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

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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 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 −70C. 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 −70C.

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 μg/μl, 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

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