

REVIEW

Urinary biomarkers for prostate cancer: a review

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Although the routine use of serum prostate-specific antigen (PSA) testing has undoubtedly increased prostate cancer (PCa) detection, one of its main drawbacks is its lack of specificity. As a consequence, many men undergo unnecessary biopsies or treatments for indolent tumours. PCa-specific markers are needed for the early detection of the disease and the prediction of aggressiveness of a prostate tumour. Since PCa is a heterogeneous disease, a panel of tumour markers is fundamental for a more precise diagnosis. Several biomarkers are promising due to their specificity for the disease in tissue. However, tissue is unsuitable as a possible screening tool. Since urine can be easily obtained in a non-invasive manner, it is a promising substrate for biomarker testing. This article reviews the biomarkers for the non-invasive testing of PCa in urine.

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INTRODUCTION

Annually, 241 740 men in the United States and 338 700 men in Europe are newly diagnosed with prostate cancer (PCa) and around 28 170 US and 70 800 European men die from this disease.^{1,2} Early detection of PCa relies on serum prostate-specific antigen (PSA) testing or digital rectal examination (DRE). Since its first clinical application, serum PSA has been a valuable tool in the detection, staging and monitoring of this disease. Although the routine use of serum PSA testing has undoubtedly increased PCa detection, one of its main drawbacks has been its lack of specificity resulting in a high negative biopsy rate.³ The early detection of many indolent PCas has resulted in treatment of tumours that would not have become life-threatening to a patient.

Serum PSA has a low specificity because it is not a PCa-specific event; elevated levels can also be detected in men with benign prostatic hyperplasia (BPH) and prostatitis. Methods to enhance PSA specificity have assisted clinicians in deciding which patients should undergo biopsy, but have not necessarily improved diagnostic accuracy or facilitated optimal therapeutic decision-making. More accurate tests that can stratify patients according to their risk of developing PCa, identify men who require repeat prostate biopsy and stratify men at risk for aggressive disease are needed.

Many biomarkers have been identified and some of them are promising due to their specificity for the disease in tissue. However, tissue is unsuitable as substrate for biomarker testing because of its invasiveness and expensiveness. Therefore, testing of disease-related biomarkers in body fluids that can be obtained in a non-invasive manner seems a good alternative as possible screening tool. Because of the ease of collection, and the fact that prostate cells are directly released into the urethra through prostatic ducts after DRE, urine has become the future for non-invasive biomarker testing. This review focusses on the available data concerning the applicability of promising DNA or

RNA-based urinary biomarkers in the early detection and prediction of aggressiveness of PCa (summarized in **Table 1**).

EPIGENETIC MODIFICATIONS

Alterations in DNA, without changing the order of bases in the sequence, often lead to changes in gene expression. These epigenetic modifications include changes such as DNA methylation and histone acetylation/deacetylation. Many gene promoters contain GC-rich regions also known as CpG islands. Hypermethylation of CpG islands results in decreased transcription of the gene into mRNA. Recently, it was suggested that the DNA methylation status may be influenced in early life by environmental exposures, such as nutritional factors or stress, and that this leads to an increased risk of cancer in adults.⁴ Changes in DNA methylation patterns were observed in many human tumours.⁵ A technique known as methylation-specific PCR (MSP) is used for the detection of promoter hypermethylation. In contrast to microsatellite or loss of heterozygosity analysis, this technique requires a tumour-to-normal ratio of only 0.1%–0.001%. This means that using this technique, hypermethylated alleles from tumour DNA can be detected in the presence of 10⁴–10⁵ excess amounts of normal alleles.⁶ Therefore, DNA methylation can serve as a useful marker in cancer detection. Recently, there have been many reports on hypermethylated genes in human PCa. Two of these genes are glutathione S-transferase P1 (*GSTP1*) and Ras-association domain family protein isoform A (*RASSF1A*).

GSTP1

The most described epigenetic alteration in PCa is the hypermethylation of the *GSTP1* promoter. *GSTP1* belongs to the cellular protection system against toxic effects and as such, is involved in the detoxification of many xenobiotics. *GSTP1* hypermethylation was reported in approximately 6% of the proliferative inflammatory atrophy lesions

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Table 1 Biomarkers and their potential application in the urinary detection of prostate cancer

Goal	Biomarker	Substrate	Method	References
Reduce the number of unnecessary biopsies	<i>GSTP1</i>	DNA	MSP	9–11
	<i>GSTP1+RASSF1A+RARB+APC</i>	DNA	MSP	18,19,21
	<i>GSTP1+HIST1HK4+TFAP2E</i>	DNA	MSP	22
	<i>PCA3^a</i>	RNA	TMA	27–32
	<i>PCA3+TMPRSS2-ERG</i>	RNA	Quantitative RT-PCR	46,47
	<i>PCA3+TMPRSS2-ERG+SPINK1+GOLPH2</i>	RNA	Quantitative RT-PCR	52
	<i>AMACR</i>	Protein	Immune reactivity assay	60,61
	<i>AMACR</i>	Protein	Western blot analysis	62
	<i>AMACR+PCA3</i>	RNA	Quantitative RT-PCR	64
	<i>PSMA</i>	RNA	Quantitative RT-PCR	69
	<i>PSMA+PCA3+PSGR</i>	RNA	Quantitative RT-PCR	69
	<i>PSMA+PSM'</i>	RNA	Quantitative RT-PCR	65,68
	miR-107, miR-574-3p	microRNA	Quantitative RT-PCR	75
	Distinguish the aggressive tumours from the indolent ones	<i>RASSF1A</i>	DNA	MSP
<i>TMPRSS2-ERG</i>		RNA	Quantitative RT-PCR	49–51
<i>AMACR</i>		RNA	Quantitative RT-PCR	63

Abbreviations: *AMACR*, α -methylacyl-CoA racemase; *APC*, adenomatous polyposis coli; *GOLPH2*, golgi phosphoprotein 2; *GSTP1*, glutathione S-transferase P1; MSP, methylation-specific PCR; *PSMA*, prostate-specific membrane antigen; *RARB*, retinoic acid receptor β 2; *RASSF1A*, Ras-association domain family protein isoform A; RT-PCR, reverse transcription-PCR; *SPINK1*, serine peptidase inhibitor Kazal type 1; TMA, transcription-mediated amplification; *TMPRSS2*, transmembrane-serine protease gene.

^a FDA approved molecular marker for prostate cancer: ProgenSA PCA3 test.

and in 70% of the prostate intraepithelial neoplasia (PIN) lesions.⁷ It was shown that some proliferative inflammatory atrophy lesions merge directly with PIN and early carcinoma lesions, although additional studies are necessary to confirm these findings. Hypermethylation of *GSTP1* was detected in more than 90% of prostate tumours, whereas no hypermethylation was observed in BPH and normal prostate tissues.⁸

Hypermethylation of the *GSTP1* gene was detected in 50% of ejaculates from PCa patients but not in ejaculates of men with BPH. Due to the fact that ejaculates are not always easily obtained, hypermethylation of *GSTP1* was determined in urinary sediments obtained from PCa patients after prostate massage. Cancer could be detected in 77% of these sediments.⁹ Moreover, hypermethylation of *GSTP1* was found in post-prostate massage urinary sediments of 68% of patients with early confined disease, 78% of patients with locally advanced disease, 29% of patients with PIN and 2% of patients with BPH. These findings resulted in a specificity of 98% and a sensitivity of 73%. The negative predictive value of this test was 80%, indicating that this assay bears potential to reduce the number of unnecessary biopsies. Recently, these results were confirmed and a higher frequency of *GSTP1* methylation was observed in the urine of men with stage 3 vs. stage 2 disease.¹⁰ Because hypermethylation of *GSTP1* has a high specificity for PCa, the presence of *GSTP1* hypermethylation in urinary sediments of patients with negative biopsies (33%) and patients with atypia or high-grade PIN (67%) suggests that these patients may have occult PCa.¹¹

RASSF1A

RASSF1 has two major transcripts, termed *RASSF1A* and *RASSF1C*, which are transcribed from different CpG island promoters. The promoter of *RASSF1A* is often hypermethylated in breast, kidney, liver, lung and PCa, whereas the promoter region of *RASSF1C* is never methylated. *RASSF1A* reduces tumour growth *in vivo* and *in vitro*.¹² This supports a role for *RASSF1A* as a tumour suppressor gene. It was shown that *RASSF1A* binds to microtubules and protects cells from microtubule destabilizing agents.¹³ This interaction contributes to cell cycle regulation and mitotic progression.

Initially no *RASSF1A* hypermethylation was detected in normal prostate tissue.^{14,15} Recently, methylation of the *RASSF1A* gene was observed in both premalignant PINs and benign prostatic epithelia.¹⁶ *RASSF1A* hypermethylation has been observed in 60%–74% of prostate tumours and in 18.5% of BPH samples. Furthermore, the methylation frequency is clearly associated with high Gleason score and stage.^{14,15,17} These findings suggest that *RASSF1A* hypermethylation may distinguish the more aggressive tumours from the indolent ones.

Multiplexed assays

Multiplexed assays consisting of three or more methylation markers may provide better specificity and sensitivity. Abberant methylation of *GSTP1*, *RASSF1A*, retinoic acid receptor β 2 (*RARB*) and adenomatous polyposis coli (*APC*) in urinary cells, obtained after prostate massage, discriminated malignant from non-malignant cases with 86% sensitivity and 89% specificity.¹⁸ In urine sediments obtained from catheterized urine specimens (collected during radical prostatectomy), the combined sensitivity of the methylated genes *GSTP1*, *RASSF1A* and *RARB* for PCa was 82%.¹⁹ The most informative biomarkers for PCa were the hypermethylated promoters of *RASSF1A* and *RARB*. The *RASSF1A* promoter was the most commonly methylated with a frequency of 71%. *RARB* hypermethylation was more common in men with PCas of high Gleason scores. *GSTP1*, *RARB* and *RASSF1A* are known to accumulate low-level promoter methylation in normal cells of ageing individuals.²⁰ Therefore, these genes should be investigated in age-matched negative controls to establish a quantitative cutoff point for the amount of methylation that would indicate the presence of cancer.

Using an assay for *GSTP1*, *RARB* and *APC* on urine samples from patients with serum PSA concentrations $\geq 2.5 \mu\text{g l}^{-1}$, a good correlation of *GSTP1* hypermethylation with the number of PCa-positive biopsy cores was observed.²¹ Samples that contained methylation for either *GSTP1* or *RARB* correlated with higher tumour volumes. DNA methylation of the genes *GSTP1*, *RASSF2*, *HIST1HK4* and *TFAP2E* had a higher sensitivity for PCa in post-prostate massage

urine samples compared to plasma samples of the same patients. Furthermore, hypermethylation of the genes *RASSF2*, *HIST1HK4* and *TFAP2E* was individually as good as *GSTP1* in the diagnosis of PCa.²²

DNA hypermethylation is highly specific for cancer and this epigenetic event is frequent, abundant and stable. Hypermethylated genes have the potential to provide a new generation of cancer urinary biomarkers, although prior to clinical utilisation, these findings require validation in prospective clinical studies.

GENES UNIQUELY EXPRESSED IN PCA

Microarray studies have been very useful and informative in identifying genes that are consistently up- or downregulated in PCa compared to benign prostate tissue.²³ These genes can provide PCa-specific biomarkers and provide a greater insight into the aetiology of the disease. For the molecular diagnosis of PCa, genes that are highly upregulated in PCa and have low or normal expression in normal prostate tissue are of special interest. Such genes could enable the detection of one tumour cell in a background of normal cells and thus be applied as a diagnostic marker in PCa detection.

PCA3

PCA3, formerly known as *DD3*, was identified using differential display analysis. *PCA3* was found to be overexpressed in prostate tumours compared to normal prostate tissue of the same patient using northern blot analysis.²⁴ Moreover, *PCA3* was found to be strongly overexpressed in more than 95% of primary PCa specimens and in PCa metastasis. The expression of *PCA3* is restricted to prostatic tissue, that is, no expression was found in other normal human tissues.^{25,26} The gene encoding for *PCA3* is located on chromosome 9q21.2. Open reading frame analysis revealed that the *PCA3* exons are populated by an unusual number of stop codons. A gene that codes for proteins will typically possess one long open reading frame delimited by a stop codon. The multiplicity of stopcodons across the three reading frames of *PCA3* and the lack of an extended open reading frame indicates that *PCA3* does not encode a protein and functions as a non-coding RNA.²⁴

In the absence of a protein, RT-PCR assays were developed for *PCA3*. A time-resolved fluorescence-based quantitative RT-PCR assay showed a median 66-fold upregulation of *PCA3* in PCa tissue compared to normal prostate tissue.²⁶ Moreover, a median upregulation of 11-fold was found in prostate tissues containing less than 10% of PCa cells. This indicated that *PCA3* was capable of detecting a small number of tumour cells in a background of normal cells. This hypothesis was tested on post-DRE voided urine samples obtained from a group of 108 men who were indicated for prostate biopsies based on a total serum PSA value of >3 ng ml⁻¹. This test had 67% sensitivity and 83% specificity using prostatic biopsies as a gold-standard for the presence of a tumour. Furthermore, this test had a negative predictive value of 90%, which indicates that the quantitative determination of *PCA3* transcripts in urinary sediments obtained after DRE bears potential in the reduction of the number of invasive transrectal ultrasound-guided biopsies in this population of men.²⁶

The tissue specificity and the high overexpression in prostate tumours indicate that *PCA3* is the most PCa-specific gene described so far. *PCA3* has been extensively reviewed.^{27,28} Gen-probe Inc. (Hologic Gen-Probe Inc., San Diego, CA, USA) has the exclusive worldwide licence to the *PCA3* technology and has successfully transferred the technology to its APTIMA platform.²⁹ APTIMA uses transcription-mediated amplification (TMA), which is an RNA transcription

amplification system using RNA polymerase and reverse transcriptase to drive the isothermal reaction. This test was released commercially in Europe under the name ProgenSA PCA3. In several studies using large patient cohorts, *PCA3* appears to be superior to serum PSA.³⁰⁻³² A urine *PCA3* score of >35 had an average sensitivity of 66% and specificity of 76% for the prediction of PCa at biopsy, whereas serum PSA had a specificity of only 47% at about the same sensitivity (65%).³³ The *PCA3* score is not influenced by age, prostatitis, prostate volume and 5 α -reductase inhibitors. In men with elevated serum PSA levels and one or two previous negative biopsy results, elevated *PCA3* scores were correlated to a positive repeat biopsy. ProgenSA *PCA3* was approved by the FDA as a diagnostic test in men with elevated serum PSA and previous negative biopsy result. *PCA3* has become the first basis for the molecular diagnostics in clinical urological practice.

Studies on the predictive value of *PCA3* for aggressive disease have been contradictory. No significant association between *PCA3* score and any prognostic parameter (including stage, Gleason score, tumour volume or extraprostatic extension) was found in three independent studies.³⁴⁻³⁶ Other studies dispute this, and found a correlation between high *PCA3* scores and Gleason score ≥ 7 PCa, extracapsular extension and tumour volume.³⁷⁻³⁹ Recently, *PCA3* score was found to be a valuable predictor of low-volume, insignificant cancer.^{38,40} As such, *PCA3* could be of help in selecting patients for active surveillance. However, in men with significant PCa, *PCA3* may not differentiate the aggressiveness of a tumour. Thus, a biomarker indicative of tumour aggressiveness is still an unmet need in PCa.

Gene fusions of *TMPRSS2* with *ETS* family members

Differential gene expression analysis has been successfully used to identify PCa-specific biomarkers by comparing PCa tissues to non-malignant prostate tissues. Recently, a new biostatistical method called cancer outlier profile analysis (COPA) was used to identify genes that are differentially expressed in a subset of PCas.⁴¹ COPA identified strong outlier profiles for v-ets erythroblastosis virus E26 oncogene (*ERG*) and ets variant gene 1 (*ETV1*) in 57% of PCa cases.⁴² This was in concordance with the results of a study where PCa-associated *ERG* overexpression was found in 72% of PCa cases.⁴³ In $>90\%$ of the cases that overexpressed either *ERG* or *ETV1*, a fusion of the 5' untranslated region of the prostate-specific and androgen-regulated transmembrane-serine protease gene (*TMPRSS2*) with these ETS family members was found. Recently, another fusion between *TMPRSS2* and an ETS family member has been described, the *TMPRSS2-ETV4* fusion, although this fusion is sporadically found in PCas.⁴⁴ Furthermore, a fusion of *TMPRSS2* with *ETV5* was found. Overexpression of *ETV5 in vitro* was shown to induce an invasive transcriptional programme.⁴⁵ These fusions can explain the aberrant androgen-dependent overexpression of ETS family members in subsets of PCa because *TMPRSS2* is androgen-regulated.⁴² The discovery of the *TMPRSS2-ERG* gene fusion and the fact that *ERG* is the most frequently overexpressed proto-oncogene described in malignant prostate epithelial cells suggests its role in prostate tumourigenesis.

Combination of *TMPRSS2-ERG* gene fusions with *PCA3*

Recently, it was shown that non-invasive detection of *TMPRSS2-ERG* fusion transcripts is feasible in urinary sediments obtained after DRE using an RT-PCR-based research assay. Due to the high specificity of the test (93%), the combination of *TMPRSS2-ERG* fusion transcripts with *PCA3* improved the sensitivity from 62% (*PCA3* alone) to 73% (combined) without compromising the specificity for detecting PCa.⁴⁶ Another study confirmed that *PCA3* has the highest sensitivity

(93%) and *TMPRSS2-ERG* fusion transcript has the highest specificity (87%) in predicting PCa diagnosis. Combining *PCA3*, *TMPRSS2-ERG* and serum PSA in a multivariable algorithm, PCa could be predicted in urine with 80% sensitivity and 90% specificity.⁴⁷ In another study, no *TMPRSS2-ERG* fusion transcripts were found in urine samples obtained from females, healthy young men and post-radical prostatectomy patients.⁴⁸ In 34.8% of the urine samples from PCa patients, *TMPRSS2-ERG* fusion transcripts were found compared to 18.2% of the urine specimen from men with negative biopsies. Although this study is limited in sample size, it confirms that this marker can be used as a diagnostic tool for PCa.

Using real-time PCR, a positive correlation was found between *TMPRSS2-ERG* fusion transcripts in urine collected after prostate massage and a high serum PSA, pathological stage and Gleason score.⁴⁹ *TMPRSS2-ERG* gene fusions were highly associated with aggressive Gleason score ≥ 7 and PCa-related death.⁵⁰ Elevated urine *TMPRSS2-ERG* was also associated with the presence of PCa and Epstein criteria for significant PCa (Gleason score, tumour volume, % of cancer per biopsy core and number of positive cores).

It was shown that men with extremes of *TMPRSS2-ERG+PCA3* have different risks of cancer and aggressiveness of the cancer on biopsy. In combination with other clinical pathological parameters (combined in the multivariate prostate cancer prevention trial risk calculator), urine *TMPRSS2-ERG+PCA3* may guide the urgency of biopsy after the detection of an elevated serum PSA.⁵¹

Serine peptidase inhibitor Kazal type 1 (*SPINK1*), golgi phosphoprotein 2 (*GOLPH2*) and *TMPRSS2-ERG* were, like *PCA3*, independent predictors of PCa upon repeat biopsy.⁵² By combining *PCA3* with these markers in a quantitative multiplexed RT-PCR analysis, the ROC AUC value improved from 0.66 (*PCA3* alone) to 0.76. This multiplexed urine-based assay had 66% sensitivity and 76% specificity for detecting PCa in repeat biopsies. In men with elevated serum PSA, urine *TMPRSS2-ERG* in combination with *PCA3* may aid in decision-making on biopsy and management of the disease. By combining *SPINK1* with *TMPRSS2-ERG* and *PCA3* in a multiplexed panel, the risk stratification of PCa may be further improved.

α -methylacyl-CoA racemase (AMACR)

The gene coding for *AMACR* on chromosome 5p13 was found to be consistently upregulated in PCa. This enzyme plays a critical role in peroxisomal β -oxidation of branched chain fatty acid molecules obtained from dairy and beef.⁵³ Interestingly, the consumption of dairy and beef has been associated with an increased risk of PCa.⁵⁴

In clinical PCa tissue, a ninefold overexpression of *AMACR* mRNA was found compared to normal prostate tissue. Immunohistochemical studies and western blot analyses have confirmed the upregulation of *AMACR* at the protein level. Furthermore, it was shown that 88% of PCa cases and both untreated metastases and castration resistant PCas were strongly positive for *AMACR*.⁵⁵ *AMACR* expression was not detected in atrophic glands, basal cell hyperplasia and urothelial epithelium or metaplasia. Immunohistochemical studies also showed that *AMACR* expression in needle biopsies had a 97% sensitivity and a 100% specificity for PCa detection.⁵⁶ Combined with a staining for p63, a basal cell marker absent in PCa, *AMACR* greatly facilitated the identification of malignant prostate cells.^{57,58} Currently, the accuracy and specificity of *AMACR* in the detection of PCa in biopsy specimens is regarded as an improvement over the serum PSA test.⁵⁹ Its high expression and cancer cell specificity implies that *AMACR* may also be a candidate for the development of molecular

probes that may facilitate the identification of PCa using non-invasive imaging modalities.⁵⁵

There have been many efforts to develop a body fluid-based assay for *AMACR*. First, an *AMACR* activity assay used on extracts derived from prostate needle biopsy specimen had 92.3% sensitivity and 89.2% specificity for PCa detection.⁶⁰ Second, an *AMACR* immune reactivity assay showed 71.8% specificity and 61.6% sensitivity in distinguishing the sera of PCa patients from those of healthy controls, and could be used in combination with serum PSA to reduce the number of unnecessary biopsies.⁶¹ Third, *AMACR* protein western blot analysis on urine samples obtained after prostate massage had a sensitivity of 100%, a specificity of 58%, a positive predictive value of 72% and a negative predictive value of 88% for PCa.⁶² Fourth, a small study indicated that *AMACR* mRNA-based quantitative real-time PCR analysis on urine samples obtained after prostate massage has the potential to exclude the patients with clinically insignificant disease when *AMACR* expression is normalized for PSA.⁶³ Fifth, when *AMACR* transcripts were measured and combined with *PCA3* scores in post-DRE urinary sediments of 43 men with and 49 men without PCa, this duplex test had 81% sensitivity and 84% specificity for the detection of PCa.⁶⁴ Therefore, *AMACR*-based assays for the detection of PCa in urine specimen are promising, although additional investigation is needed to validate the clinical usefulness of this urinary biomarker.

Prostate-specific membrane antigen (PSMA)

PSMA is a transmembrane glycoprotein that is expressed on the surface of prostate epithelial cells. It was shown that PSMA is upregulated in PCa tissue compared to benign prostate tissues. No overlap in PSMA expression was found between BPH and PCa, indicating that PSMA is a very promising diagnostic marker.⁶⁵ Recently, it was shown that high PSMA expression in PCa cases correlated with tumour grade, pathological stage, aneuploidy and biochemical recurrence. Furthermore, increased *PSMA* mRNA expression in primary PCas and metastasis correlated with PSMA protein overexpression.⁶⁶ Its clinical utility as a diagnostic or prognostic marker for PCa has been hindered by the lack of a sensitive immunoassay for this protein. However, a combination of ProteinChip (CIPHERGEN Biosystems, Fremont, CA, USA) arrays and surface-enhanced laser desorption ionisation/time-of-flight (SELDI-TOF) mass spectrometry has led to the introduction of a protein biochip immunoassay for the quantification of serum PSMA. It was shown that the average serum PSMA levels for PCa patients were significantly higher compared to those of men with BPH and healthy controls.⁶⁷ These findings implicate a role for serum PSMA to distinguish men with BPH from PCa patients. However, further studies are needed to assess its diagnostic value.

RT-PCR studies have shown that *PSMA* in combination with its splice variant *PSM'* could be used as a prognostic marker for PCa. In the normal prostate, *PSM'* expression is higher than *PSMA* expression, whereas in PCa tissues, the *PSMA* expression is more dominant. Therefore, the ratio of *PSMA* to *PSM'* is highly indicative for disease progression. Designing a quantitative PCR analysis which discriminates between the two PSMA forms could yield another application for *PSMA* in diagnosis and prognosis of PCa.^{65,68}

In men with serum PSA values between 4 and 10 ng ml⁻¹ with no previous biopsies, *PSMA* mRNA measured in post-prostate massage urinary sediments was superior to *PCA3* and *PSGR* in predicting cancer upon biopsy. At 70% specificity, the sensitivity for *PSMA*, *PCA3* and *PSGR* was 64%, 46% and 61%, respectively. A combination of the three markers resulted in 68% sensitivity at 70% specificity.⁶⁹

Because of its specific expression on prostate epithelial cells and its upregulation in PCa, *PSMA* has also become the target for therapies. The proposed strategies range from targeted toxins and radio nuclides to immunotherapeutic agents. First-generation products have entered clinical testing.⁷⁰

MICRORNAS (MIRNAS)

Mature miRNAs are non-coding RNAs that play key roles in the regulation of gene expression. They are short, single-stranded RNA molecules of ~22 nucleotides in length that bind to complementary sequences in the 3' UTR of multiple target mRNAs, usually resulting in their silencing. miRNAs have shown to be involved in a wide range of biological processes such as cell cycle control, apoptosis and several developmental and physiological processes. miRNAs have also been implicated in a number of diseases including a broad range of cancers, heart disease and neurological diseases. Their small size and the fact that they are secreted within microvesicles protect the miRNAs from RNase.^{71,72} Consequently, the detection of miRNAs in body fluids represents a promising non-invasive diagnostic utility for PCa. miRNAs are mostly studied in serum and plasma. Independent studies have shown that there are two promising miRNAs in the blood circulation, miR-141 and miR-375. In patients with metastatic PCa plasma miR-141 levels were 46-fold higher compared to the healthy individuals.^{73,74} Elevated plasma levels of miR-141 and miR-375 could discriminate men with metastatic disease from men without metastases.⁷⁵ Elevated serum levels of these two miRNAs were associated with higher Gleason score and positive lymph node status.⁷⁶ In microvesicle and exosome portions taken from sera from men with metastatic disease, both miRNAs were significantly increased in both portions compared to those obtained from men with non-recurring disease.⁷⁵

Since changes in miRNA levels occur in serum and plasma of PCa patients, there may be a possibility that some of these changes may also occur in urine. Until now, only one study for miRNA in urine was reported.⁷⁵ Five of the miRNAs that were differentially quantified in PCa patients compared to controls (miR-107, miR-574-3p, miR375, miR200b and miR-141) were successfully quantified in urine of men with cancer. Although miR-141 and miR-375 were useful serum and plasma biomarkers for metastatic disease, they were unable to discriminate men with PCa compared to healthy controls in urine. However, miRNAs miR-107 and miR-574-3p were significantly higher in the urine of men with PCa when compared to controls. They appeared to be even better than *PCA3* normalized to PSA in identifying the presence of PCa in urine samples.

Several miRNA biomarkers have been described to be associated with PCa onset or progression. However, the analysis on circulating miRNAs for PCa is still in its infancy. The analysis of miRNAs in body fluids is a challenge with respect to the extraction of RNA from limited sources of biomaterial. Although many of the miRNA studies as potential biomarkers for PCa were done on serum and plasma specimen, the single study in urine holds promise that they can be used for blood-based as well as urine-based assays. The promising results from these initial studies need to be validated in larger patient cohorts with good controls (e.g., healthy males, men with BPH and/or prostatitis) and comprehensive follow-up data using robust methods and standards for miRNA extraction and detection.⁷⁷

EXOSOMES

Exosomes are small tissue-derived vesicles with a diameter of 50–150 nm that are shed by many mammalian cell types, including

malignant cells. The protein and RNA content of exosomes represent their tissue origin and associate with the original cellular conditions.⁷⁸ Exosomes are enriched in unique mRNA transcripts specific to tumour cells compared to the tissue donor cells. This can be explained by the fact that the exosomes contain less ribosomal RNA, thereby increasing the relative amount of mRNA.⁷⁹ Exosomes are present in different body fluids (e.g., urine, semen, blood and malignant ascites). The celltype specificity of these exosomes makes them an attractive source of new tissue or disease-specific biomarker targets such as proteins, mRNAs and miRNAs.^{80–83} For PCa, the urinary exosomes hold promise as a non-invasive source of biomarkers.

Recently, it was shown that it is technically feasible to study urinary exosomes of PCa patients for evaluation of the clinical status of disease, although the current extraction protocols have been labour-intensive and not optimal.^{79,84,85} PCa patients had higher levels of urinary exosomes than the healthy donors. Prostate markers (e.g., *PSA* and *PSMA*) were not expressed in specimen from healthy donors, suggesting that few exosomes in healthy donor urines are of prostatic origin.⁸⁵ The membranes of exosomes are resistant to the osmolytic and proteolytic activity of urine. This indicates that exosomes are quite stable in this body fluid.

PSA, *TMPRSS2-ERG* gene fusion and *PCA3* mRNA transcripts can be detected in the urinary exosomal fraction after a mild prostate massage.⁸⁵ Prostate massage is necessary to increase the exosomal secretion into the urethra and subsequently into the collected urine fraction which is in concordance with earlier published findings of shedding prostate tumour cells in the urine.^{26,33,52}

After a mild prostate massage, *TMPRSS2-ERG* gene fusions were detected in 2/4 patients with high Gleason scores and in none of the patients with low Gleason scores. *PCA3* transcripts were detected in all of the patients. None of the patients under androgen deprivation therapy (ADT) had detectable levels of *PSA*, *TMPRSS2-ERG* gene fusion or *PCA3* mRNA transcripts. This loss of expression of these androgen-regulated biomarkers correlated with tumour regression and a positive response to the ADT.⁸⁵ Independently, others showed that there is a decrease in the amount of urinary exosomes under ADT.⁸⁴

Although these results are promising, more studies are necessary to assess the clinical value of urinary exosomes. Modifications in collection, storage and handling of urine samples can improve the isolation of the urinary exosomes providing a simple enrichment of potential disease biomarkers.

CONCLUSION

There is an urgent need to discover more accurate non-invasive tests for PCa diagnosis and to allow the stratification of patients with life-threatening PCa. Because of the ease of collection, and the fact that prostate cells are directly released into the urethra through prostatic ducts after DRE or prostate massage, urine has become the future of non-invasive biomarker testing. Many studies have shown the feasibility of urine for the non-invasive detection of PCa. Recently, it was shown that RNA biomarkers can also be detected in urinary exosomes, making them promising for biomarkers research as well.

Biomarker research is in focus at many laboratories and several biomarkers are promising due to their specificity for the disease in tissue. To date, only few of these biomarkers have shown to be useful as urinary marker. Two PCa-specific RNA-based biomarkers have been identified (*PCA3* and *TMPRSS2-ERG* gene fusions). The recent FDA approval of *PCA3* has led to its introduction in clinical practice and the combination of both markers has been marketed for clinical use as

well. Compared to single biomarkers, the combination of several biomarkers considerably improves the prediction of PCa in urine samples which is consistent with the heterogeneity of the disease.

In the era of individualized therapy, the biomarker combinations are necessary to not only predict PCa at biopsy, but also the aggressiveness of the cancer. Preliminary results show that the PCA-specific *TMPRSS2-ERG* gene fusion may be indicative of aggressiveness of cancer upon biopsy, although further studies are warranted. In PCA biomarker development, the greatest unmet need remains: a biomarker that stratifies men at risk of aggressive PCa, eventually leading to a reduction of unnecessary interventions.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

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