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RESEARCH ARTICLE

The combination of urine DD3^{PCA3} mRNA and PSA mRNA as molecular markers of prostate cancer

Ettore Mearini[1], Cinzia Antognelli[2], Chiara Del Buono[2], Giovanni Cochetti[1], Antonella Giannantoni[1], Emanuela Nardelli[2], and Vincenzo N. Talesa[2]

[1]University of Perugia, Department of Medical-Surgical Specialties and Public Health, Division of Urology and Andrology, via Brunamonti 06126, Perugia, Italy, and [2]University of Perugia, Department of Experimental Medicine, via del Giochetto 06123, Perugia, Italy

Abstract

The identification of reliable molecular biomarkers in prostate cancer early diagnosis is clinically desirable. We quantitatively detected prostate cancer specifically overexpressed genes, DD3 and PSA, in urine sediments of men suffering from prostate cancer or benign prostate hyperplasia, after prostatic massage. As both genes are exclusively expressed in androgen receptor expressing human prostate carcinoma cell lines, we further investigated the possible effect of androgens on PSA and DD3 gene expression. DD3 and PSA mRNA levels were measured by real-time polymerase chain reaction. The combined markers test had a sensitivity of 80.2% and a specificity of 100%. Both gene transcripts were significantly upregulated by androgens. Results indicated the clinical usefulness of the combination of DD3 and PSA as molecular markers in the early diagnosis of prostate cancer and the need of combining as many as possible analytical data with the clinical and demographic ones to achieve the maximum level of diagnostic accuracy.

Keywords: *Prostate cancer; DD3 mRNA; PSA mRNA; molecular markers; benign prostatic hyperplasia; urine sediments*

Introduction

Prostate-specific antigen (PSA) is currently the most important biochemical marker for the diagnosis of prostate cancer (PCa). Because of the limited specificity of PSA, clinically irrelevant tumours and benign abnormalities are also detected that potentially lead to over-treatment (Frydeberg et al. 1997). Identification of new markers and new target therapy would represent a considerable advance in the treatment of PCa. In particular, molecular assays usually produce more qualitative (categorical) results with higher sensitivity and reproducibility than the continuous data typically produced by biochemical serologic assays (Anderson & Anderson 2002).

Differential display 3 (DD3), also known as prostate cancer gene 3 (*PCA3*), is a novel prostate-specific gene that is markedly upregulated in almost all PCa tissues compared with benign prostate (Bussemakers et al. 1999, de Kok et al. 2002). It was found to be undetectable in non-prostate normal and tumour tissues (including bladder and testis) and cancer cell lines from bladder, breast, kidney and ovaries (Bussemakers et al. 1999, Gandini et al. 2003, Sokoll et al. 2008). Therefore, DD3^{PCA3} is one of the most PCa-specific genes yet described, its expression being restricted to the prostate.

While several studies have identified and characterized DD3^{PCA3} as a promising marker of PCa (Bussemakers et al. 1999, de Kok et al. 2002, Schalken et al. 2003, Groskopf et al. 2006, Marks et al. 2007, Vaananen et al. 2008), others failed in finding such an association (Marangoni et al. 2006, Steuber et al. 2007). Hence, the clinical relevance of this recently introduced mRNA target remains to be fully elucidated and needs further investigation.

Address for Correspondence: Ettore Mearini, Department of Medical-Surgical Specialties and Public Health, Division of Urology and Andrology, University of Perugia, Perugia, Italy. Tel/Fax: (+39) 075-5724890. E-mail: talesa@unipg.it

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In the first part of the present study, in order to verify the potential clinical usefulness of DD3^{PCA3} gene expression as well as of PSA as molecular markers in the early diagnosis of PCa, we quantitatively analyzed DD3^{PCA3} and PSA mRNA levels in human urine sediments of men suffering from PCa and benign prostatic hyperplasia (BPH) after prostatic massage.

For comparison, the diagnostic performance of urinary DD3 and PSA tests relative to serum total (tPSA) assay was also evaluated in the overall population and in subsets of serum tPSA ranges. A stratified analysis was also performed by age at diagnosis.

Because PCa is a heterogeneous disease, it becomes clear that a combination of markers will become important in early PCa diagnosis. Therefore, as we believe that the employment of a single marker alone is often not indicative in the diagnosis of PCa, the combination of both tests in each single case was studied, to better predict a clinical diagnosis. Hence, for the first time, we report on the diagnostic usefulness of DD3^{PCA3} transcripts in combination with those of PSA in PCa.

DNA profiling analysis pointed out that mRNA of both PSA and DD3^{PCA3} has been exclusively detected in androgen receptor (AR) expressing human prostate carcinoma cell lines, such as LNCaP, while androgen-independent human prostate carcinoma cells, such as PC3, appear to be PSA and DD3^{PCA3} mRNA-negative (van Bokhoven et al. 2003).

Furthermore, basic research has shown that PSA gene expression is controlled by androgens, acting via the AR (Ruckle & Oesterling 1993). However, other *in vivo* studies found conflicting results (Mostaghel et al. 2007). Therefore, the biological effects of androgens on PSA expression remain controversial.

On the other hand, at present, no consistent literature exists about the effect of androgens on DD3^{PCA3} expression. Hence, in the second part of the study, we, firstly, quantitatively measured DD3^{PCA3} and PSA mRNA levels at baseline in LNCaP and PC3 cells, in order to verify the selective DD3^{PCA3} and PSA mRNA expression in responsive cell lines. Secondly, both cell lines were used to further investigate the possible effect of testosterone, the main circulating androgen, on PSA gene expression and to evaluate the possible modulation of DD3^{PCA3} gene expression after the same steroid hormone exposure.

Materials and methods

Patients

Ninety-six age-matched patients, with different serum tPSA level and/or abnormal digital rectal examination (DRE), who were referred to our department for prostate biopsy, were included in this study. Approval was obtained from the Institutional ethics committee and informal consent from each patient. Bioptic examination revealed that 26 patients had benign BPH and 70 PCa. The patients with BPH were clinically diagnosed as highly symptomatic BPH. Mean age of the BPH group was 70.3 years (range 53–77), mean age of the PCa cohort was 68.7 years (range 53–83). The tPSA values ranged from 1.08 to 22.0 ng ml^{-1} for men with BPH and from 2.71 to 172.0 ng ml^{-1} in the PCa group. Among the PCa patients, 45/70 (64.3%) had Gleason score <7, 19/70 (27.1%) = 7 and 6/70 (8.6%) >7. Patients had not had any transurethral manipulation, radiotherapy, an indwelling catheter or acute infection before biopsy and were not on hormonal therapy.

Collection of urine samples after prostate massage

Immediately prior to biopsy an attentive digital rectal palpation was performed as described previously (Tinzl et al. 2004). Patients were then asked to void spontaneously and urine (20–30 ml first catch) was collected for analysis. Immediately thereafter, a standardized ultrasound-guided transrectal biopsy was performed. The urine samples were immediately cooled on ice. Upon centrifugation at 4°C and 700 rpm for 10 min, urine sediments were obtained, washed twice with ice-cold phosphate-buffered saline (again at 4°C and 700 rpm for 10 min), snap-frozen in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), and stored at -70°C until subsequent use.

Cell culture and hormone/inhibitor treatments

Androgen-responsive human LNCaP cells were a kind gift of Prof. P. Limonta (Milan, Italy). Androgen-independent human PC3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Both cell lines were maintained routinely at 37°C in 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated (1 h at 56°C) fetal bovine serum, 1x L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids, 100 units ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin (Invitrogen, Carlsbad, CA, USA). Both cells were seeded into 6-well plates at a density of 10^5 cells per well and cultured in whole serum. Subsequently, to deplete endogenous steroids, the medium was changed with another containing 10% charcoal-stripped fetal bovine serum (CSS) (Migliaccio et al. 1993), 3 days before treatment with testosterone (Sigma, Milan, Italy). To ascertain that the observed biological effects were really due to testosterone, we also performed experiments in combination with letrozole (a kind gift of Novartis Pharma AG, Basel, Switzerland),

that is capable of blocking the aromatase activity for the transformation of androgens into estrogens. Stock solutions of testosterone and letrozole were prepared in 0,05% ethanol. The same ethanol concentration was used in controls and ligand-treated samples. At subconfluence, cells were incubated with either vehicle (controls) and testosterone or testosterone and letrozole in combination for 2 days at the concentrations shown in the appropriate Figures (1A, 1B, 1C). Four independent cultures of cells were prepared and assayed in quadruplicate.

RNA isolation and real-time PCR analysis

Total cellular RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was synthesized employing all the isolated total RNA from urinary sediments or 1 μg RNA from cells, with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and random primers system (Invitrogen). DD3^{PCA3} and PSA mRNA expression was detected by real-time TaqMan polymerase chain reaction (PCR) analysis on a LightCycler instrument (MX3005P System, Stratagene, La Jolla, CA, USA). The sequences of oligonucleotide primers and TaqMan probes for DD3^{PCA3}, PSA and β-actin (the housekeeping gene chosen for normalization following previous verification of its constant gene expression levels in all samples) (Khimani et al 2005) as well as the PCRs conditions used in this study are listed in Table 1. The amplification reactions were performed in quadruplicate for each sample. For comparative analysis of gene expression, data were obtained using the ΔΔCt method (User Bulletin ABI Prism 7700 Sequence Detection System 1997). For the quantitative analysis, in order to transform the Ct values

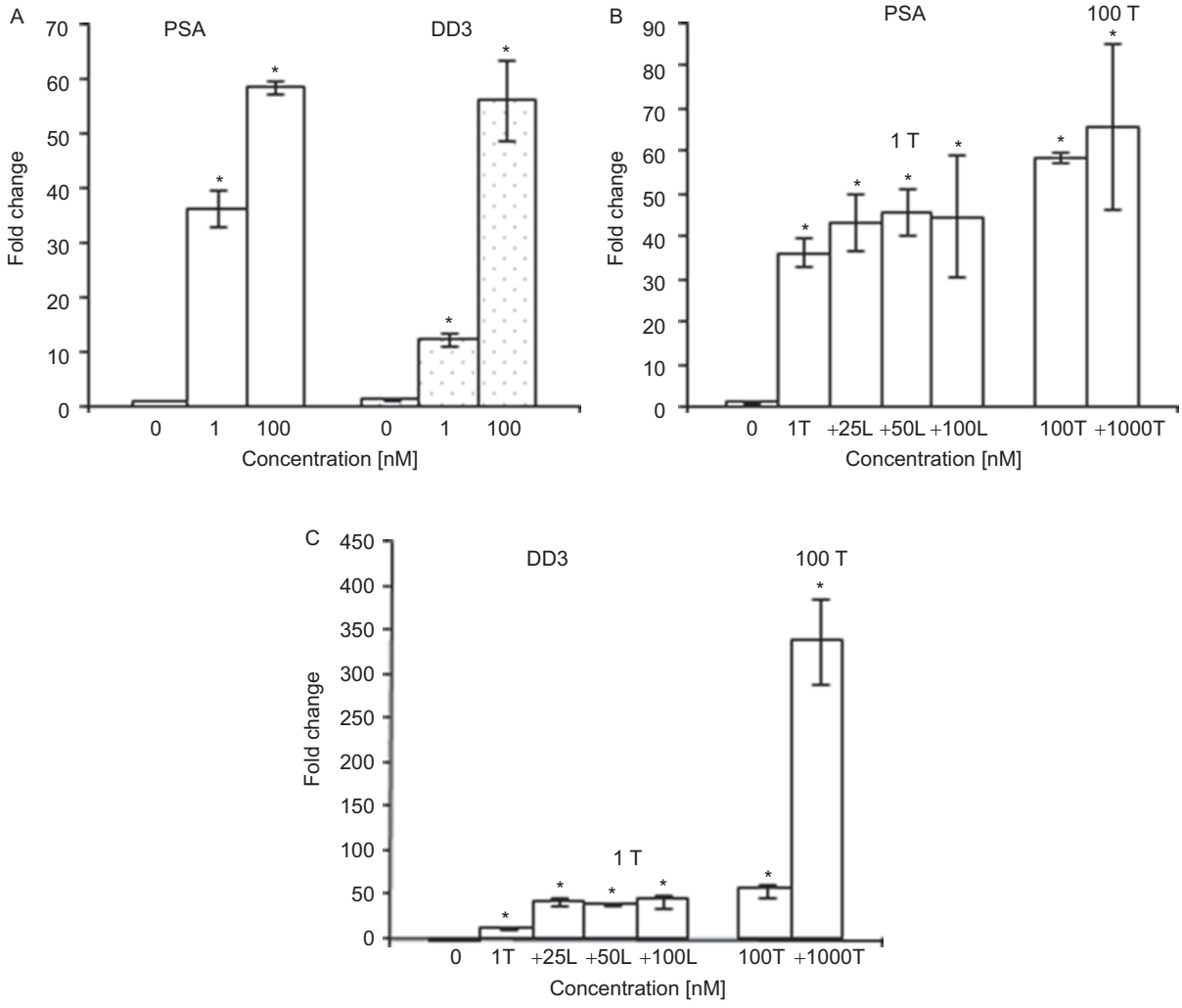

Figure 1. Dose-response of testosterone (T) induction of PSA and DD3^{PCA3} mRNA expression in LNCaP cells. Test agent was added either alone (A) or simultaneously with letrozole (L) (B and C), for 2 days in charcoal-stripped serum (CSS) medium. mRNA levels were measured by real-time RT-PCR analysis. All the data are given as fold increment related to negative controls (represented by non-stimulated cells). Graphs illustrate mean ± SE values averaged from three experiments. *Significantly different from the negative control ($p < 0.001$).

Table 1. Primers, probes sequences for TaqMan RT-PCR analysis and PCR conditions.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-3′)	Amplicon size (bp)
DD3^{PCA3}	GGTGGGAAGGACCTGATGATAC (located in exon 1) (600 nM)	GGGCGAGGCTCATCGAT (located in exon 4a) (600 nM)	AGAAATGCCCGGCCGCCATC[a] (100 nM)	262
PSA	GACCACCTGCTACGCCTCA (400 nM)	GGAGGTCCACACTGAAGTTTC (400 nM)	CAG CAT TGAACCAGAGGAGTTCTT GACCC[a] (100 nM)	83
β-Actin	CACTCTTCCAGCCTTCCTTCC (600 nM)	ACAGCACTGTGTTGGCGTAC (600 nM)	TGCGGATGTCCACGTCACACTTCA[b] (200 nM)	120
Cycles	1 cycle: 95°C for 10 min	45 cycles: 95°C for 20 s 60°C for 1 min		

[a]Labelled with FAM fluorochrome; [b]labelled with Cy5 fluorochrome.

into absolute mRNA concentrations we generated three calibration curves that were prepared from a dilution series of linearized plasmids containing DD3^{PCA3} (a kind gift of Prof. J. B. De Kok, HB Nijmegen, the Netherlands), PSA (a kind gift of Dr M Rissanen, Turku, Finland) and β-actin (pTRI-β-Actin-Human Antisense Control Template, Ambion), respectively. In our experiments, the calibration curves consisted of at least five points and each concentration was run in triplicate. Only calibration curves with a R^2 of 0.985–0.995 and an efficiency between 90 and 100% were considered (User Bulletin ABI Prism 7700 Sequence Detection System 1997).

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS; version 10.00; SPSS Inc., Chicago, IL, USA). The distributions of normalized DD3^{PCA3} and PSA mRNA levels were characterized by their median values and ranges. Differences in these markers between BPH and PCa were tested for statistical significance with the non-parametric Mann–Whitney *U* test. To visualize the efficacy of DD3^{PCA3} and PSA as markers to discriminate PCa from BPH, we summarized the data in a receiver operating characteristic curve (ROC). The area under the curve (AUC) and its 95% confidence interval (CI) were calculated as a single measure for the discriminative efficacy of the tested marker. When a marker has no discriminative value, the ROC curve will lie close to the diagonal and the AUC is close to 0.5. When a test has strong discriminative power, the ROC curve will move up to the upper left-hand corner and the AUC will be close to 1.0.

Logistic regression analysis was used as the basis of determining if the combination of both markers better predicted a clinical diagnosis. The accuracy of the model was assessed as the percentage of patients who were correctly classified.

The statistical significance of differences between testosterone- (and/or letrozole-) treated and untreated cells, were assessed by analysis of variance (ANOVA). All reported numerical data are expressed as the mean ± standard error (SE). Differences between means were significant when $p < 0.05$.

Results

Quantification of DD3^{PCA3} and PSA transcripts in urinary sediments after prostate massage – diagnostic performance of DD3^{PCA3} and PSA mRNA expression as prostate tumour markers

DD3^{PCA3} and PSA mRNA expression was quantitatively evaluated measuring their concentration in the urine samples of BPH and PCa patients. Median upregulation of DD3^{PCA3} from BPH (median 0.561×10^{-3} pg μl^{-1}; range 0 to 7.46×10^{-3} pg μl^{-1}) to PCa (median 20.8×10^{-3} pg μl^{-1}; range $10^{-2} \times 10^{-3}$ to $10^{6} \times 10^{-3}$ pg μl^{-1}) samples was 37-fold ($p < 0.0001$). As for PSA, the median concentration was also significantly different between BPH (median 6.07×10^{4} pg μl^{-1}; range 0.54×10^{4} to 26.4×10^{4} pg μl^{-1}) and PCa (median 43×10^{4} pg μl^{-1}; range 0.67×10^{4} to 7123×10^{4} pg μl^{-1}; $p < 0.0001$) patients. Median upregulation of PSA from BPH to PCa was 7-fold.

A ROC curve was constructed for both markers (Figure 2). AUC-ROC values, cut-off levels, sensitivity and specificity of both DD3^{PCA3} and PSA are shown in Table 2.

Comparison between the diagnostic performance of serum tPSA and urinary DD3^{PCA3} transcripts or urinary PSA mRNA levels

For comparison, the performance of the serum PSA assay on the population stratified by serum tPSA was also evaluated (Table 3). In the overall population the serum tPSA level has a comparable diagnostic value for this subject population. The differences between the areas under the curves for DD3^{PCA3}, PSA or serum tPSA were not statistically significant (DD3^{PCA3} vs serum tPSA, $p = 0.08$; urinary PSA mRNA vs serum tPSA, $p = 0.305$). However, when selecting the population for serum tPSA ranges, all the three markers appear to have the same diagnostic performance only in the range of serum tPSA of 4 to 10 ng ml^{-1}. Instead, at serum tPSA ranges ≤4 ng ml^{-1} or >10 ng ml^{-1}, the molecular markers

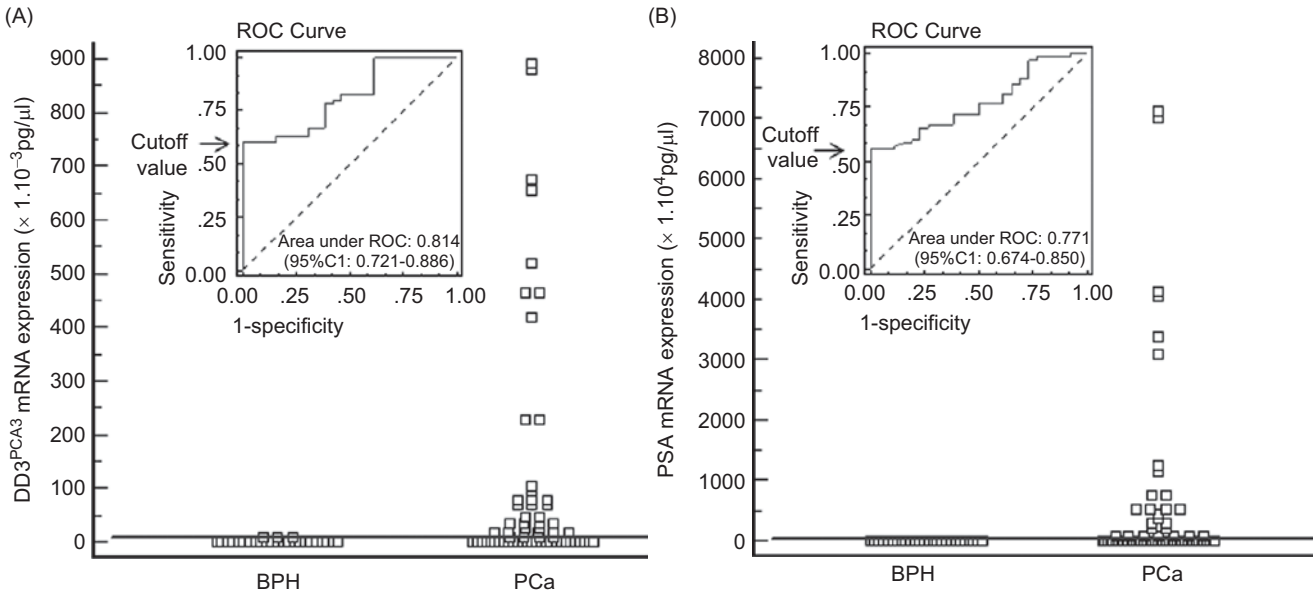

Figure 2. Expression of DD3^{PCA3} mRNA (A) and PSA mRNA (B) in urine samples of patients suffering from benign prostate hyperplasia (BPH) or prostate cancer (PCa). To visualize the diagnostic efficacy of the DD3^{PCA3} (A) or PSA-based assay (B) of urine samples in the absence of an arbitrary cut-off value, the data were summarized using a receiver operating characteristic curve (ROC) (inserts). Based on this ROC curve, cut-off levels of 7.46×10^{-3} pg µl^{-1} for DD3^{PCA3} and 26.4×10^{4} pg µl^{-1} for PSA were determined (arrows). Six DD3^{PCA3} extreme values are not shown, but considered in the calculation of the ROC curve.

Table 2. Sensitivity, specificity and AUC/ROC for urinary DD3^{PCA3} and PSA in the overall population.

	% Sensitivity	% Specificity	Cut-off value (pg µl^{-1})	AUC-ROC (95% CI)	*p*-Value
DD3^{PCA3}	60	100	$>7.46 \times 10^{-3}$	0.814 (0.721–0.886)	0.0001
PSA	56	100	$>26.4 \times 10^{4}$	0.771 (0.674–0.850)	0.0001

AUC/ROC, area under curve/receiver operating characteristic curve; CI, confidence interval.

Table 3. Comparison between the diagnostic performance of serum tPSA and that of urinary DD3^{PCA3} and PSA mRNA levels.

tPSA range (ng ml^{-1})	AUC/ROC (95% CI)	Sensitivity (%)	Specificity (%)	*p*-Value
Serum tPSA				
≤4[a]	0.575 (0.228–0.872)	100	50	0.71
4–10[b]	0.796 (0.661–0.895)	69.4	87.5	0.0001
>10[c]	0.580 (0.402–0.744)	31	100	0.547
Overall[d]	0.696 (0.594–0.786)	77.1	69.2	0.0005
Urinary DD3^{PCA3}				
≤4[a]	0.900 (0.530–0.980)	100	75	0.0004
4–10[b]	0.743 (0.603–0.854)	52.8	100	0.0005
>10[c]	0.862 (0.703–0.954)	69	100	0.0017
Overall[d]	0.814 (0.721–0.886)	60	100	0.0001
Urinary PSA				
≤4[a]	1.0 (0.662–1.0)	100	100	0.000001
4–10[b]	0.737 (0.596–0.849)	50	100	0.0007
>10[c]	0.782 (0.610–0.903)	55.2	100	0.0017
Overall[d]	0.771 (0.674–0.850)	56	100	0.0001

AUC/ROC, area under curve/receiver operating characteristic curve; CI, confidence interval; [a]benign prostatic hyperplasia (BPH), $n=9$ and prostate cancer (PCa), $n=12$; [b]BPH, $n=9$ and PCa, $n=20$; [c]BPH, $n=8$ and PCa, $n=38$; [d]BPH, $n=26$ and PCa, $n=70$.

have a clinical diagnostic value far higher. In particular, for serum tPSA ≤4 ng ml^{-1}, $AUC_{DD3^{PCA3}} = 0.900$ (0.530–0.980) and $AUC_{PSA} = 1.0$ (0.662–1.0), versus $AUC_{serum\ tPSA} = 0.575$ (0.228–0.872); for serum tPSA >10 ng ml^{-1}, $AUC_{DD3^{PCA3}} = 0.862$ (0.703–0.954) and $AUC_{PSA} = 0.782$ (0.610–0.903), versus $AUC_{serum\ tPSA} = 0.580$ (0.402–0.744). The differences between the AUCs for DD3^{PCA3}, PSA or serum tPSA were statistically significant ($p < 0.05$).

Diagnostic performance of DD3^{PCA3} and PSA mRNA expression as prostate tumour markers in the population stratified by age and a range serum tPSA of ≤4 ng ml^{-1}

As we observed the best clinical performance of both urinary markers at serum tPSA ≤4 ng ml^{-1}, we decided to choose this range of serum tPSA values to analyze the performance of the markers according to different ages. In particular, two categories for each criteria were performed: age ≤65 or >65 years, serum tPSA ≤4 or >4 ng ml^{-1} and a combination of both (age ≤65 years and serum tPSA ≤4 ng ml^{-1}, or age >65 years and tPSA >4 ng ml^{-1}). Results are shown in Table 4.

Use of combined DD3^{PCA3} and PSA biomarkers for PCa diagnosis

It was possible to discriminate between BPH and PCa groups with DD3^{PCA3} or PSA markers using univariate analysis. However, as there was considerable variation of gene expression within the sample groups, the use of a single marker to diagnose PCa in individuals would not be sufficiently discriminating. A combination of both markers would be expected to enhance the diagnostic power of the assays. Using the logistic regression analysis, we determined whether the two markers together or the use of them singly was more useful in establishing a difference between cancer and BPH (Table 5). The combination of both markers remarkably increased the sensitivity for detection of PCa from 60% (DD3^{PCA3} test alone) to 80.2%. Percentage of samples correctly categorized rose to 90.5% when stratifying by age (≤65 years) and to 100% by serum tPSA (≤4 ng ml^{-1}). When also adding age and serum tPSA to the logistic regression analysis, analysis of this complete set of data predicted the correct classification of 84.37% of all samples (Table 5). Therefore, an integrative use of DD3^{PCA3}, PSA, age and serum tPSA is considered as the best multivariate predictive model.

Quantitative DD3^{PCA3} and PSA mRNA expression levels at baseline in LNCaP and PC3 cells

DD3^{PCA3} and PSA mRNA expression was quantitatively measured in LNCaP and PC3 cells by real-time TaqMan PCR analysis. In LNCaP cells, DD3^{PCA3} transcript level was $5.23 \pm 0.217 \times 10^{-4}$ pg μl^{-1}, while PSA transcript level was $4.5 \pm 0.82 \times 10^{5}$ pg μl^{-1}. PC3 cells expressed none of either of the two markers.

Effects of testosterone on PSA and DD3^{PCA3} gene expression in LNCaP cells and PC3 cells

Testosterone produced a dose-dependent induction of both PSA and DD3^{PCA3} gene expression in LNCaP cells (Figure 1A). In particular, the androgen increased PSA mRNA levels about 36-fold ($p < 0.0001$) at 1 nM, to

Table 4. Sensitivity, specificity AUC/ROC for urinary DD3^{PCA3} and PSA in the population stratified for age and serum tPSA.

	% Sensitivity	% Specificity	Cut-off value (pg μl^{-1})	AUC/ROC (95% CI)	*p*-Value
DD3^{PCA3}					
Age (years)					
≤65[a]	81.2	100	$>1.855 \times 10^{-3}$	0.900 (0.690–0.984)	0.0001
>65[b]	55.6	100	$>7.46 \times 10^{-3}$	0.791 (0.682–0.876)	0.0001
tPSA (ng ml^{-1})					
≤4[c]	100	75	>0	0.900 (0.530–0.980)	0.0004
>4[d]	60	100	$>7.46 \times 10^{-3}$	0.795 (0.695–0.874)	0.0001
Age (years) + tPSA (ng ml^{-1})					
≤65 and ≤4[e]	66.7	100	$>7.36 \times 10^{-3}$	0.857 (0.659–0.962)	0.0001
>65 and >4[f]	58.2	100	$>7.46 \times v10^{-3}$	0.789 (0.691–0.868)	0.0001
PSA					
Age (years)					
≤65[a]	81.2	100	$>5.03 \times 10^{4}$	0.900 (0.690–0.984)	0.0001
>65[b]	51.9	100	$>26.4 \times 10^{4}$	0.730 (0.615–0.826)	0.0001
tPSA (ng ml^{-1})					
≤4[c]	100	100	$>25.7 \times 10^{4}$	1.0 (0.662–1.00)	< 0.00001
>4[d]	52.3	100	$>26.4 \times 10^{4}$	0.764 (0.661–0.848)	0.0001
Age (years) + tPSA (ng ml^{-1})					
≤65 and ≤4[e]	72.2	100	$>25.7 \times 10^{4}$	0.857 (0.659–0.962)	0.0001
>65 and >4[f]	53.7	100	$>26.4 \times 10^{4}$	0.757 (0.655–0.840)	0.0001

AUC/ROC, area under curve/receiver operating characteristic curve; CI, confidence interval. [a]Benign prostatic hyperplasia (BPH), $n = 12$ and prostate cancer (PCa), $n = 36$; [b]BPH, $n = 14$ and PCa, $n = 34$; [c]BPH, $n = 9$ and PCa, $n = 12$; [d]BPH, $n = 8$ and PCa, $n = 38$; [e]BPH, $n = 11$ and PCa, $n = 17$; [f]BPH, $n = 12$ and PCa, $n = 40$.

Table 5. Statistical analysis of biomarkers and parameters (age, serum tPSA).

Marker (parameter) combinations	Sample group	Samples correctly categorised (%) (95% CI)	*p*–Value
Multivariate analysis			
DD3^{PCA3}	BPH	61.5 (37.8–78.6)	<0.0001
PSA	PCa	87.1 (57.1–93.6)	
	Overall	80.2 (57.1–93.6)	
DD3^{PCA3}	BPH	80.0 (56.7–92.4)	<0.0001
PSA	PCa	93.7 (79.8–95.6)	
age ≤65 years	Overall	90.5 (79.7–96.9)	
DD3^{PCA3}	BPH	100.0 (81.5–100)	0.0021
PSA	PCa	100.0 (89.1–100)	
serum tPSA ≤4 ng ml^{-1}	Overall	100.0 (92.9–100)	
DD3^{PCA3}	BPH	76.92 (52.1–92.5)	<0.0001
PSA	PCa	87.14 (69.2–88.8)	
age	Overall	84.37 (72.6–91.8)	
serum tPSA			
Univariate analysis			
DD3^{PCA3}	BPH	38.46 (22.6–61.8)	<0.0001
	PCa	82.86 (70.6–81.8)	
	Overall	70.83 (47.8–88.7)	
PSA	BPH	23.08 (17.8–46.7)	<0.0001
	PCa	97.14 (85.1–99.9)	
	Overall	77.08 (52.8–92.7)	
Age	BPH	0	0.3
	PCa	100.0 (92.8–100)	
	Overall	72.92 (47.8–88.7)	
Serum tPSA	BPH	0	0.002
	PCa	100.0 (92.8–100)	
	Overall	72.92 (47.8–88.7)	

BPH, benign prostatic hyperplasia; PCa, prostate cancer.

peak at about 58-fold ($p < 0.0001$) at 100 nM over control. Increasing concentrations of testosterone (1 nM and 100 nM) strongly upregulated DD3^{PCA3} mRNA levels at both the used doses, maximum effect (about 56-fold) being at 100 nM (Figure 1A). To ascertain that the observed biological effect was really due to testosterone, experiments in combination with the aromatase inhibitor letrozole, capable of blocking the aromatase activity for the transformation of androgens into estrogens, were also performed. The results of separate experiments, carried out in the presence of both testosterone and letrozole, suggest that the observed increase in both PSA and DD3^{PCA3} transcript levels after testosterone exposure, are effectively due to testosterone (Figure 2B, 2C). At the used concentrations, letrozole alone did not exert any significant effect on PSA and DD3^{PCA3} mRNA expression (data not shown). Testosterone exposure did not induce either PSA or DD3^{PCA3} gene expression in PC3 cell line.

Discussion

In the present study, we quantitatively detected DD3^{PCA3} and PSA mRNA levels in human urine sediments of patients suffering from PCa or BPH after prostatic massage. Our results confirm the clinical usefulness of evaluating DD3^{PCA3} gene expression in the early diagnosis of PCa, in agreement with a large part of the literature (Bussemakers et al. 1999, de Kok et al. 2002, Schalken et al. 2003, Groskopf et al. 2006, Marks et al. 2007, Vaananen et al. 2008, Shappell et al. 2008). In particular, a very similar, but multicentre study was recently carried out by Shappell et al. (2008), who confirmed the high specificity of DD3^{PCA3} gene expression in the diagnosis of PCa, suggesting that in patients with high serum PSA (tPSA) levels and negative biopsy findings, DD3^{PCA3} testing might be useful in choosing between repeat biopsy and a more conservative follow-up. However, in comparison with the study by Shappell et al., our results provide additional information regarding the importance of a concomitant evaluation of PSA gene expression, as a molecular marker in the early diagnosis of PCa. Furthermore, besides the clinical usefulness of the combination of urinary DD3^{PCA3} and PSA as molecular markers in the early diagnosis of PCa, our findings additionally indicate the need of combining as many analytical data as possible with the clinical (serum tPSA) and demographic data to achieve the maximum level of diagnostic accuracy.

Recent reports have cited the use of single molecular markers in the diagnosis and prognosis of PCa (Hessels

et al. 2003, Harden et al. 2003, Kattan 2003). Although all of these individual markers have been able to discriminate between cancer and BPH as sample groups, none seems to be sufficient for predicting the presence of PCa in individual patients with total reliability (Kattan 2003). We demonstrated here that the combination of DD3^{PCA3} and PSA markers, along with clinical and demographic data, is highly predictive (84%, $p < 0.0001$) in distinguishing between PCa and BPH.

For comparison, the diagnostic performance of urinary DD3^{PCA3} and PSA tests relative to serum tPSA assay was also evaluated in the overall population and in subsets of serum PSA ranges. For the DD3^{PCA3}, the area under the ROC curve was 0.814 (95% CI 0.721–0.886) and for PSA mRNA 0.771 (95% CI 0.674–0.850). The serum tPSA yielded an AUC of 0.696 (95% CI 0.594–0.786), indicating that in the overall population the serum tPSA level has a comparable diagnostic value for this subject population. However, when selecting the population for serum tPSA ranges, all three markers appear to have the same diagnostic performance only in the range of serum tPSA of 4–10 ng ml^{-1}, thus suggesting that these tests could complement each other. Instead, at serum tPSA ranges ≤4 or >10 ng ml^{-1}, the molecular markers have a clinical diagnostic value far higher than serum tPSA, thus suggesting that urinary DD3^{PCA3} and PSA mRNA levels may be even more useful tests in PCa detection when the total serum PSA level is ≤4 or >10 ng ml^{-1}.

Comparison between the diagnostic performance of the combination of urinary DD3^{PCA3} and PSA tests relative to serum tPSA assay indicated that one of the most promising characteristics of our test may be its high accuracy in men with a serum tPSA level <4 ng ml^{-1}. In this range, in fact, the combination of urinary DD3^{PCA3} and PSA has a 100% sensitivity (tPSA sensitivity 73%). Therefore, this type of non-invasive test may be particularly attractive, also, to identify those at high risk of PCa among the large population of men with a PSA level between 2.5 and 4 ng ml^{-1}.

Exposure of LNCaP and PC3 prostate cancer cell lines to testosterone indicated a marked upregulation of DD3^{PCA3} and PSA transcripts only in AR-positive LNCaP cells. The present study provides further evidence supporting the concept that PSA is an androgen-inducible gene in concordance with earlier published data (Young et al. 1991, Montgomery et al. 1992, Lin et al. 2000). Multiple functional androgen response elements have been identified in the promoter region of human PSA gene, conferring androgen inducibility (Cleutjens et al. 1996, Schuur et al. 1996).

As to DD3^{PCA3} mRNA expression, our results suggest a possible regulatory role of DD3^{PCA3} by androgens with an AR-mediated mechanism. In the literature, the effect of androgens on DD3^{PCA3} expression has not been consistently described. A group observed that if 'serum-starved' LNCaP cells were kept in the presence of dihydrotestosterone, DD3^{PCA3} increased precipitously, thus suggesting that there might be an interaction, similar to that with steroid receptor RNA activator (Schalken et al. 2003). The same group performed immunoprecipitation with AR, also utilizing a number of controls; indeed, it appeared that DD3^{PCA3} was decreasing along with the AR. Although they could not duplicate the results of that experiment, these data seem to agree with our hypothesis.

The demonstration that either DD3^{PCA3} or PSA are encoded by androgen-inducible genes indicates, once again, the need of combining as many as possible analytical data with the clinical and demographic data to achieve the maximum level of diagnostic accuracy for PCa (Steuber et al. 2007). In particular, serum testosterone levels should be included in the logistic regression analysis, as also suggested by a recently published paper demonstrating that patients positive for PCa have statistically lower levels of serum total testosterone compared with patients diagnosed as suffering from BPH (Sofikerim et al. 2007). At the time when our study was planned, it was not possible to obtain enough suitable samples to test testosterone levels. The small number of samples collected was taken to make such a correlation, and, indeed, the results seemed to improve both markers test accuracy (data not shown), even though, as expected, they did not reach statistical significance. At present, sampling of a significant number of patients for serum testosterone level measurements is being carried out.

In conclusion, our results indicate that the combination of urinary DD3^{PCA3} mRNA and PSA mRNA is indeed a promising marker for the early diagnosis of PCa. Furthermore, we have shown that a combination of markers with clinical and demographic data greatly enhance sensitivity of molecular diagnosis of PCa. Certainly, further extensive research in this field is required.

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