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ARTICLE

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A prospective study of a urine and plasma biomarker test for the prediction of gleason $\geq 3+4$ prostate cancer in a mixed cohort

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ABSTRACT

Purpose: Definitive diagnosis of prostate cancer is based on biopsies, a procedure associated with side-effects. The use of biomarkers in blood and urine could potentially help clinicians select patients for whom biopsies are needed. The aim of the study was to test a new urine and plasma biomarker test in detecting medium and high grade prostate cancer.

Materials and methods: Blood and urine samples were prospectively collected from 41 patients prior to prostate biopsy or TUR-P and again after 3 months. The cohort included patients with suspicion of prostate cancer and patients with prior prostate cancer diagnosis. The mRNA expression of ten selected genes measured by PCR were used together with clinical data in multiple algorithms for prediction of medium-high grade prostate cancer in prostate biopsies. The testing was originally developed and validated in the USA. The method was transferred to a local Danish laboratory. Medium and high grade cancer was defined as Gleason score $\geq 3+4$.

Results: Using the biomarker test, prior to any prostate procedures, the sensitivity for detecting medium-high grade prostate cancer was 100% and the specificity was 56% and 63%, depending on the cut-off point used. When using the biomarker test, following biopsy or TUR-P, the sensitivity and specificity were reduced to 89% and 28–34% respectively. When comparing results, there was a significant difference ($p < 0.05$), favoring the test performed prior to the procedures.

Conclusions: We were able to predict the presence of medium-high grade prostate cancer, thereby confirming earlier findings of the biomarker test.

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Prostate cancer; biomarker; liquid biopsy; diagnosis; prognostic test

Introduction

At the time of diagnosis, prostate cancer may be categorized as indolent/low grade or aggressive/medium-high grade. For the vast majority of the patients, a low grade prostate cancer will never progress either locally or to a metastatic stage. In other words, low grade prostate cancer may never harm the patient and as a consequence the detection of the cancer provides no value to the patient [1,2]. The detection of a low grade cancer will, however, have a negative impact on the patient. Clinically, the detection of prostate cancer involves prostate biopsies which puts patients at risk of complications such as bleeding, infections, and even death [3]. The psychological aspect of being diagnosed with cancer is tremendous, and for the majority of the patients, it represents a major life crisis, and the quality of life may be affected lifelong [4–7]. The detection and treatment of the medium-high grade prostate cancer is, on the other hand, of pivotal importance. The traditional clinical tools includes prostate specific antigen (PSA), rectal digital examination, trans-rectal ultrasound, and biopsies have shortcomings, and may underestimate or miss the medium-high grade cancer, again causing harm to the patient due to understating and prolonged time to treatment [8,9].

The introduction of a biomarker test may facilitate and improve the diagnosis of prostate cancer, where patients with a medium-high grade prostate cancer can be distinguished from patients with no cancer or a low grade cancer. In the recent series of publications, by our group, we have shown that our urine and plasma test may pinpoint patients with medium-high grade prostate cancer, defined by Gleason score $\geq 3+4$. We have achieved a sensitivity of 86–97%, and a specificity of 36–57% depending on the cut-off point of the test: low, standard, or high. The area under the ROC curve plotting specificity against sensitivity was 81.5% [10]. In addition, we recently showed that the urine and blood biomarker test performed better than prostate biopsies using the prostatectomy specimen as the reference, achieving sensitivity of 92–97%, depending on cut-off, while the sensitivity of the prostate biopsies was 78% with regard

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to detection of medium-high grade prostate cancer (Gleason score $\geq 3+4$) [11]. Therefore, the introduction of the urine and plasma biomarker test may facilitate and improve the diagnosis of medium-high grade prostate cancers and at the same time patients with no cancer or a low grade cancer may be spared diagnostic procedures and negative consequences.

The aim of the study was to perform an external validation of the clinical utility of the urine and plasma biomarker test for prediction of medium-high grade prostate cancer. In addition, we wanted to assess the efficiency of the test after prostate procedures.

Materials and methods

Study design and patients

The biomarker test used, was based upon the expression levels of 10 different genes (NeoLAB Prostate®, Florida, US) in the urine and peripheral blood plasma, and thereby predict the presence of medium-high grade prostate cancer. The selection of genes was based on evaluation of existing mRNA based studies. As a control, we quantified the levels of two housekeeping genes (GAPDH and B2M) [12–14]. We have defined medium-high grade prostate cancer by the presence of a Gleason score of $3+4$ or above. The gene panel consists of *PDLIM5*, *HSPD1*, *PSA*, *IMPDH2*, *PCA3*, *TMPRSS2*, *ERG*, *UAP1*, *PTEN*, and *AR* [15,16]. Using machine learning techniques, we have optimized the number of input features and identified the most accurate scoring algorithm, combining the urine and plasma analyses with the clinical history.

In this blinded prospective diagnostic study, we collected plasma and urine samples from 41 patients undergoing prostate biopsy or transurethral resection of the prostate (TUR-P) and again after 3 months. We included consecutive patients from our out-patient clinic with an indication for prostate biopsy due to: (A) suspicion of prostate cancer on the basis of an elevated PSA; (B) suspicion of prostate cancer due to a digital rectal palpation; or (C) as part of the active surveillance of patients. In addition, we included patients who were planned for TUR-P, with no prior prostate cancer diagnosis. The exclusion criteria were: (A) PSA >20ng/mL; or B) patients not eligible for curative treatment of prostate cancer. The study was approved by the Regional Ethics Committee (Approval number S-20080004) and the Danish Data Protection Agency (Approval number 2012-58-0018). The study was registered at ClinicalTrials.gov (NCT00957606). All participants gave informed consent to participate.

Urine and plasma processing

Urine and plasma samples were collected from patients prior any digital examination and stored on ice. Peripheral blood from one 4 mL blood collection tube was fractionated by centrifugation at 800 g for 10 min at 4 °C; 1000 µL plasma was used for nucleotide extraction. Urine components above 3 kDa were concentrated from 35–75mL urine *via* repeated rounds of centrifugation using Amicon Ultra-15 centrifugal filter units with 3 kDa membrane (Millipore, Billerica, MA) at 3220 g and 4 °C, into a final volume of 1200 µL. 1000 µL urine concentrate was used for nucleotide extraction. Total nucleic acids were extracted from urine and plasma using the NucliSENS® extraction kit (BioMerieux, Durham, NC) within 36 h of sampling.

Quantitative reverse transcription-polymerase chain reaction

The RNA levels of prostate cancer specific genes, *PDLIM5*, *HSPD1*, *PSA*, *IMPDH2*, *PCA3*, *TMPRSS2*, *ERG*, *UAP1*, *PTEN*, and *AR*, and the housekeeping *B2M* and *GAPDH* genes were quantified in urine and plasma using quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) as previously detailed [11,15,16]. Briefly, qRT-PCR was performed using the RNA Ultrasense One-Step Quantitative RT-PCR on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following thermocycler conditions: hold stage of 50 °C for 15 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. The primers and probes were purchased as TaqMan Gene Expression. In all assays, an equal amount of plasma was used for RNA extraction, dissolved in equal amount of water, and an equal amount of RNA solution was used in each assay. Similarly, for urine, all voided urine was concentrated to 1 mL and RNA was extracted from total concentrate of urine and dissolved in equal amount of water. Equal amount of RNA solution was used in each assay.

Scoring system

A previously developed algorithm was used to calculate the probability of having medium-high grade prostate cancer with a Gleason score $\geq 3+4$. In this algorithm, urine/plasma biomarkers are combined with prostate size, history of prior prostate biopsies, clinical history, and PSA to generate a score used to determine the risk of having a Gleason score $\geq 3+4$. Three cut-off points (standard, high, and low) were used in this study [11,15,16].

Statistical analyses

Standard statistical tests were used to evaluate correlations between variables including the Wilcoxon Matched Pairs Test. Sensitivity and specificity was calculated using standard formulae. Statistical significance considered at p-value $< .05$.

Results

Patient characteristics

At inclusion, patients had a mean age of 68 years (range 54–75), median PSA of 6.8 µg/L (range 1.3–37), and mean prostate volume of 51 ml (range 24–82). Of the 41 patients, 33 patients had a prostate biopsy and 8 had TUR-P. Prior to this study, 17 of the patients planned for a prostate biopsy,

had previously been diagnosed with prostate cancer and were in a surveillance program. None of the patients planned for TUR-P had a prior diagnosis of prostate cancer. Following the prostate biopsy and TUR-P, 22 patients were found to have prostate cancer, 9 patients had a Gleason score of 7 (3 + 4), 13 had a Gleason score of 6 (3 + 3), and the remaining 19 patients had benign pathological findings (Table 1).

Prediction of medium-high grade cancer prior to prostate procedures (biopsy or TUR-P)

Urine and plasma samples were analyzed and the algorithm was applied using the three pre-defined cut-offs; standard, high, and low. As shown in Table 2, the urine and plasma test predicted the presence of medium-high grade prostate cancer (Gleason score ≥3 + 4) with a sensitivity of 100% for all three cut-off points. Specificity was 56% using the high sensitivity cut-off point and 63% for both standard and low cut-off points. The negative predictive value (NPV) was 100%, and positive predictive value (PPV) was 39% for high sensitivity cut-off and 43% for the low and standard cut-off points. In Figure 1, the receiver operating characteristic (ROC) curve is depicting the area under the curve (AUC) of serum PSA, and the biomarker test. ROC for PSA and biomarker test were 0.730 (95% CI: 0.574–0.886) and 0.816 (95% CI: 0.714–0.918), respectively.

Table 1. Characteristics of the patients.

Age (years)	
Mean (range)	68.0 (54–75)
PSA (ng/mL)	
Median (range)	6.8 (1.3–37)
<4	9 (22.0%)
4–10	19 (46.3%)
>10	13 (31.7%)
Prostate size (mL)	
Mean (range)	51 (24–82)
Race	
Caucasian	41 (100%)
Pathological findings, Gleason score	
Benign	19
3 + 3	13 (59.1%)
3 + 4	9 (40.9%)
No. clinical stage of cancer patients (%)	
T1	15 (68.2%)
T2	5 (22.7%)
T3	2 (9.1%)

Prediction of medium-high grade cancer after prostate procedures (biopsy or TUR-P)

To test the effects of prostate procedures on the urine and plasma markers, the samples were collected once again 3 month later, following the biopsy or TUR-P. As shown in Table 2, the urine and plasma test predicted the presence of medium-high grade prostate cancer (Gleason score ≥3 + 4) with sensitivity of 89%, specificity of 28–34%, negative predictive value of 90–92%, and positive predictive value of 26–28%, depending on cut-offs used.

Discrepancy between the analyses performed prior to and after the prostate biopsy or TUR-P

The results of the urine and plasma samples performed three months after the prostate biopsy or TUR-P, compared to the results of the urine and plasma samples performed prior the prostate or TUR-P, differed significantly with a *p*-value of <.05. The differences were more pronounced with the cut-

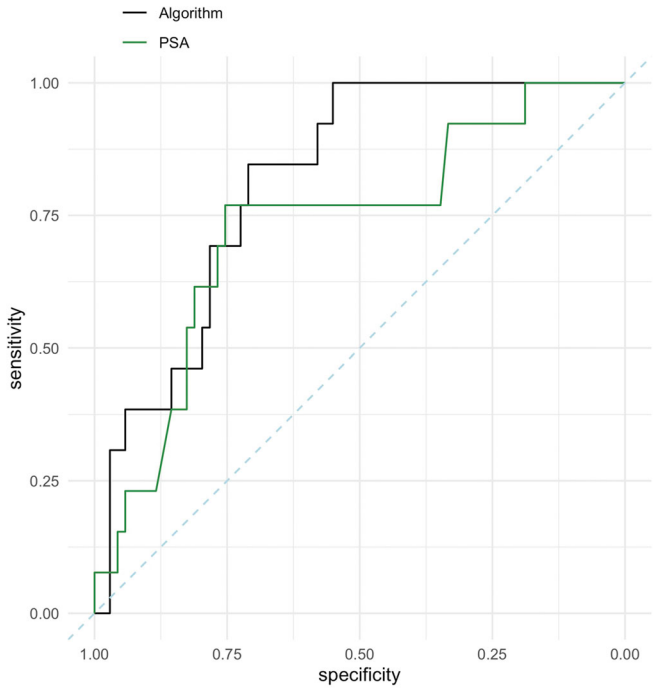

Figure 1. Receiver operating characteristic (ROC) curve showing the area under the curve (AUC) of serum PSA, and the algorithm that incorporate the urine and plasma biomarkers and clinical information.

Table 2. Sensitivity, specificity, positive, and negative predictive values (PPV and NPV) of the urine and plasma test of predicting Gleason score ≥ 3 + 4 in prostate biopsy and TUR-P.

	High sensitivity Value (95% CI)	Standard sensitivity Value (95% CI)	Low sensitivity Value (95% CI)
Pre-Biopsy and TUR-P			
Sensitivity	100% (63–100%)	100% (63–100%)	100% (63–100%)
Specificity	56% (38–73%)	63% (44–78%)	63% (44–78%)
PPV	39% (20–61%)	43% (23–66%)	43% (23–66%)
NPV	100% (78–100%)	100% (80–100%)	100% (80–100%)
Post-Biopsy and TUR-P			
Sensitivity	89% (51– 99%)	89% (51–99%)	89% (51–99%)
Specificity	28% (14–47%)	28% (14–47%)	34% (19–53%)
PPV	26% (13–45%)	26% (13–45%)	28% (13–47%)
NPV	90% (54–99%)	90% (54–99%)	92% (60–100%)

Table 3. D'Amico risk classification for prostate cancer.

	T-stage	Gleason score	PSA	Final D'Amico risk classification
Low risk	15	13	14	9
Intermediate risk	5	9	4	7
High risk	2	0	4	6

off set at high and low, with p values of $<.01$ and $<.03$, respectively, whereas for the cut-off set to standard value the p value was 0.07.

Predicting D'Amico classification based on urine and plasma test

We applied the D'Amico risk classification for prostate cancer to our cohort after the pathology results of the prostate biopsy and TUR-P [17]. We found that for the 22 patients with prostate cancer, 13 were classified as intermediate or high risk group patients, whereas 9 were low risk group patients (Table 3). Of the 13 patients with intermediate or high risk according to D'Amico, our urine and plasma test predicted medium-high grade prostate cancer in 12 of the patients. The results were similar using all three sensitivity cut-offs in our test. The one patient our urine and plasma test did not predict intermediate or high risk prostate cancer was a 71 year old patient with PSA 7.8 ng/mL, cT2 stage, and Gleason score of $3+3$. Notably, in this patient, the cancer was only located in 1 of 12 biopsies and the total cancer length within the biopsies was 1 mm.

Discussion

The present study demonstrated the robustness of the urine and plasma biomarker test (NeoLAB Prostate®) in predicting the presence of medium-high grade prostate cancer and in detecting patients in whom biopsies might not be warranted. With this study, we were able confirm the previous findings of the urine and plasma test. The test was previously developed and tested in the USA, and with this study, we were able to transfer the biomarker test to our laboratory and perform the analyses locally.

Over the last decade more blood and urine analyses have appeared both for the detection of prostate cancer and for distinguishing medium-high grade/aggressive from low grade/indolent cancer [18,19]. The Stockholm 3 model, combines plasma proteins, SNPS and clinical data, the prostate health index (PHI) combines total PSA, free PSA, and [–2] proPSA, the 4 K; four-kallikrein panel with the total PSA, free PSA, intact PSA, and human kallikrein levels [20,21]. The blood-based biomarkers, have been proven to accurately predict the presence of medium-high grade/aggressive prostate cancer [22]. The urine based biomarkers for prediction of medium-high grade/aggressive prostate cancer include PCA3; prostate cancer antigen 3, transmembrane serine 2-*ERG* gene fusion, ExoDx prostate IntelliScore (combines exosomal RNA of *PCA3*, *ERG*, and *SPDEF*), and SelectMDx (*HOXC6* and *DLX1*) [23–26]. With the addition of clinical data and the detection of more than one gene or protein, the performance of the tests has improved significantly [18,19], however, none of the tests have reached a wide clinical use.

Looking at the existing non-invasive tests from a distance, the tests widely depend on either PSA (including isoforms of PSA) or on analyzing biomarkers in urine alone, which may cause problems. A PSA based method lacks the information within the genetic changes of the cancer and a purely urine based test will be affected by dilution effects.

Our test is unique in that it utilizes biomarkers in both urine and blood together with the clinical data. Our test uses 10 different biomarkers in addition to two housekeeping genes and combines them with clinical data for generating a risk score. The use of so many biomarkers most likely overcomes the problems associated with a dependence on PSA or its isoforms or on few genes.

The agreement of our urine and plasma biomarker test with the D'Amico risk classification was remarkable. While the D'Amico risk classification relies partly on biopsies, we were able to achieve the same results without biopsies for all but one patient. The one patient where there was discrepancy had only one positive biopsy, with a 1 mm of prostate cancer lesion Gleason score of $3+3$, out of 12 biopsy samples analyzed. This patient may very well only harbor a low risk cancer, in accordance with the findings of our urine and plasma test.

Another key finding was the results with regard to the performance of the test three months after any procedures to the prostate. Plasma and urine biomarkers clearly remain disturbed even three months after a prostate procedure and manipulation. The urine and plasma test performed significantly worse at the three months time point as compared to the performance of the test prior to prostate procedures. As a consequence, the test should be performed prior to performing a prostate biopsy or TUR-P, since the procedures may have an influence on the balance of the biomarkers in the urine and plasma.

Putting our findings in perspective, we were able to reproduce the robustness of the urine and plasma test with regard to detection of medium-high grade prostate cancer at our local laboratory within a mixed population of patients with benign prostate lesions as well as low and high grade prostate cancers. As a consequence, the NeoLAB Prostate® test proved, in our cohort, to be useful prior to prostate biopsy to select patients for whom prostate biopsy might not be warranted.

Our study has a number of shortcomings. The cohort was limited in size and the cohort was mixed. Therefore, further validation is needed. The next step in the validation should be a randomized controlled trial for directly measuring the health impact of a biomarker strategy. Our endpoint was Gleason score calculated from prostate biopsy results, rather than Gleason score calculated from prostatectomy tissue, which has been shown to be more reliable. Using only one point in time for the analyses of the plasma/urine marker after the prostate biopsies or TUR-P, we were unable to further categorize the use of our markers after the prostate procedures, and were not able to provide a sufficient time point

after prostate manipulation when test may be safely used again.

Conclusions

In an everyday setting we were able to predict the presence of medium-high grade prostate cancer and in detecting patients, for whom biopsies might not be warranted with the use of the biomarker test; thereby confirming the earlier findings of the test.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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