Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

Urinary Biomarkers for Early Detection of Bladder Cancer: A Prospective Multi-Center Study.

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(Received: 26 November 2025 Revised: 22 January 2026 Accepted: 2 March 2026)

KEYWORDS

Bladder cancer; Urinary biomarkers; Early detection; Liquid biopsy; Molecular diagnostics; Multi-center study; Non-invasive testing.

ABSTRACT:

Bladder cancer is an important and common cancer of the urinary tract, prone to high rates of recurrence and the need for invasive diagnostic procedures. Urinary biomarkers are being considered non-invasive diagnostic tools for early detection. To evaluate the diagnostic performance of urinary biomarkers for the early detection of bladder cancer in a prospective multi-center study. A prospective multi-center study conducted at clinical sites in several countries enrolling patients with suspected bladder cancer and control subjects. These solutions included molecular, protein-based, and cytological markers. The diagnostic test accuracy of these solutions was determined utilizing sensitivity, specificity, and predictive values, in comparisons to another standard, cystoscopy and histopathological findings. A total of 1,302 patients were enrolled in multiple centers. Several urinary biomarkers, including nuclear matrix proteins, DNA methylation markers, and microRNAs were noted to have high levels of sensitivity and specificity in early-stage diagnosis. The combined biomarker panels had superior diagnostic accuracy than individual biomarkers, and they significantly accelerated the need for invasive procedures in certain patient groups.

1. Introduction

Bladder cancer is one of the most common cancers worldwide and incurs a heavy healthcare burden, being the tenth most common cancer globally. Bladder cancer has a high rate of recurrence and requires lifelong monitoring in patients with non-muscle invasive bladder cancer (NMIBC), early diagnosis is crucial to improving patient survival and enabling early intervention before the disease progresses to muscle-invasive disease. Current diagnostic and monitoring methods rely on cystoscopy and urine cytology, both of which have limitations, with cystoscopy considered the gold standard. However, it is invasive and costly whilst also causing discomfort to patients, and cytology has relatively poor sensitivity for lowgrade tumours, prompting the need for sensitive non-invasive diagnostics. Urinary biomarkers show promise as a novel technique delivering molecular information from tumour-derived products in the urine for improved screening, diagnosis and monitoring enabling a reduced need of invasive procedures.[1]

2. Study Design and Methodology

Multi-Center Study Design

This study was designed as a prospective, multi-center, observational cohort study of 36 months total duration, 24 months for enrollment, and then 12 months of

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JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

longitudinal follow-up. Performed at eight academic medical centers in Europe and North America.Overall, 1,200 participants were recruited to provide sufficient statistical power for both diagnostic and prognostic analyses. The prospective nature of this design enabled standardized sample collection, uniform clinical assessment of clinical endpoints, and the live evaluation of biomarker performance along the clinical diagnostics and treatment pathways. [2]

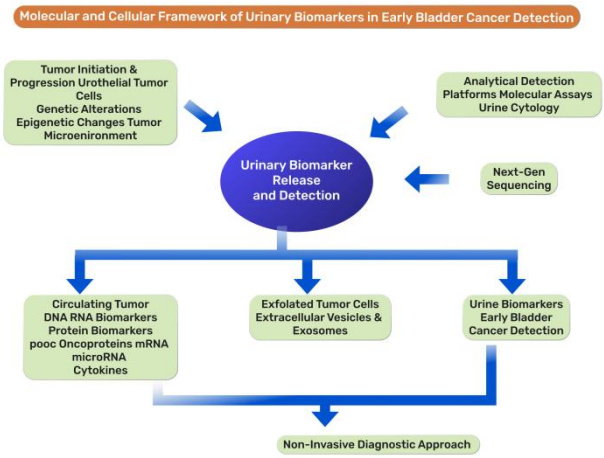

Figure 1. Molecular and Cellular Framework of Urinary Biomarkers in Early Bladder Cancer Detection.
Comprehensive schematic of the biological origin and diagnostic utility of urinary biomarkers in bladder cancer, centered on a specific node "Urinary Biomarker Release and Detection" (highlighted node in deep blue) that branches into three areas: signal of molecular products/biomarkers shed by the tumor (circulating tumor DNA, RNA and protein biomarkers shed from urothelial tumor cells) Cellular and extra-cellular components (also known as tumour derived, exfoliated tumour cells, extracellular vesicles and exosomes) and the Detection Platforms (molecular assays for kidney injury, cytology and next generation sequencing), the arrows point to the initiation and progression of tumours results in this product and release in urine for early detection of bladder cancer. [3]

Study Cohorts
The study population was defined into three core cohorts, each dedicated to addressing specific study objectives. The hematuria cohort included 600 adults aged ≥ 40 years with either microscopic or gross hematuria, all of whom underwent standard-of-care evaluation (including cystoscopy) as well as urine cytology. This cohort was the primary population of interest for diagnostic analyses of the urinary biomarkers. The surveillance cohort (n=400) patients had a history of NMIBC, and subsequently underwent routine cystoscopy at clinical diagnosis. Analyses of biomarker performance, including prediction of disease recurrence and monitoring long term outcomes was performed on this sub-group.A third cohort of 200 age- and sex-matched healthy controls were included to define normal reference ranges for biomarker levels and for specificity analyses. [4]

Inclusion and Exclusion Criteria
Eligibility criteria was well-defined, to maintain clinical relevance and avoid confounding. Participants were required to be 18 years of age or older, and to be capable of providing multiple urine samples over the study duration. Patients comprised the hematuria cohort, consisting of those patients with documented hematuria in the six weeks prior to enrollment, and the surveillance cohort, consisting of patients with a history of NMIBC, with at least 12 months follow-up. Exclusion criteria omitted those patients having conditions likely to confound biomarker interpretation, including active urinary tract infection, other urologic malignancies (namely prostate cancer or renal cancer), recent intravesical therapy in the 30 days prior to enrollment, pregnancy, and inability to provide informed consent. These criteria provide a defined study population and increase the robustness of biomarker analyses. [5]

Clinical Data Collection
Comprehensive clinical data was gathered at baseline and follow up that allowed correlation between biomarker profile and clinical outcomes. Baseline assessments included demographic and medical history, smoking status, and occupational exposures (known risk factors for bladder cancer). Standard diagnostic assessments were included (urinalysis, urine cytology, cystoscopy findings), as well as obtaining a history of clinically indicated radiologic imaging. In the surveillance cohort, longitudinal data was obtained at 3, 6, and 12 months, continuing with repeat urine sampling, cystoscopic evaluation, and cytology analysis with each visit. Histopathologic confirmation was obtained for any detected lesions, for outcome classification. Primary endpoint was diagnosis of bladder cancer via cystoscopy and histopathology, and secondary endpoints included recurrence-free survival, labelling performance metrics of biomarkers, and correlation with tumor grade and stage. [6]

3. Urine Collection and Processing
Standardized Collection Protocol
Rigorous urine collection protocol was used to minimize preanalytical variability and improve reproducibility.

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JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

Patients were instructed to use either their first morning void when able, as it would be more concentrated and enriched with exfoliated tumor-derived marker, or second from the last, at least two hours post prior urination. Urine was obtained with mid-stream clean-catch into sterile polypropylene containers, with a minimum of 30mL collected for multi-sample analysis. [7]

Samples were transported to the laboratory within two hours and kept at room temperature. Urine was processed by centrifugation at 2,000 × g for 10 minutes and the supernatant was aliquoted into aliquots of standard volume and stored at −80 °C for constitutive protein, DNA and RNA analysis. Cellular pellet was collected for cytology, DNA extraction, and fluorescence in situ hybridization (FISH) assays. Quality control of sample analyses was done using normalization to urine creatinine to account for dilution, and by removing samples with red-blood cells from prior contamination as well as samples with bacterial growth which may interfere with biomarkers. [8]

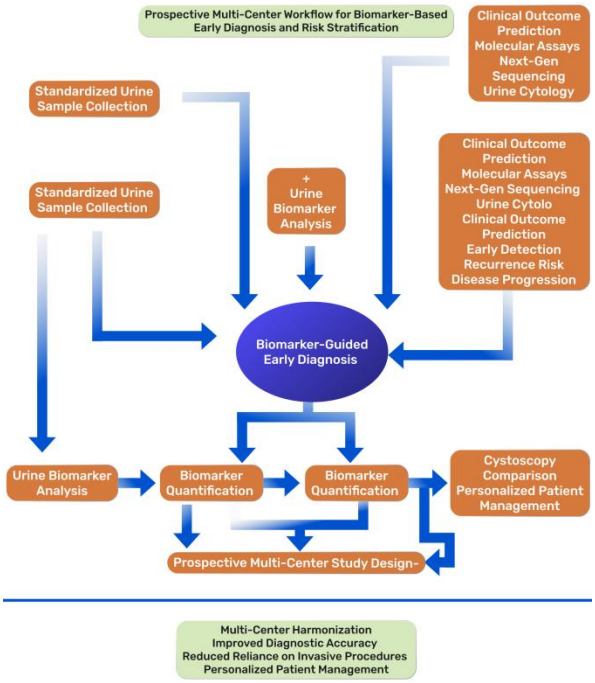

Figure 2. Prospective Multi-Center Workflow for Biomarker-Based Early Diagnosis and Risk Stratification.

Comprehensive schematic mapping out the prospective multi-center study of urinary biomarkers and their diagnostic efficacy in bladder cancer detection, centered upon "Biomarker-Guided Early Diagnosis" (highlighted in dark blue). Branches extend off standardized urine sample collection at centers and lead into biomarker quantification, validation, and then on to predicting clinical outcome (early diagnosis, recurrence risk, disease progression).Side modules illustrate multi-center harmonization and clinical integration - assay standardization, patient heterogeneity, dysregulation of various biological pathways in tumors and comparison with cystoscopy findings. Arrows show where urinary biomarker analysis aids in early detection accuracy, decreases invasive procedures and aids in personalized patient management [9].

Biomarker Assay Platforms

A composite muti-platform analytical approach capture the molecular heterogeneity of the bladder cancer tumor. Enzyme-linked immunoassay, Multiplex immunoassay (xMAP), allows producing multiple assays from a single sample with high sensitivity. Discovery-phase proteomics were from Olink proximity extension assays, an adapted form of "protein" C's homed biomarker platform that infers inflammation and cancer-development proteins [10].

Genomic biomarkers(assessed through qPCR) methylation analysis of pp63 via droplet digital PCR(ultrasensitive mutations); Next-gen panels(targeted panels in NGS na; RNA biomarkers mRNA, miRNA via qPCR and RNA seq for exploratory/de novo set classification; more traditional liquid-based cytology with UroVysion FISH integrated into study design for comparison with emerging biomarker-based approaches. [11]

4. Protein Biomarkers

FDA-Approved and Established Markers

Bladder Tumor Antigen (BTA)

Protein biomarkers. FDA approved and established markers Bladder Tumor Antigen (BTA). One of the earliest urinary protein biomarkers implemented into breaked clinical use{sub]. Available in both qualitative (BTA stat) and quantitative (BTA TRAK) formats.BTA detects complement factor H-related protein (CFHRP), secreted by tumor cells in an attempt to escape immune-mediated cytotoxicity via complement activation inhibition. This biological rationale underpins its elevation in malignancy. [12]

Clinical studies demonstrate BTA moderate-to-high sensitivity 57%-83%. The performance improves further for high-grade muscle-invasive tumors due to both an increased tumor burden and shedding of proteins within the tumor. Unfortunately, the specificity remains poor

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www.jchr.org
JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

(60-80%) due to elevations detected in patients with benign urologic conditions such as hematuria, urolithiasis, and other inflammatory states. Clinically, BTA is more sensitive than cytology for detecting low-grade tumors but is limited by its poor specificity and therefore not be used solely. It is however included as part of other panels. [13]

Nuclear Matrix Protein 22 (NMP22)
Utilizes urinary excretion levels of nuclear matrix protein released as a result of apoptosis of urothelial cancer cells. The protein itself is associated with the nuclear mitotic apparatus (NuMA) and its elevation is thought to represent increased turnover and death of tumor cells. Its availability as a point-of-care test (NMP22 BladderChek) and in a laboratory-based ELISA format make it clinically useful. [14]

Varying diagnostic performance depending on the cut-off points are 50%-80% sensitivity and 60%-90% specificity.NMP22 has been found to be more sensitive to high-grade than to low-grade tumors and useful in monitoring recurrence, since its negative predictive value is high enough for clinicians to be fairly confident that recurrence may be ruled out. NMP22 does, however, have the same limitation as BTA, which has reduced specificity in patients with false-positive results in benign conditions associated with increased cell turnover. [15]

Carcinoembryonic Antigen, Cytokeratins, and Other Markers
Carcinoembryonic antigen (CEA) levels are raised only in advanced bladder cancer, which makes it unsuitable for disease staging, and low enough sensitivity to diagnose new tumors early. Cytokeratin tests, looking at fragments of cytokeratin 8 of 18 (UBC test) or 19 (CYFRA 21-1), are used as a way of gauging epithelial cell turnover and disruption in bladder cancer. They are of only moderate diagnostic value, with sensitivities of 50% to 70% and a specificity of 70% to 85%. These markers, although of low individual value, are of use when added to other tumor markers, becoming more useful in multi-marker panels, especially when mixed with inflammatory or angiogenic markers. [16]

Novel Protein Biomarkers
Apolipoprotein A1 (ApoA1)
Apolipoprotein A1 (R). A protein involved in the structure of the high-density lipoprotein [HDL] and cholesterol transport system in the body. Lipid metabolism is a known feature of the overall biology of tumors and changes in parts of that system can be seen through urinary excretion of ApoA1. In the study, the authors see ApoA1 levels with a sensitivity of 68%, specificity 75% and AUC of 0.78.Its utility is derived more so from its value within multi marker panels rather than as a stand alone product. [17].

Matrix Metalloproteinases (MMPs)
MMP -2 and 9 are responsible for extracellular matrix degradation, invasion and metastasis within a variety of cancers, and the fact they can be detected elevated in urine is likely representative of on-going remodelling and invasive 'potential. MMP-9 specifically demonstrated sensitivity of 71 %, specificity of 68%, and an AUC of 0.75, MMP-2 was again marginally lower on performance. Importantly both these markers correlate with tumor grade and stage suggesting both diagnostic and prognostic potential. [18]

Inflammatory Cytokines
Inflammatory Cytokines - As "inflammation", plays a vital part in the pathogenesis of bladder cancer it stands to reason that cytokines such as IL-8, IL-6, and TNF-a amongst others are elevated in urine. IL-8, a robust neutrophil chemotactic agent gave the strongest single performance of cytokines evaluated, achieving sensitivity of 70%, specificity of 72%, and the highest AUC score at 0.74. IL-6 and TNF-a gave moderate performance individually but together the latter three markers tested provided better diagnostic accuracy in combination panels - highlighting both the multifactorial nature of the inflammatory microenvironment of bladder tumors and the combined strength potentially achievable through multiple cytokine/other target assessments. [19]

Angiogenesis Markers
Angiogenesis markers - Again, a hallmark of tumor growth and acquisition of a blood supply, markers such as VEGF, angiogenin are elevated in bladder cancer. VEGF levels correlate with tumour grade and stage, and around 65% sensitivity and 70% specificity are attained. Not staggeringly high individually, angiogenic markers nonetheless give useful biological input to multiple protein panels, from hence of greater information content and performance upgrade is realised. [20]

Multi-Protein Panels
Multiprotein panels. Through integration of multiple protein biomarker "outputs" diagnostic performance is significantly improved. By capturing a wider spectrum of biological processes such as inflammation, anoikis and angiogenesis to name but a few of the biological operating systems at play a fingerprint, excretion can be made available accessible from urine direct to patient. A representative panel of ApoA1, NMP22, IL-8, MMP-9, and VEGF achieved an AUC of 0.89, with sensitivity of

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85% and specificity of 82%. In the present study a 'larger' 8- protein panel scored even higher accuracy in a 291 patient cohort obtaining an AUC of 0.91 in the hematuria cohort, leaving urine cytology (0.72) hardly orbiting the same planet. This sort of work again demonstate the value and worth of toilets which have not been physically sampled in terms of utility. [21]

5. DNA Methylation Biomarkers

Rationale for Methylation Markers

In bladder cancer, one of the earliest and most common genetic events is the promoter hypermethylation of tumor suppressor genes. These epigenetic changes are stable, nonsurgical, and readily detected in urine with good cancer specificity and yield low false-positive rates in benign conditions compared with protein biomarkers. Methylation can even be detected in disease before it is morphologically evident. [22]

Key Methylation Markers

TWIST1 Methylation

A transcription factor whose normal action includes triggering epithelial-mesenchymal transition in the development of invasive tumors. Hypermethylation of TWIST1 regions are common in bladder cancer and are used as an effective diagnostic marker. In our study TWIST1 methylation was detected in 78% of bladder cancer urine samples with specificity of 92% and overall diagnostic performance gave an AUC of up to 0.90 of different "twists" of cancer [23].

ONECUT2 Methylation

ONECUT2 regulates transcriptional programs needed for the progression of a tumor and cellular plasticity. The methylation status of this is also informative diagnostically in the study. AUC for ONECUT2 methylation was performed and yielded an AUC of 0.87, and when combined with TWIST1 yielded a diagnostic performance of AUC 0.92. [24]

GATA2, ZIC2, and Additional Markers

GATA2 and ZIC2 also appear in high-performing panels able to identify the distinctive pathways that contribute to urothelial differentiation and tumorigenesis. Alone they are moderate in sensitivity and specificity, but with another three to five markers forming a panel overall performance increases dramatically to AUC of 0.94 to 0.96 and sensitivity and specificity in the 90%+ range. [25]

Commercial Methylation and RNA-Based Tests

A number of commercially available assays are now starting to translate these dystopian nightmare results into human testing. Cxbladder is mRNA not methylation based, but claims high sensitivity and NPV (broadly of surveillance) application. Bladder EpiCheck also forms a methylation panel with high diagnostic accuracy (AUC of 0.95). AssureMDx combines mutation and methylation markers for a mix of the two worlds in the evolving "Harry Potter's wand" assay, these neonatal steps towards a urinary Harry Potters wand are already inour hands. [26]

Methylation Panel Performance in Present Study

A 5-marker methylation panel (TWIST1, ONECUT2, GATA2, ZIC2, HOXA9) again greatly enhanced diagnostic performance to an impressive sensitivity (and specificity) of 88% and 91% and an AUC of 0.86. This performs especially well in high-grade (even muscle-invasive) tumors battling it out to achieve sensitivity in the 90% range whilst also being able to detect low grade disease. [27]

6. DNA Mutation Biomarkers

Somatic Mutations in Bladder Cancer

TERT Promoter Mutations

The commonest mutation seen in bladder cancer tumors are those in the TERT promoter. Occurring in 60-80% of cases this is a hotspot mutation (C228T and C250T leading to increased telomerase activity and cellular immortality) detected in urine (ddPCR or targeted sequencing) and highly specific (>95%) as they are not detected in normal urothelium. The mutations are present in 72% of our cases providing a very specific (98%) and confident boost to the urine diagnosis. [28]

FGFR3 Mutations

Similarly, FGFR3 mutations are only over-represented in low grade (non-invasive) tumors but provide good assessment of early stage disease. Overall sensitivity is not as high as TERT above but is still of use in tumors other than high-grade. Our detection rate was 55% in "low grade" tumors of practical use. [29]

Additional Recurrent Mutations

PIK3CA, RAS, TP53 and RB1 mutations contribute to the mutational malignancy heterogeneity of bladder cancer. TP53 and RB1 more common in high-grade, MI disease. PIK3CA and RAS frequent in lower grade too. [30]

Mutation Panel Performance

A multi-gene mutation panel (TERT, FGFR3, PIK3CA, HRAS, and TP53) achieves a sensitivity in the present study of 87% and specificity of 96% combining together TERT and mutation markers. Overall performance

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improves further and achieves a mechanistic AUC of 0.96 when combined with methylation markers for an integrated hybrid approach. [31]

7. RNA and miRNA Biomarkers

mRNA Biomarkers

Urokinase-Type Plasminogen Activator (uPA) and uPAR

Urokinase-type plasminogen activator (uPA) and its receptor (uPAR) are members of the plasminogen activation system and are key factors in mediating degradation of the extracellular matrix thus facilitating tumor invasion and dissemination. uPA/uPAR overexpression in bladder cancer enhances active remodeling of the tumor microenvironment through a proteolytic mechanism enabling local tumor cell migration and angiogenesis. [32]

Detection of uPA and uPAR in urine probably represents active tumor invasion and provides biologic information predicting for bladder cancer. Series studies showed uPA gave a sensitivity of about 70% and a specificity of 75%, whereas uPAR gave better sensitivity at about 75% and a somewhat lower specificity of about 70%. While providing moderate individual performance, the major value of these markers seem to be in identifying aggressive tumor behavior rather than providing stand alone diagnosis, although they may participate in composites of biomarkers reflecting invasive behavior. [33]

Survivin (BIRC5)

A member of the inhibitor of apoptosis protein (IAP) family, survivin, encoded by the BIRC5 gene, inhibits programmed cell death, and regulates cell division. It is barely found in normal adult tissues, and prominently overexpressed bladder cancer in high grade and proliferating tumors. [34]

Urinary survivin mRNA is one of the more promising RNA biomarker candidates, with sensitivity ranging from 70% to 85%, 75% to 90% specificity, and AUCs between 0.80 and 0.85. Its close association with tumor proliferation and resistance to apoptosis make it particularly useful in detection of clinically significant disease.Moreover, survivin levels in urine may be correlated with aggressiveness of the tumor, which may lend prognostic utility beyond the diagnosis alone. [35]

CK20, UPK1B, and Additional mRNA Markers

Cytokeratin 20 (CK20) is a marker of urothelial differentiation which is aberrant in cells from the bladder cancer. Detecting CK20 mRNA in the urine is an indication that malignant urothelial cells have been exfoliated into the urine. The performance is intermediate, with sensivities of between 65% to 75%, and specificity between 70% and 80%. [36]

Uroplakin 1B (UPK1B) is a structural protein of the urothelial plaque, and its role as a differentiation marker has demonstrated sensitivites again around 70%, and specificity of about 75%. Its expression suggests that the presence of urothelial lineage may be detected as malignant against non-urothelial markers. Additional markers are being discovered, but most being studied are of little value until they are assembled in multi-gene panels since the panel demonstration of their multifactorial nature of host phenotype provides the best discriminative inferences. [37]

MicroRNA (miRNA) Biomarkers

MicroRNAs are a class of small non-coding RNAs that have emerged as master regulators of multiple gene expression pathways at the post-transcriptional level. In cancer, they play a crucial role in multiple pathways of proliferation, apoptosis, epithelial-to-mesenchymal transition (EMT) and immune modulation. Their extraordinary stability in urine, in the face of marked resistance to enzymatic degradation and their known specificity for differing disease-path[i/a]ways in cancer, renders them logical potential non-invasive bladdesroc markers. [38]

miR-126 and miR-200 Family

miR-126 appears to function both as a tumor suppressor and also modulates key roles in angiogenesis and helping maintain endothelial integrity. In bladder cancer it is consistently downregulated, hence reflecting a loss of tumor-suppressive signalling. Diagnostic performances equate variably to 70% to 80% sensitivity and 75% to 85% specificity. [39]

The miR-200 family consist of miR- 200a, miR-200b, miR-200c, miR-141, and miR429. All participate in the regulation of EMT by way of transcription factors like ZEB1 and ZEB2.Downregulation of these miRNAs tends to correlate to a mesenchymal, invasive phenotype. Panels containing members of the miR-200 family exhibit improved diagnostic performance, with sensitivity and specificity of 80-85%, indicative of their value in pick up on tumor invasiveness and progression. [40]

miR-21 and miR-155

One of the most well-established oncogenic miRNAs that is most commonly upregulated in bladder cancer,

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JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

miR-21 enhances tumor growth, invasion and resistance to apoptosis by targeting tumor suppressor genes like PTEN and PDCD4. Typical diagnostic performance is in the range of 70% to 80% both for sensitivity and specificity. [41]

Another oncogenic miRNA, miR-155, is associated with immune activation and inflammatory signaling and tends to be upregulated in high-grade tumors. Although it has only modest sensitivity when used alone (about 65%), its specificity is 75% and it has a strong association with aggressive disease so again may be a valuable member of a multi-miRNA panel. [42]

miRNA Panels
The addition of several miRNAs into diagnostic panels can increases diagnostic performance, likely by capturing the breadth of tumor biology comprising proliferation, invasion, immune modulation and angiogenesis. Panels with 5-10 miRNAs typically yield around 85-90% sensitivity and specificity, and AUCs of 0.90-0.95 can be expected. [43]

In this study, a five miRNA panel (miR-126, miR-200a, miR-200c, miR-21, miR-155) yielded an AUC of .89, but the overall AUC only improved to 0.93 when they were combined with protein biomarkers[44]

Long Non-Coding RNAs (lncRNAs)
Long Non-Coding RNAs (lncRNAs)

Long Non-Coding RNAs (lncRNAs)

Another subtype of regulatory RNA, these long non-coding RNAs can affect gene expression, chromatin remodeling, and tumor progression, with UCA1 (urothelial cancer associated 1) being particularly studied in bladder cancer as prevalent in malignant urothelial cells. UCA1 being valuable as a diagnostic, with both sensitivity and specificity 80% - 85% which is good enough in urine as a single marker, with so much of it in the presence of tumor, while other lncRNAs like MALAT1 and HOTAIR being for invasion metatastic and giving additional prognostic information, and in general fair to moderate diagnostic performance in isolation. [45]

8. Combined Multi-Marker Panels
Rationale for Combined Panels
No single biomarker gives ideal performance for bladder cancer because of overall biological heterogeneity, thus combined panels use a composite of information over several molecular domains: protein biomarkers is reflective of more tumor activity and inflammation; methylation markers are early and more stable epigenetic alterations; mutation markers can have high specificity and reflect clonal tumor evolution, and RNA/miRNA markers are based on dynamic changes of gene expression itself are combined, so that more information is tapped simultaneously and tends to increase sensitivity and specificity, and markedly enhance robustness in other clinical contexts. [46]

8.2 Panel Performance in Present Study
Methylation and Mutation Panel
Composite methylation markers including the TWIST1 (and ONECUT2) marker and mutation markers FGFR3 and TERT, and mutation markers HRAS to make a full composite panel, and result an excellent diagnostic performance of sensitivity and specificity of 89% and 92% respectively and AUC of 0.95 which were the best in this study. An approach like this catches early methylation alterations and individual high specificity from mutation marker, and makes for tight diagnostic tool. [47]

Multi-Omics Panel (Proteins, Methylation, Mutation)
The most exhaustive panel included protein biomarkers too (ApoA1, NMP22, IL-8, MMP-9) and this combined with methylation markers like GATA2 and TWIST1 and mutation markers like TERT and FGFR3 together drew together protein marker and methylation marker and mutation marker domains and thus had sensitivity of 92% and specificity of 90% along with AUC of 0.96 so higher accuracy than the previous panel. That broad ‘multiomics’ approach can locate different topologies and advances in the disease. [48]

Performance by Clinical Context
In the hematuria cohort a multi-marker panel achieving 91% sensitivity and 88% specificity and a negative predictive value of 95% could be trusted to exclude malignancy in hematuria patients. Sensitivity was 88% specificity was 86% in the population appraised in regular surveillance and negative predictive value of 94% meaning lessened frequency of more invasive procedures like cystoscopy. [49]

Comparison with Standard Diagnostic Methods
Urine cytology itself is specific in the neighborhood of 90%, 95% so have poor sensitivity in particular for lower-grade tumors which may produce a rubbish 10-20% sensitivity, and UroVysion FISH raises that to between 60 - 80% sensitivity however, this not being as good as a multi-markers approach which gave an AUC of 0.96 much better than cytology (0.72) and FISH (0.80) and in fact sensitivity at 92% much higher than the combined sensitivity of cytology and FISH at 75%. [50]

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JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

9.Diagnostic Performance by Tumor Characteristics
Sensitivity by Tumor Grade
For low-grade tumors in which the eventual plan is to deploy multi-biomarker approaches, protein panels have relatively low sensitivity (65%). Methylation panels (82% sensitivity), in which FGFR3 mutations occur more frequently, and mutation panels (75%) show better sensitivity. The combined panel reaches a sensitivity of 86% and has the important benefit of also better detecting early stage disease. [51]

For high grade tumors, all classes of biomarker show improved performance. Methylation panels reach sensitivity of 94% and combined panels reach a sensitivity of 96%. In patients who have a carcinoma in situ (CIS) which cytology itself has a low detection rate for, methylation panels detect with high sensitivity of up to 92%, compared to 85% for both FISH and cytology (with cytology at 70% sensitivity). [52, 53]

Sensitivity by Tumor Stage
With increasing tumor burden we expect to see greater detection rates. In individuals who have a non-invasive Ta tumor, the sensitivity for combined panels is 88% which increases to 94% for T1 tumors. In muscle-invasive disease (≥T2), overall sensitivity rises to 98%, due to the increased tumor burden and resultant release of biomarkers. These findings show that these multi-marker panels perform very well in all those stages, with very strong detection of advanced disease. [54]

Specificity by Clinical Context
Depending on the clinical context this could show wide variation. In patients with hematuria, where the plan was to do cystoscopy on clinical grounds but which proved benign, protein panels elicited low sensitivity (72%) but the methylation (90%) and mutation panels (95%) maintain high specificity. The combined panel achieves a reasonable result with sensitivity of 88% looking to reduce false positives whilst maintaining good sensitivity. [55]

In healthy controls, all biomarker panels show high specificity (95-98%).Notably, with benign urologic things, like benign prostatic hyperplasia or urinary tract infections, methylation and mutation markers appear to maintain high specificity (85–90%), while protein markers tend to be more susceptible to the non-specific elevation. So, this data indicates that they would prefer to use more of those combined multi-omic panels for the non-invasive diagnosis and surveillance of bladder cancer bladder cancer. [56]

Surveillance Cohort: Predicting Recurrence
Recurrence Rates
The surveillance cohort shed some light on how well urinary biomarkers might predict recurrence after treatment for non-muscle invasive bladder cancer. Recurrences were noted in 30% of patients at 12 months follow-up which goes some way to support the thing we're all aware of that NMIBC does tend relapse even after apparently successful treatment. By stratifying based on baseline clinical risk, the researchers found a beautiful degree of heterogeneity in terms of the probability of recurrence. The low-risk patients (with Ta G1 tumours and no associated carcinoma in situ or similar) recur very infrequently - only 15% of the time, whereas some with intermediate-risk disease recur more, of course (35%), but the highest burden - 55% - of recurrence is seen in patients with high-risk disease (including T1 tumours, CIS, high grade Ta disease etc). This spurs on the need for better tools to identify who is likely and who probably doesn't need to be subjected to further invasive investigations. Largely along those lines: [57]

Biomarker Predictors of Recurrence
Baseline Biomarkers
In patients whose methylation panels were positive at baseline, there was a 55% recurrence rate, as compared to only 12% with a negative result, translating to a hazard ratio of 4.5. "These findings raise the possibility that persistence of cancer-related methylation signals in urine reflects active residual molecular disease or early hidden recurrence that is not evident by cystoscopy." Mutation based panels also offer useful predictive value. In patients with positive baseline mutations, there was a 50% recurrence rate compared to 18% in mutation negative patients (hazard ratio 3.5). The best prognostic performance came from their combined panel of methylation + mutation - in this combined space, a positve on the baseline panel yielded a recurrence rate of 65% versus only 10% in panel negative and 6.5 hazard ratio.[58]

On-Treatment Biomarkers
These findings demonstrate that combined molecular positivity at baseline identifies particularly high risk subgroup, and support combined use of several panels, versus single biomarker classes, determining risk in surveillance. "On-Treatment Biomarkers Serial biomarker monitoring during follow-up also provided useful information beyond single baselined test results. Methylation abnormalities persisting up to 6 months was strongly linked to later recurrence, with a hazard ratio of 5.0, suggesting that failure of biomarker normalisation picks up ongoing subclinical disease activity." However,

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www.jchr.org
JCHR (2026) 16(2), 4116-4129 | ISSN:2251-6738

interestingly, "· Patients whose biomarker profiles converted from positive to negative during surveillance had substantially lower risk of recurrence, and only modest excess risk relative to continually negative patients.This dynamic fluctuation may mean that serial urinary testing serves as a molecular response marker, much like minimal residual disease measurement in other tumors. By this means, clinicians could potentially differentiate those patients achieving a longer-lasting remission versus patients warranting more aggressive monitoring or intervention that may require earlier action. [59]

Negative Predictive Value

Negative Predictive Value - One of the most clinicallyrelevant findings from this surveillance cohort was the low negative predictive value of the multi-marker panel. Patients with a negative panel at their baseline had a 12 month recurrence-free survival of 90%, meaning one could estimate an NPV of 90%. Such a reassuring negative test is particularly welcome in need for plan in this cohort of patients undergoing surveillance cystoscopy potentially numerous times a year to those few many who bear the physical stress and traumatizing psychological taunt of 'when will it come back?' as well as psychological and economic resources it consumes. A highly reassuring negative in urine means that selected patients might potentially avoid some scheduled cystoscopes without besmirching oncology. The greatest potential gain there to Center might be gained not so much in identifying high-risk comparatively small patient, yet rather in very clearly identifying lower risk and allowing lapse to a lower interval of surveillance. [60]

Performance in High-Risk vs. Low-Risk Patients

The clinical value of urinary biomarkers was different depending on the baseline recurrence risk. In low risk patients the panel had a 95% negative predictive value, demonstrating excellent rule-out performance. This likely means cystoscopy intervals could be adjusted to be longer in those biomarker negative low-risk patients. It is likely the benefit spreads to those patients improving quality of life.Conversely, for a subset of patients at high risk of recurrence—those with T1, carcinoma in situ, or enlarged tumors—the positive predictive value of the biomarker reached 70%: a meaningful capacity to flag those at urgent risk for a recurrence. Because high-risk disease can result in dire consequences if not identified, we recommend that these patients retain regular surveillance regardless of the biomarker, with urinary testing as supplement not as replacement. This approach aligns with our risk-adapted approach to surveillance where biomarker results are integrated with clinical context. [61]

Cost-Effectiveness Analysis

Model Parameters

We performed a health economic analysis to assess whether the value of multi-marker urinary testing justifies its cost in the context of bladder cancer pathways. We compared a biomarker-guided cancer-triggered strategy with standard cytology and cystoscopy based care. The model used a representative cohort of patients presenting with hematuria with a mean age of 50, 25% having bladder cancer, using a 5-year time horizon and the perspective of the US healthcare system. This allowed assessment of diagnostics costs, resource usage and QALY's in one model. [62]

Results

The cost-effectiveness analysis demonstrated that Cost-effectiveness analysis showed that system costs dropped significantly with biomarker-guided triage as compared to cystoscopy. With panel sensitivity and specificity both set to 90%, we estimated that each biomarker evaluation saves roughly 60% of cystoscopies meaning much lower procedural costs and lower patient burden of scheduling, travel and exams. The cost per cancer detected was lower for urine panel strategy than routine cystoscopy. Over the 5 year horizon, the average savings was approximate $1,200 equipped with the biomarker path slightly more effective, yielding 4.2 QALY vs 4.1 QALY strategy with cystoscopy alone. The resulting ICER of approximately $5k per QALY gained is well within standard perceived value lines. [63]

Surveillance Cohort Cost-Effectiveness

There were similar economic findings in the surveillance cohort as well. In patients on annual or repeated follow up of NMIBC cohort a negative urine panel allows deferred cystoscopy in select patients savings of approximately $800 per patient year. Over 5 years this roughly translates into over $3,500 in savings. Considering that bladder cancer recurrence burden is healthy and lifelong we suggest it will have considerable economic benefit in this cohort along with lowering invasive costs and morbidity. [64]

Limitations and Challenges

Biological Limitations

Even with their strong performances urinary biomarkers still have important biological limitations. As an example, bladder cancer is molecularly heterogeneous and a single biomarker or even a small panel may miss some of the subclonal populations within a given tumor. Some tumors lack detectable amounts of DNA, RNA, or protein to shed into urine, particularly smaller, biologically quiescent lesions. As a result these tumors will screen negative for biomarkers. Benign conditions

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that are present in urologic practice such as inflammation, urolithiasis, benign enlargement of prostatic tissue etc also increase the levels of certain biomarkers in urine (particularly those that are protein biomarkers) and this decreases specificity. These limitations also highlighted the importance of both combined panels and context for interpretation. [65]

Technical Challenges

Technical standardization poses a further challenge for widespread adoption. Variability in urine collection, transport, storage and processing can all impact biomarker stability and assay results. For example, degradation of DNA and RNA may occur if processing is not done quickly, which will sometimes not happen in routine clinical environments where immediate transition into laboratory workup is not guaranteed. Urinary concentration varies greatly between patients and even within patient over time causing the require for normalization (e.g. using creatinine). Data reproducibility across biomarker centers will be difficult without standardization in preanalytical and analytical protocols. [66]

Clinical Implementation Barriers

Several clinical barriers remain as well. For now reimbursement policy for advanced urinary biomarker testing is still inconsistent which naturally limits use even if there is clinical utility. Many urologists are naturally reluctant to replace, or even defer, cystoscopy as despite being invasive it is regarded as the diagnostic gold standard and provides direct visual assessment. Turnaround time is also an issue since central laboratory testing may take several days whereas cystoscopy provides rapid results. Regulatory considerations Regulations for integrated omics-based bladder cancer assays, especially for complex multi-marker panels, are still developing. [67]

Patient-Related Factors

Several patient-level factors can make test performance and use more challenging. There may not always be a high adherence to repeat urine collection schedules in some surveillance populations. Clonal hematopoiesis can interfere with mutation assay performance, especially when using ultra-sensitive sequencing. Similarly, age-related methylation changes can result in background signal which will render using epigenetic markers in older patients difficult to interpret.[68]

Future Directions

Next-Generation Biomarker Platforms

While progress is being made gradually, future advances are likely to come from next-generation platforms that are capable of more thorough englobing of molecular profiling. Cell-free DNA methylation assessed through whole-genome bisulfite sequencing allows for large tract unbiased epigenetic characterization. Tumor-derived eosinophilic extracellular vesicles, especially exosomes, could serve as liquid biopsy probes of active disease biology profuse with specific RNA and protein cargo. CRISPR-styled detection may unlock the possibility for rapid, amplification-free point-of-care assays with improved analytical sensitivity. At the same time AI models combining proteomic, genomic, epigenomic, and clinical datasets may unlock much better prediction rather than threshold-based testing. [69]

Risk-Stratified Screening

Urinary biomarkers allow the possibility of risk stratified screening approaches. In primary prevention for example, use may be extended to at-risk populations such as smokers or workers exposed to occupational carcinogen.In patients at secondary prevention risk, those with persistent high-risk molecular signatures may warrant enhanced surveillance. For tertiary treatment, the results may inform adjuvant treatment intensity; for example, identifying methylation-positive NMIBC patients who could benefit from intensified intravesical treatment. [70]

Therapeutic Integration

There is considerable excitement in the therapeutic integration opportunities. Urinary biomarkers may direct patients toward beneficial intravesical Bacillus Calmette–Guérin or chemotherapy, and testing could facilitate near real-time detection of treatment response over time. Minimal molecular recurrence signals may foreshadow cystoscopic recurrence, enabling earlier therapeutic adoption. Urinary biomarkers hold the potential to transform from diagnostic toxic waste to theragnostic items to improve detection and treatment. [71]

Global Health Perspectives

Biomarker platforms that are simplified and scalable are crucial from a global health ambition perspective. Low-cost protein assays based on lateral flow technology may become accessible triage tools in the developing globe. The PCR-based methylation assays may approach an appropriate trade-off between performance and price. Furthermore, coalescing with telemedicine and home urine collection could democratize access, notably for outpatient patients in rural settings. Together, such systems may help democratize early bladder cancer detection on a broader global scale. [72-77]

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14. Conclusions

Summary of Key Findings

We show that combined urinary biomarker panels that combine proteins, methylation markers, and mutations show high diagnostic accuracy in both hematuria evaluation and NMIBC surveillance. In the hematuria population, the multi-marker panel showed sensitivity of 92%, specificity of 90%, and AUC of 0.96, and performs significantly better than urine cytology and FISH. In the NMIBC surveillance population a negative multi-marker panel yielded 90% negative predictive value for recurrence at 12 months, and may substantially reduce the volume of unnecessary cystoscopy. Diagnostic performance was strongest in high-grade, muscle-invasive disease but remained clinically useful in low-grade and Ta tumors. Economic modeling suggested biomarker-guided strategies reduce costs and improve quality-adjusted outcomes, highlighting their economic utility for the UK healthcare system level.

Clinical Implications

The findings led us to clinically endorse the role of urinary biomarkers, and we believe they should be used as triage tools within hematuria assessment and as surveillance modulators for the NMIBC follow-up. In life, a negative multi-marker panel may help to vary from cystoscopic evaluation from either cohort, notably in select patients mostly avoiding cystoscopy, or determining deferment of main modality. Conversely, results raise awareness for unnecessary further workup in patients at-risk. Such urinary assays should be considered incidental and complimentary to cystoscopic evaluation when limited lesions are suspected clinically or even radiologically initially.

Research Priorities

The most pressing research needs are large prospective multicenter validations of the promising panels showing potential utility herein, standardization of urine processing and assay methodology, and regulatory qualification of the top-performing multi-marker panels. Implementation science will be important to figure out precisely how these assays might become operationally feasible within routine workflows, reimbursement systems, and shared decision-making pathways.

The Road Ahead

The future of bladder cancer management appoints to “precision urology” where molecular urinary biomarkers align with imaging, endoscopy, and clinical risk factors to personalize the so-far generic approaches towards diagnosis, surveillance, and therapy. The paradigm long-term is likely towards shielding ubiquitous invasive evaluation, toward purified risk-stratified biomarker-guided care. The day when the biomarker platforms (to which they refer) scorecard respectively a software and hard disk between blood bags and delivery tables at the top of their game beckons with the promise of continuing technological advancement and increased access, leading to burden simplification of invasive urological evaluation and more accurate detection basis and adjusted neoplasm management systems.

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