Urine-based bladder cancer diagnostic: Oncuria™

Abstract
Bladder cancer is a major healthcare issue in the US. For the avoidance of invasive procedures, naturally voided urine is the most clinically relevant biospecimen for the discrimination of patients with bladder cancer. Voided urine cytology, the gold standard urine-based assay for the detection of bladder cancer, has been used since the 1940's with little modification, despite significant limitations in the clinic. Oncuria™, a multiplex immunoassay for the detection of a bladder cancer protein signature, is a validated assay for the detection of bladder cancer from a voided urine sample. Here, we provide information supporting the development of Oncuria™ in the clinic to assist with the non-invasive identification of bladder cancer patients.

Discovering and Developing Breakthrough Diagnostics for Human Diseases

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Contents

Bladder cancer

Epidemiology

Bladder cancer is among the most commonly diagnosed cancers worldwide [1]. Urothelial carcinomas are the most common bladder tumors in Western countries, constituting approximately 95% of all cases [2]. Risk factors associated with the development of urothelial carcinomas are mainly environmental exposure to known carcinogens in tobacco smoke [3], and to chemical compounds in the color and rubber industries [4]. Urothelial carcinoma has a 5-times higher prevalence among men than women and has a 2-times higher prevalence in Caucasians than other racial groups [2,5]. In the US, an estimated 83,730 newly diagnosed cases of bladder cancer and 17,200 deaths from bladder were reported in 2021 [5]. Alarmingly, both the absolute numbers of cases and deaths from bladder cancer have increased by 45 and 35%, respectively, since 2002 [6]. Moreover, bladder cancer has one of the highest recurrence rates of any tumor type. Due to the prolonged natural history of bladder cancer, it is estimated that >700,000 Americans are burdened with bladder cancer each year. Bladder cancer has the highest lifetime diagnostic and treatment cost of all cancers [7], with estimated expenditures of approximately $187,000 per case and an annual cost of approximately $4 billion to the healthcare system [7,8]

Treatment

When diagnosed early as a non-muscle invasive bladder cancer (NMIBC, e.g., Ta/T1/Tis stage lesion), cure by transurethral resection (TUR) with immediate post-operative intravesical chemotherapy instillation is possible in a high percentage of cases: 5-year survival rate >94%. Guidelines for NMIBC with adverse features (number of tumors, tumor size, prior recurrence rate, clinical T stage, presence of carcinoma in situ, and grade) recommend adjuvant intravesical instillation of Bacillus Calmette-Guerin (BCG), a live attenuated tuberculosis vaccine that acts as a non-specific immune system stimulant, which has proven to assist in the eradication of residual disease, reducing recurrence rates and decreasing the progression to muscle-invasive lesions [9,10]. However, despite intravesical treatment, more than half of patients fail to respond and 20% experience disease progression to muscle invasive bladder cancer (MIBC) within a 5-year period [11,12]. Failure to intervene with definitive radical cystectomy prior to progression to MIBC is associated with a significant reduction in long-term survival [13,14]. Patients with MIBC (>T2 lesion) treated with either radical cystectomy or radiation therapy have 5-year survival rates of approximately 50% [15,16]. The field of bladder cancer therapeutics has advanced recently with the approval of immune checkpoint inhibitors and reagents that target specific signaling molecules [17], but the early detection and monitoring of disease state remain paramount.

Molecular pathogenesis of bladder cancer

A broad range of research efforts has provided insight into the genetic alterations that lead to the development of bladder cancer [18], and recent studies from US and European consortia have begun to classify bladder cancer into molecular subtypes based on gene expression profiles [19]. Comparisons across independent cohorts have begun to reveal the underlying biology associated with various classification systems, but significant biologic subgroup heterogeneity remains, and more work is needed before a unified classification system can gain wide acceptance.

Current urine-based diagnostics

Table 1 lists the urine-based assays available for the detection of bladder cancer. There are advantages to using urine as a biological sample for biomarker discovery and detection. Tumors in the bladder wall are continuously bathed in urine, facilitating the release of protein, DNA and RNA, as well as intact exfoliated urothelial cells. Urinalysis also has the advantage of easy repeat sampling. This enables the collection of reliable replicate samples (which cannot be achieved with excised tissue) and allows for temporal sampling which may be useful for investigating disease status post-treatment or at recurrence. A brief description of currently available urinary assays for bladder cancer detection follows.

Voided urinary cytology

The most widely used urine-based analysis for the non-invasive detection of bladder cancer is voided urine cytology (VUC), first reported in 1945 [20]. Since its inception, VUC has changed very little. VUC can be a challenging test to perform since it is dependent on the skills and experience of highly trained cytopathologist. The sensitivity reported for VUC is 30-92% with an accompanying specificity of 93-97% [21-25]. The lack of sensitivity achieved by voided urine cytology is skewed by low-grade and early stage tumors, because these

lesions tend to shed fewer cancer cells into the urine for analysis. It is widely known in the urology field that VUC have insufficient predictive power to be applied to the management of individual patients. Because of these substantial limitations, only approximately 10% of patients presenting with hematuria (blood in the urine) are currently evaluated with VUC [26].

Table 1 Performance of current FDA approved/cleared urine-based assays for the non-invasive detection of bladder cancer [24,25]

Urine Test	Analyte(s)	Sensitivity (%)	Specificity (%)	Assay Platform	Quantitative	Multi-sample capability
Cytology	None	30-92	93-97	Microscopy	No	No
ImmunoCyt	Mucin, CEA	32-100	33-91	Microscopy	No	No
BTA TRAK	Complement factor H	52-78	63-86	ELISA	Yes	Yes
BTA stat	Complement factor H	32-100	63-92	Point of Care	No	N/A
NMP22 Test	NMP22	33-100	41-92	ELISA	Yes	Yes
BladderChek	NMP22	38-86	78-96	Point of Care	No	N/A
UroVysion	Aneuploidy 3,7, 17 and loss of 9p21	51-100	55-100	Complex FISH	No / Yes	No
Oncuria™	ANG, A1AT, APOE, CA9, IL8, MMP9, MMP10, PAI1, SDC1, VEFGA	93	93	Multiplex Protein	Yes	Yes

BTA assays & NMP22 assays

The Bladder tumor antigen (BTA) test is a quantitative ELISA (BTA TRAK) or a qualitative point of care (BTA stat) protein assay that detects urinary complement factor H. Sensitivity reported for BTA is 32-100% with an accompanying specificity of 63-92% [24,25,27-29]. The NMP22 Test and BladderChek test measure urinary NMP22, a nuclear mitotic apparatus protein. The reported sensitivity is 33-100% and specificity is 41-96% [24,25,30-35]. The specificity of these assays may be impacted by other non-cancerous conditions, specifically, hematuria without cancer or inflammation [36-38]. Previous studies have demonstrated a correlation between hematuria and test positivity [39,40]. Rather than detecting a specific bladder cancer biomarker, these assays may be quantifying biomarkers introduced into the urine by a variety of benign conditions, *e.g.,* infection, inflammation, or instrumentation.

UroVysion FISH Assay™

The UroVysion™ Bladder Cancer Kit, is a multicolor, multiprobe fluorescence *in situ* hybridization (FISH) assay that monitors four specific chromosomal alterations in exfoliated urothelial cells. The sensitivity reported for the FISH assay is 51-100% and specificity is 55-100% [24,25,41-49]. Similar with VUC, the lack of sensitivity achieved by UroVysion™ is skewed by the poor detection of low-grade and early stage tumors.

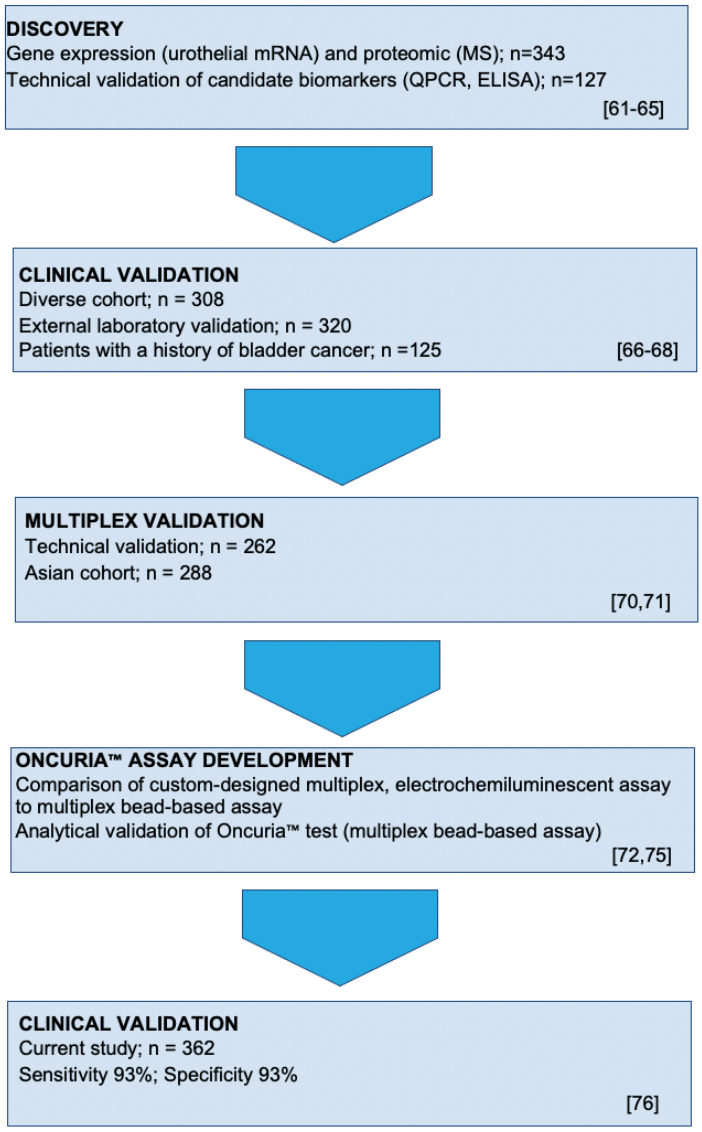
ImmunoCyt Test

The ImmunoCyt/uCyt+ assay is designed to supplement VUC through the detection of cellular biomarkers on cytology slides using fluorescent monoclonal antibodies. The sensitivity reported for ImmunoCyt/uCyt+ is 32-100% with a specificity of 33-91% [24,25,50-53]. As a cell-based assay, ImmunoCyt/uCyt+ is less impacted by hematuria and inflammatory conditions, but, as with cytology, the assay depends on specimen stability and handling, and may be influenced by inter-observer variation.

Multiplex assays

Single biomarkers are limited by the fact that not all bladder cancer tumors, or even those in one category of lesion (*e.g.,* low stage or low-grade) will harbor any single molecular change. Accordingly, the concept that the presence or absence of one molecular biomarker will aid clinical evaluation has not proved to be the case for bladder cancer, or for other cancers. Investigators have begun to identify diagnostic biomarker panels, or

Figure 1. Phased approach for the development and validation of Oncuria™



MS – mass spectrometry; qPCR – quantitative polymerase chain reaction; ELISA – enzyme-linked immunosorbent assay

signatures. For example, through analysis of nine gene promoters, Hoque *et al.* found that 69% of bladder cancer patients had specific methylation in at least one of four genes; CDKN2A, ARF, MGMT, GSTP1 [54]. By combining the data from all 4-genes, a logistic prediction model was derived that achieved a sensitivity of 82% and a specificity of 96%. Chung *et al.* tested the hypermethylation state of 10 genes in voided urine samples and identified a multigene predictive model comprised of five target genes (*MYO3A, CA10, NKX6-2, DBC1*, and *SOX11*). Sensitivity and specificity of this model were 85% and 95%, respectively [55]. Examples of diagnostic mRNA signatures include those proposed by Hanke *et al.* [56] and Mengual *et al.* [57]. However, these studies have evaluated small and limited populations (*i.e.,* few benign confounding conditions included) and have not undergone extensive validation. Only Holyoake *et al.* have reported extensively on the discovery [58] and validation of a multiplex urinary RNA signature (Cxbladder™) to date [59,60].

Derivation of a bladder cancer signature

The methodological approach we deployed to identify a diagnostic bladder cancer signature is depicted in **Figure 1**. Two complementary techniques were applied to profile urine samples from patients with or without bladder cancer; gene expression (mRNA) of shed urothelia [61,62], and glycoproteomics profiling of urine supernatant [63,64]. Using sophisticated bioinformatics, the two datasets were combined, and a cancer-associated signature comprised of 19 candidate biomarkers was identified.

The potential clinical utility of the candidate protein biomarkers was monitored in voided urine samples from an independent cohort using commercial ELISA kits. In a cohort of 127 patients (64 with bladder cancer), a 10-protein biomarker signature (ANG; apolipoprotein E, APOE; alpha-1 antitrypsin, A1AT; carbonic anhydrase 9, CA9; interleukin 8, IL8; matrix metallopeptidase 9, MMP9; matrix metallopeptidase 10, MMP10; plasminogen activator inhibitor 1, PAI1; syndecan 1, SDC1 and vascular endothelial growth factor A, VEGFA) achieved a diagnostic sensitivity of 92% and specificity of 97% [65]. Appreciating that benign conditions can adversely affect the performance of urinary biomarkers, signature association was confirmed in a cohort comprised of 108 bladder cancer patients and 202 controls, including patients with urinary tract infection, hematuria and no cancer, kidney stones and moderate to severe voiding symptoms [66], and the potential utility was validated by

an independent laboratory [67]. The diagnostic signature was also confirmed to perform equally well for the detection of recurrent bladder cancer in a cohort of 125 patients on disease surveillance. The assay outperformed both UroVysion™ and VUC in this context [68]. The analysis of cumulative data from over 1,100 patients confirmed the diagnostic power of the multi-factor signature over individual biomarkers, regardless of histological grade or disease stage of tumors [69], so the development of a robust multiplex assay was initiated. Early prototypes of a multiplex immunoassay were tested in two large independent cohorts. In a US cohort of 200 patients (100 with bladder cancer), the immunoassay achieved a diagnostic sensitivity of 85% and specificity of 81% [70], and in a Japanese cohort of 78 patients (211 with bladder cancer), the next iteration of the immunoassay achieved a diagnostic sensitivity of 85% and specificity of 81% [71]. Furthermore, we have tested and compared the performance of the multiplex assay on alternative technology platforms [72].

Decisions in cancer care depend on certain predictions. Ideally, such predictions or "risks" are personalized, and benefits outweigh the risks in terms of harm or toxicity [73]. Clinical nomograms or risk calculators can provide a probability for certain outcomes, e.g., cancer detection, recurrence, progression, or death. Leveraging the utility of key clinical features (*e.g.,* age, race and gender) in stratifying at-risk patients of harboring bladder cancer, we developed a diagnostic nomogram that was comprised of the 10 biomarker signature plus key clinical features. The addition of molecular data into a clinical nomogram improved the predictive performance, i.e., a hybrid nomogram performed better than demographic or biomarker data alone [74]. Thus, the multiplex immunoassay can be viewed as an In Vitro Diagnostic Multivariate Index Assay (IVDMIA), which combines multiple variables (biomarkers and clinical) using an interpretation function to yield a single, patient-specific result (e.g., a "risk score"), that is intended for use in the diagnosis of disease, in this case bladder cancer.

Oncuria™

The 10-biomarker test has now been developed by BioTechne into a commercial multiplex immunoassay (Oncuria ™) for the early detection of bladder cancer in patients presenting with hematuria or with a history of bladder cancer on disease surveillance. The immunoassay is performed using Luminex xMAP technology. The physical components of the BioTechne multiplex assay, a library of detection and capture antibodies, and the secondary reagents have undergone extensive optimization for consistent implementation. Analytical validation of the test has assessed selectivity, sensitivity, specificity, accuracy, linearity, dynamic range, and detection threshold, using voided urine as the test matrix [75]. Lower and upper limits of quantification (LLOQ and ULOQ), antigen cross-reactivity, and the effect of potential interference of the assay by matrix substances has been defined. Clinical validation of the optimized test was performed using urine samples obtained from a cohort of 362 patients (46 with bladder cancer) [76]. In this study, mid-stream voided urine samples were collected from the 362 subjects presenting to outpatient Urology clinics at two sites within the US and were analyzed using the Oncuria™ test. The median age of bladder cancer subjects was 69 years (range 38-87 years), 76.1% were men and 67.4% were Caucasian. Of the 46 bladder cancer cases, 61.4% were classified NMIBC; stages Ta, Tis, T1), and 38.6% were MIBC; stage ≥T2, 19.6% cases were reported as low-grade cancer and 80.4% cases as high-grade. Urinary concentrations of all 10 test analytes were elevated in patients with bladder cancer compared to

Table 2 Mean urinary (±SD) concentrations of 10 biomarkers assessed by Oncuria™ in cohort of 362 subjects

		Bladder Cancer		Non-Cancer Control		
Biomarker	**Detectable**	**N = 46**		**N = 316**		
(pg/mL)	% of samples	Mean	SD	Mean	SD	**P**
MMP9	64.3	1,237.2	2,191.7	143.0	1,304.3	**0.002**
IL8	84.4	681.0	1,376.4	90.3	582.5	**0.006**
VEGFA	88.6	1,003.9	2,743.3	127.8	261.3	**0.04**
CA9	40.6	8,979	35,518	0.843	2.016	0.09
SDC1	99.3	9,461	6,415	8,707	4,455	0.44
PAI1	71.7	1,169.8	2,803.0	29.8	132.9	**0.009**
APOE	95.7	16,627	35,895	1,014	2,001	**0.005**
A1AT	93.2	179,562	236,921	33,742	67,463	**0.0001**
ANG	81.8	1,800.4	3,170.3	194.7	464.7	**0.001**
MMP10	57.7	52.79	200.47	4.92	8.88	0.12

those without evidence of bladder cancer (**Table 2)** with statistical significance reached for individual analytes MMP9, IL8, VEGFA, PAI1, APOE, A1AT, and ANG.

A combinatorial analysis of all ten biomarkers achieved a sensitivity of 87% and a specificity of 92%. This was further improved upon when three key clinical factors (e.g., age, race, and gender) were included in the clinical nomogram which achieved a sensitivity of 93%, a specificity of 93% and a negative predictive value of 99%.

Bladder cancer is a common cancer with a high rate of recurrence and progression, and the recurrence phenomenon makes it one of the most prevalent cancers worldwide. The development of a robust non-invasive, urine-based assay for the detection of bladder cancer would clearly have a positive impact on the clinical management of patients with bladder cancer. Oncuria™ is a reliable, validated assay which can provide physicians valuable assistance with the management of patients at risk of harboring bladder cancer. The detection of urinary proteins through multiplex array platforms has the potential to be relatively simple to perform and interpret, and affordable. Currently, four large multicenter, international prospective clinical trials (NCT 03193515, 03193528, 03193541 and NCT04564781) are underway to establish the potential role of Oncuria™ in the physicians' armamentarium.

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