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Molecular Diagnostic Trends in Urological Cancer: Biomarkers for Non-Invasive Diagnosis

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Abstract

The early detection of urological cancers is pivotal for successful patient treatment and management. The development of molecular assays that can diagnose disease accurately, or that can augment current methods of evaluation, would be a significant advance. Ideally, such molecular assays would be applicable to non-invasively obtained body fluids, enabling not only diagnosis of at risk patients, but also asymptomatic screening, monitoring disease recurrence and response to treatment. The advent of advanced proteomics and genomics technologies and associated bioinformatics development is bringing these goals into focus. In this article we will discuss the promise of biomarkers in urinalysis for the detection and clinical evaluation of the major urological cancers, including bladder, kidney and prostate. The development of urine-based tests to detect urological cancers would be of tremendous benefit to both patients and the healthcare system.

Keywords

Biomarkers; body fluids; diagnosis; non-invasive; urinary; urological cancer

INTRODUCTION

The urologic cancers include cancers of the bladder, kidney, prostate and testicles, and as a group these conditions are highly prevalent. Prostate cancer is the second most common cancer in American men, second only to skin cancer, with some 250,000 new cases estimated to be diagnosed in 2011. Bladder cancer is the fifth most common cancer among men and the ninth most common among women in the United States, with over 50,000 new cases of bladder cancer each year. There are also over 50,000 newly diagnosed kidney cancer cases per year in the United States. All of these cancers are associated with age, the majority of cases being diagnosed in patients >55 years old [1]. Conversely, testicular cancer is most common in young or middle-aged men, and is relatively rare with some 8,000 new cases per year in the United States.

As is the case for all diseases, the identification of biomarkers that can be informative of phenotype or biological function could greatly facilitate the detection and diagnosis of a specific urologic condition. While histopathology of excised tissue or biopsy material remains the gold standard for clinical diagnosis, non-invasive diagnostic techniques are desirable, and are thus a major focus of current research. For the urological cancers, the

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CONFLICTS OF INTEREST NOTIFICATION

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accessibility of urine is a major advantage. The sample can be obtained non-invasively, avoiding patient discomfort and potential complications from an invasive procedure, copious amounts of sample can be obtained for analysis, and repeat sampling is easily achievable. Once accurate and robust assays are developed, urinalysis is amenable to screening at risk or asymptomatic populations.

Detecting urological cancers using diagnostic markers remains a challenge. The inadequate power of single markers may partly explain this. The concept that the presence or absence of one molecular marker will aid clinical evaluation has not proved to be the case. This makes sense when one considers the complex interactions between various molecules within a single pathway, the cross-talk between molecular pathways, and the oligoclonality of many tumors. There needs to be an evolution from single-marker research to a more global assessment strategy and the development of multiplex biomarker assays. The advent of new high-throughput genomic and proteomic techniques is driving biomarker discovery forward. These approaches are being used to derive biomarkers of disease status, but they may also provide insight into mechanistic information and reveal therapeutic targets. In this article, we review the advances in urinalysis for the diagnosis of bladder, prostate and kidney cancer, highlighting some of the techniques used for biomarker discovery.

SECTION 1

URINARY DIAGNOSTIC BIOMARKERS FOR BLADDER CANCER

Transitional cell carcinomas (TCCs) are the most common form of bladder cancer (BCa) in Western countries and constitute approximately 95% of all cases [2]. Risk factors associated with the development of BCa include carcinogens in tobacco smoke, and to a lesser extent exposure to chemical compounds in the color and rubber industries. The disease has a 5-times higher prevalence among men than women and the median age at diagnosis is 65 years. At presentation, more than 80% of BCa are non-muscle invasive tumors (stages pTa or pT1). The remaining 20% of tumors show muscle invasion at the time of diagnosis and have a much less favorable prognosis (5-year survival < 50%). When detected early, the 5-year survival rate for BCa is approximately 94%, thus timely intervention can dramatically increase the probability of patient survival. While radical surgery is required for muscle invasive BCa, non-muscle invasive BCa can be treated more conservatively by transurethral resection of the tumor. However, more than 70% of patients with Ta/T1 BCa will have a recurrence during the first two years after diagnosis. If left untreated these initially non-muscle invasive BCa can progress to being muscle invasive [3]. The recurrence phenomenon of non-muscle invasive BCa makes BCa one of the most prevalent cancers worldwide. Once treated, BCa patients are under continued surveillance with routine cystoscopy examinations and cytology for early detection of new BCa development. Consequently, the development of a non-invasive urine based assay using reliable diagnostic markers would be of tremendous benefit to both patients and the healthcare system.

The gold standard for initial clinical diagnosis of BCa involves cystoscopic examination of the bladder. Cystoscopy is an unpleasant invasive procedure, which may involve anesthetizing the patient followed by biopsies for histopathological diagnosis and staging. For the non-invasive evaluation of patients with possible BCa, voided urinary cytology (VUC) currently remains the method of choice [4, 5]. VUC relies on the microscopic visualization of shed cancer cells in voided urine. Low-grade tumors and low stage tumors (Ta-T1) shed less cancer cells into the urine, and so the sensitivity for detecting these early-stage tumors by VUC typically ranges from 20 to 40% [6, 7]. Furthermore, results are not available rapidly, it is prone to inter-observer variation [8], and it is relatively expensive. Understandably, voided urine cytology is rarely the single test performed during a clinical workup to investigate potential bladder cancers [9].

Commercially Available Bladder Cancer Urinary Assays—A number of commercial molecular tests have been developed to detect putative tumor-associated molecules that are released into the urine from malignant lesions. Tests include the measurement of bladder tumor antigen (BTA) [10] by BTASat, an immunochromographic, qualitative point-of-care assay, or by BTATrak, an ELISA test that measures the human complement factor H-related protein in a quantitative fashion [11]. BTASat is FDA approved for surveillance (monitoring) of bladder cancer in conjunction with cystoscopy, but not for screening/diagnosis. Monitoring of nuclear matrix protein 22 (NMP22/NUMA1) [12, 13] has also developed into an immunochromographic, qualitative point-of-care assay. The NMP22 assay is a major advancement in the identification and adoption of an inexpensive urine-based tumor marker into today's clinical practice, but specificity can be compromised by the fact that NMP 22 is also elevated in pyuria, urolithiasis, in the presence of foreign bodies, hematuria or cystitis [14, 15]. Unfortunately, there is no protein test that has been developed to date that can replace cystoscopy or VUC [16, 17]. The analysis of naturally shed urothelial cells can be enhanced by combining VUC with molecular assays. The ImmunoCyt assay uses fluorescent monoclonal antibodies to detect mucin antigens and a BCa glycoform of the carcinoembryonic antigen that can be applied to urine cytology slides [18, 19]. Urovysion™ is FDA approved as a urine marker for the diagnosis of bladder cancer as well as surveillance of bladder cancer. Previously, cytogenetic studies have demonstrated aneuploidy of chromosomes 3, 7 and 17, and loss of 9p21 locus in bladder cancers [20]. A multicolored, multiprobe FISH assay has been developed by Abbott Laboratories (Abbott Park, IL, USA) that stains exfoliated cells in urine specimens for these chromosomal alterations [21]. The criteria for detecting bladder cancer include finding more than five urinary cells with gains of more than two chromosomes, or more than ten cells with a gain of a single chromosome, however, these techniques can also be complex, and require skillful interpretation. These currently available tests have been reviewed in detail elsewhere [15] and are summarized in Table 1.

Candidate Urinary Biomarkers for Bladder Cancer Detection—The search for more accurate, non-invasive urine tests for more reliable diagnosis is warranted, and some of the experimental markers being identified in targeted studies are described below. Hyaluronic acid (HA) and enzymes that metabolize this tissue matrix component have been investigated as potential urinary markers for the detection of bladder cancer. HA has been reported to be elevated in a number of cancers, including bladder cancer, and urinary HA has been shown to be indicative of BCa [22]. HA is synthesized by HA synthases HAS1-3 and degraded by hyaluronidase (HAase) an enzyme produced by the liver. Factors in the HA metabolism system have been shown to have effects on the behavior of bladder tumor cells [23, 24], and as biomarkers for BCa detection. When used in combination, The monitoring of HA and HAase using immunoassays achieved a combined diagnostic sensitivity of 83% and specificity of 78%, outperforming Immunocyt and cytology [18]. Levels of HA family transcripts in exfoliated urothelial cells may be associated with disease recurrence [25].

The application of proteomic and nucleic acid screening approaches has, to date, primarily focused on solid tissue specimens [26], and many deregulated mRNA transcripts, miRNAs, and proteins have been identified as being associated with BCa. Due to the lack of normal tissue for comparison, such studies have tended to identify factors that can classify tumors or predict patient outcome or disease recurrence, but biomarkers identified in solid tissue may be subsequently translated to a serum or urinary test, and some have shown promise as urinary diagnostic markers. Examples from proteomic analyses include psoriasin for squamous cell carcinoma [27] and cystatin B [28]. Proteomic analysis of the urine is more likely to reveal promising urinary biomarkers.

Proteins as Bladder Cancer Urinary Biomarkers—Two-dimensional electrophoresis (2-DE) of proteins has been the conventional method for biomarker assessment in urological proteomics [29, 30]. Irmak *et al.* identified two proteins, orosomucoid (ORM) and zinc-alpha glycoprotein (ZAG) which were increased in urine samples of tumor-bearing patients in comparison with samples from a few healthy volunteers [31], and Pinero *et al.* used 2D-DIGE coupled with mass spectrometry to identify regenerating protein-1 (Reg-1) and keratin 10 as being associated with bladder cancer [32]. Saito *et al.* [33] used a 2-D PAGE approach, but focused specifically on proteins of the extracellular matrix and matrix metalloproteinases in urine. Samples enriched with gelatin-affinity beads revealed that MMP-2, MMP-9, and fibronectin (FN) fragments were present in cancer patients, but not in healthy individuals [33]. To date, a limited number of proteomic studies have utilized MS technologies for the analysis of urological cancers, largely because of the lack of defined methodologies that can reduce the complexity of the sample and rapidly and accurately identify specific proteins. A number of studies have used SELDI-TOF to identify peptide signatures associated with bladder cancer, but validation of specific targets has not been forthcoming. Theodorescu *et al.* employed online coupling of capillary electrophoresis (CE) to an electrospray time-of-flight mass spectrometer (ESI-TOF-MS) to identify a 22-peptide signature that could discriminate between samples of bladder cancer and healthy subjects [34]. The signature was able to correctly classify 31 bladder cancer patients from a cohort that included 149 samples from healthy or non-malignant urological diseases. A recent study used an iTRAQ (isobaric tag for relative and absolute quantitation) technique to discover proteins that were differentially expressed between pooled urine samples and non-tumor controls. This strategy identified 55 candidate biomarker proteins. Orthologous techniques confirmed that the level of apolipoprotein A-I (APOA1) was significantly elevated in urine samples from bladder cancer patients. Using a commercial ELISA assay, APOA1 was confirmed to have high diagnostic potential in an extended sample set [35]. Using a glycoprotein enrichment strategy to profile urine samples from 100 subjects (54 cancer cases), we identified a panel of glycoproteins associated with BCa [36]. The most discriminatory protein in that study was alpha-1-antitrypsin (A1AT), also known as SERPINA1. In an independent validation cohort of 70 subjects, A1AT measurement by ELISA achieved a sensitivity of 74% and a specificity of 80% [36]. Collectively, these studies show the power of MS-based urinary analysis for the discovery of biomarkers.

Although they can outperform cytology in many cases, there is an apparent trend that limits single biomarkers to sensitivity and specificity thresholds around 70–80%. Multiples of protein biomarkers may be able to raise the diagnostic performance to higher levels. Based on the fact that cancer often involves inflammatory processes, Margel *et al.* monitored a panel of immune modulators in urine to investigate biomarker potential [37]. The panel included 15 heat shock proteins and cytokines, monitored by commercial ELISA assays. The combined urinary concentrations of HSP60 and IL-13 significantly improved the performance over any single factor. Abogunrin *et al.* tested the performance of 22 urinary biomarkers in combination with BTA and NMP22 commercial tests. Algorithms that combined demographic information with NMP22, epidermal growth factor or thrombomodulin were able to achieve 91% sensitivity and 70–80% specificity in a cohort of 157 cases [38].

Transcriptomics and Bladder Cancer Urinary Diagnosis—The advent of high-throughput microarray gene expression technology has greatly enabled the search for disease biomarkers. Numerous exploratory studies have demonstrated the potential value of gene expression signatures in tumor diagnosis and classification [39, 40], and in assessing the risk of post-surgical disease recurrence [41–43]. Gene expression profiling studies of urological clinical material have focused on the analysis of excised solid tumor tissue. These studies have identified gene signatures that are associated with tumor stage [44, 45], disease

recurrence and outcome prediction [44–46], and subtype classification [45]. The fact that follow-up studies have validated some of the biomarkers in independent tissue collections shows the potential utility of microarray profiling of solid tissue source materials [47, 48], however, the molecular analysis of solid tissue is most applicable to the development of assays that will aid the histological evaluation of biopsy or excised tumor material. Normal tissue is not available for comparison for obvious reasons. In order to progress towards the development of novel molecular assays for non-invasively obtained material, the more clinically appropriate material for profiling is the urine, more specifically, the transitional urothelia that are naturally shed into the urine. We have tested the feasibility of genome-wide profiling (~47,000 transcripts) of the urothelial component of urine, and showed that the approach can identify tumor-associated profiles with high diagnostic accuracy [49]. We have subsequently expanded the study to profile samples from over 90 cases. Analyses of the resulting dataset using our recently developed feature selection algorithms [41–43, 50] revealed an optimal gene signature for discriminating between cancer and non-cancer groups. We went on to validate this gene signature using targeted quantitative PCR in an independent cohort of 81 cases. A urothelial cell signature comprised of 14-genes was able to detect bladder cancer with 100% specificity at 90% sensitivity. In comparison, cytological evaluation of this cohort diagnosed only 35% of tumor cases correctly. Of the genes in our diagnostic signature some have been implicated in BCa previously (*MMP12*, *CA9*, *ERBB2*, *MMP9*, *VEGF*) but many have not (*CCL18*, *AGT*, *CRH*). Mengual *et al.* reported the results of a study utilizing a similar strategy [51]. Their approach monitored 384 genes in urothelial samples using quantitative RT-PCR. The 384 genes were selected from a previous study that profiled only 12 solid bladder tissue samples using a cDNA microarray platform. Analyses identified a 12-gene signature that achieved high accuracy (89% sensitivity and 95% specificity) in diagnosing BCa in a cohort of 211 subjects. Despite significant differences between the studies with respect to the biomarker discovery phase, both groups were able to derive molecular signatures that could accurately classify BCa samples. This demonstrates that a multiplex quantitative PCR test on voided urine sample holds promise as a non-invasive urine-based assay in the evaluation of patients being investigated for BCa. Although a quantitative PCR test has some upfront processing requirements, it has the advantage of being developed into an assay that can be automated and highly standardized for consistency between laboratory sites.

RNA-based Bladder Cancer Urinary Biomarkers—Another component available for measure in urine is soluble RNA, including both mRNA or microRNA (miRNA) targets. Holyoake *et al.* also published a study that used molecular profiling of solid tissues to identify genes over-expressed in tumor stages Ta, T1 or >T1, relative to non-tumor epithelial tissues and inflammatory cells [52]. Using this strategy, *CDC2*, *MDK*, *IGFBP5*, and *HOXA13* were selected for development of a quantitative RT-PCR urine assays for TCC detection and disease risk stratification of patients. RNA for the assay was extracted from voided urine samples, but it is not clear whether this extraction would have included urothelial cells or only soluble urinary RNA. The combination of RNAs detected BCa at a sensitivity of 85% and a specificity of 80% across all stages, with the best performance with stages >T1 and tumors >1cm in diameter [52]. Hanke *et al.* analyzed the expression of a selected panel of mRNAs as biomarkers of BCa in whole urine, cell pellets and clarified urine. In a cohort of 98 subjects, they found that the ratio of v-ets erythroblastosis virus E26 oncogene homolog 2 (*ETS2*) to urokinase plasminogen activator (*PLAU*) in whole urine facilitated the detection of BCa with a sensitivity of 75% at 100% specificity [53]. Other mRNA-based diagnostic urinalyses have targeted *BIRC5* (survivin), *HYAL1*, *KRT20* and *MUC7* [54–56]. These targets performed similarly at sensitivities between 62–90%, and indications of combinations of 2–3 mRNA markers performing better than single target

assays. Going forward it may be beneficial to focus on either cell pellets or cleared urine and to normalize to cell content or other stable, non-cellular housekeeping transcripts.

MicroRNAs (miRNAs) are key post-transcriptional regulators of gene expression [57]. To date, >1500 human miRNAs have been identified and characterized to some extent. Each miRNA controls the expression of multiple genes, and so this molecular family may represent an opportunity to identify biomarkers of a higher order. Array-based profiling and deep-sequencing approaches for miRNA analysis are becoming routine technically, and studies targeting miRNAs as potential diagnostic biomarkers are increasing accordingly. Excised tissue profiling of 290 miRNAs identified miR-21 as being most associated with BCa, and miR-129 as being most associated with poor outcome [58]. Analysis of miRNAs by deep sequencing of bladder tumors identified miR-96 as most associated with urothelial carcinoma [59]. On the urinalysis front, Hanke et al examined the expression of 157 miRNAs in exfoliated urothelial cells using quantitative RT-PCR. The study found that the ratio of miR-126 to miR-182 achieved 72% sensitivity and 82% specificity [60] in a cohort of 47 samples.

DNA-based Bladder Cancer Urinary Biomarkers—The DNA present in tumor cells is also a resource for diagnostic biomarker discovery. Beyond the detection of gross chromosomal aberrations used for urothelial cell FISH analyses, such as UroVysion (described above), alterations to DNA copy number, sequence, methylation status provide multiple targets for potential development. Bladder cancer, with tumor cells being bathed in urine perhaps provide the best potential use of DNA markers, but there may be opportunities in renal and prostate cancer also. A comparative genome hybridization (CGH) chip, including loci proposed to be associated with BCa was designed by Larre *et al.* for the assessment of bladder tissues. The CGH data was used to derive a test that could be used on urothelial cell pellets. This test achieved an overall diagnostic accuracy of 91% in a cohort of 44 samples [61]. The detection of specific urothelial gene mutations is also applicable to disease evaluation. A subset of bladder tumors are reported to have *FGFR3* mutations (mt), and these can be detected in urothelial cell pellets by PCR. Zuiverloon *et al.* recently reported the optimization of *FGFR3* mt analysis in 440 urine samples. The test can be very sensitive, but is only perfect with large tumors, and is only applicable to the *FGFR3* mt subset of BCa cases [62]. Finally, the DNA methylation status of specific gene promoter regions in bladder tumor cells has been proposed as markers for primary diagnosis and for detection of recurrence. Experiments with BCa cell lines and analyses of excised tissues were used to identify a list of novel methylation candidates [63]. The methylation status of 3 genes (*GDF15*, *TMEFF2* and *VIM*) were identified as epigenetic biomarkers for BCa, and achieved very high accuracy (94% sensitivity, 100% specificity) when used as a panel in analysis of urine sediments. The same group has recently reported that the methylation of 2 genes (*TCF21* and *PCDH17*) is associated with all urological cancers. In urine samples, the sensitivity was 60%, 32%, and 26% for bladder, renal, and prostate tumors, respectively [64]. Using a 4-gene panel of methylation probes, Zuiverloon *et al.* were able to predict BCa recurrence in 268 cases with sensitivities and specificities between 52% to 72% [65]. The methylation assays require further development, but appear to hold promise.

Metabolomics in Bladder Cancer Urinary Diagnosis—The most recent developments in biological fluid analyses have come in the field of metabolomics. Application of urine-based metabolomics, using high pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) may identify specific metabolites or profiles that can aid diagnosis of BCa [66]. Two groups have used spectrometry to show the proof-of-principle that global urinary profiles could distinguish BCa cases from controls with very high accuracy [67, 68], however, there are major problems with urinary metabolomic profiling. The analytes are small metabolites that are not always filtered by the

kidney, so confounding factors such as polypharmacy or even recent dietary intake can create large variations between individuals and so such studies require large cohorts.

Summary: Bladder Cancer Urinary Biomarkers—The impetus for the search for bladder cancer biomarkers comes from the idea that an accurate biomarker can reduce the number of cystoscopies performed each year, and thus, cut down the frequency of this invasive and costly procedure. The emergence of high-throughput technology promises to identify tumor-associated molecules that are available in non-invasively obtained urine samples. The biomarkers may be soluble proteins, metabolites or nucleic acids, or may be specific changes detectable within the genome and/or transcriptome of urothelial cells that are shed from the bladder tumor and can be readily recovered from urine samples (Table 2). The trend towards panels of biomarker rather than single target assays is encouraging, and it may well be that the optimal tests of the future will consist of combinations of not only multiple targets, but of mixed molecular classes.

SECTION 2

URINARY DIAGNOSTIC BIOMARKERS FOR PROSTATE CANCER

Screening for prostate cancer involves both a digital rectal examination (DRE) and measurement of the serum PSA level [69]. The American Cancer Society recommends that men who choose to undergo screening should begin at age 50, but they also recommend that screening with PSA and DRE be stopped at age 75, given the low likelihood of death from de novo prostate cancer after this age. Screening of asymptomatic individuals with the PSA test has been the focus of mounting criticism of late, primarily due to concerns of unnecessary biopsies and potential overtreatment. The debate would become moot if research efforts can identify more robust biomarkers amenable to testing in serum or better yet, non-invasively obtained urine. Recent efforts have identified promising protein-based, metabolite-based and nucleic acid-based urine biomarkers and these are under further study to determine potential clinical utility. Novel tests may outperform the PSA test, but may be more valuable as an adjunct to the PSA test. As described for bladder cancer above, candidate biomarkers will be discussed in groups with respect to molecular class, soluble proteins DNA methylation, mRNA, microRNA etc.

The most developed urinary biomarkers for prostate cancer (PCa) detection are RNA markers, and the most prominent of those is prostate cancer antigen 3 (*PCA3*), a non-protein coding RNA. Differential display analyses of prostate tumor and adjacent non-malignant tissue identified a prostate-specific gene that was highly expressed in PCa [70]. The higher level of *PCA3* associated with malignancy can be accurately measured using quantitative PCR assay and has proven useful in excised tissue analysis, biopsy, and more recently in urinalysis. Assays require DRE in order to encourage the shedding of prostate tumor cells into the urine. Isolation of the shed cells and/or exosomes [71], *PCA3* and *PSA* transcripts are measured and the ratio between the two is used to produce a 'PCA3 score' [72]. Sokoll *et al.* tested the performance of the test in a multicenter study and found high correlation (97%) between the PCA3 score and biopsy-positive men [73]. The assay was developed into a commercial assay called ProgenSA in 2006 by GenProbe, San Diego, CA [74]. Numerous studies since the development of the commercial assay have supported the fact that it is superior to PSA testing for the diagnosis of PCa [75], however, the sensitivity and specificity values are not overly impressive for *PCA3* alone. It is the combination of the PCA3 score with established factors (PSA, DRE result, prostate volume, and biopsy history) in risk nomograms that provides the most power [76, 77].

A biomarker that is currently used as an adjunct to pathology may also hold promise as an RNA marker. Alpha-methylacyl coenzyme A racemase (AMACR) is a standard

immunostaining tool for needle biopsy evaluation, and so it is a natural candidate for urinalysis using either protein or mRNA detection strategies. Using RT-PCR, a AMACR-score can be derived in the same way as the PCA3 score described above. Ouyang *et al.* have shown that the AMACR-score was superior to serum PSA for the detection of PCa, and the combination of PCA3 and AMACR scores achieved a sensitivity of 81% and specificity of 84% [78]. Combination of AMACR with PCA3 and several other candidate RNA markers revealed that a quadruplex model that included *TRPM8* and *MSMB* transcripts outperformed *AMACR* or *PCA3* analysis alone [79].

Other promising RNA markers are the transcripts of fusion genes. Fusion between two genes occurs through chromosomal rearrangements, and some of these become stable and produce a viable mRNA during transcription. If the transcripts are translated to form a functional hybrid protein, this has multiple consequences for the cell. Fusions between the androgen-regulated transmembrane protease serine 2 gene and (*TMPSS2*) and *ETS* transcription factors were reported several years ago to be present in prostate cancer cells [80]. The androgen-regulated *TMPRSS2-ETS* fusion proteins have downstream effects that are not subject to the normal tight regulation of native *ETS* transcription factors [81]. The most common fusion detected in PCa is between *TMPRSS2* and the *ETS* family gene *ERG* [82]. These fusion gene transcripts have been tested as biomarkers of PCa in tissue and in post-prostatic massage urine samples. In a study of 108 PCa cases, detection of the fusion transcript by RT-PCR in urine sediments had high specificity (93%) but poor sensitivity (37%), and no relationship with Gleason score was evident [83]. A study of 237 men confirmed these findings [84]. Promisingly, combining data on the expression level of *PCA3* transcripts and the presence of the *TMPRSS2-ERG* fusion transcript achieved more acceptable sensitivities [83]. The mRNA/fusion gene multiplex approach has been extended to include panels of RNA markers. Laxman *et al.* used PCR to analyze seven candidate RNA biomarkers in 257 post-DRE urine sediment samples. Multivariate analysis identified transcript levels of *SPINK1*, *PCA3*, *GOLM1* and *TMPRSS2-ETS* to be predictors of PCa [85]. Most recently, the combination of *TMPRSS2-ERG* with *PCA3* and serum PSA data in a study of 45 patients achieved a specificity of 90% and sensitivity of 80% [86].

Protein-based Prostate Cancer Urinary Biomarkers—A number of soluble urinary proteins have been reported to be associated with prostate cancer. PSA itself has been investigated, most often as a serum:urine ratio, but conflicting results have reduced enthusiasm for this biomarker [87]. Matrix metalloproteinases (MMPs), especially MMP-9, have been proposed as potential urinary biomarkers. Sensitivities >80% and specificities >70% have been reported for PCa detection [88]. Another protein candidate is ANXA3, a calcium binding protein that has been shown to be decreased in prostate cancer tissue. It is difficult to develop a biomarker that is decreased in disease for detection, but when combined with PSA, urinary ANXA3 measurement did improve performance over PSA alone [89]. The problem with these latter examples is the fact that the prostate is not the only source for these proteins. If elevated levels of these proteins are found in the urine, it indicates disease of the urinary tract, including cancer, but the source of the biomarker is not evident. The prostate-specific expression of PSA, *PCA3* and other protein biomarkers provides a major advantage over ubiquitous cellular proteins.

DNA-based Prostate Cancer Urinary Biomarker—As described for BCa, the methylation status of various gene loci has been proposed as an indicator of PCa. Glutathione-S-transferase P (*GSTP1*) expression has been shown to be reduced by promoter hypermethylation in prostate cancer cells [90], and this can be monitored in the urine after prostate massage. The methylation status of *GSTP1* alone showed promise, but once again, inclusion in a panel was far more powerful. Using methylation-specific PCR assays, analysis of panels of three or four gene promoters achieved specificities of 89%–100% and

sensitivities of ~85% [91–93]. The common denominator in these studies, and most often the best performing individual biomarker was *GSTP1*.

Metabolomics in Prostate Cancer Urinary Diagnosis—The urine is an obvious resource for metabolomic profiling. As described for BCa, there are many problems faced when teasing out the metabolites that are truly associated with the disease status when there is so much variation and noise from individual's dietary and lifestyle choices. But if one can achieve this, it can provide biomarker opportunities and insight into the biochemical pathways involved in urological cancers. Through comparison of tissue, serum and urine samples, the levels of sarcosine, a derivative of glycine, were reported to be associated with advanced disease [94]. With such a labile biomarker, the need for DRE, and the association to date being with advanced disease, it is not surprising that the utility of this biomarker is still in question, but follow up studies have developed improved methods for urinary sarcosine quantitation [95, 96], and have prompted the analysis of other amino acid metabolites for PCa evaluation. In one study, urinary cysteine, a methionine metabolite, was also found to be elevated in patients with recurrent PCa, but they reported that serum metabolite concentrations were of more predictive value than urine levels [97].

Summary: Prostate Cancer Urinary Biomarkers—Urinary biomarkers for prostate cancer detection are the focus of much current research (Table 3). The incentive for this research is to reduce the number of biopsies and to avoid potential overtreatment. There are some promising results, but anatomical considerations may always limit the accuracy of urinary assays. The need for prior prostatic massage, a procedure that often differs between clinics, and the focal nature of prostate cancer, can cause a high rate of false-negatives, and so sensitivity of urine tests for PCa are unacceptably low to date. For these reasons, PCa urinary tests are less likely to be amenable to mass screening, and are not useful for post-surgical disease recurrence monitoring. Combinatorial assays, and inclusion of data into risk nomograms that use established factors such as PSA, age, biopsy history etc., are likely to be the best option going forward.

SECTION 3

URINARY DIAGNOSTIC BIOMARKERS FOR KIDNEY CANCER

Renal cell carcinoma (RCC) often progresses asymptotically, with diagnosis regularly occurring incidentally when patients are undergoing ultrasound for unrelated problems, so many tumors are not detected until they are advanced and often metastatic. If the tumor is confined within the capsule, at diagnosis, the survival rate can exceed 70% [98]. Thus, earlier detection of RCC would greatly facilitate effective patient management. Furthermore, RCC progression and treatment response are unpredictable, so the identification of molecular markers that can both diagnose and/or classify tumors with respect to treatment options is also highly desirable. At this time, there are no existing biomarkers for kidney cancer diagnosis or for population screening. More in line with prostate cancer than bladder cancer, the majority of biomarker studies on RCC have to date focused on serum biomarkers for molecular detection, but a number of recent studies have investigated the value of urinary biomarkers in this context. The majority of RCC tumors originate within the tubule epithelium such that cells and their contents will be shed or secreted into the tubular lumen, or urinary space, providing an opportunity to discover diagnostic biomarkers in the urine.

As with the other urological cancers, both mRNA and microRNAs (miRNA) are under extensive investigation in RCC tissues. There are many reports of transcriptome profiling of excised kidney tissues using high-throughput microarray platforms. A comprehensive summary of such studies is available elsewhere [99], but due to the limitations of tissue analyses described above for BCa, it is fair to say that these studies have been focused on

identifying biomarkers for prognosis and survival, or for classification through association of gene expression with stage and grade, or subtype. A whole-exon deep sequencing analysis of tissue suggests that ccRCC can be divided into two groups with respect to a hypoxia signature [100]. This is of interest because hypoxia-induced factors may represent an opportunity for biomarker derivation. While some of the tissue-based profiling findings have translated to potential serum biomarkers, none have yet been followed up in a study of urine samples from patients with kidney disease [99]. A similar situation exists for miRNA studies in kidney tissues. Several specific targets have been reported as being upregulated in RCC tissue relative to non-malignant tissue samples [57, 101–103], but to date, no urinary miRNA biomarkers have been associated with RCC.

Gene Promoter Methylation in Kidney Cancer Urinalysis—There are reports of urinary DNA methylation markers with some promise in RCC detection. In a study that matched tumor, serum and urine samples from 17 patients with RCC and 91 controls, the methylation status of nine gene promoters was examined quantitatively. Promoter hypermethylation of at least 2 of the 9 genes studied was detected in 16 of 17 RCC cases. As expected, aberrant methylation in the urine and serum was accompanied by methylation in the matched tumor tissue [104]. An independent study of 50 RCC patients looked at some of the same gene loci targets and found that at least one of six promoters were hypermethylated in all 50 cases. Such methylation was not observed in the control cohort samples [105]. Such biomarkers have been investigated through a candidate gene approach, monitoring gene loci known to be involved in cancer. The field awaits a more global epigenome approach that may be better suited to the identification of optimal promoter panels for RCC evaluation or screening [106].

Metabolomics in Kidney Cancer Urinary Diagnosis—There has been some application of metabolomic profiling to RCC urine samples. Tumor cells often shift their metabolic pathway utilization to glycolysis in order to accommodate their need for rapid growth in an environment with scarce blood supply. The metabolites that are excreted from tumor cell proliferation offer unique biomarkers if they can be measured accurately in non-invasively obtained urine. Studies have identified carnitine derivatives as being significantly altered in the urine of RCC patients. Carnitine mediates fatty acid and branched chain amino acid metabolism. Acylcarnitines are required for fatty acid transport into the mitochondria, and these compounds have been shown to appear at higher quantities in 29 RCC patient urines, and these findings were confirmed in a murine xenograft model [107]. Another study identified 3 metabolites, quinolate, 4-hydroxybenzoate and gentisate, as being present at different levels in the urine of RCC patients [108]. These metabolites are involved in amino acid metabolism, and quinolate was subsequently shown to increase proliferation in 2 of 3 kidney cancer cell lines [108]. Metabolomic analyses do suffer from magnified variability. The genetic background, the age, the recent dietary intake can all alter the urine metabolome significantly. As the small metabolite molecules are highly labile, sample handling and processing can also introduce variability. Once standard methods of collection are developed, specific metabolites that have clinical utility may be identified, but the approach can also identify potential targets/pathways for therapeutic perturbation.

Protein-based Kidney Cancer Urinary Biomarkers—Protein targets are perhaps the most promising urinary biomarkers for RCC. Soluble proteins are relatively stable in the urine and can be detected with a variety of straightforward antibody or ligand-based detection techniques. The urinary bladder cancer biomarker NMP 22 (described above) has been touted to be a potential marker of RCC also. In a study of 41 patients, 60% of the RCC patients had a positive urinary NMP22 test, compared with only 13% in the control group [109]. Similarly, others reported preoperatively urinary NMP22 levels significantly higher in

each of their respective RCC groups than in healthy volunteers [110, 111]. Protein-based investigations of surgically excised renal tissue have also highlighted potential biomarkers of RCC. Studies identified aquaporin-1 (AQP1) and adipophilin (ADFP) as being upregulated in malignant tissues, and these have been followed up as biomarkers in urine. Morrissey *et al.* monitored the levels of these two proteins in urine samples from patients undergoing nephrectomy for a presumed diagnosis of RCC [112]. The levels of both proteins were significantly elevated in ccRCC and papillary kidney cancer relative to other kidney cancer subtypes and control cases. Known urinary biomarkers of chronic kidney disease have been investigated as indicators of RCC. In a study of 122 cases (67 with RCC), urinary NGAL and KIM-1 proteins were monitored using ELISA. The findings supported a potential role for KIM-1 in RCC detection, but the specificity may be a problem given the fact that its presence may reflect multiple kidney diseases or trauma [113]. Through a proteomics approach of cyst fluid, Minamida *et al.* identified 14-3-3 protein beta/alpha (YWHAB) as being associated with RCC, and analysis of this protein in urine revealed that it was accurate (area under ROC curve of 0.88) in the diagnosis of RCC [114]. Rogers *et al.* used surface enhanced laser desorption ionization (SELDI) mass spectrometry to derive protein profiles from 116 urine samples (48 with ccRCC). Neural network models were derived based on presence/absence and/or peak intensity values, and optimal models reached sensitivities and specificities of >98%. However, in an independent validation cohort of 80 cases, the performance of the peptide profile was significantly weaker [115]. Other potential biomarkers have been identified in tissue using SELDI approaches [116], but these have either not been tested in urine, or the proteins comprising a diagnostic peak pattern have not been identified [117]. Although the number of urine samples was small, Teratani *et al.* did find that the transcript of a tissue biomarker, fatty acid-binding protein (FABP), was detectable only in urine samples from RCC patients [118].

CONCLUSION

The development of an accurate, non-invasive urologic cancer detection assays would clearly benefit both the patient and the health system. Studies that leverage advanced high-throughput technologies are identifying cancer biomarkers in voided urine samples that achieve higher values of sensitivity and specificity than previously reported for any current or proposed test. Technologies are also extending the range of molecular classes that may serve as potential biomarkers. Beyond the cellular and proteinaceous milieu, mRNA and microRNA, DNA markers that include mutated sequence or methylation information, and metabolite concentrations can all provide valuable information and are amenable to urinalysis. If assays of these biomarkers are accurate and reliable enough, then they would be applicable for not only diagnosing and surveillance of urologic cancers, but also for screening at risk, asymptomatic individuals or even for routine screening of the population. Larger, confirmatory studies are required in order to further modify and validate proposed biomarker panels prior to the development of clinically applicable urine-based assays, but the field is in a good position to provide much needed improvements in the non-invasive diagnosis of urological cancers. Even when novel urine-based assays are not accurate enough to be stand-alone tests, they may improve clinical evaluation by augmenting current histological and imaging evaluation procedures.

Challenges include the standardization of urine sample collection and processing, and standardization of specific analytical techniques. Variability of baseline data between individuals is an ongoing problem, especially for metabolomic studies that are affected by often uncontrollable patient activities. Urine is not as complex a biological fluid as serum or tissue lysates, but both discovery and analytical assays can benefit from complexity reduction. Isolation of RNA, DNA, protein classes such as glycoproteins, or subsets of metabolites is likely to be a fruitful approach. Given the heterogeneity of cancer, it may be

most efficient to study subtypes of disease initially in order to identify robust biomarkers. At the validation stage, promising biomarkers can be tested on an expanded cohort that includes all subtypes and non-malignant conditions. Finally, it is becoming clear that panels of biomarkers, or molecular signatures are far more powerful than single or small combinations of biomarkers. It may well be that the most robust diagnostic signatures may be multifaceted, including a mix of molecular types and classes.

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Table 1

Commercially available bladder cancer urinary assays.

	Commercial Analyte Description	Genes / Proteins assayed	Assay Type	Ref.
BTA stat	BTA (Bladder Tumor Antigen) a.k.a. Complement factor H-related protein	CFHR1 CFHR2 CFHR3 CFHR4 CFHR5	Colorimetric Ag-Ab reaction (Point of care)	[10]
BTA trak	BTA (Bladder Tumor Antigen) a.k.a. Complement factor H-related protein	CFHR1 CFHR2 CFHR3 CFHR4 CFHR5	Sandwich Immunoassay	[11]
NMP22	NMP22 (Nuclear Matrix Protein)	NUMA1	Colorimetric Ag-Ab reaction (Point of care) or Sandwich Immunoassay	[13]
ImmunoCyt™/ uCyt+™	High MW form of glycosylated CEA & MUCIN-like antigens	Not identified	Fluorescent antibody cytology	[19]
UroVysion™	Copy number: Chromosome 3 Chromosome 7 Chromosome 17 Locus 9p21	N/A	FISH (Fluorescence In Situ Hybridization)	[21]

Table 2

Candidate urinary biomarkers for bladder cancer detection.

Markers / Signature Components	Type of analyte/ Process analyzed	Reference
HA (Hyaluronic Acid) HYAL1	Glycosaminoglycan & protein levels	[24]
APOA1	Protein levels	[35]
SERPINA1	Protein levels	[36]
Ratio: HSPD1 (HSP60) to IL-13	Ratio of protein levels	[37]
EGF NMP22 (NUMA1) & Demographic variables	Protein levels	[38]
ORM1 -ORM2 & AZGP1	Protein levels	[31]
REG1A -REG1B KRT10	Protein levels	[32]
MMP-2, MMP-9 FN1	Protein levels	[33]
22-peptide signature	Peptide levels	[34]
14-gene signature	Transcript levels	[49]
12+2 gene signature:	Transcript levels	[51]
CDK1 MDK IGFBP5 HOXA13	Transcript levels	[52]
Ratio: ETS2 to PLAU	Ratio of messenger RNA transcript levels	[60]
Ratio: miR-126 to miR-182	Ratio of micro RNA transcript levels	[53]
Mutation: FGFR3	Gene mutations	[62]
Methylation: GDF15 TMEFF2 VIM	Methylation of gene promoter	[63]
Methylation: TCF21 PCDH17	Methylation of gene promoter	[64]
Metabolomic profiles	Metabolites levels	[67, 68]

Table 3

Candidate urinary biomarkers for prostate cancer detection.

Markers / Signature Components	Type of analyte/ Process analyzed	Ref.
PSA	Protein levels / mRNA levels	[69]
PCA3	Non-Coding RNA	[74]
AMACR	Protein or RNA levels	[78]
Fusion: TMPRSS2-ETS	Fusion transcript levels	[84]
SPINK1 PCA3 GOLM1 TMPRSS2-ETS	Transcript levels	[85]
MMP-9	Protein levels	[88]
ANXA3	Protein levels	[89]
Methylation GSTP1	Methylation of gene promoter	[90]
Sarcosine	Metabolite levels	[94]
Cysteine	Metabolite levels	[97]

Table 4

Candidate urinary biomarkers for kidney cancer detection.

Markers / Signature Components	Type of analyte/ Process analyzed	Reference
Methylation: VHL CDKN2A CDH1 APC MGMT RASSF1A GSTP1 p16 RAR- β 2 TIMP3	Levels of gene promoter methylation	[104, 105]
Acylcarnitines	Metabolite levels	[107]
Quinolate 4-hydroxybenzoate Gentisate	Metabolite levels	[108]
NMP22 (NUMA1)	Protein levels	[109]
AQP1 ADFP	Protein levels	[112]
NGAL KIM-1	Protein levels	[113]
YWHAB	Protein levels	[114]
FABP	Transcript levels	[118]