Development of a voided urine assay for detecting prostate cancer non-invasively: a pilot study

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Objective
To validate a hypothesis that prostate cancer can be detected non-invasively by a simple and reliable assay by targeting genomic VPAC receptors expressed on malignant prostate cancer cells shed in voided urine.

Patients/Subjects and Methods
VPAC receptors were targeted with a specific biomolecule, TP4303, developed in our laboratory. With an Institutional Review Board exempt approval of use of de-identified discarded samples, an aliquot of urine collected as a standard of care, from patients presenting to the urology clinic (207 patients, 176 men and 31 women, aged ≥21 years) was cytopspun. The cells were fixed and treated with TP4303 and 4,6-diamidino-2-phenylindole (DAPI). The cells were then observed under a microscope and cells with TP4303 orange fluorescence around the blue (DAPI) nucleus were considered ‘malignant’ and those only with a blue nucleus were regarded as ‘normal’. VPAC presence was validated using receptor blocking assay and cell malignancy was confirmed by prostate cancer gene profile examination.

Results
The urine specimens were labelled only with gender and presenting diagnosis, with no personal health identifiers or other clinical data. The assay detected VPAC positive cells in 98.6% of the men with a prostate cancer diagnosis (141), and none of the 10 men with benign prostatic hyperplasia. Of the 56 ‘normal’ patients, 62.5% (35 patients, 10 men and 25 women) were negative for VPAC cells; 19.6% (11, 11 men and no women) had VPAC positive cells; and 17.8% (10, four men and six women) were uninterpretable due to excessive crystals in the urine. Although data are limited, the sensitivity of the assay was 99.3% with a confidence interval (CI) of 96.1–100% and the specificity was 100% with a CI of 69.2–100%. Receptor blocking assay and fluorescence-activated cell sorting (FACS) analyses demonstrated the presence of VPAC receptors and gene profiling examinations confirmed that the cells expressing VPAC receptors were malignant prostate cancer cells.

Conclusion
These preliminary data are highly encouraging and warrant further evaluation of the assay to serve as a simple and reliable tool to detect prostate cancer non-invasively.

Keywords
imaging prostate cancer, targeting VPAC receptors, urinary assay, optical imaging, #ProstateCancer, #PCSM

Introduction
Increased understanding of human diseases at the cellular and molecular levels has paved the way for development of several novel life-sciences technologies. Detecting circulating tumour cells (CTC) in human blood is one. The CTC-associated liquid biopsy approach has drawn considerable attention and is advancing into clinical applications [1,2]. Broadly, such approaches are based upon collection of body fluids, cell isolation, followed by multiplex genomic profiling and identification of disease-specific fingerprints. Although innovative and state of the art, such approaches can be technically complex, expensive, and subject to errors leading to inconsistent results [1,2].

Among men worldwide, prostate cancer is the most common malignancy. With increasing lifespan, the incidence of prostate cancer is on the rise. In the USA alone, every hour of each day in 2016, >25 new cases of prostate cancer were identified and more than three men died from prostate
cancer [3–5]. Prostate cancer is a heterogeneous disease in its presentation, biochemistry and even in its histology. Despite the recent advances in understanding of the molecular basis of the origin of prostate cancer, its diagnosis by non-invasive or minimally invasive methods has continued to be challenging. With the serum PSA test there is a considerable controversy, with no consistent recommendations from major medical organisations with the best approach to screening [5,6]. DRE remains a commonly performed physical examination but is unreliable and accurate diagnosis of prostate cancer requires histological identification of cancer cells in invasive prostate biopsy tissue. More than 66% of prostate biopsy procedures reveal benign pathology without evidence of malignancy [7–9].

The era of molecular profiling has led investigators to new discoveries in the field of prostate cancer. One minimally invasive diagnostic test approved recently by the USA Food and Drug Administration (FDA) is the urinary prostate cancer antigen 3 (PCA3) multiplex gene test. It targets a prostate cancer molecular signature, and requires that urine must be collected after prostate massage performed in a specific manner during DRE [10]. The PCA3 sensitivity ranged from 62% to 94%, specificity from 37% to 99%, positive predictive value from 42% to 98%, and negative predictive value was 36–96%. As a result, the predictive accuracy of PCA3 test has been questioned and its clinical utility when examined in the context of its high cost is considered controversial [10].

Another relatively recent test, now performed by the commercial OPKO Laboratory (Nashville, TN, USA) is the 4Kscore® test. Based on measurements of specific kallikrein markers in blood serum of patients, the test determines the serum concentration of: (i) total PSA, (ii) free PSA, (iii) intact PSA and (iv) human kallikrein (hK2). Although early results of the 4Kscore test appear promising, its general clinical acceptance as a prostate cancer screening test remains undetermined [11]. Therefore, there is a clear need for a non-invasive examination that will improve accurate detection of prostate cancer.

Over the past few years, we have targeted VPAC1 genomic receptors that are overexpressed on the surface of malignant cells at the onset of cancers such as those of the breast, prostate, and lung. The human VPAC1 receptor, named for the combined vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) family of cell surface receptors, encodes a G protein-coupled receptor that recognises with high affinity both VIP- and PACAP-related peptides. It has been shown that VPAC1 receptors are expressed in men with prostate cancer irrespective of their hormonal status (68/68 men with prostate cancer, 100%) including 15 men with metastatic lesions [12]. High expression of VPAC1 receptors (10^4/cell) on prostate cancer cells has been confirmed by others [13–15]. Both VIP and PACAP have high affinity for VPAC (VPAC1 and VPAC2) receptors. VIP is a 28-amino acid peptide initially isolated from porcine intestine [16]. VIP, whose structure is conserved in humans, pigs and rats, is a hydrophobic, basic peptide containing three lysines (no. 15, 20 and 21) and two arginines (no. 12 and 14). From the essential histidine residue at the N-terminus to the amidated C-terminus, all 28 amino acids of VIP are required for high affinity binding and biological activity [17].

Using a 64Cu-labelled VPAC1-specific peptide designed in our laboratories, we imaged breast and prostate cancers in humans with >97% sensitivity [18,19]. Encouraged by these results, we hypothesised that cells shed in voided urine of patients with prostate cancer could be imaged optically, by targeting the VPAC1 receptors with the same peptide labelled with a fluorophore. We report on a pilot feasibility study examining VPAC on shed urinary cells.

Patients/Subjects and Methods

Peptide Synthesis

To validate the hypothesis, the peptide was synthesised on solid state TG Sieber resin as described previously with added cystein at the N-terminus [20–23]. The peptide was cleaved off the resin, purified by HPLC, and purity was examined by matrix-assisted laser desorption ionisation (MALDI) spectroscopy. To the peptide dissolved in 1:1 water acetonitrile was added as a maleimide moiety, near infrared fluorophore PSVue 794, (MTTI, Westchester, PA, USA), in 1:1 molar ratio. The pH was adjusted to 8 and stirred at 22 °C for 2 h. After HPLC purification, fractions were collected, lyophilised and characterised using MALDI spectroscopy. The product, with >98% purity was named TP4303 after its molecular weight 4 303 (observed 4 304). The Kd value (dissociation constant, the molar concentration of radioligand which, at equilibrium, occupies 50% of the receptors) for such a sequence of peptide was determined to be 3.3 × 10^-9 M [22,23]. TP4303 was dissolved in deionised water and 0.5 mg/mL fractions were stored at −80 °C until use.

Patient Population

From patients (men and women), aged ≥21 years (207 patients, 176 men and 31 women) presenting to the Urology clinic at our institution, 10–20 mL of discarded, de-identified urine was obtained for this pilot investigation on an Institutional Review Board (IRB) exemption waiver. If the protocol processing did not begin within 4 h of urine collection the samples were stored at −10 °C for up to 72 h. For up to 4 h the samples were kept at 22 °C and processed at 22 °C.
Processing Urine Sample

No patient health information was provided to the laboratory personnel. The samples were identified only by the patient gender, date of collection, and presenting diagnosis.

Samples were centrifuged at 2 000g for 10 min and all but ~250 µL of supernatant was discarded. The cells were then suspended, and cytocentrifuged (Shandon Cytospin 4; Thermo Fisher Scientific, Philadelphia, PA, USA), and fixed in 97% ethyl alcohol. TP4303 solution (0.5 µg) was added to the cells to cover the entire cell area, ~1 cm in diameter. The slide was then kept in dark, at 22 °C for ~20 min and then thoroughly rinsed with deionised water and air dried. The slide was then kept in dark, at 22 °C for ~20 min and then thoroughly rinsed with deionised water and air dried. On the cells was then added, 20 µL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Fisher Scientific, Philadelphia, PA, USA), which strongly binds to A–T rich region of DNA in the cell nucleus. A coverslip was then placed and slide was observed using an inverted confocal microscope (excitation: 630 nm, emission: 730 nm; LSM 510 Carl Zeiss, Oberkochen, Germany). Cells with TP4303 interaction presented themselves with dark orange fluorescence around the nucleus and thereby indicated the presence of VPAC receptor molecules around the cell surface. In the absence of VPAC receptors, only the DAPI-bound cell nucleus was seen in dark blue. Normal epithelial cells that may only have minimal or no expression of VPAC therefore do not interact with TP4303 and show only the cell nucleus.

Statistical Analysis

As the assay determines only 'yes' (orange fluorescence positive) or 'no' (orange fluorescence negative) malignant cells, no area under the curve analysis was performed. Instead for these relatively small sample sizes, Clopper-Pearson exact 95% CIs were computed for sensitivity and specificity.

Validating the Presence of VPAC Receptors and Cell Malignancy

Validation that TP4303 truly targeted VPAC receptors

Human prostate cancer cell line PC3 was obtained from the American Type Culture Collection (ATCC) and grown in tissue culture (10% fetal bovine serum [FBS] containing Roswell Park Memorial Institute medium [RPMI] 1 640). The confluent cells were detached, washed with 0.9% PBS, 10 × 10^5 cells were suspended in 1 mL PBS and cytocentrifuged on two glass slides A and B. The cells on slides were then fixed with 97% ethanol and to the cells on slide A, VPAC receptor-specific peptide (10 µg) that was not conjugated with the fluorophore was added and incubated for 10 min. The slide was washed, air dried and 0.5 µg of TP4303 was added to the cells on both slides, A and B, incubated for 20 min and prepared similarly for microscopic examination.

Fluorescence-activated cell sorting

To further show that VPAC receptors are truly targeted by TP4303 and that they are cell surface receptors, PC3 cells were subjected to fluorescence-activated cell sorting (FACS) analysis before and after receptor blocking. PC3 cells were grown as stated above, washed with PBS and fixed with 97% ethanol. In three separate test tubes (A, B, C), ~50 × 10^3 cells were dispensed. Cells in A, served as controls, cells in B were incubated with 2 µg TP4303 for 10 min, washed with PBS and subjected to (APC-CY7) FACS using excitation 750 nm and emission 810 nm filters. Cells in test tube C were incubated first for 10 min, with 10 µg of VPAC receptor-specific peptide that was not conjugated with the fluorophore washed, and then incubated for 10 min with 2 µg TP4303. The cells were then washed with PBS and subjected to FACS as for cells in B. Cells in A were also analysed by FACS similarly.

Validation that the VPAC1 expressing cells were malignant

The purpose was to ascertain that the cells that do express VPAC1 receptors and to which TP4303 selectively binds, are true malignant prostate cancer cells. Prostate cancer is heterogeneous disease and the molecular basis of it’s clinical, pathological and genetic heterogeneity remains poorly understood. However, to determine the cell malignancy, we chose to examine mutation in gene expression on these cells, as compared to the genes in urine cells, collected from young normal males. Non-DRE urine was collected from four patients diagnosed with prostate cancer, and from three normal male volunteers. Urine was centrifuged for 10 min at 2 000g, cells were fixed, washed with PBS and suspended in 0.5 mL PBS. To the suspension was added 1 µg TP4303, incubated at 25 °C for 10 min, cells were washed and re-suspended. The cells were then subjected to orange FACS (Becton Dickinson, Franklin Lakes, NJ, USA).

A pure cell population with TP4303 orange fluorescence was selectively collected, centrifuged, and the supernatant was discarded. To the cells was then added 250 µL of cell lysis buffer provided with the Norgen Biotek RNA purification kit. Following the kit procedure step-by-step, RNA was extracted from the cells and quantified using a nanodrop ND-100 spectrophotometer followed by RNA quality assessment analysis using Agilent 2200 Tapestation (Agilent Technologies, Palo Alto, CA, USA). Using pure RNA, and GeneChip WT Pico Kit (Affimax, Santa Clara, CA, USA) fragmented biotin-labelled complementary DNA (cDNA) was synthesised. Affymetrix gene chips, Human Transcriptome Array 2.0
failures were due to hypercalciuria or sediment. The deposit
was similar to those in tumours were seen in the blood of a
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Table 1 Urine analysis (N = 207).

<table>
<thead>
<tr>
<th>Presenting diagnosis</th>
<th>Total number</th>
<th>VPAC positive</th>
<th>VPAC negative</th>
<th>Technically unclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH (10 men)</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
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<tr>
<td>Prostate cancer (141 men)</td>
<td>141</td>
<td>139</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non-oncology controls (25 men and 31 women)</td>
<td>56</td>
<td>11</td>
<td>35</td>
<td>10</td>
</tr>
</tbody>
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growing tumour, sheds nearly 0.4% \((3.4 \times 10^6\) cells\) every 24 h \([27]\). Parts of these cells, in the case of prostate cancer, through prostatic ducts, pass into the urine, 15–20 mL of which we used upon natural voiding without performing DRE.

At the time of this writing, urine samples studied from >141 patients with prostate cancer have correctly identified prostate cancer cells with 98.5% sensitivity. In urine from 10 patients with BPH, no prostate cancer cells were detectable. We have further validated our present data by (i) receptor blocking studies using VPAC-expressing human prostate cancer cells \(\text{PC}_3\), and (ii) demonstrated that these genomic receptors are expressed on the cell surface. Furthermore, we performed prostate cancer gene expression profiling studies using VPAC-expressing malignant cells, separated from voided urine of patients with known prostate cancer. These data strongly showed that not only did TP4303 specifically target, cell surface VPAC receptors but also confirmed the cells we identified as prostate cancer cells, were truly malignant.
The VPAC genomic biomarker belongs to the superfamily of G-protein-coupled surface receptors, which are expressed in high density \((10^4 – 10^5/cell)\) at the onset of oncogenesis, and prior to the alterations in cell morphology [12–14]. VPAC receptors are also expressed in breast and lung cancer [12]. On stroma, normal cells and benign masses, VPAC1 receptors are minimally present [15,28].

The VPAC1 receptor-specific peptide was designed and thoroughly evaluated in our laboratory [18–23]. When labelled with a positron-emitting radionuclide \(^{64}\text{Cu} (\beta^+ 19\%\), half-life 12.7 h), it permits us to image spontaneously grown breast cancer in mouse mammary tumour virus (MMTV)-nue transgenic mice, and spontaneously grown prostate cancer in transgenic adenocarcinoma of mouse prostate...
(TRAMP) mice [20–23]. These findings have now been transformed into successful imaging of breast and prostate cancers in humans [18,19]. This agent is named Cu-64-TP305. These investigations showed, the ability of the peptide to image with high sensitivity only malignant lesions, but not benign masses and prompted us to hypothesise that the VPAC1 receptor-specific peptide, covalently bound to a fluorophore (TP4303), would allow us to optically image prostate cancer cells, but not the normal epithelial cells shed in voided urine.

Fig. 3 This figure demonstrates VPAC1 receptor specificity of TP4303. As described in the text, the two separate images shown in A and B, VPAC1 receptors of the human prostate cancer, PC3 cells were blocked (pre-separated) using TP4303 without the fluorophore attached the TP4303 molecules. This resulted in seeing only the cell nucleus (blue) but no orange fluorescence around them. In image C and D, were the PC3 cells in which the receptors were not blocked, and incubated with fluorescence labeled TP4303 show the customary, orange fluorescence indicating the presence of VPAC receptors.
The present feasibility study was performed under an IRB exempt waiver using discarded urine specimens from de-identified subjects with limited clinical information. The study therefore restricts us to rigorously evaluate the data statistically. However, the study with its sensitivity for prostate cancer ($N = 140$) detection was 99.3% (CI 96.1–100%), and specificity for BPH ($N = 10$) was 100% (CI 69.2–100%) validates our hypothesis that prostate cancer malignant cells shed in voided urine without prostate stimulation, can be correctly identified for a reliable detection of prostate cancer. Although the numbers of observations in the present pilot study are limited, the results warrant a further evaluation to scrutinise if it could serve as a much needed simple and reliable assay to detect prostate cancer non-invasively. Our long-term goal therefore is to: (i) fully and statistically validate our hypothesis in patients known to have elevated PSA levels and prostate cancer as confirmed by histology, (ii) substantiate our initial observations in normal volunteers (aged $\geq 21$ years) and those with BPH. Furthermore, the goal (iii) is to determine a quantitative threshold above which a physician can distinguish with $>95\%$ confidence that a patient with elevated PSA levels has either prostate cancer or BPH. Our long-term goal is to minimise the number of unnecessary invasive biopsies, sparing the patient from anxiety and trauma and to reduce the healthcare expenditure.

As lifespan continues to increase, the number of prostate cancer cases will increase dramatically in the ageing population worldwide. Today, for diagnosis of prostate cancer however, histology remains a ‘gold standard’ that requires invasive biopsy, which is morbid, and 66% of the time finds benign pathology.

Although great strides have been made in detecting CTCs and urinary proteins, to diagnose prostate cancer, the tests are either cost prohibitive, controversial or have not yet to become widely acceptable for routine clinical practice. Due to the rarity of CTC ($10^{-4}–100$ malignant-cells/mL blood) the detection, separation, and characterisation of CTC remains a major challenge. Furthermore, the most commonly used method for identifying CTC is based upon enumeration of epithelial cells using anti-epithelial cell adhesion molecule (EpCAM) antibody and subsequent staining for cell visualisation. However, $\sim 40\%$ of the cancers have low EpCAM expression and 20% of cancers are EpCAM negative. These parameters lead to low sensitivity of CTC detection [29]. There remains therefore an unmet need for a simple and reliable test that can (i) screen a patient, (ii) serve as a diagnostic test, (iii) reduce the number of unnecessary

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**Fig. 4** Fluorescence-activated cell sorting (FACS) analysis of human prostate cancer, PC$_3$ cells (A control) depicts that 100% of the PC$_3$ cells express VPAC cell surface receptors (B), and that these receptors can be selectively blocked by the receptors specific peptide TP4303 (C).

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biopsies that can minimise patient morbidity and decrease the healthcare cost, and (iv) play a vital role in the determination of the effectiveness of therapeutic intervention.

We believe that the present optical imaging assay promises to be simple, innovative, and specific for detecting VPAC1 expressing malignant prostate cancer cells and shows a potential to address the three, above stated important parameters needed for the management of genitourinary conditions of the ageing population in North America and beyond.

As this optical urinary assay requires only voided urine and does not need any patient physical contact, the assay has a fair probability to be of a clinical use provided that the results of the current evaluation continues to demonstrate >95% sensitivity and ability to distinguish between malignant and benign conditions. Once the urine optical imaging is positive, further VPAC-based imaging tests can be performed to localise the disease.

VPAC receptors are known to express in many solid tumours, such as those of the breast, lung and colorectal. Success of such an optical screening liquid biopsy test may pave the way to detect shed malignant cells in nipple discharge to detect breast cancer, lung lavage to determine lung cancer, saliva to screen cancers of the head and neck, and stool sample to detect colorectal cancer to name a few.

**Conclusion**

In patients with prostate cancer, malignant cells are shed in voided urine. These cells express VPAC receptors. Targeting
VPAC receptors with high sensitivity, for optical imaging of these cells, may represent a novel diagnostic assay for an early, accurate and simple non-invasive detection of these malignancies. Such an assay once fully validated may play a vital role in the management of prostate cancer.

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Author Contributions

M.L. Thakur conceived, demonstrated and established that VPAC genomic receptors can be targeted for scintigraphic and optical imaging of oncological diseases. M.L. Thakur and L. Gomella conceptualised that prostate cancer cells shed in urine can be imaged by targeting VPAC receptors. M.L. Thakur gathered, coordinated the team of investigators and designed the protocol. S.K. Tripathi performed all the laboratory work including RNA extraction. M.L. Thakur and S.K. Tripathi blinded to patient condition analysed the data and presented the data for validation, to L. Gomella and E.J. Trabulsi, who made the urine samples available. C. Solomides facilitated cytospun urine slides and participated in frequent discussion of results. E. Wickstrom supervised synthesis of TP4303. M. L. Thakur and E.J. Trabulsi wrote the manuscript.

Conflicts of Interest

M.L. Thakur and L. Gomella hold a pending patent through their employer, Thomas Jefferson University. At the time of this submission, M.L. Thakur is a consultant to NuView Life Sciences Inc. The other authors disclose no current conflict of interest.

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Abbreviations: cDNA, complementary DNA; CTC, circulating tumour cells; DAPI, 4,6 dimidino-2-phenylindole, dihydrochloride; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; IRB, Institutional Review Board; MALDI, matrix-assisted laser desorption ionisation; PACAP, pituitary adenylate cyclase-activating peptide; PCA3, prostate cancer antigen 3; VIP, vasoactive intestinal peptide.

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