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Performance of a Single Assay for Both Type III and Type VI *TMPRSS2:ERG* Fusions in Noninvasive Prediction of Prostate Biopsy Outcome

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Abstract

BACKGROUND—*TMPRSS2:ERG* fusions are promising prostate cancer biomarkers. Because they can occur in multiple forms in a single cancer specimen, we developed a quantitative PCR test that detects both type III and type VI *TMPRSS2:ERG* fusions. The assay is quantified from a standard curve determined with a plasmid-cloned type III *TMPRSS2:ERG* fusion target.

METHODS—We collected expressed prostatic secretion (EPS) under an institutional review board–approved, blinded, prospective study from 74 patients undergoing transrectal ultrasound-guided biopsy for prostate cancer. We compared the characteristic performance of the test for type III and type VI *TMPRSS2:ERG* fusions in predicting biopsy outcome and distinguishing between high and low Gleason scores with similar tests for the expression of *PCA3* and DNA methylation levels of the *APC*, *RARB*, *RASSF1*, and *GSTP1* genes. We used logistic regression to analyze the effects of multiple biomarkers in linear combinations.

RESULTS—Each test provided a significant improvement in characteristic performance over baseline digital rectal examination (DRE) plus serum prostate-specific antigen (PSA); however, the test for type III and type VI *TMPRSS2:ERG* fusions yielded the best performance in predicting biopsy outcome [area under the curve (AUC) 0.823, 95% CI 0.728–0.919, $P < 0.001$] and Gleason grade >7 (AUC 0.844, 95% CI 0.740–0.948, $P < 0.001$).

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CONCLUSIONS—Although each test appears to have diagnostic value, PSA plus DRE plus type III and type VI *TMPRSS2:ERG* provided the best diagnostic performance in EPS specimens.

Prostate cancer screening with serum prostate-specific antigen (PSA)⁴ and other indicators has been successful in the early detection of prostate cancer. These indicators [e.g., serum PSA >4 $\mu\text{g/L}$ and/or abnormal digital rectal examination (DRE)] require a prostate biopsy for definitive diagnosis. On average, about 62% of these biopsies will initially be negative, and about 8% of patients who have initially negative results will undergo repeat biopsy (1). Thus, about 434 000 biopsies will be performed to detect the estimated 186 320 new cases of prostate cancer diagnosed in the US during 2008 (2).

Clearly the number of initial and repeat biopsies required could be diminished if additional noninvasive biomarkers were available. For this reason, a number of biomarkers are being studied as supplements to PSA and DRE. Serum testing for early prostate cancer antigen 2 (EPCA-2) (3) and urine testing for DNA methylation (4) have been shown to be effective in detecting prostate cancer in previously diagnosed patients but have not yet been applied to the prediction of biopsy outcome in undiagnosed groups. *PCA3* (prostate cancer antigen 3),⁵ a well-characterized RNA expression biomarker for prostate cancer (5–7), has been successfully applied to the prediction of biopsy outcome (8) in urine specimens obtained after attentive DRE. Another well-characterized expression marker comprises the various chromosome 21 fusions that can occur in prostate cancer between *TMPRSS2* (transmembrane protease, serine 2) and *ERG* [v-ets erythroblastosis virus E26 oncogene homolog (avian)]^(9) and *TMPRSS2* and *ETV1* (ets variant 1) (10). Fusions of this type appear to be unique to prostate cancer. Among these fusions, several of the subtypes (11) have been detected in multiple chromosomal copies in prostate cancer cells (12). Selection for multiple copies is reminiscent of gene amplification during the evolution of drug resistance (13,14) and therefore suggests that

⁴Nonstandard abbreviations:

PSA	prostate-specific antigen
DRE	digital rectal examination
PSA3	prostate cancer antigen 3
QPCR	quantitative PCR
EPS	expressed prostatic secretion
AUC	area under the curve

⁵Human Genes:

PCA3	prostate cancer antigen 3
TMPRSS2	transmembrane protease, serine 2
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ETV1	ets variant 1
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
KLK3	kallikrein-related peptidase 3
GSTP1	glutathione S-transferase π 1
APC	adenomatous polyposis coli
RARB	retinoic acid receptor β
RASSF1	Ras association (RalGDS/AF-6) domain family member 1

increased levels of expression of these particular gene fusions confer a strong selective advantage on prostate cancer cells.

One of the *TMPRSS2:ERG* variants (type III) has been successfully applied in mixed populations of patients undergoing biopsy for cancer diagnosis or previously diagnosed patients undergoing surgery for prostate cancer (15,16). Characteristic performance of *TMPRSS2:ERG* type III as a biomarker for prostate cancer was enhanced by inclusion of *PCA3* in a multiplex quantitative PCR (QPCR) system (16). However, this combined system has not yet been applied to the prediction of biopsy outcome in an undiagnosed group.

In previous work (17), we noted that expressed prostatic secretion (EPS) might have some advantages over urine as a source of prostate cancer biomarkers. Since that time, *PCA3* tests have been shown to yield accurate results when applied to either EPS or urine obtained after attentive DRE (7). In the work we report here, we compare the performance of TaqMan® QPCR assays designed to quantify prostate cancer biomarkers by reference to cloned standards. We compared biomarkers for DNA methylation, *PCA3*, and *TMPRSS2:ERG* fusions for their effectiveness in predicting biopsy outcome. Our results suggest that a single test designed to detect both the type III and type VI variants of the several *TMPRSS2:ERG* fusions is superior in performance to bisulfite-mediated DNA methylation testing or *PCA3* testing in expressed prostatic secretion.

Patients and Methods

GENERAL PROCEDURES

Specimen collection and storage—Men who were undergoing prostate biopsy for evaluation of prostate cancer gave consent for EPS specimen collection under an institutional review board–approved, blinded, prospective study protocol. Before biopsy for prostate cancer, a digital rectal examination was performed, followed by prostatic massage and milking of the urethra to collect prostatic secretions. Each specimen was immediately placed on ice and transported to the laboratory, where it was suspended in 3 mL chilled PBS. The resuspended specimen was dispensed into 1.5-mL screw-capped microcentrifuge tubes in 3 1-mL aliquots. The aliquots were sedimented at 8000g for 5 min at 4 °C. Supernatant fluid was discarded, and the tubes containing EPS sediment were stored at –80 °C until use.

Nucleic acid preparation—We used 1 of the 3 stored aliquots to prepare cDNA for RT-PCR assays on each of the genes studied. DNA was prepared from the other 2 aliquots. Of the 74 specimens, only the 63 that yielded ≥ 300 ng total nucleic acid were used for DNA methylation analysis.

PCR data—Data are expressed as number of copies detected in 25- μ L reaction volume by comparison to an appropriate standard curve (18). Data points were assigned zero in the methylation analyses if amplification was not observed during the 50-cycle PCR reaction.

Assay controls for the DNA methylation assays have been described (18). We performed similar controls demonstrating the log-linearity of the standard curves and dynamic range of each assay for each of the RNA detection assays (see Supplemental Fig. 1, which accompanies the online version of this article at <http://www.clinchem.org/content/vol54/issue12>, for representative data on the *TMPRSS2:ERG* fusion assay). Standard curves were very reproducible. The average (SD) R^2 value for all data sets was 0.9979 (0.0019), and the average slope for all data sets was –3.4893 (0.1328).

Serum PSA measurement—We measured serum PSA concentrations immunometrically using the Vitros Immunodiagnostics Total PSA system (Ortho-Clinical Diagnostics).

DRE—We used current standard-of-care methods in performing DREs. Reported results were analyzed as a dichotomous variable segregating data into DRE: normal or DRE: suspicious for malignancy.

DNA methylation detection with methylation-sensitive TaqMan QPCR—Primers and cloned standards used in the analysis are listed in (18), as are details of the bisulfite treatment and the PCR conditions.

Cloning and isolation of TaqMan QPCR standards—Overlap sequences (18) used in cloning standards for PSA, *PCA3*, and *TMPRSS2:ERG* TaqMan RNA detection systems are described in Fig. 1.

REVERSE TRANSCRIPTION TAQMAN QPCR

RNA isolation and cDNA preparation—RNA was isolated using the RNAqueous Kit (Ambion) according to the manufacturer's instructions. We added total RNA to a 20- μ L cDNA reaction containing 1 unit Omniscript RT (Qiagen), 2 μ L 10 $\times$ RT buffer, 1 μ mol/L random hexamer primers, 0.5 mmol/L dNTPs, and 20 units Superscript-III (Applied Biosystems). The reaction was incubated at 37 °C for 1 h, then 2 μ L of the cDNA product was used as template for QPCRs.

QPCR conditions—Plasmids containing cloned standards appropriate to each reaction were linearized by restriction digestion and serially diluted from stock solutions as described (18). Standards were run in parallel in the same rotor as the unknowns.

1. *TMPRSS2:ERG*: Final reaction conditions: 1.25 units Hotstar *Taq* polymerase, 1 $\times$ Hotstar PCR buffer, 800 μ mol/L MgCl₂, 2 μ L Q solution, 0.9 μ mol/L forward and reverse primers, 320 μ mol/L dNTPs, 0.25 μ mol/L probe, and 2 μ L cDNA in a total volume of 25 μ L. PCR cycling conditions: 1 $\times$ (95 °C 10 min); 50 $\times$ (95 °C 15 s, 60 °C 30 s, 72 °C 30 s). The dynamic range of the assay obtained with the linearized plasmid-cloned target sequences is depicted in online Supplemental Fig. 1.

2. *PCA3*: Final reaction conditions: 1.25 units Hotstar *Taq* polymerase, 1 $\times$ Hotstar PCR buffer, 400 μ mol/L MgCl₂, 1 μ L Q solution, 0.9 μ mol/L forward and reverse primers, 320 μ mol/L dNTPs, 0.25 μ mol/L probe, and 2 μ L cDNA in a total volume of 25 μ L. PCR cycling conditions: 1 $\times$ (95 °C 10 min); 50 $\times$ (95 °C 20 s, 56 °C 40 s, 72 °C 40 s).

3. *PSA (KLK3)* and *GAPDH*: We collected data on *GAPDH* and *PSA (KLK3)* RNAs primarily to determine whether our RNA preparations contained amplifiable amounts of RNA.

4. *PSA (KLK3)*: Final reaction conditions: 1.25 units Hotstar *Taq* polymerase, 1 $\times$ Hotstar PCR buffer, 400 μ mol/L MgCl₂, 1 μ L Q solution, 0.9 μ mol/L forward and reverse primers, 320 μ mol/L dNTPs, 0.25 μ mol/L probe, and 2 μ L cDNA in a total volume of 25 μ L. PCR cycling conditions: 1 $\times$ (95 °C 10 min); 50 $\times$ (95 °C 15 s, 60 °C 60 s).

5. *GAPDH*: Final reaction conditions: 1.25 units Hotstar *Taq* polymerase, 1 $\times$ Hotstar PCR buffer, 800 μ mol/L MgCl₂, 2 μ L Q solution, 0.9 μ mol/L forward and reverse primers, 320 μ mol/L dNTPs, 0.25 μ mol/L probe, and 2 μ L cDNA in a total volume of 25 μ L. PCR cycling conditions: 1 $\times$ (95 °C 10 min); 50 $\times$ (95 °C 20 s, 60 °C 40 s, 72 °C 40 s). *GAPDH* forward primer, 5'-GAAGGTGAAGGTCGGAGT-3'; reverse primer, 5'-GAAGATGCTGATGGGATTTC-3'; probe, 5'-Cy5-CAAGCTTCCCGTTCTCAGCC-BHQ-3'.

STATISTICAL ANALYSIS

We used logistic regression models to examine the association of biomarker levels with the outcome of biopsy, as well as low- or high-grade tumors as measured by Gleason sum at biopsy. To evaluate the performance of single biomarkers, we simply varied the cutoff points and calculated the true- and false-positive rates in predicting positive biopsy for prostate cancer (or high-grade tumor) based on the value of biomarker. ROC curves were then plotted, and the area under the curve (AUC) was calculated using the Mann–Whitney *U*-statistic. We constructed 95% CIs to test whether the AUC was significantly different from 0.5 (the value at which a useless biomarker is defined). Mann–Whitney tests were used to evaluate *P* values.

To evaluate the incremental discrimination power of the biomarker over baseline serum PSA and DRE, we constructed logistic regression models with all 3 variables. The full model was then compared to the model with only serum PSA and DRE. The related ROC curve was based on the linear predictor obtained from the logistic regression model. The Wald statistic was computed to obtain a 1-tailed *P* value.

We also used the sum of *GSTP1*, *APC*, *RARB*, and *RASSF1* methylated copies as a single marker to evaluate the performance of the combination of methylation markers. This value was then entered into the logistic regression model together with serum PSA and DRE. The combination of expression markers was based on the linear predictor for *PCA3* and *TMPRSS2:ERG* RNA.

When there were 2 or more continuous markers in the logistic regression model, we used spline covariate structure for ROC analysis for reasons of flexibility.

To further check whether methylation and expression biomarkers were complementary to each other, we did a forward stepwise model selection starting with just the baseline model containing only PSA and DRE, then added individual markers to the model, evaluating the statistical significance of their regression coefficients using the Akaike information criterion.

All analyses were carried out using statistical software R 2.4.1.

Results

PATIENT POPULATION

Biomarker data were collected in a blinded fashion on 86 specimens. Based on biopsy pathology, 35 patients were diagnosed with prostate cancer, 12 with high-grade prostatic intraepithelial neoplasia (HGPIN), and 39 as normal (benign). Those patients with HGPIN were excluded from further analysis. The characteristics of the study population are given in Table 1.

BIOMARKER TEST PERFORMANCE IN PREDICTING BIOPSY OUTCOME

We measured the number of methylated copies of promoter targets observed for *GSTP1*, *RARB*, *RASSF1*, and *APC* from the standard curve generated with their respective plasmid-cloned target. We also used the sum of the copy numbers observed from all 4 genes in evaluating methylation test performance. Copy numbers for the single marker *PCA3* RNA were effective in predicting biopsy outcome, as were measured values for *TMPRSS2:ERG* fusion RNAs. The data summarized in online Supplemental Table 1 suggest that, based on AUCs, effectiveness of the single biomarkers in predicting biopsy outcome was ordered as follows: methylation sum $\leq$ *PCA3* < *TMPRSS2:ERG*. However, only the *TMPRSS2:ERG* test was statistically significant in predicting biopsy outcome from EPS. The inclusion of the serum PSA values and DRE results for each patient improved the effectiveness of each biomarker (Fig. 2 and

Table 2). Each biomarker contributed significantly to the baseline model, with $P < 0.05$ for each model. The effectiveness of the tests was ordered as suggested by the single-marker data.

It is important to note that the smaller patient population (63 patients) used for methylation studies completely overlapped with the larger population (74 patients) used for RNA expression analyses. When AUC calculations for RNA analyses were restricted to those 63 patients, we found insignificant variations of <0.046 in AUC values computed from the ROC analyses. Thus the methylation data can be compared directly with the RNA expression data.

PERFORMANCE IN DIFFERENTIATING BETWEEN GLEASON SUM RANGES

It was also of interest to determine whether the methylation and RNA expression markers were effective in differentiating between high-grade and low-grade tumors as measured by Gleason sum at biopsy.

This analysis can be approached in 2 ways. One can confine the data set to those patients with prostate cancer; alternatively, the patients with a benign diagnosis can be included in the analysis. We performed both analyses. Including the benign patients is preferable, however, since the envisioned use for a test of this type is the prediction of dangerous cancer before biopsy. The data summarized in online Supplemental Table 2 show that only *TMPRSS2:ERG* was effective as a statistically significant single marker, with an AUC value of 0.773, $P < 0.05$. The data summarized in Fig. 3 and Table 3, however, show that each marker in combination with standard PSA and DRE results added statistically significant value to diagnostic performance of the model over the baseline model with only PSA and DRE values incorporated. Here again, each biomarker contributed significantly to the baseline model ($P < 0.05$ for each model), and the order of the effectiveness of the tests was methylation sum $\approx$ *PCA3* $<$ *TMPRSS2:ERG* based on AUC values.

SCREENING PERFORMANCE: SINGLE MARKER SCREENING PARAMETERS

Although the characteristic performance of the biomarkers permits the objective ranking of these tests implemented in EPS specimens, the utility of each biomarker in screening is best gauged at optimal cutoff values. We explored the ability of these markers to perform in EPS screening for prostate cancer. By choosing a cutoff that yields a 90% chance that the full test (PSA plus DRE plus marker) will be negative in patients who have a negative biopsy (90% specificity), we can compute the probability that a patient has the disease if the test is positive (positive predictive value). The positive predictive values again ranked the *TMPRSS2:ERG* test as the most effective in predicting biopsy outcome (online Supplemental Table 3) or in differentiating between high and low Gleason sums (online Supplemental Table 4) as a single marker. However, the rankings were not statistically significant with this small sample size, $P > 0.05$.

Alternatively, one can choose the cutoff value so that the test will be positive in 90% of the patients with a positive biopsy (90% sensitivity) and compute the probability that a patient will not have a positive biopsy when the test is negative (negative predictive value). Again, the negative predictive values ranked the *TMPRSS2:ERG* test as most effective in predicting biopsy outcome (online Supplemental Table 3) or in differentiating high and low Gleason sums (online Supplemental Table 4). Here again, however, the rankings were not statistically significant with this small sample size, $P > 0.05$.

Discussion

Although the PCR system and plasmid standard developed for the detection of *TMPRSS2:ERG* fusions comprises a single QPCR analysis, the test is designed to detect 2 of

the known fusions: type III and type VI (Fig. 1A). Of the various fusion types described by Wang et al. (11), type III is the most common, with *TMPRSS2* fusions of any type present in 40%–80% of prostate cancers (15). Based on the reported cloning frequencies (11), the single PCR for type III and type VI used here should detect the majority of the fusions in prostate cancer specimens, since these two isoforms are found in 86% and 26%, respectively, of the prostate cancers that express a fusion. (Note that the sum of these numbers exceeds 100% because many cancers express >1 fusion type.) Moreover, as noted in the introduction, duplication of *TMPRSS2:ERG* fusions is associated with poor outcome (12), suggesting (by analogy with gene amplification during drug resistance) that high levels of expression of the fusion are selected during tumor progression. For example, Tomlins et al. (10) reported that 95% of prostate cancers that overexpress *ERG* possess an inframe *TMPRSS2:ERG* fusion. Thus, given the specificity of the expression of the fusion for prostate cancer, its expression in EPS could be a marker of both prostate cancer and aggressiveness.

Studies employing PCR analysis of postmassage urines (i.e., urines obtained from patients after attentive DRE) have appeared. Tests for type III *TMPRSS2:ERG* fusions have been studied in postmassage urine using cohorts comprising patients with a diagnosis of prostate cancer (15). In the latter study, samples were collected from 19 patients, including 11 prebiopsy patients whose diagnosis was positive and 8 patients who were previously diagnosed and tested before radical prostatectomy. The TaqMan QPCR test for type III fusions *TMPRSS2:ERG* was demonstrated to detect many of the cancers (42%), thus validating the marker (15). A second study used semiquantitative RT-PCR to study an atypical patient population comprising 78 patients with positive prostate cancer biopsies and 30 with negative biopsies (19).

Since the completion of the present study, a third report has appeared in which the TaqMan QPCR on type III fusions was studied in postmassage urines (16). After excluding patients with low yields of PSA RNA, the study population contained 52 previously diagnosed men undergoing radical prostatectomy and 182 patients undergoing biopsy of whom 86 were positive on diagnosis and 96 were not. The AUC value reported for *PCA3* as a single marker (0.661) in Laxman et al. (16) was comparable to that reported here (0.600). For the assay detecting type III *TMPRSS2:ERG* fusions in postmassage urine specimens (16), only the sensitivity (40.6%) and specificity (72.9%) were reported. These values were lower than the corresponding 63% sensitivity and 80% specificity (data not shown) we observed for the type III plus type VI *TMPRSS2:ERG* assay applied to EPS. Unfortunately, it is not possible to isolate the relative effects of the 2 noninvasive specimen types (postmassage urine vs EPS) or assay types (type III assay vs the type III plus type VI assay) from the available data.

Nevertheless, the present study allowed us to rank the effectiveness of comparably implemented tests for *PCA3*, DNA methylation, and *TMPRSS2:ERG* fusion tests in a single cohort of previously undiagnosed patients with a single noninvasive specimen type. Based on AUC performance characteristics, our analyses ranked DNA methylation testing (sum of methylated copies) as roughly comparable to *PCA3* testing. In terms of ease of application, however, *PCA3* RNA expression is likely to be more cost effective and less time consuming than DNA methylation testing, since methylation testing requires inspecting multiple single-copy genes for changes in methylation state to determine whether any gene has become hypermethylated. Thus, our results are consistent with those of others (4) in suggesting that multiple genes must be tested for methylation to obtain results comparable to those obtained with single RNA expression markers like *PCA3*. Moreover, all of the methods reported thus far in which *TMPRSS2* fusions have been employed show clear improvement over serum PSA alone (15,16,19), and the combined use of serum PSA, DRE, and type III and type VI *TMPRSS2:ERG* fusion levels reported here is exceptional in that regard.

Given the potential relationships reported between the presence of *TMPRSS2:ERG* fusions and aggressiveness, we asked whether the biomarkers tested here could distinguish between patients with biopsy Gleason sums <7 and those with Gleason sums ≥7 (Table 2). In this application, when coupled with serum PSA and DRE, each test had value, but *TMPRSS2:ERG* was clearly the most effective (Fig. 3A).

Combining these markers into panels may also be of value; however, combining serum PSA, DRE, *PCA3*, and *TMPRSS2:ERG* data did not appear to improve the diagnostic performance of the tests in EPS over that observed with serum PSA, DRE, and *TMPRSS2:ERG* alone. Even so, it is worth noting that the performance of the combination of biomarkers reported in this article may have optimism bias because we evaluated the markers based on the same data used to fit the model. Adjusting the bias is beyond the current scope of our analysis. However, the single-marker evaluations (online Supplemental Tables 1 and 2) do not have the potential for optimism bias because there is no modeling involved.

Ideally, noninvasive testing based on EPS screening could be used clinically to determine the necessity of biopsy in patients being evaluated for prostate cancer following general diagnostic screening or in patients in watchful waiting (expectant management) programs. For this purpose, positive and negative predictive values of the test at logical cutoffs are most informative. Although limited by the relatively small sample size of the present study, the data in online Supplemental Tables 3 and 4 suggest that EPS screening using *TMPRSS2:ERG* fusion analysis as described here may well serve as a useful adjunct to current screening modalities when combined with serum PSA and DRE values in both general diagnosis and expectant management programs.

In summary, our findings suggest that each of the 3 tests studied here (sum of methylated DNA copies in the *GSTP1*, *APC*, *RARB*, *RASSF1* panel; *PCA3* RNA expression; and *TMPRSS2:ERG* fusion RNA expression) has diagnostic value in determining biopsy outcome and relative Gleason Sum with noninvasively obtained EPS specimens. Of these, the simple 3-marker panel, serum PSA, DRE, and type III plus type VI *TMPRSS2:ERG* expression, tested as described here has significant potential in this regard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. O'Dowd GJ, Miller MC, Orozco R, Veltri RW. Analysis of repeated biopsy results within 1 year after a noncancer diagnosis. *Urology* 2000;55:553–9. [PubMed: 10736500]
2. National Cancer Institute. Prostate cancer. [Accessed November 2008]. <http://www.cancer.gov/cancertopics/types/prostate>
3. Leman ES, Cannon GW, Trock BJ, Sokoll LJ, Chan DW, Mangold L, et al. EPCA-2: a highly specific serum marker for prostate cancer. *Urology* 2007;69:714–20. [PubMed: 17445657]

4. Hoque MO, Topaloglu O, Begum S, Henrique R, Rosenbaum E, Van Criekinge W, et al. Quantitative methylation-specific polymerase chain reaction gene patterns in urine sediment distinguish prostate cancer patients from control subjects. *J Clin Oncol* 2005;23:6569–75. [PubMed: 16170165]
5. Reynolds MA, Kastury K, Groskopf J, Schalken JA, Rittenhouse H. Molecular markers for prostate cancer. *Cancer Lett* 2007;249:5–13. [PubMed: 17303324]
6. Marks LS, Fradet Y, Deras IL, Blase A, Mathis J, Aubin SM, et al. *PCA3* molecular urine assay for prostate cancer in men undergoing repeat biopsy. *Urology* 2007;69:532–5. [PubMed: 17382159]
7. van Gils MP, Cornel EB, Hessels D, Peelen WP, Witjes JA, Mulders PF, et al. Molecular *PCA3* diagnostics on prostatic fluid. *Prostate* 2007;67:881–7. [PubMed: 17440939]
8. Groskopf J, Aubin SM, Deras IL, Blase A, Bodrug S, Clark C, et al. APTIMA *PCA3* molecular urine test: development of a method to aid in the diagnosis of prostate cancer. *Clin Chem* 2006;52:1089–95. [PubMed: 16627561]
9. Perner S, Demichelis F, Beroukhi R, Schmidt FH, Mosquera JM, Setlur S, et al. *TMPRSS2:ERG* fusion-associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res* 2006;66:8337–41. [PubMed: 16951139]
10. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of *TMPRSS2* and *ETS* transcription factor genes in prostate cancer. *Science (Wash DC)* 2005;310:644–8.
11. Wang J, Cai Y, Ren C, Ittmann M. Expression of variant *TMPRSS2/ERG* fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res* 2006;66:8347–51. [PubMed: 16951141]
12. Attard G, Clark J, Ambroisine L, Fisher G, Kovacs G, Flohr P, et al. Duplication of the fusion of *TMPRSS2* to *ERG* sequences identifies fatal human prostate cancer. *Oncogene* 2008;27:253–63. [PubMed: 17637754]
13. Stark GR, Debatisse M, Giulotto E, Wahl GM. Recent progress in understanding mechanisms of mammalian DNA amplification. *Cell* 1989;57:901–8. [PubMed: 2661014]
14. Singer MJ, Mesner LD, Friedman CL, Trask BJ, Hamlin JL. Amplification of the human dihydro-folate reductase gene via double minutes is initiated by chromosome breaks. *Proc Natl Acad Sci U S A* 2000;97:7921–6. [PubMed: 10859355]
15. Laxman B, Tomlins SA, Mehra R, Morris DS, Wang L, Helgeson BE, et al. Noninvasive detection of *TMPRSS2:ERG* fusion transcripts in the urine of men with prostate cancer. *Neoplasia* 2006;8:885–8. [PubMed: 17059688]
16. Laxman B, Morris DS, Yu J, Siddiqui J, Cao J, Mehra R, et al. A first-generation multiplex biomarker analysis of urine for the early detection of prostate cancer. *Cancer Res* 2008;68:645–9. [PubMed: 18245462]
17. Crocitto LE, Korn D, Kretzner L, Shevchuk T, Blair SL, Wilson TG, et al. Prostate cancer molecular markers *GSTP1* and *hTERT* in expressed prostatic secretions as predictors of biopsy results. *Urology* 2004;64:821–5. [PubMed: 15491741]
18. Munson K, Clark J, Lamparska-Kupsik K, Smith SS. Recovery of bisulfite-converted genomic sequences in the methylation-sensitive QPCR. *Nucleic Acids Res* 2007;35:2893–903. [PubMed: 17439964]
19. Hessels D, Smit FP, Verhaegh GW, Witjes JA, Cornel EB, Schalken JA. Detection of *TMPRSS2-ERG* fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. *Clin Cancer Res* 2007;13:5103–8. [PubMed: 17785564]

Fig. 1. TaqMan QPCR

(A), Targeting *TMPRSS2:ERG* type III and type VI fusions. Eight well-characterized fusions between *TMPRSS2* and *ERG* are known to occur in prostate cancer (11). Two of these, type III and type VI, occur in 86% and 26% of cloned representatives from prostate cancer specimens, respectively (11). These 2 variants differ only in the precise position of the breakpoints and can be detected with the single TaqMan system depicted. In type III fusions, exon 1 of *TMPRSS2* is fused to the beginning of exon 4 of *ERG* (11). In type VI fusions, exon 1 and exon 2 of *TMPRSS2* are fused to the beginning of exon 4 of *ERG* (11). Thus by choosing primer sites in exon 1 of *TMPRSS2* and exon 4 of *ERG*, both fusion types are amplified. (B), Cloning and linearization of standards. The PCR conditions used to produce fragments for blunt-end cloning and the cloning vectors were as follows. PSA overlap PCR: 1.65 units Hotstar *Taq* polymerase (Qiagen), 1× Hotstar Buffer (Qiagen), 1 mmol/L $MgCl_2$, 5.0 μ L Q solution, 1.08 μ mol/L upper and lower overlap primers, and 400 μ mol/L dNTPs in a total reaction volume of 25 μ L. Cycling conditions: 1× (95 °C 10 min), 5× (94 °C 30 s, 60 °C 30 s, 72 °C 30 s), 5× (94 °C 30 s, 58 °C 30 s, 72 °C 30 s), 15× (94 °C 30 s, 56 °C 30 s, 72 °C 30 s), 1× (72 °C 3 min). Vector and length: TOPO 2.1, 3999 bp. Cloned standard linearized with: *R.ScaI*. *PCA3* overlap PCR: 1.65 units Hotstar *Taq* polymerase, 1× Hotstar buffer, 1 mmol/L $MgCl_2$, 5.0 μ L Q solution, 1.08 μ mol/L upper and lower overlap primers, and 400 μ mol/L dNTPs in a total reaction volume of 25 μ L. Cycling conditions: 1× (95 °C 10 min), 5× (94 °C 30 s, 60 °C 30 s, 72 °C 30 s), 5× (94 °C 30 s, 58 °C 30 s, 72 °C 30 s), 15× (94 °C 30 s, 56 °C 30 s, 72 °C 30 s), 1× (72 °C 3 min). Vector and length: TOPO 2.1, 4031 bp. Cloned standard linearized with: *R.ScaI*. *TMPRSS2:ERG* overlap PCR: 1.25 units Hotstar *Taq* polymerase, 1× Hotstar buffer, 2.5 μ L Q solution, 300 μ mol/L dNTPs, and 0.9 μ mol/L upper and lower overlap primers in a final reaction volume of 25 μ L. Cycling conditions: 1× (95 °C 10 min) 15× (95 °C 30 s, 56 °C 30 s, 72 °C 30 s). Vector and length: Bluescript, 3041 bp. Cloned standard linearized with: *R.NcoI*. oligo(s), oligonucleotide(s).

Fig. 2. ROC curves comparing *PCA3*, *TMPRSS2:ERG*, or sum of promoter methylation levels with baseline PSA and DRE results in predicting biopsy result

ROC data comparing incremental increases in diagnostic performance over baseline covariates serum PSA + DRE results. (A), *PCA3* or *TMPRSS2:ERG* for the RT-PCR data set: $n = 74$, benign = 39, prostate cancer = 35. Performance for baseline covariates PSA + DRE are plotted as a broken blue line (AUC 0.645, 95% CI 0.519–0.771). Performance for PSA + DRE + *PCA3* are plotted as a broken red line (AUC 0.692, 95% CI 0.571–0.813 compared to 0.600, 0.469–0.732 for the single marker itself). Performance for PSA + DRE + *TMPRSS2:ERG* are plotted in green (AUC 0.823, 95% CI 0.728–0.919 compared to 0.778, 0.671–0.886 for the single marker itself). (B), Sum of promoter methylation levels for the bisulfite mediated PCR data set: $n = 63$, benign = 33, prostate cancer = 30. Performance for baseline covariates PSA + DRE are plotted as a solid green line (AUC 0.645, 95% CI 0.519–0.771). Performance for PSA + DRE + sum of promoter methylation levels are plotted as a broken red line (AUC 0.721, 95% CI 0.595–0.847 compared to 0.576, 0.432–0.720 for the sum evaluated as single marker itself).

Fig. 3. ROC curves comparing *PCA3*, *TMPRSS2:ERG*, or sum of promoter methylation levels with baseline PSA and DRE results in predicting Gleason sum ≥ 7

ROC data comparing incremental increases in diagnostic performance over baseline covariates Serum PSA + DRE results. (A), *PCA3* or *TMPRSS2:ERG* for the RT-PCR data set: $n = 74$, Gleason sum $< 7 = 60$, Gleason sum $\geq 7 = 14$. Performance for baseline covariates PSA + DRE are plotted as a broken blue line (AUC 0.688, 95% CI 0.552–0.824). Performance for PSA + DRE + *PCA3* are plotted as a broken red line (AUC 0.751, 95% CI 0.638–0.864 compared to 0.510, 0.350–0.670 for the single marker itself). Performance for PSA + DRE + *TMPRSS2:ERG* are plotted as a solid green line (AUC 0.844, 95% CI 0.740–0.948 compared to 0.733, 0.640–0.880 for the single marker itself). (B), Sum of promoter methylation levels for the bisulfite mediated PCR data set: $n = 63$, Gleason sum $< 7 = 52$, Gleason sum $\geq 7 = 11$. Performance for baseline covariates PSA + DRE are plotted as a solid green line (AUC 0.684, 95% CI 0.531–0.838). Performance for PSA + DRE + sum of promoter methylation levels are plotted as a broken red line (AUC 0.733, 95% CI 0.585–0.880).

Table 1
Selected descriptive parameters for patients undergoing biopsy for prostate cancer.

Parameter	Benign	Prostate cancer	Total
n	39	35	74
Mean age, years (SD) ^a	63.82 (7.65)	67.06 (6.66)	65.35 (7.33)
Serum PSA concentration, ng/dL ^a			
Mean (SD)	7.07 (5.03)	9.83 (15.93)	8.38 (11.55)
Range	1.1–15.74	1.1–98.6	1.1–98.6
DRE, n (%) ^a			
Normal	25 (64.10)	20 (57.14)	45 (60.81)
Not completed	4 (10.26)	4 (11.43)	8 (10.81)
Suspicious for malignancy	10 (25.64)	11 (31.43)	21 (28.38)
Ethnicity, n (%) ^a			
White	35 (89.74)	31 (88.57)	66 (89.19)
Other	4 (10.26)	4 (11.43)	8 (10.81)
Gleason sum, n (%) ^b			
<7	39 (100)	21 (60)	
≥7	0	14 (40)	

^a *t* Test or χ^2 test, *P* > 0.05.

^b *t* Test or χ^2 test, *P* < 0.001.

Table 2

Prediction measures, benign vs prostate cancer diagnosis.

Serum PSA + DRE + marker	Odds ratio ^a (95% CI)	AUC (95% CI) ^b
Benign (n = 33) vs prostate cancer (n = 30)		
None	—	0.630 (0.491–0.770)
<i>GSTP1</i> methylation	0.390 (0.050–1.121)	0.688 (0.557–0.820) ^c
<i>APC</i> methylation	0.890 (0.446–1.563)	0.662 (0.527–0.796) ^c
<i>RARB</i> methylation	2.064 (1.030–6.551) ^c	0.705 (0.576–0.835) ^d
<i>RASSF1</i> methylation	1.284 (0.754–2.396)	0.671 (0.535–0.807) ^c
Methylation sum ^e	1.584 (0.884–3.603)	0.721 (0.595–0.847) ^d
Benign (n = 39) vs prostate cancer (n = 35)		
None	—	0.645 (0.519–0.771) ^c
<i>PCA3</i> RNA	0.809 (0.260–1.391)	0.692 (0.571–0.813) ^d
<i>TMPRSS:ERG</i> RNA	4.239 (1.858–11.870) ^f	0.823 (0.728–0.919) ^f

^aThe odds ratio tests the improvement in goodness of fit obtained by including the additional predictor when the baseline model with PSA and DRE is compared to the corresponding augmented model. Markers were standardized to have mean 0 and SD 1. *P* values indicate significance of difference between the baseline and augmented model.

^bThe AUC and its 95% CI are reported for each marker coupled with the baseline covariate markers serum PSA and DRE. *P* values indicate significance of difference between the baseline and augmented model.

^c*P* < 0.05.

^d*P* < 0.01.

^eThe methylation sum is defined as the sum of all methylated copies at *GSTP1*, *APC*, *RARB*, and *RASSF1* in a given specimen taken as a single marker.

^f*P* < 0.001.

Table 3

Prediction measures, benign plus Gleason sums <7 and ≥7.

Serum PSA + DRE + marker	Odds ratio ^d (95% CI)	AUC (95% CI) ^b
Benign + Gleason sum <7 (n = 52) vs Gleason sum ≥7 (n = 11)		
None	—	0.684 (0.531–0.838)
<i>GSTP1</i> methylation	0.769 (0.082–1.577)	0.701 (0.551–0.851) ^c
<i>APC</i> methylation	0.005 (0–1.156)	0.754 (0.616–0.892) ^d
<i>RARB</i> methylation	0.786 (0.226–1.551)	0.764 (0.618–0.910) ^d
<i>RASSF1</i> methylation	0.961 (0.405–1.784)	0.696 (0.539–0.852) ^c
Methylation sum ^e	0.739 (0.206–1.493)	0.733 (0.585–0.880) ^c
Benign + Gleason sum <7 (n = 60) vs Gleason sum ≥7 (n = 14)		
None	—	0.688 (0.552–0.824)
<i>PCA3</i> RNA	0.092 (0–1.283)	0.751 (0.638–0.864) ^d
<i>TMPRSS:ERG</i> RNA	1.084 (0.571–1.857)	0.844 (0.740–0.948) ^f

^aThe odds ratio tests the improvement in goodness of fit obtained by including the additional predictor when the baseline model with PSA and DRE is compared to the corresponding augmented model. Markers were standardized to have mean 0 and SD 1. *P* values indicate significance of difference between the baseline and augmented model.

^bThe AUC and its 95% CI are reported for each marker coupled with the baseline covariate markers serum PSA and DRE. *P* values indicate significance of difference between the baseline and augmented model.

^c*P* < 0.05.

^d*P* < 0.01.

^eThe methylation sum is defined as the sum of all methylated copies at *GSTP1*, *APC*, *RARB*, and *RASSF1* in a given specimen taken as a single marker.

^f*P* < 0.001.