HHS Public Access

Author manuscript

Mod Pathol. Author manuscript; available in PMC 2014 October 01.

Evaluation of Tissue *PCA3* Expression in Prostate Cancer by RNA in situ Hybridization - A Correlative Study with Urine *PCA3* and *TMPRSS2-ERG*

Joshua I. Warrick, M.D.[2], **Scott A. Tomlins, M.D., Ph.D.**[1,2,3,4], **Shannon L Carskadon, M.S.**[1,2], **Allison M Young, M.D.**[2], **Javed Siddiqui, M.S.**[1,2], **John T Wei, M.D.**[4], **Arul M. Chinnaiyan, M.D., Ph.D.**[1,2,3,4,5], **Lakshmi P. Kunju, M.D.**[1,2,3,*,#], and **Nallasivam Palanisamy, M.Sc.,M.Phil., PhD.**[1,2,3,*,#]

[1]Michigan Center for Translational Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

[2]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

[3]Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, USA

[4]Department of Urology, University of Michigan Medical School, Ann Arbor, MI, USA

[5]Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI, USA

Abstract

PCA3 is a prostate-specific non-coding RNA, with utility as 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 were 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.

#**Corresponding Author** Lakshmi P. Kunju, M.D., Department of Pathology, 2G332 UH, 1500 E. Medical Center Drive, Ann Arbor, Michigan 48104-5054, Phone: 734/647-9125, Fax: 734/763-4095, lkunju@med.umich.edu.
*These senior authors have contributed equally
#Nallasivam Palanisamy, M.Sc., M.Phil., Ph.D., Michigan Center for Translational Pathology, 2900 Huron Parkway, Traverwood IV, Suite 100, Ann Arbor, MI 48105, Phone: 734-615-1417, Fax: 734 232 0805, nallasiv@med.umich.edu

Keywords

PCA3; RNA *in-situ* hybridization; Prostate cancer

INTRODUCTION

PCA3 is a noncoding RNA specifically overexpressed in >90% of prostate cancers(1, 2). The urine assay for *PCA3* (PROGENSA, Gen-Probe, San Diego, CA) has recently been approved by the Food and Drug Administration for predicting prostate cancer on rebiopsy. As *PCA3* is a noncoding 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).

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 is 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 (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 (12–14, 15). The PROGENSA *PCA3* assay (Gen-Probe Inc, San Diego, CA, USA) was used to determine *PCA3* RNA and *PSA* mRNA urine levels. *PCA3* score was determined as a ratio of *PCA3* RNA to PSA mRNA.

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 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 LPK and JIW. 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(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 to 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 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 >4mm 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 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 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 area

of strongest staining in a given tumor focus. *PCA3* expression was considered 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 0–4, *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) 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 one hour. RNA *in situ* hybridization was performed using *PCA3* RNA probe (NR_015342 region 1683–2816- http://www.ncbi.nlm.nih.gov/nuccore/nr_015342) and RNAscope FFPE Reagent Kit 2.0 (Advanced Cell Diagnostics, Hayward, CA) according to manufacturer's instructions. Briefly, tissues were deparaffinized by immersing in xylene twice for 15 minutes each with periodic agitation. The slides were then immersed in 100% ethanol twice for 3 minutes each with periodic agitation then air-dried for 5 minutes. Tissues were circled using a pap pen (Vector, #H-4000), allowed to dry and treated with Pretreatment 1 buffer for 10 minutes. Slides were rinsed in deionized water and then boiled in 1× Pretreatment 2 buffer for 15 minutes. Slides were rinsed again in deionized water and then treated with Pretreatment 3 buffer for 30 minutes 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 negative control, for 2 hours at 40°C in a humidity chamber. Slides were then washed in 1× Wash Buffer twice for 2 minutes. Slides were then treated with Amp 1 solution for 30 minutes, Amp 2 solution for 15 minutes, Amp 3 solution for 30 minutes and Amp 4 solution for 15 minutes, all at 40°C in a humidity chamber with 2 washes in 1× Wash Buffer for 2 minutes after each step. Slides were then treated with Amp 5 solution for 30 minutes and Amp 6 solution for 15 minutes at room temperature in a humidity chamber with 2 washes in 1× Wash Buffer for 2 minutes 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 minutes. Slides were washed twice in deionized water and then immersed in a 50% hematoxylin (Fisher, #SH26-4D) solution for 2 minutes. 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 5 times in 70% ethanol twice, immersing 5 times in 95% ethanol twice, immersing twice in 100% ethanol for 5 seconds each and in xylene for 5 seconds. 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 in order 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 20× magnification. Scoring for an entire tumor focus was based on the highest *PCA3* intensity using these criteria. Based on the number of dots/cell, we established five grading levels ranging from 0–4: tumor foci with no staining or less than 1 dot/cell under 20× magnification were scored as zero; foci with1–3 dots/cell in more than 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 more than 5% of tumor were scored as 2; tumor foci with more than 10 dots/cell with less than 10% of positive cells having dot clusters in more than 5% of tumor were scored as 3; tumor foci with more than 10 dots/cell with more than 10% of positive cells having dot clusters in more than 5% of tumor were scored as 4 (Figure 1). Expression was considered 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 *PCA3*-positive, while tumor foci showing scores 0–1 were considered *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 to tissue *PCA3* expression by *in situ* hybridization, tissue *PCA3* expression values by *in situ* hybridization were compared to 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 specimen transport medium, heated in a 60° C waterbath for 30 minutes with occasional swirling, then placed on ice for 5 minutes. Separate sterile swabs were used to remove solidified and floating paraffin. Specimen tubes were then capped, mixed by inverting 5 times, and stored at −70° C until testing. Prior to analysis, samples were warmed to 60° C in a water bath for <20 minutes. *PCA3* scores were 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. Wilcox 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 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 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 cm 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 and/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 (SD 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 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 *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; Wilcox rank sum test) (Table 2).

In our cohort, 5 patients (12%) had no *PCA3*-positive tumor focus by RNA *in situ* hybridization (total 9 tumor foci). The median urine *PCA3* score in these patients was 34.8 (range 3.2–82.9). Similarly, 2 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 7 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 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 (Wilcox 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 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 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 to 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%2013;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 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 semi quantitative 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 approximately 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 immunohistochemsitry 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.

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 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 *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. Lastly, 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. Based on 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* and/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 over-expression 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

REFERENCES

1. Bussemakers MJ, van Bokhoven A, Verhaegh GW, et al. DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res. 1999; 59:5975–5979. [PubMed: 10606244]

2. Schmidt U, Fuessel S, Koch R, et al. Quantitative multi-gene expression profiling of primary prostate cancer. Prostate. 2006; 66:1521–1534. [PubMed: 16921506]

3. Popa I, Fradet Y, Beaudry G, et al. Identification of PCA3 (DD3) in prostatic carcinoma by in situ hybridization. Mod Pathol. 2007; 20:1121–1127. [PubMed: 17873893]

4. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005; 310:644–648. [PubMed: 16254181]

5. Tomlins SA, Laxman B, Dhanasekaran SM, et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature. 2007; 448:595–599. [PubMed: 17671502]

6. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nat Rev Cancer. 2008; 8:497–511. [PubMed: 18563191]

7. Mosquera J-M, Perner S, Genega EM, et al. Characterization of TMPRSS2-ERG fusion high-grade prostatic intraepithelial neoplasia and potential clinical implications. Clin Cancer Res. 2008; 14:3380–3385. [PubMed: 18519767]

8. Rubin MA, Maher CA, Chinnaiyan AM. Common gene rearrangements in prostate cancer. J Clin Oncol. 2011; 29:3659–3668. [PubMed: 21859993]

9. Tomlins SA, Bjartell A, Chinnaiyan AM, et al. ETS gene fusions in prostate cancer: from discovery to daily clinical practice. Eur Urol. 2009; 56:275–286. [PubMed: 19409690]

10. Park K, Tomlins SA, Mudaliar KM, et al. Antibody-based detection of ERG rearrangement-positive prostate cancer. Neoplasia. 2010; 12:590–598. [PubMed: 20651988]

11. Tomlins SA, Palanisamy N, Siddiqui J, et al. Antibody-based detection of ERG rearrangements in prostate core biopsies, including diagnostically challenging cases: ERG staining in prostate core biopsies. Arch Pathol Lab Med. 2012; 136:935–946. [PubMed: 22849743]

12. Young A, Palanisamy N, Siddiqui J, et al. Correlation of Urine TMPRSS2:ERG and PCA3 to ERG + and Total Prostate Cancer Burden. Am J Clin Pathol. 2012; 138:685–696. [PubMed: 23086769]

13. Tomlins SA, Aubin SMJ, Siddiqui J, et al. Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA. Sci Transl Med. 2011; 3 94ra72.

14. Cornu J-N, Cancel-Tassin G, Egrot C, et al. Urine TMPRSS2:ERG fusion transcript integrated with PCA3 score, genotyping, and biological features are correlated to the results of prostatic biopsies in men at risk of prostate cancer. The Prostate. 2012; 73:242–249. [PubMed: 22821767]

15. Lin DW, Newcomb LF, Brown EC, et al. Urinary TMPRSS2:ERG and PCA3 in an active surveillance cohort: results from a baseline analysis in the Canary Prostate Active Surveillance Study. Clin Cancer Res. 2013; 19:2442–2450. [PubMed: 23515404]

16. Stephan C, Jung K, Semjonow A, et al. Comparative assessment of urinary prostate cancer antigen 3 and TMPRSS2:ERG gene fusion with the serum prostate-specific antigen-based prostate health index for detection of prostate cancer. Clin Chem. 2013; 59:280–288. [PubMed: 23213079]

17. Wise AM, Stamey TA, McNeal JE, et al. Morphologic and clinical significance of multifocal prostate cancers in radical prostatectomy specimens. Urology. 2002; 60:264–269. [PubMed: 12137824]

18. Marks LS, Fradet Y, Deras IL, et al. PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. Urology. 2007; 69:532–535. [PubMed: 17382159]

19. Braun M, Goltz D, Shaikhibrahim Z, et al. ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer--a comparative study of two monoclonal antibodies. Prostate Cancer Prostatic Dis. 2012; 15:165–169. [PubMed: 22231490]

20. Falzarano SM, Zhou M, Carver P, et al. ERG gene rearrangement status in prostate cancer detected by immunohistochemistry. Virchows Arch. 2011; 459:441–447. [PubMed: 21773753]

21. He H, Magi-Galluzzi C, Li J, et al. The diagnostic utility of novel immunohistochemical marker ERG in the workup of prostate biopsies with "atypical glands suspicious for cancer.". Am J Surg Pathol. 2011; 35:608–614. [PubMed: 21383613]

22. Jenkins RB, Qian J, Lee HK, et al. A molecular cytogenetic analysis of 7q31 in prostate cancer. Cancer Res. 1998; 58:759–766. [PubMed: 9485032]

23. Qian J, Bostwick DG, Takahashi S, et al. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. Cancer Res. 1995; 55:5408–5414. [PubMed: 7585610]

24. Vukovic B, Park PC, Al-Maghrabi J, et al. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. Oncogene. 2003; 22:1978–1987. [PubMed: 12673203]

25. Kunju LP, Chinnaiyan AM, Shah RB. Comparison of monoclonal antibody (P504S) and polyclonal antibody to alpha methylacyl-CoA racemase (AMACR) in the work-up of prostate cancer. Histopathology. 2005; 47:587–596. [PubMed: 16324196]

26. Auprich M, Bjartell A, Chun FK-H, et al. Contemporary role of prostate cancer antigen 3 in the management of prostate cancer. Eur Urol. 2011; 60:1045–1054. [PubMed: 21871709]

27. Groskopf J, Aubin SMJ, Deras IL, et al. APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. Clin Chem. 2006; 52:1089–1095. [PubMed: 16627561]

28. Ploussard G, Durand X, Xylinas E, et al. Prostate cancer antigen 3 score accurately predicts tumour volume and might help in selecting prostate cancer patients for active surveillance. Eur Urol. 2011; 59:422–429. [PubMed: 21156337]
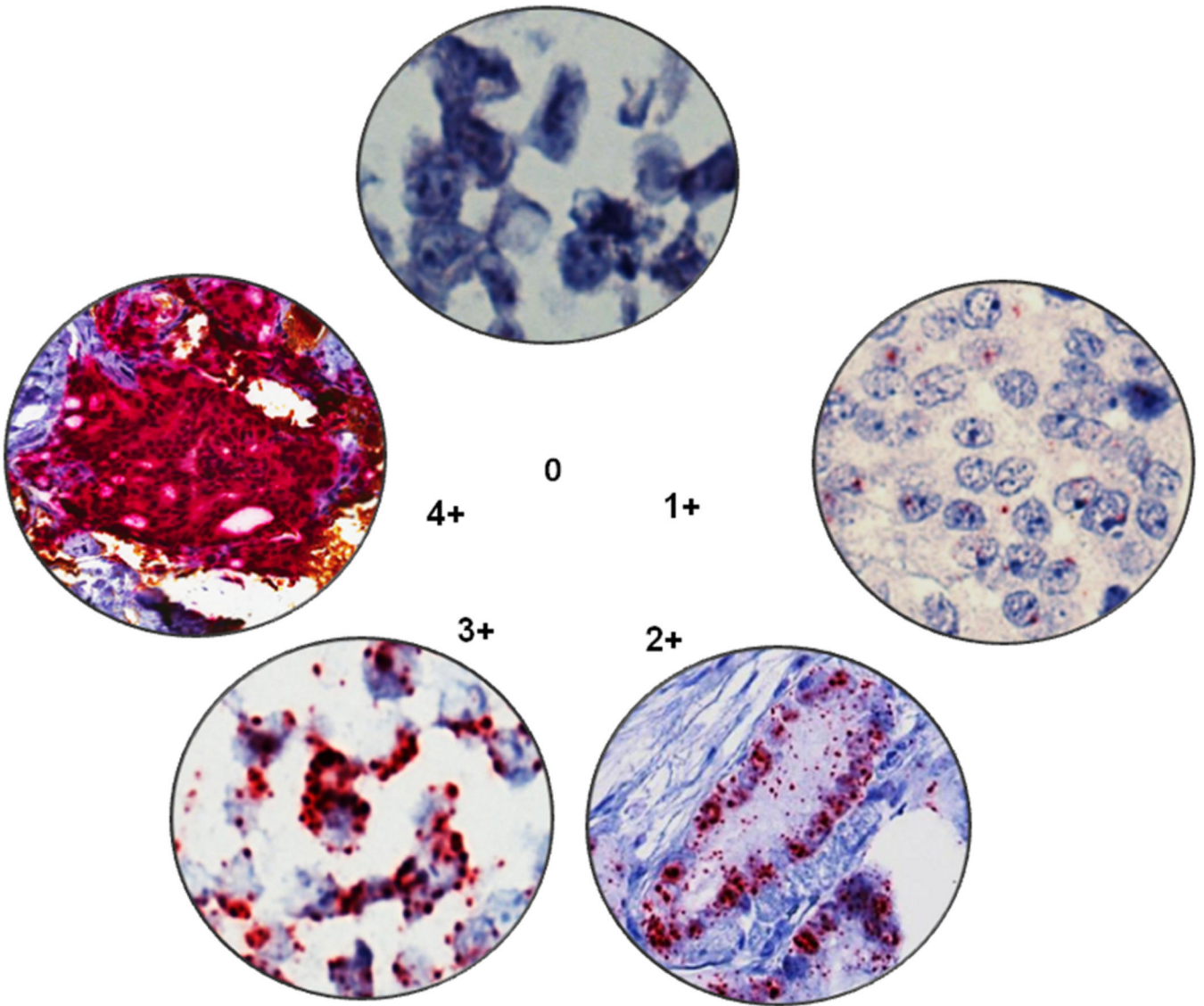
Figure 1.
PCA3 intensity scores; tumor foci with no staining or less than 1 dot/cell under 40× magnification were scored as zero; foci with1–3 dots/cell in more than 5% of the tumor were scored as 1; tumor foci with 4–10 dots/cell with no or very few dot clusters (fused overlapping dots) in more than 5% of the tumor were scored as 2; tumor foci with more than 10 dots/cell with less than 10% of positive cells having dot clusters in more than 5% of the tumor were scored as 3; tumor foci with more than 10 dots/cell with more than 10% of positive cells have dot clusters in more than 5% of the tumor were scored as 4.
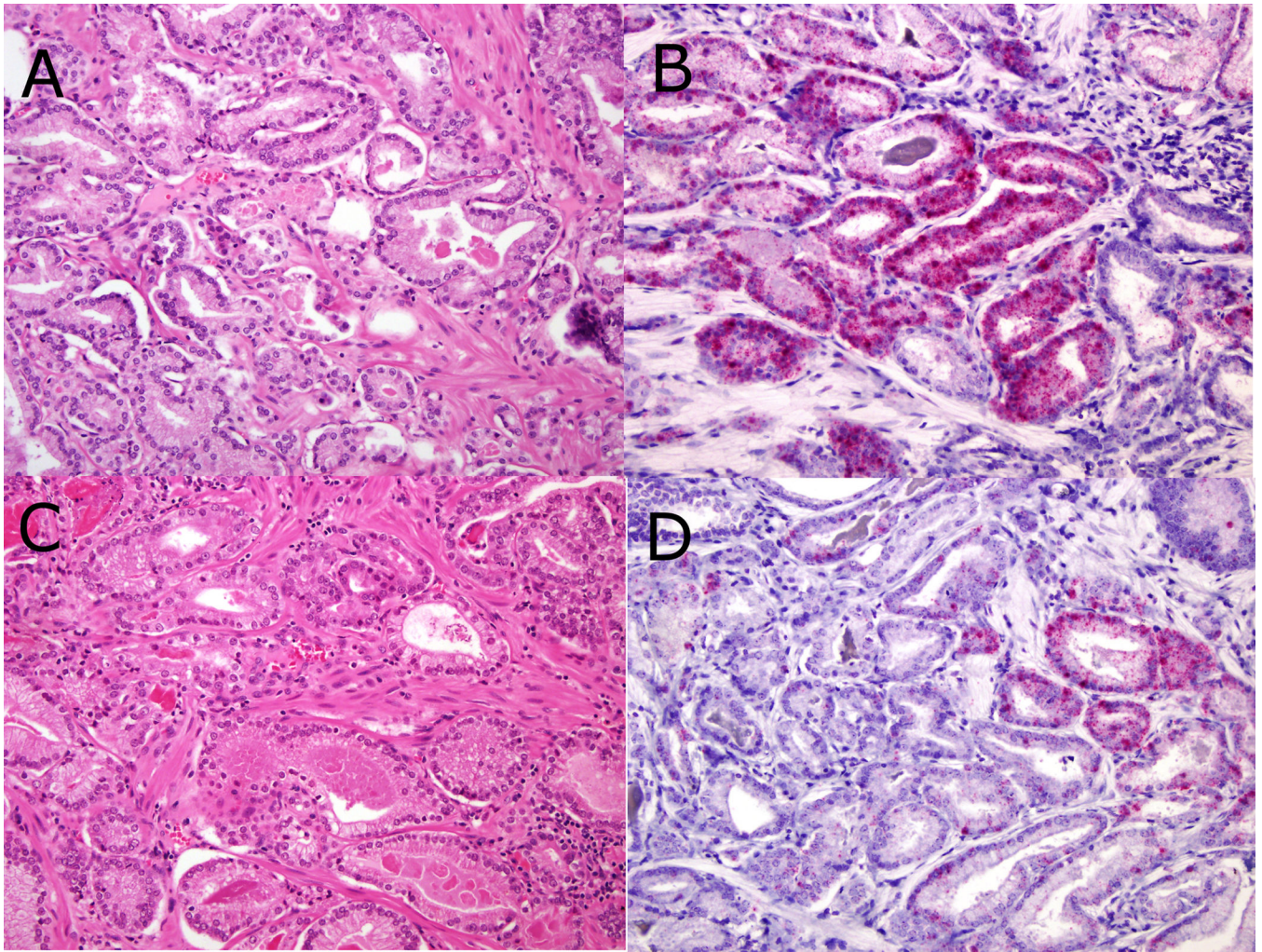
Figure 2.
PCA3 (RNA *in-situ* hydridization) expression with corresponding H&E stained sections.
Diffuse expression with maximum intensity score 4 (2A H&E, 2B *PCA3* RNA *in-situ*
hybridization; both 200× magnification). Focal expression with intensity score 4 at right side
of image and 0 at left side of image (2C H&E, 2D *PCA3* RNA *in-situ* hybridization; both
200× magnification).

ERG and *PCA3* Status of Prostate Cancer Foci, n=138

ERG-/*PCA3*+
17% (24 foci)

ERG-/*PCA3*-
32% (44 foci)

ERG+/*PCA3*-
12% (17 foci)

ERG+/*PCA3*+
38% (53 foci)
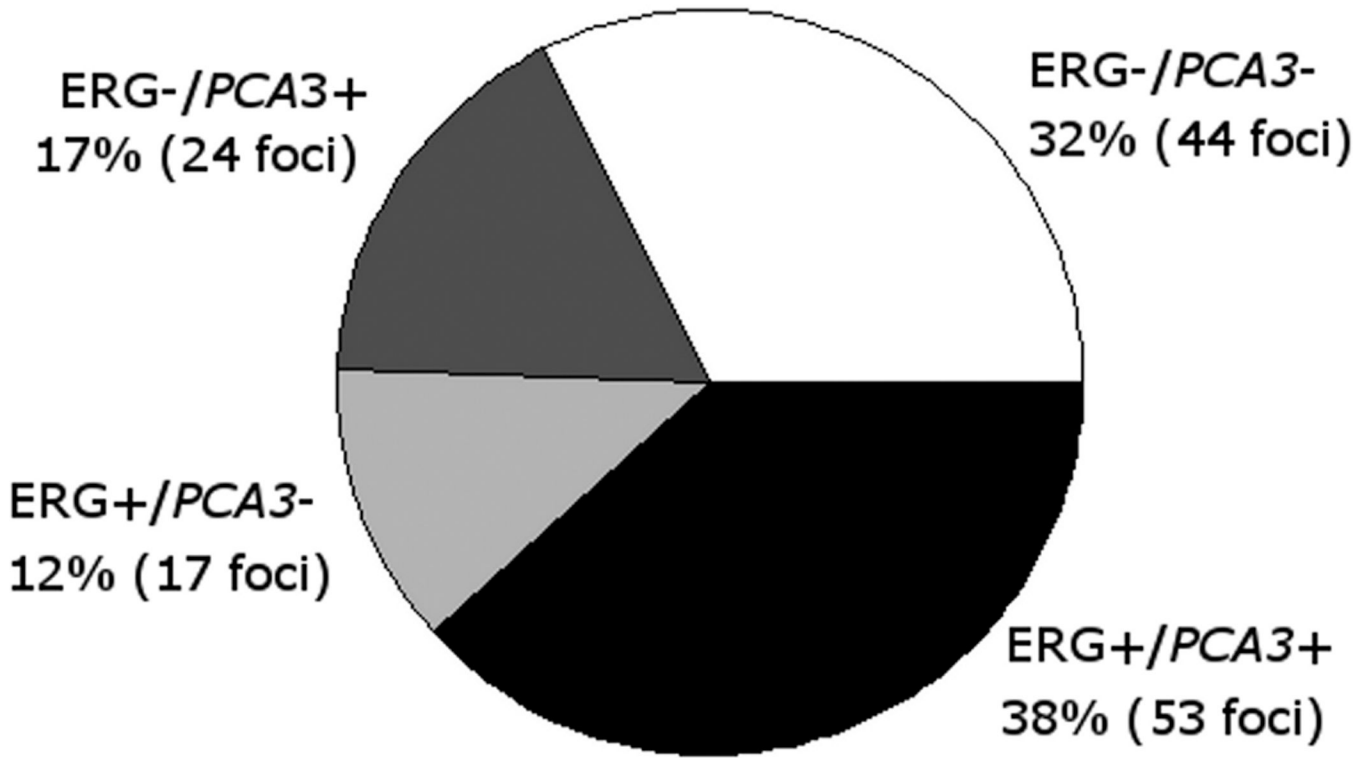
Figure 3.
PCA3 RNA *in-situ* hybridization and ERG immunohistochemistry status of all tumor foci.
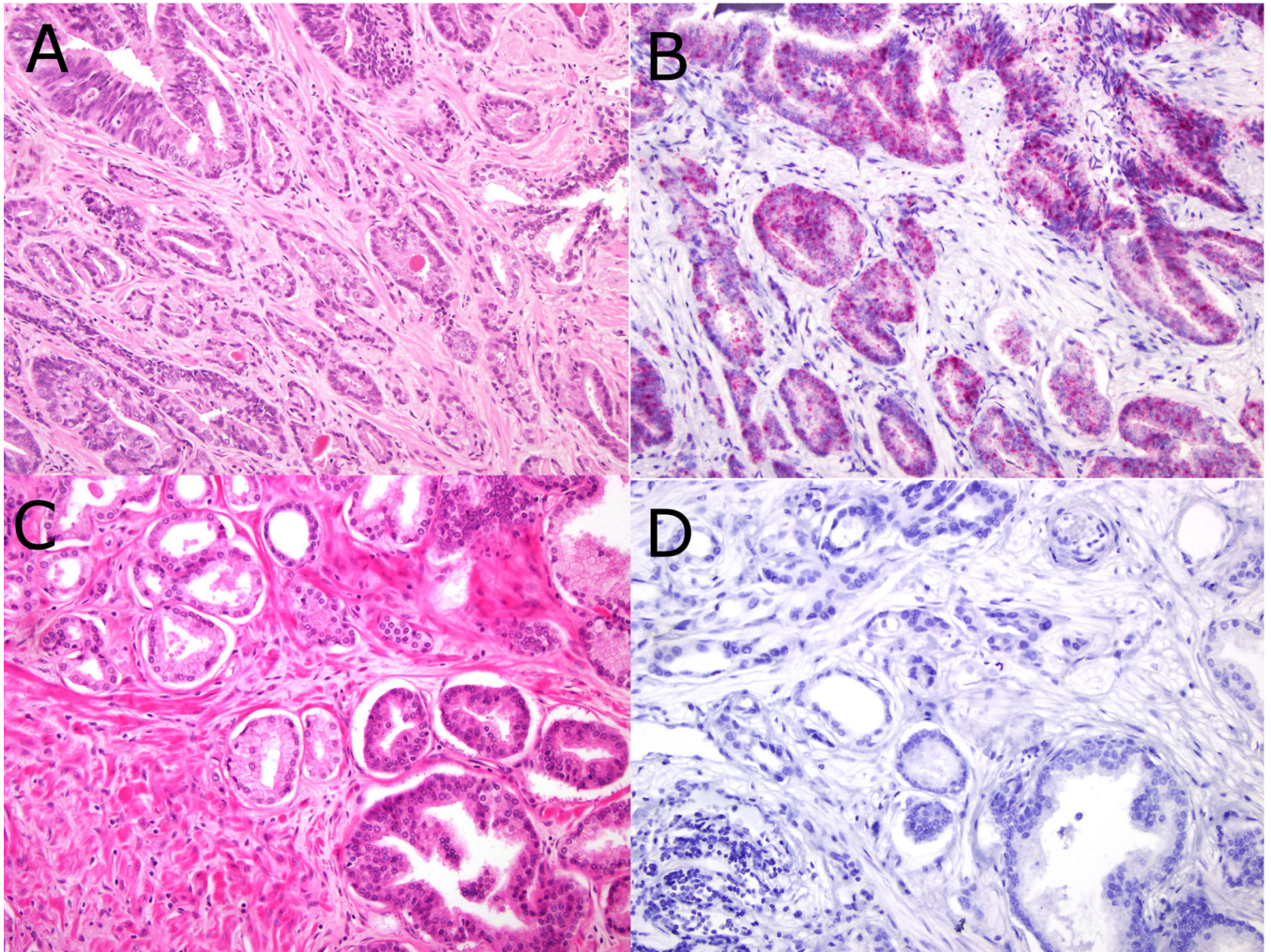
Figure 4.
PCA3 RNA in-situ hybridization in prostate cancer and adjacent high grade high grade prostatic intraepithelial neoplasia showing concordant positive (3A H&E, 3B *PCA3* RNA *in-situ* hybridization; both 200× magnification) and negative (3C H&E, 3D *PCA3* RNA *in-situ* hybridization; both 200× magnification) *PCA3* status.

Cancer *PCA*3 Status vs Adjacent High Grade Prostatic Intraepithelial Neoplasia *PCA*3 Status

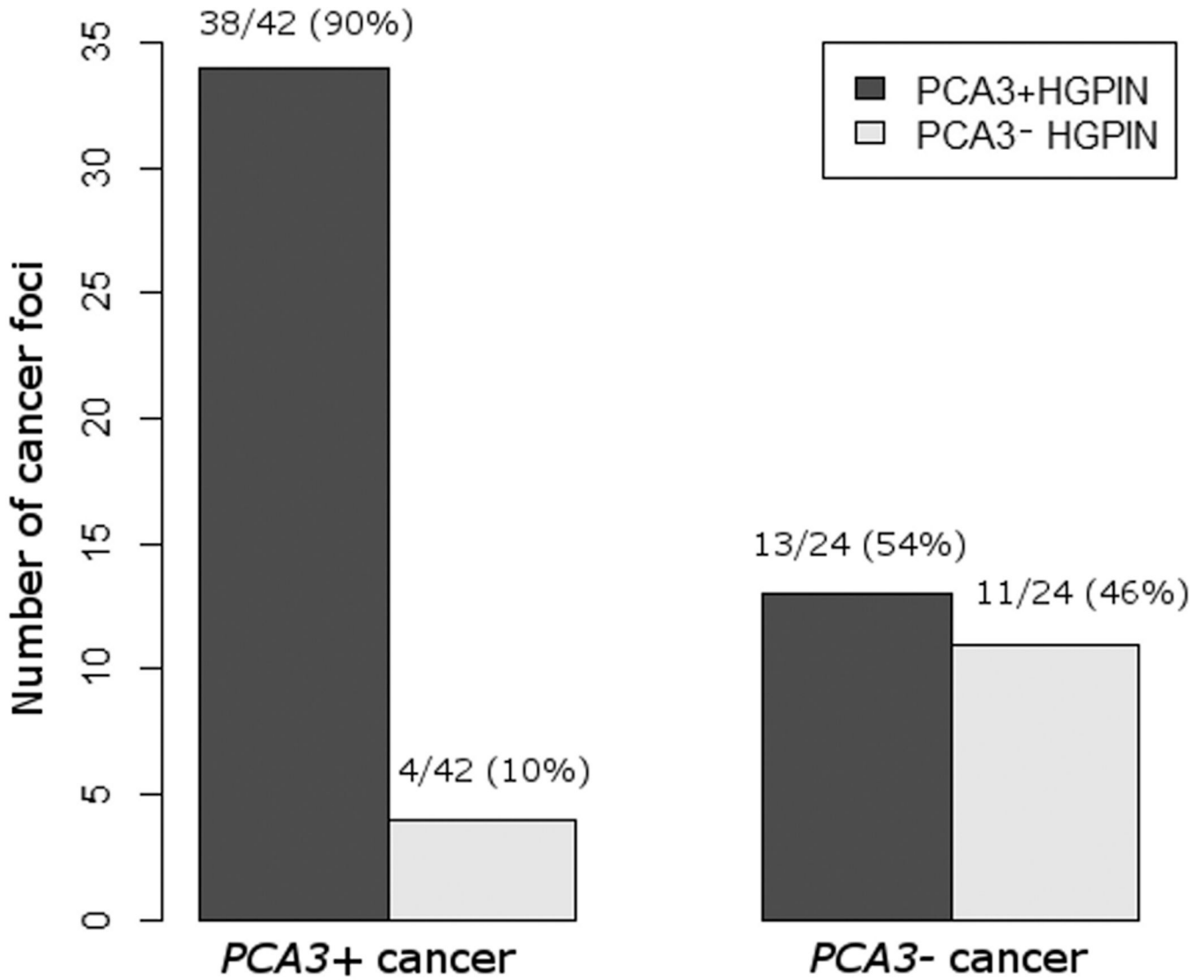**Figure 5.**
PCA3 RNA *in-situ* hybridization status of prostate cancer and adjacent high grade prostatic intraepithelial neoplasia.

Table 1

Pathologic data and *PCA3* scores for all case.

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 cancer foci	Total number 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

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 cancer foci	Total number PCA3+ cancer foci	Urine PCA3 score
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

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%)

Table 3

PCA3 and PSA RNA values in copies/mL, as measured by transcription mediated amplification

Case number[#]	PCA3 RNA value	PSA mRNA value	PCA3:PSA ratio[*]	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	2,360	556,125	4	0
17 - index tumor	5,932	2,539,530	2	0
30 - non-index tumor	2,364	920,239	3	0
41 - non-index tumor	4,282	1,542,647	3	0
41 - non-index tumor	3,977	1,167,430	3	0

[*] PCA3:PSA ratio calculated as $1,000 \times$ (PCA3 copies/mL) / (PSA copies/mL).

[#] Case number refers to numbering in Table 1.