Urine markers and prostate cancer

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ABSTRACT

Prostate cancer (PCa) is globally the most common cancer in men, with an estimated prevalence of more than two million cases. Given the poor success rate in treating advanced PCa, intervention in early stages may reduce the progression of a small, localized carcinoma to a large metastatic lesion, thereby reducing disease-related deaths. Urine is readily available and can be used to detect either exfoliated cancer cells or secreted products. The major advantages of urine-based assays are their noninvasive character and ability to monitor PCa with heterogeneous foci. The aim of this review was to summarize the current evidence regarding performance characteristics of tests proposed for urine-based prostate cancer detection.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer deaths in men. The risk of developing PCa increases with age, with about 60 percent of cases diagnosed in men over age 60. Other risk factors include a family history of prostate cancer on either the mother's or father's side. Diagnosis and management are confounded by the lack of symptoms, which typically present when the cancer has progressed, and the lack of proven CaP-specific diagnostic techniques available during the early stages of the disease. There are also unique problems for the treatment and prognosis of PCa owing to its frequent histologic heterogeneity. In reality, this carcinoma is genetically multicentric and histologically multifocal [1, 2]. Prostate cancer is indeed curable if detected early, while still localized within the capsule. Novel approaches for the detection and control of this cancer are therefore extremely important. The identification of molecular markers is one of the most challenging goals for the early detection of PCa because available noninvasive methods have neither sufficient sensitivity nor specificity to be suitable for routine use. The development of urinary markers for the detection of prostate cancers is a dynamic field. So the perfect marker should have high sensitivity and high specificity, must have no inter-observer variability, and must be easy to perform [1-5].

Prostate cancer is the only one of the four solid tumor types (breast, lung, and colorectal cancer) that has a clinically useful protein biomarker for diagnostics and follow-up after treatment. Prostate-specific antigen (PSA) has shown reasonable sensitivity for detection of incipient cancer and can also predict response to treatment. One of the drawbacks with PSA is its low specificity, such that benign hyperplasic conditions can also be associated with a PSA increase. Thus, additional PCa biomarkers are needed; especially the ones that give information about the severity of the disease and can predict high or low risk for future metastases [6].

Currently, no single marker can guide us in surveillance. Whether the use of a set of markers will be the answer will have to be studied. The primary aim of this review was to summarize the current evidence regarding performance characteristics of tests proposed for urine-based prostate cancer detection.

Advantages and prospects of the urine studies

Urine is readily available and can be used to detect either exfoliated cancer cells or secreted products. The major advantages of urine-based assays are their noninvasive character and ability to monitor PCa with heterogeneous foci. The purpose of urine-based screening tests for prostate cancer is to find cancer cells from which markers can be extracted or to find released proteins or nucleic acids that are modified compared with the forms in healthy men. Urine, with less complexity than serum and relatively high thermodynamic stability, is a promising study medium for the discovery of novel biomarkers in prostate cancer [7].

Two types of microvesicles are present in prostate secretions: (1) prostasomes (150-500 nm), produced by prostatic ductal epithelial cells that are a normal component of seminal fluid and play a role in male fertility [8]; and (2) exosomes (30-100 nm), specialized nanovesicles with a cup shaped morphology, which are actively secreted by a variety of normal and tumor cells. An elevated exosome secretion has been found in malignancy effusions as well as serum and urine from cancer patients [9]. Certain RNA transcripts are enriched several 100-fold in the exosomes compared with the donor cells, supporting a specific packing mechanism [10]. The exosomes essentially lack all of the ribosomal RNA, which represent ~80% of the total RNA in cells, and thus contain mainly mRNAs and microRNAs (miRNAs). Therefore, exosomes are enriched in unique transcripts specific to tumor cells that may be below detectable levels even in the tumor cells themselves [10]. Therefore, analyzing the transcriptome in secreted PCa exosomes in urine has the advantages of being both noninvasive and informative as to the overall tumor malignancy status, including: tumor-specific splice variants and mutations as well as mRNA and miRNA levels known to be diagnostic for PCa [11].

Conventional and innovative urine markers

8-hydroxy-2'-deoxyguanosine (8-OHdG)

The most important oxygen-free radical causing damage to basic biomolecules (proteins, membrane lipids, and DNA) is the hydroxyl radical ($HO^{\bullet}$). The interaction of $HO^{\bullet}$ with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanosine; (8-OHdG) [12,13]. Determination and analysis of 8-OHdG can be performed in animal organs and in human samples (urine, human organs, and leukocyte DNA). As a result of these studies 8-OHdG has been established as an important biomarker of oxidative stress, of cancer risk to

humans by mechanisms of oxygen-free radicals, of aging processes including degenerative diseases, and in general as a biological marker of lifestyle and the effect of diet [13].

AMACR

Alpha-methylacyl CoA racemase (AMACR) is located at 5p13.3, a gene region found to be important in prostate cancer in several genomewide scans. AMACR, an α-methylacyl coenzyme A racemase (also known as P504S) is involved in β-oxidation of branched-chain fatty acids and fatty acid derivatives. AMACR is consistently up-regulated at both the mRNA and protein levels in prostate tissue, and several studies have also analyzed its presence in the urine of PCa patients [14]. Western blot analysis for AMACR was used on voided urine after TRUS and biopsy, showing a 100% sensitivity and 58% specificity for PCa detection in the group of patients with negative biopsy findings. In another study, the quantification of AMACR transcripts normalized to PSA transcripts in prostate secretions was predictive of PCa. A meta-analysis of expression microarrays found that AMACR is consistently overexpressed in prostate cancer with high specificity (79%-100%) and sensitivity (82%-100%) [15].

Annexin A3

Annexin A3 (ANXA3), belongs to a family of calcium and phospholipid binding proteins that are implicated in cell differentiation and migration, immunomodulation, and bone formation and mineralization in PCa metastasis. The presence of ANXA3 in urinary exosomes and prostasomes might be the reason for its remarkable stability in urine [16, 17]. ANXA3 has been quantified by western blot and immunohistochemistry studies in the urine samples [18]. ANXA3 has an inverse relationship to cancer and the staining pattern of ANXA3 in prostatic tissue was reported to correlate with Gleason score and was able to differentiate lower and higher malignant cases. Moreover, staining had also shown an apparent correlation during the whole process of prostatic transformation, ranging from benign prostatic hyperplasia via prostatic intraepithelial neoplasia (PIN) to the various stages of PCa [18].

δ-Catenin

δ-Catenin is an adhesive junction associated protein in the β-catenin superfamily. δ-Catenin is a unique β-catenin superfamily protein primarily expressed in the brain, but is up-regulated in human prostatic adenocarcinomas. δ-Catenin, caveolin-1, and CD59 were all detected in cell-free human voided urine prostasomes. δ-Catenin immunoreactivity was significantly increased in the urine of prostate cancer patients [19].

Endoglin

Endoglin is a transmembrane glycoprotein that is otherwise known as CD105. While the expression of this protein is not prostate restrictive or even prostate cancer selective, it has been shown by a number of groups to be expressed by vascular endothelial cells and therefore found to be elevated in cancerous states. Investigators have examined the ability to detect endoglin in the plasma as well as the urine of men with prostate cancer [20]. Urinary levels of endoglin are increased in men with prostate cancer compared to levels in men without prostate cancer, and serum endoglin levels appear to correlate with increasing prostate cancer stage. It has weak or negative expression in normal tissues [21, 22]. Immunohistochemical analysis has shown endoglin to be expressed not only by endothelium associated with prostate cancer, but also by some prostatic intraepithelial neoplasia (PIN) and prostate cancer epithelial cells and associated stromal components [23].

Estrogen metabolites

Kosti et al., [24] evaluated urinary estrogen metabolites as a biomarker of prostate cancer risk. Using a liquid chromatography–tandem mass spectrometry method, urinary concentrations of 15 estrogen metabolites were determined. The two main pathways for metabolism of estrogens are 16α-hydroxylation and 2-hydroxylation. The major estrogen metabolites excreted in urine are the parent estrone (E1) and 17β-estradiol (E2), 2-hydroxy products [2-hydroxyesterone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 2-methoxyesterone (2-MeOE1)], and 16α–hydroxy products [estriol (E3) and 16α-hydroxyestrone (16α-OHE1). The 16α-metabolites are considered the dominant biologically active metabolites, while the 2-OHE1 is less estrogenic. Association between the urinary 16-ketoestradiol (16-KE2) and 17-epiestriol (17-epiE3)—metabolites with high estrogenic activity—and prostate cancer risk was detected. Both 16-KE2 and 17-epiE3 are products of the 16α-hydroxylation pathway and derive from the biologically active 16α-OHE1 metabolite. Men in the lowest quartile of 16-KE2, had a 4.6-fold risk of prostate cancer, compared with those in the highest quartile.

GOLM1

GOLM1 (Golgi membrane protein 1 alias GP73 or GOLPH2) is a resident cis-Golgi membrane protein of unknown function. The first evidence of its up-regulation was shown in the hepatocytes of patients with acute and chronic forms of hepatitis and hepatocellular cancer. GOLM1 has a single N-terminal transmembrane domain and an extensive C-terminal, coiled-coil domain that faces the luminal surface of the Golgi apparatus. N-terminal cleavage by a furin proprotein convertase resulted in the release of the C-terminal ectomain and its appearance in serum [25].

GOLM1 transcript levels were measured in urine sediments using quantitative PCR on a cohort of patients presenting for biopsy or radical prostatectomy. It was found that urinary GOLM1 mRNA levels were a significant predictor of prostate cancer [26]. Authors have suggested, that GOLM1 had the best discriminatory power in distinguishing between urine from prostate cancer patients and control populations representing a sensitivity and specificity of 75% and 72%, respectively.

LOH

LOH (loss of heterozygosity) might be the most common deletion event in prostate cancer. Cussenot et al. assessed LOH at 7q, 8p, 13q, and 16q chromosome arms using two microsatellite markers (CA repeats) per chromosomal hot spot deletion region, and obtained a sensitivity of 73% (39-94%) and a specificity of 67% (43-85%) for LOH at one or more of the locations [27]. Using additional microsatellite markers localized on chromosome arms 12p13 and 18q21, Thuret et al. obtained a sensitivity and specificity of LOH detection at 86.7% (72-96%) and 44% (29-60%), respectively [28]. Authors suggested that the presence of LOH of urinary prostatic cells obtained after prostatic massage is significantly associated with PCa on biopsy and may potentially help to identify a set of patients who are candidates for further prostate biopsies.

Met

Met, a receptor tyrosine kinase, has received much attention for its overexpression and/or mutation in a number of malignancies. Multiple lines of evidence point to the importance of met in PCa initiation and progression. *In vitro* studies have shown that met expression is inversely correlated with androgen receptor expression [29]. In prostatectomy specimens, met expression was shown to correlate with progressive disease. A subsequent study showed similar results reporting uniform met positivity in prostate

bone metastasis. Thus, both experimental as well as clinical data have associated met activity with PCa progression. Russo et al., [30] investigated met as a biomarker of disease progression, and urinary met expression was evaluated *via* ELISA in localized and metastatic prostate cancers. Met distribution was significantly different between the metastatic group and the group with localized prostate cancer and people with no evidence of cancer. Urinary met may provide a noninvasive biomarker indicative of metastatic prostate cancer and may be a central regulator of multiple pathways involved in prostate cancer progression [30].

Methylation

One type of epigenetic aberration is DNA methylation—the addition of a methyl group to the 5'-carbon of cytosine in CpG islands catalyzed by DNA methyltransferases (DNMTs). Hypermethylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes correlates with loss of gene expression in human tumors [31-35]. Hypermethylation of regulatory sequences at the detoxifying GSTP1 gene locus is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues nor in benign hyperplasia of the prostate [36]. GSTP1 methylation is thus the most common genetic alteration thus far described in prostate cancer, and this assay sensitivity was between 19% and 76%, and specificity ranged from 56% to 100% [36].

Other candidate genes have been examined for hypermethylation along with GSTP1. Two recent studies looked at a panel of 10 candidate genes (APC, DAPK, ECDH1, GSTP1, MGMT, p14 [ARF], p16, RARβ2, RASSF1a, and TIMP3) [32, 37]. In the first study, the four most common hypermethylated genes were GSTP1, p16, ARF, and MGMT. In the second study, the four genes with the greatest difference (GSTP1, APC, RASSF1a, and RARβ2) had sensitivity for prostate cancer detection of 86% and a diagnostic accuracy of 89% [38].

Payne et al., using real-time PCR, measured four DNA methylation biomarkers: GSTP1, RASSF2, HIST1H4K, and TFAP2E in sodium bisulfite-modified DNA. GSTP1 methylation was found in 81% of PCa urine samples and in 39% of PCa plasma samples. Methylation of the remaining genes was found in 92-100% of PCa urine samples and in 18-31% of PCa plasma samples. The biomarkers were also detected in negative controls, but at a lower frequency with the exception of TFAP2E in urine samples [39].

MMPs

Matrix metalloproteinases (MMPs) have been implicated in invasion and metastasis of human malignancies. MMP9 yielded better sensitivity (64%) than MMP2 (39%) for PCa whereas specificities (84 and 98%, respectively) were calculated from controls of both sexes. Also the ~140, >220, and ~190 kDa gelatinase species were identified as MMP9/TIMP1 complex, MMP9 dimer, and ADAMTS7, respectively [40]. Also, Di Carlo et al. detected that MMP-9 activity is enhanced in the urine from patients with benign prostate hyperplasia compared with cancer patients [41].

PCA3

The PCA3 (prostate cancer antigen-3) gene, also known as DD3 (differential display 3) consists of four exons whereas exon 2 is deleted from most transcripts (present in only 5% of the transcripts) and alternative polyadenylation can occur at three different positions in exon-4. There is a high density of stop codons in all three open reading frames, and thus, PCA3 belongs to the class of noncoding RNAs. PCA3 encodes a prostate-specific mRNA that has shown promise as a PCa diagnostic tool. Measurement of PCA3 mRNA normalized to PSA mRNA in urine has been proposed as a marker for PCa [42].

The PCA3 gene is highly specific for prostate cancer and is detectable in prostate cancer cells shed into urine after rectal palpation, compared with BPH tissue. A noncoding messenger RNA is expressed by the PCA3 gene in epithelial prostate cells and is overexpressed in prostate cancer tissue samples compared with nonmalignant tissue [14].

Three diagnostic tests have been developed that measure PCA3. The first was a dual time resolved fluorescence-based RTPCR assay used during the primary study in the Netherlands, and the second was uPM3™ (Bostwick Laboratories, Glen Allen, VA), a lab-developed test using nucleic acid sequence based amplification. The third is APTIMA® (Gen-Probe Incorporated; San Diego, CA), which uses transcription mediated amplification and is the only reagent currently available commercially [43].

Appropriate PCA3 scores provided a relatively high level of sensitivity (range 61-82%) and specificity (44-46%). The addition of PCA3 to the urologist's diagnostic tools will not result in a state of certainty, however, the diagnostic sensitivity, specificity, and predictive value are incrementally improved by its inclusion [47].

Sarcosine

Sarcosine is a derivative of the amino acid glycine, formed by the enzymes glycine N-methyl transferase (GNMT) or dimethylglycine dehydrogenase (DMGDH), and converted back into glycine via sarcosine dehydrogenase (SARDH). Sarcosine, an isomer of *L*-alanine, has been proposed as a prostate cancer progression biomarker by Sreekumar et al., [48]. The technique used to assess sarcosine levels in urine was gas chromatography-mass spectrometry (GC-MS). Both compounds are detected in urine, where the measured sarcosine/alanine ratio has been found to be higher in the prostate biopsy-positive group versus controls. These data have shown that sarcosine is not only a novel and predictive biomarker, but also a key element of a potentially promising target pathway for the treatment and control of prostate cancer development [49].

Also Martínez-Lozano and Rus successfully explored the ability of a differential mobility analysis-mass spectrometry (DMA-MS) system to discriminate the isomers *L*-alanine and sarcosine in urine [50]. However, Jentzmik et al. (2010) suggested that sarcosine in urine after digital rectal examination cannot be considered as a suitable marker to differentiate between patients with and without PCa [51].

TERT

Telomerase reverse transcriptase (TERT) maintains the telomeric ends of chromosomes and if telomerase is active, cancer cells may escape cell cycle arrest and replicative senescence. Several groups have measured telomerase activity with the telomeric repeat amplification protocol assay and obtained sensitivities of 58, 90, and 100% and specificities of 100, 87, and 89%, respectively [15]. However, Crocitto et al. measured TERT mRNA expression by reverse transcription-PCR and obtained a sensitivity and specificity of 36 and 66%, respectively [52].

Fusion in the ETS gene family

It has recently been shown that the majority of prostate cancers harbor a chromosomal rearrangement that fuses the gene for an androgen-regulated prostate-specific serine protease, TMPRSS2, with members of the ETS family - ERG [v-ets erythroblastosis virus E26 oncogene homolog (avian)] (21q22.2) and ETV1 (ets variant 1) (7p21.1). These are among the most common genetic alterations in any human solid tumor. This knowledge may provide us with clues to prostate carcinogenesis, and may lead to the development of important molecular-based biomarkers for patients with local-

ized prostate cancer. The most common variant is fusion between the 5'-untranslated region of TMPRSS2 and the 3' region of ERG [53-55].

The consequence of the most common gene fusion is to generate a hybrid transcript that attaches the prostate-specific promoter sequence of the TMPRSS2 gene to the ERG oncogene open reading frame (ORF). The proteins sequences have been predicted from the sequence of the fusion ORFs [54]. Clark et al., studied cDNAs prepared from ERG mRNAs isolated from prostate cancers. They reported that of the 14 different fusion transcripts identified from the cDNA sequence, five would be predicted to generate premature stop codons and would be unlikely to encode for a functional ERG protein. In most cases, no amino acid sequence derived from TMPRSS2 is integrated in the hybrid ORF, and therefore a fusion protein is not created [56].

The fusion of TMPRSS2 with ERG or ETV1 only occurred in cases with overexpression of the respective ETS gene, and fusions were not detectable in benign prostate tissues. Analysis of TMPRSS2:ERG-positive and TMPRSS2:ERG-negative prostate cancer cell lines showed that the TMPRSS2:ERG fusion resulted in androgen-regulated expression of ERG. Thus, the androgen-responsive elements that normally restrict the expression of TMPRSS2 to the prostate drove the aberrant overexpression of 50 truncated ETS oncogenes [57].

Two studies have been conducted on DNA specimens isolated from urine from men known to have prostate cancer with a gene rearrangement. The sensitivity of the urine test was only 37% and the specificity was 93% [58]. The fusion of these genes is seen in 40-80% of prostate cancer patients, approximately 20% of prostatic intraepithelial neoplasia (PIN) cases, and rarely in benign prostatic tissue [59]. It is possible that future assays will have comparatively better sensitivity or that the presence of the fusion gene in urine could supplement a panel of markers in a screening setting [60].

Multiplex Biomarker Analysis

Although urine-based testing for PCA3 expression has already been documented in large screening programs [44], the feasibility of testing based on other markers has not been rigorously evaluated. Importantly, single marker tests, such as those based on PCA3, ignore the heterogeneity of cancer development and may only capture a proportion of cancer cases. To overcome this limitation, multiplexing, or combining, biomarkers for cancer detection can improve testing characteristics. Laxman et al., measured the expression of seven putative prostate cancer biomarkers (AMACR,ERG, GOLPH2, PCA3, SPINK1, TFF3, and TMPRSS2:ERG) in sedimented urine using quantitative PCR. By univariate analysis, they found that increased GOLPH2, SPINK1, and PCA3 transcript expression and TMPRSS2:ERG fusion status were significant predictors of prostate cancer. The sensitivity and specificity for the multiplexed model were 65.9% and 76.0%, respectively [61].

Cao et al., determine that the multiplex model adds even more to the diagnostic performance for predicting PCa than the single biomarker assay. Using quantitative PCR, Western blot, and liquid chromatography-mass spectrometry, they examined expression patterns of PCA3, TMPRSS2: ERG, annexin A3, sarcosine, and PSA in urine samples. The expression patterns of studied genes and a panel including these biomarkers were significant predictors. Employing ROC analysis, the areas under the curve for the panel of both of these cohorts were 0.840 and 0.856, respectively, which outperform that of any single biomarker (PCA3: 0.733 and 0.739; TMPRSS2: ERG: 0.720 and 0.732; annexin A3: 0.716 and 0.728; sarcosine: 0.659 and 0.665, respectively). The authors suggested that, compared with single biomarker, the multiplex model, which includes PCA3, TMPRSS2:ERG, annexin A3, and sarcosine, adds even more to the diagnostic precision for predicting PCa [62].

In another study Jiang and coworkers using liquid chromatography with tandem mass spectrometry examined sarcosine, proline, kynurenine, uracil, and glycerol 3-phosphate concentrations in metastatic prostate cancer urine samples. The authors suggested that this technique can be used successfully for quantifying these five metabolites in urine samples for potential early cancer screening [63].

Talesa et. al., investigated the diagnostic value of five different genes, associated with PCa carcinogenesis, encoding for prostate-specific membrane antigen (PSMA), serine protease hepsin, PCA3, UDP-N-acetyl-alpha-D-galactosamine transferase (GaINAC-T3), and prostate-specific antigen (PSA). They demonstrated that the diagnostic potential of the combined urinary PSA and PSMA level was significantly better than that of each singularly considered marker, including total serum PSA [64].

Prior and coworkers analyzed expression of AMACR and MMP-2 proteins, and GSTP1/RASSF1A methylation status, in addition to PSA levels. Sensitivity, specificity, area under the ROC (AUROC) curves, and discriminant function analysis were assessed to determine the diagnostic potential of each variable alone or in combination. Sensitivity and specificity for methylation status were 53.3 and 45.9%, respectively. Sensitivity, specificity, and positive and negative predictive values for the combination of all biomarkers were 57.1, 96.6, 88.9, and 82.4%, respectively, and the authors conclude that analysis of this combination of biomarkers in body fluids significantly improves the diagnosis of PCa compared to the PSA test [65].

CONCLUSIONS

One of the biggest challenges in the management of cancer remains the lack of prognostic and predictive biomarkers that can help design a therapeutic strategy as well as monitor its tumor response. Current clinical practice in oncology has a growing impetus on early diagnosis, proper prognostication and screening for malignancy in asymptomatic groups. Tumor markers are assuming a growing role in all aspects of cancer care, starting from screening to follow-up after treatment. Important clinical decisions are increasingly likely to be made on the basis of these results, whether for diagnosis, screening, prediction or treatment monitoring. The emerging urine tests should help in both early diagnosis of prostate cancer and identifying aggressive tumors for radical treatment. Of the numerous tumor markers identified, described and extensively researched upon, only a handful of them are used in routine clinical practice; and even of these, only a few have established consensus guidelines for use in the day-to-day care of patients. It is hoped that the use of panels of markers can improve PCa diagnosis and prognosis and help predict the therapeutic response in PCa patients.

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