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Correlation of Urine *TMPRSS2:ERG* and *PCA3* to ERG+ and Total Prostate Cancer Burden

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Abstract

ERG rearrangements, (most commonly *TMPRSS2*: ERG [*T2:ERG*] gene fusions), have been identified in approximately 50% of prostate cancers (PCa). Quantification of *T2:ERG* in post-DRE urine, in combination with *PCA3*, improves the performance of serum PSA for PCa prediction on biopsy Here we compared urine *T2:ERG* and *PCA3* scores to ERG+ (determined by immunohistochemistry) and total prostate cancer burden in 41 mapped prostatectomies. Prostatectomies had a median of 3 tumor foci (range: 1–15) and 2.6 cm of summed linear tumor dimension (range: 0.6–7.1 cm). Urine *T2:ERG* score most correlated with summed linear ERG+ tumor dimension and number of ERG+ foci (r_S=0.68 and 0.67, respectively, both p<0.001). Urine *PCA3* score showed weaker correlation with both number of tumor foci (r_S=0.34, p=0.03) and summed linear tumor dimension (r_S=0.26, p=0.10). In summary, we demonstrate a strong correlation between urine *T2:ERG* score and total ERG+ PCa burden at prostatectomy, consistent with high tumor specificity.

Keywords

TMPRSS2:ERG; prostate cancer; *PCA3*; urine

Introduction

Recently discovered chromosomal rearrangement in prostate carcinoma (PCa), resulting in fusion of the 5′ translated region of the androgen-regulated gene *TMPRSS2*

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Conflict of interest:
The University of Michigan has been issued a patent on the detection of ETS gene fusions in prostate cancer, on which A.M.C. and S.A.T. are listed as co-inventors. The University of Michigan licensed the diagnostic field of use to Gen-Probe, Inc, which sublicensed rights to Ventana Medical Systems, Inc. Neither company played a role in data collection, interpretation or analysis, and did not participate in the study design or the decision to submit for publication. N.P. has served as a consultant for Ventana Medical Systems. A.M.C. has served as consultant to Gen-Probe, Inc. and Ventana Medical Systems. S.A.T. has received honoraria from Ventana Medical Systems.

(*transmembrane protease, serine 2*) with members of the ETS family of transcription factors, including *ERG* or *ETV1*[1], are promising new biomarkers to aid in the detection of PCa[2–6]. ETS fusions have been reported in approximately 50% of PSA screened prostate cancers and fusions between *TMPRSS2* and *ERG* represent 90% of all ETS fusions; by fluorescence in situ hybridization (FISH) or RT-PCR, *ERG* rearrangements (as a surrogate for *TMPRSS2:ERG* fusion) are very specific for prostate cancer or HGPIN immediately adjacent to cancer, and hence, the ability to detect this fusion can potentially be utilized for the detection of prostate carcinoma[2–10]. More recently, monoclonal antibodies against ERG have been developed which detect the truncated ERG protein product of *TMPRSS2:ERG* fusions[11,12]. By immunohistochemistry (IHC), these antibodies are strongly correlated with *ERG* rearrangement as detected by FISH, and stain ~50% of prostate cancers and ~15% of HGPIN (immediately adjacent to ERG+ cancer), with exceptionally rare staining in non-neoplastic prostate tissue[3,11–16].

Recently, our group has evaluated a clinical grade, transcription-mediated amplification (TMA) assay that quantifies *TMPRSS2:ERG (T2:ERG)* mRNA in post-DRE (digital rectal exam) urine[17]. This assay is based on the same technology as the PROGENSA PCA3 assay, a urine based assay for the quantification of the non- coding transcript *PCA3*[18,19], which is FDA approved for predicting prostate cancer on rebiopsy. In a prospective study of over 1,300 men, we showed that urine *T2:ERG* score, used in combination with urine *PCA3* score, enhances the utility of serum PSA to predict prostate cancer risk on biopsy; urine *T2:ERG* score was also significantly correlated with the number of involved cores and maximum % core involvement at biopsy, and maximum index tumor dimension at prostatectomy[17].

PCA3 encodes a non-translated transcript over-expressed in >95% of all prostate cancers with high prostate specificity[20–22]. The PROGENSA™ PCA3 Assay has demonstrated utility for predicting prostate biopsy outcome, and urine PCA3 score has shown an association with tumor volume[23–28] and multifocality[29] in prostatectomy cohorts. However, as *PCA3* encodes a non-coding transcript, precluding IHC based detection, only a single *in situ* based evaluation of *PCA3* expression has been reported *PCA3* expression in the majority of prostate cancers and HGPIN lesions[30].

As FISH and IHC studies have shown that *ERG* rearrangements and protein expression are exceptionally rare in benign prostate tissue or cancer mimickers, we hypothesized that urine *T2:ERG* should be strongly correlated with the total ERG+ prostate cancer burden in a given patient. Likewise, published studies indicate that urine *PCA3* score is correlated with overall tumor burden. Thus, here we compared urine *T2:ERG* and *PCA3* scores to ERG+ and overall cancer burden at prostatectomy to assess the cancer specificity of these urine biomarkers.

Materials and Methods

Study Cohort

The prostatectomy cohort studied was identified from a cohort of 301 men referred for prostate needle biopsy at the University of Michigan Health System (UMHS), who were all assessed by transcription mediated amplification (TMA) for urine *T2:ERG* and urine *PCA3* scores as described below. Forty one men who subsequently underwent prostatectomy at our institution between 2008 and 2011 were included in the study. None of the patients received preoperative radiation or androgen deprivation therapy. Clinicopathologic characteristics including age of patient, ultrasound volume at biopsy, pre-biopsy PSA levels, PSAD and biopsy details (total number of biopsy cores, number of positive cores and percentage of

cores positive) were obtained from our clinical database. All biopsy and prostatectomy cases and urine specimens were obtained with Institutional Review Board approval.

Urine *T2:ERG* and *PCA3* assays

Assessment of urine *T2:ERG* and *PCA3* were performed essentially as described[17]. Urine specimens were obtained immediately after attentive DRE, refrigerated, and processed within 4 hours by mixing with an equal volume of urine transport medium and stored above −70 C until analysis. Amounts of *T2:ERG* and *PSA* mRNA were determined by transcription mediated amplification (TMA). To generate a *T2:ERG* score, the amount of *T2:ERG* mRNA is normalized to the amount of *PSA* mRNA, which is calculated utilizing the formula: 100,000 × average urine *TMPRSS2:ERG* copies/ml)/ (average urine *PSA* copies/ml). Samples with average *PSA* copies/ml > 20,000 were considered informative. Patients in the current study were assessed with a third generation, final clinical TMA assay, which is highly correlated to first and second generation assays (Spearman correlation (r_s) = 0.86, p<0.001) described in [17]. The PROGENSA PCA3 assay (Gen-Probe Inc, San Diego, CA, USA) similarly quantitates *PCA3* and *PSA* mRNA in post- DRE urine. The *PCA3* score was calculated utilizing the formula: 1,000 × (average urine *PCA3* copies/ml)/ (average urine *PSA* copies/ml). Samples with average PSA copies/ml > 7,000 were considered informative. Identical primers for quantifying *PSA* are used in the PROGENSA *PCA3* assay and *T2:ERG* assay.

Prostatectomy Evaluation

Fresh prostates removed after surgery were weighed, measured, inked, and fixed in 10% neutral formalin. Seminal vesicles, apex, and base were amputated, and the remaining prostate was serially sectioned at 4 mm to 5 mm intervals perpendicular to the long axis of the gland from the base to apex and quartered. All prostatectomy specimens were reviewed by the study pathologists. Tumor maps were generated by tracking each section and reconstructing them as a whole-mount section. A cancer focus was considered spatially separate or multifocal if it was 3 mm or more from the closest cancer in any single section or 4mm or more from the closest cancer on the adjacent section above or below, as described previously[31]. The largest tumor focus was designated as the index tumor and additional smaller tumors were labeled as multifocal tumors. For each prostatectomy, the total number of tumor foci, linear dimension and Gleason score of the index focus, and linear tumor dimension and Gleason score of all tumor foci was documented. As the greatest linear dimension of the index focus is reported clinically at UMHS rather than index focus volume, we used the greatest linear dimension of all foci as a cancer volume measurement, which has been validated previously[32]. Immunohistochemistry for ERG (see below) was performed on sections representing all index and multifocal foci from each case. As ERG staining was uniformly nuclear, strong and diffuse except as noted, we assigned all tumor foci as ERG+ or ERG−, and tumor foci with heterogeneous ERG staining were considered ERG+. The index tumor focus showed the highest Gleason score in the majority of cases. In the rare cases where a smaller multifocal focus had a higher Gleason score compared to the index tumor, the smaller multifocal tumor focus with the highest Gleason score was considered as the index tumor. The summed linear tumor dimension was calculated by summing the largest dimension of the index focus and the largest dimension of all multifocal tumor foci. Likewise, the summed ERG+ linear tumor dimension was calculated by summing the largest dimension of all ERG+ tumor foci, including the index tumor when ERG+.

ERG Immunohistochemistry (IHC)

IHC on unstained formalin fixed, paraffin-embedded levels of all tumor foci from the prostatectomy specimen blocks was performed using a monoclonal antibody against ERG, clone EPR 3864 (Epitomics, Burlingame, CA), using the automated Discovery XT staining

platform (Ventana Medical Systems, Tucson, AZ) as described[12,33]. ERG staining was evaluated by the study pathologists. Staining of vessels was used as a positive control and slides without staining of vessels were excluded from further analysis. All immunostains were reviewed by study pathologists.

Statistical Analysis

Associations between urine *T2:ERG* score, urine *PCA3* score and clinicopathological data were assessed using GraphPad Prism 5 (Graph Pad Software). Comparisons of the number of ERG+ and ERG− foci, and summed ERG+ and total tumor dimension per case, were assessed by paired t-tests. Correlations between urine *T2:ERG* and *PCA3* scores and continuous and categorical clinicopathologic variables were assessed with Spearman's rho (r_s) and the Wilcoxon rank-sum test, respectively. Linear regression analysis was also performed to assess the association between urine biomarkers, and between urine biomarkers and ERG+ and total tumor volume. Urine *T2:ERG* scores were log transformed (log(*T2:ERG*+1) to minimize the impact of outliers, which resulted in increased R^2 values for all associations compared to non-transformed *T2:ERG* scores. Two-tailed tests were used for all comparisons and p values <0.05 were considered statistically significant.

Results

Prostatectomy cohort

The 41 prostatectomies included in the study had a median of 3 tumor foci (range 1–15) and 2.6 cm of summed linear tumor dimension (range 0.6–7.1 cm). The index focus showed the highest Gleason score in 39/41 (95%) cases. In two cases (cases 12 and 38), a smaller multifocal focus showed higher Gleason grade than the larger index focus (4+3 and 3+4 in the smaller multifocal focus vs. 3+3 respectively) and was considered as the index focus for analysis. The vast majority of cases in this study were confined to prostate (pT2, 37/41, 90%), with index tumor Gleason scores of 7 (31/41, 76%). Pathological data for all cases are shown in Table 1.

A total of 159 tumor foci were evaluated for ERG staining (including index foci), of which 78 tumor foci (49%) were ERG+. Tumor foci, when positive, showed strong nuclear staining with ERG in all cancerous glands within the tumor focus, except for 3 foci where the index tumor showed heterogeneous ERG expression (moderate to strong staining, considered ERG+ for analysis). ERG was expressed in cancerous glands in 32 of 41 cases (78%) within at least one tumor focus, while the remaining 9 of 41 cases (22%) lacked ERG expression in all tumor foci. ERG expression in the index tumor was noted in 24 (73%) of cases. Representative ERG+ and ERG− foci are shown in Figure 1. The pathological data, ERG IHC, and urine *T2:ERG* and *PCA3* scores are summarized in Table 1.

The median summed linear dimension of ERG+ cancer was 1.2 cm (range 0–5.0 cm). There was no significant difference between the number (mean 1.9 vs. 2.0, paired t-test, p=0.89) or summed linear tumor dimension (mean 1.6 cm vs. 1.3 cm, paired t-test, p=0.52) of ERG+ and ERG− foci per case. Given the low frequency of >T2 disease and Gleason scores < or >7, association of index focus ERG status and Gleason grade and stage were not assessed.

Overall, across 169 total sections, ERG staining was extremely specific for prostatic adenocarcinoma. Although vessels and lymphocytes stain with ERG, this represents expression of wild-type ERG, and does not represent *ERG* rearrangements leading to *TMPRSS2:ERG* over-expression[12]. When positive for ERG staining, HGPIN was always present adjacent to ERG+ cancer, with the exception of one ERG+ HGPIN focus not immediately adjacent to ERG+ cancer (this HGPIN gland was 0.4 cm from ERG positive cancer). ERG positivity in benign glands was extremely rare, with ~ 35 ERG+ glands noted

in 8 cases across 169 total sections. Only one focus composed of two benign acini was greater than 0.4 cm from ERG positive cancer. Using the estimated number of benign glands per prostatectomy section by Furusato *et al.*[34], the overall specificity of ERG staining for prostate cancer is >99.99%. Representative ERG+ HGPIN and benign glands not immediately adjacent to ERG+ carcinoma are shown in Figure 2.

Urine *T2:ERG* and PCA 3

All 41 patients had sufficient urine PSA mRNA expression to evaluate the *T2:ERG* score. The median *T2:ERG* score was 41 (range 0–6,032). As shown in Table 2, of patients with 0 cm, >0.1 to 1.0 cm, 1.1 to 2.0 cm and >2.0 cm of summed ERG+ linear tumor dimension, 1/9 (11%), 4/9 (44%), 6/8 (75%) and 15/15 (100%), respectively, had urine *T2:ERG* scores >30, which is associated with an ~70–75% risk of prostate cancer on biopsy[17]. Of patients with 0 cm, >0.1 to 1.0 cm, 1.1 to 2.0 cm and >2.0 cm of summed ERG+ linear tumor dimension, 1/9 (11%), 7/9 (78%), 8/8 (100%) and 15/15 (100%), respectively, had urine *T2:ERG* scores >10, below which is associated with an ~30–35% risk of prostate cancer on biopsy[17].

All 41 patients had sufficient urine PSA mRNA expression to evaluate the *PCA3* score. The median *PCA3* score was 40 (range 3–187). As shown in Table 2, of patients with 0.1 to 1.0 cm, 1.1 to 2.5 cm, 2.6 to 3.5 cm and >3.5 cm of summed total linear tumor dimension, 3/5 (60%), 6/12 (50%), 6/13 (46%) and 9/11 (82%), respectively, had urine *PCA3* scores>35, which has been proposed as an optimal cutoff for the detection of cancer on biopsy[35]. Of patients with 0.1 to 1.0 cm, 1.1 to 2.5 cm, 2.6 to 3.5 cm and >3.5 cm of summed total linear tumor dimension, 3/5 (60%), 7/12 (58%), 10/13 (77%) and 9/11 (82%), respectively had urine *PCA3* score>25, the FDA approved cutoff for predicting the presence of prostate cancer after initial negative biopsy.

We next addressed our hypothesis that urine *T2:ERG* should be strongly correlated with total ERG+ prostate cancer burden, and urine *PCA3* should be strongly correlated with total prostate cancer burden. As shown in Table 3, comparing correlations of urine *T2:ERG* with various clinicopathological parameters, urine *T2:ERG* most significantly correlated with the summed linear dimension of ERG+ cancer and the number of ERG+ tumor foci (r_S=0.68 and r_S= 0.67, respectively, both p<0.0001). There was no significant association with other parameters, including summed total linear tumor dimension (r_S=0.24, p=0.13) and urine *PCA3* score (r_S=0.22, p=0.18). Similarly, summed linear dimension of ERG+ cancer was most significantly associated with the total number of ERG+ tumor foci and urine *T2:ERG* score (r_S=0.86 and r_S= 0.68, respectively, both p<0.0001), as shown in Table 4. Urine *T2:ERG* was significantly associated with ERG+ vs. ERG− index focus status (median 130 vs. 6.1, Wilcoxon rank-sum test, p=0.002), and a trend of association with index Gleason score >6 vs. 6 was observed (median 51 vs. 22, p=0.11), however the small number of Gleason 6 cases limited this analysis.

Urine *PCA3* score was most correlated with the total number of tumor foci and summed total linear tumor dimension (r_S=0.34, p=0.03 and r_S=0.26, p=0.10), however these correlations were substantially weaker than the correlation between urine *T2:ERG* score and the number of ERG+ tumor foci or summed ERG+ linear tumor dimension (Table 3). There was no significant correlation between urine *PCA3* score and urine *T2:ERG* score or other clinical parameters (Table 3). Likewise, as shown in Table 4, summed total linear tumor dimension was significantly correlated with a number of indicators of total tumor burden (as well as ERG+ tumor burden) at biopsy and prostatectomy, however, there was no significant correlation with urine *PCA3* or *T2:ERG* scores, which showed nearly equal correlations (0.26 and 0.24, respectively). Lastly, urine *PCA3* score was not significantly associated with

ERG+ vs. ERG− index focus status (median 42 vs. 039, Wilcoxon rank-sum test, p=0.78) or index Gleason score >6 vs. 6 (median 37 vs. 41, p=0.74).

Linear regression analysis also supported the above associations of urine *T2:ERG* and *PCA3* with ERG+ and total tumor burden. *T2:ERG* was not significantly associated with summed total linear tumor dimension (R^2=0.07, p=0.10), while *PCA3* showed a statistically significant correlation with summed total linear tumor dimension, however it explained little of the variation in total tumor dimension (R^2=0.15, p=0.01). *T2:ERG* showed a strong correlation with summed ERG+ linear tumor dimension (R^2=0.43, p<0.0001), while *PCA3* was not significantly associated with summed ERG+ linear tumor dimension (R^2=0.08, p=0.08). Lastly, there was no significant association between *T2:ERG* and *PCA3* scores (R^2=0.06, p=0.12).

Discussion

In this study, through assessing both urine and prostatectomy tissues, we have demonstrated a strong correlation between urine *T2:ERG* score and total ERG+ tumor burden, supporting the very high cancer specificity of this biomarker in urine and tissue. Recurrent *T2:ERG* fusions, which occur in approximately 50% of PSA-screened PCa, result in over-expression of a truncated *ERG* protein [4,11,12,16,33]. This rearrangement can be confidently detected at the chromosomal level by FISH studies, as has been demonstrated in numerous studies (reviewed in [2,4,6]). We and others have more recently evaluated ERG protein expression in prostatectomy specimens using a monoclonal antibody against ERG (EPR3864) and documented excellent concordance of ERG staining by IHC compared with FISH for *ERG* rearrangements in >1,000 tumors[3,12–16,33,36,37]. Similar concordance between another monoclonal antibody against ERG and FISH for *ERG* rearrangement has been reported recently[3,11,16]. Likewise, we have previously confirmed the high concordance (92%) between the TMA *T2:ERG* assay and the presence of *ERG* rearrangements by FISH in prostate needle biopsy cores[17]. Importantly, by IHC, ERG expression is extremely rare in benign prostatic acini[11,12,33] and is nearly 100% specific for prostate cancer or HGPIN immediately adjacent to prostate cancer in tissue studies[3,11–16,33,36,37], which we confirmed here (>99.99% specificity for cancer). Although, the protein product of *T2:ERG* fusion cannot be detected in serum, our group has recently evaluated a clinical grade, urine based TMA assay for quantifying *T2:ERG* fusion mRNA[17]. This assay is based on the same technology as the FDA approved urine-based PROGENSA PCA3 assay which in conjunction with serum PSA has proven to be useful for prostate cancer detection and has been shown to be correlated with features of clinically significant disease[18,19,25–29,35,38–41].

We have previously shown that urine *T2:ERG* and *PCA3* scores show moderate, but significant correlation with the greatest linear dimension of the index focus at prostatectomy (n=187, r_s=0.26 and r_s=0.30, both p<0.001)[17]. However, PCa is frequently multifocal and shows multiple separate tumor foci in addition to the index tumor[31]. Heterogeneity amongst the multifocal tumor foci with respect to both histology and Gleason grade has been well described[42,43]. Similar to previous results[42], we found that the majority of our cases (37/41, 90%) had multifocal tumor foci, with 18/37 (49%) cases with multifocal tumors demonstrating heterogeneity in Gleason scores between index and multifocal tumor foci. Recently, multiple groups have confirmed the heterogeneity of ETS gene fusions status (as indicated by FISH for *TMPRSS2* or *ERG* rearrangements) between tumor foci in multifocal PCa[44–46]. For example, our group analyzed 93 multifocal PCa foci from 43 radical prostatectomy specimens and found that 70% of cases harbor *TMPRSS2* rearrangement, of which 70% were discordant in at least one tumor focus, consistent with multifocal PCa arising from multiple, independent clonal expansions[46].

Although we have previously shown significant correlation between urine *T2:ERG* and the greatest linear dimension of the index tumor at prostatectomy (r_S=0.26)[17], we hypothesized that measuring all multifocal tumor foci and stratifying ERG+ and ERG− tumor foci would demonstrate more significant correlation between urine *T2:ERG* score and ERG+ tumor burden. Importantly, our study confirms a strong correlation between urine *T2:ERG* and the summed total dimension and number of ERG+ tumor foci (r_S=0.68 and 0.67). In our present study, there was no significant correlation between urine *T2:ERG* score and greatest dimension of the index tumor focus (r_S=0.21, p=0.19) or summed total linear tumor dimension (r_S=0.24, p=0.13), however the correlation coefficients are similar to those seen in our previous study regarding index tumor size[17], suggesting that the smaller size of our current cohort may explain the lack of statistical significance. Linear regression analysis also demonstrated similar findings. Importantly, we recently evaluated ERG protein expression in a full spectrum of lesions encountered in routine diagnostic prostate needle biopsies, including diagnostically challenging biopsies, and showed ERG positivity in 44% of PCa and 18% of HGPIN; ERG expression was not observed in benign mimics of cancer such as adenosis and partial atrophy and was also exceedingly rare in benign glands[33]. These results are consistent with those observed in our current prostatectomy cohort and other studies showing that nearly the entire burden of ERG+ prostate tissue (as a surrogate for *ERG* rearrangements and *TMPRSS2:ERG* transcript) is carcinoma or HGPIN adjacent to carcinoma[3,11–16,33,36,37]. Thus, the total amount of ERG+ protein in a given prostate is nearly entirely ERG+ cancer, which our study demonstrates is strongly correlated with the urine *T2:ERG* score. Thus, while a limitation of *T2:ERG* as a biomarker is that it is not expressed in all tumor foci, it is extremely specific for prostate cancer, and there is no known mechanism for markedly elevated *T2:ERG* in the urine other than prostate cancer.

Interestingly, the correlation between urine *PCA3* score and the summed total linear tumor dimension (r_S=0.26, p=0.10) is weaker than the correlation between urine *T2:ERG* score and summed linear ERG+ tumor dimension (r_S=0.68, p<0.0001). Although the small size of our current cohort may explain the lack of statistical significance between urine *PCA3* score and summed total linear tumor dimension, we observed a similar correlation between urine *PCA3* score and the largest dimension of the index tumor at prostatectomy in our previous study (r_S=0.30, p<0.001)[17], and our current results are consistent with previously published correlations of urine *PCA3* scores and total tumor volume at prostatectomy (r and r_S= 0.27–0.41)[24–26,28]. Linear regression analysis also identified a significant correlation between *PCA3* and summed total linear tumor dimension, however *PCA3* scores accounted for a limited amount of the variation in total tumor dimension (~15%).

PCA3 has been shown to be markedly over-expressed in >95% of prostate cancers and gene expression studies support prostate specificity, suggesting that the lower correlation between urine *PCA3* and total cancer burden compared to urine *T2:ERG* and ERG+ cancer burden is not due to lack of (or variation in) *PCA3* expression in some prostate cancer foci. Importantly, given that *PCA3* is a non-coding transcript that does not produce a protein product that can be detected by an antibody, there is a lack of studies that have systematically evaluated the specificity of *PCA3* across precursor lesions and benign mimics of prostate cancer at the tissue level, unlike ERG. Only one reported study has evaluated *PCA3* expression *in situ* in prostatic tissues. Evaluating 24 and 26 cases by chromogenic and radioactive in situ hybridization detection methods, respectively, Popa *et al.* showed that while the majority of PCa (92–96%) showed at least focal cytoplasmic *PCA3* expression, the majority of HGPIN (71–96%) as well as a subset of benign glands (29–33%) also showed *PCA3* expression[30]. Hence, in a single study, *PCA3* tissue expression appears similar to that of AMACR, a sensitive and specific prostate cancer marker with utility in tissue based diagnosis, but also with expression in the majority of HGPIN foci[47–53].

Additional studies will be needed to determine if *PCA3*, like AMACR, is expressed in a subset of benign mimickers of prostate cancer as well[48,54,55].

The expression of *PCA3* in the majority of HGPIN lesions (based on a single tissue based study) may contribute to the lower correlation of urine *PCA3* scores and total tumor burden compared to urine *T2:ERG* scores and ERG+ cancer burden. A number of studies have correlated urine *PCA3* score with the presence of HGPIN at biopsy, with conflicting results. For example, Deras *et al.* found no difference in *PCA3* score for HGPIN vs. no evidence of abnormal pathology[18], while Haese *et al.* found increased *PCA3* scores in men with HGPIN[56]; these two studies yielded equivalent diagnostic accuracy for biopsy-detectable cancer. However, these studies were based on HGPIN identified at biopsy, and did not assess the entire prostatic HGPIN burden. Nevertheless, urine *PCA3* has clear utility in predicting the presence of prostate cancer at biopsy and is significantly associated with indicators of aggressive disease at prostatectomy, and multiplexing urine *PCA3* and *T2:ERG* will likely allow for more complete assessment of prostate cancer risk and evaluation of prostate cancer burden[17,24–26,28,29,35,39–41,57,58].

The current study has some limitations. As our series is rather small, does not include the full spectrum of pathology (i.e. Gleason scores and stage) seen at prostatectomy and lacks long term follow up, associations with outcome measures are limited and will require additional studies. Additionally, although urine was prospectively collected prior to biopsy, our study does not represent a prospectively defined prostatectomy cohort. Lastly, although ERG expression by IHC has been highly correlated to *ERG* rearrangement by FISH, and *TMPRSS2* is the 5′ partner in the vast majority of *ERG* rearranged prostate cancers, other 5′ partners can pair with *ERG*, such as *NDRG1*[59], which would not be detected by the urine *T2:ERG* assay but would result in ERG protein expression. However, a strength of our study, was the ability to directly compare urine *T2:ERG* and *PCA3* scores to tissue based ERG+ and total cancer burden, and correlations observed between urine *T2:ERG* scores and index tumor dimension and *PCA3* and index dimension and total tumor volume are consistent with previous reports[17,25,26,28]. Although the clearly stronger correlation of urine *T2:ERG* with total ERG+ cancer burden supports the very high specificity of ERG (and urine *T2:ERG*) for *T2:ERG* positive prostate cancer, our findings will need to be validated in larger series.

In summary, by comparing urine *T2:ERG* and *PCA3* scores to ERG+ and total cancer burden at prostatectomy, our results confirm the extraordinary specificity of prostatic tissue ERG expression for prostate cancer (>99.99%) and demonstrate strong concordance of total ERG+ prostate cancer burden with urine *T2:ERG* score. This strong correlation supports the potential utility of *T2:ERG* in a variety of clinical situations which can now be prospectively addressed, including risk stratifying men with elevated serum PSA, those with prior negative biopsy and those considering active surveillance (as high urine *T2:ERG* scores is strongly associated with a large volume of ERG+ prostate cancer). Urine *T2:ERG* score may also have utility in predicting upgrading on prostatectomy, as high urine *T2:ERG* score but low tumor volume on biopsy may indicate undetected ERG+ cancer.

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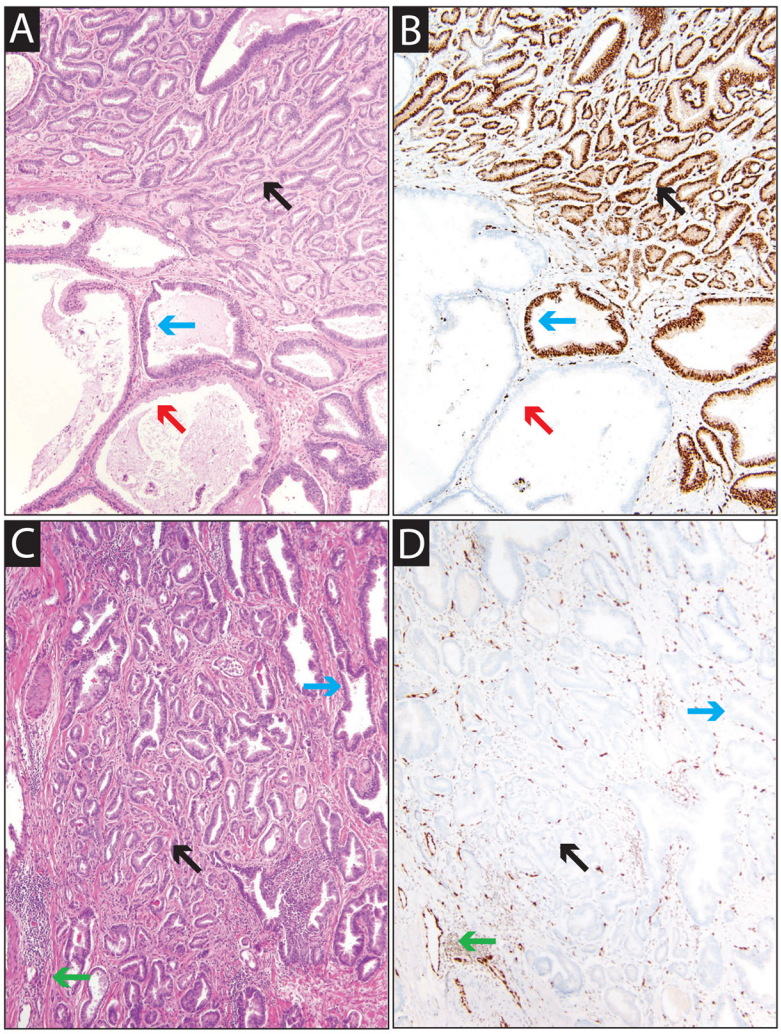

Figure 1. Mapping ERG+ and ERG− tumor foci in prostatectomy specimens

Prostatectomy specimens (n=41) were mapped, and the index focus and all multifocal foci were identified (see Methods). Immunohistochemistry for ERG was performed on sections representing all index and multifocal foci from each case, and each focus was classified as ERG+ or ERG−. Staining of vessels was used as a positive control and sections without staining of vessels were excluded and staining repeated. **A&B**. Hematoxylin and eosin (H&E, **A**) and ERG (**B**) stained sections of an ERG+ index focus (case 31). Areas of benign glands, HGPIN and carcinoma are indicated by red, blue and black arrows, respectively. **C&D**. H&E (**C**) and ERG (**D**) stained sections of an ERG− multifocal focus (case 6). Endothelial cells and lymphocytes serve as an internal positive control (green arrows). All images are 10× original magnification.

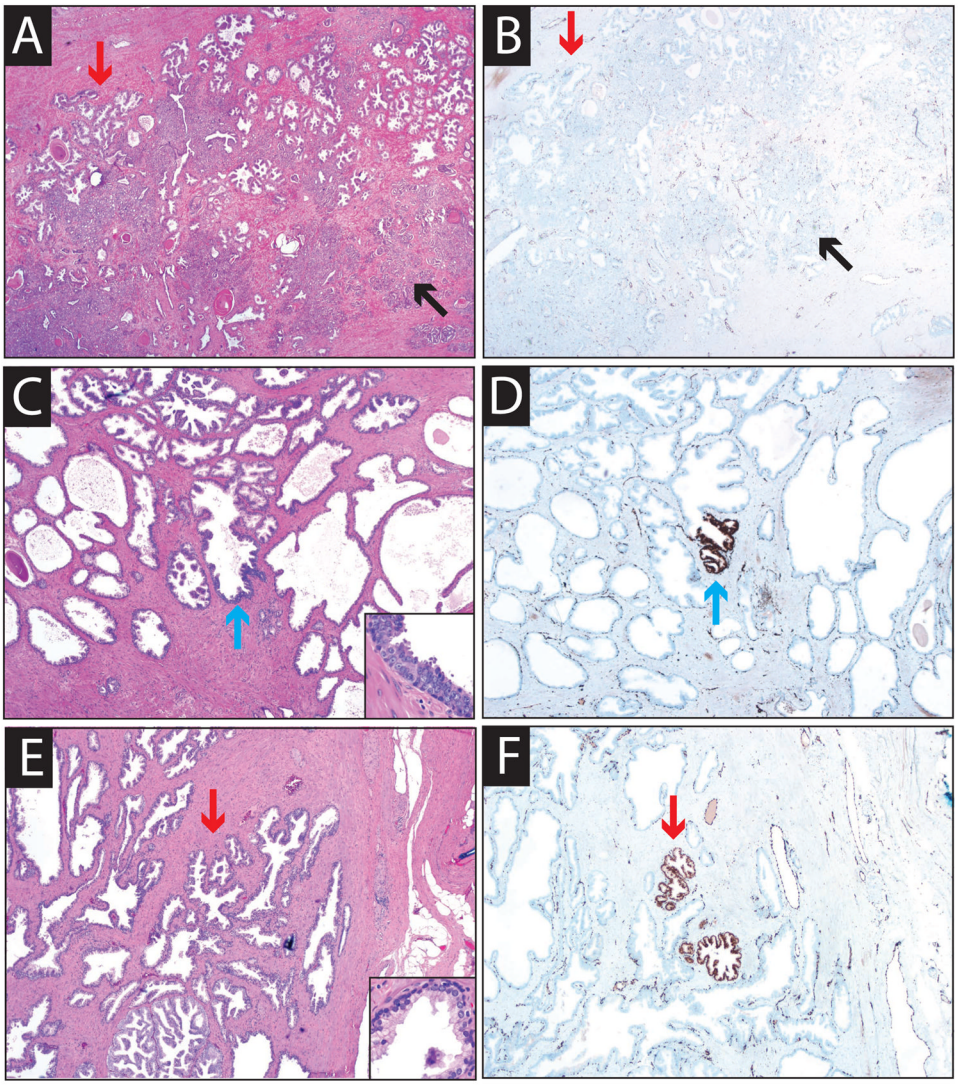

Figure 2. ERG staining is specific for PCa and immediately adjacent HGPIN

ERG staining was evaluated in 169 prostatectomy sections as described in Fig. 1. Across all 169 sections, only one focus of ERG+ high grade prostatic intraepithelial neoplasia (HGPIN) and approximately 2 ERG+ benign glands not immediately adjacent to ERG+ carcinoma were identified. **A&B**. Hematoxylin and eosin (H&E, **A**) and ERG (**B**) stained sections from an ERG− cancer focus showing no expression in cancerous or benign glands. **C&D**. H&E (**C**) and ERG (**D**) stained sections from case 38 demonstrating ERG+ HGPIN not immediately adjacent to cancer. **E&F**. H&E (**E**) and ERG (**F**) stained sections from case 35 demonstrating ERG+ morphologically benign glands not immediately adjacent to cancer. Areas of benign glands, HGPIN and carcinoma are indicated by red, blue and black arrows, respectively. Staining of vessels was used as a positive control.

Table 1

Pathological data and urine *TMPRSS2:ERG* (T2:ERG) and *PCA3* scores for prostatectomy patients (n=41)

Case	Summed tumor dimension (cm)	Summed ERG + tumor dimension (cm)	Index nodule dimension (cm)	ERG status of index nodule	Gleason score of index nodule	Total number of tumor nodules	Total number of ERG+ nodules	Highest Gleason score of multifocal nodules	Urine *T2:ERG* score	Urine *PCA3* score
1	3.4	0.0	1.2	Negative	3+3	3	0	3+3	0	35
2	2.6	0.0	1.4	Negative	3+4	3	0	3+4	0.1	27
3	3.2	0.0	1.8	Negative	4+3	2	0	3+4	0.5	3
4	0.8	0.0	0.8	Negative	3+3	1	0	NA	1.3	66
5	5.1	0.0	1.0	Negative	3+4 w/ 5	5	0	3+4	2.4	60
6	2.9	0.0	1.5	Negative	4+3	5	0	3+3	3.7	19
7	1.4	0.0	0.7	Negative	3+4	2	0	3+3	6.1	74
8	0.9	0.0	0.6	Negative	3+4	2	0	3+4	96.4	50
9	0.9	0.1	0.8	Negative	3+3	2	1	3+3	96.3	13
10	3.4	0.2	2.8	Negative	4+3 w/5	3	1	3+3	39.6	74
11	2.4	0.4	2.0	Negative	3+4	3	2	3+3	860.8	42
12	2.4	0.0	1.9	Negative	4+3	2	0	3+3	2.6	86
13	2.4	0.5	0.7	Negative	3+3	6	2	3+3	14.1	22
14	0.6	0.6	0.6	Positive	4+4	1	1	NA	0.1	3
15	0.9	0.6	0.6	Positive	3+3	3	1	3+3	21.5	83
16	1.8	0.9	0.9	Negative	3+4	2	1	3+3	25.2	15
17	2.6	0.9	0.7	Negative	3+4	6	3	3+4	1951.5	72
18	4.3	1.0	1.6	Negative	3+4	5	2	3+3	6.1	55
19	2.2	1.1	1.1	Positive	3+4	3	1	3+3	40.7	6
20	1.1	1.1	1.1	Positive	3+4	1	1	NA	53.0	8
21	2.0	1.2	1.1	Positive	3+4	5	2	3+3	19.2	38
22	1.9	1.3*	1.2	Positive	3+4	5	2	3+3	97.7	12
23	1.3	1.3	1.3	Positive	3+4	1	1	NA	288.7	31
24	3.8	1.5	1.6	Negative	3+4	3	1	3+4	21.6	17
25	2.6	1.5	0.8	Positive**	3+4	5	2	3+4	446.5	104
26	2.3	1.9	1.0	Positive	3+3	6	3	3+3	31.1	95
27	3.2	2.1	2.1	Positive	4+3	2	1	3+4	343.6	36

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Case	Summed tumor dimension (cm)	Summed ERG + tumor dimension (cm)	Index nodule dimension (cm)	ERG status of index nodule	Gleason score of index nodule	Total number of tumor nodules	Total number of ERG+ nodules	Highest Gleason score of multifocal nodules	Urine *T2:ERG* score	Urine *PCA3* score
28	2.3	2.3	1.0	Positive	3+3	4	2	3+3	288.3	37
29	2.7	2.4	1.6	Positive	3+4	4	2	3+3	48.9	126
30	2.6	2.6	1.9	Positive	3+4	2	2	3+4	37.4	32
31	4.9	2.6	2.0	Positive**	3+4	6	4	3+4	167.4	75
32	3.2	2.7	2.1	Positive	3+4	4	3	3+3	37.9	11
33	5.5	2.7	1.5	Positive	3+4	4	2	3+4	34.1	47
34	2.9	2.8	1.0	Positive	3+4	8	7	3+3	112.9	33
35	4.9	3.3	1.6	Positive**	3+4	5	3	3+4	147.6	69
36	3.5	3.4	2.4	Positive	3+4	3	2	3+3	707.7	42
37	3.7	3.7	2.0	Positive	3+4	3	3	3+3	6031.6	11
38	3.8	3.8*	0.7	Positive	4+3	7	6	3+3	236.3	43
39	3.8	3.8	1.9	Positive	3+4	3	3	3+4	762.5	105
40	7.1	4.6	0.8	Positive	3+4	15	8	3+3	1261.4	186
41	5.0	5.0	2.1	Positive	4+3	3	3	3+4	301.8	40

*A tumor focus was lost on deeper sections, precluding IHC staining for ERG status.

**Heterogenous ERG staning, designated ERG+ for analysis.

Table 2

Association of summed ERG+ and total tumor dimension with urine *TMPRSS2:ERG* (*T2:ERG*) and *PCA3* scores

	Summed ERG+ tumor dimension (cm)			
	0 cm	**0.1–1.0 cm**	**1.1–2.0 cm**	**>2.0 cm**
***T2:ERG* score >10**	1/9 (11%)	7/9 (78%)	8/8(100%)	15/15 (100%)
***T2:ERG* score > 30**	1/9 (11%)	4/9 (44%)	6/8 (75%)	15/15 (100%)

	Summed tumor dimension (cm)			
	0.1–1.0 cm	**1.1–2.5 cm**	**2.6–3.5**	**>3.5 cm**
***PCA3* score >25**	3/5 (60%)	7/12 (58%)	10/13 (77%)	9/11 (82%)
***PCA3* score >35**	3/5 (60%)	6/12 (50%)	6/13 (46%)	9/11 (82%)

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Table 3

Clinicopathological data and associations with urine *TMPRSS2:ERG* (*T2:ERG*) and *PCA3* scores for prostatectomy patients (n=41)

Parameter	(*n*)	Prostatectomy Patients	*T2:ERG* score		*PCA3* score	
			r_s (95% CI)	*p*	r_s (95% CI)	*p*
Sum ERG+ tumor dimension (cm)	41	1.2 (0.2–2.6)	0.68 (0.46 to 0.82)	*< 0.0001*	0.14 (−0.19 to 0.44)	0.39
ERG+ tumor foci (n)	41	2 (1–3)	0.67 (0.45 to 0.81)	*< 0.0001*	0.24 (−0.08 to 0.52)	0.13
Prostate Weight (g)	40	50 (42–58)	−0.25 (−0.53 to 0.08)	0.12	0.02 (−0.30 to 0.34)	0.91
Sum tumor dimension (cm)	41	2.6 (2–3.7)	0.24 (−0.08 to 0.52)	0.13	0.26 (−0.06 to 0.53)	0.10
Tumor foci (n)	41	3 (2–5)	0.23 (−0.09 to 0.51)	0.15	0.34 (0.03 to 0.59)	*0.03*
T2:ERG score	41	41 (14–288)	NA	NA	0.22 (−0.11 to 0.50)	0.18
PCA3 score	41	40 (19–72)	0.22 (−0.11 to 0.50)	0.18	NA	NA
Index tumor dimension (cm)	41	1.2 (0.8–1.9)	0.21 (−0.12 to 0.49)	0.19	−0.06 (−0.37 to 0.26)	0.73
PSAD (ng/ml / g)[1]	40	0.096 (0.074–0.136)	0.19 (−0.14 to 0.48)	0.24	0.01 (−0.31 to 0.33)	0.94
Bx maximum core + (%)	41	50% (20%–70%)	0.18 (−0.14 to 0.47)	0.26	−0.16 (−0.46 to 0.16)	0.31
Age (yr)	41	59 (55–63)	0.17 (−0.15 to 0.46)	0.28	0.06 (−0.26 to 0.37)	0.70
PSA (ng/ml)	41	5.4 (4.5–6.4)	0.10 (−0.22 to 0.41)	0.52	−0.02 (−0.33 to 0.30)	0.92
Bx cores + (%)	41	33% (14%–54%)	0.08 (−0.25 to 0.38)	0.63	−0.02 (−0.33 to 0.30)	0.90

Parameter	n	Prostatectomy Patients	*T2:ERG* score		*PCA3* score	
			median (IQR)	*p*	median (IQR)	*p*
Index tumor status:						
ERG −	41	17 (41%)	6.1 (1.9–68)	*0.002*	42 (18–69)	0.78
ERG +		24 (59%)	130 (38–330)		39 (17–81)	
Index tumor Gleason score:						
6	41	7 (17%)	22 (1.3–96)	0.11	37 (22–83)	0.74
>6		34 (83%)	51 (16–310)		41 (17–72)	
Race:						
White	41	33 (80%)	41 (20–290)	0.88	40 (14–74)	0.99
Not White		8 (20%)	64 (6.1–290)		39 (25–65)	
Bx Epstein status[2]:						
Insignificant	41	5 (12%)	31 (11–270)	0.58	83 (20–100)	0.31

Parameter	n	Prostatectomy Patients	*T2:ERG* score		*PCA3* score	
			median (IQR)	*p*	median (IQR)	*p*
Significant		36 (88%)	45 (8.1–292)		39 (17–68)	

Number (n) of patients with data and median (IQR) or # (%) at prostatectomy, or biopsy (bx), are given. Spearman's rho (r_S) (95% CI) and *p* value for correlation with urine T2:ERG or *PCA3* score, or median *T2:ERG* or *PCA3* score (95% CI) and *p* values from Wilcoxon Rank-Sum tests are shown.

[1] Serum PSA/prostatectomy weight.

[2] Epstein criteria: any >T1c, PSAD>=0.15ng/ml, Gl >6, >=3 cores + or >50% greatest core as significant.

Table 4

Associations of summed ERG+ and total tumor dimension for prostatectomy patients (n=41)

Parameter	(*n*)	Summed ERG+ tumor dimension		Summed tumor dimension	
		R_s (95% CI)	*P*	R_s (95% CI)	*P*
ERG+ tumor foci (n)	41	0.86 (0.75 to 0.92)	*< 0.0001*	0.44 (0.15 to 0.66)	*0.004*
T2:ERG score	41	0.68 (0.46 to 0.82)	*< 0.0001*	0.24 (−0.09 to 0.52)	0.13
Sum tumor dimension (cm)	41	0.51 (0.23 to 0.71)	*0.0007*	NA	NA
Sum ERG+ tumor dimension (cm)	41	NA	NA	0.51 (0.23 to 0.71)	*0.0007*
Tumor foci (n)	41	0.38 (0.07 to 0.62)	*0.02*	0.50 (0.22 to 0.71)	*0.0008*
Bx maximum core + (%)	41	0.33 (0.01 to 0.58)	*0.04*	0.43 (0.13 to 0.65)	*0.005*
Index tumor dimension (cm)	41	0.32 (0.00 to 0.58)	*0.04*	0.53 (0.26 to 0.72)	*0.0004*
Bx cores + (%)	41	0.29 (−0.03 to 0.55)	0.07	0.45 (0.15 to 0.67)	*0.004*
PSAD (ng/ml / g)	40	0.28 (−0.05 to 0.55)	0.09	0.41 (0.10 to 0.64)	*0.009*
Prostate Weight (g)	40	−0.17 (−0.47 to 0.15)	0.28	0.01 (−0.31 to 0.33)	0.95
PCA3 score	41	0.14 (−0.19 to 0.44)	0.39	0.26 (−0.06 to 0.53)	0.10
PSA (ng/ml)	41	0.13 (−0.19 to 0.43)	0.41	0.30 (−0.02 to 0.56)	0.06
Age (yr)	41	0.07 (−0.25 to 0.38)	0.65	0.13 (−0.19 to 0.43)	0.40

Number (n) of patients with data and Spearman's rho (r_S) (95% CI) and *p* value for correlation with sum of ERG+ tumor dimension and total tumor dimension are shown.

[1] Serum PSA/prostatectomy weight.