# Clinical Utility of Prostate Carcinoma Molecular Diagnostic Tests

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*Instead of relying on serum prostate-specific antigen (PSA) to identify patients for prostate biopsy, new laboratory tests are needed that have improved specificity for prostate carcinoma (CaP), allow accurate classification of clinically insignificant CaPs, allow for detection of clinically significant CaP in patients without elevated serum PSA, and allow for identification of aggressive forms of CaP, which may warrant adjunctive or even molecularly targeted therapy in the future. Over the last several years, high-throughput gene expression profiling and proteinomics have led to the identification of genes and proteins that are specifically overexpressed in CaP. Molecular diagnostic techniques readily translated to the clinical laboratory have been incorporated into the development of new tests based on these novel molecular alterations in CaP. Some of these tests already have well-documented clinical utility, such as in facilitating prostate biopsy decisions, and are routinely available. The current review focuses on the biological, clinical, and laboratory aspects of the most promising of these current and near-future molecular CaP tests.*

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The use of serum prostate-specific antigen (PSA) to identify patients for prostate biopsy for early prostate carcinoma (CaP) detection is well known to urologists.[1,2] So too, are the deficiencies inherent in the use of total serum PSA, in particular as a consequence of its low specificity for CaP. The large number of negative and, hence, potentially unnecessary prostate biopsies is recognized as a clinical problem in the current approach to prostate patient evaluation. Patients with persistently elevated PSA and negative biopsies present difficult

management issues. The detection and quantitation of different forms of PSA, such as free PSA, can improve the specificity for the use of PSA as a CaP biomarker.[1,2] The biology, clinical use, and laboratory aspects of PSA and different forms of PSA have been reviewed,[1,2] and are not specifically addressed herein.

New technologies have facilitated the discovery of novel genetic alterations in CaP. As these molecular alterations are specific to the malignant versus the benign prostate, it is expected that tests based on these new targets will have markedly improved specificity for CaP detection, as well as other potential applications in CaP management. It can be anticipated that their use will augment the current applications of PSA testing in prostate patient care.

*Test Platforms, Sources, and Targets*

Testing methodology affects not only how the clinic collects the sample and how the laboratory performs the test, but also may affect potential diagnostic sensitivity and specificity. Test sources for new molecular diagnostics in CaP include tissue, blood, and urine. Each specimen source has potential advantages and disadvantages, including its amenability to particular test molecules (Table 1).

The test targets addressed in this review include genes that are expressed normally in some tissues, but are increased in CaP and some other

**Table 1**
**Comparison of Test Sources for Molecular Diagnostics in Prostate Carcinoma, Including Application to Tests in Commercial Development**

| Test Source | Theoretical Advantages | Theoretical Disadvantages | Example Test Candidates | Possible Test Application* |
|---|---|---|---|---|
| Tissue | • FFPE biopsy amenable to protein detection by IHC<br>• FFPE amenable to DNA extraction<br>• Appropriate for CaP prognostic markers on biopsy-documented tumor | • Requires biopsy<br>• FFPE not ideal for RNA extraction and analysis<br>• Limited sample | (a) EPCA IHC<br>(b) DNA promoter methylation QMSP<br>(c) FISH (eg, TMPRSS2:ERG fusion) | (a) IHC of benign glands in negative biopsy<br>(b) Analysis of DNA extracted from negative biopsy<br>(c) Detection of targeted genetic changes in biopsy-sampled CaP |
| Blood | • Amenable to protein detection and quantitation, such as by antibody-based approach, including ELISA<br>• Amenable to DNA analysis<br>• Easy sample to collect | • Not as readily amenable to RNA analysis<br>• Sensitivity for detection of CaP depends on access of target molecule to systemic circulation (possibly stage dependent)<br>• Subject to nonprostate sources, possibly impacting specificity | (a) EPCA ELISA<br>(b) EPCA-2 ELISA | (a) CaP detection/diagnosis<br>(b) CaP detection/diagnosis; staging/prognosis |
| Urine | • Amenable to protein, DNA, and RNA analysis (whole urine and/or cell pellet)<br>• Easy sample to collect<br>• More specific to prostate/GU tract than blood | • Cancer-specific markers require access to excretory system, possibly requiring DRE and/or prostatic massage | (a) AMACR<br>(b) PCA3<br>(c) DNA promoter methylation<br>(d) TMPRSS2:ERG fusion transcripts | (a) CaP detection/diagnosis<br>(b) CaP detection/diagnosis; prognosis/treatment decision<br>(c) CaP detection/diagnosis<br>(d) CaP detection/diagnosis; prognosis/treatment decision |

*Matched to letter-designated assay in previous column (discussed in text).
AMACR, alpha-methylacyl-CoA racemase; CaP, prostate carcinoma; DRE, digital rectal examination; ELISA, enzyme-linked immunosorbent assay; EPCA, early prostate cancer antigen; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; GU, genitourinary; IHC, immunohistochemistry; PCA3, prostate carcinoma antigen 3; QMSP, quantitative methylation-specific polymerase chain reaction.

carcinomas, such as alpha-methylacyl-CoA racemase (AMACR); proteins that are increased in the nucleus of CaP, such as early prostate cancer antigen (EPCA) and EPCA-2; genes that are specific to the prostate and expressed minimally in benign prostate, but are markedly overexpressed in CaP, such as PCA3; epigenetic changes that may alter transcription of tumor suppressor and other genes in CaP, such as promoter hypermethylation; and stable gene rearrangements that result in the fusion of androgen-regulated genes and those encoding transcription factors, such as TMPRSS2:ERG and related gene fusions (Table 2).

Proteins in tissue or blood can be detected by techniques currently employed in prostate testing, such as immunohistochemistry (IHC) on core biopsies and serum enzyme-linked immunosorbent assay (ELISA). Some genetic alterations may be detected using fluorescence in situ hybridization (FISH) or other ISH approaches, which urologists may be familiar with from the use of UroVysion™ (Abbott Molecular Inc., Des Plaines, IL) FISH in diagnosing urothelial carcinoma (UC) in urine specimens. Other genetic alterations forming the basis for new molecular tests are detected by techniques such as polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR), as well as quantitative RT-PCR (qRT-PCR) and other quantitative nucleic acid amplification techniques, including transcription-mediated amplification (TMA).

### *Clinical Use of Analyte-Specific Reagent Tests*

All of these techniques are already routinely used in diagnostic laboratories, forming the basis for a variety of clinical tests in areas such as microbiology, hematology, and oncology. This should facilitate their laboratory incorporation into new urology-directed tests. A practical issue to address is that none of the tests described herein is yet US Food and Drug Administration (FDA) approved. Some of these tests, however, are already available for clinical use, offered by laboratories as tests classified as analyte-specific reagents (ASRs). As per laboratory regulations governing ASRs, it is up to the individual testing laboratory to validate the assay performance. For example, several laboratories in the United States offer PCA3 testing for clinical use, and tissue-based EPCA IHC and promoter methylation have been licensed to laboratories for clinical use.

Urologists should not be hesitant to order ASR tests, as long as they understand the test applications and potential use as substantiated in the literature. ASR tests are used extensively in clinical medicine, not only by pathologists for supporting diagnoses (such as non–FDA-approved IHC testing on tissues), but also by clinicians, particularly in hematology and oncology, for diagnosis, prognosis, treatment decisions, and patient monitoring. In the rapidly evolving field of molecular diagnostics, the ability to offer ASR tests can lead to faster improvements in patient management.

As these biomarker targets and test strategies are discovered and developed based on their specific increase or alteration in CaP, and as many or all of these molecular and genetic alterations may actually contribute to CaP development or progression, they may eventually become the targets or indications for specific molecularly targeted therapies.

### *Scope of Review*

This detailed review of new molecular CaP tests includes only those biomarkers known to the author as being in commercial development with intended translation to clinical utility. They are presented in alphabetical order. Each biomarker test section is divided into four parts: a description of the CaP pathobiology of the involved gene or genes, including their discovery and characterization of alterations in CaP versus the benign prostate; a description of research efforts indicating possible suitability as a clinical test target; a description of the current status of a commercial test product, including further validation and application of the specific test platform and reagents; and a brief description of future directions, including additional validation research needs and/or possible test applications. Any omissions are unintentional, and opinions expressed are those of the author and not necessarily the commercial developer or manufacturer of the test.

As not all of the biomarkers described herein are available currently for clinical testing, but may become so with further development, the material presented herein is also intended to provide a frame of reference for the ongoing evaluation of the potential clinical application of these tests.

## Alpha-Methylacyl-CoA Racemase (AMACR)

### *Prostate Cancer Pathobiology*

**Increased expression of AMACR in CaP.** AMACR (P504S) was identified as a gene overexpressed in CaP versus the benign prostate by high-throughput genomic expression profiling.[3,4] Low levels of AMACR expression were detected in 9 of 9 benign prostatic hyperplasia (BPH) specimens, but AMACR was overexpressed relative to a common reference an average of 5.7-fold in 13 of 16 CaP samples.[4] In subsequent cDNA microarray analyses, AMACR mRNA was increased in 20 of 23 CaP specimens[5] and AMACR mRNA was increased relative to the benign prostate 3.1-fold in localized

Table 2
Summary of Current and Possible Future Applications of Prostate Carcinoma Molecular Diagnostic Tests*

| Biomarker: Gene/Protein; Description | Possible Test Source; Target | Possible Test Application†,* | Comments | Reference Number |
|---|---|---|---|---|
| AMACR (alpha-methylacyl CoA racemase); enzyme involved in branched chain fatty acid oxidation, increased in CaP and some other carcinomas | Tissue; IHC (P504S antigen, same as AMACR) | (a) *Interpretation of small atypical gland foci on prostate bx*<br>(b) Staining of benign glands in negative bx to predict repeat bx | (a) Positive staining supports CaP dx; in absence of definitive CaP dx, does not provide information on risk of CaP in subsequent bx<br>(b) Unlikely to translate into useful clinical test | (a) 21<br>(b) 23 |
| | Blood; mRNA | qRT-PCR (or other nucleic acid amplification); CaP dx or prognosis | Positive in ~ 45% patients with clinically organ-confined CaP; dx, utility not established; prognosis, if patients with elevated AMACR blood mRNA have circulating tumor cells and/or adverse outcomes, could have utility | 27 |
| | Urine; protein | Western blot or other techniques; CaP dx | Urine AMACR detected in all patients with CaP, 42% patients with negative bx; quantitative techniques could improve specificity | 25 |
| | Urine; mRNA | (a) qRT-PCR; CaP dx<br>(b) **TMA test in commercial development (Gen-Probe Inc., San Diego, CA); CaP dx** | (a) Shows promise as dx test (~ 70% of patients with CaP bx above cutoff for positive test); specificity much greater than serum PSA<br>(b) Prototype TMA assay ~ 60% sensitivity, 70% specificity for CaP on bx | (a) 26, 27<br>(b) 29 |
| EPCA (early prostate carcinoma antigen); nuclear matrix protein, increased in CaP | Tissue; IHC | *Staining of benign glands in negative bx to predict repeat bx; Commercially available (Onconome, Seattle, WA)* | (a) Preliminary studies suggest unacceptably high false-negative rate; may be difficult to establish reliable reference ranges for IHC intensity in benign glands<br>(b) Possible targeted application to bx with HGPIN, PIA for predicting repeat bx not reported to date | (a) 31, 37<br>(b) 35 |
| | Blood; protein (ELISA) | **CaP dx; in commercial development (Onconome, Seattle, WA)** | Preliminary studies suggest high sensitivity and specificity for CaP; needs to be validated in samples from patients undergoing subsequent prostate bx | 36 |
| EPCA-2 (early prostate carcinoma antigen-2); nuclear matrix protein, increased in CaP | Blood; protein (ELISA) | **In commercial development (Onconome, Seattle, WA)**<br>(a) CaP dx<br>(b) Prognosis | (a) Potentially high sensitivity (including in patients with PSA < 2.5 ng/mL) and specificity for CaP; needs to be validated in samples from patients undergoing subsequent prostate bx<br>(b) Preliminary studies suggest possible differentiation of organ-confined CaP vs CaP with ECP; requires confirmation | (a) 32, 38<br>(b) 32 |

**Table 2**
**(Continued)**

| Biomarker: Gene/Protein; Description | Possible Test Source; Target | Possible Test Application[†,‡] | Comments | Reference Number |
|---|---|---|---|---|
| **Gene promoter methylation** (including GSTP1, APC); stable genetic change occurring in variety of genes in most CaPs | Tissue; DNA extracted from FFPE negative bx | *QMSP for multiple genes to predict repeat bx; commercially available (Veridex, LLC)* | Testing on negative bxs may have insufficiently low sensitivity for clinical utility; studies planned for high-risk bx-negative patients | 55, 59 |
| | Urine; DNA | **QMSP testing for multiple genes; CaP dx; in commercial development (Veridex, LLC)** | QMSP for GSTP1 and APC shows promise as dx test; prototype assay ~ 60%-75% sensitivity, 75%-90% specificity for CaP on bx | 60 |
| PCA3 (prostate carcinoma antigen 3); prostate-specific gene markedly upregulated in CaP | Urine; mRNA | *Quantitation (relative to PSA mRNA) in post-DRE urine (see Table 3); commercially available (Gen-Probe)*<br>(a) CaP dx<br>(b) CaP prognosis | (a) For CaP on initial or repeat bx, sensitivity ~ 60%-80%, specificity ~ 70%-90% (see Table 4); useful in facilitating bx decision in patients with elevated PSA, including after initial negative bx<br>(b) Possible future application to prognosis (eg, predicting clinically insignificant CaP after bx CaP dx) | (a) See Tables 3, 4<br>(b) 53 |
| TMPRSS2:ERG gene fusions; stable recurrent genetic rearrangements occurring in ~ 50% CaPs | Tissue; FISH or other ISH | Classification of diagnosed CaP as ERG fusion positive or negative for prognostication | FISH break-apart probes on bx or RP CaP to identify ERG fusion–positive CaPs, which may have adverse prognosis; positive test may indicate need for further classification based on mRNA isoforms (see below) | See Table 5 |
| | Urine; mRNA | **TMA-based test in commercial development (Gen-Probe);**<br>(a) CaP dx<br>(b) CaP prognosis | (a) Detection of TMPRSS2:ERG fusion mRNA in urine, alone or in combination with other gene targets, for dx<br>(b) Detection of specific mRNA isoforms in urine (or tissue) of patients with CaP on bx for prognosis | (a) 79<br>(b) See Table 5 |

*See corresponding sections of text for full details of these and other topics for each candidate biomarker.
†Matched to letter-designated descriptions and references in subsequent columns.
‡Tests described in bold are targeted for commercial development and clinical application; those described in italics are already available commercially (see text for more details).
bx, biopsy; CaP, prostate carcinoma; DRE, digital rectal examination; dx, diagnosis; ECP, extraprostatic penetration; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; HGPIN, high-grade prostatic intraepithelial neoplasia; IHC, immunohistochemistry; ISH, in situ hybridization; PIA, proliferative inflammatory atrophy; PSA, prostate-specific antigen; QMSP, quantitative methylation-specific polymerase chain reaction; RP, radical prostatectomy; TMA, transcription-mediated amplification.

CaP and 1.67-fold in metastatic CaP.[6] In a more direct comparison, AMACR mRNA was increased in 9 of 12 (75%) CaP samples versus matched normal prostate from the same patient.[5] By qRT-PCR, AMACR mRNA levels were an average of 8.8-fold higher in 8 samples of CaP versus 8 samples of benign prostate.[5]

Increased AMACR protein in CaP versus benign prostate has also been shown by Western blot and particularly by IHC.[5,6] AMACR was increased by IHC in the vast majority of 168 primary CaP cases and was also variably increased in high-grade prostatic intraepithelial neoplasia (HGPIN).[5] AMACR epithelial IHC score cutoffs could be established by which 95.6% of CaPs versus only 3.5% of benign prostates were immunopositive.[5] On

tissue microarrays including 108 benign prostate, 75 prostatic intraepithelial neoplasia (PIN), 116 clinically localized CaP, and 17 metastatic CaP samples and with scoring of IHC from 0 to 4, AMACR was significantly increased in clinically localized CaP versus benign prostate, with mean scores of 3.2 versus 1.3, respectively.[6] There was no significant association between AMACR IHC score in CaP and Gleason score, tumor stage, or PSA recurrence following radical prostatectomy (RP).[5,6]

**Biology of AMACR and possible role in prostate carcinogenesis.** Biomarkers based on causal molecular alterations may be more likely to translate to clinical utility. AMACR is an enzyme that functions in peroxisomal beta oxidation of branched chain fatty acids.[7] AMACR is normally expressed in certain human tissues, including liver hepatocytes.[7] The main sources of branched chain fatty acids are dairy products and beef, the consumption of which has been associated with an increased risk for CaP.[8] Paralleling increased gene expression and protein levels, AMACR enzymatic activity is also increased in CaP versus benign prostate.[9]

Sequence variants of AMACR have been linked to CaP risk in hereditary CaP, but not necessarily sporadic CaP.[10,11] The functional significance of AMACR allelic variants regarding catalytic activity is unknown.

Further understanding of any role for AMACR in prostate carcinogenesis could have important epidemiological and preventive implications for CaP, in addition to facilitating diagnostic applications of AMACR.

**Other biologic issues potentially complicating application of AMACR testing in CaP.** *Alternatively spliced variants of AMACR mRNA*. Several different splice variants of AMACR mRNA have been observed in CaP.[12] The predominant AMACR IA transcript is the only one encoding a protein likely targeted to peroxisomes.[12] By qRT-PCR and IHC using specific antibodies, it appears that both A (5 exons) and B (lacking exon 3) forms are co-upregulated in CaP.[12] At present, the potential biologic significance of different AMACR splice variants remains unknown in CaP. However, clinical investigators considering diagnostic applications of AMACR, whether in tissue or fluids, need to be aware of these potential issues and which specific mRNA or protein products the assay reagents are detecting.

*Increased AMACR expression in nonprostatic neoplasms*. AMACR is not a prostate-specific gene, and increased expression of AMACR in human neoplasia is not limited to CaP, which could influence specificity for CaP detection using either blood or urine. In addition to more than 90% of CaPs, AMACR is increased in approximately 75% of hepatocellular carcinomas, papillary renal cell carcinomas, and colon adenocarcinomas.[13-15] In one study, 9 of 29 (31%) UCs were positive for AMACR immunostaining,[13] whereas in a subsequent report, a small number of UCs studied were all AMACR IHC negative.[15]

Even for blood-based testing, other carcinomas are unlikely to be more than rare confounding factors in CaP testing in patients lacking symptoms/signs related to such tumors. However, in tissue microarray studies, AMACR immunostaining was equally frequent (~ 70%) in colorectal adenomas and carcinomas.[14] Whether these more prevalent benign/precursor lesions could cause false-positive results due to increased serum levels overlapping those in CaP in a blood-based AMACR assay for CaP diagnosis remains to be investigated.

Urine-based testing for CaP would likely be more specific for a gene target such as AMACR. Regarding renal tumors, AMACR expression increases in papillary renal cell carcinomas, which comprise approximately 15% of renal carcinomas. The expected uncommon occurrence of renal neoplasms, particularly papillary renal cell carcinomas, involving the collecting system in patients undergoing CaP screening makes this unlikely to be a significant factor in compromising urine-based mRNA testing for CaP. However, possible increased AMACR expression in bladder carcinoma or benign reactive urothelium (e.g., in common bladder or prostate conditions) as a potential confounding factor in urine-based AMACR testing for CaP likely needs to be further addressed.

*Further understanding of any role for alpha-methylacyl-CoA racemase (AMACR) in prostate carcinogenesis could have important epidemiological and preventive implications for prostate carcinoma (CaP), in addition to facilitating diagnostic applications of AMACR.*

*Possible hormone modulation of AMACR and impact of routinely used antiandrogen treatments*. Multiple studies have reported reduced AMACR immunostaining in CaP in RP specimens following neoadjuvant antiandrogen therapy.[16] In vitro experiments using the AMACR-expressing androgen-responsive LNCaP CaP cell line indicated that AMACR is not directly regulated by androgens (eg, in contrast to PSA).[17] These results suggest that reduced expression in intact prostates from antiandrogen therapy may be indirect, such as mediated by the hormonally responsive stroma.

Regardless of the precise mechanisms involved, possible modulation of AMACR expression in CaP by antiandrogen therapy has potential impact on clinical application of laboratory tests for CaP based on AMACR, similar to that well known for PSA. For example, if 5-alpha-reductase inhibitors also reduce AMACR expression in prostate, it may become necessary to define alternate reference ranges for patients on these treatments for BPH or male pattern baldness.[18]

*Towards a Clinical Test Based on AMACR*

**Use of AMACR IHC in prostate biopsy interpretation by surgical pathologists.** Several studies have supported the diagnostic utility of AMACR IHC in the interpretation of prostate needle biopsy specimens that are diagnostically challenging due to a small focus of atypical glands.[5,6,19,20] It is now common practice to utilize IHC for AMACR (P504S) in addition to IHC for basal cell markers, such as high-molecular-weight cytokeratin (HMWCK) and/or p63, as an adjunct to hematoxylin and eosin (H&E) histopathology in the interpretation of small foci suspicious but not diagnostic of adenocarcinoma.[21] The presence of basal cells on IHC for HMWCK and/or p63 supports a benign mimic, whereas absence of basal cells supports a diagnosis of CaP.[21,22] In contrast, increased AMACR (P504S) IHC staining compared to clearly benign glands in the same biopsy can support a diagnosis of CaP.[20-22]

Of the biomarkers included in this review, AMACR is the only one with a currently established role in support of histology in the pathology interpretation of actual prostate tissue biopsies. However, this use applies to the interpretation of already sampled lesions, and is in essence an extension of histopathology interpretation. This is quite distinct from the development of a biomarker test that can be used to indicate the potential need for an initial or repeat biopsy. Of note, in addition to urine- or blood-based tests that may allow for more global prostate sampling, such a test could be applied to benign prostate glands sampled in an initial negative biopsy, in which increased expression could occur due to a so-called field effect (Tables 1,2).

**AMACR IHC of benign glands in prostate biopsy as possible predictor of subsequent biopsy outcome.** Whether AMACR could be increased in benign prostate glands adjacent to CaP, potentially representing genetic alterations in a field effect, and whether AMACR expression in benign prostate glands could have predictive value for repeat biopsy outcomes was investigated by IHC.[23] In RP tissues, benign glands near a CaP focus had significantly higher mean AMACR immunostaining compared to those that were distant in the same specimen or benign prostate glands in cystoprostatectomy specimens without CaP.[23]

In a series of HGPIN biopsies, there was significantly higher mean AMACR immunostaining per benign gland in 23 cases later having CaP than in 22 with subsequent negative biopsies (mean, .29 vs. .21 on a scale of 0 to 3; 3 represents diffuse strong immunostaining). In contrast, differences in IHC staining were not noted in the HGPIN compartment.[23] Although such results suggest the possibility of a field effect, the low level of IHC staining in benign glands and the very small absolute difference in score between cases with and without later CaP argues against ready translation to a useful clinical test for which reproducible reference ranges could be established by which a positive result would support the need for repeat biopsy. In addition, this study included only cases with HGPIN on initial biopsy. Whether a similar result could be seen with AMACR IHC in benign glands from more common negative prostate biopsies (without HGPIN) has not been shown.

**Blood- and urine-based approaches to AMACR testing for CaP detection.** In contrast to tissue-based approaches that are potentially limited by sampling and require an initial prostate biopsy, precluding their application to screening, urine and blood may allow for more consistent sampling of products of CaP foci that enter the excretory or circulatory systems.

Using a combination of protein microarrays, immunoblots, and ELISA assays, Chinnaiyan's laboratory at the University of Michigan showed that immunoreactivity against AMACR was significantly higher in sera from patients with CaP than in controls.[24] In patients with serum PSA 4 to 10 ng/mL, immunoblot analysis showed that the antibody response against AMACR was more sensitive and specific for CaP than PSA (sensitivity and specificity of 77.8% and 80.6% vs. 45.6% and 50%, respectively; area under the receiver operating characteristic [ROC] curve of 0.789 vs. 0.492; $P < .001$).[24] Validation of a serum auto anti-AMACR antibody assay based on prospective testing and correlation with biopsy results has not been reported.

Investigators at Johns Hopkins examined AMACR protein by Western blot in urine specimens obtained after prostate biopsy for suspected CaP.[25] AMACR protein was detected in the urine of 18 of 26 patients (69%), including 12 of 12 (100%) patients with biopsy-confirmed CaP, 1 of 2 with atypia on biopsy, and 5 of 12 (42%) patients with negative concurrent biopsies.[25] Whether at least some patients with elevated AMACR protein and negative biopsy could represent those with unsampled CaP, whether

other tissue sources such as bladder urothelium can contribute to immunoreactive AMACR in urine, and whether quantitative protein assays could improve specificity of this approach are unknown.

qRT-PCR for AMACR mRNA was performed on total cellular RNA extracted from post–prostatic massage urine specimens from 21 patients, including 10 with CaP, 2 with HGPIN, and 9 cancer-free individuals. Similar methodology quantitated PSA mRNA in order to verify prostate cell–derived RNA recovery, and normalized AMACR mRNA in urine to PSA mRNA for a relative AMACR score. Using cutoffs defined by the cancer-free control group, 7 of 10 (70%) with CaP had scores above the cutoff.[26] Interestingly, 2 of the 3 false-negative cases showed limited Gleason score 6 on biopsy and a single small focus (< 5% tissue) of Gleason score 6 CaP in subsequent RP, raising possible implications for pretreatment prognostication. The 2 patients with HGPIN were also above the positive cutoff for AMACR score.[26] Whether this represents the presence of unsampled CaP or increased expression of AMACR in HGPIN is unknown. HGPIN by virtue of its location in normal prostatic glands/ducts communicating with the prostatic excretory system could particularly shed AMACR-expressing cells into urine under such testing conditions.

AMACR mRNA levels were determined in blood using quantitative RT-PCR and were normalized to a non–prostate-specific housekeeping gene. Cutoff values for blood testing were established using 76 samples from non–age-matched normal donors. Normalized AMACR mRNA levels were above the cutoff values in the blood of 28 of 58 (48%) patients with known metastatic CaP who were undergoing treatment.[27] In 39 of 88 (44%) patients with presumed organ-confined CaP, AMACR mRNA was detectable in blood.[27] AMACR mRNA transcripts in blood were detected at levels classified as borderline positive in 3 of 9 (33%) patients with BPH, 10 of 20 (50%) patients with prostatitis, and 3 of 12 (25%) patients with other urological disorders, such as kidney stones or nephritis.[27] These comparisons indicate the potential contribution of non-CaP sources to blood AMACR transcripts and the requirement for quantitative assays for any potential blood test applications.

The same study analyzed post–prostatic massage urine samples using the qRT-PCR AMACR assay in samples from 7 patients with CaP, 3 with BPH, and 1 with prostatitis. Urine sediment samples demonstrated elevated normalized AMACR mRNA in 4 of 6 stage T1 CaP patients and in the 1 patient with stage T2 CaP.[27]

These preliminary studies support the application of urine mRNA quantitation for genes increased in CaP, including AMACR, for predicting findings on prostate biopsy and potentially indicating the need for initial or repeat prostate biopsy.

*Current Status of Commercial AMACR Testing for CaP*

The rights to further develop and commercialize CaP diagnostic tests based on AMACR transcripts were licensed by Gen-Probe, Incorporated (San Diego, CA) from Corixa Corporation (Seattle, WA). Gen-Probe has begun to characterize the performance of a quantitative urine mRNA AMACR test for potential CaP diagnostic applications based on its proprietary technologies of target capture, TMA, and detection of amplified target based on hybridization protection.[28]

Results of AMACR mRNA quantitation in urine were recently compared to diagnostic prostate biopsy findings in 232 patients, 71 (30.6%) of which had CaP on biopsy. First-catch urine samples were collected after digital rectal examination (DRE) and total RNA was extracted from urine sediments. Samples were also analyzed using identical methodology for hypoxanthine phospho ribosyl transferase (HPRT) to confirm the presence of amplifiable mRNA and to normalize AMACR mRNA expression levels. The average normalized AMACR mRNA copy level was 1416 per reaction for men with CaP on biopsy versus 434 per reaction for those with negative biopsy.[29] In a ROC curve analysis for AMACR/HPRT ratio against biopsy result, the area under the ROC curve was 0.69.[29] With a cutoff of AMACR/HPRT of 0.73, the sensitivity and specificity for CaP on biopsy for the AMACR TMA assay were 57% and 70%, respectively (vs 92% and 14% for serum PSA of > 4.0 ng/mL, respectively).[29]

*Future Directions*

The potential improvement in specificity over serum PSA for CaP detection by the urine AMACR mRNA assay is encouraging, suggesting that such an assay could have utility in reducing the number of prostate biopsies, especially repeat biopsies following initial negative biopsies in patients with persistently elevated serum PSA.

Although it is crucial to include some sort of housekeeping gene for normalization and verification of recovery of intact RNA (eg, to exclude false-negative results), the inclusion of a prostate-specific gene, such as PSA, may improve assay performance. Quantitating PSA mRNA can correct for the amount of prostate cell (benign or malignant)–derived RNA in the sample, as the expression of mRNA for PSA is not markedly altered in malignant versus benign prostate cells.[1]

Based on preliminary results with the AMACR/HPRT mRNA scores and correlation with biopsy results, the Gen-Probe AMACR TMA assay may not perform as well as the similarly

based urine mRNA test for PCA3, as detailed below.

Another potential testing approach is to use a combination of CaP markers by quantitating mRNA for multiple CaP genes in urine. However, the increment in sensitivity and/or specificity needs to be substantial to justify the added expense and complexity inherent in testing for multiple versus single gene targets.

In addition to these potential diagnostic applications, it should be noted that analysis of some gene targets following a definitive CaP diagnosis in tissue could provide meaningful prognostic information. Urine- or tissue-based assays in conjunction with biopsy information could thus potentially influence treatment decisions. For example, there is some data to support altered AMACR expression levels in tissues as a prognostic factor in CaP,[30] although the optimal approaches for any clinical applications for testing remain to be further resolved.

## Early Prostate Cancer Antigen (EPCA), Early Prostate Cancer Antigen-2 (EPCA-2)

*Prostate Cancer Pathobiology*

EPCA and EPCA-2 are nuclear structural proteins that have been identified as expressed in CaP, but not in other normal tissues or cancer types.[31,32] Changes in nuclear matrix proteins are associated with carcinogenesis in a variety of tissues. The nuclear matrix proteins of the Dunning rat model of CaP were identified as different from those of the normal rat prostate.[33] In an analysis of the nuclear matrix proteins in human prostate tissues, 1 protein (designated PC-1) was identified in 14 of 14 of the CaP nuclear matrix preparations, but was not detected in similar preparations of any of 13 benign prostate specimens or 13 BPH specimens.[34]

EPCA is a nuclear matrix protein that is reportedly the human counterpart of the rat protein Am-1.[33,35] In contrast to some other biomarkers described herein that were discovered by high-throughput analyses of mRNA expression patterns, the nuclear matrix proteins such as EPCA that may be unique to CaP were discovered by proteinomic approaches, including 2-dimensional (2D) gel electrophoresis.[33,34] Partial peptide sequencing of EPCA indicated a novel protein showing some regions common to other known proteins.[33,34]

*Towards a Clinical Test*

**EPCA tissue IHC.** A polyclonal antibody generated against peptides of gel-purified EPCA protein has been used in tissue IHC[31] and a blood-based ELISA test (see below).[36] EPCA immunostaining was noted in CaP foci from RPs as well as in HGPIN and benign prostate glands in foci adjacent to CaP in RP specimens, but was not seen in BPH samples or in benign prostate glands from organ donors without CaP.[31] In a series of negative biopsies from patients eventually detected as having CaP, EPCA immunostaining was noted in the benign glands of the negative biopsies, as well as in the benign glands of subsequent CaP-positive biopsies from the same patients, with even stronger staining in the cancer glands. In nonblinded analysis, IHC staining intensity of 1+ or less was seen in 23 of 27 CaP-free prostates from organ donors. In contrast, 21 of 25 negative prostate biopsies from patients later diagnosed with CaP had staining intensity of more than 1+ (on a scale of 0-3).[31] These preliminary results suggest that upregulation of EPCA is an early event, perhaps as part of a field effect, in prostate carcinogenesis.

The observations of EPCA immunostaining in epithelium of glands adjacent to CaP versus benign glands from prostates without CaP was subsequently confirmed, with some qualification, by a separate group of investigators.[35] In prostate samples from 50 patients with localized CaP versus 10 from patients with UC, EPCA immunostaining was noted in 94% of CaP samples, but was completely negative in benign prostates from cystoprostatectomies of UC patients.[35] EPCA immunostaining was positive in noncancerous glands adjacent to CaP in 86% of the RPs. However, most EPCA-positive glands adjacent to CaP were affected by PIN or proliferative inflammatory atrophy (PIA), both implicated as precursor lesions for CaP.[35]

*In contrast to some other biomarkers described herein that were discovered by high-throughput analyses of mRNA expression patterns, the nuclear matrix proteins such as early prostate cancer antigen that may be unique to CaP were discovered by proteinomic approaches, including 2-dimensional gel electrophoresis.*

A recent study evaluated the potential application of tissue-based EPCA IHC on initial negative biopsy for predicting CaP risk in subsequent biopsy in a small number of more clinically relevant actual biopsy cases. Using the commercially available antibody (Onconome Inc., Seattle, WA) described below, anti-EPCA IHC was performed on biopsies of 39 patients with first-time negative biopsy (no repeat available), 24 patients with persistently negative biopsies, 8 patients with initially negative biopsy subsequently diagnosed with CaP on repeat biopsy, and 27 negative biopsies from patients with CaP in other concurrent biopsy sites.[37] EPCA IHC was blindly assessed

by two different investigators, who classified intensity (0-3) and extent (1-3), with any intensity of 3 considered positive for primary analysis, and extent also analyzed secondarily. The proportion of EPCA immunopositivity in benign prostate was highest in the patients with CaP on repeat biopsy (6/8; 75% positive). It was lowest in the negative biopsies of patients with persistently negative biopsies (7/24; 29% positive). However, EPCA was only positive in 59% of negative biopsies of patients with concurrent CaP on other cores, and was positive in 59% of patients with initial negative biopsies (no repeat biopsy; certainly a higher percentage of patients than would be expected to have CaP on repeat biopsy). Similar results were reportedly noted when extent of EPCA immunostaining was analyzed.

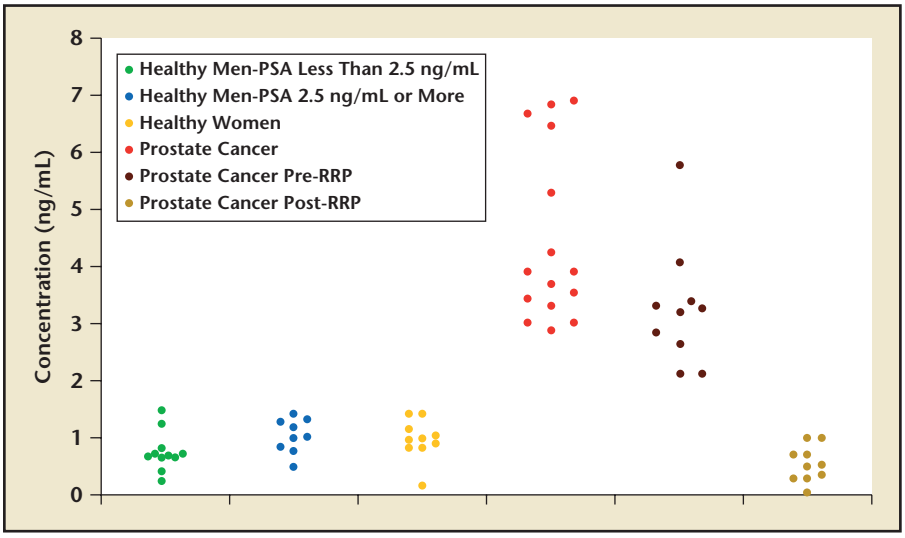
Whether any sort of IHC analysis based on extent, intensity, and perhaps number of biopsy sites/case immunostained could be used to generate reproducible cutoffs for predicting CaP versus benign on repeat biopsy clearly remains to be established. The 41% false-negative results for absent immunostaining in benign tissues of patients with concurrent CaP raises concerns for the biologic validity of this approach for future development of a useful diagnostic assay.

**EPCA ELISA.** The anti-EPCA antibody has also been used by investigators in the Getzenberg laboratory in an indirect ELISA assay that showed promise for detecting circulating EPCA antigen in the plasma of CaP versus control patients.[36] An ELISA absorbance cutoff was predetermined with a training set of samples from CaP patients and healthy controls. Using this value of 1.7, EPCA was increased in the plasma of 11 of 12 CaP patients, but was not increased in the plasma of any of 16 non–age-matched normal donors or 7 spinal cord injury patients.[36] EPCA was increased in the plasma of 2 of 6 UC patients, but not in that of 3 other cancer patients (2 colon, 1 renal) or 2 patients with prostatitis.

Although only 2 of 34 non-CaP patients had EPCA ELISA results above cutoff, the fact that 2/6 or 33% of UC patients had elevated levels is of some concern regarding potential specificity. For blood-based tests using antibodies, and especially polyclonal antibodies, it is not only relevant whether other benign or malignant lesions in other organs can express the specific targeted protein, but also whether the antibody can show even minor crossreactivity with other antigens that can be expressed at various levels. The UC patients studied were also closer to the CaP patients in age, such that age could also be a factor to consider in future analysis of test performance.

**EPCA-2 ELISA.** Getzenberg and colleagues at Johns Hopkins have also recently reported data on a serum ELISA test for CaP based on another nuclear antigen, EPCA-2, which is unrelated to EPCA.[33] An indirect ELISA was developed using a polyclonal antibody to EPCA-2.22, 1 of 3 sequenced epitopes of the EPCA-2 protein. They established assay cutoffs in an initial pilot set of 10 men, each with negative PSA, organ-confined CaP, and non–organ-confined CaP. When applied to 50 μL of banked serum samples, none of the samples from patients without evidence of prostate disease or the other controls had EPCA-2 levels above the positive cutoff. However, 8 of 35 (23%) patients with BPH had a serum EPCA-2 greater than the cutoff. Interestingly, in patients with serum PSA less than 2.5 ng/mL and with biopsy-documented CaP, the EPCA-2 ELISA was positive in 14/18 (78%). The EPCA-2 ELISA test was positive in 36/40 men with organ-confined CaP and 39/40 men with non–organ-confined CaP. Further, the EPCA-2.22 assay was able to separate those men with organ-confined CaP versus those with non–organ-confined CaP (EPCA-2.22 [mean ± SD] 33.90 ± 4.18 vs 42.81 ± 6.74, respectively), a difference that persisted after adjustment for Gleason score and PSA.[33] Within the same report, assay modifications for optimizing the EPCA-2 ELISA were described.[31] The assay was run on similar patient groups, in a distinct set of samples (Figure 1).

Figure 1. *Preliminary optimization of EPCA-2 assay. In evaluating additional 55 serum samples, optimized EPCA-2 assay resulted in minimal reactivity in noncancer populations, with increased discrimination from cancer samples. RRP, radical retropubic prostatectomy. Reprinted from* Urology, *Leman ES et al., EPCA-2: a highly specific serum marker for prostate cancer, 714-720, Copyright 2007, with permission from Elsevier.*



In a follow-up study,[38] the indirect ELISA for EPCA-2.22 was used to analyze 189 serum samples, including from 33 healthy men with PSA less than 2.5 ng/mL, 30 healthy men with PSA higher than or equal to 2.5 ng/mL, 35 men with BPH, 33 men with chronic prostatitis/chronic pelvic pain syndrome, 18 men with CaP with PSA lower than 2.5 ng/mL, and 40 men with CaP and PSA higher than 2.5 ng/mL. With the previously established cut-off of 30 ng/mL,[32] the reported sensitivity and specificity for separating men with CaP (regardless of PSA level) from healthy men, men with BPH, or men with prostatitis were 91% and 94%.[38]

*Current Status of Commercial Test Development and Application*

The tissue EPCA IHC test is commercially offered by Onconome (Seattle, WA; http://www.onconome.com/index.php), formerly Tessera, Inc., as an ASR for use by pathology laboratories to assist in evaluation of prostate biopsies in patients being evaluated for possible CaP. It is available as a test at UniPath, LLC (Denver, CO; http://www.unipathllc.com/index.html).

The license for test development based on EPCA-2 has also been transferred to Onconome (ProstaMark® EPCA-2 Serum Assay), which is reportedly optimizing the EPCA-2 serum assay within its own laboratories, including for additional clinical trials and possible FDA approval (http://www.onconome.com/products_prostamark.htm).

A commercial EPCA or EPCA-2 ELISA is not available for use by clinicians at present.

*Future Directions*

The specific antigen recognized by the anti-EPCA antibody has not been fully characterized at the sequence level (e.g., cDNA identified or reported). Of note, IHC staining in normal and malignant prostate glands is cytoplasmic and membranous, which is perhaps unexpected for a supposedly nuclear matrix protein.[31] Studies on biopsy tissues to date have not supported the ready translation to a reliable clinical test for predicting need for repeat biopsy. Any potential clinical application awaits further validation in appropriate patient subsets.

Regarding EPCA and/or EPCA-2 ELISA tests, it is crucial to validate these assays in the actual populations and settings for which a diagnostic test is intended. In this case, if a major goal (as stated by the developing investigators) is to improve on the specificity of serum PSA for making initial or repeat biopsy decisions, then it should be straightforward to study the ELISA test in the actual samples generated when patients have PSA testing done. Large serum banks likely exist from samples collected for PSA, the results for which prostate biopsy was performed, and/or such samples can be generated readily in prospective fashion. It can then be determined whether EPCA or EPCA-2 ELISAs predict prostate biopsy results better than PSA.

It is conceivable that such validation studies are awaiting assay improvements from the laboratory perspective. As suggested by preliminary studies, if any diagnostic applications are ultimately demonstrated for patients with serum PSA less than 2.5 ng/mL,[32] these reference ranges could be different. Similarly, if any prognostic information can be further substantiated for patients with diagnosed CaP regarding preoperative assessment of tumor stage,[32] it would be expected that the reference ranges would be different than for initial diagnostic applications.

A serum- or plasma-based ELISA for EPCA or EPCA-2 is not commercially available at present.

## Prostate Cancer Antigen 3 (PCA3)

*Prostate Cancer Pathobiology*

PCA3 is a prostate-specific gene that is highly upregulated in the vast majority of CaPs. In terms of commercial assay product development, demonstration of reproducible laboratory performance, and substantiated validation in appropriate patient populations for clinical application, PCA3 mRNA testing in urine by TMA is the most established of the biomarkers being considered in this review.

The DD3 gene (differential display gene 3, subsequently renamed PCA3 to reflect its association with CaP) was identified as overexpressed in CaP versus benign prostate by differential display.[39] By Northern blot analysis, DD3 (PCA3) mRNA was upregulated 10- to 100-fold in CaP versus benign in 53/56 RP specimens, with only low or no expression detected in benign prostate or BPH tissue.[39] Using more sensitive RT-PCR, DD3 (PCA3) mRNA was detected in only CaP tissues or tissues of benign prostate or BPH. PCA3 mRNA was not detected in other benign tissues, including normal bladder, seminal vesicles, or testis.[39] PCA3 mRNA was not detected in tumors or tumor cell lines of other tissues, including testis, bladder, or kidney.[39]

The PCA3 gene is located on chromosome 9q21.1, and is composed of four exons. Alternate splicing, particularly involving exon 2, and multiple polyadenylation sites in exon 4 can lead to multiple distinct mRNA transcripts. By qRT-PCR, similarly low levels of PCA3 were detected in benign prostate as well as in BPH tissues. In contrast, there was a median 34-fold increase in PCA versus benign/BPH specimens.[40] This striking level of upregulation in CaP can be

compared to those noted above (ie, ~ 5- to 10-fold) for AMACR mRNA. Initial studies quantitating PCA3 mRNA and correlating with histopathology of examined prostate specimens suggested that PCA3 could be overexpressed in the small number of HGPIN specimens analyzed.[40] Recent detailed ISH studies demonstrated that PCA3 is overexpressed in the vast majority of HGPIN lesions, at least in cases associated with invasive CaP.[41]

The PCA3 gene is highly unusual compared to others considered in this review, including that there is an unusually high number of stop codons, the mRNA does not include any extended open reading frames, and the mRNA is not translated into a protein.[42] The function of PCA3 mRNA in the prostate and the mechanism that increased expression could contribute to CaP development or progression are unknown.[42]

*Towards a Clinical Test*

As PCA3 mRNA is not translated into protein, assay methodologies such as tissue IHC or serum ELISA are not applicable, and mRNA is the only target. The potential diagnostic application of DD3 (PCA3) mRNA quantitation in urine sediments was initially explored using qRT-PCR.[43] mRNA was also quantitated for PSA in order to verify prostate cell recovery and to normalize expression of PCA3. Following prostatic massage, voided urine was collected and total RNA was extracted from urine sediments from 108 patients scheduled for prostate biopsy for PSA higher than 3 ng/mL. Based on correlating mRNA ratios with biopsy results, the area under the ROC curve for DD3 (PCA3)/PSA was 0.72. At the optimal DD3 (PCA3)/PSA $\times 10^{-3}$ cutoff of 200, the sensitivity was 67%, and the specificity was 83%, which represents a substantial improvement over the specificity for serum PSA.[43]

Urine-based testing for PCA3 mRNA with normalization to PSA mRNA was originally offered as a

**Table 3**
**Evolution of Nucleic Acid Amplification–Based Testing for PCA3, Including Current Status of Commercial Availability in the United States**

| Test Type | RNA Source | RNA Extraction | Amplification | Primer Targets | Detection | Clinical Availability in the United States | Manufacturer | Laboratories Offering Testing in United States |
|---|---|---|---|---|---|---|---|---|
| TRF–RT-PCR | Urine sediment | Total RNA (TRIzol) | PCR | For: Exon 1 Rev: Exons 3/4 | Single time point; fluorescence (Eu3+- and Tb3+-labeled probes) | NA | NA | NA |
| uPM3™ | Urine sediment | Boom | NASBA | — | Fluorescence (molecular beacons), 2 h | NA | NA | NA |
| PCA3 | Whole urine | Target capture | TMA | For: Exon 3 Rev: Exon 4 | Single time point; luminescence (hybridization acridinium ester-labeled probes, luminometer) | 2nd generation | Gen-Probe (San Diego, CA) | Bostwick Laboratories; LabCorp/ Dianon (Glen Allen, VA); Molecular Profiling Institute (Phoenix, AZ); Mosaic Diagnostics (Dallas, TX) |

NA, not available; NASBA, nucleic acid sequence–based amplification; PCA3, prostate carcinoma antigen 3; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; TMA, transcription-mediated amplification; TRF, time-resolved fluorescence.

commercially available ASR diagnostic test by DiagnoCure (Quebec City, Canada) as uPM3™. This test involved collection of voided urine following prostatic massage, buffer stabilization of urine RNA, RNA extraction from pelleted urine via Boom extraction,[44] isothermal amplification of PCA3 and PSA based on nucleic acid sequence–based amplification (NASBA),[45] and fluorescence determination of amplification products. Table 3 summarizes different assay approaches employed to date for urine PCA3 mRNA.

Multiple clinical research trials characterized the performance of the uPM3 test. Importantly, these studies were performed in a patient setting for which the test was actually targeted, that is, in patients undergoing initial or repeat prostate biopsies primarily for elevated serum PSA. In a multicenter trial in Canada, uPM3 testing was performed on 443 evaluable urine samples from patients undergoing prostate biopsy. Serum PSA was less than 4.0 in 21%, 4-10 in 55%, and more than 10 in 24%. One hundred fifty (34%) prostate biopsies were positive for CaP.[46] Based on ROC curve analysis for PCA3/PSA mRNA versus prostate biopsy result, the overall sensitivity and specificity of uPM3 for biopsy CaP detection were 67% and 89%.[46] In patients biopsied with serum PSA of less than 4, 4-10, and greater than 10, respectively, sensitivity/specificity of uPM3 were 78%/91%, 58%/91%, and 80%/80%, respectively. A subset of 146 of the 443 patients also had free PSA determined. With a free PSA cutoff of 0.15 or less, sensitivity and specificity of free PSA were 72% and 55%.[46] Although free PSA showed expected greater specificity for CaP detection than total PSA, uPM3 performed even better than free PSA, with improved specificity.[46] These results and those of several other trials comparing urine PCA3 mRNA to biopsy using different methods are summarized in Table 4.

### *Current Status of Commercial Test Development and Application*

The DiagnoCure uPM3 assay was offered in the United States by only one laboratory prior to the transfer of further development and commercialization of PCA3-targeted testing to Gen-Probe (Table 3). The uPM3 assay required

**Table 4**
**Summary of Representative Clinical Trials Showing Prediction of Prostate Biopsy Results of PCA3 Testing on Various Laboratory Formats, Including TMA PCA3 Test Currently Offered in the United States**

| Study | PCA3 Test Type | bx Patients (n) | PSA (ng/mL) Criteria | PCA3 Test Informative Rate | Prostate-bx Positive | PCA3/ PSA mRNA (×1000) Cutoff | PCA3 Test Sensitivity | PCA3 Test Specificity | Reference Number |
|---|---|---|---|---|---|---|---|---|---|
| Hessels et al, 2003 | RT-PCR | 108 | > 3.0 | – | 22% | 200 | 67 | 83 | 43 |
| Van Gils et al, 2007 | RT-PCR | 534 | 3.0-15.0 | 92% | 33% | 58 | 65 | 66 | 80 |
| Tinzl et al, 2004 | uPM3™ | 201 | > 2.5 | 79% | 39% | * | 82 | 76 | 82 |
| Fradet et al, 2004 | uPM3™ | 517 | > 2.5 | 86% | 34% | * | 66 | 89 | 83 |
| Groskopf et al, 2006 | TMA PCA3 | 70 | > 2.5 | 97% | 24% | 50 | 69 | 79 | 48 |
| Marks et al, 2007 | TMA PCA3 | 233 | > 2.5, prior negative bx | 97% | 27% | 35 | 58 | 72 | 49 |

*Qualitative assay; fluorescence detection curve analysis with classification tree indicating probability > 0.5 (range, 0-1.0). bx, biopsy; PCA3, prostate carcinoma antigen 3; PSA prostate-specific antigen; RT-PCR, reverse transcriptase-polymerase chain reaction; TMA, transcription-mediated amplification.

rigorous prostatic massage. In addition, broader application of the NASBA-based uPM3 test was complicated in part by an unacceptable rate of uninformative test results, reflecting the inability to detect even amplifiable PSA mRNA. The uPM3 test was nondiagnostic (insufficient) in as many as 20% or more of clinical samples (Table 4).

The modified Gen-Probe assay was developed using patented technologies, including target capture and TMA (http://www.gen-probe.com/pdfs/tma_whiteppr.pdf).[28] Target capture during sample processing prior to amplification involves use of magnetic particles with oligonucleotides specific for the targeted transcripts (in this case, PCA3 or PSA). In addition to augmented capture of specific RNA molecules, this allows for washing steps without loss of target that may help remove other factors that could interfere with subsequent amplification.[28] Amplified PCA3 and PSA products are detected by chemiluminescence based on hybridization protection using specific acridinium ester-labeled probes. These technologies are well established and already utilized in high-volume laboratory tests produced by Gen-Probe for nonquantitative nucleic acid amplification, including the FDA-approved APTIMA Combo 2® test for the detection of *Neisseria gonorrhoeae* (GC) and *Chlamydia trachomatis* (CT), which can test voided male urine.[47]

Groskopf and colleagues, from Gen-Probe, reported on a prototype PCA3 molecular urine test for quantitating PCA3 mRNA, normalized to PSA mRNA.[48] Of note, the assay reagents described have been incorporated by multiple laboratories now offering PCA3 testing as an independent laboratory–validated ASR test that is available for clinical use (Table 3). Urine was collected for PCA3 testing following a DRE performed as three strokes per prostate lobe, with pressure firm enough to depress the prostate surface about 1.0 cm being applied from base to apex and from lateral to median line. First-catch post-DRE urine (20-30 mL) was collected and kept on ice and processed within 4 hours by mixing equal volume of urine (eg, 2 mL) with a detergent-based RNA stabilization buffer (Urine Transport Medium [UTM], the same formulation commercially offered as the APTIMA® Urine Specimen Collection Kit for Male and Female Urine Specimens) prior to storage until analysis. PCA3 and PSA mRNAs were analyzed separately from the same samples. Each assay utilizes just 400 μL of the urine/stabilization buffer mix (equivalent to 200 μL of whole urine). Calibrators of known amounts of PCA3 and PSA mRNA analyzed at the same time were used to create standard curves. PCA3 mRNA copy numbers in urine were 20- to 30-fold lower than those for PSA. Hence, the PCA3 score was calculated as PCA3 mRNA/PSA mRNA $\times$ 1000.[48]

In this initial report of the Gen-Probe PCA3 test, PCA3/PSA ratios were determined in 3 groups of patients: 70 men scheduled for prostate biopsy for serum PSA 2.5 ng/mL or higher and/or abnormal DRE (mean PSA, 7.7 ng/mL), 52 healthy men aged 45 years or less without elevated PSA and no CaP risk factors, and 21 men who were post-RP for CaP. The PCA3 test was informative in 98%.[48] The median ratios of PCA3/PSA $\times$ $10^{-3}$ for the healthy men, biopsy-negative men, and biopsy-positive men were 4.5, 27.0, and 81.8, respectively. On ROC curve analysis of prebiopsy PCA3 score versus biopsy result, the area under the curve was 0.746.[48] With a data-defined optimal cutoff of a PCA3 mRNA/PSA mRNA $\times$ $10^{-3}$ of 50, the sensitivity for CaP on biopsy was 69% and the specificity was 79%.[48] For 44 patients specifically in the PSA "gray zone" (PSA levels 2.5-10 ng/mL), the sensitivity was 69% and specificity was 83%.[48] Of 21 postprostatectomy samples, 20 had PCA3 and PSA mRNA signals at or near background levels (ie, undetectable). One specimen yielded a PCA3/PSA $\times$ $10^{-3}$ ratio score of 55. This patient had biopsy-documented CaP recurrence.[48]

Subsequent studies have confirmed the performance of the Gen-Probe PCA3 assay in clinically relevant prostate patient biopsy settings, including the high test informative rate and the marked improvement in specificity compared to PSA. Marks and colleagues recently reported on the application of urine PCA3 mRNA testing to patients with persistently elevated PSA ($>$ 2.5 ng/mL) and at least 1 prior negative prostate biopsy.[49] In 233 men enrolled at three different North American institutions, the PCA3 test informative rate was 97%.[49] For the PCA3 score, the area under the ROC curve was 0.678 compared to only 0.524 for serum PSA. A PCA3 score cutoff of 35 achieved an optimal combination of sensitivity and specificity. With 35 as a cutoff, the sensitivity for CaP diagnosis in the repeat biopsy was 58% and the specificity was 72%.[49] Importantly, the risk of a positive biopsy increased in a continuous fashion with increasing PCA3 score ranges.[49] Patients with a PCA3 score lower than 5 had CaP on biopsy in only 12%, whereas in patients with PCA3 scores higher than 100, the risk of a positive biopsy was 50% (Figure 2).

**Urine PCA3 score is independent of prostate volume.** One well-recognized problem with the application of serum PSA for CaP screening is the relationship of total serum PSA to prostate volume.[1,2] In contrast, preliminary results indicate that the

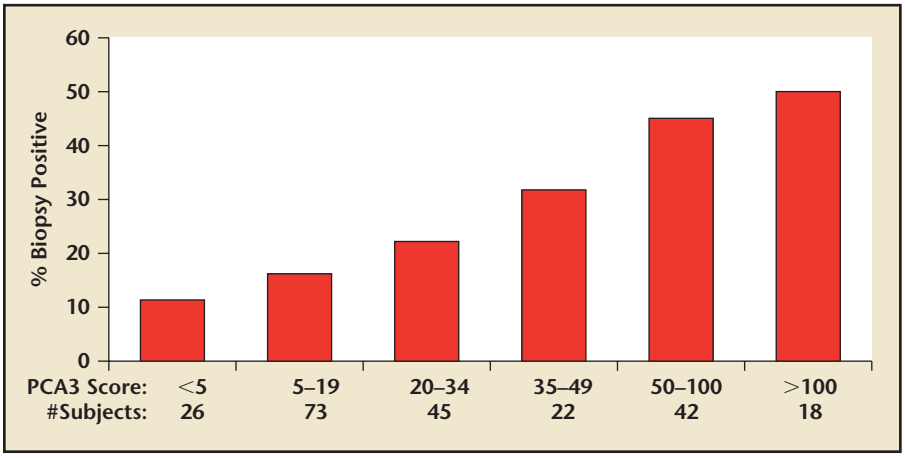



Figure 2. *Probabilities of positive biopsy findings at different PCA3 score ranges. Number of subjects in each range shown at bottom. Reprinted with permission from* Urology, *Volume 69, Marks LS et al., PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy, pp. 532–525, Copyright Elsevier 2007.*

urine PCA3 score is not related to prostate volume.[50] In 529 men scheduled for prostate biopsy, urine PCA3 score was correlated with prostate volume determined by transrectal ultrasound at the time of biopsy. Prostate volume was divided into three categories: less than 30 cc, 30-50 cc, and greater than 50 cc. In contrast to serum PSA, PCA3 scores did not increase with prostate volume. The mean PCA3 scores for the three groups were 45, 38, and 43, respectively. These encouraging results suggest that age- and volume-related effects that complicate application of serum PSA in CaP screening, particularly affecting specificity of mild PSA elevations, will not similarly be encountered with PCA3 testing.

**Urine PCA3 score may synergize with other clinical parameters in prediction of biopsy outcome.** Groskopf and colleagues showed that the risk of a positive biopsy was 14% in patients with a PCA3 score lower than 5, but increased to 69% in patients with a PCA3 score of higher than 100.[50] Logistic regression (LR) and artificial neural network (ANN) approaches incorporating PCA3, age, prostate volume, and serum PSA showed that the area under the ROC curve improved from 0.734 with PCA3 alone to 0.774 and 0.783 for LR and ANN, respectively, when other patient parameters were included.[50]

**Laboratory issues of current PCA3 testing.** *Testing laboratories and sample processing.* PCA3 urine mRNA testing with the Gen-Probe (http://www.gen-probe.com) reagents as described herein is available for routine clinical use (ie, not just for research purposes) as an ASR test offered by several laboratories in the United States (Table 3). The urine PCA3 test is also offered for clinical use in Europe. PROGENSA™ PCA3 is available as a CE-marked product in Europe (http://www.PCA3.org).

First-catch urine should be collected after an attentive DRE,[48] and the specimen should either immediately have 2 mL transferred into APTIMA UTM urine transport tubes or be placed on ice for up to 4 hours. After pipetting of 2 mL into at least 1 urine transport tube, the fixed specimen containing tubes should be stored at −20°F (routine freezer) until shipment to the testing laboratory with a cold pack.

*Test performance.* PCA3 samples collected from urologists are informative in approximately 98% of specimens (S. B. Shappell, unpublished observations). Although based on sophisticated technologies and instrumentation, this test is robust from the laboratory perspective. In addition to good intra-assay and interassay agreement within an individual laboratory,[48] high interlaboratory concordance has been noted as well, with about 95% concordance when negative and positive results are based on similar reference ranges using cutoffs as reported[49] and high correlation when PCA3 score is regarded as a continuous variable (S. B. Shappell and B. Wright, unpublished observations).

Preliminary results suggest that PCA3 values are stable in individual patients over at least short time periods. Partin and colleagues reported on urine PCA3 scores on weekly samples over a 4-week time frame for 25 patients participating in an expectant management program following a biopsy diagnosis of CaP.[51] The mean and median percentage coefficient of variation (CV) for the tests over the 4-week period were about 20%.[51] These CVs are quite good for quantitative mRNA assays and not markedly different than those noted for intra- and interassay CV for the same sample. These results suggest that there is little biologic variation in urine PCA3 scores over these relatively short periods. Additional results will be needed to further these observations, including in non–CaP-diagnosed patients.

*Current indications.* Perhaps the most obvious indication based on the validation studies performed to date is facilitating repeat biopsy decision in patients with persistently elevated serum PSA (ie, 2.5-10 ng/mL) and a negative prior biopsy.

Particularly given the rates of false-negative first prostate biopsies performed for elevated PSA, one mode of application of this PCA3 testing approach is to collect a urine sample for possible PCA3 testing at the time of

prostate biopsy for "reflex" PCA3. If the biopsy is negative, urine PCA3 testing can be performed. If the PCA3 test is positive, it supports that CaP is present and was missed on biopsy due to sampling issues. The patient may warrant a rapidly scheduled repeat biopsy (eg, at 3-4 weeks, somewhat analogous to the detection of an atypical small acinar proliferation [ASAP], suspicious but not diagnostic for CaP on initial biopsy).[52] If the PCA3 test is negative, it supports that the mild elevations of PSA may be due to prostate enlargement (including BPH) or prostatitis. As no test has a 100% negative predictive value, a negative PCA3 test in this setting does not exclude CaP, but such patients are less likely to harbor CaP and could be followed conservatively, depending in part, of course, on other clinical and laboratory results, such as PSA velocity.[1,2]

Similarly, in patients with elevated PSA who have had past negative biopsies and in whom there is uncertainty regarding management, including possible repeat biopsy, PCA3 testing (ie, collected independently of biopsy) may be useful. As with the above scenario, an elevated PCA3 supports the presence of CaP and the need for biopsy. Our own experience with biopsies performed shortly after a positive PCA3 test in patients with prior negative biopsies supports this approach (S. B. Shappell, unpublished observations). A negative PCA3 test may favor non-neoplastic origins for mild PSA elevations, argues against the need for current repeat biopsy, and supports ongoing conservative follow-up. Again, patients should be managed based on the results of all available clinical and laboratory data.

We report PCA3 test results as negative or positive based on the PCA3 score of 35 for optimal sensitivity and specificity established by the multicenter trial reported by Marks and colleagues.[49] Ongoing correlation with biopsies performed at the time of PCA3 sample collection or following a PCA3 test allows for additional prospective validation. However, our report includes an explanation of what results mean, including reference to the fact that risk for CaP on biopsy increases with PCA3 score, with reference to published risk results (Figure 2).[49] Urologists should communicate with the laboratory that they use for PCA3 testing as to how the test is performed, how it is validated, how it is reported, what the results mean, and what the basis for such claims are.

*Future Directions*

As expected, urine PCA3 scores do not correlate with serum PSA[49] (S. B. Shappell, unpublished observations). As PCA3 is much more of a cancer-specific gene and biomarker than PSA, it will be of clinical interest to see if PCA3 testing can also be used to detect clinically significant CaP in patients without elevated serum PSA (eg, in patients with PSA $<$ 2.5 ng/mL). Studies published to date correlating prebiopsy PCA3 scores with biopsy results have been with patients scheduled for biopsy because of elevated PSA (or abnormal DRE). Application of PCA3 for detection of CaP in patients without elevated PSA will require dedicated clinical trials, including those in which patients undergo biopsy regardless of serum PSA, either because of a positive PCA3 or regardless of PCA3 score to allow correlation with all ranges of PCA3 results.

*As prostate cancer antigen 3 (PCA3) is much more of a cancer-specific gene and biomarker than prostate-specific antigen (PSA), it will be of clinical interest to see if PCA3 testing can also be used to detect clinically significant CaP in patients without elevated serum PSA (eg, in patients with PSA $<$ 2.5 ng/mL).*

The normalization of PCA3 mRNA to that of PSA mRNA raises some concerns regarding possible effects on PCA3 scores of antiandrogen therapy, such as 5-alpha-reductase inhibitors used for BPH or even male pattern baldness. In androgen-responsive cell lines, PSA mRNA is markedly downregulated by antiandrogens, and effects on serum PSA of such agents are well known, necessitating correction factors for use of serum PSA in antiandrogen-treated patients.[18] The potential regulation of PCA3 transcription by androgens[42] and effects of antiandrogens on urine mRNA levels of PCA3 versus PSA are not known. If antiandrogen therapy lowers PSA levels (the denominator in determining PCA3 scores) more than PCA3 levels, it could result in false-positive results. Ongoing trials are addressing the possible effects of antiandrogen treatment on PCA3 scores and whether altered reference ranges would be required for patients being treated with antiandrogens.

In addition to its emerging role in facilitating CaP diagnosis and biopsy decisions, as elevations in PCA3 are more specific for CaP than those of PSA, PCA3 testing could also be useful in monitoring patients following therapy, such as radiation treatment or radical prostatectomy. In such patients, minor elevations of PSA could also reflect contributions of residual benign prostate, whereas this would not be expected for PCA3.

Finally, a recent study showed that PCA3 scores correlated with tumor Gleason score, but inversely with tumor volume, in totally submitted RP specimens, such that values may be established that correlate with clinically insignificant CaP (ie, organ

confined; < 0.5 cc; ≤ Gleason 6).[53] These results support the possible application of urine PCA3 scores for prognostication and treatment decisions in patients with biopsy-diagnosed CaP. If future studies support that PCA3 can be used, either alone or in combination with other clinical and laboratory data, to adequately predict clinically insignificant CaP, PCA3 testing (or perhaps serial PCA3 testing) could be incorporated into watchful waiting protocols for patients with CaP.

## Promoter Hypermethylation of GSTP1 and Other Genes in CaP

*Prostate Cancer Pathobiology*

Promoter methylation has been extensively studied in CaP. A large number of genes have been identified as hypermethylated in the majority of CaPs, including genes affecting cell growth and proliferation, apoptosis, cell adhesion, steroid hormone receptors, inflammation, and carcinogen metabolism.[54]

DNA methylation is a covalent modification through the bonding of a methyl group to cystosine in CpG dinucleotides, catalyzed by DNA methyltransferases, and reversed by demethylases or potentially by drugs, such as 5-azacytidine.[54] Some CpG dinucleotides occur in so-called CpG islands, 200-2000 base pair (bp) lengths of DNA with more than 50% GC content, which occur in the 5′ region, including the promoter, of approximately half of human genes.[54] These promoter CpG islands are typically unmethylated, which allows gene expression. Promoter methylation is an epigenetic change that can result in reduced gene transcription, which in the case of putative tumor suppressor genes, can contribute to carcinogenesis.[54]

Gene promoter methylation can be detected by methylation-specific PCR of extracted DNA,[54] amenable to quantitation, using so-called quantitative methylation-specific PCR (QMSP).[55] GSTP1, the gene for glutathione S-transferase-pi, which functions in the metabolic detoxification of potentially carcinogenic reactive oxygen metabolites, is the most extensively characterized gene that is methylated in CaP.[54] GSTP1 promoter methylation is accompanied by the loss of GSTP1 protein and is observed in 75% to 100% of CaPs, as well as in approximately 70% of HGPIN lesions, suggesting that it is an early event in prostate carcinogenesis.[54] GSTP1 promoter methylation is not present in benign prostate epithelium, but was detected in 6% of PIA lesions.[56] Other genes that have been reported as having promoter methylation in the majority of CaPs in multiple studies or which have otherwise been studied as potential targets for diagnostic CaP assays include APC, RARbeta2, RASSF1A, p16INK4a, MGMT, p14ARF, EDNRB, CDH1, and TIMP3.[54,57]

*Towards a Clinical Test*

**Tissue-based testing.** Tissue-based approaches for gene promoter methylation analysis for CaP diagnosis could include testing DNA from biopsy paraffin blocks already processed for routine histology and negative for CaP, in order to identify patients who may harbor an unsampled CaP (ie, still in their prostate) and thus warrant timely repeat prostate biopsy. Application of molecular analysis to benign glands sampled in negative biopsies in order to detect CaP predicates on genetic changes in benign glands that precede morphological changes and that would be a marker for fully developed, pathologically diagnosable CaP elsewhere in the organ, a so-called field effect, as has been alluded to above in discussion on AMACR and EPCA.

In paraffin tissues from 37 RPs, ex vivo core tissue samples were obtained from the CaP and serial 1-mm distances of benign prostate. Methylation ratios to the housekeeping gene beta-actin were determined by fluorogenic multiplex QMSP assays for GSTP1, APC, RARbeta2, and RASSF1A.[55] In initial studies on 51 pairs of malignant versus benign prostate tissues, 62% of CaP versus only 2% benign tissues showed GSTP1 methylation. For APC, RARbeta2, and RASSF1A, the percentages of CaP versus benign specimens showing promoter methylation were 69% versus 0%, 58% versus 0%, and 58% versus 11%, respectively.[55] In the analyses on the spatial magnitude of any field effect, only 4 of the 37 CaP cases showed promoter methylation in any of the benign samples serially spaced up to 4 mm away from adjacent positive CaP cores, including 0, 1, 3, and 2 being positive for GSTP1, APC, RARbeta2, and RASSF1A promoter methylation, respectively. Only 3 of 37 cases had promoter methylation in benign glands 2 mm or further away from the CaP focus.[55] Hence, the spatial separation of the affected benign glands from CaP is so small and the frequency of these changes is so sufficiently low that it can be anticipated that detection of promoter methylation in sampled benign glands on negative biopsies will have inadequate sensitivity for clinical utility in predicting repeat biopsy outcomes in patients with mild elevations of PSA and negative prostate biopsies.

**Urine-based testing.** In contrast, urine-based testing should allow for more extensive prostate sampling. No single gene is promoter methylated uniformly in all CaPs, even in tissue, and issues related to CaP cells gaining access to the excretory ducts and urine could reduce sensitivity for urine-based tests. Sensitivity for CaP diagnosis based on gene promoter methylation may be expected to be

greater for analysis of combinations of genes. Further, promoter methylation has been noted for some candidate genes at varying frequencies in benign prostate and/or BPH.[54] Specificity may also be expected to be increased by including multiple genes in the analysis, and particularly by using quantitative techniques (ie, QMSP) that should allow for optimization of thresholds for CaP versus benign prostate.

Illustrating some of these points, in a study using conventional (nonquantitative) methylation-specific PCR, GSTP1 promoter methylation was detected in only 27% of urine samples from patients with GSTP1 methylation in the corresponding CaP tumor tissue.[58] In a subsequent study using QMSP for 9 gene promoters in testing urine sediments from 52 patients with CaP, 21 matched CaP tumor tissues, and 91 age-matched controls, investigators in Sidransky's laboratory at Johns Hopkins found GSTP1 was methylated in 48% of the urine samples.[57] GSTP1 promoter methylation was detected in the urine of only 8 of 19 (42%) of the patients with GSTP1 promoter methylation in the primary tumor. Likewise, urine promoter methylation was noted in 9 of 20, 7 of 17, 2 of 8, and 3 of 7 samples from patients whose tumors had promoter methylation for RARbeta2, p16, MGMT, and ARF, respectively. As expected, sensitivity of urine testing was markedly improved when considering panels from the 9 genes analyzed using QMSP. Promoter methylation of at least 1 of the genes was detected in the urine sediments of all 52 (100%) of the CaP patients. Methylation-positive urine samples from CaP patients ranged from 19% for MGMT to 77% in CDH1, and 42/52 (81%) of the CaP urine samples were positive for at least 3 genes. Of the 91 age-matched controls, which included 66 males and several patients with potentially confounding prostate or bladder conditions, promoter methylation in urine by QMSP was seen in 4% to 11% for APC, CDH1, RARbeta2, TIMP3, and RASSF1A. Of note, most of the controls with promoter methylation of urine samples were patients with BPH. Importantly, based on analysis of a combination of only 4 genes (p16, ARF, MGMT, and GSTP1), with a positive result being promoter methylation of at least 1 of the 4, sensitivity and specificity for CaP detection by urine testing would have been 87% and 100%, respectively.[57] These results demonstrate the strong potential for urine-based promoter methylation analysis of a combination of genes for CaP diagnosis.

*Current Status of Commercial Test Development and Application*

OncoMethylome Sciences (http://www.oncomethylome.com) owns proprietary technology and holds patents related to the development of promoter methylation–based tests for CaP applications. In January 2005, OncoMethylome Sciences licensed genes that could be utilized in a tissue- or urine-based test to Veridex LLC (Warren, NJ), a Johnson & Johnson company.

Data have recently been presented characterizing the performance of both tissue- and urine-based assays for CaP detection, with intention towards utilization in certain prostate patient subsets. In May 2007, Veridex granted a license to LabCorp® (Burlington, NC), which permits LabCorp to offer commercial testing using tissue-based promoter methylation analysis. Urine-based testing has not been licensed to any commercial laboratories in the United States.

**Tissue-based promoter methylation assay.** OncoMethylome Sciences initially developed simplex assays, in which quantitative methylation-specific polymerase chain reaction (QMSP) is performed for individual genes in separate assay mixtures, with the ABI 7900 as the readout platform. More recently, Veridex constructed a multiplexed, Scorpion™-based assay for QMSP on the Cepheid SmartCycler® II real-time instrument.[55]

With the simplex assays for QMSP for GSTP1, APC, and RARbeta2 normalized to beta-actin on 142 formalin-fixed, paraffin-embedded RP tissues and CaP-negative biopsies, sensitivity for CaP was 88%, 55%, and 54% for GSTP1, APC, and RARbeta2, respectively. Specificity was 97%, 99%, and 100% for GSTP1, APC, and RARbeta2 promoter methylation, respectively.[59]

To determine if promoter methylation in benign prostate biopsies could predict CaP on repeat biopsy, these tests were applied to 85 negative initial biopsies from patients who had CaP on repeat biopsy. The sensitivity for predicting CaP on repeat biopsy was 32%, 13%, and 14%, for the GSTP1, APC, and RARbeta2 promoter methylation assays, respectively.[59] As testing was not reported on cases with negative repeat biopsy, the potential for false-positive results based on cutoffs utilized is not known.

In a multiplex assay, in which GSTP1 and APC promoter methylation are analyzed simultaneously, results obtained with cancer and BPH specimens were used to set assay cutoffs to yield the highest specificity. DNA from negative initial biopsies was analyzed from 68 patients for which a second biopsy was positive. This included 39 patients with "suspicious cells" on the first negative biopsy. Twenty of these 68 patients were found to be positive for methylation of GSTP1 and/or APC in the first biopsy, for a sensitivity of 29% for detection of CaP on second biopsy.[59] As ASAP (as biopsies with

"suspicious cells" may be regarded) on an initial biopsy predicts CaP on repeat biopsy in about 35% to 45% of specimens,[52] it would be of interest to know how the promoter methylation assay performed in patients with only benign glands on initial biopsy versus those with suspicious cells. The specificity for a negative multiplex promoter methylation test for 84 negative biopsies was 94%.[59]

These sensitivities for a tissue-based approach for predicting CaP on repeat biopsy are relatively low. In theory, anywhere from 68% to 87% (based on results of single gene assays) and 71% (based on results of the multiplex assay) of the CaPs would have been missed if repeat biopsy decisions had been based only on the promoter methylation assay results. It may be expected that these tests would be specific. If such is the case, in patients with an elevated PSA and a negative biopsy, a positive tissue promoter methylation test may strongly indicate the need for repeat biopsy, but a negative test would essentially provide no useful information as a high percentage of such patients could still have unsampled CaP. This is likely a limitation of the biology, as described in the discussion of the field effect in surgical specimens above. In contrast, urine may be expected to allow for improved sampling, not only of benign glands that may be closer to undetected CaP glands, but also of actual cancer cells in foci of CaP that were missed on biopsy.

**Urine-based promoter methylation assay.** Investigators recently characterized the performance of a urine-based assay using QMSP technology. First morning, post-DRE, and postbiopsy urine samples were collected (for sample type comparisons), and following preparation of cell pellets, DNA was extracted and subjected to QMSP for a panel of genes. In the first sample set, 114 men undergoing prostate biopsy for elevated PSA (4-10 ng/mL) were analyzed. Fifty-one percent of prostate biopsies were positive for CaP. Based on results for promoter methylation of GSTP1, p14, p16, RARbeta2, and RASSF1A single PCR reactions, the postattentive DRE urine samples were superior, and demonstrated a sensitivity and specificity for CaP of 74% and 75%, respectively.[60]

In a second sample set of 52 cases from patients with PSA levels of 2.5 to 4 ng/mL, for which 48% of prostate biopsies were positive for CaP, post-DRE urine samples subjected to multiplex QMSP for GSTP1, RARbeta2, and APC showed a sensitivity of 58% and a specificity of 88%.[60]

Although the positive biopsy rates for the respective PSA ranges may appear a little high in these studies, the results for the urine-based tests are extremely encouraging. The high specificity of this approach compared to that of serum PSA suggests that this assay could potentially reduce the number of unnecessary initial or repeat biopsies in patients with persistently elevated PSA.

*Future Directions*

A prospective clinical trial is anticipated to determine if gene methylation markers can improve the negative predictive value over histopathology alone in high-risk patients, described as those with PSA higher than 8.0 ng/mL or abnormal DRE, or HGPIN or ASAP on negative initial biopsy.[59] As this proposed trial does not include most patients with mildly elevated PSA and negative initial biopsy, it will not be able to demonstrate potential utility in these patients. However, it may provide definitive conclusions for the patient subsets targeted.

There is a need for prospective trials for the encouraging urine-based assay, particularly testing of patients with elevated PSA scheduled for prostate biopsy, including those with prior negative biopsy.

*The high specificity of urine-based testing compared to that of serum PSA suggests that this assay could potentially reduce the number of unnecessary initial or repeat biopsies in patients with persistently elevated PSA.*

## TMPRSS2:ERG Gene Fusions

*Prostate Cancer Pathobiology*

**Discovery and characterization of TMPRSS2 gene fusions with ETS transcription factors in CaP.** Extensive data have emerged over the last few years to indicate that approximately 50% to 60% of clinically detected CaPs harbor gene rearrangements in which the 5′ region of the androgen-regulated TMPRSS2 gene (located at chromosome 21q22.3) is fused with one of multiple genes belonging to the ETS family of transcription factors, most commonly ERG (also located on the long arm of chromosome 21 at 21q22.2), as well as occasionally ETV1 (7q21.2) and ETV4 (17q21).[61-63] TMPRSS2 is an androgen-regulated transmembrane serine protease that is expressed in normal prostate epithelium, with increased expression reported in CaP.[64] ERG is a member of the ETS family of transcription factors, which contribute to the regulation of expression of genes that could be involved in carcinogenesis or tumor progression, and which are known to be involved in oncogenic transformations in Ewing's sarcoma and myeloid leukemias.[65] These gene fusions presumably result in the increased expression of ETS transcription factors under the control of the androgen-response elements present in the 5′ region of TMPRSS2.[61]

Recurrent chromosomal rearrangements are common in hematopoietic malignancies. In addition to providing diagnostic and prognostic information, diagnosis by FISH or PCR of specific genetic changes can also confirm the presence of a target for molecularly targeted therapy. The first and best known example is the treatment of patients with chronic myelocytic leukemia (CML) with imatinib, which targets the bcr-abl kinase that results as a consequence of the t(9;22)(q34;q11.2) or Philadelphia chromosome.[66] Investigators in the laboratories of Arul Chinnaiyan (University of Michigan Medical School, Ann Arbor, MI) and Mark Rubin (Harvard Medical School, Boston, MA) postulated that gene expression data in CaP could harbor clues to gene rearrangements that could similarly drive marked overexpression of oncogenes in CaP.

Using a novel bioinformatics approach termed *cancer outlier profile analysis* (COPA), Tomlins and colleagues reported recurrent gene fusions of the 5′ untranslated region of TMPRSS2 to ERG or ETV1 in CaP tissues.[62] In the CaP cell line MET28-LN, molecular biology techniques identified a fusion of the complete exon 1 of TMPRSS2 with the beginning of exon 4 of ERG (referred to as TMPRSS2:ERGa). In 42 cases of clinically localized CaP chosen based on overexpression of ERG or ETV1, quantitative PCR demonstrated that TMPRSS2:ERG and TMPRSS2:ETV1 fusions were found only in CaP cases that overexpressed ERG or ETV1, respectively. These results were extended to 29 CaP cases selected independently of ERG or ETV1 expression using tissue microarrays and FISH. Because of the proximity of TMPRSS2 and ERG on chromosome 21 (complicating use of fusion probes that can indicate when two different genes are brought together), a break-apart probe strategy was utilized in which probes spanning the 5′ and 3′ region of ERG could detect when an ERG rearrangement occurred, compatible with a TMPRSS2:ERG fusion (Figure 3). In contrast, fusion probes were used to detect translocation between TMPRSS2 and ETV1. Of 29 CaP cases, 23 (79%) showed evidence of either an ERG rearrangement, consistent with TMPRSS2:ERG fusion (16/23), or a TMPRSS2:ETV1 fusion (7/23).[61] Overall, in three independent microarray data sets, ERG or ETV1 was markedly overexpressed in 57% of 165 CaP cases.[61]

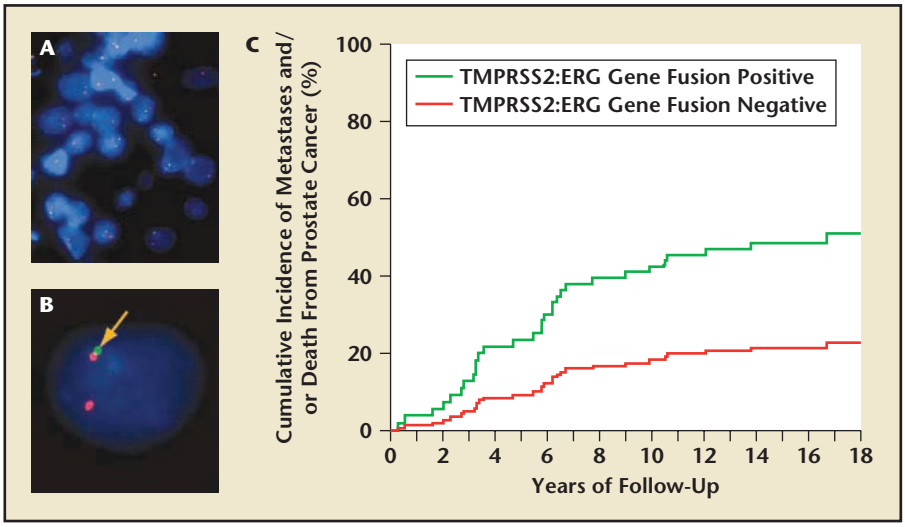



**Figure 3.** *Low magnification* **(A)** *with ~25 nuclei and high magnification* **(B)** *of single nucleus showing fluorescence in situ hybridization (FISH) assay with breakapart probes to detect ERG rearrangement (consistent with TMPRSS2:ERG fusion) in prostate carcinoma (CaP). Normal nuclei (without ERG gene rearrangement) have two signals composed of closely spaced (juxtaposed) red and green signals (from probes flanking either side of ERG), which often give a yellow "fusion" signal* **(arrow, B)**. *In cancer cells from tumor with ERG rearrangement* **(B)**, *one red-green fusion signal is lost due to breakapart of the probes. In this case, a single isolated red signal is seen (bottom of nucleus). The green probe signal is lost, reflecting deletion of the intervening DNA between TMPRSS2 and ERG (gene fusion by deletion of intervening DNA between the two genes on chromosome 21; see text).* **(C)** *Cumulative incidence of metastases and/or CaP-related death in patients having CaP with or without ERG fusions, followed by watchful waiting (difference was significant; see text and reference). Reprinted by permission from Macmillan Publishers Ltd:* Oncogene, *Demichelis et al,*[72] *copyright 2007.*

Subsequently, TMPRSS2 fusions with a third ETS family member, ETV4, were demonstrated in CaP in conjunction with observations that ETV4 was overexpressed in 2 of 98 cases.[62]

**Frequency of TMPRSS2:ETS family member gene fusions in clinically localized CaP.** The frequency of TMPRSS2:ERG, TMPRSS2:ETV1, and TMPRSS2:ETV4 fusions and potential clinicopathologic correlations of the most common ERG fusions have been systematically investigated in CaP. Using break-apart FISH probes on tissue microarrays, Perner and colleagues observed ERG rearrangements consistent with TMPRSS2:ERG fusions in 115/237 (48.5%) of clinically localized high-risk CaP cases.[67] TMPRSS2:ERG fusions can occur through deletion of intrachromosomal intervening DNA between the two genes on the same chromosome or by translocation between the two different chromosomes 21.[68] Based on FISH results in this series, 71 of the 115 ERG fusion cases appeared to arise via deletion, whereas 44/115 showed TMPRSS2:ERG fusion through translocation, which may have prognostic implications[68] (Table 5).

TMPRSS2:ERG fusions were detected in 5/26 (19%) of HGPIN foci studied. Positive HGPIN foci in close association with invasive CaP showed the same ERG fusion as the

**Table 5**
**Prognostic Significance of TMPRSS2:ERG Gene Fusions in Prostate Carcinoma**

| Patient Population and Sample Description | Targets and Methodology | Nonsignificant Prognostic Findings | Significant Prognostic Findings | Reference Number |
|---|---|---|---|---|
| RP cases: 118 patients, clinically localized CaP, partially PSA-screened high-risk European cohort (44% Gleason score 7, 50% Gleason score > 7; 78% pT3; 72% positive SM; 56% pelvic LN mets; 51% PSA recurrence) | FISH (paraffin) for ERG rearrangement as indication of TMPRSS2:ERG fusion | No correlation with grade (but very few Gleason score ≤ 6) | TMPRSS2:ERG rearrangement through deletion (see text) associated with higher pT stage and pelvic LN mets, trend for higher PSA recurrence vs CaPs without any ERG fusion | 68 |
| RP cases: 96 patients, clinically localized CaP; 67% pT2, 19% pT3a, 5% pT3b; 35% PSA recurrence | FISH (paraffin) for TMPRSS2, ERG, ETV4 rearrangements; TMPRSS2:ETV1/ETV4 fusions | TMPRSS2 and/or ERG rearrangements not associated with PSA recurrence | TMPRSS2 rearrangements associated with high pathologic stage | 63 |
| RP cases: 59 patients, clinically localized CaP; 18 early (< 1 year), 16 late (1-5 years) PSA recurrences; 20 nonrecurrent (5 years) | RT-PCR for different mRNA splice variants (isoforms) of TMPRSS2:ERG fusion (see text) | See text/reference | Expression of TMPRSS2:ERG fusion mRNAs with native translation initiation codons in frame with ERG associated with aggressive CaP (see text) | 69 |
| Laser capture microdissection of CaP vs benign prostates in 114 patients | ERG mRNA by quantitative RT-PCR | See text/reference | High ERG expression associated with lower risk of PSA recurrence, lower tumor grade, lower pT stage, negative SM status | 71 |
| TURP cases: 111 patients; watchful waiting study | FISH (paraffin) for ERG rearrangement as indication of TMPRSS2:ERG fusion | See text/reference | TMPRSS2:ERG fusion associated with CaP metastasis and/or CaP specific death (when adjusted for Gleason score, no longer significant) | 72 |
| RP cases: 106 evaluable; clinically localized CaP; < 5% clinical T3; ~16% PSA recurrence | FISH (paraffin) for TMPRSS2 rearrangement, TMPRSS2:ERG fusions (TMPRSS2:ETV1, ETV4, or FLI1 fusions if ERG negative) | No correlation with stage or PSA recurrence | ERG rearrangements associated with grade; positive in ~ 7% of well differentiated (Gleason pattern 2) vs ~ 40% of moderately and poorly differentiated (Gleason patterns 3, 4, 5) CaP | 77 |

CaP, prostate carcinoma; FISH, fluorescence in situ hybridization; LN, lymph node; PSA, prostate-specific antigen; RP, radical prostatectomy; RTR-PCR, reverse transcriptase-polymerase chain reaction; SM, surgical margins; TURP, transurethral resection of prostate.

corresponding invasive CaP.[67] Of note, 0 of 15 BPH samples, 0 of 38 atrophy/PIA samples, and 0 of 47 benign prostate samples showed ERG rearrangements.[67]

Using break-apart FISH probes, Mehra and colleagues at the University of Michigan found TMPRSS2 rearrangements in 37/57 (65%) of evaluable cases, ERG rearrangements in 36/65 (55%) of cases, ETV1 rearrangements in only 1/53 (2%) of cases, and ETV4 rearrangements in only 1/58 (2%) of cases. Rearrangements in both TMPRSS2 and ERG, indirectly supporting TMPRSS2:ERG fusions, were found in 30/56 (54%) of cases.[63] A TMPRSS2:ETV1 fusion was found in only 1/53 (2%) of cases, and no cases had TMPRSS2:ETV4 fusions.[63] Based on FISH results compatible with

intronic loss of DNA between the two genes, approximately 40% of the TMPRSS2:ERG fusion cases arose by intrachromosomal deletion.[63]

**Potential prognostic implications of TMPRSS2:ERG fusions, including alternately spliced variants.** Ittmann's laboratory at Baylor College of Medicine (Houston, TX), analyzed the expression of TMPRSS2:ERG fusion mRNAs by RT-PCR in RP tissue samples of 59 nonrandom patients with clinically localized CaP.[69] Fifty-nine percent of the CaPs expressed the TMPRSS2:ERG fusion gene. However, there was prominent variation among tumors in the expression of different alternately spliced isoforms. Expression of an isoform in which the native ATG of exon 2 of the TMPRSS2 gene was in frame with exon 4 of ERG was found in 26% of ERG fusion-expressing CaPs and was significantly associated with early PSA recurrence and seminal vesicle invasion. Expression of two different isoforms (with and without exon 2 of ERG) in which the native ERG ATG in exon 3 was the first in frame ATG was found in 20% and 11% of ERG rearranged CaPs, respectively, and were both associated with seminal vesical invasion.[69]

The association of specific isoforms with adverse tumor pathology parameters/patient outcome may be related to their translation into higher levels of ERG protein from the native ATG translation initiation codons.[69] Increased ERG expression could subsequently result in higher levels of ETS target genes, which could function in a variety of ways to contribute to CaP progression.[70] However, not all studies have found that increased ERG expression is correlated with adverse outcome in CaP.[71] Potential prognostic significance of ERG fusions is shown in Table 5.

Demichelis and colleagues recently reported on the prognostic significance of TMPRSS2:ERG fusions in a population-based cohort of men with localized CaP followed by watchful waiting in Orebro, Sweden.[72] In contrast to the surgical-based series, in which the frequency of ERG rearrangements in CaPs is about 50%, based on FISH analysis on tissue microarrays, the studied population-based cohort had ERG rearrangements consistent with TMPRSS2:ERG fusions in only 15% (17/111) of the CaPs.[72] There was a statistically significant association between TMPRSS2:ERG fusion and CaP metastases and/or CaP-specific death (cumulative incidence ratio 2.7; Figure 3).[72]

Although these intriguing observations suggest that TMPRSS2:ERG fusion–positive CaPs may be more aggressive, the studied cohort of patients were all diagnosed with CaP between 1977 and 1991 by transurethral resection of prostate (TURP) or transvesical enucleation of transition zone (TZ) BPH adenoma for symptomatic BPH. Hence, there was likely a majority of TZ tumors, which are not uncommonly found in TURPs for BPH.[73] TZ tumors have characteristic morphology, commonly represented in Gleason pattern 2 or well-differentiated CaP.[73,74] Of course, TZ-originating tumors can also show Gleason patterns 3, 4, and 5.[75,76] In addition, TZ tumors may have different origins and gene-expression patterns than peripheral zone (PZ) tumors, including potential association with adenosis and BPH nodules, and lack of association with HGPIN, which is primarily/almost exclusively a PZ lesion.[73,74] In addition, patients with TZ tumors, at least following surgical treatment, appear to have a better prognosis in at least some studies.[75]

Whether the lower incidence of TMPRSS2:ERG fusions in these TURP-diagnosed CaPs reflects the origin of many in the TZ and whether this could account for some/all of the prognostic differences noted remains to be determined. Of note, 13 of 17 (76%) of the ERG fusion–positive CaPs were Gleason score 7 or higher, whereas 57% of the ERG fusion–negative CaPs were Gleason score 6 or lower.[72] When adjusted for Gleason score, the association between ERG fusion status and metastases or CaP-specific death was no longer significant.[72]

Indirect support that the lower incidence of TMPRSS2:ERG fusions in TURP-diagnosed CaPs could be related to biological differences in usual TZ (commonly Gleason pattern 2) versus usual PZ (commonly Gleason pattern $\geq$ 3) tumors comes from a recent study correlating the presence of ERG fusions with tumor grade.[77] Using tissue microarrays on which 106 CaP cases from RPs were evaluable, researchers detected TMPRSS2:ERG fusions in 19/46 (41.3%) Gleason pattern 3 CaPs, 9/24 (37.5%) Gleason pattern 4 CaPs, and 7/16 (43.8%) of Gleason pattern 5 CaPs. In contrast, TMPRSS2:ERG fusions were detected in only 1/15 (6.7%) Gleason pattern 2 CaPs.[77]

*Towards a Clinical Assay*

**Urine TMPRSS2:ERG mRNA detection.** Using qRT-PCR with primers and Taqman probes targeted to detect only the most common TMPRSS2:ERG fusion product (TMPRSS2:ERGa, present in about 85% of TMPRSS2:ERG gene fusion–positive CaPs), Chinnaiyan's laboratory analyzed urine from 19 CaP patients (11 prebiopsy, 8 pre-RP samples).[78] Based on preliminary considerations of sensitivity, total RNA from urine was first amplified prior to qPCR analysis.[78] Eight of 19 (42%) of the urine samples from CaP patients had detectable TMPRSS2:ERGa fusion mRNA, including the 7 samples with the highest levels of ERG mRNA.[78] CaP tissues from 3 patients with detectable TMPRSS2:ERG mRNA in their urine were positive for ERG rearrangement by FISH, whereas

2 CaP tissue samples from patients without TMPRSS2:ERG mRNA in their urine were negative for ERG rearrangement by FISH. As not all of the CaP tissues were examined for possible ERG rearrangement, the actual sensitivity of the urine mRNA assay approach to detect ERG fusions when present in CaP is not known.

**Urine-based analysis of TMPRSS2:ERG fusion mRNA in combination with other CaP gene targets.** Hessels and colleagues used RT-PCR followed by Southern blot hybridization to detect possible TMPSS2:ERG mRNAs in urine sediments following DRE in 78 patients with CaP on prostate biopsy versus 30 men with negative biopsy.[79] In the same specimens, qRT-PCR was used to determine PCA3 score, as reported previously.[80] RT-PCR on urine-sediment RNA was done using forward primers targeted to exon 1 of TMPRSS2 and a reverse primer targeted to exon 4 of ERG. Southern blot with radiolabeled probes was not needed for sensitivity of detection, but was used to identify specifically TMPRSS2:ERG amplimers in the PCR products obtained. Multiple specific amplimers were detected, corresponding to alternately spliced forms involving exons 1 and 2 of TMPRSS2 and exons 2 to 4 of ERG. The urinary sediments of 29 of the 78 (37%) CaP patients and 2 of the 30 men with negative biopsies (7%) harbored TMPRSS2:ERG fusion transcripts. The mRNA transcript involving TMPRSS2 exon 1 fused with ERG exon 4 was found in 27 of 31 (87%) fusion transcript–positive cases; variant fusion transcripts were found along with this most common form in 3 patients and were present alone, without the major form, in another 4 positive specimens. For any TMPRSS2:ERG mRNA transcript detected in the urine, sensitivity for CaP in biopsy was 37% and the specificity was 93%.

Using a PCA3 score cutoff of 58, as established in a recent Dutch multicenter trial,[80] Hessels and colleagues showed that 48/78 CaP biopsy patients had a positive PCA3 test.[79] However, 9 of 10 men who had a negative PCA3 test but were positive for TMPRSS2:ERG fusion transcripts in the urine had CaP on biopsy. This combined test detection of 57 CaPs increased the sensitivity to 73%.

*Current Status of Commercial Test Development and Application*

The license to develop commercial CaP diagnostic tests based on TMPRSS2:ERG and related gene fusions is owned by Gen-Probe. Testing strategies may include mRNA detection and/or quantitation based on Gen-Probe technologies of target capture, TMA, and hybridization protection, as described above for AMACR and PCA3, as well as FISH or other in situ hybridization formats.

For urine mRNA testing, the inclusion of primers and probes that can detect other TMPRSS2:ERG rearrangement isoforms besides just the most common TMPRSS2:ERGa could be expected to improve sensitivity.[79] As RT-PCR without prior RNA amplification can detect ERG fusion transcripts in the urine,[79] it can be expected that the application of Gen-Probe technologies of target capture followed by TMA should allow ready analysis of fusion transcripts in whole urine or urine sediments, similar to the successful approach to quantitation of AMACR and PCA3 mRNA in post-DRE urine as described above.

FISH or other ISH approaches could be directed to a variety of specific TMPRSS2/ETS fusion targets and include use of break-apart or fusion probes as described above, and could be applied to prostate cells captured in urine or, even more readily, to biopsy tissue sections containing CaP and for which ERG fusion status is to be determined, as described below.

*Future Directions*

**CaP diagnosis.** Possible applications for TMPRSS2:ERG testing include CaP diagnosis and prognostication, as well as indication for specific molecularly targeted therapy. As ERG rearrangements are only present in about 50% to 60% of CaP, urine mRNA testing by definition would not be able to detect those CaPs without ERG rearrangements. Thus, sensitivity of TMPRSS2:ERG testing alone would not be expected to be very good, as supported by the study described above.[79] However, as TMPRSS2:ERG fusions have not been detected in benign prostate, one might expect that patients with fusion-positive urine testing and negative biopsies had CaP missed due to biopsy sampling error, and would warrant repeat biopsy. As reported, testing based on combinations of gene targets such as TMPRSS2:ERG and PCA3 should increase sensitivity for CaP.[79]

*Possible applications for TMPRSS2:ERG testing include CaP diagnosis and prognostication, as well as indication for specific molecularly targeted therapy.*

**CaP prognosis.** The bulk of the data to date supports the hypothesis that TMPRSS2:ERG fusion–positive CaPs are more aggressive (Table 5). Confirmation of prognostic differences based on ERG fusion status in PSA or other screening detected CaPs and possible translation to more aggressive treatment for ERG fusion–positive CaPs awaits future clinical trials.

If ERG-positive CaPs emerge as clinically distinct and crucial to identify versus ERG-negative CaPs, urine-based testing could have utility, either

in an initial screening setting or after CaP has been diagnosed on biopsy. To characterize a CaP diagnosed on biopsy as ERG fusion–positive or –negative, a tissue-based approach could be applied, particularly with FISH or other ISH methodology. This would be analogous to the current situation with essentially every newly diagnosed breast carcinoma, for which possible HER-2/neu overexpression is tested for by FISH or a combination of IHC possibly reflexed to FISH. These results indicate not only prognosis, but also potential response to stage-indicated adjunctive therapy, including Herceptin, a therapeutic antibody specifically targeting the HER-2 protein.[81] If it turns out that prognostication in ERG fusion–positive CaPs requires identification of specific alternatively spliced mRNA isoforms, a urine- or tissue-based mRNA approach with specific primers and probes may be necessary. A combination of tissue ISH reflexed to mRNA analysis on urine or tissue for ERG fusion–positive CaPs could evolve.

**Targeted therapy in CaP.** The discovery of likely causative gene rearrangements in at least half of CaPs ushers in a new era for CaP diagnosis, prognosis, and possible treatment. Characterization of overexpressed genes as a downstream consequence of ERG overexpression may identify candidate drug targets, analogous to the BCR/ABL kinase in CML[66] and

## Main Points

- High-throughput expression profiling and other research techniques utilizing prostate carcinoma (CaP) tissues have allowed discovery of genes and proteins that are overexpressed in CaP and that represent targets for new CaP diagnostic tests that can be expected to have much greater specificity for CaP than serum prostate-specific antigen (PSA) levels.
- Depending on the specific biomarker, new CaP molecular diagnostic tests may target proteins, mRNA, or genetic alterations in tissue, blood, or urine.
- Alpha-methyl-CoA racemase (AMACR) is a gene overexpressed in more than 90% of CaPs and a few other carcinomas. Immunohistochemistry (IHC) on prostate biopsies is already used in interpretation of small suspicious foci. Urine mRNA testing shows improved specificity for CaP detection on subsequent biopsy compared to serum PSA. Future testing may include assay modifications, combination testing with other genes, or prognostic applications. It is not commercially available at present.
- Early prostate cancer antigen (EPCA), a nuclear matrix protein, increases in CaP. Tissue IHC is commercially available, but likely has inadequate sensitivity for clinical utility when applied to negative initial biopsies for predicting CaP on repeat biopsy. EPCA-2 is a nuclear matrix protein that is increased in CaP. An EPCA-2 enzyme-linked immunosorbent assay shows promise for diagnosing CaP and is in commercial development. It needs to be further characterized and validated in appropriate patient subsets, and is not clinically available for routine use at present.
- Prostate cancer antigen 3 (PCA3) is a prostate-specific gene that is markedly upregulated in the vast majority of CaPs. Urine mRNA quantitative testing has markedly higher specificity than serum PSA and has documented clinical utility in predicting CaP on initial and repeat biopsies in patients with elevated PSA. It is commercially available for routine clinical use through multiple laboratories in the United States. Future research may show other clinical applications.
- Promoter methylation of genes such as GSTP1 and APC is a stable genetic change that occurs in most CaPs. It is amenable to detection and quantitation in DNA extracted from tissue or urine. Quantitative methylation-specific polymerase chain reaction for targeted genes on DNA from prostate biopsy tissues is commercially available, but likely has inadequate sensitivity for clinical utility when applied to negative initial biopsies for predicting CaP on repeat biopsy. Urine testing has shown good sensitivity and much higher specificity than PSA for predicting CaP on biopsy. Ongoing validation in appropriate patient subsets is needed. It is not clinically available for routine use at present.
- TMPRSS2:ERG gene fusions are stable genetic rearrangements that are likely causal events in about 50% of CaPs, and hence represent the most common genetic change in cancers in humans. Urine mRNA testing would have inadequate sensitivity for detecting both ERG fusion–positive and –negative CaPs, but may have diagnostic utility when used in combination with other gene targets. ERG fusion CaPs may have worse prognosis. In situ hybridization or mRNA testing in tissue or urine may have utility for prognosis and treatment indication in the future.
- The era of molecular testing for CaP diagnosis has arrived in urology. New tests can allow for improvements in prostate patient management. For example, urine PCA3 score has documented utility in predicting the need for initial and repeat prostate biopsy in patients with elevated serum PSA. Urologists should not hesitate to utilize new molecular tests, as long as they understand the clinical and laboratory issues relevant to their effective use. As new molecular tests, such as those described herein, are further characterized, they will likely achieve new applications for diagnosis, prognosis, and treatment decisions in prostate cancer patients.

HER-2 in breast carcinoma.[81] In addition to potential diagnostic applications, identification of patients with more adverse prognosis and/or warranting future ERG fusion–indicated targeted therapies is an exciting prospect for future CaP patient management. ■

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