

# Mass Spectrometric Markers of Prostate Cancer

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## Abstract

Prostate cancer (PCa) is the most predominant male cancer in North America, with an estimated 230,000 cases and 30,000 deaths per year in the United States. Currently, the most widely used diagnostic tool for the early detection of prostate cancer is prostate specific antigen (PSA). Although screening with PSA comes recommended from the American Cancer Society, it is still an imperfect means for the detection of prostate cancer. Its low specificity has forced clinicians to rely on complementary diagnostic tools, specifically biopsy, for a positive diagnosis. This results in control patients being subjected to unnecessary testing, increasing the financial and emotional burden of a prostate cancer diagnosis. We report here the discovery of several clinical sample components that are better able to differentiate PCa from non-PCa patients over the current screening methods. These components were characterized by mass spectrometry from a sample population of 184 men (66 diagnosed with PCa, 42 diagnosed with benign prostatic hyperplasia (BPH) and 76 healthy/asymptomatic controls). Possible confounding factors such as age, medication usage and secondary conditions do not appear to be as strongly associated with the most predictive of these components.

## Introduction

PCa is the most common cancer diagnosed in men in Western countries and is the third most common cause of cancer death. It is estimated that approximately 230,000 men are diagnosed per year with PCa in the United States with 30,000 associated deaths<sup>1</sup>. Total PSA testing is the current standard screening method for prostate cancer<sup>2,3</sup>. A total PSA level above 4.0 ng/mL is considered to indicate an elevated risk of PCa<sup>4</sup>. This may be refined to account for a number of factors, such as age<sup>5</sup>, but total PSA is an imperfect means of diagnosis and is not indicative of pathological stage<sup>6,7</sup>. This results in healthy patients being subjected to unnecessary testing, increasing the financial and emotional burden of a prostate cancer diagnosis. Complements to total PSA screening include digital-rectal examination (DRE)<sup>8</sup>, but prostate biopsy remains the means of ultimate confirmation of diagnosis, despite significant rates of complications<sup>9</sup>. Given the shortcomings of total PSA testing, there remains a need for a simple and reliable (specific and sensitive) screening test to identify clinically relevant PCa. The objective of this study was to (a) discover novel target proteins and peptides in urine to differentiate prostate cancer from non-prostate cancer patients, and (b) to apply these markers to a preliminary diagnostic test.

## Methods

**Sample Collection.** Mid-stream urine samples (184 total, see Table 1) were collected from males in sterile collection cups prior to freezing for shipment on dry ice to Miraculins Inc. Upon receipt samples were thawed and divided into 1 mL aliquots for long-term storage at -20°C. Patients were approximately age matched, and > 95% of patients from each recruitment site were caucasian. Medical history information was transcribed into a relational database for ease of reference.

Table 1. Summary of sample type and recruitment site distribution used during biomarker discovery.

	Prostate Cancer	BPH	Control	Total
Alberta	42	15	34	91
Manitoba	24	27	42	93
Total	66	42	76	184

**Marker Discovery:** Single 1 mL aliquots of each sample was thawed and centrifuged to remove precipitate matter immediately prior to the experiment. Urine samples and appropriate positive and negative controls were applied to randomly selected spots on NP20 ProteinChip® arrays, washed with HPLC-grade water and co-crystallized with CHCA. Each sample was applied in duplicate, controls were applied to each array to monitor inter-array bias. Samples were assayed using a PCS4000 SELDI-TOF MS over a mass range of 0 to 80,000 m/z. Sample spectra were normalized for total ion current prior to automatic peak detection between 1,500 and 80,000 m/z. Peaks were retained only if detected in at least 10% of all spectra. Peaks from different spectra were paired and average peak intensities were compared between patient groups. The diagnostic utility of statistically significant peaks was evaluated using ROC-AUC statistics and confirmed through the use of attribute evaluation algorithms in the Waikato Environment for Knowledge Analysis (WEKA). Possible confounding factors (e.g. recruitment location, presence of non-prostate disease, medication usage, age, height, weight, body mass index, ethnicity) were tested to determine if statistically significant peaks were better diagnostic tools for these factors than for prostate cancer.

**Diagnostic Test Development.** Markers MI-0005, MI-0045, MI-0180, MI-0360, MI-0750 and MI-0900 were used to generate diagnostic tests using several classification algorithms provided in WEKA. A total of 134 randomly selected samples (67 PCa, 67 BPH or control/healthy) were used to train the diagnostic tests using 10-fold cross-validation.

**Materials.** CiphergenExpress™ software, ProteinChip® arrays, CHCA, and PCS-4000 SELDI-TOF MS; Ciphergen Biosystems Inc. WEKA software: <http://www.cs.waikato.ac.nz/ml/weka/> (the Waikato University Machine Learning Project). Miscellaneous chemicals: Sigma-Aldrich and Fisher.

Table 2. Summary of peaks capable of discriminating urine samples obtained from prostate cancer patients from other patients in the 1,500 to 80,000 m/z range. P values are given where P < 0.05 and where the peak is detected on the first or second pass by the automated peak detection algorithm of the CiphergenExpress™ software.

Marker	P value for comparison of PCa vs... Ctrl	BPH	Non-PCa	Intensity elevated in... PCa
MI-0005	3.4x10 <sup>-7</sup>	0.032	2.3x10 <sup>-7</sup>	√
MI-0015	-	7.2x10 <sup>-7</sup>	0.010	√
MI-0045	-	0.033	0.025	√
MI-0050	-	0.016	0.025	√
MI-0180	-	-	0.036	√
MI-0340	8.8x10 <sup>-7</sup>	3.7x10 <sup>-7</sup>	1.5x10 <sup>-7</sup>	√
MI-0360	9.6x10 <sup>-7</sup>	-	3.0x10 <sup>-7</sup>	√
MI-0385	0.014	0.021	5.2x10 <sup>-7</sup>	√
MI-0520	-	1.4x10 <sup>-7</sup>	-	√
MI-0530	-	0.027	-	√
MI-0560	0.016	4.4x10 <sup>-7</sup>	9.2x10 <sup>-7</sup>	√
MI-0635	2.6x10 <sup>-7</sup>	-	7.7x10 <sup>-7</sup>	√
MI-0750	1.7x10 <sup>-7</sup>	3.7x10 <sup>-7</sup>	2.7x10 <sup>-7</sup>	√
MI-0900	0.041	-	-	√

Table 3. Summary of peak intensity distributions for peak MI-0750, indicating the observed median peak intensity and observed peak intensities for the lower and upper five percent of samples from PCa, BPH and control/healthy patients.

	MI-0750 Intensity...		
	Lower 5%	Median	Upper 5%
PCa	0.38	1.77	11.96
BPH	1.10	5.83	179.80
Control/Healthy	0.99	4.39	147.31

Table 4. Relative discriminatory power of marker MI-0750 for prostate cancer (PCa) and potential confounding conditions

Comparison	P	ROC-AUC
PCa vs Non-PCa	2.04x10 <sup>-7</sup>	0.78
Diabetic vs Non-Diabetic	2.03x10 <sup>-7</sup>	0.66
Age < 60 years vs Age > 60 years	7.12x10 <sup>-7</sup>	0.63
Hypertensive vs Non-Hypertensive	0.53	0.53

Table 5. Evaluation of the sensitivity, specificity and rate of correct diagnosis for diagnostic tests based on multiple urine biomarkers of prostate cancer in comparison to total PSA testing based on the analysis of a training set of 134 samples (67 PCa, 67 non-PCa).

Test	TRAINING (n=134)		
	Sensitivity (%)	Specificity (%)	% Correct
Algorithm 1	82.26	93.75	88.10
Algorithm 2	90.48	92.06	91.27
Algorithm 3	91.94	93.75	92.86
Algorithm 4	88.33	87.88	88.10
Algorithm 5	96.72	74.24	85.04
**Total PSA	30.30	64.06	46.92

\*\*Total PSA. Values for sensitivity, specificity and rate of correct diagnosis when applying the current clinical standard (4 ng/mL cutoff) for prostate cancer screening to total PSA values obtained from training set patients at time of sample collection.

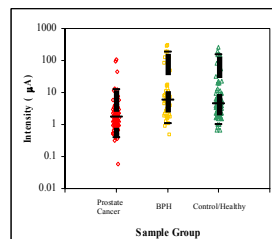


Figure 1. Distribution of average peak intensities for peak MI-0750 based on sample group. Peak intensities for MI-0750 were averaged from duplicate spectra when present, and this value plotted according to the patient group (prostate cancer (red diamonds), BPH (amber squares), control/healthy (green triangles)) the sample arose from. The median peak intensity for each sample group, and corresponding upper and lower 5% percentiles, are depicted as horizontal lines.

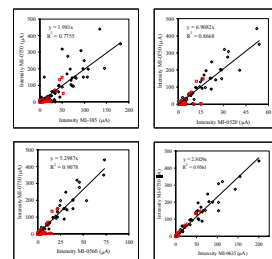


Figure 3. Correlation of intensity of peak MI-0750 and other peaks discriminatory for prostate cancer. Intensity values for a peak are the average of two replicate spectra for a single patient, except for those samples where one spectrum was discarded from statistical analysis due to excessive normalization factor (see Table 3). Regression curves are for the entire patient population and are forced through the origin. Patients diagnosed with prostate cancer are represented by hollow red squares, BPH and control patients by hollow black diamonds.

## Results

**Marker Discovery.** Statistical analysis of these spectra not excluded because of excessive normalization factor indicated that several potentially useful markers for the diagnosis of prostate cancer exist in the 1,500 to 80,000 m/z range. These include markers that can distinguish patients with BPH from those with prostate cancer, control patients from those with PCa, or non-PCa patients from those with PCa. Furthermore, there is overlap between the markers obtained for each of these indications (Table 2). The detailed analysis of one of these biomarkers, MI-0750, is presented. This marker is shown to have significantly reduced expression in PCa compared to non-PCa samples ( $P$  of between  $3.7x10^{-7}$  and  $2.7x10^{-10}$  (Table 2, Table 3, Figure 1); is not an artefact of recruitment location (data not presented); and is more discriminatory for prostate cancer than other confounding factors, the most significant of over 20 parameters tested being age, the reporting of diabetes or the reporting of hypertension by the patient (Table 4). Positive correlation of expression was observed between markers MI-0750, MI-0385, MI-0520, MI-0560 and MI-0635 (Figure 2).

**Diagnostic Test Development.** Several different rule and tree-based algorithms were used to generate diagnostic tests. Ten-fold cross-validation was used to promote test robustness. These diagnostic tests show a significant improvement over current best clinical practices applied to total PSA testing conducted on patients at the time of sample collection (Table 5).

## Conclusions

This work describes the discovery of several urine biomarkers for prostate cancer. We also demonstrate that several of these have correlated expression, and use the case of MI-0750 to outline methods to determine that the biomarkers described here are not artefacts of confounding factors such as age, recruitment site, medication usage or disease history.

Diagnostic tests developed with these biomarkers are significantly better than current screening methods. Specifically, the diagnostic tests described here are approximately three times as sensitive and 50% more specific than the total PSA test currently recommended for frontline prostate cancer screening by the American Cancer Society<sup>1</sup>. Quality assurance of the biomarkers described here using naive samples, as well as validation of the diagnostic tests developed, are currently underway.

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YZ prepared arrays, conducted mass spectrometric analysis, and assisted in experiment design and data evaluation. KS designed experiments and assisted in the preparation of arrays and in data evaluation. DSB secured samples and medical history information, conducted data analysis and assisted in data evaluation.

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