Prominent examples of aberrant phosphorylation of proteins found in cancer studies include extra-cellular signal-regulated kinase 1/2 in breast cancer (33) and androgen receptor in prostate cancer (34).
PCA cell lysates exhibited similar protein profiles underscoring the phenotypic similarity of these two disease states. Three peaks at 4036, 4361, and 4749 Da showed increased abundance in PIN and PCA lesions when compared with matched benign cell profiles. Two other peaks at 11,744 and 28,442 Da (free PSA) were decreased in BPH, PIN, and PCA cell extracts, and one marker at 14,696 Da was overexpressed in 56% of the PIN samples and only 29% of matched PCA samples. Interestingly, a few of these peaks, based on closely matched molecular masses, were found to be present in serum and seminal plasma from two of the patients donating tissue for this study (data not shown). Because these body fluid profiles were generated using the IMAC surface pretreated with CuSO4, they may represent the same proteins. However, additional samples will need to be examined to confirm this result. If the markers discovered in the tumor cell lysates can be detected in serum or seminal plasma, they may aid in the early detection/diagnosis of prostate cancer. The identification of these peptides or proteins and their use as possible markers of early detection is currently under investigation in our laboratory.

In this study, most of the markers found in the PCA profiles were also present in the PIN profiles, and thus, the ability to discriminate between these two cell types was difficult. However, better discrimination could be achieved between the benign cell types (normal, BPH) and the diseased cell types (PIN or PCA combined). Because it is well established that multiple foci of PIN and PCA arise independently within the same prostate and prominent genetic heterogeneity is a common feature of prostate disease, a panel of biomarkers is the most likely solution to improvements in early detection and diagnosis. In maximizing the use of our approach, we explored a combination of biomarkers with the most significant differential expression. Combining markers 4361 and 4749 Da improved the sensitivity to 100% for the detection of PIN and PCA while maintaining 87% specificity. When we incorporated the seven most differentially expressed proteins in a logistic regression analysis, a predictive equation resulted in 93.3% sensitivity and 93.8% specificity for PIN or PCA. One of the seven peaks (4,639 Da) and an additional peak (24,184 Da) were found only in PCA cell lysates. Each of these markers was expressed in 43% of tumor samples profiled. Because the majority of our tissue samples were moderately differentiated cancer (combined Gleason scores of 6 or 7), no correlation could be made to Gleason grade of tumor with regard to the expression of the peaks we found to be differentially expressed in PCA. Future studies involving the protein profiles of poorly differentiated and metastatic prostate cancer samples would determine whether any of the selected biomarkers represent markers of metastatic potential. Identifying if a patient has a clinically significant or insignificant cancer could feasibly be determined by an antibody array analysis of the patient’s biopsy (i.e., lysate) using antibodies to selected biomarkers.

Differences were observed in the expression of a 28,400-Da peak, which is consistent with intracellular-free PSA. The molecular mass identified in our study closely matches the observed molecular mass of free PSA (28,430 Da), determined using ion spray MS (29). Likewise, this peak was absent from matched adjacent stroma cell lysates, indicating its specific expression in epithelial cells. Furthermore, data obtained from our immunoassay studies, also performed using the SELDI platform (16), have identified this peak from LCM cell lysates as PSA based on immunoaffinity (data not shown). In this study, normal epithelia of the prostate expressed large amounts of PSA, whereas the diseased cell types (PIN and PCA) had reduced expression levels. Normal epithelia microdissected directly adjacent to the tumor foci also expressed high levels of PSA. This decrease in intracellular PSA in PCA cells is in agreement with other studies. For example, Jung et al. (38) found tissue PSA levels lower in cancerous than in normal tissue from the same prostate gland, and a study by Weir et al. (39) found immunohistochemical staining intensity of PSA inversely correlated with histological grade of tumor. Furthermore, significant inverse correlations have been found between tissue PSA expression levels and serum PSA values (40). Interestingly, a recent report by Pawletz et al. (15) also found a 28-kDa protein peak (not identified as PSA) via SELDI to be down-regulated in microdissected PCA cells when compared with matched normal epithelia. Our results are consistent with the hypothesis that the increase in serum PSA in men with prostate cancer is not because of increased production of PSA by the tumor cells but rather an increased leakage of PSA from the tumor tissue into the circulation as a result of a breakdown of tissue architecture.

The BPH protein profiles also displayed some notable differences. There was an increase in abundance of peaks at 3448, 4413, and 5666 Da in the BPH lesions. Of special interest was the 5666-Da peak, found to be overexpressed in 86% of the BPH cell lysates with a specificity of 88%. Only 22% of the PIN lesions and none of the PCA lesions overexpressed this marker. Efforts are under way to characterize this protein. A biomarker indicative of BPH alone may, if secreted into serum or seminal plasma, be useful in the reduction of biopsies in patients with elevated PSA.

In conclusion, differential SELDI protein profiles were observed for cell lysates prepared from microdissected normal, BPH, PIN, and PCA epithelial cells. Several small molecular mass species were found to be overexpressed in PIN, and because they were also overexpressed in PCA, these proteins may represent early signals or signatures of a developing cancer. Additionally, one marker at 5666 Da was found to be increased in BPH and may have the ability to distinguish BPH from PCA. A combination of markers was effective in distinguishing normal/BPH from PIN/PCA with a sensitivity and specificity of 93%. Additional studies are under way to identify and characterize these potential peptide/protein biomarkers using liquid chromatography tandem MS. Once identified, characterization of their function and biological role in prostate oncogenesis may lead to their potential use as diagnostic and prognostic biomarkers as well as conceivable therapeutic targets.

ACKNOWLEDGMENTS
We thank Dr. Michael Doviak for statistical analysis.

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