Prostate Cancer Biomarker Profiles in Urinary Sediments and Exosomes

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Abbreviations and Acronyms

BDL = below analytical detection limit

DNase = deoxyribonuclease

DRE = digital rectal examination

Pca = prostate cancer

PCA3 = Pca antigen 3

PCR = polymerase chain reaction

PSA = prostate specific antigen

TMPRSS2 = transmembrane protease serine 2

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Purpose: Urinary biomarker tests for diagnosing prostate cancer have gained considerable interest. Urine is a complex mixture that can be subfractionated. We evaluated 2 urinary fractions that contain nucleic acids, ie cell pellets and exosomes. The influence of digital rectal examination before urine collection was also studied and the prostate cancer specific biomarkers PCA3 and TMPRSS2-ERG were assayed.

Materials and Methods: Urine samples were prospectively obtained before and after digital rectal examination from 30 men scheduled for prostate biopsy. Cell pellet and exosomes were isolated and used for biomarker analysis. Analytical and diagnostic performance was tested using the Student t-test and ROC curves.

Results: Unlike the exosome fraction, urinary sediment gene expression analysis was compromised by amorphous precipitation in 10% of all specimens. Digital rectal examination resulted in increased mRNA levels in each fraction. This was particularly relevant for the exosomal fraction since after digital rectal examination the number of samples decreased in which cancer specific markers were below the analytical detection limit. Biomarker diagnostic performance was comparable to that in large clinical studies. In exosomes the biomarkers had to be normalized for prostate specific antigen mRNA while cell pellet absolute PCA3 levels had diagnostic value.

Conclusions: Exosomes have characteristics that enable them to serve as a stable substrate for biomarker analysis. Thus, digital rectal examination enhances the analytical performance of biomarker analysis in exosomes and cell pellets. The diagnostic performance of biomarkers in exosomes differs from that of cell pellets. Clinical usefulness must be prospectively assessed in larger clinical cohorts.

Key Words: prostate, prostatic neoplasms, biological markers, exosomes, gene expression

PROSTATE cancer is the most common malignancy in males in developed countries and the third leading cause of cancer related death in this population.¹ The gold standard of Pca diagnosis is based on histopathological

examination of prostate biopsies. The indication for prostate biopsies primarily relies on serum PSA and/or suspicious DRE. The introduction of serum PSA testing led to a considerable increase in the number of

prostate biopsies, which in turn led to an increase in the PCa incidence. Although serum PSA has low specificity for detecting PCa, it is currently the only biomarker used in clinical practice for PCa diagnosis. However, it does not differentiate between indolent and clinically significant PCa. Therefore, better diagnostic and monitoring tools are urgently needed.

A new biomarker should ideally meet certain criteria. It should be a noninvasive test that is produced by tumor tissue only and has the ability to detect PCa at an early stage. Thus, it should differentiate aggressive from indolent tumors with high specificity and sensitivity.

To date several urinary biomarkers for PCa have been investigated. In 1999 Bussemakers et al noted that the prostate specific noncoding RNA DD3, better known as PCA3, is highly over expressed in prostate tumor tissue.² Further research recently led to Food and Drug Administration approval of the commercially available PCA3 urine test, calculated as $\text{PCA3 mRNA/PSA mRNA} \times 1,000$, as a decision making aid for repeat biopsy. Another biomarker strongly associated with malignant prostate epithelial cells is the TMPRSS2-ERG gene fusion transcript.³ This androgen regulated gene fusion is found in almost 50% of patients with PCa and it is absent in nonPCa specimens.⁴ Each biomarker can be measured noninvasively in urinary samples.

Urinary samples can be a noninvasive substrate for biomarker analysis using various components of urine. In most previous studies biomarkers were analyzed in whole urine or urinary sediments. Recent findings revealed that small tissue derived vesicles called exosomes are a component of urine and contain a wide variety of proteins and RNAs that represent the tissue of origin.^{5,6} However, few groups have examined the role of these exosomes as a novel substrate for PCa biomarkers.⁷⁻⁹

Biomarker expression in urinary samples is expected to be higher after performing DRE, considering that prostate manipulation mobilizes cancer cells, if present, via the prostatic ductal system into the urethra. Subsequently, first catch urine contains the highest concentration of prostate secretions, including cells. This hypothesis was investigated in previous series.^{10,11} In a pilot study Nilsson et al noted that mild prostate manipulation increased exosomal secretion into the first catch urinary fraction.⁸

We further investigated urinary exosomes as a substrate for PCa biomarkers by assaying PCA3 and TMPRSS2-ERG in cell pellets and exosomes before and after DRE. PSA mRNA levels were determined for normalization as a prostate reference gene. The diagnostic value of PCA3 in exosomes

and cell pellets for biopsy outcome prediction was also evaluated.

MATERIALS AND METHODS

Data Collection

Urinary samples were prospectively taken from 30 patients at the outpatient clinic of 2 university hospitals in The Netherlands in an almost equal ratio. Approval was obtained from the institutional review boards in accordance with all medical ethical requirements. Patients were scheduled for prostate biopsies based on PSA (3 ng/ml or greater) and/or abnormal DRE. After obtaining written informed consent first catch urine collection was done without DRE. Standardized DRE was then performed with firm pressure to the prostate from base to apex and from the lateral to the medial side.¹² Directly after DRE a second first catch urine sample was collected and transrectal ultrasound guided prostate biopsies were obtained according to the local protocol (8 to 16 cores). Pathology results and all other clinical data were collected prospectively.

Cell Pellet and Exosome Isolation

Coded 50 ml transfer tubes containing 4 ml 0.5 M ethylenediaminetetraacetic acid were used for urine collection. After collection samples were immediately cooled and processed within 48 hours to maintain optimal sample quality. Analysis was done at a central laboratory.

Cell pellet and exosome isolation were performed according to a validated procedure.¹³ Cell pellets were separated from supernatant at $1,800 \times$ gravity for 10 minutes at 4C, washed twice with ice-cold buffered sodium chloride solution, snap frozen in liquid nitrogen and stored at -70°C . Cellular debris was removed from supernatant containing exosomes by centrifuging at $3,200 \times$ gravity for 90 minutes at 4C, followed by filtration using a 0.8 μm filter. The concentrate containing exosomes was obtained by filtration through a 100 kDa filter using a Vivaspin® centrifuge. The acquired exosome content was washed twice with ice-cold buffered sodium chloride solution, snap frozen in liquid nitrogen and stored at -70°C .

Real-Time PCR RNA Extraction and Gene Expression Analysis

RNA was extracted from exosomes and cell pellets using a modified TriPure® Reagent protocol (catalogue No. 11667165001). GlycoBlue™ (15 $\mu\text{g}/\mu\text{l}$, catalogue No. AM 9515) served as the carrier to co-precipitate RNA. RNA samples were treated with DNase for 10 minutes before the amplification protocol using DNase I enzyme (catalogue No. 18068-015, Invitrogen™).

Total RNA was used to generate amplified sense strand cDNA using the Whole Transcriptome Analysis Kit (catalogue No. 4411974, Ambion®) according to the manufacturer protocol. PSA, PCA3 and TMPRSS2-ERG expression levels were analyzed by quantitative real-time PCR, normalized to the amount of urine used and expressed in copies per ml. The supplementary table (<http://jurology.com/>) lists the designed primer pairs and hydrolysis probe sequences used. Two μl of each cDNA

Figure 1. Immunoelectron microscopy shows exosome isolated from urine by ultrafiltration. Black dots attached to exosome indicates exosomal surface marker CD63. Scale bar indicates 100 nm.

sample were amplified in a 20 μ l PCR reaction containing 10 μ mol of each primer, 2 μ mol hydrolysis probe and 1 $\times$ Probes Master mix (Roche, Indianapolis, Indiana). Control samples served as the referent. Amplification conditions were 95C for 10 minutes followed by 50 cycles at 95C for 10 seconds and 60C for 30 seconds with cooling at 40C for 55 seconds using a LightCycler® LC480. LightCycler 480 SW 1.5 software was used to determine crossing point values. Calibration curves with a wide linear dynamic range (10 to 1,000,000 copies) were generated using plasmid serial dilutions. We converted sample crossing point values to concentrations by extrapolation in the generated calibration curve.

The cutoff value for an adequate amount of prostate content was set at 1,000 copies of PSA mRNA.¹⁴ Copy numbers below this cutoff were assumed to contain an insufficient amount of prostate specific transcripts and be less accurate and reliable. Therefore, they were excluded from further diagnostic analysis. To test the analytical performance no exclusion was done based on the mentioned criteria since the amount of prostate specific transcripts measured could possibly have been influenced by DRE.

Electron Microscopy

Exosome isolation for electron microscopy imaging was done in 1 urine sample according to the mentioned methods. For immunoelectron microscopy exosome identification we used the protocol described by Lässer et al.¹⁵ Figure 1 shows a urinary exosome.

Statistical Analysis

Data were analyzed using SPSS®, version 18.0.2. To test for differences in biomarker expression before vs after DRE we used the paired sample t-test after log transformation of data. ROC curve analysis and the AUC were applied to calculate diagnostic accuracy.

RESULTS

Patient Characteristics

A total of 30 patients were included in study, from whom a total of 60 urinary samples were collected. For analysis we used a urinary sample obtained before and after DRE from each patient. All men subsequently underwent transrectal ultrasound guided prostate biopsies. PCa was found in the biopsy specimen in 14 patients (47%). Table 1 lists patient characteristics.

The amount of urine collected before DRE was similar to the amount collected after DRE (mean 36.8 and 39.2 ml, respectively, $p = 0.255$). In 3 samples (10%) before DRE and 3 (10%) after DRE no RNA was extracted from the cell pellet due to precipitation of impurities (crystals). Three samples (5%) did not fulfill the predetermined cutoff values for a sufficient amount of prostate specific transcripts, including 2 samples before and 1 after DRE. Another sample was lost in the RNA extraction process. For urinary exosomes no precipitation occurred. However, 9 samples (30%) before DRE and 6 (20%) after DRE did not fulfill the predetermined cutoff values for a sufficient amount of prostate specific transcripts. Therefore, for PCA3 mRNA diagnostic evaluation 25 post-DRE cell pellet samples and 24 post-DRE exosome samples remained. Table 2 lists the number of samples with mRNA levels below BDL and nonassessable samples. BDL was defined as PSA less than 1,000 copies per ml and PCA3 less than 10 copies per ml.

Table 1. Patient characteristics

	Prostate Ca		No Prostate Ca		Overall
No. pts (%)	14	(46.7)	16	(53.3)	30
Mean age (range)	67	(58–75)	65	(55–75)	66 (55–75)
Mean ng/ml serum PSA (range)	9.11	(4.2–32.6)	9.85	(2.9–27.7)	9.51 (2.9–32.6)
No. Gleason score (%):					
6	6	(42.9)	—		—
Greater than 6	8	(57.1)			
Mean cc prostate vol (range)	49.7	(23–107)	66.1	(24–124)	58.5 (23–124)

Table 2. Nonassessable and BDL samples of 60 cell pellet and exosome samples

	No. PSA (%)		No. PCA3 (%)	
	Before DRE	After DRE	Before DRE	After DRE
Cell pellet:				
Nonassessable	3 (10)	3 (10)	3 (10)	3 (10)
BDL	2 (6.7)	1 (3.3)	7 (23.3)	2 (6.7)
Exosome BDL*	9 (30)	6 (20)	17 (56.7)	5 (16)

* No exosomes were nonassessable.

RNA Analysis

In total RNA from urinary sediments (cell pellets) we noted prominent 18S and 28S peaks, representing rRNA. However, analysis of exosomal RNA

showed a small, pronounced RNA peak between 25 and 200 nucleotides, and little or no ribosomal RNA. After treating the exosomal fraction with ribonuclease and DNase the extra-exosomal RNA and DNA were removed, resulting in exosome specific transcript preparations.

DRE and Biomarker Levels

Figure 2 shows the influence of DRE on biomarker expression levels in cell pellets and exosomes isolated from urinary samples. In the cell pellet fraction after DRE an increase in PSA mRNA was noted in 24 of 30 samples (80%), 22 of 30 (73.3%) showed an increase in PCA3 mRNA and 5 of 6 (83.3%) were

Figure 2. PSA mRNA in cell pellet (A) and exosomes (B), PCA3 mRNA in cell pellet (C) and exosomes (D), and TMPRSS2-ERG mRNA in cell pellet (E) and exosomes (F) before and after DRE.

Table 3. Differences in biomarker expression in cell pellets and exosomes before and after DRE after log transformation of number of copies of mRNA/ml

	Mean $\pm$ SD Before DRE	Mean $\pm$ SD After DRE	p Value
Pellets:			
PSA	10.3 $\pm$ 3.8	12.8 $\pm$ 2.5	0.003
PCA3	6.5 $\pm$ 4.6	9.5 $\pm$ 3.4	0.001
TMPRSS2-ERG	0.3 $\pm$ 1.5	1.4 $\pm$ 2.6	0.032
Exosomes:			
PSA	7.7 $\pm$ 4.0	10.3 $\pm$ 4.6	0.002
PCA3	2.9 $\pm$ 3.6	7.4 $\pm$ 4.2	0.000
TMPRSS2-ERG	0	1.0 $\pm$ 2.6	0.047

positive for TMPRSS2-ERG. In exosomal isolation samples an increase in PSA mRNA after DRE was observed in 22 of 30 (73.3%), 23 of 30 (76.7%) showed an increase in PCA3 and 4 of 4 (100%) were positive for TMPRSS2-ERG. Mean biomarker levels were significantly higher in cell pellets and exosomes after DRE (table 3).

Biomarker Diagnostic Performance

To assess the diagnostic performance of PCA3 we calculated ROC curves for biopsy outcome prediction (histologically confirmed PCa). As a continuous

variable, PCA3 mRNA copies showed an AUC of 0.67 (95% CI 0.45–0.89) in cell pellet samples before DRE and 0.81 (95% CI 0.64–0.98) after DRE. No reliable AUC could be calculated on exosome samples before DRE since in most samples mRNA levels were below the detection limit and, thus, were excluded from this analysis (table 2). However, after DRE when more samples could be included in analysis, the AUC of absolute PCA3 mRNA was 0.52 (95% CI 0.28–0.76). When the quantitative PCA3 mRNA level was normalized to PSA mRNA (PCA3 mRNA/PSA mRNA $\times$ 1,000), the AUC increased to 0.64 (95% CI 0.41–0.88) for exosomes. For cell pellets the AUC changed to 0.67 (95% CI 0.46–0.89) in samples obtained after DRE (fig. 3).

DISCUSSION

Although progress has been made, validating new biomarkers in serum and urine remains a challenge. Our data show that first catch urine after DRE results in a clear increase in biomarker levels and, therefore, could contribute in PCa diagnosis. This increase was observed in cell pellets and exosomes.

Figure 3. PCA3 expression in cell pellets (A) and exosomes (B) by positive biopsy outcome (PCa+) after DRE compared to negative biopsy outcome (PCa-). Horizontal lines represent mean. ROC curves after DRE for PCA3 mRNA in cell pellets without normalization for PSA mRNA and after normalization (AUC 0.81 and 0.67) (C), and for exosomes (AUC 0.52 and 0.64, respectively) (D).

Exosomes were first identified in human urine in 2004 and recent findings revealed that these small, tissue derived vesicles contain various RNAs, representing their tissue origin.^{5,6,16} Exosomes are the internal vesicles of multivesicular bodies, suggesting that exosomal RNA would be protected and better preserved than RNA in whole cells. This difference between RNA from exosomes and RNA derived from cells in urine was previously described.¹³ It corresponds with our results showing that microvesicles can resist ribonuclease and DNase digestion, and still protect the nucleic acids inside. In addition to this finding, the main difference between cell pellets and exosomes is that each exosome sample was assessable for RNA extraction, ie none of the exosome samples contained (in)organic precipitate while 3 cell pellet samples (10%) could not be assessed due to precipitation. No biomarker analysis can be performed in samples that contain precipitation and the clinical consequence of this would be resampling. The cell pellet informative rate in this study is in accordance with that in some earlier studies of PCA3 mRNA, emphasizing the more stable nature of exosomes over cell pellets.^{14,17}

On the other hand, lower mRNA levels were measured in exosomes and a significant number of samples before DRE showed mRNA expression that was BLD, including 17 samples in exosomes vs 7 in cell pellets for PCA3 (table 2). However, this improved significantly after DRE, that is 5 samples in exosomes vs 2 in cell pellets. This supports the value of DRE for determining biomarkers in urine. Nevertheless, 16% to 20% of exosome samples did not achieve the analytical detection limit. In clinical practice these samples would be interpreted as a negative test. Therefore, low analytical sensitivity can lead to false-negative test results. This is a detriment of exosomes and makes them not yet suitable as a biomarker source for PCa diagnosis in the clinical setting.

Despite our small sample size PCA3 analysis of urinary sediments had diagnostic performance similar to that in previous studies.^{12,14,18} When exosomal RNA was analyzed, the levels of PCA3 and TMPRSS2-ERG were significantly higher after DRE. However, these biomarker levels in exosomes seemed to have an insufficient correlation with the prostate biopsy outcome. A hypothesis to explain

this finding may be that urinary microvesicles from patients with PCa have a different content than those from healthy donors and, therefore, they contain less RNA, as previously described by Nilsson et al.⁸ This would also explain the fact that more exosome samples than cell pellet samples had biomarker levels that were BDL. Notably, absolute exosomal PCA3 copy numbers showed no diagnostic value. However, when PCA3 levels were normalized to PSA levels in the exosomes, the diagnostic value improved. This is in agreement with the Progenesa® PCA3 test in whole urine, which is also based on the PCA3/PSA ratio. Since PSA mRNA expression is relatively constant in normal prostate cells and PCa cells, PSA mRNA expression is used for normalization to noncancerous prostate cells.^{12,19}

Our control group (negative biopsies) can be considered a limitation since it was not representative of healthy men in the normal population. Patients were selected for prostate biopsies based on increased PSA and/or abnormal DRE. Thus, they were at higher risk for a false-negative biopsy outcome than healthy peers. Diagnostic data might have been biased due to this limitation.

CONCLUSIONS

Comparative analysis of biomarkers in urinary sediment (cell pellets) and exosomes showed that exosomes seem to be a more robust source of biomarkers, although exosomes have lower analytical sensitivity. Furthermore, DRE resulted in higher biomarker levels in first catch urine for cell pellets and exosomes. However, the diagnostic performance of PCA3 in exosomes appeared different from that in cell pellets, ie PCA3 mRNA levels had to be normalized to PSA mRNA to achieve diagnostic improvement. Although we report that measuring biomarkers in exosomes is feasible and results seem promising, clinical usefulness and diagnostic value must be prospectively explored in larger cohorts.

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