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Prostate-based biofluids for the detection of prostate cancer: A comparative study of the diagnostic performance of cell-sourced RNA biomarkers

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ABSTRACT

Background: Prostate cancer (PCa) diagnosis requires improvement with the aid of more accurate biomarkers. Postejaculate urethral washings (PEUW) could be a physiological equivalent to urine obtained following rectal prostatic massage, the current basis for the prostate cancer antigen 3 (PCA3) test. The aim of this study was to investigate if PEUW contained prostate-based material, evidenced by the presence of prostate specific antigen (PSA), and to evaluate the diagnostic performance of PEUW-based biomarkers.

Methods: Male patients referred for elevated serum PSA or abnormal digital rectal examination provided ejaculate and PEUW samples. PSA, PCA3, and β2-microglobulin (β2M) were quantified in ejaculate and PEUW and compared with absolute and clinically significant (according to D'Amico criteria) PCa presence, as determined by biopsies. Diagnostic performance was determined and compared with serum PSA using receiver operating characteristic analysis.

Results: From 83 patients who provided PEUW samples, paired analysis with ejaculate samples was possible for 38 patients, while analysis in an unpaired, extended cohort was possible for 62 patients. PSA and PCA3 were detected in PEUW, normalized to β2M, and PCA3:PSA was calculated. In predicting absolute PCa status, PCA3:β2M in ejaculate [area under the curve (AUC) 0.717] and PEUW (AUC 0.569) were insignificantly better than PCA3:PSA (AUC 0.668 and 0.431, respectively) and comparable with serum PSA (AUC 0.617) with similar trends observed for the extended cohort. When considering clinically significant PCa presence, serum PSA in the comparison (AUC 0.640) and extended cohorts (AUC 0.665) was comparable with PCA3:β2M (AUC 0.667) and PCA3:PSA (AUC 0.605) in ejaculate, with lower estimates for PEUW in the comparison (PCA3:β2M AUC 0.496; PCA3:PSA AUC 0.342) and extended (PCA3:β2M AUC 0.497; PCA3:PSA AUC 0.469) cohorts. The statistical analysis was limited by sample size.

Conclusion: PEUW contains prostatic material, but has limited diagnostic accuracy when considering cell-derived DNA analysis. PCA3-based markers in ejaculate are comparable to serum PSA and digital rectal examination–urine markers.

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1. Introduction

The detection of prostate cancer (PCa) is fraught with difficulties that include limitations of currently available biomarkers, access to imaging and tissue sampling. Total serum prostate specific antigen (PSA) is currently the single most widely used marker clinically for identifying men at risk of PCa, but it is a nonspecific indicator of prostatic pathology that includes PCa among others so that population and opportunistic screening is discouraged due to over-investigation and over-detection, resulting in overtreatment. Contemporary PCa detection approaches have included, amongst others, advocacy for a biomarker panel, the Prostate Health Index and the 4-kallikrein protein test.

Although imaging modalities, such as multiparametric magnetic resonance imaging and even prostate specific membrane antigen (PSMA) positron emission tomography, are being integrated into the detection strategy for triaging patients with an elevated PSA and may improve detection of clinically significant PCa, there are limitations that detract from their widespread use. The false negative rate (~15–20%) for multiparametric magnetic resonance imaging suggests a significant proportion of clinically significant tumors may be missed, which is also observed with PSMA-positron emission imaging of tumors that do not express PSMA. That imaging is establishing a niche in detection strategies is undeniable; however, it is the cost of such imaging methods that really limits application to mainstream clinical practice. Improved patient selection for imaging with accurate biomarkers is likely to optimize their practical application clinically.

Prostate-specific biofluids are an ongoing source for investigation using new analytical platforms. Prostate cancer antigen 3 (PCA3), a long noncoding RNA, collected in the first void of urine following a vigorous digital rectal examination (DRE) or prostatic massage, has been examined over the past 20 years, with studies reporting to improve detection in men undergoing repeat biopsy, but its role clinically remains uncertain. Modifications of PCA3 as well as a combination with TMPRSS2-ERG fusion gene have been described to improve detection but have not been accepted as a useful addition in routine patient testing. Other approaches utilizing exosomes, proteomics, and metabolomics have the potential to improve early diagnosis of localized disease. Indeed molecular and metabolomic markers in ejaculate or seminal fluid have been reported to improve diagnosis compared to serum PSA. Indeed the postmassage urine concept, collection of urine following ejaculation, or postejaculate urethral washings (PEUW) potentially represents a new source of prostate-specific biomarkers for PCa detection and characterization, providing a physiologically produced alternative to serum or digital prostatic massage effluent for further biomarker discovery and development.

The aim of this study was to investigate the diagnostic potential of selected molecular markers in PEUW in detecting PCa and comparing diagnostic accuracy with the same markers in ejaculate, or postejaculate urethral washings (PEUW). The hypothesis tested was that the diagnostic performance of molecular markers in postejaculate urine would be comparable with those in ejaculate or serum PSA.

2. Materials and methods

2.1. Patients

A clinical cohort of men (n = 83) being investigated for PCa on the basis of an abnormal DRE and/or elevated serum PSA provided specimens of ejaculate and PEUW into sterile micro-ureine jars, containing 20 mL Hanks’ Balanced Salt Solution (Gibco, Life Technologies Australia Pty Ltd, Scoresby, Victoria, Australia) and empty urine jars, respectively, between January 2007 and December 2009. As previously reported, all specimens were processed within 2 hours of production after being delivered to the hospital campus without cooling. All specimens were collected prior to or at least 1 month following transrectal ultrasound guided biopsy (TRUSbx) or transperineal template biopsy (TPBx).

Ethical approval to conduct this study was obtained from the University of Queensland Medical Research Ethics Committee, Brisbane, Australia (Project No. 2006000262) and the Royal Brisbane and Women’s Hospital, Human Research Ethics Committee, Brisbane, Australia (HREC/09/QRBW/320, HREC/09/QRBW/305 together with 1995/088B).

2.2. Clinical data

All patient data were prospectively collected following recruitment and included clinical details such as age, family history, and serum PSA. Initial and updated TRUSbx/TPBx/radical prostatectomy (RP) histology specimens were reviewed by D.P., J.P.-K., and H.S. and reported according to the 2005 International Society of Urological Pathology classification, including standard biopsy (number of cores taken, number and percentage of positive cores, Gleason score) and RP (gland size, Gleason score, pathological stage, extracapsular status, and margin status) parameters.

In order to identify the patients for whom active treatment would be recommended, risk stratification for biopsies in determining clinically significant PCa presence was performed using the D’Amico criteria recommended in the American Urological Association Guidelines. The clinically significant PCa category included patients defined as intermediate- and high-risk according to the D’Amico criteria, while the absence of clinically significant PCa was defined as low risk patients according to the D’Amico criteria or those without PCa. The most accurate classification of clinically significant PCa, based on histopathology from TRUSbx, TPBx, and/or RP, was used given established disparity between TRUSbx and RP histopathology. As previously reported, patients were subsequently placed in one of two clinical groups based on classification scheme used. Because of the imprecise nature of TRUSbx in particular, patient follow-up was pursued for up to 7 years to ensure that those designated as negative for prostate cancer really were negative.

2.3. Specimen processing and cellular isolation

Ejaculate specimens combined with 20 mL Hanks’ Balanced Salt Solution were layered over 10 mL isotonic Percoll (GE Healthcare–Pharmacia) and centrifuged at 974 g for 30–60 minutes at 4 °C, with supernatants subsequently collected in 1 mL aliquots, snap-frozen on dry ice and stored at –80 °C. The epithelial cell layer at the Percoll interface, present as a discrete band suspended between supernatant above and sperm and noncellular components below, was then pipetted, washed with 25 mL phosphate-buffered saline or Hanks’ and centrifuged at 1,258 g for 10 minutes at 4 °C.

2.4. RNA preparation

Total RNA from collected cells was isolated using TRIzol reagent (Invitrogen) then subjected to on-column DNase treatment and clean-up with the RNeasy kit (Qiagen). Low yield samples were amplified using the SenseAmp kit (Gensisphere).

2.5. cDNA synthesis and quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qPCR) was undertaken using the QIAGEN Quantitect SYBR green
qPCR Mastermix (QIAGEN, Germany) on a Corbett Rotorgene machine 3000/6000 (Corbett Research, Australia). cDNA synthesis was performed with 200–500 ng of total RNA reverse transcribed using Superscript III (Invitrogen) and random hexamer primers (Promega). The synthesized cDNA was diluted 10-fold and 5 μL was used for the assay in the presence of 7.5 μL Quantitect SYBR green mastermix (Qiagen) and 5 pmol gene specific forward and reverse primers. Each reaction was performed in triplicate for both patient samples and calibrator. Reaction conditions were 95°C for 15 minutes followed by 45 cycles of 20 seconds at 95°C, 20 seconds at 58°C, and 20 seconds at 72°C. Data for each cycle were acquired at the 72°C step.

The genes that were characterized were β2-microglobulin (β2M), PCA3, and PSA using the following primers (Sigma-Aldrich, Australia) for qPCR: β2M (forward: 5’-AGCAGGAGATGAAAGTCAAA-3’, reverse: 5’TGGCTGCAACATCTCTCG-3’); PCA3 (forward: 5’-GGAAGGACCCTGATGATACAGAGGTGAG-3’, reverse: 5’-CACAGGGCGAGGTCATCG-3’); PSA (forward: 5’-CATCAGGAAACAAAGCTG-3’, reverse: 5’-CTGAGGGAATCGATTCTTCA-3’).

Standardized processing (including standard curve fitting, dynamic tube, slope correct) was performed for all runs using Rotorgene 6000 Series Software version 1.7 (Corbett Research, Australia). To maintain quality control, specimens with atypical melt curves or quantitation curves below threshold for any single target (with normal melt curves) were excluded. A standard threshold value was manually set for each individual gene across all samples and used to calculate cycle threshold (Ct) values, which were exported to Excel (Microsoft Corporation, Redmond, WA, USA). Based on average Ct (CtAv) and standard deviation (CtSD) values of triplicates observed across all genes (see Fig. S1), cutoff points defined for exclusion were CtAv > 35 and CtSD > 1.5 for PEUW and ejaculate, as previously described. Analysis of PSA, PCA3, and β2M in ejaculate and PEUW specimens was possible for 38 patients, with these markers detected in PEUW only from 62 patients.

2.6. Relative gene expression determination

Relative gene expression was calculated using the method previously described by Pfaffl, which uses the following equation:

\[
\text{Expression (R)} = \frac{E^{ΔCt}_{GOI}(\text{Calibrator}–\text{sample})}{E^{ΔCt}_{RG}(\text{Calibrator}–\text{sample})}
\]

\(ΔCt\) (Calibrator-sample) estimates the amplification (Ct values) difference between the calibrator reaction (uniform template quantity to standardize all runs) and the target gene transcription of the sample reaction (unknown). The reaction efficiency (E) of the gene of interest (GOI) and endogenous reference gene (RG) are considered without the required use of a standard curve in every run, based on the assumption that reaction efficiency between different runs was consistent and normalized by the calibrator used.

2.7. Reference gene variation

β2M, a known housekeeping gene, was used as the endogenous reference gene with subsequent relative gene expression calculated for PSA and PCA3. The commercial use of PCA3, which, unlike PSA, is highly overexpressed in prostate cancer, requires PSA to be used as the reference gene on the basis of reports that PSA expression is relatively constant and considers only cells of prostatic origin so the expression of PCA3 relative to PSA was also calculated.

2.8. Data analysis

Relative gene expression results were analyzed considering two clinical classifications, absolute PCa status (cancer vs. no cancer) and clinically significant (present vs. absent) PCa status, as determined using the D’Amico classification. Univariate analysis was conducted with the nonparametric Mann–Whitney U test.

Receiver operating characteristic (ROC) analysis was performed for each marker and compared to each other and serum PSA, with binomial exact confidence interval and optimal cut points for each marker calculated in Stata Statistical Software 13 (StataCorp. College Station, TX, USA) using the Liu method. Significance thresholds were Bonferroni corrected (<= 0.05) to adjust for multiple comparisons (n = 4), and all reported P values <0.0125 were considered statistically significant.

3. Results

3.1. Clinical characteristics

From 83 potential patients with adequate clinical data who donated PEUW samples between January 2007 and December 2009, relative gene expression determination for PSA, PCA3, and β2M was performed. Strict exclusion criteria (see Fig. S2) were applied, resulting in sample exclusion due to unsatisfactory qPCR analyses (atypical melt or quantitation curves, n = 1), insufficient cDNA to reach detection threshold (n = 5), CtAv or CtSD outside determined cutoffs (n = 14) or coupled with an ejaculate sample excluded for a similar reason (n = 25). Of the remaining 38 patients, the relationships observed for the entire cohort with respect to median (interquartile range) age [62 (57–68)] years and serum PSA [6.7 (4.75–9.15)] ng/mL were preserved in this group, with a median age [62 (57–69)] years (P < 0.0125) and serum PSA 6.3 (4.9–8.9) ng/mL (P > 0.0125). Within the included 38 patients were 25 participants having a prostate biopsy positive for cancer, with 21 deemed to have clinically significant PCa.

The demographic information of the cohort including median (interquartile range) age, serum PSA, and relative expression ratios of RNA markers for each group, absolute and clinically significant PCa presence, are presented in Tables 1 and 2, respectively. Men were of comparable ages in each classification group. Serum PSA was similar for men diagnosed with PCa compared with men in the no PCa group. This relationship was preserved for serum PSA when comparing men with clinically significant PCa against those without clinically significant PCa. Similar trends were observed in the expanded cohort, which comprised 62 patients with 36 participants with a positive prostate biopsy and 30 deemed to have clinically significant PCa (Tables 1 and 2).

3.2. Biomarker performance

3.2.1. Absolute PCa status

When considering absolute PCa status (Table 1) and compared to a chance area under the curve (AUC) of 0.500, serum PSA (AUC 0.617; P = 0.217) and PSA:β2M (AUC = 0.600; P = 0.353) in PEUW samples provided similar diagnostic performance. PCA3:β2M (AUC = 0.569; P = 0.522) and PCA3:PSA (AUC = 0.431; P = 0.528) in PEUW demonstrated inferior predictive ability. Similar results were not observed in the expanded cohort, with serum PSA (AUC = 0.610, P = 0.128) performing better than PSA:β2M (AUC = 0.506; P = 0.935), PCA3:β2M (AUC = 0.550; P = 0.531), and PCA3:PSA (AUC = 0.529; P = 728).
Median and interquartile range are shown for age and each marker in postejaculate urethral washing and ejaculate samples. All comparisons using the Mann-Whitney U test (2-tailed; using a Bonferroni corrected threshold) were non-significant (P > 0.0125).

In ejaculate, best diagnostic performance was observed for PCA3:PSA (AUC = 0.717; P = 0.033), followed by PCA3:PSA (AUC = 0.468; P = 0.078), followed by less impressive performance for serum PSA (AUC = 0.617; P = 0.217), and poor performance of PSA:PSA (AUC = 0.486; P = 0.895).

### 3.2.2. Clinically significant PCa

When considering clinically significant PCa (Table 2), similar performance was observed for serum PSA (AUC = 0.640; P = 0.124), PSA:PSA (AUC = 0.608; P = 0.269), and PCA3:PSA (AUC = 0.342; P = 0.093). The performance of PCA3:PSA (AUC = 0.496; P = 0.966) was poorer and less predictive of clinically significant PCa. Within the expanded cohort, only serum PSA (AUC = 0.665; P = 0.018) performed as well, with poorer performance for the PEUW-based PSA:PSA (AUC = 0.525; P = 0.740), PCA3:PSA (AUC = 0.503; P = 0.967), and PCA3:PSA (AUC = 0.469; P = 0.681).

In ejaculate samples, PCA3:PSA (AUC = 0.667; P = 0.083) was similar to serum PSA, and PCA3:PSA (AUC = 0.605; P = 0.263). The performance of PSA:PSA (AUC = 0.521; P = 0.828) was less impressive. For graphical purposes, comparison ROC curves are available in Figs. S3 and S4 including the comparison P values against the serum PSA AUC.

### 4. Discussion

PEUW could potentially be a new source of prostate-specific biomarkers for PCa detection and characterization, providing an alternative to serum and urine for further biomarker discovery and development. Thus, we investigated in this study the utility of prostatic cells in PEUW as a physiological source of PCa biomarkers. We have shown that the diagnostic performance of the mRNA-based marker PCA3, normalized to PSA or J2M, in PEUW is likely to be inferior to these markers in ejaculate, which were comparable to serum PSA. Overall, the performance is similar to PCA3 in post-massage urine in isolation (AUC 0.62),23 as well as in ejaculate in an expanded cohort (AUC 0.625).22 PCA3 performed best in this cohort in detecting absolute PCa status compared with clinically significant PCa, in accordance with previous reports.23 This preliminary investigation helps to build on current PCa biomarker research literature.

The use of PEUW as a prostate-based biofluid is advantageous for a number of reasons. First, it contains prostatic effluent following ejaculation, indicated here by the presence of PSA. In addition, there is no requirement for patient discomfort, in contrast to prostate massage. Furthermore, there is the potential for tumor disruption and dissemination of malignant cells.24
given known elevations in serum PSA after TRUSbx and DRE. Reports regarding serum PSA elevation after ejaculation are mixed, with levels reported to return to normal after 48 hours, thus PEUW following global contraction of the prostate gland with ejaculation can be considered a physiological equivalent of nonphysiological postmassage urine. PEUW sampling enables postcoital donation, which may be more acceptable for some men and has been used to investigate infertility. The combination of urine and ejaculatory components in PEUW allows for assessment of markers reflecting local (ejaculate) and systemic (urine) pathology. While this enables use in clinical scenarios where systemic biological alterations are important to monitor, such as active surveillance and metastatic disease, it is also a potential drawback of PEUW, because the local pathology markers in the ejaculate component can be confounded by the systemic contributions from the urine component. These may contribute to the lower diagnostic performance of PEUW compared to ejaculate described here.

The benefits that we have previously outlined for the use of ejaculate as a prostate-based biofluid also apply to PEUW. Specifically, ejaculate contains malignant prostatic epithelial cells, with cell-derived molecular markers PCA3 and hepsin shown to be complementary biofluids diagnostically with PCA3 in post-massage urine. Analysis of microRNAs in cell-derived mRNA in ejaculate has been reported to improve PCA detection, with miR-200b combined with serum PSA (AUC = 0.751) significantly better than serum PSA alone (AUC = 0.555). We have previously reported the use of a composite score, created using contributions from serum PSA, and ejaculatory micro RNAs-125b, and -200c, to significantly improve PCa detection (AUC = 0.869) compared with serum PSA alone (AUC = 0.672; P < 0.05). The ability to provide an ejaculate sample may also indicate a favorable performance status and consequent survival benefit, with a high and significantly better overall and PCA-specific survival benefit observed for these patients at 10 years, 15 years, and 20 years. Incorporation of exosome and metabolome analysis may improve predictive accuracy using these non-invasively obtained biofluids, reducing anxiety and uncertainty for clinicians and patients managed by active surveillance, in addition to assisting with PCa testing. Use of PEUW may be more favorable than ejaculate as PEUW samples can be provided in the comfort of the home environment and postintercourse, a strategy more likely to be used by men than the sterile surroundings of the clinic setting. Similar to urine cytology, the sample could be stored overnight in a refrigerator and brought to the clinic the morning after, assuming RNA integrity is maintained.

The aim of this comparative study was to investigate the diagnostic potential of selected molecular markers in PEUWs in detecting PCa on the basis of the D’Amico classification benchmark, widely used to stratify in the past. However, the goal posts of clinicians and patients managed by active surveillance have been extended to include some Gleason 3 + 4 (ISUP 2) tumors regarded as favorable by the National Comprehensive Cancer Network (NCCN) for men with a life-expectancy < 10 years with this change supported by the American Society of Clinical Oncology. By inference, this means that not all intermediate-risk tumors can be considered as clinically significant. However, for the purpose of comparison, risk classifications such as that proposed by D’Amico remain relevant until the entity of clinically significant can be defined better.

The limitations of this preliminary, exploratory study include the small sample size resulting in large confidence intervals and low statistical power. Biologically, the potential for low ejaculatory contribution or dilution reducing the prostate-specific RNA yield may impair results. PCA3:PSA levels in PEUW were lower in men with PCa, both in the comparison and expanded cohorts, resulting in ROC estimates below 0.5, which was the inverse of that observed for ejaculate samples and other published reports. Although the sample drop-out rates were similar for PEUW and ejaculate, with positive PSA signals suggesting the presence of prostatic material in both sample sets, these results suggest malignant cells may dominate in the ejaculate. A potential explanation for this are changes in cell adhesion molecules, which have been recognized for some time in PCa, with most attention focused on E-cadherin. Loss of E-cadherin is particularly evident in more aggressive tumors with cadherin switching also recently described. Thus, disaggregated cells or cell clusters from aggressive tumors first appear in ejaculate as a result of global contraction of prostatic smooth muscle following accumulation in acini prior to ejaculation. This in turn may cause relatively fewer cancerous cells to be present in the urethra from the latter part of the ejaculate, which would be dislodged with subsequent voiding. As a result, PEUW may contain fewer cancerous cells but does contain prostate epithelial cells, evidenced here by the presence of PSA. Thus, in patients suspected to harbor nonaggressive PCa, the presence of prostatic cells in PEUW with low expression of PCA3 may help determine which patients have nonaggressive PCa amenable to active surveillance or watchful waiting.

In conclusion, we introduced and investigated PEUW as a physiological source of PCa biomarkers. We found that PEUW contains prostatic cells, as evidenced by PSA signal. However, significantly upregulated PCA3 levels, consistent with those reported for malignant tissue, were only observed in ejaculate specimens. PEUW may yet prove to be a useful source of cell-free secreted markers, as opposed to cell-derived markers. Further biomarker development using these prostate-specific biofluids may result in improved diagnosis and monitoring of PCa, reducing anxiety and doubt for the benefit of clinicians and patients.

Conflicts of interest

All authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.prnil.2016.04.002.

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