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Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA

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Abstract

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Author contributions: S.A.T. and A.M.C. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. S.A.T., S.M.J.A., A.B., Y.P., M.G.S., M.A.R., D.R.R., H.R., J.G., J.T.W., and A.M.C. designed the study. S.M.J.A., S. Miick, J.R.D., S.W., P.H., J.M., H.R., and J.G. developed and performed the TMA assays. J.L.S., L.S.-M., D.W., B. Hollenbeck, K.S., J.S., Y.F., J.B.A., S. Meyers, and J.T.W. obtained, evaluated, and processed specimens. R.V. maintained clinical information. B. Han, L.W., and N.P. performed FISH assays. S.A.T. and R.J.L. analyzed the data. S.A.T. wrote the first draft of the manuscript, which was reviewed by all authors.

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More than 1,000,000 men undergo prostate biopsy each year in the United States, most for “elevated” serum prostate specific antigen (PSA). Given the lack of specificity and unclear mortality benefit of PSA testing, methods to individualize management of elevated PSA are needed. Greater than 50% of PSA-screened prostate cancers harbor fusions between the transmembrane protease, serine 2 (TMPRSS2) and v-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG) genes. Here, we report a clinical-grade, transcription-mediated amplification assay to risk stratify and detect prostate cancer noninvasively in urine. The TMPRSS2:ERG fusion transcript was quantitatively measured in prospectively collected whole urine from 1312 men at multiple centers. Urine TMPRSS2:ERG was associated with indicators of clinically significant cancer at biopsy and prostatectomy, including tumor size, high Gleason score at prostatectomy, and upgrading of Gleason grade at prostatectomy. TMPRSS2:ERG, in combination with urine prostate cancer antigen 3 (PCA3), improved the performance of the multivariate Prostate Cancer Prevention Trial risk calculator in predicting cancer on biopsy. In the biopsy cohorts, men in the highest and lowest of three TMPRSS2:ERG+PCA3 score groups had markedly different rates of cancer, clinically significant cancer by Epstein criteria, and high-grade cancer on biopsy. Our results demonstrate that urine TMPRSS2:ERG, in combination with urine PCA3, enhances the utility of serum PSA for predicting prostate cancer risk and clinically relevant cancer on biopsy.

INTRODUCTION

Although the use of serum prostate-specific antigen (PSA) to screen for prostate cancer is widespread clinically (1), PSA has several limitations as an early detection biomarker. PSA is highly specific for tissue of prostatic origin, but is not cancer-specific. Moreover, serum levels are frequently elevated in benign conditions. Currently, whereas most men undergo needle biopsy when levels of PSA are more than 4.0 ng/ml, less than half of these biopsies result in a diagnosis of prostate cancer (2, 3). PSA also has sensitivity limitations, as shown by the Prostate Cancer Prevention Trial (PCPT), which demonstrated that 15% of men with PSA of 0 to 4.0 ng/ml have prostate cancer, of which 15% have high Gleason grade disease (4, 5). Despite the development of multivariate models, such as the PCPT risk calculator that incorporates PSA and other clinical factors in an attempt to provide an individual risk estimate (6), men are commonly referred for biopsy in the United States on the basis of their serum PSA concentration alone.

Moreover, screening with PSA has probably led to the overdiagnosis of prostate cancer—an estimated 23 to 44% of all screening-detected cancers would never have caused symptoms (7)—and overtreatment. Two randomized trials evaluating the effect of PSA screening on prostate cancer mortality showed that during the first decade of follow-up, PSA screening has a modest effect on prostate cancer mortality, with substantial risks of negative biopsy and over diagnosis and overtreatment of indolent cancer (cancer that would not cause symptoms in a lifetime) (3, 8). Together, these results highlight the limitations of the current serum PSA-based paradigm of prostate cancer early detection. Recognizing these limitations, several groups, including the United States Preventative Task Force, the American Cancer Society, and the American Urological Association, have advocated for individualized decision making between a patient and his physician regarding PSA screening and/or proceeding to biopsy (9). Biomarkers to assist this process, however, are lacking.

Several modifications of serum PSA, including free PSA, rate of PSA change (PSA velocity), various PSA isoforms, and related proteins, have been proposed as prostate cancer biomarkers that can be used to help PSA-screened men make more informed decisions about proceeding to biopsy (10). These strategies, however, rely on surrogate biomarkers that are

tissue-specific and not intrinsically cancer-specific. An alternative approach is to develop clinically robust assays for cancer-specific biomarkers that have been identified through genomic and transcriptomic studies (11).

Recently, chromosomal rearrangements were identified in prostate cancer that fuse the 5' untranslated region of the androgen-regulated gene transmembrane protease, serine 2 (TMPRSS2) with v-etserythroblastosis virus E26 oncogene homolog (avian) (ERG) or ets variant 1 (ETV1); ERG and ETV1 are both members of the erythroblastosis virus E26 transformation-specific (ETS) transcription factor family (12). Subsequent studies confirmed ETS gene fusions in about 50% of PSA screened prostate cancers (13). Fusions between TMPRSS2 and ERG, which result in a truncated ERG protein product, represent about 90% of all ETS gene fusions (13). Fusion of TMPRSS2 and ERG loci at the chromosomal level [as detected by fluorescence in situ hybridization (FISH)] and subsequent overexpression of the TMPRSS2:ERG transcript and truncated ERG protein product are essentially 100% specific for the presence of prostate cancer in tissue-based studies (13–15). Additionally, multiple studies have demonstrated that TMPRSS2:ERG fusions are only detectable in about 15% of high-grade prostatic intraepithelial neoplasia (PIN) lesions, invariably adjacent to fusion-positive cancer (16–18). In vitro and in vivo functional studies have also demonstrated a functional role for TMPRSS2:ERG fusions in prostate cancer oncogenesis (13, 17, 19, 20). Together, TMPRSS2:ERG gene fusions are highly specific biomarkers that define a distinct molecular subtype of prostate cancer.

The protein product of the TMPRSS2:ERG fusion is neither chimeric nor known to be secreted, which precludes the possibility of antibody-based detection in serum (as for PSA). However, a clinical grade, urine-based assay for the noncoding transcript prostate cancer antigen 3 (PCA3) [a prostate-specific gene overexpressed in greater than 95% of prostate cancers (21)] has been developed and has proven useful as an adjunct to serum PSA for prostate cancer detection (22, 23). In addition, research-grade, reverse transcription–polymerase chain reaction (RT-PCR)–based assays have shown that TMPRSS2:ERG mRNA is indeed detectable in urine (24–28).

To translate these findings to clinical practice, we have developed a clinical-grade, transcription-mediated amplification (TMA) assay for quantifying TMPRSS2:ERG mRNA, which is normalized to the amount of PSA mRNA by TMA (which controls for the abundance of prostate cells and prostate mRNA) to generate a TMPRSS2:ERG “score.” The assay is based on the same technology as the PCA3 assay. We tested prospectively collected, post–digital rectal exam (DRE) urine from men presenting for biopsy and/or prostatectomy. We then correlated urine TMPRSS2:ERG levels with clinicopathologic features, including indicators of clinically significant cancer. We also measured PCA3 in the same urine specimens and report the combined performance of TMPRSS2:ERG and PCA3 for prostate cancer risk stratification of PSA-screened men. This work represents an initial step in using a panel of cancer-specific biomarkers for early detection of prostate cancer.

RESULTS

TMPrSS2:ERG assay accuracy To assess the accuracy of the TMPRSS2:ERG TMA assay, we compared it to FISH for ERG rearrangement on 208 needle biopsy cores from 122 patients in the University of Michigan Health System (UMHS) biopsy cohort. On the basis of sufficient PSA expression (PSA copies/ml >3000), 206 of 208 (99%) cores were informative for further TMPRSS2:ERG evaluation. As shown in table S1, 40 of 79 (51%) informative cancerous cores were positive for ERG rearrangement by FISH; all informative benign cores evaluated by FISH (n = 72) were negative. By TMA, 41 of 79 (52%) cancerous and 5 of 127 (4%) benign cores were positive for TMPRSS2:ERG, with an overall

concordance between FISH and TMA in informative cores of 92%. These results further support the cancer specificity of TMPRSS2:ERG and demonstrate the accuracy of the TMA assay.

Urine TMPRSS2:ERG in men undergoing prostatectomy

To assess associations between urine TMPRSS2:ERG and clinicopathological parameters in prostate cancer, we first examined a cohort of 218 men presenting for radical prostatectomy at UMHS. This cohort was selected because prostatectomy provides a more complete assessment of tumor pathology than biopsy, and offers the opportunity to compare biopsy and prostatectomy pathology (see fig. S1 for flow chart of all urine samples in the study). Of 218 men, 187 (86%) had informative urine, based on sufficient urine PSA expression (average PSA copies/ml >15,000), for further TMPRSS2:ERG score evaluation. Clinicopathological characteristics for informative men are shown in Table 1. Consistent with the notion that biopsy often under samples disease burden, 35 of 70 (50%) informative men with biopsy Gleason 6 disease were upgraded to Gleason 7 at prostatectomy, similar to previous reports (29).

An ideal early detection biomarker not only would distinguish patients with and without cancer but would also be associated with clinically significant cancer. At prostatectomy, significant cancer is most commonly defined on the basis of tumor size, high Gleason score (>6), and non-organ-confined disease (pathologic tumor >2). Similarly, models, which commonly include tumor size and Gleason score, have been developed for defining significant cancer in men with positive biopsies (30). In prostatectomy patients, TMPRSS2:ERG score was positively associated with markers of tumor volume, including number of positive cores and maximum percentage of tumor involvement of a single core in the preceding biopsy (Table 1).

TMPRSS2:ERG score was also positively associated with maximum tumor dimension at prostatectomy, but was not associated with prostate weight, serum PSA, or PSA density (PSAD) at prostatectomy (Table 1). TMPRSS2:ERG score was significantly higher in men with high prostatectomy Gleason score (>6 versus 6) and was associated with upgrading at prostatectomy from biopsy (6 to >6 versus 6 to 6) (Table 1). Finally, consistent with associations with indicators of significant pathology at biopsy and prostatectomy, TMPRSS2:ERG score was significantly higher in men with significant versus insignificant cancer at prostatectomy and preceding biopsy.

Urine TMPRSS2:ERG in an academic biopsy cohort

Of the 623 men in the academic biopsy cohort [combined UMHS, University of Laval (UL), and the Veterans Administration San Diego (VASD) Medical Center cohorts], 606 (97%) had informative urine based on sufficient urine PSA expression (average PSA copies/ml >15,000) for TMPRSS2:ERG score evaluation. Prebiopsy clinicopathological characteristics for these men are shown in table S2. On biopsy, 269 of the 606 (44%) informative men were diagnosed with prostate cancer. TMPRSS2:ERG was higher in men diagnosed with cancer compared to those with noncancerous diagnoses (Table 2). TMPRSS2:ERG score was positively associated with direct markers of tumor volume, including maximum percentage of tumor involvement of a single core and number of positive cores (Table 2). TMPRSS2:ERG score was significantly higher in men with significant versus insignificant cancer on biopsy (Table 2). TMPRSS2:ERG score was correlated with increasing age (Table 2) and was higher in men at initial biopsy compared with repeat biopsy, but was not significantly correlated or associated with other prebiopsy clinicopathological parameters, including indicators of prostate size (Table 2).

Urine TMPRSS2:ERG in a community biopsy cohort

To demonstrate the utility of urine TMPRSS2:ERG outside of academic medical centers, we also evaluated a community biopsy cohort. In this cohort, owing to ongoing assay optimization, we used a second generation TMPRSS2:ERG TMA assay (which uses TMPRSS2:ERG primers different from that of the initial assay). Of 471 men in the community biopsy cohort, 463 (98%) had informative urine based on sufficient urine PSA expression (average PSA copies/ml >10,000) for TMPRSS2:ERG score evaluation, using the second-generation assay. Prebiopsy clinicopathological characteristics for these men are shown in table S3

On biopsy, 204 of the 463 (44%) informative men in the community biopsy cohort were diagnosed with prostate cancer. TMPRSS2:ERG score was higher in informative men diagnosed with cancer compared to those with noncancerous diagnoses (Table 3). In informative men with cancer, TMPRSS2:ERG score was positively associated with maximum tumor involvement of a single core, percentage of total involvement, and number of cores positive (Table 3), consistent with findings in the prostatectomy and academic biopsy cohorts. Finally, TMPRSS2:ERG score was higher in men with Gleason score >6 versus 6 (Table 3). All associations between clinicopathological parameters in patients with benign and cancerous biopsies and TMPRSS2:ERG and PCA3 scores are given in Table 3.

TMPRSS2:ERG score for individualizing cancer risk in men with elevated serum PSA undergoing biopsy

On the basis of associations with the presence of cancer and significant pathology, we explored several clinically applicable models for using urine TMPRSS2:ERG to individualize prostate cancer risk in PSA-screened men presenting for biopsy. We first compared receiver operating characteristic (ROC) curves for TMPRSS2:ERG score and serum PSA. Among men with informative urine (where urine PSA expression was sufficient for TMPRSS2:ERG score evaluation) with measured serum PSA, TMPRSS2:ERG had significantly increased area under the curve (AUC; the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one) compared to serum PSA in the academic biopsy cohort (n = 606; 0.71 versus 0.61) (fig. S2A). In the community cohort, although TMPRSS2:ERG had increased AUC compared to serum PSA (particularly at high specificity), this difference was not significant (n = 460; 0.65 versus 0.59)(fig. S2B).

Combined TMPRSS2:ERG and PCA3 scores for predicting the presence of prostate cancer on biopsy

Although nearly 100% specific for the presence of prostate cancer in tissue-based studies, TMPRSS2:ERG gene fusions occur in only ~50% of PSA-screened prostate cancers. Previous studies using research-grade assays have demonstrated that measuring both PCA3 and TMPRSS2:ERG in urine outperforms either marker alone for predicting the presence of prostate cancer on biopsy (24, 25). Thus, to optimize performance, we analyzed the performance of urine TMPRSS2:ERG in combination with PCA3 (both measured using TMA assays from the same urine sample). PCA3 was available for all evaluable men in both the academic prostatectomy and the biopsy cohorts, and in 459 of 463 (99%) of men in the community biopsy cohort. Among informative men with measured TMPRSS2:ERG, PCA3, and serum PSA, the TMPRSS2:ERG+PCA3 score had a significantly increased AUC compared to serum PSA in both the academic (Fig. 1A) and the community biopsy cohorts (Fig. 1B).

We next assessed the ability of TMPRSS2:ERG+PCA3 score to increase the AUC of the multivariate PCPT risk calculator for predicting prostate cancer diagnosis on biopsy. Among

informative men in the academic and community biopsy cohorts, 463 and 439, respectively, had not been diagnosed with prostate cancer previously and the clinical information required for the PCPT risk calculator was available. The PCPT risk calculator showed significantly increased AUC compared to serum PSA alone in both the academic ($n = 463$; 0.64 versus 0.60) and the community ($n = 439$; 0.66 versus 0.61) cohorts (fig. S3). To evaluate the performance of TMPRSS2:ERG in combination with PCA3 in the academic cohort, we used the subset of UL and VASD patients ($n = 202$) to train a model incorporating TMPRSS2:ERG+PCA3 score into the PCPT risk calculator (see Materials and Methods). We then tested this model on the remaining UMHS patients ($n = 261$). In the independent UMHS testing set, incorporation of TMPRSS2:ERG+PCA3 score significantly increased the AUC of the PCPT risk calculator (Fig. 1C). To explore the potential clinical benefit of incorporating TMPRSS2:ERG+PCA3 score into the PCPT risk calculator, we performed decision curve analysis (31) on the same modeled data. Incorporation of TMPRSS2:ERG+PCA3 into the PCPT risk calculator resulted in net benefit across all threshold probabilities (the probability of cancer on biopsy at which an individual would choose to undergo biopsy) from ~15 to 90% (fig. S4), thus demonstrating improved clinical outcome.

Among informative men in the community biopsy cohort, we were able to perform the PCPT risk calculation and derive a PCA3 score for 436 men. Incorporation of TMPRSS2:ERG+PCA3 score significantly increased the AUC of the PCPT risk calculator (Fig. 1D). Similarly, we also explored the ability of TMPRSS2:ERG+PCA3 score to improve an alternative nomogram (which requires free PSA) for predicting cancer on initial biopsy developed by Chun et al. (32). Although this nomogram had increased AUC compared to serum PSA alone, this difference was not statistically significant (0.71 versus 0.64) (fig. S5A). Nevertheless, incorporation of TMPRSS2:ERG+PCA3 into the nomogram resulted in significantly increased AUC compared to the nomogram alone (0.77 versus 0.71) (fig. S5B). The effect of incorporating TMPRSS2:ERG score alone into the PCPT risk calculator is shown in fig. S2, C and D.

TMPRSS2:ERG+PCA3 score groups stratify prostate cancer risk on biopsy

We next evaluated whether stratifying patients on the basis of TMPRSS2:ERG and PCA3 scores could individualize prostate cancer risk in men with elevated serum PSA. Because the TMPRSS2:ERG assays used in the academic biopsy and prostatectomy (initial assay) and community biopsy (second generation) cohorts were different, direct comparison of TMPRSS2:ERG scores was not possible (PCA3 scores were directly comparable across all cohorts). We therefore applied a quartile-based approach using TMPRSS2:ERG and PCA3 scores in our prostatectomy cohort (where pathology was better defined than in the biopsy cohort) to identify cutoffs that binned men into three distinct TMPRSS2:ERG+PCA3 score groups (lowest, intermediate, or highest) in each biopsy cohort. As shown in Table 4, 363 (34%), 346 (32%), and 356 (33%) of 1065 men in the combined biopsy cohorts were in the lowest, intermediate, and highest TMPRSS2:ERG+PCA3 score groups, respectively. Biopsy resulted in a cancer diagnosis in 21%, 43%, and 69% of men in the lowest, intermediate, and highest groups, respectively. Moreover, 7%, 20%, and 40% of men in the respective lowest, intermediate, and highest groups were diagnosed with Gleason score >6 cancer (Table 4). Of the 966 men with required clinical information for determining Epstein criteria for significance of cancer on biopsy (see Methods), 15%, 33%, and 61% of men in the lowest, intermediate, and highest groups, respectively, had Epstein criteria-defined significant cancer (table S4). Results for the individual biopsy cohorts are also shown in Table 4 and table S4.

TMPRSS2:ERG+PCA3 score groups compared to PCPT risk calculator

These results demonstrate that men stratified by TMPRSS2:ERG+PCA3 scores have markedly different risks of cancer, high-grade cancer, and clinically significant cancer on biopsy. To be useful clinically, however, TMPRSS2:ERG+PCA3 score-based stratification should add to currently used clinicopathological information. Thus, we compared PCPT risk calculator and PCPT high-grade risk calculator scores for men in the lowest, intermediate, and highest TMPRSS2:ERG+PCA3 score groups from the biopsy cohorts. As shown in Fig. 2, calculated PCPT risks of cancer (or Gleason score >6 cancer) differed markedly from the actual risks observed in the lowest and highest TMPRSS2:ERG+PCA3 score groups of men. For example, academic patients in the lowest TMPRSS2:ERG+PCA3 score group had a 20% actual risk of prostate cancer on biopsy, but of men evaluable for PCPT risk score calculation, 95% had PCPT risk scores greater than 20%; similarly, 90% of PCPT-evaluable men in the highest TMPRSS2:ERG+PCA3 score group had PCPT risk scores less than their actual risk of cancer on biopsy (72%) (Fig. 2A). Results were similar with regard to high-grade cancer (Gleason score >6) risk in the academic cohort (Fig. 2C), and in the community biopsy cohort for cancer (Fig. 2B) and high-grade cancer risk (Fig. 2D).

DISCUSSION

We report the development of a quantitative, urine-based TMA assay for TMPRSS2:ERG, which is associated with indicators of significant cancer at biopsy and prostatectomy. We demonstrate the utility of this assay alone and in combination with PCA3 expression in urine for individual risk stratification of serum PSA-screened men presenting for prostate biopsy in two multicenter cohorts. Development, validation, and clinical implementation of early detection biomarkers for prostate cancer, particularly specific markers of “aggressive” cancer, are complicated by several aspects of prostate cancer biology and current clinical management. First, the gold standard for detecting prostate cancer based on a biomarker, needle biopsy, is not image-guided and is limited by substantial undersampling. This has been demonstrated by ex vivo studies on prostates at autopsy, which showed that extended (≥ 10 cores) and saturation biopsy schemes miss about 40% of all cancers (including ~20% of significant cancers) detectable when the entire prostate is pathologically examined (33, 34). Second, given the high prevalence of indolent prostate cancer [~30 to 40% among men aged 50 to 80 years (35)], a 100% specific and sensitive test for prostate cancer is unlikely to be achieved and clinically undesirable. Third, localized prostate cancer is often multifocal—with heterogeneous TMPRSS2:ERG status between individual foci (13)—and individual foci cannot currently be followed definitively by imaging or biopsy. Therefore, little is known about the natural history of “aggressive” prostate cancer; furthermore, it is unclear whether an ideal biomarker would need to detect tiny foci of high-grade aggressive cancers (which are rarely detected with current PSA screening and biopsy paradigms). Alternatively, if aggressive cancers progress from low-grade cancers, the ideal biomarker would identify foci of low-grade cancer that might require acquisition of additional aberrations, such as those that might only develop in disseminated cells years after supposedly curative treatment. Finally, defining cutoffs or algorithms for reporting and subsequent clinical decision making will be a balance between sensitivity and specificity, which is complicated in prostate cancer by the above factors.

To increase sensitivity for predicting biopsy outcome, we combined TMPRSS2:ERG with the noncoding transcript PCA3 for individual risk stratification. We envision the first step toward a more specific early detection strategy for prostate cancer to be improved management of men with elevated serum PSA. We have shown that in multiple cohorts, elevated urine TMPRSS2:ERG is associated with the presence of prostate cancer and features of aggressiveness. Moreover, men with extremes of TMPRSS2:ERG+PCA3 have different risks of cancer (and high-grade cancer) on biopsy. Thus, in combination with other

clinical parameters, urine TMPRSS2:ERG+PCA3 may be useful initially in guiding the urgency of biopsy after the detection of elevated serum PSA.

Urine TMPRSS2:ERG+PCA3 score may have additional utility for common scenarios encountered in the early diagnosis of prostate cancer. For example, men in the highest TMPRSS2:ERG+PCA3 score group with a negative biopsy might benefit from close follow-up, because their chances of having cancer are high (table S5). Similarly, men in the highest TMPRSS2:ERG+PCA3 score group enrolling in active surveillance may wish to consider more extensive biopsy, because their risk of having significant disease that was under sampled on initial biopsy is high. Inclusion of other ETS fusions or aberrantly expressed genes that define additional molecular prostate cancer subtypes, such as SPINK1 (25), into a multiplexed panel, may improve accuracy. Last, in light of increasing evidence that ETS fusion-positive and ETS fusion-negative cancers are distinct molecular subtypes with different biological behavior and response to therapy (13, 36), the clinically robust assay described here might have potential prognostic utility.

A limitation of this study is that most patients in the study (>85%) were Caucasian, and additional studies will be required to determine whether our findings extend to all men. Furthermore, because men in this study were PSA-screened (and elected to undergo biopsy), studies in non-PSA-screened cohorts will be required to determine the potential utility of TMPRSS2:ERG+PCA3 in that clinical context.

In summary, we report a novel TMA-based urine assay for TMPRSS2:ERG, a highly specific tissue biomarker for prostate cancer that also has a documented role in driving prostate cancer tumorigenesis. We demonstrate that urine TMPRSS2:ERG score is associated with the presence of cancer, tumor volume, and clinically significant cancer in prostatectomy and biopsy patients. The combination of TMPRSS2:ERG and PCA3 improves on the multivariate PCPT risk calculator, suggesting utility for risk stratification beyond currently measured clinicopathological parameters. Last, we demonstrate that stratification by TMPRSS2:ERG+PCA3 scores identifies groups with markedly different risks of cancer, high-grade cancer, and clinically significant cancer on biopsy. Given the uncertainty currently surrounding the utilization of serum PSA for the early detection of prostate cancer, urine TMPRSS2:ERG in combination with PCA3 score may provide an opportunity to help men and their physicians make more informed decisions about early detection, biopsy, and management of prostate cancer in the context of elevated serum PSA.

MATERIALS AND METHODS

Patient cohorts

Post-DRE urine was prospectively collected with informed consent from 623 men referred for needle biopsy at three academic centers: the UMHS (n = 317); the UL, Quebec, Canada (n = 213); and the VASD Medical Center (n = 93). Prospective post-DRE urine was also collected with informed consent from 471 men presenting for needle biopsy at seven community clinics throughout the United States. Specimens from UL and VASD were collected from July 2006 to October 2008. Specimens from UMHS were collected from March 2008 to June 2009 and are part of an Early Detection Research Network (EDRN) biopsy cohort. Specimens from the community clinics were collected from August 2007 to June 2008. Post-DRE urine was prospectively collected with informed consent from 218 men before radical prostatectomy at UMHS, including 48 men who had their diagnostic biopsy performed at UMHS (and are also included in that biopsy cohort) and 170 men who presented for prostatectomy. See fig. S1 for a flow chart of all urine specimens.

Clinicopathological information was determined at each academic institution, needle biopsy pathology was reviewed at UMHS for all prostatectomy patients, and a central pathologist reviewed needle biopsy pathology from the community clinics. Men with previous treatment for prostate cancer (prostatectomy, radiation, hormone therapy, and chemotherapy), men with surgical treatment of the prostate within 6 months of urine collection (or previous biopsy within 6 weeks), men taking 5 α -reductase inhibitors or testosterone within 3 months of urine collection, or men with prostatitis at the time of urine collection were excluded. For the prostatectomy cohort, men whose urine was collected >6 months before prostatectomy were excluded.

TMA of TMPRSS2:ERG in urine specimens

Urine specimens were obtained immediately after DRE (involving three sweeps of each lateral prostate lobe), refrigerated, and processed within 4 hours by mixing with an equal volume of urine transport medium (detergent-based stabilization buffer; PROGENSA PCA3 Urine Specimen Kit, Gen-Probe Inc.) and stored above -70°C until analysis. Samples not processed according to protocol were excluded. Amounts of TMPRSS2:ERGA and PSA mRNA (the latter to control for the abundance of prostate cells and prostate mRNA in the urine) were determined by TMA, an isothermal nucleic acid amplification method (23). Briefly, in separate procedures, TMPRSS2:ERGA and PSA mRNAs were captured from processed urine specimens through hybridization to oligonucleotide complements coupled to magnetic micro particles. The targets were amplified by TMA. Products were detected with chemiluminescent DNA probes with the hybridization protection assay. Aliquots of the same specimen were assessed for PCA3 expression by TMA with the PROGENSA PCA3 assay (Gen-Probe Inc.) according to the manufacturer's instructions (22, 23). Urine samples were tested by TMA for TMPRSS2:ERG and PSA in triplicate and duplicate, respectively. TMPRSS2:ERG and PSA mRNA copy levels were interpolated from calibration curves derived by assaying in vitro transcripts. For each specimen, the TMPRSS2:ERG score was calculated as follows: $100,000 \times (\text{average urine TMPRSS2:ERGA copies/ml})/(\text{average urine PSA copies/ml})$. PCA3 scores were calculated as $1000 \times (\text{average urine PCA3 copies/ml})/(\text{average urine PSA copies/ml})$.

Initial and second-generation TMPRSS2:ERG TMA assays

Academic biopsy and prostatectomy specimens were assayed with a TMPRSS2:ERG TMA assay using TMPRSS2:ERGA primers that span the junction between TMPRSS2 (NM_005656.3, exon 1) and ERG (NM_004449.4, exon 4), producing an amplification product of 86 nucleotides. PSA primers were the same as those used in the PROGENSA PCA3 assay (23). Samples with average PSA copies/ml >15,000 were considered informative.

Owing to ongoing assay optimization, community biopsy specimens were assayed with a second-generation assay using TMPRSS2:ERGA primers that also span the junction between TMPRSS2 and ERG, but produce an amplification product of 117 nucleotides; PSA primers were the same as those used in the PROGENSA PCA3 assay (23). Samples with average PSA copies/ml >10,000 were considered informative.

Tissue specimens

Formalin-fixed biopsy cores (n = 153; 74 benign, 79 cancerous) from 105 patients in the UMHS needle biopsy cohort with ERG rearrangement status determined by FISH were used for comparison with TMA for TMPRSS2:ERG expression. Because all benign cores were negative by FISH for ERG rearrangement, TMA for TMPRSS2:ERG expression was performed on an additional 55 benign cores (presumed negative for ERG rearrangement for

analysis), resulting in a total of 208 cores (129 benign and 79 cancerous) from 122 patients evaluated by TMA.

Eight 10-mm sections from each evaluated block were cut; the first and last sections were evaluated by hematoxylin and eosin to ensure homogeneity and presence of cancerous or benign tissue. FISH for ERG rearrangement was performed on the seventh section as previously described (37). For TMA testing, the remaining sections were placed into a single transport tube containing 3 ml of specimen transport media (buffered detergent solution, part number 101768, Gen-Probe Inc.), heated to 60°C in a water bath for 30 min, and placed on ice for 5 min. Paraffin was removed with a sterile swab. The transfer tube was then capped, inverted, and stored at $\leq -70^{\circ}\text{C}$ until testing. The initial TMA assay was then performed on 300 ml of sample added to 100 ml of specimen transport medium, and assays were performed in singlicate for TMPRSS2:ERG and PSA. Specimens with PSA copies/ml >3000 were considered informative and those with TMPRSS2:ERG score >50 were considered positive.

Statistical analysis

Statistical analyses were performed with R, version 2.10.1 (R Foundation for Statistical Computing, <http://www.R-project.org>). Two-tailed tests were used for all comparisons and P values <0.05 were considered statistically significant.

For biopsy cohorts, Gleason score was assigned from the highest scoring single core and clinically significant cancers were defined by the Epstein criteria (38, 39) [any clinical stage $>\text{T1c}$, PSAD (serum PSA/prostate volume on ultrasound) ≥ 0.15 ng/ml per cubic centimeter, Gleason score >6 , three or more cores positive, or $>50\%$ greatest single core involvement as significant]. Eight evaluable men in the academic biopsy cohort with a previous diagnosis of cancer (on active surveillance) with Gleason score 6 disease who had a negative biopsy in the current study were considered to have clinically insignificant cancer. PCPT risk factor scores and PCPT high-grade risk factor scores (6) were calculated as described (<http://deb.uthscsa.edu/URORiskCalc/Pages/figure.jsp#>) for all biopsy patients without a previous prostate cancer diagnosis who had all required clinicopathological information (DRE, first-degree family history of prostate cancer, PSA, and history of previous negative biopsy; or DRE, PSA, history of previous negative biopsy, African-American race, and age). Chun et al. nomogram (32) risk percentages were calculated with the online calculator (http://www.nomogram.org/Prostate/pros_calc.php) for all community biopsy patients presenting for initial biopsy who had all required clinicopathological information (age, DRE status, serum PSA, percentage free PSA, prostate volume by ultrasound, and number of biopsy cores).

Significant cancers at prostatectomy were defined by the Epstein criteria as those with maximum tumor dimension >1.0 cm (equivalent to tumor volume >0.5 cm³, because tumor volume is not routinely estimated at UMHS), Gleason score >6 , and non-organ-confined disease (seminal vesicle involvement, positive lymph nodes, or extraprostatic extension) (39, 40). In the prostatectomy cohort, for determining significance at previous biopsy, the above Epstein biopsy criteria were used, except PSAD was calculated with prostate weight at prostatectomy (38), because ultrasound volume at biopsy was not always available.

Associations between cancer status (or TMPRSS2:ERG and PCA3 scores) and clinicopathological variables were assessed with Fisher's exact test, Wilcoxon rank-sum test, or Spearman's rho, depending on variable type (continuous or categorical). Confidence intervals on the median were computed with standard distribution-free methods (41). Diagnostic potential was quantified with sensitivity, specificity, likelihood ratio, and AUC. Comparisons between AUCs for different markers on the same collection of patients were

performed with a nonparametric approach that accounts for the correlation between markers (42).

The ability of various combinations of biomarkers (TMPRSS2:ERG, PCA3, PCPT risk percentage, Chun et al. nomogram, and/or serum PSA) to predict biopsy status was assessed through the use of multivariate logistic regression models. TMPRSS2:ERG, serum PSA, and PCA3 were logarithmically transformed [with the transformation $\log_2(1 + x)$] to avoid inappropriate influence of outlying values on prediction. For analysis of PCPT risk percentage in the academic cohort, we divided informative biopsy patients without a previous prostate cancer diagnosis who had all required clinicopathological information into training (VASD and UL patients) and testing (UMHS patients) sets, fit the models of interest to the training set, and used the estimated coefficients of these models to compute the predicted probability of positive biopsy for patients in the test set. The statistical significance of the difference in AUCs was then assessed (42). In all other cases, when training and testing sets were not explicitly constructed, the predictive ability of multivariate combinations of biomarkers was assessed with 10-fold cross-validation. Decision curve analysis to explore potential clinical net benefit was performed as described (31).

To assess for cancer risk in men with extremes of TMPRSS2:ERG and PCA3 scores, we first computed quartiles of TMPRSS2:ERG and PCA3 scores for the men in the prostatectomy cohort. We then used these quartile cutoffs to divide men into three combined TMPRSS2:ERG+PCA3 score groups (lowest, intermediate, and highest), as follows. Men were classified into the highest TMPRSS2:ERG+PCA3 group if at least one of the two individual biomarker scores was in the highest quartile, and into the lowest group if one biomarker score was in the lowest quartile and the other no higher than the second quartile; all other men were classified as intermediate risks. These prostatectomy cohort–derived cutoffs were then used to classify men in the academic biopsy cohort, because the two cohorts used the same TMPRSS2:ERG and PCA3 assays. To classify men in the community biopsy cohort, we used the same PCA3 score cutoffs, but because prostatectomy quartiles for TMPRSS2:ERG scores could not be directly used in this cohort (given the different assays used), we used sample quartiles of TMPRSS2:ERG determined from the community biopsy cohort to determine cutoffs. Rates of cancer, high-grade cancer (Gleason score ≥ 6), and clinically significant cancer (by Epstein criteria) in each TMPRSS2:ERG+PCA3 score group were then calculated. For determining clinically significant cancer rates, all VASD patients were excluded from the academic cohort (because the number of positive cores and maximum percentage involvement of individual cores was not recorded), and patients with cancer on biopsy where Epstein significance could not be determined owing to missing clinical information were excluded from both the academic and the community biopsy cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Table 1
Clinicopathological data, including parameters at preceding biopsy (bx) and associations with *TMPRSS2:ERG* score for informative prostatectomy (RRP) patients (*n*=187)

The number of patients with data for each parameter is given. (Top) The median (with interquartile range [IQR]) for each parameter, Spearman's rho (r_s) and *P* value (Spearman rank correlation) for the correlation of each parameter with urine *TMPRSS2:ERG* score are shown. (Bottom) The number (with percentage) for each parameter, median *TMPRSS2:ERG* score (with 95% confidence interval [CI]) and *P* values from Wilcoxon Rank-Sum tests are shown.

Parameter	Total number of patients (<i>n</i>)	Median (IQR)	<i>TMPRSS2:ERG</i> score (r_s)	<i>P</i> value
Age (years)	187	60 (56–65)	0.06	0.45
Time to prostatectomy (days)	187	55 (24–75)	0.11	0.13
PSA	187	4.9 (3.9–6.6)	−0.06	0.43
PSAD ¹	185	0.11 (0.08–0.16)	0.07	0.33
Number of bx cores with cancer	187	3 (2–5)	0.29	<0.001
Percentage of bx cores with cancer	181	25 (13–42)	0.30	<0.001
Greatest cancer involvement of a single core (%)	187	35 (10–60)	0.22	0.002
Prostate weight (g)	185	44.3 (35.7–55.0)	−0.14	0.05
Maximum tumor dimension (cm)	187	1.5 (1–2)	0.26	<0.001
Parameter	Total number of patients (<i>n</i>)	Number of patients (%)	Median <i>TMPRSS2:ERG</i> score (95% CI)	<i>P</i> value
Race				
White	187	166 (89%)	18 (9–35)	0.63
Not white		21 (11%)	17 (0–88)	

Parameter	Total number of patients (n)	Median (IQR)	TPRS2:ERG score (r _s)	P value
Family history				
Negative	187	126 (67%)	13 (5–25)	0.07
Positive		61 (33%)	44 (20–81)	
Number of bx cores				
≤12	181	131 (72%)	18 (7–35)	0.99
>12		50 (28%)	15 (5–76)	
Bx Gleason score				
6	187	70 (37%)	20 (7–47)	0.82
>6		117 (63%)	17 (8–35)	
Bx clinical stage				
T1	186	142 (76%)	17 (17–27)	0.05
>T1		44 (24%)	49 (7–107)	
Bx significance ²				
Insignificant	186	35 (19%)	5 (0–18)	0.004
Significant		151 (81%)	27 (13–56)	
Bx/RRP Gleason				
6/6	70	35 (50%)	7 (0–28)	0.008
6/>6		35 (50%)	81 (17–124)	
RRP Gleason score				
6	187	39 (21%)	5 (0–25)	0.009
>6		148 (79%)	27 (14–47)	
pT stage				

Parameter	Total number of patients (n)	Median (IQR)	TMPRSS2:ERG score (r _s)	P value

¹ Serum PSA/prostatectomy weight.

² Epstein criteria: any Bx clinical stage >T1, PSAD≥0.15 ng/ml, Bx Gleason score >6, ≥3 cores positive or greatest cancer involvement of a single core >50% as significant.

³ Any Gleason score >6, maximum tumor dimension >1.0 cm, or non-organ-confined disease as significant.

Table 2
Associations between urine *TMPRSS2:ERG* score and clinicopathological parameters among informative academic biopsy (bx) patients (*n*=606)

The number of patients with data for each parameter is given. (Top) Spearman's rho (r_s) and *P* value (Spearman rank correlation) for the correlation of each parameter with urine *TMPRSS2:ERG* score are shown. (Bottom) The median *TMPRSS2:ERG* score (with 95% confidence interval [CI]) and *P* values from Wilcoxon Rank-Sum tests are shown.

Parameter	Number of patients (<i>n</i>)	<i>TMPRSS2:ERG</i> score (r_s)	<i>P</i> value
Age	606	0.16	< 0.001
PSA	606	0.03	0.45
Ultrasound volume	602	−0.04	0.27
PSAD	602	0.06	0.14
PCPT cancer risk%	463	0.09	0.06
Number of bx cores with cancer ¹	229	0.18	0.005
Percentage of bx cores with cancer ¹	229	0.20	0.003
Greatest cancer involvement of a single core (%) ¹	231	0.24	<0.001
Parameter	Number of patients (<i>n</i>)	Median <i>TMPRSS2:ERG</i> score (95% CI)	<i>P</i> value
Diagnosis			
Non-cancer	337	4 (2–6)	<0.001
Cancer	269	40 (30–61)	
Diagnosis ²			
Atypia and/or PIN	89	6 (2–18)	0.02
Other benign	248	3 (1–5)	
Race			
White	522	12 (8–17)	0.79
Not white	84	8 (5–31)	
Family history			
Negative	401	11 (6–15)	0.35
Positive	114	12 (5–28)	
DRE			
Normal	418	11 (7–18)	0.28

Parameter	Number of patients (n)	TMPRSS2:ERG score (r _s)	P value
Abnormal	171	15 (8–24)	
Previous bx ³			
No	395	15 (9–20)	0.02
Yes	169	5 (2–14)	
Number of bx cores			
≤12	251	12 (6–24)	0.39
>12	265	8 (5–16)	
Gleason score ¹			
6	127	35 (17–57)	0.16
>6	142	49 (30–79)	
Clinical stage ¹			
T1	187	47 (33–63)	0.83
>T1	76	35 (17–77)	
Bx significance ^{1,4}			
Insignificant	49	23 (4–38)	0.007
Significant	187	49 (33–77)	

¹ Only patients with cancerous biopsies (bx).

² Only patients with benign bx.

³ Excluding patients on active surveillance.

⁴ Epstein criteria: any Bx clinical stage >T1, PSAD≥0.15 ng/ml, Bx Gleason score >6, ≥3 cores positive or greatest cancer involvement of a single core >50% as significant.

Table 3
Associations between urine *TMPRSS2:ERG* score and clinicopathological parameters among informative community biopsy (bx) patients (*n*=463)

The number of patients with data for each parameter is given. (Top) Spearman's rho (r_s) and *P* value (Spearman rank correlation) for the correlation of each parameter with urine *TMPRSS2:ERG* score are shown. (Bottom) The median *TMPRSS2:ERG* score (with 95% confidence interval [CI]) and *P* values from Wilcoxon Rank-Sum tests are shown.

Parameter	Number of patients (n)	TMPRSS2:ERG score (r _s)	P value
Age	463	0.10	0.03
PSA	460	0.06	0.17
Ultrasound volume	430	−0.02	0.74
PSAD	427	0.08	0.10
Free PSA (%)	457	−0.03	0.48
PCPT cancer risk%	439	0.00	0.95
Number of bx cores with cancer ^I	204	0.26	< 0.001
Percentage of bx cores with cancer ^I	204	0.24	< 0.001
Greatest cancer involvement of a single core (%) ^I	204	0.29	< 0.001
Total cancer involvement of all cores (%) ^I	203	0.30	< 0.001
Parameter	Number of patients (n)	TMPRSS2:ERG score (median, 95% CI)	P value
Diagnosis			
Non-cancer	259	2 (1–3)	<0.001
Cancer	204	14 (5–23)	
Diagnosis ²			
Atypia and/or PIN	125	7 (2–10)	<0.001
Other benign	134	0 (0–1)	
Race			
White	384	5 (3–7)	0.06
Not white	79	2 (0–5)	
Family history			
Negative	349	5 (2–7)	0.18

Parameter	Number of patients (n)	TMPRSS2:ERG score (r _s)	P value
Positive	93	3 (1–6)	
DRE			
Normal	338	5 (3–7)	0.04
Abnormal	125	2 (0–5)	
Previous bx ³			
No	354	4 (3–6)	0.40
Yes	109	2 (1–8)	
Number of bx cores			
≤12	386	3 (2–6)	0.05
>12	77	6 (2–21)	
Gleason score ¹			
6	106	5 (3–14)	0.005
>6	98	27 (15–52)	
Clinical stage ¹			
T1	142	17 (6–31)	0.14
>T1	62	5 (1–19)	
Bx significance ^{1,4}			
Insignificant	31	5 (0–16)	0.12
Significant	167	15 (5–28)	

¹ Only patients with cancerous biopsies (bx).

² Only patients with benign bx.

³ Excluding patients on active surveillance.

⁴ Epstein criteria: any Bx clinical stage >T1, PSAD ≥ 0.15 ng/ml, Bx Gleason score >6, ≥3 cores positive or greatest cancer involvement of a single core >50% as significant.

Risk of cancer (and high-grade cancer) on biopsy based on stratification by *TPMRSS2:ERG+PCA3* score

Number (*n*) of patients in groups defined by *TPMRSS2:ERG+PCA3* scores, and the number with cancer or high-grade cancer (Gleason grade >6) in each group are given. Risk ratios (with 95% CI) between highest and lowest *TPMRSS2:ERG+PCA3* groups and Fisher's exact test *P* values are shown.

Academic cohort ¹ (<i>n</i> =606)	<i>n</i> (%)	Cancer (%)	Risk ratio (95% CI)	<i>P</i> Value	Gleason >6 (%)	Risk ratio (95% CI)	<i>P</i> Value
<i>TPMRSS2:ERG+PCA3</i> group							
Lowest	235 (39%)	48 (20%)	3.5 (2.7–4.6)	<0.001	14 (6%)	7.4 (4.4–12.7)	<0.001
Intermediate	193 (32%)	93 (48%)			49 (25%)		
Highest	178 (29%)	128 (72%)			79 (44%)		
Community cohort ² (<i>n</i> =459)	<i>n</i> (%)	Cancer (%)	Risk ratio (95% CI)	<i>P</i> Value	Gleason >6 (%)	Risk ratio (95% CI)	<i>P</i> Value
<i>TPMRSS2:ERG+PCA3</i> group							
Lowest	128 (28%)	30 (23%)	2.8 (2.0–3.9)	<0.001	11 (9%)	4.2 (2.3–7.7)	<0.001
Intermediate	153 (33%)	56 (37%)			21 (14%)		
Highest	178 (39%)	116 (65%)			65 (37%)		
Combined cohort ³ (<i>n</i> =1065)	<i>n</i> (%)	Cancer (%)	Risk ratio (95% CI)	<i>P</i> Value	Gleason >6 (%)	Risk ratio (95% CI)	<i>P</i> Value
<i>TPMRSS2:ERG+PCA3</i> group							
Lowest	363 (34%)	78 (21%)	3.2 (2.6–3.9)	<0.001	25 (7%)	5.9 (3.9–8.8)	<0.001
Intermediate	346 (32%)	149 (43%)			70 (20%)		
Highest	356 (33%)	244 (69%)			144 (40%)		

¹ Highest: *TPMRSS2:ERG*>106.85 or *PCA3*>72.69; lowest: *TPMRSS2:ERG*<0.81 and *PCA3*<44.24 or *TPMRSS2:ERG*<17.57 and *PCA3*<18.81; intermediate: all other *TPMRSS2:ERG* and *PCA3* score combinations.

² Highest: *TPMRSS2:ERG*>30.29 or *PCA3*>72.69; lowest: *TPMRSS2:ERG*<3.89 and *PCA3*<18.81; intermediate: all other *TPMRSS2:ERG+PCA3* score combinations.

³ Combined analysis after risk groups were assigned in each cohort.