Urinary Biomarkers in Bladder Cancer

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ABSTRACT

AIM:

This narrative review aims to describe established and emerging urinary biomarkers in the diagnosis and surveillance of non-muscle invasive bladder cancer (NMIBC). It provides a comprehensive account of classical, FDA-approved protein biomarkers and discuss their limitations. Further, we discuss the role that epigenetic, genetic and exosomal markers can play to enhance sensitivity and specificity of the available tests.

BACKGROUND:

The initial diagnosis and surveillance of BC involves a combination of cystoscopy, upper urinary tract imaging and urine cytology. Despite high specificity, cytology is limited by low sensitivity. There are currently six urinary assays approved by the FDA to enhance diagnosis and surveillance of BC. While these have improved diagnosis and surveillance when combined with cytology, these tests are still not sufficiently sensitive and false positives often occur in benign conditions which result in inflammation of the urinary tract. Advancements in laboratory techniques have produced significant advancements in epigenetic and genetic markers, as well as extracellular vesicles, with DNA- and RNA-based markers dominating the research in this area in recent years.

METHODS:

We identified relevant published data, using the PubMed/ Medline search engines as well as Google Scholar. We performed an online search using the terms ‘bladder cancer’, ‘NMIBC’ in combination with ‘urine biomarkers’ and limited articles in English published up to February 2020. This review consolidated on all available narrative and systematic reviews published in the five years in this field, while also reviewing the original data of each clinical trial or observational study which led to the development of the biomarkers.

CONCLUSION:

The development of laboratory techniques and understanding urine-based biomarkers in BC has fuelled the use of non-invasive liquid-based biomarkers to complement urine cytology. Nonetheless, none are sufficiently effective when used in isolation, and cytology remains the gold standard in many practices.

Future efforts will be focused on using these markers in combination as a predictive signature, and moving on to validating them for use in everyday clinical practice.

INTRODUCTION

Bladder cancer (BC) is the eight most common cancer worldwide, with over 550,000 cases diagnosed worldwide in 2018 (1). Eighty percent of patients with BC present with non-muscle invasive bladder cancer (NMIBC), with the remainder presenting as muscle-invasive BC (MIBC). Up to 50% of NMIBC cases eventually recur despite an initial radical resection of bladder tumour ~~radical treatment,~~ and up to 30% of them experience disease progression to an MIBC (2). Due to its high recurrence rate, surveillance cystoscopy is recommended at an interval dictated by the initial grade and stage of the disease. In cases of high-grade disease, cystoscopy may be required up to three-monthly intervals (3).

The initial diagnosis and surveillance of BC usually requires a combination of cystoscopy, upper urinary tract imaging and urine cytology. Cystoscopy and imaging have limited sensitivity in the detection of small lesions of the urinary tract. In these cases, there is a reliance on urine cytology, the most widely used non-invasive test for the detection and surveillance of BC. Despite its high specificity (approximately 86%), the utility of cytopathology is hindered by low sensitivity (48%) as well as interobserver variation (4), limiting its use especially in low-grade tumours (5, 6).

The reliance on invasive procedures as well as the limited sensitivity and specificity of current investigation modalities represent a clinical unmet need both in the diagnosis and surveillance of patients with BC. ~~In addition,~~The ~~the~~ requirement for cystoscopy represents a significant cost to healthcare services in diagnosing BC (7). Urinary biomarkers for BC represent an area of considerable research tested in both patients presenting with hematuria and patients with NMIBC requiring surveillance cystoscopy. There are currently six urinary assays approved by the US Food and Drug Administration (FDA) for clinical use in conjunction with cystoscopy. NMP22 (ELISA), NMP22 BladderChek, and UroVysion have FDA approval for diagnosis and surveillance; immunocyte (UCyt+), BTA-TRAK and BTA-STAT have been approved only for bladder surveillance following the diagnosis of a primary tumour. This review will summarise the current data on all FDA-approved and commercially available assays and cover a range of emerging biomarkers for detection and surveillance of BC, as depicted in Figure 1. In this era of precision medicine, the performance

of any single biomarker is limited by methodological issues, and therefore none of them are approved for diagnosis or screening when used in isolation. This review will not cover biomarkers in relation to screening for BC, which is a distinct topic in its own right.

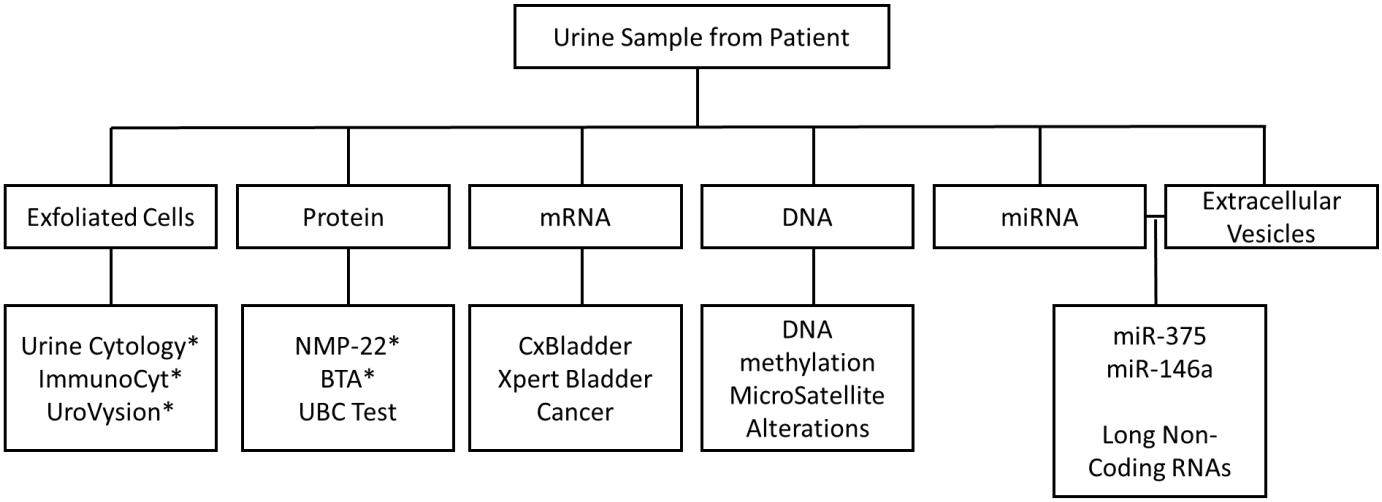

Figure 1: Potential of Urine Based Liquid-Biopsy Biomarker Testing. Selected representative examples denoted for each category of testing. *represent FDA approved assays. mRNA = messenger RNA, miRNA = micro RNA, NMP22 = Nuclear Matrix Protein 22, UBC = Urinary Bladder Cancer, BTA = Bladder Tumour Antigen

CLASSIC FDA-APPROVED BIOMARKERS

Nuclear Matrix Protein 22

Nuclear matrix proteins (NMPs) are a family of proteins that play a crucial role in the structure of the nucleus and are involved in every step of its function, ranging from DNA replication to regulation of gene expression. Several of the NMPs are overexpressed in urothelial tumours and are released into the urine upon apoptosis of the tumour cells. Of these, NMP22 has been the most extensively investigated, and assays for the antigen are used both in the context of diagnosis and monitoring for cancer recurrence.

NMP22 Bladder Cancer ELISA-Test and NMP22 BladderChek tests have been approved by the US Food and Drug Administration (FDA). The former is often referred to as the quantitative NMP22 test which is performed in a laboratory, while the qualitative BladderChek test is a point-of-care (POC) test. These tests

have approval both in the context of diagnosis and surveillance. The performance of the NMP22 assays has been evaluated in several meta-analyses. In 2015, Chou et al performed a meta-analyses on NMP22, demonstrating a sensitivity of 69% and a specificity of 77% for the quantitative ELISA test. The corresponding values for the qualitative POC test was 58% for sensitivity and 88% for specificity (8). In 2017, Wang et al conducted a separate meta-analysis of 19 studies looking at NMP22 POC test encompassing 5291 patients. It demonstrated a sensitivity of 52-59% and a specificity of 87-89% (9).

NMP22 remains one of the most well-studied biomarkers to date. While relatively specific, most meta-analyses concur that the NMP22 assay is insufficiently sensitive when used in isolation. Like many available biomarkers, the test has a particularly lower sensitivity to detect low-grade tumours (10). NMP22 assays measure the cellularity or amount of cell turnover that may be introduced into the urine by a variety of conditions, which includes surface shedding from bladder tumours. Hence, false-positive results are common in patients with benign bladder conditions such as infection, stones, inflammation and hematuria.

Bladder Tumour Antigen (BTA) Assays

BTA Stat/BTA TRAK test are in vitro immunoassays, which detect the presence of human complement factor H-related protein (hCFHrp) in the urine of patients with BC. BTA-stat is a qualitative bedside point-of-care assay with results available within 5 minutes, whereas BTA-TRAK is a specialised quantitative enzyme-linked immunosorbent assay (ELISA). These tests have been approved by the FDA only for monitoring BC recurrence in combination with cystoscopy.

In a meta-analysis of 13 studies of BTA Stat, Guo et al found that the test had a higher sensitivity (67%, 95% CI 64-69%) than urine cytology (43%, 95% CI 40-46%), but the specificity, likelihood ratios and area under the curve were inferior to urine cytology (11). Like other biomarkers, BTA Stat was found to have a much higher sensitivity for high-grade tumours (74%) than low grade tumours (25%), with a specificity of 77% (12).

In general, the sensitivity of BTA Stat ranges from 57-82%, with a specificity of 68-93% (13-15), whereas BTA TRAK has a sensitivity of 66-77% and a specificity ranging from 5-75% (16, 17). These figures generally point towards a higher sensitivity than cytology, but like NMP22, the BTA assays suffer from a higher false positive rate in patients with inflammatory disease in the urinary tract.

UroVysion

The UroVysion test is a multicolour fluorescent in situ hybridisation (FISH) assay which detects aneuploidy of chromosomes 3,7 or 17 or loss of the 9p21 locus. It has received FDA approval for urothelial BC diagnosis and surveillance. The criteria set for detecting BC by UroVysion are at least one of the following (18):

(a) ≥4 cells (of 25) with gains of ≥2 chromosomes in the same cell

(b) ≥10 cells with a gain of a single chromosome

(c) ≥10 cells with tetrasomic signal patterns

(d) Homozygous deletion of the 9p21 locus in 20% or more cells

The sensitivity of this test ranges between 69-87% with a specificity between 89-96% (19, 20). The UroVysion test has demonstrated excellent sensitivity to detect Carcinoma In Situ and high-grade tumours, with sensitivities ranging between 83-100% (18). It is also a useful adjunct to cytology as it maintains the specificity of this test but simultaneously increases sensitivity (21, 22) (45.8% vs. 72.2%). A key advantage of this test is its high specificity, as the assay is not affected by hematuria, inflammation and other conditions which may give false-positive readings with some other tumour markers. There is data suggesting its use for monitoring patients with NMIBC for response to intravesical therapy (23). Indeed, a positive test at the end of BCG treatment in patients with superficial disease indicates a higher risk of progression to muscle invasive cancer (24).

ImmunoCyt Test

The ImmunoCyt assay (also marketed as uCyt+) uses three fluorescently labelled monoclonal antibodies to detect carcinoembryonic antigen (CEA) and sulphated mucin glycoproteins that are expressed on most bladder cancer cells, but not on normal cells. The sensitivity of this assay varies widely among studies, ranging from 60-100%, with a specificity of 75-84% (25-27). In a meta-analysis, ImmunoCyt showed the highest sensitivity at evaluating symptoms and for surveillance (8). It is approved for bladder surveillance following diagnosis of a primary tumour.

However, ImmunoCyt has been shown to be significantly affected by urinary tract infections, urolithiasis, and benign prostate hyperplasia. Other difficulties causing the low uptake of this test is the need for technical

expertise, substantial interobserver variability and a high rate of test failure due to inadequate specimen cellularity.

As of early 2020, the uCyt+/ImmunoCyt test is currently off the market due to the unavailability of the antibody. However, this immunocytological testkit has been unique in employing a cytology-only strategy, and may warrant reinstating into the market, perhaps with a newer, more BC-specific antibody.

LIMITATIONS WITH CURRENT URINARY BIOMARKERS

The FDA-approved biomarkers are collectively the most studied biomarkers to date, with multiple meta-analyses to support their clinical utility. The results of the meta-analyses must be interpreted with caution due to inter-study heterogeneity between the study populations. Selected meta-analyses have also failed to take key confounding factors influencing test performance into consideration, such as the proportions of subjects who smoked in the NMP22 BladderChek meta-analyses reported by Wang et al (9). Many of the meta-analyses described here have also excluded non-English language articles, with variability as to whether they took non-peer reviewed meeting abstracts into account.

Nonetheless, most of these studies concur that currently FDA-approved biomarkers suffer from a high rate of false positive cases by nature of its assay design. Urinary biomarkers may yield false-positive results in 12-26% of patients without bladder cancer. This is coupled with its limited sensitivity when used in isolation, leading up to a missed diagnosis in up to 43% of patients with bladder (8). A consideration of the patient's pre-test probability, assimilating the patient's clinical history and investigations where necessary (especially cystoscopy and cytology), will be required for accurate interpretation of the results.

Considering the high false-positive and false-negative rates of the approved markers, multiple biomarker assays have been studied to provide additional molecular information to guide individualised surveillance and therapy. These will be described in the remainder of the review. While the mechanism of detection of recurrence or diagnosis are novel, the majority have had variable consistency at detecting cancer and are lacking in high quality studies and meta-analyses.

ADDITIONAL PROTEIN MARKERS DETECTABLE IN THE URINE

Several immunological assays have been developed to detect the presence of cytokeratin fragments in the urine. Cytokeratins form part of the cytoskeleton of epithelial cells, and urothelial cytokines are released into the urine after cell death and can be predictive of the presence of cancer. Cytokeratins 8,18,19 and 20 have been associated with BC (28).

For instance, Urinary Bladder Cancer (UBC) ELISA and UBC immunoradiometric assay (IRMA) have been developed to detect the presence of fragments of cytokeratin 8 and 18 in the urine (29). CYFRA21-1 is an ELISA which measures soluble fragments of CK19 in the urine. While a standardised cut-off is unavailable, studies usually employ normalisation to urine creatinine. Detection sensitivities of cytokeratin immunoassays for low-grade bladder tumours could be as low as 13 percent, and the specificity can be particularly low in individuals where urinary tract infections are present (30).

Recently, URO17™ urine test for BC utilizing another member of cytokeratin family, Keratin 17 (K17), was shown to be a promising urine test for BC. A study by Babu et al. (31) used immunocytochemistry to detect presence of K17 in 112 urine specimens. The results showed that K17 was significantly elevated in BC specimens with a sensitivity of 100% and specificity of 96% in BC detection from urine samples. Analysis of histological tissue sections showed that K17 is elevated in both low-grade and high-grade tumours, and urothelial cancer. Significance of elevated level of K17 in cancer cells was described in another study that showed that K17 binds to $p27^{kip1}$ in the nucleus and aid in transporting $p27^{kip1}$ to cytoplasm where it is degraded (32). Degradation of $p27^{kip1}$ allows the cancer cell to bypass G_1-S phase cell cycle control thus leading to cell proliferation which could explain specific association of K17 elevation and BC and high sensitivity and specificity of URO17™ test. Interestingly, the current data suggest that URO17 could be a sensitive and specific test to detect PUNLMP and both papillary and non-papillary carcinomas which could potentially providing diagnostic utility in cases where it could help identify lesions that can be easily missed by traditional urine cytology. Furthermore, the data also showed that URO17 test was able to detect BC in renal pelvis that was missed by urine cytology and cystoscopy which suggest that URO17 test could be used to augment and increase the accuracy of cystoscopy and traditional urine cytology in monitoring patients for recurrence.

Two transcription factors, BLCA-1 and BLCA-4, have also shown promise as biomarkers. They are protein components of the nuclear matrix which are present in the urothelium of patients with bladder tumours. BLCA-

1 is not expressed in non-malignant urothelium (33), whereas BLCA-4 is expressed in both the tumour and adjacent benign areas of the bladder, but not in malignant bladders (34). BLCA-4 may represent the field effect observed at the molecular level in normal tissues adjacent to tumours. The reported sensitivity of BLCA-4 is in the order of 89-96% with a specificity 95-100% (35). These markers appear to show a degree of promise as an adjunct to diagnosing early tumours, and further validation is warranted.

In addition, the CellDetect assay is a novel histochemical staining platform which allows for the discrimination between normal and malignant cells on the basis of colour and morphological discrimination – based on the higher metabolic activity in cancer cells (36). An Israeli study across nine hospitals employing urine smears found that the overall sensitivity of this test was 84%, and the specificity was also 84% for patients undergoing routine surveillance by cystoscopy (37). This test is currently gaining importance by using a cell based assay in clinical practice.

In patients with hematuria, aurora A kinase (AURKA) may be particularly helpful. The AURKA gene encodes a serine/threonine kinase associated with aneuploidy and chromosomal instability, and has been explored in urine sediment by FISH. In a case-control study involving patients with BC, patients with benign conditions and normal individuals, the AURKA-FISH test reported an 87% sensitivity and 96.6% specificity (38).

EPIGENETIC ALTERATIONS

DNA Methylation

The most well characterised epigenetic phenomenon is DNA methylation. Hyper- and hypomethylated regions of DNA are identified in BC and in premalignant lesions. DNA methylation status can be assessed in cell free DNA fragments and tumour cells shed in urine. A significant prevalence of methylated genes, for example APC and cyclin D2, was elevated compared to benign cases (39). Hypermethylation of selected genes, including GSTP1, APC, RARb2 have been identified in patients with urothelial BC (40).Table 1 summarises some of the key DNA-based urine biomarkers investigated in recent years, along with their accompanying sensitivities and specificities. Although the specificities of these markers are highly encouraging, the molecular genetic techniques required to detect these are expensive, time consuming and highly specialised.

Gene	Sensitivity	Specificity

Diagnosis of bladder cancer		
GSTP1, RARb2, APC(40)	62	89
TWIST1 and NID2 (41, 42)	79	63
POU4F2 and PCDH17 (43)	90	94
CFTR, SALL3/TWIST1(44)	84	68
HDAC3(45)	89	63
Surveillance of bladder cancer recurrence		
SOX-1, IRAK3 and Li-MET(46)	86	89
HS3ST2, SEPTIN9, SLIT2/FGFR3 (47)	98	85
Delineation of bladder cancer grade and stage		
APC/Cyclin 2 (39)	55	100

Table 1: Tumour-derived DNA methylation status as urine biomarker of urothelial bladder carcinoma diagnosis and/or surveillance

Histone Tail Modifications

Histone modifications represent a diverse set of epigenetic markers involved in both dynamic cellular processes and the stable maintenance of chromatin. In BC, the levels of histone methylation are lower in advanced tumours and correlated to poor survival. For instance, high levels of H3K27me3 correlated with poorer prognosis post-cystectomy in pT1-3 and node negative patients with BC (48).

GENETIC ALTERATIONS

DNA Mutational Analysis

Analysis of tumour-derived DNA via cell-free DNA (cfDNA) can reveal mutations and serve as non-invasive biomarkers. Amongst the mutations which have been analysed include urinary TERT promoter mutations, FGFR3 and telomere length. Telomerase reverse transcriptase (TERT) maintains the integrity of telomeres and mutations in the TERT promoter are frequent in BC. Descotes et al reported that an assay analysing the

TERT promoter mutation in urine showed an overall sensitivity of 80.5% and specificity of 89.8% in diagnosis of BC, and that TERT mutations significantly predicted recurrence of NMIBC ($p<0.0001$) (49). TERT, in combination with FGF3 and OTX1 also showed high sensitivity of diagnosis of NMIBCs as well as in pT1 tumours (50). Mutations in FGF-3 are seen in approximately half of BC patients, with an elevated incidence (60-70%) in low-grade tumours. Recent studies have suggested that partial replacement of cystoscopy with FGFR3 mutational analysis during surveillance can be safe and cost effective (51).

Microsatellite Analysis

Microsatellites are polymorphic repeating units of 1-6 base pairs in length in human DNA. Microsatellite analysis is a PCR analysis of DNA in exfoliated urine cells. One of the most common genetic changes in BC is loss of heterozygosity in chromosome 9 (52). Chromosomes 4p, 8p, 9p, 11p and 17p also often display LOH in patients with BC. Generally, the sensitivities of these markers range from 72-97% and the overall specificity between 80-100% (53, 54).

URINARY TUMOUR RNAs

MiRNAs

MicroRNAs are small 21-23 nucleotide long non-protein coding RNAs that regulate gene expression by pairing to the 3' untranslated region of their target mRNAs. They can be found in body fluids as free circulating miRNAs, bound to ribonucleoprotein complexes or in extracellular vesicles such as exosomes (55). Changes in miRNA expression in cancer tissues exhibit tissue specificity with a high level of stability and detectability. Due to their short length, miRNAs are less vulnerable to degradation than mRNA chains and can be stored for up to 48 hours at room temperature (56). Hence miRNA expression analysis is considered a potential biological marker for both detection and surveillance.

Urinary microRNA can be derived from a range of specimens – voided urine, urine sediment or supernatant. In a systematic review, Kutwin et al showed that miRNA from urine supernatant have the greatest sensitivity (78.4%) followed by urine sediment (75.6%) and voided urine (74.3%). Urinary supernatant also has the highest specificity amongst the three at 79.4% (57).

To date, twelve studies have reported the diagnostic performance of microRNA (miRNA). Of the miRNA panels, four have a sensitivity and specificity above 80% or more, and employed miRNA arrays or next generation sequencing (NGS) to identify targets. MiRNA was then quantified by real time PCR.

Author	Marker	Specimen	Proportion of Low Grade (%)	Sensitivity	Specificity	PPV	NPV	AOC
Mengual et al 2013	6 miRNAs: miR-187 + miR-18a + miR-25 + miR-142-3p + miR-140-5p + miR204	Not specified	38	85	87	88	83	0.92
Zhang et al 2014	miR-99a + miR-125b	Urine supernatant	30	87	81	92	71	0.88
Eissa et al 2015	miR-96 + Cytology	30-60ml void	80	87	87	86	80	
Urquidi et al 2016	25 target diagnostic miRNA signature	Mid-stream void	16	87	100			

Table 2: Study characteristics and diagnostic accuracy of urinary miRNA for the diagnosis of bladder cancer: Selected multi-miRNA studies have sensitivity and specificity of 80% or more.

Urinary-based mRNA assays

Circulating messenger RNAs (mRNAs) reflect the status of intracellular processes. Despite the majority of them being degraded by RNAses, they are still detectable in the urine of BC patients and may represent potential biomarkers. For instance, the Urine Ubiquitin Conjugating Enzyme E2C (UBE2C) and isoleucine glutamine motif-containing GTAase-activating proteins (IQGAP3) mRNA levels are higher in BC patients than in controls (58, 59). In practical terms, commercially available mRNA-based urine biomarkers combine multigene panels, which are described below.

Multigene Panels

Several groups have investigated the utility of multigene panels in the detection of BC from urine samples. Of these, Cxbladder, which quantifies mRNA biomarkers is the most well-known. The test suite includes assays to potentially rule out the presence of BC in low-risk patients with hematuria (Cxbladder Triage), complement cystoscopy for BC detection in the presence of hematuria (Cxbladder Detect), and complement cystoscopy for surveillance in the context of recurrence (Cxbladder Monitor). Other tests, along with their accompanying sensitivity and specificity as well as validation studies (wherever relevant) are found in Table 3.

Commercial Test	**Genes Involved**	**Sensitivity**	**Specificity**	**Additional Notes**
mRNA tests				
Cxbladder (60)	IGFBP5, HOXA13, MDK, CDK1 and CXCR2	82% in patients with hematuria	90%	Large study comparing Cxbladder with FDA approved markers showed superior sensitivity and NPV(61)
XpertBC(62)	UPK1B, IGF2, CRH, ANXA10 and ABL	46.2%	77%	Study of 140 patients showed Xpert BC outperofmrs cytology at sensitivity and NPV even in low grade tumours, with no reduction of specificity(63).
DNA-based tests				
Assure MDx(64)	FGFR3, TERT and HRAS in combination with methylation analysis of OTX1, ONECUT2 and TWIST1	97%	83%	Follow-up validation study demonstrated 93% sensitivity and 86% specificity(65)

UroSEEK(66)	Mutations in 11 genes or presence of abnormal number of chromosomes	96%	88%	
Uromonitor (67)	FGFR3 hotspot and TERT promoter mutations	73.5%	93.2%	
DNA Methylation Assays				
EpiCheck(68)	15 proprietary DNA methylated genes	68.2%	88.0%	

Table 3 Multigene Panels in the Diagnosis and Surveillance of Bladder Cancer involving DNA, mRNA and Epigenetic Targets

EXTRACELLULAR VESICLES AND EXOSOMES

Exosomes are membrane vesicles secreted at an elevated level in cancer patients – they participate in intercellular communication through transferring biologically active molecules (including RNA, DNA and proteins) (69). Extracellular vesicle (EV) enrichment has been observed in the urine of patients with BC, and analysis has demonstrated specific patterns of protein and miRNA (microRNA). For instance, an interesting micro-fluidic chip-based system has been employed to analyse EVs from patients with and without BC, demonstrating that the concentration of EVs in urine from patients with BC was significantly higher compared to healthy controls. This technique depicted a sensitivity of 81% and specificity of 90% for accurately diagnosing BC (70).

In addition to evaluating concentration of EVs, a parallel research strategy has been to categorise the cargoes contained within the EVs to determine whether there is a profile predictive of BC. One proteomic analysis of urinary EVs identified 2 proteins – alpha-1-anti-trypsin and H2B1K, which are enriched in EVs isolated from patients with BC (71). There has simultaneously been focus on the genetic cargo, specifically long-non-coding RNAs (lncRNAs), in urinary exosomes. Berrondo et al (72) demonstrated that lncRNA levels were elevated in exosomes of patients with BC. The HOX transcript antisense RNA (HOTAIR) together with other

lncRNA, such as HOX-AS-2, ANRIL and linc-RoR, were increased in urinary exosomes from high grade MIBC patients.

A separate study sought to analyse the profile composition of miRNAs and proteins associated with urinary EVs in patients with BC (73). Using a microarray platform of >850 different miRNAs, the authors aimed to investigate dysregulation of particular miRNA and its association with the presence of BC. They found that 26 miRNAs were dysregulated in patients with high-grade BC. Real-time PCR analysis indicates that miR-375 is a biomarker for high-grade BC while miR-146a could identify low grade patients.

Although extracellular vesicles represent an interesting source of biomarkers, the lack of accurate isolation and detection affects their uptake in clinical practice. However, the diverse exosome cargo represents a rich source of biomarkers, and the development of more sensitive capture platforms will increase its incorporation into clinical practice.

THE PRACTICAL VALUE OF URINARY BIOMARKERS

From a practical standpoint, the variety of test systems can be broadly categorised into two distinct characteristics. Two different approaches could be employed in laboratory test marketing – (a) the specialised system, where test systems employ complex techniques and elaborate pre-analytics that have high test qualities, but are limited to specialised centres and expensive, or (b) easy to perform assays that are cheaper, but test results are of limited value as less specific. The value of urinary biomarkers and its clinical utility depends on the clinician's ability to estimate pretest probability of the disease, the importance to patients (and their treating clinician) of relatively small changes in the probability of bladder cancer, and the acceptable threshold and clinical consequences of missed or delayed diagnoses and false-positive results.

The potential benefit of urinary biomarkers depends on the situation in which it is employed. For instance, a urinary biomarker used a diagnostic tool in a patient with haematuria will require a high negative predictive value and specificity to avoid false positive results. Patients with haematuria should be categorised by gross and microscopic hematuria, with the former receiving cystoscopy. For patients with only microscopic haematuria, urinary markers can be an important adjunct to nomograms leading to more accurate evaluation of their disease status (74).

The clinical applicability of urinary biomarkers in the context of surveillance is arguably more complex, and dependent heavily on the initial tumour grading. Following a transurethral tumour resection, markers may be a useful surveillance tool reducing the frequency of cystoscopies in a low grade tumour. Due to the low probability of recurrence, an acceptable threshold for recurrence can be agreed with the individual patient to allow urinary markers and sonography to guide follow-up investigations. The UroFollow trial, which studies the use of noninvasive marker-based follow-up with standard of care, will provide some answers for patients with pTa G1-2/low-grade NMIBC(75). In the context of high grade tumours, it is unlikely that urologists will rely on biomarkers solely (in isolation or in combination) in the near future, and are likely to instead fall on more conventional methods like cystoscopy and cytology.

Another area of unmet clinical need is the assessment of tumour aggressiveness to help guide treatment intensification and planning. In the first study investigating the combined use of urine markers to predict aggressiveness, Todenhofer et al demonstrated that the presence of simultaneously positive urine cytology and NMP22 was associated with a 20-fold risk for G3/CIS (76). From a genetic perspective, a 12 + 2 gene-set panel based on qRT-PCR developed by Mengual et al has demonstrated ability to predict tumour aggressiveness. With a sensitivity of 79% and specificity of 91% in voided tumour samples, they devised and validated a panel of molecular markers that could help guide the intensity of a follow-up schedule for patients(77, 78). In aggressive tumours with a higher number of genetic mutations, urinary markers could indicate the need to switch from receiving intravesical therapy to an early cystectomy.

DISCUSSION AND CONCLUSION

Urine cytology is useful and remains the current standard for the detection of high-grade tumours. Most of the other available markers are characterised by low positive predictive values that limit their application in routine clinical practice (79). The FDA-approved biomarkers almost uniformly suffer from high false positive rates as a result of benign inflammatory conditions. While the novel genetic markers have shown initially promising results, the enthusiasm is often dampened by similar shortcomings. For instance, urinary DNA methylation markers produced many false positive results in symptomatic men with sexual infections (80). This low specificity remains one of the greatest limitations of urine biomarkers in clinical practice.

The UroFollow study, by nature of its multi-panel design, will hopefully guide de-intensification of follow-up for low-grade tumours through non-invasive monitoring methods. For high grade tumours, urine cytology (and cystoscopy) are likely to remain common practice in the near future. The question then is how we can best combine the array of available biomarkers, taking into consideration their different utilities and limitations, to help guide surveillance and treatment. A comprehensive systematic review by Tan et al (81) reinforces that single target assays have limited value regardless of their '-omics' class. Only 4 single target urinary biomarkers achieved a sensitivity and specificity of 90% or more -ie the protein markers orosomucoid 1 (ORM1) and HtrA1, the epigenetic marker POU Class 4 Homeobox 2, and the transcriptomic marker long non-coding RNA urothelial carcinoma associated-1. There is an increasing appreciation that the use of multi-target biomarkers is increasing and that these biomarkers have better diagnostic performance. At present, despite an expanding field of urinary biomarkers, none of these reported have displaced cystoscopy as the gold standard for diagnosis and surveillance. The lack of field testing, validation studies, diverse thresholds of normal ranges, and complex interplay of different 'omics' each present a range of challenges in biomarker development and validation.

Whereas established test systems often employ common features of cell degeneration or proliferation for detection (eg cytokeratins), modern assays already use BC specific features – though these have to undergo larger studies to validate their utility. Therefore, we propose that the requirements of an optimal BC urine assay include (i) an assay that may detect BC-specific features (exclusive from normal urothelium), (ii) expansion of the gold-standard cytology technique with these BC-specific features, thereby combining modern developments while maintaining the important contribution of microscopy.

Funding

None

Conflict of Interest

Regarding the work under consideration for publication, there are no conflicts of interest for any of the authors. Regarding relevant financial activities outside of the submitted work, there are the below declarations: Anand Sharma and Nikhil Vasdev – no conflicts of interests. Kenrick Ng has received honoraria and travel grants from TESARO and GSK. Kenrick Ng is currently funded by Cancer Research UK under a research grant under a Training Fellowship (Award Number 549580). Arnulf Stenzl has received fees from Ipsen Pharma, Janssen, Alere, Bristol-Myers-Squibb,

Stebabiotech, Synergo, Ferring, CureVac, Astellas, Amgen and Sanofi Aventis. Arnulf Stenzl has also received grants from Johnson and Johnson, Roche, Cepheid, Amgen, Bayer AG. Immatics Biotechnologies GmbH, GemeDX Biosciences, Novartis AG and Karl Storz AG. Regarding intellectual property, patents and copyrights, there are no conflicts of interest for any of the authors. No authors declared any other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what was written in the submitted work

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