

Research Paper

# Potential Urinary Protein Biomarker Candidates for the Accurate Detection of Prostate Cancer among Benign Prostatic Hyperplasia Patients

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## Abstract

Globally, Prostate cancer (PCa) is the most frequently occurring non-cutaneous cancer, and is the second highest cause of cancer mortality in men. Serum prostate specific antigen (PSA) has been the standard in PCa screening since its approval by the American Food & Drug Administration (FDA) in 1994. Currently, PSA is used as an indicator for PCa - patients with a serum PSA level above 4ng/mL will often undergo prostate biopsy to confirm cancer. Unfortunately fewer than ~30% of these men will biopsy positive for cancer, meaning that the majority of men undergo invasive biopsy with little benefit. Despite PSA's notoriously poor specificity (33%), there is still a significant lack of credible alternatives. Therefore an ideal biomarker that can specifically detect PCa at an early stage is urgently required. The aim of this study was to investigate the potential of using deregulation of urinary proteins in order to detect Prostate Cancer (PCa) among Benign Prostatic Hyperplasia (BPH). To identify the protein signatures specific for PCa, protein expression profiling of 8 PCa patients, 12 BPH patients and 10 healthy males was carried out using LC-MS/MS. This was followed by validating relative expression levels of proteins present in urine among all the patients using quantitative real time-PCR. This was followed by validating relative expression levels of proteins present in urine among all the patients using quantitative real time-PCR. This approach revealed that significant the down-regulation of Fibronectin and TP53INP2 was a characteristic event among PCa patients. Fibronectin mRNA down-regulation, was identified as offering improved specificity (50%) over PSA, albeit with a slightly lower although still acceptable sensitivity (75%) for detecting PCa. As for TP53INP2 on the other hand, its down-regulation was moderately sensitive (75%), identifying many patients with PCa, but was entirely non-specific (7%), designating many of the benign samples as malignant and being unable to accurately identify more than one negative.

Key words: Prostate Cancer. Benign Prostatic Hyperplasia. Urine. Proteins. Biomarkers.

## Introduction

Globally, PCa is the second most common, non-cutaneous cancer among men (1, 2). Various studies have observed a positive correlation between age and incidence of PCa (2, 3, 4). Environmental factors, particularly diet, have also been reported to

potentially play a role in a man's risk for PCa (5). A number of techniques are currently available for assessing prostate health, the most common of which include digital rectal exam (DRE), trans-rectal ultrasonography (TRUS), and prostate specific anti-

gen-based testing (PSA) (6, 7).

Since PSA is primarily produced in the prostate and is therefore relatively prostate specific, it was proposed that a sharp but consistent increase in serum PSA was indicative of exponential prostate cell growth, as might occur in PCa (8). PSA test had been validated for detecting PCa using any serum level above the empirically determined cutoff of 4ng/mL (9). PSA's use as a marker for PCa was soon limited by the many instances of BPH that were also associated with an increased serum PSA above the 4ng/mL cutoff, and especially in the range of 4-10ng/mL (10).

Serum PSA testing for the early detection of PCa has a sensitivity of around 86% and a specificity of approximately 33%, depending on patient's age and local prevalence of disease (11, 12). These problems have formed the basis for much controversy surrounding PSA's use as a regular blood-based screening tool for PCa (13, 14). As such, the search for novel PCa biomarkers, especially from non-invasive fluids, has been ongoing (15, 16).

As a promising powerful biological research technology, proteomics has recently become a chief tool in the identification of disease biomarkers (12). One major advantage of proteomics is its ability to concurrently check the whole proteome or sub-proteomes such that differentially expressed or modified proteins corresponding to a disease condition can be identified. This enables researchers to merge several protein markers together to form panels of protein markers with higher sensitivity and specificity for the detection and screening of a disease. Because a cancer proteome is an exceptionally complex biological sample which contains information from almost all the biological activities that take place in cancer cells, cancer tissue microenvironment, and cancer cell-host interaction (17), 2D SDS-PAGE is not suitable as it is time-consuming, technically challenging, requires special concern to achieve tolerable comparability/reproducibility and is not ideal for the analysis of smaller polypeptides (<10 kDa) (18, 19).

As an alternative to 2D SDS-PAGE, the shotgun proteomics "bottom-up approach" has evolved. Shotgun proteomics involve the proteolytic digestion of the protein into relatively small peptides whose m/z can be accurately determined by mass spectrometry. The generated peptides are then separated in one or more successive steps of liquid chromatography, analyzed by tandem MS (MS/MS), and the information is processed with bioinformatic tools to obtain the protein composition of the original sample (20, 21). A major advantage for this approach is that it will simplify the complexity of the cancer proteome as all the proteins will be cleaved into smaller peptides and fractionated, and then the identity of the proteins

present in the sample is revealed either according to their sequence or to their m/z spectra through peptide mass fingerprinting (PMF).

Urine is considered a specific filtrate of blood; the protein components of urine are qualitatively similar to those of blood but quantitatively more diluted (22). Generally, it contains highly soluble proteins and peptides of low molecular weight (<30 kDa) which facilitate analyzing such polypeptides in their natural state (23). An advantage for urine over blood is that urinary polypeptides are stable and do not undergo significant proteolysis within several hours of collection in contrast to blood, where activation of proteases and generation of proteolytic breakdown products takes place within minutes of collection (24). Urinary proteomics presents an attractive approach to cancer biomarker discovery, not only for kidney/urological malignancies (25, 26) but for other systemic malignancies (27, 28). Urinary biomarkers offer a great chance for the development of novel, non-invasive assays for the diagnosis, monitoring and the accurate diagnosis of Pca. Many advantages favor the use of urine for cancer biomarker discovery over blood and tissues samples, including the fact that urine-based tests are absolutely noninvasive, and urine is non-infectious for HIV and less infectious for many other pathogens (29).

In this study, we compared the urinary proteomic profile of 8 PCa patients, 12 BPH patients and 10 healthy males. Shotgun proteomics "bottom-up approach" was used to identify unique differentially expressed urinary proteins by pooling urine samples from each group. Two proteins (Fibronectin, and TP53INP2) were found to be de-regulated in both PCa and BPH patients. We evaluated the deregulation of these two proteins as potential non-invasive urinary biomarkers for the accurate diagnosis of prostate cancer among Benign Prostatic Hyperplasia Patients.

## Materials and Methods

### Urine sample collection.

After approval from the Research Ethics Board at Brock University (St. Catharines, ON, Canada), urine samples were collected from 30 Egyptian males present at Alexandria University's General Hospital (Alexandria, Egypt). Urine samples were collected into 50mL Corning tubes containing Norgen's Urine Preservative Solution (Cat# 18126) (Norgen Biotek, Thorold, ON, Canada). The samples were acquired from among three groups of individuals, each described by a questionnaire completed by the patients' attending physician. Group I urine samples were collected from eight individuals diagnosed with Prostate Cancer (PCa). Group II urine samples were collected

from twelve individuals with Benign Prostatic Hyperplasia (BPH). Urine samples from Group III were collected from ten healthy males (Table 1). Urine samples collected from men with other ailments, including Diabetes mellitus, kidney disease, and/or infections such as chronic HBV, HBV/HCV co-infection or Schistosomiasis, were excluded from participation. Each man included in the study was also given a PSA test as part of sample collection. Furthermore, urine samples were collected from the same patients two years from their initial participation in the study; a PSA test was also performed for this second collection.

**Table 1.** Clinical parameters regarding the males involved with this study

	Healthy Males	Prostate Cancer Patients	Benign Prostatic Hyperplasia Patients
Number of males	10	8	12
PSA Level (ng/mL)	(0.8 ± 0.6)	(7.9 ± 3.0)	(4.9 ± 1.4)
HCV or HBV Status	-Ve	-Ve	-Ve
Diabetes mellitus	-Ve	-Ve	-Ve
Schistosomiasis	-Ve	-Ve	-Ve
Kidney diseases	-Ve	-Ve	-Ve

### Isolation of total proteins from urine.

Total proteins were isolated from 1mL of urine using the Urine Protein Concentration Micro Kit (Cat# 17400) (Norgen Biotek, Thorold, ON, Canada). The isolation was performed according to the manufacturer's instructions.

### Isolation of total RNA from urine.

Total RNA was isolated from 2.5mL of urine using the Urine Total RNA Purification Maxi Kit (Slurry Format) (Cat# 29600) (Norgen Biotek, Thorold, ON, Canada). The isolation was performed according to the manufacturer's instructions.

### Profiling of Pooled Urinary Proteins by LC-MS/MS.

Before analysis, pooled protein samples were first digested in 25mM ammonium bicarbonate, using 0.1µg of trypsin in a total volume of 50µL. After an overnight incubation at 37°C, the peptide solution was then lyophilized by SpeedVac centrifugation and subsequently re-suspended in 20µL of 0.1%TFA (Tri-Fluoro Acetic Acid). The LC/MS-MS experiment was performed at Sick-Kids Hospital (Proteomics Facility, Toronto, ON, Canada) using an on-line LC-MS/MS setup that employed an Agilent 1100 capillary liquid chromatography system (Palo Alto, CA, USA) that was fitted to an LTQ ion trap mass spec-

trometer (Thermo Electron, San Jose, CA, USA). A Carbon-18 pre-column (150µm i.d. x 5.0cm length) and a µLC analytical column (75µm x 10cm), which also served as a µESI emitter, were used for the separation of the digested proteins.

The samples were first injected into the pre-column for de-salting and then eluted into the analytical column for further separation. For the reverse-phase chromatography (C-18 column), a 60 minute gradient elution from water to acetonitrile, and always containing 0.1% TFA, was performed at a flow rate of 0.2~0.3µL/min. The mass spectrometer was set to data-dependent mode, which automatically cycled through the acquisition of full-scan mass spectrums. Six further spectra were then sequentially recorded on the six most abundant ions present upon the initial scan. A dynamic exclusion time of 90 seconds was used throughout.

The LC-MS/MS data were initially obtained in \*.RAW format, which were then converted into mzXML, basically a list of mass peaks. The lists were then used as input in a search of the human NCBI-NR database using the GPM XE ([www.thegpm.org](http://www.thegpm.org)) search tool. Search results were then assessed in Scaffold ([www.proteomesoftware.com](http://www.proteomesoftware.com)). Acquired MS/MS data was also compared against three other databases: NCBI's non-redundant database, SwissProt and the MDSB database, all using the Mascot search tool (Matrix Science, London, UK).

### Relative expression profiling for candidate proteins using RT-qPCR.

Relative mRNA expression levels for the 3 candidate proteins (Cadherin 1, Fibronectin, and TP53INP2), determined from the shotgun proteomic analysis were analyzed among the PCa group, BPH group and the health male group. Candidate proteins were reversibly-transcribed using a 3 µl RNA template and 0.5 µl 0.5µg/µL Oligo (dT)18 Primer. The reaction was then made up with RNase-free water to 10µL then incubated for 5 mins at 70°C then held at 4°C. During the 4°C incubation, a mix of 4µl 5X first strand Buffer, 2µl of 0.1mM DTT, 1µl of 10mM dNTPs, 0.5µl SuperScript III (Invitrogen) and 2.5µl nuclease-free water were added to the initial reaction for a final reaction volume of 20µL. The final reaction was then incubated at 25°C for 5 min, 50°C for 30 min, 70°C for 15 min with a final hold at 4°C. All samples were analyzed using the iCycler iQ real time PCR detection system with iQ SYBR Green Supermix (BioRad Laboratories, Mississauga, ON, Canada). For each sample, 3µl from its corresponding RT reaction were mixed in a 10µl iQ SYBR Green Supermix, 6.76µl nuclease-free water and 0.12µl Cadherin 1 (F) 5'-AAGAAGCTGGCTGACATGTACGGA -3', Cadherin

1 (R) 5'- CCACCAGCAACGTGATTTCTGCAT -3' / Fibronectin (F) 5'- AAACCTGTCATCTGGAGGCAAAA CCC -3', Fibronectin (R) 5'- AGCTCTGATCAGCATG GACCACTT -3' / TP53INP2 (F) 5'- AGGCCCTGAAA TCTGAAGGGCTTA -3' and TP53INP2 (R) 5'- AGTTCTAGCACCTTGGGCTTGTGA -3' in a total of 15 $\mu$ l reaction. All RT-qPCR experiments were carried out in triplicate and the expression levels were normalized to 5S rRNA levels. The relative expression level (fold change) for each candidate miRNA within each group was then calculated using the equation  $2^{-\Delta\text{Ct}}$  test /  $2^{-\Delta\text{Ct}}$  control.

## Results

### LC-MS/MS Shotgun Analysis

Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS) analysis of the proteins pooled from the healthy male group, the PCa group and the BPH group resulted in 2062, 1514 and 2527 spectra for each of the respective samples. By analyzing the different spectra generated by the HPLC-MS/MS analysis with MASCOT, X! Tandem on-line search engines and the Scaffold proteome software, a total of 28 individual proteins in the healthy control sample, 41 in the BPH and 31 in the PCa sample were identified. Proteins were then classified based on the likelihood of their presence within each of the three-pooled samples. Of the identified proteins, 10 were common to all three groups: uromodulin, variants of Alpha-1 anti-trypsin, Alpha-2-glycoprotein-1, Ig lambda chain V region, Immunoglobulin light chain constant region, Fibrinogen gamma chain, Glutaminyl-peptide cyclotransferase, Hemopexin, Human Ferritin L Chain and Leucine-rich  $\alpha$ -2-glycoprotein-1

Furthermore, there were sixteen proteins unique to the PCa group: Human Serum Albumin Complexed with Myristate and Aspirin; Apolipoprotein-D; Prostaglandin H2 D-isomerase; Ig A1 Bur; G-protein coupled receptor 115; Cadherin-1;  $\alpha$ -N-acetylglucosaminidase; Pro-epidermal growth factor; Apolipoprotein-J precursor; Human Beta-Defensin-1; Matrilin-4; CD14 antigen; Chemokine-like factor superfamily-5 isoform a; Fibronectin precursor; Immunoglobulin-kappa light chain variable region; and SLURP1. Another sixteen proteins were found to be unique to the BPH group, they were:  $\alpha$ -1-microglobulin/bikunin pre-pro-protein;  $\alpha$ -1 acid glycoprotein-2 precursor; Pepsinogen-3; Kininogen-1; Phosphoinositide-3-kinase interacting protein-1; Immunoglobulin heavy chain; Coagulation factor-II (thrombin); IgA-H; Amylase; Apolipoprotein-H; VASN protein; CD44 molecule; Protein-C inhibitor; EPH receptor B3; CD59 antigen pre-pro-protein; and

Vitronectin. Ten proteins were identified in both the BPH and control groups and were notably absent from the PCa group, they were: hCG-1996769; complement component-3 precursor; retinol binding protein-4; myosin VA; haptoglobin; keratin-13; defensin HNP1; delta-globin; PRO-2675; and hCG-1817845

There were eight proteins found in the control group that were notably missing from both the PCa and BPH samples, they were: Lipopolysaccharide binding protein; Janus kinase and microtubule interacting protein-2; Polyamine modulated factor-1 binding protein-1;  $\alpha$ -mannosidase-II; Apolipoprotein-A-I; Zinc finger protein; ACSF3 protein; and Chloride intracellular channel-1. Another five proteins were absent from the control sample but were present in both the pooled PCa and BPH samples, they were: Apo-Human Serum Transferrin (Non-Glycosylated); Nidogen 1 precursor; N-ethylmaleimide-sensitive factor attachment protein; CTCL tumour antigen; and Tumour Protein 53 Inducible Nuclear Protein 2 (TP53INP2).

### Determining the Expression of Cadherin-1, Fibronectin and TP53INP2 mRNA among individual Prostate Cancer samples by RT-qPCR

Employing RT-qPCR, mRNA levels among individual samples were compared to the average expression levels of the healthy control group. Transcript expression for each sample was first normalized against the sample's own content of 5S rRNA. Relative expression was then calculated as  $\text{LOG}_2$  fold change values according to equation:  $\text{LOG}_2 (2^{-\Delta\text{Ct}}(\text{target mRNA}) \text{ PCa} / 2^{-\Delta\text{Ct}}(\text{target mRNA}) \text{ Mean Healthy Control})$  (Table 2).

**Table 2.**  $\text{LOG}_2$  fold change values for the expression of: Cadherin-1, Fibronectin and TP53INP2 mRNAs among initially collected PCa samples.

Sample ID	LOG2 Fold Change (PCa / Average Healthy Control)		
	Cadherin-1	Fibronectin	TP53INP2
PCa 1	4.91	-4.24	-9.36
PCa 2	-7.39	-0.04	-14.76
PCa 3	-4.39	-10.14	-23.86
PCa 4	-1.49	-2.94	0.34
PCa 5	-6.69	-7.14	-4.76
PCa 6	-1.09	-2.44	-0.96
PCa 7	N/A	-2.64	0.04
PCa 8	0.81	-6.84	0.24

(Red - Two fold or more up-regulated, Green - Two fold or more down-regulated and Black - Minimally Deregulated) (Relative to average healthy group expression)

In contrast to mass spectrometry data that showed Cadherin-1, Fibronectin and TP53INP2 proteins to be present in the pooled PCa sample and not in the healthy control sample, mRNA levels for the

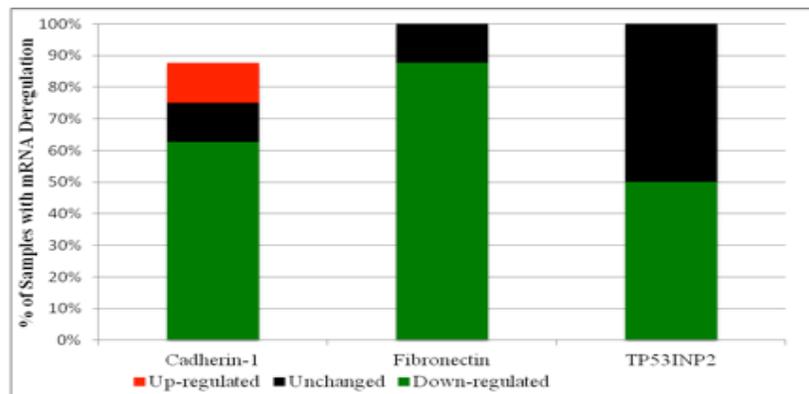
corresponding proteins (especially Fibronectin and TP53INP2) were widely down-regulated amongst individual PCa samples. In particular, Fibronectin mRNA was found to be down-regulated in seven of the eight PCa samples. TP53INP2 mRNA was found to be down-regulated in half of PCa samples. Cadherin-1 mRNA on the other hand was found to be up-regulated in one sample, unchanged in one and down-regulated in the remaining five PCa. Figure 1 illustrates the percentage of initially collected PCa samples with either up, down or minimal deregulation, for each of the three target mRNAs.

### Screening for Deregulation of Fibronectin and TP53INP2 among BPH Samples by RT-qPCR

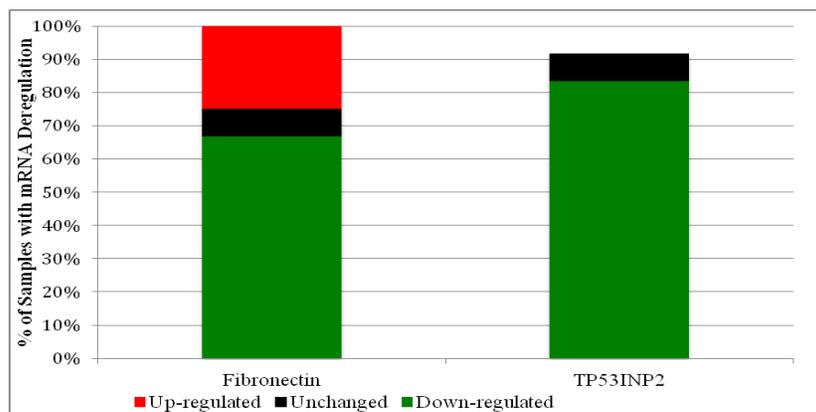
Since both Fibronectin and Cadherin-1 proteins were specifically identified by mass spectrometry in the pooled PCa sample, but only Fibronectin mRNA (and not Cadherin-1 mRNA) was consistently down-regulated among initially collected PCa samples it was decided that only Fibronectin mRNA ex-

pression would be evaluated in the initially collected BPH samples. Analysis then revealed that Fibronectin mRNA was variably expressed in BPH, down-regulated in eight of the twelve initially collected BPH samples (67%), unchanged in one (8%) and up-regulated in three (25%) (Table 3).

Since the TP53INP2 protein was originally identified by mass spectrometry in both PCa and BPH, and since its mRNA was considerably down-regulated in half the PCa samples, its expression was also evaluated among individual BPH samples. Expression analysis among the initially collected BPH samples revealed that TP53INP2 mRNA was predominantly down-regulated. TP53INP2 mRNA was down-regulated in ten of the twelve samples (83%) (Table 3). Figure 2 illustrates the percentage of initially collected BPH samples with either up, down or minimal deregulation, for both Fibronectin and TP53INP2 mRNA.



**Figure 1.** Percentage of initially collected PCa samples with either up, down or minimal deregulation in the expression of mRNAs for: Cadherin-1, Fibronectin or TP53INP2.



**Figure 2.** Percentage of initially collected BPH samples with up, down or minimal deregulation in the expression of mRNAs for: Fibronectin or TP53INP2.

**Table 3.** LOG<sub>2</sub> fold change values for the expression of: Fibronectin and TP53INP2 mRNAs among initially collected BPH samples.

Sample ID	LOG <sub>2</sub> Fold Change (BPH/ Average Healthy Control)	
	Fibronectin	TP53INP2
BPH1	-5.14	-0.96
BPH2	-5.04	-21.36
BPH3	-6.34	-16.56
BPH4	-2.94	-1.96
BPH5	3.06	-12.86
BPH6	-2.14	-3.26
BPH7	-6.24	-21.76
BPH8	-6.74	-6.56
BPH9	-0.54	N/A
BPH10	1.26	-13.96
BPH11	1.86	-12.06
BPH12	-2.69	-10.56

(Red - Two fold or more up-regulated, Green - Two fold or more down-regulated and Black - Minimally Deregulated) (Relative to average healthy group expression)

### Re-evaluating Fibronectin and TP53INP2 mRNA expression in PCa samples after two years

Since both Fibronectin and TP53INP2 mRNA appeared to be down-regulated in at least half of the initially collected, PCa and BPH samples, it was decided to re-evaluate expression levels for the PCa samples as part of the two year follow-up. Expression levels were again determined by RT-qPCR, on RNA isolated from urine samples collected two years subsequent to the initial collection. For every sample, both transcripts were normalized against the sample's own content of 5S rRNA. Differential expression was then determined by comparing expression against the average healthy group's level. Relative expression was then calculated as LOG<sub>2</sub> fold change values according to the equation: LOG<sub>2</sub> (2-ΔCt (target mRNA)

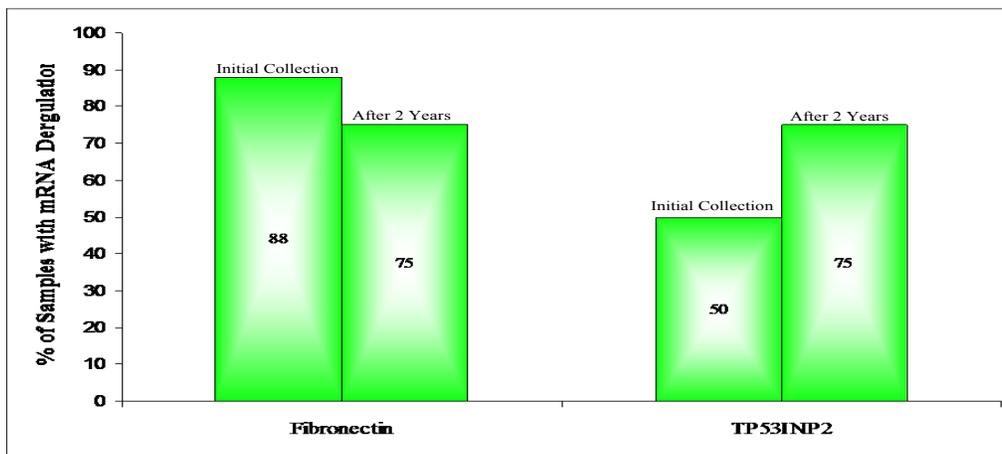
PCa / 2-ΔCt (target mRNA) Mean Healthy Control) (Table 4).

Analysis revealed that both Fibronectin and TP53INP2 mRNA were down-regulated in six of the eight PCa samples (75%). For Fibronectin mRNA this was a drop from the seven out of eight samples observed previously to be down-regulated (Figure 3). Interestingly however, despite this drop in frequency (of down-regulation), after two years many of the same PCa samples (PCa 3, 5, 6, 7, 8) had maintained their initially observed down-regulation of Fibronectin mRNA. Only PCa samples 1 and 4 had changed, becoming up-regulated or unchanged (respectively) with respect to the mean level of the original healthy control group. For TP53INP2 mRNA levels, down-regulation was more frequent after two years (75% versus 50%), occurring in three additional samples, PCa 4, 6 and 8. Meanwhile some of the other patients (PCa 1, 2, 5) maintained their down-regulation in TP53INP2 mRNA.

**Table 4.** LOG<sub>2</sub> fold change values for the expression of: Fibronectin and TP53INP2 mRNAs among PCa samples collected after two years.

Sample ID	LOG <sub>2</sub> Fold Change (PCa/ Average Healthy Control)	
	Fibronectin	TP53INP2
PCa 1	1.66	-7.66
PCa 2	-5.24	-4.66
PCa 3	-2.54	N/A
PCa 4	-0.54	-13.46
PCa 5	-2.74	-2.36
PCa 6	-1.34	-18.86
PCa 7	-7.34	N/A
PCa 8	-1.14	-6.86

(Red - Two fold or more up-regulated, Green - Two fold or more down-regulated and Black - Minimally Deregulated) (Relative to average healthy group expression)



**Figure 3.** Percentage of PCa samples with down-deregulation in the expression of mRNAs for: Fibronectin or TP53INP2. (Determined by RT-qPCR)

## Re-evaluating Fibronectin and TP53INP2 mRNA expression in BPH samples after two years

Since deregulation of either Fibronectin or TP53INP2 appeared to be common in PCa and BPH samples, even after two years, it was decided to re-evaluate their expression levels in the BPH samples that were collected after two years. It should be noted that of the original twelve BPH patients, only eight were successfully located for follow-up testing; of the eight, four happened to be diagnosed with PCa at this point (BPH patients 1, 9, 10 and 11).

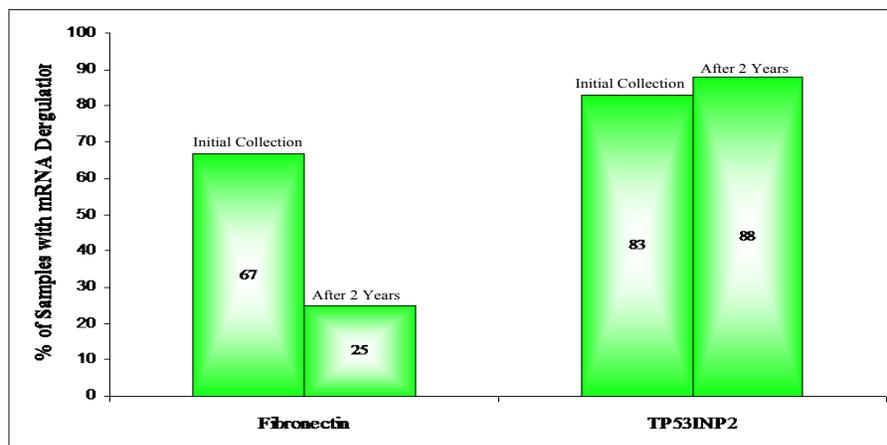
Expression analysis by RT-qPCR revealed that Fibronectin mRNA was now down-regulated in only two of the eight BPH samples (25%), down from the previous 67% (Figure 4). Interestingly, two of the four BPH patients who were found to have PCa at the second diagnosis, did in fact have down-regulation of Fibronectin mRNA in their urine after two years. The first, BPH 1, had shown such deregulation as early as two years prior, during the initial collection; BPH 10 on the other hand went from Fibronectin being 2.4

fold up-regulated to 4.7 fold down-regulated after two years (Table 5). TP53INP2 mRNA expression was, after two years found to be at least two fold down-regulated in seven of the eight BPH samples (88%) versus ten out of twelve previously (83%), a roughly consistent fraction.

**Table 5.** LOG<sub>2</sub> fold change values for the expression of: Fibronectin and TP53INP2 mRNAs among BPH samples collected after two years.

Sample ID	LOG <sub>2</sub> Fold Change (BPH / Average Healthy Control)	
	Fibronectin	TP53INP2
BPH1	-5.34	-4.36
BPH4	4.16	-1.36
BPH6	3.06	-3.36
BPH7	0.56	-9.76
BPH9	-0.74	-8.36
BPH10	-2.24	N/A
BPH11	0.06	-13.86
BPH 12	1.96	-14.06

(Red - Two fold or more up-regulated, Green - Two fold or more down-regulated and Black - Minimally Deregulated) (Relative to average healthy group expression)



**Figure 4.** Percentage of BPH samples with down-deregulation in the expression of mRNAs for: Fibronectin or TP53INP2

## Diagnostic Utility of Fibronectin & TP53INP2 for Diagnosing Prostate Cancer

To evaluate the potential for using the aberrant expression of Fibronectin or TP53INP2 mRNA for diagnosing PCa, the medical records, specifically PSA values and clinical diagnoses for all BPH patients were updated as part of the two-year follow-up. It should be noted that all BPH patients were therefore diagnosed twice, once initially and then again after two years. Interestingly, during the second assessment it was discovered that four of the BPH patients in fact had PCa, they were: BPH 1, 9, 10 and 11 (Table 6).

By comparing putative diagnoses (of PCa) made using mRNA deregulation against the actual clinical diagnoses, the sensitivity and specificity for using at least two fold down-regulation of Fibronectin and TP53INP2 (separately or in tandem) for diagnosing PCa were determined. The following criteria were applied: patients with a positive clinical diagnosis and exhibiting down-regulation of either Fibronectin or TP53INP2 or deregulation of both, were considered true positives; patients with a positive clinical diagnosis and not exhibiting down-regulation of either gene (either separately) or only one gene (in tandem) were considered false negatives; patients with a negative clinical diagnosis and exhibiting

down-regulation of either Fibronectin or TP53INP2 or deregulation of both, were considered false positives; and patients with a negative clinical diagnosis and not exhibiting down-regulation of Fibronectin or TP53INP2 or exhibiting down-regulation of only one or neither (when combined), were considered true negatives.

By applying the above-mentioned criteria, both sensitivity and specificity for using down-regulation in Fibronectin and/or TP53INP2 for diagnosing PCa were determined. Using Fibronectin mRNA down-regulation on its own to diagnose PCa resulted

in a sensitivity of 75% and a specificity of 50%, while using TP53INP2 mRNA down-regulation on its own to diagnose PCa resulted in a sensitivity of 76% and specificity of 7%. Furthermore, the sensitivity and specificity for using the tandem down-regulation of both genes for diagnosing PCa was 47% and 53%, respectively. The sensitivity and specificity for a panel employing down-regulation of both genes and an elevated serum PSA ( $\geq 4$ ng/mL) for detecting PCa was 35% and 73%, respectively. Table 7 summarizes the sensitivity and specificity values for the various assays.

**Table 6.** Diagnosing PCa among BPH Patients by Assessing Deregulation of Fibronectin and TP53INP2 mRNA.

Sample ID	Initial Urine Collection			Urine Collection After 2 Years				
	LOG <sub>2</sub> Fold Change (BPH / Avg Control)		Clinical Diagnosis for PCa	Serum PSA Levels (ng/mL)	LOG <sub>2</sub> Fold Change (BPH / Avg Control)		Clinical Diagnosis for PCa	Serum PSA Levels (ng/mL)
	Fibronectin	TP53INP2			Fibronectin	TP53INP2		
BPH1	PCa	Healthy	Healthy	4.3	PCa	PCa	PCa	3.9
BPH2	PCa	PCa	Healthy	5.6	N/A	N/A	N/A	N/A
BPH3	PCa	PCa	Healthy	3.9	N/A	N/A	N/A	N/A
BPH4	PCa	PCa	Healthy	5.8	Healthy	PCa	Healthy	9.6
BPH5	Healthy	PCa	Healthy	2.4	N/A	N/A	N/A	N/A
BPH6	PCa	PCa	Healthy	5.1	Healthy	PCa	Healthy	4.8
BPH7	PCa	PCa	Healthy	6.9	Healthy	PCa	Healthy	4.2
BPH8	PCa	PCa	Healthy	3.7	N/A	N/A	N/A	N/A
BPH9	Healthy	N/A	Healthy	4.3	Healthy	PCa	PCa	10.5
BPH10	Healthy	PCa	Healthy	5.1	PCa	N/A	PCa	9.8
BPH11	Healthy	PCa	Healthy	7.4	Healthy	PCa	PCa	4.2
BPH12	Healthy	PCa	Healthy	3.8	Healthy	PCa	Healthy	9.4

**Table 7.** Diagnostic Utility of Fibronectin and TP53INP2 for Diagnosing PCa.

Candidate Biomarker(s)	Sensitivity (%)	Specificity (%)
Fibronectin	75	50
TP53INP2	76	7
Fibronectin/TP53INP2	47	53
Fibronectin/TP53INP2/PSA	35	73

## Discussion

Proteomics studies are useful for identifying cancer biomarkers. Traditional techniques such as two-dimensional gel electrophoresis have typically been used for this purpose - in one instance to identify proteins uniquely expressed by androgen stimulation of PCa cells (30). In contrast, more modern proteomics studies rely on either protein microarray or mass spectrometry to identify specific proteins; both of which have also been successfully employed towards the study of cancer, including PCa (31). Nonetheless, the number of successful high-throughput studies that have assessed proteins, especially from the urine of patients with PCa and/or BPH has been minimal

(32). Therefore in this study, urinary proteins were evaluated by liquid chromatography coupled to tandem mass spectrometry to identify promising protein candidates for a PCa biomarker.

In order to identify those proteins and others that were differentially expressed between the groups, a "bottom-up" shotgun-based analysis using LC-MS/MS was employed. Proteins, pooled into three samples (PCa, BPH and Control) were analyzed using LC-MS/MS to reveal 1514, 2527, 2062 respective spectra. From these spectra, a total of 31 proteins were identified in the PCa sample, 41 in the BPH and 28 in the control. One protein, Tamm-Horsfall Protein (Uromodulin), was common to all three groups. Uromodulin is one of the most abundant proteins in urine (33, 34, 35) and normally runs as a monomer around 68kDa on denaturing gels. It is therefore likely that uromodulin, at least in part, formed the band that was observed near 66kDa in many of the samples from this study. Some authors have suggested that depleting abundant proteins before profiling might help to elucidate rare proteins that can be masked by the presence of proteins in such high abundance (36, 37). Unfortunately depleting highly abundant pro-

teins, which can act as transporters or carriers of other macromolecules, can also lead to the loss of other potentially important proteins (38).

Importantly, pooled analysis identified at least two proteins, found only in the cancer sample, that are acknowledged in the literature as potentially being involved with cancer: Cadherin-1 (E-Cadherin) and Fibronectin precursor proteins. In many cancers, the down-regulation or complete loss of E-Cadherin is reported to correlate to tumour malignancy (39). Lack of E-Cadherin, an epithelial glycoprotein, which mediates cell-cell adhesion (40), has even been associated with a poor prognosis in PCa patients (41, 42). Furthermore, a decrease in E-Cadherin expression has been associated with an invasive and metastatic phenotype, also in PCa (42, 43, 44). In light of its potential contribution to the development and progression of PCa, and to assess its utility as a candidate biomarker for PCa, Cadherin-1 was selected for further evaluation among individual urine samples.

Fibronectin, another protein identified via pooled analysis, is normally present in the Extracellular Matrix (ECM) (45). ECM normally also contains adhesion molecules such as laminins, fibronectins, glycoproteins, chondroitins, heparin sulfate, collagens, tenascins, proteoglycans, etc. that together complete a complex network that forms the basis of connective tissues. Furthermore these molecules are typically produced locally and by cells that are adjacent to the ECM (45). The ECM serves a diverse role, aiding in anything from the regulation of cellular differentiation to migration, tissue remodeling and even helping to maintain tissue integrity (46). The ECM and its components have also been shown to play an important role during invasion and metastasis of malignant cells throughout the body (46). Normally it is integrins (cell membrane protein) that connect cells, including prostatic epithelial cells, to the ECM by binding to fibronectin already present in the matrix (47). This interaction is required for proper assembly of fibronectin in the matrix, something that is very important for ECM organization and stability (48, 49). Cancer typically involves an epithelial to mesenchymal transition (EMT), whereby malignant cells can now break loose of their defined position within the microenvironment. This is usually associated with the loss of E-Cadherin and disruption of local ECM (50). To do this, cells not only decrease expression of E-Cadherin but also reduce their expression of integrins, and alter their secretion of ECM components; something that has been shown to occur in invasive PCas (51, 52). It has also been reported that in poorly differentiated PCas, the expression of Fibronectin is significantly reduced, thereby weakening the ECM itself (53). Loss or significant down-regulation of Fi-

bronectin has therefore been proposed as a mechanism for cancer's malignant nature, whereby cancer cells are more readily able to detach from a tumour mass before spreading elsewhere (54). In light of Fibronectin's role in cancer progression particularly with regards to published observations made in PCa, and since it was identified only in the pooled PCa sample (as assessed by mass spectrometry), Fibronectin was selected for further analysis (to assess its candidacy as a potential biomarker for PCa) among the individual urine samples collected during this study.

Another protein, tumour protein-53 induced nuclear protein-2 (TP53INP2), was notably absent from the pooled control but present among both the pooled BPH and PCa samples. TP53INP2 is thought to be a scaffold protein that is normally expressed upon induction by the p53 tumour suppressor protein (55). TP53INP2 is required for proper autophagy (56) a process that occurs when a cell recycles old materials by engulfing them into a lipid membrane and then fusing said membrane with lysosomes, ultimately catabolizing the contents (57). Autophagy is also known to occur during programmed cell death (58); one of the responses p53 is known to regulate (58). Importantly TP53INP2 is thought to possess tumour suppressor-like functionality, which might help explain why in pre- and neoplastic cervical cancers it is the target of various microRNAs that block its expression (59). Possibly as a result of the same mechanism, it was observed that in some instances of colorectal cancer p53 was unable to induce expression of TP53INP2 (60). TP53INP2 has also been observed at unusually low levels in some PCas (61). Furthermore, in a PCa cell line (LNCaP) the drug Genistein, normally used to treat PCa, was shown to up-regulate TP53INP2, among other genes (62). Moreover, it has also been proposed that the occurrence of a specific splice variant of TP53INP2 might be a necessary event in order for malignant cells to invade the ECM (63). Finally, in 2007 a patent was filed that described using measurements of TP53INP2, among other targets, as part of a multivariate signature to monitor the progression of PCa (64). Therefore based on TP53INP2's potential role in cancer, and because it was commonly identified in both pooled PCa and BPH samples, it was selected for analysis among individual samples to assess its candidacy as a potential PCa biomarker.

Based on the aforementioned evidence regarding the selected proteins (Cadherin-1, Fibronectin, TP53INP2) and their probable involvement in the development or progression of malignancy, their utility towards diagnosing PCa was investigated. The expression level of the selected candidate's mRNA (Cadherin-1, Fibronectin and TP53INP2) was assessed

on a sample-by-sample basis via RT-qPCR. The first candidate that was investigated by RT-qPCR was Cadherin-1. Initially observed only in pooled PCa by mass spectrometry, Cadherin-1 mRNA was later found to be down-regulated (relative to the average of the healthy group), among a majority of the initially collected PCa samples (63%), but remained unchanged in 13% and even up-regulated in 13%. Assuming elevated levels of mRNA correlate to elevated levels of protein, then an up-regulation of Cadherin-1 mRNA in just one patient would explain why it was observed at all in the mass spectrometry data for pooled PCa proteins. A down-regulation of Cadherin-1 is reported in the literature to correlate with general tumour invasiveness (39). Down-regulation has also been associated with metastases in PCa and is therefore (as was shown in the literature), an indicator of poor prognosis among PCa patients (42). Since down-regulation of Cadherin-1 is likely a prognostic, as opposed to diagnostic factor and because it was not strictly down-regulated in a strong majority of cancer patients, it was considered a second-rate candidate for future testing.

As for Fibronectin, differential expression profiling of its mRNA transcript among initially collected PCa samples revealed down-regulation across 88% of samples and up-regulation among none. The RT-qPCR data on Fibronectin mRNA expression would seem to agree with LC-MS/MS data suggesting that at least one PCa patient presented with fragments of fibronectin in their urine. RT-qPCR data also complements a report in the literature suggesting that down-regulation or complete loss of Fibronectin can promote tumour invasiveness by allowing malignant cells to more easily detach from a tumour mass (54). Due to its putative role in cancer progression and because Fibronectin mRNA was deregulated among a large majority of samples and up-regulated among none, it was selected for further assessment among BPH samples.

TP53INP2, reportedly down-regulated in PCa (51), was found in both PCa and BPH but interestingly not in control when pooled samples were assessed by LC-MS/MS. To investigate this trend, levels of TP53INP2 mRNA were assessed for differential expression in PCa samples by RT-qPCR. Unsurprisingly, TP53INP2 mRNA was down-regulated in 50% of the initially collected PCa samples and up-regulated in none of them. These results further agree with Poustka, who in 2007 proposed using the deregulation of TP53INP2 as part of a multivariate signature to monitor progression in PCa patients. Due to TP53INP2's likely role in PCa and because LC-MS/MS data alluded to its presence in the BPH cohort, some of whom were ultimately diagnosed

with PCa, TP53INP2 was selected for further evaluation among BPH patients.

Since down-regulation of both Fibronectin and TP53INP2 were observed among a large percentage of the initially collected PCa samples and due to their literature suggested roles in cancer, the differential expression of Fibronectin and TP53INP2 mRNA was evaluated among BPH samples. Comparing Fibronectin and TP53INP2 mRNA levels in the BPH samples to the average healthy group level revealed a down-regulation among a number of the BPH patients, some of whom were subsequently suspected of and then diagnosed with PCa (BPH1, BPH9, BPH10 and BPH11). Furthermore, differential expression profiling of both transcripts in urine collected from the same patients (both PCa and BPH) after two years seemed to confirm the observations made initially. Since deregulation of Fibronectin and/or TP53INP2 might have foreshadowed a diagnosis of PCa among some of the BPH patients, down-regulation in urine is tentatively proposed as an indicator for PCa that may ultimately be useful for diagnosing or differentiating PCa from BPH.

In order to validate the diagnostic potential of Fibronectin and TP53INP2 mRNA deregulation for predicting PCa, medical records including PSA levels and clinical diagnoses were updated as part of a two-year follow-up. Both genes differed substantially in their ability to accurately detect PCa. At least two-fold down-regulation of Fibronectin mRNA showed the most potential for diagnosing PCa; it had a sensitivity of 75% and specificity of 50%. Fibronectin mRNA deregulation was often successful at identifying PCa samples as positive, but slightly less successful at designating patients with benign disease as negatives. Altogether, Fibronectin displayed a strong ability to predict PCa. The predictive nature of Fibronectin deregulation might reflect its role in the ECM as an essential regulator of cellular differentiation, migration, and tissue remodeling and integrity (46). Interestingly, using urinary Fibronectin mRNA down-regulation to predict PCa resulted in a more specific, albeit less sensitive test than traditional PSA testing which has a sensitivity and specificity of 86% and 33% at the FDA recommended threshold of  $\geq 4$ ng/mL of blood (65). The improvement in specificity rises dramatically when compared to PSA at a cutoff of  $\geq 2.5$ ng/mL (98.2% sensitivity and 16.6% specificity) (66). As for TP53INP2 on the other hand, its down-regulation was moderately sensitive (75%), identifying many patients with PCa, but was entirely non-specific (7%), designating many of the benign samples as malignant and being unable to accurately identify more than one negative.

To improve the chances of accurately predicting

PCa, various combinations of the identified candidates were evaluated. The sensitivity and specificity for combining both Fibronectin and TP53INP2 down-regulation to detect PCa was 47% and 53%, respectively. Interestingly this combination successfully designated at least one of the malignant and 50% of benign samples within the updated BPH cohort; PSA on the other hand might have correctly designated 75% of those cancers but unfortunately would not have cleared any of the negatives.

When both mRNA-based protein candidates were combined with PSA (cutoff  $\geq 4\text{ng/mL}$ ), specificity was increased to 73%; at the same time sensitivity was reduced to 35%. Thus far the protein-based approach has identified at least one potential candidate that merits further study towards the accurate detection of PCa. And although these early results indicate a reasonable sensitivity and specificity, further studies must still be conducted using a larger pool of samples collected over a longer period of time both before and after disease has progressed, to validate their roles in PCa. Employing urine, like in this study, as the basis for a screening assay would be a convenient and non-invasive way to screen more men, and potentially reduce unnecessary biopsies by differentiating PCa from BPH, and if PCa is diagnosed earlier, possibly even reduce PCa mortality rates as well.

## Competing Interests

The authors have declared that no competing interest exists.

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