Modern Pathology (2014) 27, 609–620

Evaluation of tissue *PCA3* expression in prostate cancer by RNA *in situ* hybridization—a correlative study with urine *PCA3* and *TMPRSS2-ERG*

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***PCA3* is a prostate-specific non-coding RNA, with utility as a urine-based early detection biomarker. Here, we report the evaluation of tissue *PCA3* expression by RNA *in situ* hybridization in a cohort of 41 mapped prostatectomy specimens. We compared tissue *PCA3* expression with tissue level ERG expression and matched pre-prostatectomy urine *PCA3* and *TMPRSS2-ERG* levels. Across 136 slides containing 138 foci of prostate cancer, *PCA3* was expressed in 55% of cancer foci and 71% of high-grade prostatic intraepithelial neoplasia foci. Overall, the specificity of tissue *PCA3* was >90% for prostate cancer and high-grade prostatic intraepithelial neoplasia combined. Tissue *PCA3* cancer expression was not significantly associated with urine *PCA3* expression. *PCA3* and ERG positivity in cancer foci was positively associated ($P<0.01$). We report the first comprehensive assessment of *PCA3* expression in prostatectomy specimens, and find limited correlation between tissue *PCA3* and matched urine in prostate cancer.**

Modern Pathology (2014) **27,** 609–620; doi:10.1038/modpathol.2013.169; published online 27 September 2013

Keywords: *PCA3*; prostate cancer; RNA *in situ* hybridization

PCA3 is a non-coding RNA specifically overexpressed in >90% of prostate cancers.[1,2] The urine assay for *PCA3* (PROGENSA, Gen-Probe, San Diego, CA, USA) has recently been approved by the Food and Drug Administration for predicting prostate cancer on rebiopsy. As *PCA3* is a non-coding RNA, immunohistochemical-based detection is not feasible. Only a single study evaluating tissue *PCA3* RNA expression by *in situ* hybridization has been reported, which showed *PCA3* expression in the majority of prostate cancer foci and high-grade prostatic intraepithelial neoplasia foci, as well as a substantial subset (~30%) of benign glands.[3]

In 2005, our group discovered chromosomal rearrangements in prostate cancer resulting in the fusion of the 5′ untranslated region of the androgen-regulated gene *TMPRSS2* with members of the E26 transformation-specific family of transcription factors.[4,5] Fusions involving *ERG* represent 90% of all E26 transformation-specific fusions, and fluorescence *in situ* hybridization for the detection of *ERG* rearrangements has been shown to be highly specific for prostate cancer and high-grade prostatic intraepithelial neoplasia adjacent to prostate cancer.[6–9] Monoclonal antibodies against ERG have been used as a surrogate for *ERG* rearrangement status, and have demonstrated diagnostic utility with high sensitivity and specificity for prostate cancer with *TMPRSS2-ERG* rearrangements.[10,11]

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Received 15 May 2013; revised 19 July 2013; accepted 8 August 2013; published online 27 September 2013

Our group recently evaluated a clinical grade transcription-mediated amplification assay that quantifies *TMPRSS2-ERG* mRNA in post-digital rectal examination urine. We have shown that urine *TMPRSS22-ERG* in combination with urine *PCA3* increases the utility of serum PSA to predict the presence of prostate cancer and significant prostate cancer (per Epstein criteria) upon rebiopsy and correlates with total linear dimension of *ERG*-positive prostate cancer foci on prostatectomy specimens.[12]

Unlike *ERG*, tissue level expression of *PCA3* and its correlation with urine expression are not well understood. Therefore, to assess the tissue level expression pattern of *PCA3*, we used a novel RNA *in situ* hybridization assay for the detection of *PCA3* and evaluated its expression in prostate cancer and high-grade prostatic intraepithelial neoplasia. We also compared tissue *PCA3* expression with tissue ERG expression by immunohistochemistry, and urine expression of *PCA3* and *TMPRSS2-ERG.*

Materials and methods

Study Cohort

The Institutional Review Board approved prostatectomy cohort studied (41 men who underwent prostatectomy at our institution between 2008 and 2011) was identified from a previously reported cohort of 301 men referred for prostate needle biopsy at the University of Michigan Health System. All cases were assessed for urine *TMPRSS2-ERG* and urine *PCA3* scores by transcription-mediated amplification as previously described.[12,13] None of these patients received preoperative radiation or androgen deprivation therapy.

Urine *TMPRSS2-ERG* and *PCA3* Assays

Urine *TMPRSS2-ERG* and *PCA3* scores were determined as described previously.[12,13] In short, urine specimens were obtained immediately after attentive digital rectal examination. Expression levels of *TMPRSS2-ERG* mRNA and *PSA* mRNA were determined by a third generation, final clinical transcription-mediated amplification assay.[12–15] The PROGENSA *PCA3* assay (Gen-Probe) was used to determine the *PCA3* RNA and *PSA* mRNA urine levels. *PCA3* score was determined as a ratio of *PCA3* RNA to PSA mRNA.[16]

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 4–5 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 LPK and JIW. Tumor maps were generated by tracking each section and reconstructing them as a whole-mount section. A cancer focus was considered as spatially separate or multifocal if it was 3 mm or more from the closest cancer in any single section or 4 mm or more from the closest cancer on the adjacent section above or below, as described previously.[17] The largest tumor focus was designated as the index nodule, and additional smaller tumors were labeled as multifocal tumors. The index nodule showed the highest Gleason score in the majority of cases. In rare cases where a smaller multifocal tumor had higher Gleason scores compared with the index tumor, the smaller multifocal tumor focus with the highest Gleason scores was considered as the index nodule. For each prostatectomy, the total number of tumor foci, linear dimension of index nodule, total linear tumor dimension, and Gleason scores of all tumor foci were documented. At the University of Michigan Health System, the greatest linear dimension of the index tumor rather than index tumor volume is reported clinically. Hence, we used the summed greatest linear dimensions of all tumor foci (total tumor linear dimension) as a measurement of cancer volume. Adjacent high-grade prostatic intraepithelial neoplasia was defined as high-grade prostatic intraepithelial neoplasia ≤ 4 mm from a focus of prostate cancer; the presence of adjacent high-grade prostatic intraepithelial neoplasia was noted for all cancer foci. Isolated high-grade prostatic intraepithelial neoplasia was defined as high-grade prostatic intraepithelial neoplasia >4 mm from a focus of prostate cancer and was assessed in 15 randomly selected cases.

Immunohistochemistry for ERG (see below) was previously performed on sections representing all index and multifocal tumor foci from each case. All tumor foci were assigned as ERG positive or ERG negative. The total linear dimension of ERG-positive cancer summed the total linear size of the largest dimension of all ERG-positive tumor foci, including the index tumor when ERG was positive.[12]

PCA3 RNA *in situ* hybridization was performed on all sections representing all index and multifocal tumor foci from each case. *PCA3* score (see below) was assigned based on the area of strongest staining in a given tumor focus. *PCA3* expression was considered as focal if $<50\%$ of tumor cells in a given tumor focus were *PCA3* positive. The total *PCA3*-positive linear tumor dimension summed the total linear size of the largest dimension of all *PCA3*-positive tumor foci in each case. Total *PCA3* intensity was assigned as the sum of *PCA3* intensity scores of all tumor foci per case. Because *PCA3* expression is scored based on a ranking system ranging from 0 to 4, *PCA3*-positive tumor volume was additionally scored using *PCA3* product score (summed linear dimension of each tumor focus multiplied by its *PCA3* intensity score).

ERG Immunohistochemistry

Immunohistochemistry 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-A Roche group, Tucson, AZ, USA) and evaluated by the study pathologists JIW and LPK.[12] Staining of vessels was used as a positive control and slides without staining of vessels were excluded from further analysis.

RNA *In Situ* Hybridization

Formalin-fixed paraffin-embedded sections were baked at 60 °C for 1 h. RNA *in situ* hybridization was performed using a *PCA3* RNA probe (NR_015342 regions 1683–2816; http://www.ncbi.nlm.nih.gov/nuccore/nr_015342) and an RNAscope FFPE Reagent Kit 2.0 (Advanced Cell Diagnostics, Hayward, CA) according to the manufacturer's instructions. Briefly, tissues were deparaffinized by immersing in xylene twice for 15 min each with periodic agitation. The slides were then immersed in 100% ethanol twice for 3 min each with periodic agitation then air-dried for 5 min. Tissues were circled using a pap pen (Vector, #H-4000), allowed to dry and treated with Pretreatment 1 buffer for 10 min. Slides were rinsed in deionized water and then boiled in 1X Pretreatment 2 buffer for 15 min. Slides were rinsed again in deionized water and then treated with Pretreatment 3 buffer for 30 min at 40 °C in a humidity chamber. Slides were rinsed twice in deionized water and then incubated with target probes for *PCA3* and DapB, a bacterial gene used as a negative control, for 2 h at 40 °C in a humidity chamber. Slides were then washed in 1X Wash Buffer twice for 2 min. Slides were then treated with Amp 1 solution for 30 min, Amp 2 solution for 15 min, Amp 3 solution for 30 min, and Amp 4 solution for 15 min, all at 40 °C in a humidity chamber with two washes in 1X Wash Buffer for 2 min after each step. Slides were then treated with Amp 5 solution for 30 min and Amp 6 solution for 15 min at room temperature in a humidity chamber with two washes in 1X Wash Buffer for 2 min after each step. Color was developed by adding a 1:60 solution of Fast Red B:Fast Red A to each slide and incubated for 10 min. Slides were washed twice in deionized water and then immersed in a 50% hematoxylin (Fisher, #SH26-4D) solution for 2 min. Slides were rinsed several times in deionized water and then immersed in a 0.01% ammonium hydroxide solution. Slides were rinsed in deionized water then dehydrated by immersing five times in 70% ethanol twice, immersing five times in 95% ethanol twice, immersing twice in 100% ethanol for 5 s each and in xylene for 5 s. The slides were mounted in Cytoseal XYL (Thermo Scientific, #8312-4) for viewing under bright-field microscope. Positive controls were performed for all runs using a POLR2A gene-specific RNA probe. *PCA3* RNA-ISH was not repeated in any tumor focus showing a negative result to avoid discrepant analysis.

RNA *In Situ* Hybridization Evaluation Criteria

RNA *in situ* hybridization expression intensity scoring guidelines were established to classify tumor foci as *PCA3* positive or *PCA3* negative. *PCA3* expression by RNA *in situ* hybridization appeared as distinct cytoplasmic punctate dots. All tumor foci were evaluated and scanned at $\times 20$ magnification. Scoring for an entire tumor focus was based on the highest *PCA3* intensity using these criteria. On the basis of the number of dots/cell, we established five grading levels ranging from 0 to 4: tumor foci with no staining or <1 dot/cell at $\times 20$ magnification were scored as zero; foci with 1–3 dots/cell in $>5\%$ of the tumor were scored as 1; tumor foci with 4–10 dots/cell with no or very few dot clusters (fused overlapping dots) in $>5\%$ of tumor were scored as 2; tumor foci with >10 dots/cell with $<10\%$ of positive cells having dot clusters in $>5\%$ of tumor were scored as 3; and tumor foci with >10 dots/cell with $>10\%$ of positive cells having dot clusters in $>5\%$ of tumor were scored as 4 (Figure 1). Expression was considered as focal when dots were seen in $<50\%$ of cells in a tumor focus. For the purposes of this study, tumor foci showing scores 2–4 were considered as *PCA3* positive, whereas tumor foci showing scores 0–1 were considered as *PCA3* negative. All *PCA3* slides were reviewed by study pathologists LPK and JIW.

Tissue PCA3 Expression by *In Situ* Hybridization *vs* Transcription-Mediated Amplification

Because urine *PCA* expression by transcription-mediated amplification was compared with tissue *PCA3* expression by *in situ* hybridization, tissue *PCA3* expression values by *in situ* hybridization were compared with tissue values by transcription-mediated amplification. Nine tissue blocks were selected, on which *PCA3* RNA *in situ* hybridization had been performed—4 blocks demonstrating prostate cancer with expression intensity of 4 (diffuse), and 5 blocks with expression intensity of 0. Two 10 micron sections were taken from each block, changing the blade between blocks to prevent cross-contamination. Both sections from each block were placed in a specimen transport medium, heated in a 60 °C water bath for 30 min with occasional swirling, then placed on ice for 5 min. Separate sterile swabs were used to remove solidified and floating paraffin. Specimen tubes were then capped, mixed by inverting five times, and stored at -70 °C until testing. Before analysis, samples were warmed to 60 °C in a water bath for <20 min. *PCA3* scores were

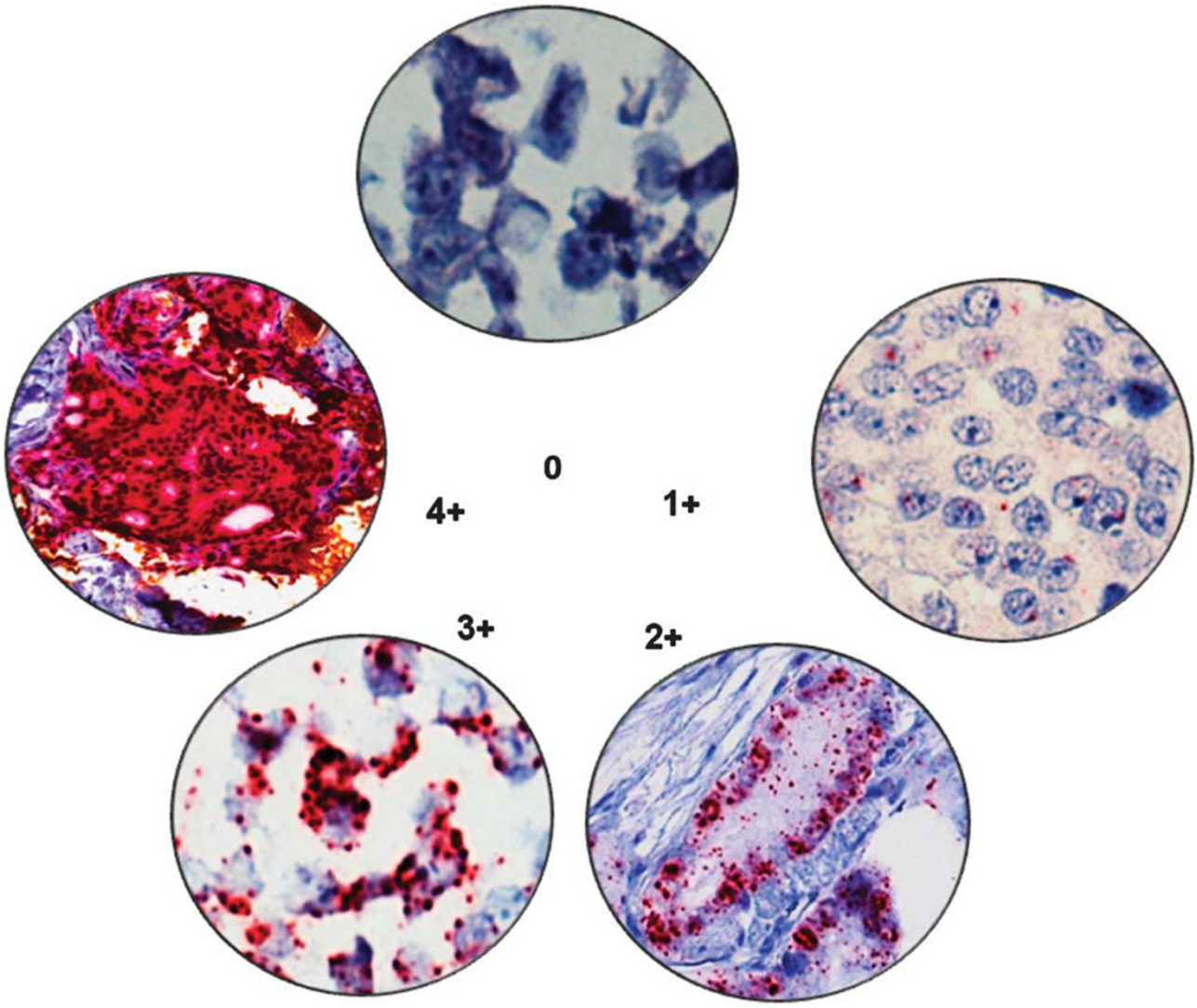

Figure 1 *PCA3* intensity scores; tumor foci with no staining or <1 dot/cell at ×40 magnification were scored as zero; foci with 1–3 dots/cell in >5% of the tumor were scored as 1; tumor foci with 4–10 dots/cell with no or very few dot clusters (fused overlapping dots) in >5% of the tumor were scored as 2; tumor foci with >10 dots/cell with <10% of positive cells having dot clusters in >5% of the tumor were scored as 3; tumor foci with >10 dots/cell with >10% of positive cells have dot clusters in >5% of the tumor were scored as 4.

determined by transcription-mediated amplification, as previously described.[13]

Statistical Analysis

All statistical analyses were performed using the Stats package in the R programming language. The two-tailed *t*-test was used to analyze tumor focus size *vs PCA3* status, and *PCA3*-positive tumor size in urine *PCA3*-high *vs PCA3*-low groups. Fisher's exact test was used to analyze ERG status *vs PCA3* status of tumor foci and, *PCA3* status of tumor foci *vs PCA3* status of adjacent high-grade prostatic intraepithelial neoplasia. Spearman ρ (r_s) was used to test associations between urine *PCA3* and total tumor linear dimension, total number of tumor foci, total *PCA3* intensity, *PCA3*-positive tumor linear dimension, and index nodule *PCA3* score. Spearman ρ (r_s) was also used to evaluate correlations between urine *TMPRSS2-ERG* and number of *PCA3*-positive tumor foci, urine *TMPRSS2-ERG*, number of ERG-positive tumor foci, number of *PCA3*-positive tumor foci, and number of ERG-positive tumor foci. Wilcoxon rank-sum test was used to analyze total *PCA3* intensity, and *PCA3* product score, in urine *PCA3*-high *vs* urine *PCA3*-low groups.

Results

Prostatectomy Cohort

As previously reported, the 41 prostatectomy specimens in this study had a median of 3 cancer foci (range 1–11) and median total linear dimension of 2.5 cm (range 0.4–5.5 cm).[12] The majority of cases were confined to the prostate (pT2, 37/41, 90%) and had an index tumor Gleason score of 7 (31/41, 76%).

PCA3 Expression by ISH and Correlation with ERG Expression by IHC

A total of 138 tumor foci were evaluated with *PCA3* RNA *in situ* hybridization. Of the 159 tumor foci identified in our previous study,[12] 21 small tumor foci were lost on deeper sectioning. Of the 138 tumor foci evaluated, 77 (56%) were *PCA3* positive. *PCA3* intensities for all tumor foci were 4 (43 foci, 31%), 3 (29 foci, 21%), 2 (5 foci, 4%), 1 (20 foci, 14%), and 0 (41 foci, 30%). The index tumor was *PCA3* positive in 25 cases (61%). *PCA3* intensities for index tumor nodules were 4 (16 tumor nodules, 39%), 3 (9 nodules, 22%), 2 (1 nodule, 2%), 1 (6 nodules, 15%), and 0 (9 nodules, 22%). Of all *PCA3*-positive cancer foci, 29 (37%) showed focal *PCA3* staining. *PCA3* expression was noted in at least one tumor focus in 36 cases (88%). *PCA3* status of index tumor foci did not associate with the Gleason score ($P=0.34$, Spearman correlation). The pathologic data, *PCA3* status, and urine *PCA3* scores are summarized in Table 1. Microphotographs demonstrating diffuse and focal *PCA3* expression are present in Figure 2.

The median total *PCA3*-positive linear tumor dimension in the 41 patients was 1.6 cm (range 0–5.1 cm). The median number of *PCA3*-positive tumor foci per case was 2 (range 0–7). The median total *PCA3* intensity across all 41 cases was 7 (range 0–26). The median *PCA3* product score was 6 (range 0–20). *PCA3*-positive tumor foci were overall larger than *PCA3*-negative tumor foci (median 0.9 *vs* 0.6 cm, respectively; two-tailed *t*-test, $P<0.01$).

ERG expression was identified in 70 tumor foci (50%), and 53 tumor foci (38%) were positive for both ERG and *PCA3*. Overall, across all tumor foci, 94 foci (68%) were positive for ERG, *PCA3*, or both (Figure 3). There was a significant positive association between *PCA3* status by RNA *in situ* hybridization and ERG status by immunohistochemistry in tumor foci ($P<0.01$, Fisher's exact test). The median *PCA3* intensity was 3+ in ERG-positive nodules and 1+ in ERG-negative nodules. Of the 41 cases, 95% (39/41) had at least one tumor focus positive for either *PCA3* by RNA *in situ* hybridization or ERG expression by immunohistochemistry.

PCA3 Expression in High-Grade Prostatic Intraepithelial Neoplasia

We identified 66 foci of high-grade prostatic intraepithelial neoplasia adjacent to cancer, of which 51 (77%) were *PCA3* positive. Tissue *PCA3* intensities for adjacent high-grade prostatic intraepithelial neoplasia were 4 (29, 44%), 3 (19, 29%), 2 (3, 5%), 1(4, 6%), and 0 (11, 17%). High-grade prostatic intraepithelial neoplasia adjacent to *PCA3*-positive cancer tended to be *PCA3* positive (90%, 38/42 foci). In contrast, high-grade prostatic intraepithelial neoplasia adjacent to *PCA3*-negative cancer was nearly equally divided into *PCA3* positive (54%; 13/24 tumor foci) and *PCA3* negative (46%; 11/24 tumor foci) (Figures 4 and 5).

We found 16 foci of isolated high-grade prostatic intraepithelial neoplasia after careful review of 15 randomly selected cases. Of these, 56% (9/16) were *PCA3* positive by RNA-ISH. *PCA3* scores for isolated high-grade prostatic intraepithelial neoplasia were as follows: 4 (4, 25%), 3 (3, 19%), 2 (2, 13%), 1 (3, 19%), and 0 (4, 25%). In total, 71% (60/84) of all identified high-grade prostatic intraepithelial neoplasia foci were *PCA3* positive.

All benign glands were examined for *PCA3* expression. Of the benign glands, 50–60 acini (on 10 slides from 9 cases; intensity range 2–3) were *PCA3* positive. These glands were frequently seen close to (<3 mm from) *PCA3*-positive tumor foci and the remaining benign prostatic glands, including all foci of atrophy and adenosis, were *PCA3* negative. Thus, across 136 tissue sections, positive *PCA3* expression by RNA *in situ* hybridization showed a specificity of >90% for combined prostate cancer and high-grade prostatic intraepithelial neoplasia.

Urine *PCA3 and Urine TMPRSS2-ERG*

All 41 patients had sufficient urine RNA for evaluation of *PCA3* and *TMPRSS2-ERG*. Median urine *PCA3* score was 40 (s.d. 38, range 3–186). Of these, 24 (59%) had urine *PCA3* scores >35, a proposed optimal cutoff for detection of cancer on biopsy,[18] and 29 (71%) had urine *PCA3* score >25, the current Food and Drug Administration approved cutoff for predicting prostate cancer after a negative biopsy. Urine *PCA3* score correlated well with number of tumor foci ($r_s=0.51$, $P<0.01$) and correlated weakly with summed total linear tumor dimension ($r_s=0.29$, $P=0.06$), consistent with our previous study using this cohort.[12] Urine *PCA3* did not correlate significantly with the Gleason score of index tumor nodule ($P=0.89$, Spearman correlation).

Urine *PCA3* score did not correlate well with any independent measure of *PCA3*-positive tumor volume, including index nodule *PCA3* score ($r_s=-0.1$, $P=0.52$), total *PCA3*-positive linear tumor dimension ($r_s=0.09$, $P=0.56$), or *PCA3* product score ($r_s=0.04$, $P=0.78$). Although urine *PCA3* did correlate with the number of *PCA3*-positive tumor foci per case ($r_s=0.34$, $P=0.03$), this correlation became non-significant on multivariate linear regression including total number of tumor foci (outcome variable urine *PCA3* score; total number tumor foci $P<0.01$, total number *PCA3*-positive tumor foci $P=0.88$). Similarly, although total *PCA3* intensity showed correlation with urine *PCA3* that approached statistical significance ($r_s=0.29$, $P=0.06$), this too was statistically non-significant on multivariate analysis (outcome variable urine *PCA3* score; total number tumor foci $P<0.01$, total *PCA3* intensity $P=0.38$). Using both 35 and 25 as cutoff values for urine *PCA3*, urine

Table 1 Pathologic data and *PCA3* scores for all cases

Case number	*Summed tumor dimension (cm)*	*Summed PCA3+ tumor dimension (cm)*	*Index nodule dimension (cm)*	*Index nodule PCA3 score (0–4)*	*Index nodule ERG status*	*Index nodule Gleason score*	*Total number of cancer foci*	*Total number of PCA3+ cancer foci*	*Urine PCA3 score*
1	2.2	0	1.2	0	Negative	3+3	2	0	34.8
2	1.6	0.1	1.4	0	Negative	3+4	3	1	27
3	3.5	3.5	1.8	4	Negative	4+3	2	2	2.6
4	1.2	0.1	0.8	0	Negative	3+3	3	1	66.3
5	2	2	1	3	Negative	3+4 (tertiary 5)	2	2	59.6
6	2.8	2.3	1.5	4	Negative	4+3	3	2	19.1
7	1.7	1	0.7	3	Negative	3+4	3	2	73.9
8	1	0	0.6	1	Negative	3+4	2	0	49.7
9	0.4	0.2	0.2	1	Negative	3+3	2	1	12.6
10	3.4	0.6	2.8	1	Negative	4+3 (tertiary 5)	3	2	74
11	2.1	2	2	4	Negative	3+4	2	1	42.4
12	2.3	2.1	1.9	4	Negative	4+3	3	2	86.3
13	1.7	0.1	0.7	0	Negative	3+3	6	1	22
14	0.6	0	0.6	0	Positive	4+4	1	0	3.2
15	0.9	0	0.6	0	Positive	3+3	3	0	82.9
16	1.8	0.9	0.9	1	Negative	3+4	2	1	15.3
17	2.3	0.6	0.7	0	Negative	3+4	6	2	71.8
18	4.5	1.6	1.6	3	Negative	3+4	5	1	54.9
19	2.2	1.7	1.1	4	Positive	3+4	3	2	6.3
20	1.1	1.1	1.1	4	Positive	3+4	1	1	7.6
21	1.3	1.2	1.1	4	Positive	3+4	3	2	38.3
22	1.3	1.2	1.2	3	Positive	3+4	2	1	12.4
23	1.3	0	1.3	0	Positive	3+4	1	0	31.2
24	3.4	3.4	1.6	4	Negative	3+4	4	4	16.9
25	3	1.5	0.8	3	Positive	3+4	4	2	104.5
26	2.3	1.9	1	4	Positive	3+3	6	3	95.2
27	3.2	2.1	2.1	4	Positive	4+3	2	1	35.8
28	2.9	2.6	1	3	Positive	3+3	4	3	37
29	2.7	1.1	1.6	0	Negative	3+4	3	2	125.9
30	3.1	2.6	1.9	4	Positive	3+4	3	2	32
31	5.1	5.1	2	4	Positive	3+4	6	6	74.5
32	2.7	2.1	2.1	4	Positive	3+4	2	1	10.5
33	5.5	4.2	1.5	3	Positive	3+4	4	3	46.9
34	2.6	1.6	1	4	Positive	3+4	5	3	32.9
35	4.9	2.8	1.6	3	Positive	3+4	5	2	68.9
36	3.5	3.5	2.4	4	Positive	3+4	3	3	41.6
37	3.4	1.4	2	1	Positive	3+4	3	2	10.6
38	4.9	4.9	1.4	3	Positive	3+3	7	7	43.2
39	3.8	3.8	1.9	4	Positive	3+4	3	3	105.1
40	5.3	1.6	0.8	1	Positive	3+4	11	3	186.3
41	5.1	2.1	2.1	2	Positive	3+4	3	1	39.6

PCA3-high and *PCA3*-low groups showed no statistically significant difference in *PCA3*-positive tumor linear dimension ($P=0.09$ for cutoff 35, $P=0.46$ for cutoff 25; two-tailed *t*-test) or *PCA3* product score ($P=0.21$ for cutoff 35, $P=0.99$ for cutoff 25; Wilcoxon rank-sum test) (Table 2).

In our cohort, five patients (12%) had no *PCA3*-positive tumor focus by RNA *in situ* hybridization (total nine tumor foci). The median urine *PCA3* score in these patients was 34.8 (range 3.2–82.9). Similarly, two patients (5%) showed no *PCA3*-positive focus by RNA *in situ* hybridization or ERG-positive focus by immunohistochemistry. Urine *PCA3* scores in these patients were 34.8 and 49.7.

Of the 41 patients, 12 (29%) had urine *PCA3*<25. Of these, 92% (11/12) had at least one *PCA3*-positive tumor focus. Median number of *PCA3*-positive tumor foci was 1 (range 0–4). Median *PCA3*-positive linear tumor dimension was 1.3 cm (range 0–3.5). *PCA3* intensities of index tumor nodules in these patients were as follows: 4 (6 nodules, 50%), 3 (1 nodule, 8%), 1 (3 nodules, 25%), and 0 (2 nodules, 17%). Of the seven index nodules with *PCA3* intensity 3 or 4, three of them demonstrated diffuse expression within the tumor focus. Median urine *PCA3* score in these seven patients with index nodule showing *PCA3* intensity 3 or 4 was 10.5 (range 2.3–19.1). Five patients with urine *PCA3* values <25 had total *PCA3*-positive linear dimension >1.6 cm (the median value).

Urine *TMPRSS2-ERG* score correlated with number of *PCA3*-positive tumor foci ($r_s=0.34$, $P=0.03$). However, number of ERG-positive tumor foci correlated much more strongly with urine *TMPRSS2:ERG* score ($r_s=0.64$, $P<0.01$), as shown in our previous study,[12] and number of ERG-positive tumor foci

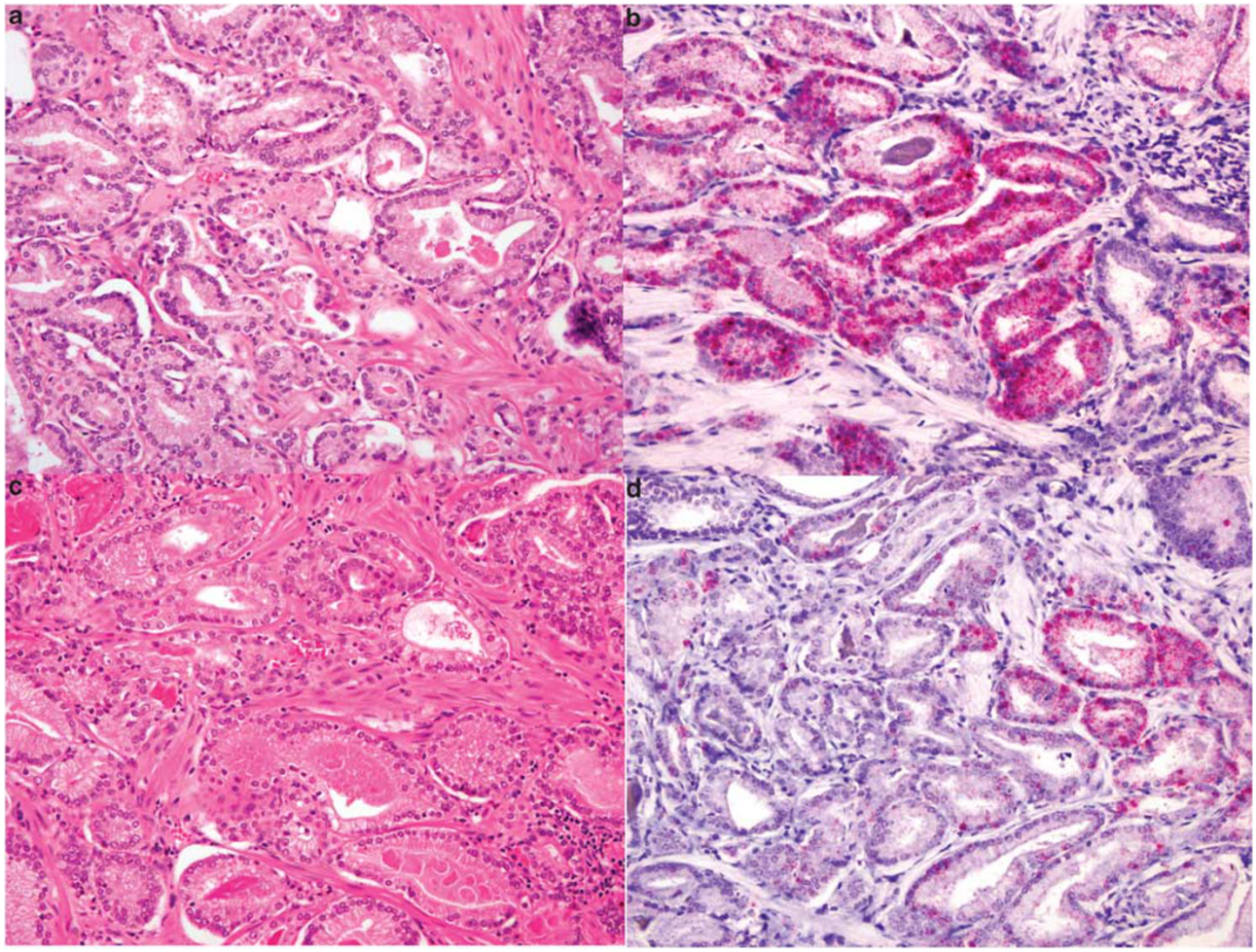

Figure 2 *PCA3* (RNA *in-situ* hydridization) expression with the corresponding H&E-stained sections. Diffuse expression with maximum intensity score 4 (**a**, H&E; **b**, *PCA3* RNA *in situ* hybridization; both at ×200 magnification). Focal expression with intensity score 4 at right side of image and 0 at left side of image (**c**, H&E; **d**, *PCA3* RNA *in-situ* hybridization; both at ×200 magnification).

correlated strongly with number of *PCA3*-positive tumor foci ($r_s = 0.63$, $P < 0.01$).

Tissue PCA3 Expression by *In Situ* Hybridization *vs* Transcription-Mediated Amplification

The four tumor foci that were strongly *PCA3* positive by RNA *in situ* hybridization (4/4 intensity) had a median tissue *PCA3* score of 10 (range 10–19) by transcription-mediated amplification performed on tissue. Conversely, the five tumors that were *PCA3* negative by RNA *in situ* hybridization (0/4 intensity) showed a median tissue *PCA3* score of 3 (range 2–4) by transcription-mediated amplification. These differences in *PCA3* score were statistically significant (Wilcoxon rank-sum test, $P = 0.02$). See Table 3 for details.

Discussion

PCA3, a non-coding RNA, was originally reported as specifically overexpressed in prostate cancer by

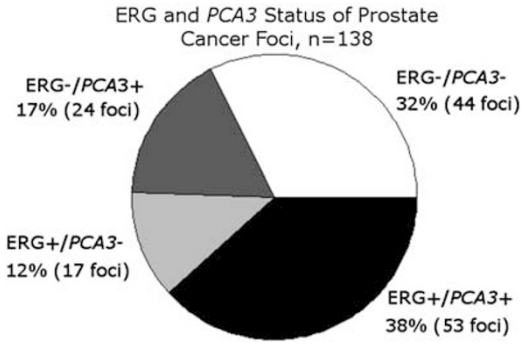

Figure 3 *PCA3* RNA *in situ* hybridization and ERG immunohistochemistry status of all tumor foci.

Bussemaker *et al*.[1] In the present study, using a novel RNA *in situ* hybridization technique to evaluate *PCA3* expression in prostate cancer, we have shown that *PCA3* RNA is significantly elevated in prostate cancer tissue relative to benign prostatic tissue. This is the first time that *PCA3* has been shown to display a strong specificity (>90%) for cancer and high-grade prostatic intraepithelial

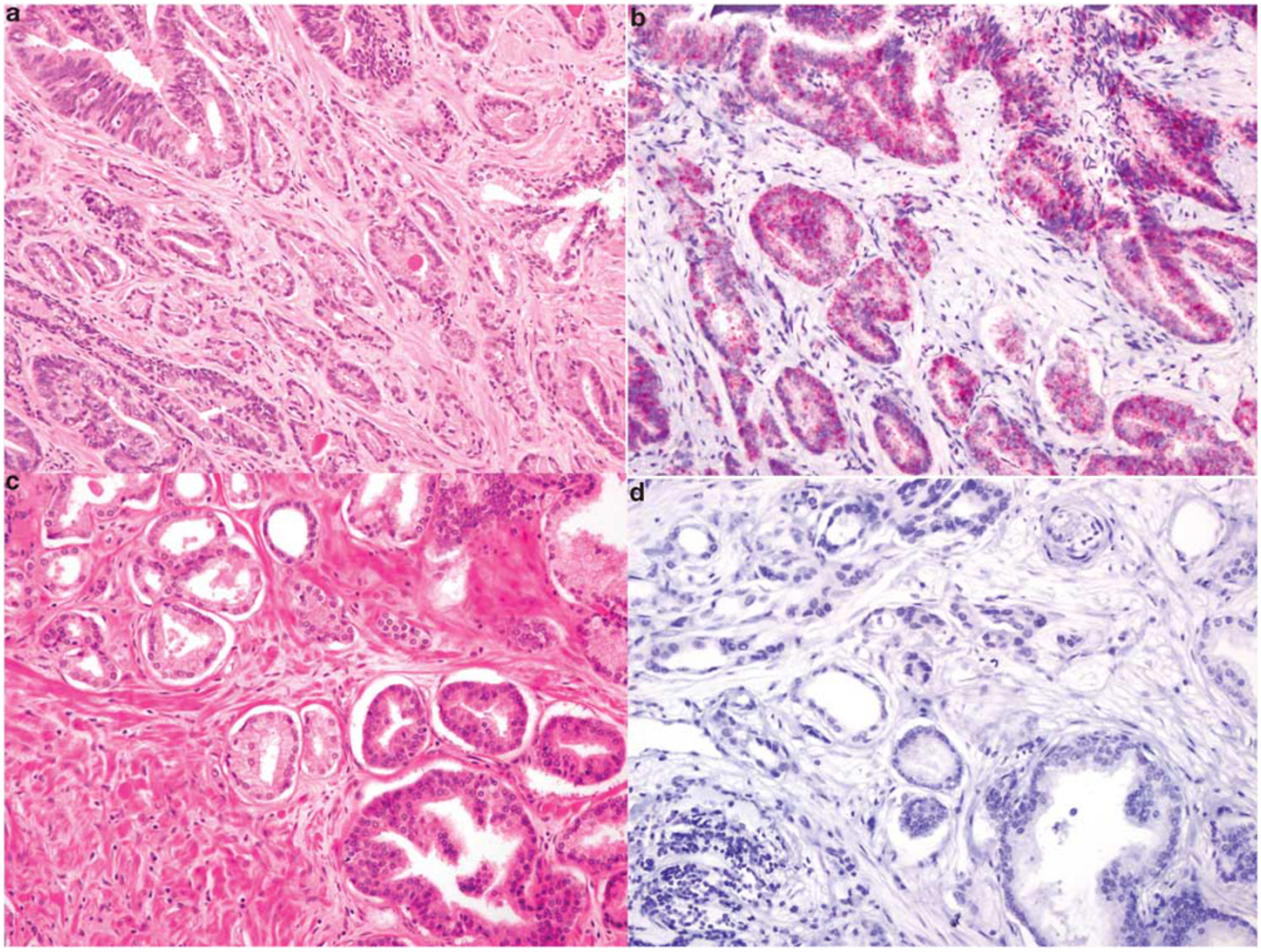

Figure 4 *PCA3* RNA *in situ* hybridization in prostate cancer and adjacent high-grade prostatic intraepithelial neoplasia showing concordant positive (**a**, H&E; **b**, *PCA3* RNA *in situ* hybridization; both at × 200 magnification) and negative (**c**, H&E; **d**, *PCA3* RNA *in situ* hybridization; both at × 200 magnification) *PCA3* status.

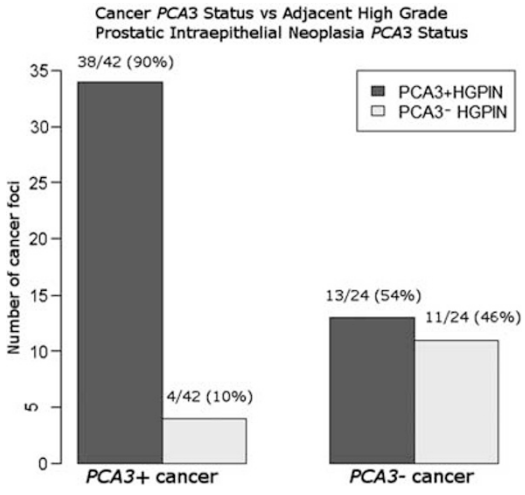

Figure 5 *PCA3* RNA *in situ* hybridization status of prostate cancer and adjacent high-grade prostatic intraepithelial neoplasia.

neoplasia in formalin-fixed paraffin-embedded radical prostatectomy specimens. In this study, we systematically mapped all the tumor foci, and found 88% of all cases in our cohort showed at least one *PCA3*-positive tumor focus. The results of the current study are consistent with previous tissue studies using PCR methods, which showed that although benign prostatic tissue displays low-level *PCA3* expression, expression in prostate cancer tissue is considerably higher in comparison with benign tissue.[1]

Due to the inability to develop an immunohistochemistry-based detection platform for *PCA3*, there have been very few studies of *PCA3* expression in tissue. To our knowledge, a study by Popa *et al*[3] is the only other study evaluating *PCA3* expression in prostate cancer tissue using *in situ* hybridization. In a series of 24 and 26 prostate cancers evaluated with chromogenic and radioactive *in situ* hybridization, respectively, their group demonstrated *PCA3* expression in the majority of prostate cancer (92–96%) and at least focal cytoplasmic expression in the majority of high-grade prostatic intraepithelial neoplasia (71–96%). *PCA3* expression was also noted in almost a third of benign glands (29–33%), showing at least focal staining, and

Table 2 Fraction of cases positive for urine PCA3 at cutoffs >25 and >35, by total tumor linear dimension (cm) and total PCA3+ tumor linear dimension (cm)

	Summed linear tumor dimension (cm)				*Summed PCA3+ linear tumor dimension (cm)*			
	0–1	*1.1–2.5*	*2.6–3.5*	*>3.5*	*0–1*	*1.1–2.5*	*2.6–3.5*	*>3.5*
Urine *PCA3* score>35	0/4 (0%)	8/16 (50%)	6/8 (75%)	8/13 (62%)	6/13 (46%)	11/18 (61%)	3/6 (50%)	4/4 (100%)
Urine *PCA3* score>25	2/4 (50%)	11/16 (69%)	8/8 (100%)	8/13 (62%)	9/13 (69%)	12/18 (67%)	4/6 (67%)	4/4 (100%)
Urine *PCA3* score<25	2/4 (50%)	5/16 (31%)	0/8 (0%)	5/13 (38%)	4/13 (31%)	6/18 (33%)	2/6 (33%)	0/4 (0%)

indicating poor specificity for prostate cancer. Some differences between our study and that of Popa *et al* bear mentioning. In the Popa *et al* study, the percentage of cells expressing *PCA3* was evaluated, and *PCA3* expression was reported as positive or negative. The presence of a single positive cell was defined as positive. In contrast, we used a commercially available well-standardized RNA *in situ* detection procedure for bright-field application. In this method, oligonucleotide-based RNA probes are designed to yield punctate dots for each RNA transcript for a semiquantitative evaluation of tissue level expression of *PCA3* RNA. Based on this, we were able to compare differences in the level of *PCA3* RNA expression among tumor foci. Therefore, we are the first to introduce the development of evaluation criteria for *PCA3* in formalin-fixed and paraffin-embedded tissue. The present study is also considerably larger, encompassing a well-characterized cohort of 138 tumor foci in radical prostatectomy specimens, in contrast to the 48 tissue blocks (28 prostatic cancers and 20 benign prostatic tissues) utilized in the previous study. Also, in contrast to the study by Popa *et al*, the present study used a non-radioactive method, thereby proposing a more clinically realistic assay.

Recurrent *TMPRSS2-ERG* fusions present in ~50% of PSA-detected prostate cancers result in overexpression of ERG protein product.[4,8–10,19] The fusion is reliably detectable using fluorescence *in situ* hybridization,[6,8,9] and immunohistochemistry using monoclonal antibodies directed against ERG has shown high concordance with fluorescence *in situ* hybridization results.[10,11,19–21] We and others have shown that ERG IHC is >99% specific for prostate cancer and high-grade prostatic intraepithelial neoplasia, showing only rare expression in benign prostatic glands.[10,11] The present study showed 55% of cancer foci were *PCA3* positive by RNA *in situ* hybridization, a fraction slightly higher than that seen for the sensitivity of ERG immunohistochemistry in the present cohort (50%) and previous studies.[8,10] Similarly, while 88% of cases had at least one *PCA3*-positive cancer focus, slightly fewer (76%) had at least one ERG-positive cancer focus. We found a strong, statistically significant positive association ($P<0.01$) between ERG status by immunohistochemistry and *PCA3* status by RNA *in situ* hybridization in prostate cancer foci. To the best of our knowledge, this is the first report of such a positive association. A subset of tumor foci (44/138 (32%)) were completely negative for both markers. Despite the strong association between *PCA3* and ERG expression in tumor foci, a subset of tumor foci (41, 29%) also showed inverse ERG and *PCA3* status. Consistent with these findings, 68% of tumor foci were positive for either *PCA3* or ERG.

Table 3 *PCA3* and *PSA* RNA values in copies/ml, as measured by transcription-mediated amplification

Case number[a]	PCA3 *RNA value*	*PSA mRNA value*	PCA3: PSA *ratio*[b]	PCA3 In situ *hybridization intensity*
12-index tumor	18 458	1 870 578	10	4
30-index tumor	32 681	1 694 140	19	4
32-index tumor	22 178	2 199 705	10	4
36-index tumor	20 544	2 093 452	10	4
1-index tumor	2360	556 125	4	0
17-index tumor	5932	2 539 530	2	0
30-non-index tumor	2364	920 239	3	0
41-non-index tumor	4282	1 542 647	3	0
41-non-index tumor	3977	1 167 430	3	0

[a]Case number refers to numbering in Table 1.
[b]*PCA3*:*PSA* ratio calculated as 1000 × (*PCA3* copies/ml)/(*PSA* copies/ml).

High-grade prostatic intraepithelial neoplasia has displayed molecular abnormalities similar to prostate cancer in numerous studies, including chromosomal losses and gains,[22,23] telomere shortening,[24] and *AMACR* overexpression.[25] Consistent with those findings, 71% of high-grade prostatic intraepithelial neoplasia foci in the present study were *PCA3* positive. Interestingly, while the majority (90%) of high-grade prostatic intraepithelial neoplasia foci adjacent to *PCA3*-positive cancer was also *PCA3* positive, slightly over half of isolated high-grade prostatic intraepithelial neoplasia (56%) and high-grade prostatic intraepithelial neoplasia adjacent to *PCA3*-negative cancer (44%) were *PCA3* positive. The relationship between ERG status of high-grade prostatic intraepithelial neoplasia and associated prostate cancer differs from that of *PCA3*, in that ERG-positive high-grade prostatic intraepithelial neoplasia has been shown to be strongly associated with adjacent ERG-positive prostate cancer.[13,17] Furthermore, in contrast to the large fraction of high-grade prostatic intraepithelial neoplasia foci showing *PCA3* expression (71%) in

the present study, several studies have shown that only a small fraction of high-grade prostatic intraepithelial neoplasia foci (15–18%) demonstrate ERG expression.[10,21] These findings favor the hypothesis that ERG-positive high-grade prostatic intraepithelial neoplasia may indicate unsampled prostate cancer or high-grade prostatic intraepithelial neoplasia that may progress to invasive carcinoma, which does not appear to be true of *PCA3*-positive high-grade prostatic intraepithelial neoplasia. In summary, while ERG-positive high-grade prostatic intraepithelial neoplasia represents the minority of high-grade prostatic intraepithelial neoplasia and may be predictive of adjacent ERG-positive prostate cancer, *PCA3*-positive high-grade prostatic intraepithelial neoplasia appears to represent the majority of high-grade prostatic intraepithelial neoplasia and does not appear to be predictive of adjacent prostate cancer.

The great majority of clinical studies of *PCA3* have been on urine measurement, which has demonstrated superior sensitivity and specificity to serum PSA for the detection of clinically significant prostate cancer on biopsy.[13,26–28] Similarly, *TMPRSS2-ERG* mRNA can be detected in the urine of patients with prostate cancer, and correlates with linear extent of ERG-positive cancer by immunohistochemistry.[19,21] In the present study, urine *PCA3* score did not correlate with any measure of *PCA3*-positive tumor burden by RNA *in situ* hybridization except the number of *PCA3*-positive tumor foci per case ($r_s = 0.34$, $P = 0.03$), which became non-significant on multivariate analysis including total number of tumor foci. In contrast, a strong correlation has been shown between urine *TMPRSS2:ERG* and both the total ERG-positive tumor dimension ($r_s = 0.68$) and the number of ERG-positive tumor foci ($r_s = 0.67$).[12]

Interestingly, 12% (5/41) of cases in the present study had no *PCA3*-positive tumor focus. Of these, 80% (4/5) had urine *PCA3* values >25, the current Food and Drug Administration approved the cutoff value. There are several possible explanations for this lack of correlation between urine and tissue *PCA3* expression levels. First, while ERG immunohistochemistry shows strong diffuse expression within positive tumor foci, *PCA3* RNA *in situ* hybridization tended to show focal expression, with variations in intensity within a given tumor focus. Thus, in contrast to ERG, in which quantification in tissue is relatively straight-forward, quantification of *PCA3* tissue expression is more challenging. Adding to this difficulty, in contrast to ERG which stains endothelial cells thereby offering a positive internal control, no internal positive control was available for *PCA3* RNA *in situ* hybridization, although successful positive controls (evaluating for the presence of RNA) were run in parallel. Third, *PCA3* RNA *in situ* hybridization was only performed on representative slides containing the majority of the index nodule. Also, although expression of *PCA3* was evaluated in high-grade prostatic intraepithelial neoplasia that happened to be on cancer-containing slides, the present study was not designed to evaluate the *PCA3* expression in all high-grade prostatic intraepithelial neoplasia within a prostate. It is possible that unmeasured high-grade prostatic intraepithelial neoplasia burden contributed to urine *PCA3*, particularly given the majority of high-grade prostatic intraepithelial neoplasia is *PCA3* positive. Finally, a small subset (13%) of small cancer foci was lost on obtaining deeper sections for *PCA3* RNA *in situ* hybridization. These lost foci may contribute to the lack of correlation between urine and tissue *PCA3* expression, to a limited extent.

Consistent with this lack of correlation between urine and tissue *PCA3* values, the majority of patients with urine *PCA3* <25 demonstrated at least one *PCA3*-positive tumor focus, and half demonstrated an index tumor nodule with strong *PCA3* expression (4/4 intensity) by RNA *in situ* hybridization. Insufficient RNA collection is an unlikely explanation for these discrepant cases, because urine prostate-specific antigen mRNA was measured in all cases, and those with insufficient values were excluded from the study. Urine *PCA3* score may reflect associations between tumor burden and overall prostate volume, with larger prostates contributing more PSA mRNA from benign prostatic tissue, thus lowering the *PCA3*:PSA ratio. This hypothesis will need to be investigated in future studies. False positive tissue *PCA3* RNA *in situ* hybridization is unlikely, as background benign prostate glands served as a successful negative control. A lack of association between *in situ* hybridization values and transcription median amplification values also appears to be unlikely, as our data show that the values between these different methodologies appear to correlate. On the basis of the preliminary observations from an independent and related study of evaluation of tissue *PCA3* expression in cases with very high and very low urine *PCA3*, we found other cases with similar discrepant results (unpublished data from our group, study in progress). It appears that the process by which *PCA3* RNA enters the urine from prostate cancer cells may be more complex than we currently realize. Further work on a large cohort of cases is needed to better understand this relationship.

The present study showed that 88% (36/41) and 76% (31/41) of prostatectomy specimens had at least one tumor focus positive for *PCA3* RNA *in situ* hybridization and ERG immunohistochemistry, respectively. Importantly, 95% (39/41) of cases had at least one tumor focus positive for either *PCA3* or ERG. Both patients with no *PCA3*-positive or ERG-positive cancer focus had urine *PCA3* scores >25. Thus, 100% of patients in this study showed overexpression of ERG or *PCA3* in tissue, urine, or both.

The current study has some limitations. This cohort does not reflect the entire spectrum of pathology seen at radical prostatectomy, including

cases with high Gleason score and high stage. Hence, our results may not necessarily reflect the performance of *PCA3* RNA *in situ* hybridization in high-grade or late-stage cancers. Our cohort also lacks follow-up information precluding the evaluation of associations with outcome, and is relatively small. Hence, our findings regarding the expression of *PCA3* by RNA *in situ* hybridization in prostate cancer need to be validated in a larger series.

Acknowledgments

This work was supported in part by the US National Institutes of Health Early Detection Research Network (U01 CA111275 and U01 CA113913), NIH SPORE (P50 CA69568), and R01 CA132874. NP and SAT are supported by University of Michigan Prostate SPORE Career Development Awards. AMC is supported by the Howard Hughes Medical Institute, the Doris Duke Foundation, and the Prostate Cancer Foundation and is an American Cancer Research Professor and a Taubman Scholar. We would like to thank Angela Wu, Ritu Bhalla and Rohit Mehra for providing help and advice.

Disclosure/conflict of interest

AMC serves on the advisory boards of Gen-Probe and Ventana Medical Systems/Roche. SAT has received honoraria from, and consults for Ventana Medical Systems. SAT and AMC are co-inventors on a patent filed by the University of Michigan covering ETS fusions in prostate cancer. The diagnostic field of use has been licensed to Gen-Probe, who has sublicensed some rights to Ventana Medical Systems/Roche. Gen-Probe and Ventana/Roche did not play a role in the design and conduct of this study, in the collection, analysis, or interpretation of the data, or in the preparation, review, or approval of the article. NP does receive research funding from Ventana/Roche but this funding was not used for the development of the assay.

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