Urine protein biomarkers for the detection, surveillance, and treatment response prediction of bladder cancer

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Abstract: The “gold standard” diagnostic procedure for bladder cancer is cystoscopy, a technique that can be invasive, expensive, and a possible cause of urinary tract infection. Unlike techniques such as histology, PCR, and staining, assays for protein biomarkers lend themselves well to the creation of efficient point-of-care tests, which are easy to use and yield fast results. A couple of urine-based tests have been approved by the U.S. FDA, but these tests suffer from low sensitivity. Hence, there is clearly a need for more reliable non-invasive biomarkers of bladder cancer. Urinary biomarkers are particularly attractive due to the direct contact of the urine with the urothelial tumor and the ease of sample collection. With these considerations, this review aims to provide a comprehensive listing of the most promising protein biomarkers of bladder cancer in urine. Biomarkers are organized by their potential role in detection, surveillance, or monitoring of treatment response. The purpose of this review is to assess progress towards the goal of identifying ideal urinary proteins for use in each of the above three biomarker applications in bladder cancer.

Keywords: Point-of-care tests, NMP22 test, tumor recurrence, proteomics, NMIBC

Introduction

Bladder cancer (BCa) is the sixth most common type of cancer in the U.S., contributing to 4.7% of cancer cases and resulting in significant morbidity and mortality [1]. BCa manifests as either muscle-invasive or non-muscle-invasive, the latter being the predominant form, comprising about 80% of cases [2]. The “gold standard” diagnostic procedure for BCa is cystoscopy, a technique that can be invasive, expensive, and a possible cause of urinary tract infection [3, 4]. Additionally, bladder mucosa irregularities and small areas of carcinoma in situ (CIS) may contribute to a significant rate of false-negatives due to operator error [5]. Because ten-year recurrence rates of non-muscle-invasive bladder cancer (NMIBC) have been found to be as high as 74.3%, cystoscopy is recommended routinely for surveillance, which contributes to increased expense and a higher risk for urologic disease in the patient [6, 7].

The most reliable non-invasive test for BCa is urine cytology. Although it has reasonably high specificity (~86%), urine cytology suffers from poor sensitivity (~48%), especially in low-grade malignancies (~16%), as well as false positives from benign conditions [8, 9]. The need for a skilled uropathologist coupled with demonstrated inter- and intra-observer variability also significantly diminish the practical utility of this technique [9, 10]. Other tests that are feasible for primary care include the urine dipstick or microscopic urinalysis to detect hematuria. However, these tests are not sufficiently sensitive and have low specificity. Hence, there is clearly a need for more reliable non-invasive biomarkers of BCa.

Urinary biomarkers are particularly attractive due to the direct contact of the urine with the urothelial tumor cells and the ease of sample collection [11]. Nuclear matrix protein 22 (NMP22) is one such urinary biomarker. The NMP22 Bladder Cancer ELISA Test and the NMP22 BladderChek point-of-care (POC) tests have been approved by the U.S. Food and Drug Administration (FDA) [12, 13]. However, these tests suffer from low sensitivity. Wang et al.
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compiled ranges in a meta-analysis of 19 studies and found that the overall sensitivity and specificity of NMP22 when used for detection of BCa were 52-59% and 87-89% respectively, with an AUC (area under the receiver operating curve) of 0.83 (Table 1). Another well-studied urinary biomarker is the bladder tumor antigen (BTA), also known as human complement factor H related protein (hCFHrp). The two FDA-approved tests for BTA detection are BTA STAT and BTA TRAK, which have 95% CI sensitivities of 64-69% and 62-71%, respectively, and 95% CI specificities of 73-77% and 45-81%, respectively (Table 1). In the presence of certain urologic conditions, particularly those associated with hematuria such as urinary tract infection and renal calculi, both the BTA STAT and BTA TRAK tests may yield false positives due to the high presence of complement factor H in blood [14]. Clearly, the current forms of non-invasive protein biomarkers of BCa are lacking in sensitivity and specificity.

Urinary biomarkers have another potential use in predicting BCa treatment response. Conventionally, NMIBC is treated with complete transurethral resection of all visible lesions, followed by induction and maintenance treatment using intravesical therapy if the BCa is classified as intermediate- or high-risk [15]. Failure of intravesical therapy in a patient often requires cystectomy, with early cystectomy resulting in significantly higher 10-year cancer-specific survival rates than deferred cystectomy [15]. Bacillus Calmette-Guerin (BCG) immunotherapy is a typical first-line intravesical therapy. However, because unpredictable failure can occur in 33% of patients after treatment with BCG, biomarkers that predict the effectiveness of BCG in a patient prior to initiating therapy would be invaluable [16]. Such predictive ability would advance the administration of key second-line therapies such as mitomycin C or indicate the need for early cystectomy, thereby reducing the risk of progression to muscle-invasive disease, which is a common outcome of unpredictable BCG failure [16].

Unlike techniques such as histology, PCR, and staining, assays for protein biomarkers lend themselves well to the creation of efficient point-of-care tests, which are easy to use and yield fast results. Testing urinary proteins is also especially convenient due to ease of sample collection, and the proximity of urine to urinary tract tumors. With these considerations taken into account, this review aims to provide a comprehensive listing of the most promising protein biomarkers of BCa in urine. In this review, biomarkers are organized by their potential role in detection, surveillance, or monitoring of treatment response. Where applicable, selected non-protein-based tests, including those that have been FDA-approved or commercialized, are surveyed for comparison. The purpose of this review is to assess progress towards the goal of identifying ideal urinary proteins to be used for each of the above three applications of biomarkers in BCa.

Methods

PubMed was searched for all relevant articles prior to January 31st, 2019. Articles were identified using the following keywords in various combinations: “bladder cancer”, “bladder carcinoma”, “urothelial cancer”, “urothelial carcinoma”, “Nuclear matrix protein 22”, “NMP22”, “Bladder tumor antigen test”, “BTA test”, “UBC”, “UBC rapid”, “effectiveness”, “reliability”, “accuracy”, “bladder cancer”, “bladder carcinoma”, “urothelial cancer”, “urothelial carcinoma”, “diagnosis”, “detection”, “surveillance”, “recurrence”, “urinary biomarker”, “treatment response”, “Bacillus Calmette-Guerin”, “BCG”, “mitomycin C”, “resection”, “TURBT”, and “outcome”. The search was limited to English language articles, but was not time-frame-limited. Only the articles providing metrics detailing the performance characteristics of the candidate biomarkers, such as sensitivity, specificity, an AUC value, or a $p$-value of an outcome association for the biomarker(s) were chosen for inclusion. For ease of interpretation and a more accurate representation of the predictive power of biomarkers, studies reporting metrics of candidate biomarkers used in combination with cystoscopy were excluded.

Results

Potential urinary protein biomarkers for the detection of BCa

Biomarkers for the detection of BCa are defined as those that can predict the presence of any type of BCa, including muscle-invasive bladder cancer (MIBC). An ideal biomarker is one with high sensitivity, specificity, positive predictive
value (PPV), negative predictive value (NPV), and AUC values. Different metrics are valuable for different applications of the biomarker: a high sensitivity could be favored for the detection of high-risk disease, while a high NPV could be favored if the objective is to rule out cystoscopy when the test is negative. Table 1 summarizes the characteristics of promising detection markers, and was limited to biomarkers found with both sensitivity and specificity greater than or equal to 85%. Biomarkers above this cutoff have comparable or superior metrics compared to the FDA approved urinary protein biomarker tests (NMP22 and BTA, which are included in the table for comparison) and thus may have the potential to be used as alternatives to these tests. PPV, NPV, and AUC values are provided when available. In Table 1, a total of 13 biomarker candidates for reliable BCa detection are listed. As a comparison, the NMP22, BTA STAT, and BTA TRAK tests demonstrated sensitivities of 52-59%, 64-49%, and 62-71%, and specificities of 87-89%, 73-77%, and 45-81% respectively (Table 1). In summary, urine Apo-A1, BLCA-4, and hyaluronidase all emerge as promising BCa detection markers, not only because they exhibit high sensitivities (89-95%, 93%, 89-100%) and specificities (85-92%, 97%, 89-91%) that equal or surpass those of the FDA-approved NMP22 and BTA biomarkers (Table 1), but also because they have been independently validated. Of promise, several additional urine proteins exhibit sensitivity, specificity, NPV, PPV and AUC values exceeding the FDA-approved tests, but have not yet been independently validated (Table 1).

Potential urinary protein biomarkers for the surveillance of BCa

Surveillance biomarkers are those that can predict the recurrence of NMIBC after initial treatment. Although all biomarkers assessed for their ability to detect BCa could theoretically be used as surveillance biomarkers, only studies that directly validated biomarkers specifically for patients at risk for recurrence are included in Table 2. When provided, the therapy used in each study is also specified in the table, given its potential influence on recurrence rates. As shown in Table 2, four candidate protein markers and marker combinations have been reported. All studies from the search that validated urinary protein biomarkers specifically for surveil lance are included. FDA-approved protein tests have also been included from studies that used them to assess their utility for surveillance. It should be stressed however that none of the candidate biomarkers listed in the table have been validated independently. Among the leading candidates are bladder tumor fibronectin (BTF), used as a single marker, that has produced a sensitivity and specificity of 91% and 88%, both of which are higher than or comparable to the FDA-approved tests (Table 2), as well as a 10-marker urine protein panel with an AUC value of 0.90, exceeding the performance of the current FDA-approved tests (Table 2). Clearly, these promising results warrant independent validation.

Potential urinary protein biomarkers for the prediction of treatment response in BCa patients

Treatment response biomarkers are defined as biomarkers that predict how a patient may respond to a specific treatment. The treatment response studies included in this review primarily present this information by testing the association between a specific post-treatment outcome with the levels of given urinary biomarkers in patients. The administered therapy, tested outcome, and statistical p value are reported for each candidate biomarker interrogated. Most of these studies assessed tumor recurrence following BCG administration (Table 3). The search yielded solely proteins, and predominantly cytokines and chemokines among them. All studies that demonstrated statistical significance (p ≤ 0.05) for potential biomarkers that could gauge response to a specific treatment are included. As listed in Table 3, a total of 12 protein markers and marker combinations have been reported as potential biomarkers of treatment response. Several of these urine biomarkers have been independently evaluated with similar results. The potential utility of urine IL-8 and IL-2 have been validated by four more independent groups each, while urine IL-6, IL-18 and TNF-α have been validated by two independent groups each (Table 3). Of the studies included, no p value above 0.0209 was observed for IL-8, making it especially promising (Table 3). The use of different statistical approaches across the different studies makes comparison between studies challenging.
Table 1. Potential Urinary Biomarkers for the Detection of BCa

<table>
<thead>
<tr>
<th>Biomolecule(s)</th>
<th>Method</th>
<th>Reference</th>
<th>Subjects</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FDA-approved</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMP22</td>
<td>NMP22 BladderChek, ELISA</td>
<td>Wang 2017 [36]</td>
<td>5291 patients total</td>
<td>52-59%</td>
<td>87-89%</td>
<td>Meta-analysis of 19 studies AUC = 0.83</td>
<td></td>
</tr>
<tr>
<td>BTA</td>
<td>BTA stat test</td>
<td>Guo 2014 [37]</td>
<td>3462 patients total</td>
<td>64-69%</td>
<td>73-77%</td>
<td>Meta-analysis of 13 studies AUC = 0.75</td>
<td></td>
</tr>
<tr>
<td>BTA</td>
<td>BTA TRAK test</td>
<td>Glas 2003 [38]</td>
<td>829 patients total</td>
<td>62-71%</td>
<td>45-81%</td>
<td>Meta-analysis of 5 studies</td>
<td></td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANG, APOE, CA-9, IL-8, MMP-9, MMP-10, PAI-1, VEGF</td>
<td>ELISA</td>
<td>Goodison 2012 [22]</td>
<td>64 BCa, 62 HC</td>
<td>92%</td>
<td>97%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>Apo-A1</td>
<td>ELISA</td>
<td>Li 2011 [39]</td>
<td>107 BCa, 49 OUC</td>
<td>92%</td>
<td>86%</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Li 2014 [40]</td>
<td>223 BCa, 153 non-BCa</td>
<td>89%</td>
<td>85%</td>
<td>87%</td>
<td>AUC = 0.948</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Chen 2010 [41]</td>
<td>126 specimens</td>
<td>95%</td>
<td>92%</td>
<td>AUC = 0.982</td>
<td></td>
</tr>
<tr>
<td>Apo-A4, IL-8, VEGF</td>
<td>ELISA</td>
<td>Goodison 2012 [22]</td>
<td>64 BCa, 62 HC</td>
<td>90%</td>
<td>97%</td>
<td>94%</td>
<td>AUC = 0.968</td>
</tr>
<tr>
<td>Apo-A4 + Coronin-1A + Dj-1/PARK7 + Gamma Synuclein + Semenogelin-2</td>
<td>Western Blot</td>
<td>Kumar 2015 [42]</td>
<td>63 T2/T3 BCa, 110 Ta/T1 BCa, 66 HC</td>
<td>94%</td>
<td>97%</td>
<td>95%</td>
<td>AUC = 0.98</td>
</tr>
<tr>
<td><strong>BLCA-4</strong></td>
<td>ELISA (8 studies) qPCR (1 study)</td>
<td>Cai 2015 [43]</td>
<td>1119 subjects total</td>
<td>93%</td>
<td>97%</td>
<td>Meta-analysis of 9 studies AUC = 0.9607</td>
<td></td>
</tr>
<tr>
<td>CCL18</td>
<td>ELISA</td>
<td>Urquidi 2012 [23]</td>
<td>64 BCa, 63 non-BCa</td>
<td>88%</td>
<td>86%</td>
<td>87%</td>
<td>PPV = 86%</td>
</tr>
<tr>
<td>CPI</td>
<td>Fluorescence spectroscopy</td>
<td>Inoue 2013 [44]</td>
<td>66 BCa, 20 HC</td>
<td>100%</td>
<td>92%</td>
<td>98%</td>
<td>Measured 8 hours after ALA administration AUC = 0.978</td>
</tr>
<tr>
<td><strong>Hyaluronidase</strong></td>
<td>Zymography</td>
<td>Eissa 2015 [45]</td>
<td>94 BCa, 60 OUC, 56 HC</td>
<td>89%</td>
<td>91%</td>
<td>90%</td>
<td>PPV = 89%</td>
</tr>
<tr>
<td></td>
<td>ELISA-like assay</td>
<td>Pham 1997 [46]</td>
<td>22 G1 BCa, 9 G2 BCa, 40 G3 BCa, 48 OUC, 20 HC</td>
<td>100%</td>
<td>89%</td>
<td>93%</td>
<td>Distinguished G2 and G3 (high-grade BCa) from G1 and controls</td>
</tr>
<tr>
<td>HtrA1</td>
<td>ELISA</td>
<td>Lorenzi 2013 [47]</td>
<td>68 BCa, 16 OUC, 68 HC</td>
<td>93%</td>
<td>96%</td>
<td>94%</td>
<td>PPV = 95%</td>
</tr>
</tbody>
</table>

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**IL-8**
- ELISA
- Rosser 2014 [48]
- 31 BCa, 42 OUC
- Sensitivity: 90%
- Specificity: 86%
- Accuracy: 88%
- PPV = 82%
- NPV = 82%
- AUC = 0.907

**ORM1**
- ELISA
- Li 2016 [49]
- 121 BCa, 21 OUC, 53 HC
- Sensitivity: 92%
- Specificity: 94%
- Accuracy: 93%
- PPV = 90%
- NPV = 93%
- AUC = 0.965

**Soluble FAS**
- ELISA
- Srivastava 2014 [50]
- 117 BCa, 46 OUC, 28 HC
- Sensitivity: 88%
- Specificity: 89%
- Accuracy: 88%
- PPV = 82%
- NPV = 85%
- AUC = 0.912

**UPI**
- Fluorescence spectroscopy
- Inoue 2013 [44]
- 66 BCa, 20 HC
- Sensitivity: 100%
- Specificity: 96%
- Accuracy: 99%
- PPV = 82%
- NPV = 85%
- AUC = 0.904

OUC = controls with other urinary conditions, HC = healthy controls. Bolded font indicates a biomarker that has been independently validated by 2+ studies. Italics indicate a sensitivity ≥ 90%, and an underline indicates specificity ≥ 90%.

**Table 2. Potential Urinary Biomarkers for the Surveillance of BCa**

<table>
<thead>
<tr>
<th>Biomolecule(s)</th>
<th>Method</th>
<th>Reference</th>
<th>Subjects</th>
<th>Treatment</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FDA-approved</strong></td>
<td>NMP22</td>
<td>NMP22 BladderChek</td>
<td>Soria 2018 [28]</td>
<td>3353 total from 14 studies</td>
<td>-</td>
<td>11-87.5% (49.5%)</td>
<td>77-100% (91.4%)</td>
<td>PPV = 18.2-100%</td>
</tr>
<tr>
<td></td>
<td>NMP22 ELISA</td>
<td>Soria 2018 [28]</td>
<td>4650 total from 15 studies</td>
<td>-</td>
<td>24-81% (64%)</td>
<td>49-100% (78.15%)</td>
<td>PPV = 31-100%</td>
<td>NPV = 60-91%</td>
</tr>
<tr>
<td><strong>BTA</strong></td>
<td>BTA STAT</td>
<td>Soria 2018 [28]</td>
<td>3064 total from 12 studies</td>
<td>-</td>
<td>40-72% (57%)</td>
<td>29-96% (86%)</td>
<td>PPV = 40-88%</td>
<td>NPV = 38-76.9%</td>
</tr>
<tr>
<td></td>
<td>BTA TRAK</td>
<td>Soria 2018 [28]</td>
<td>918 total from 6 studies</td>
<td>-</td>
<td>50-62% (61%)</td>
<td>68-87% (81.5%)</td>
<td>PPV = 45.4%</td>
<td>NPV = 88.4%</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td>ELISA</td>
<td>Rosser 2014 [51]</td>
<td>53 recurrence positive, 72 recurrence negative</td>
<td>Chemotherapy, TURBT</td>
<td>79%</td>
<td>88%</td>
<td>84%</td>
<td>PPV = 82%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NPV = 85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AUC = 0.904</td>
</tr>
<tr>
<td><strong>BTF</strong></td>
<td>Chemiluminescent immunometric test</td>
<td>Li 2008 [52]</td>
<td>126 recurrence positive, 41 recurrence negative</td>
<td>TURBT and no intravesical therapy</td>
<td>91%</td>
<td>88%</td>
<td>88%</td>
<td>PPV = 73%</td>
</tr>
<tr>
<td></td>
<td>cadherin-1, EN2, ErB2, IL-6, IL-8, VEGF-A</td>
<td>De Paoli 2016 [53]</td>
<td>27 recurrence negative, 18 recurrence positive</td>
<td>BCG, mitomycin C, TURBT</td>
<td></td>
<td></td>
<td></td>
<td>Test also includes three clinical parameters</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AUC = 0.91</td>
</tr>
</tbody>
</table>
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Table 3. Potential Urinary Biomarkers for the Prediction of Treatment Response in BCa Patients

<table>
<thead>
<tr>
<th>Biomolecule(s)</th>
<th>Method</th>
<th>Reference</th>
<th>Subjects</th>
<th>Therapy</th>
<th>Test Performance</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>CEA</td>
<td>RIA</td>
<td>Wahren 1982 [55]</td>
<td>425 BCa, 75 symptom-free, 50 healthy</td>
<td>Radiation treatment</td>
<td>Decreasing levels in patients with symptom-free survival and no local recurrence, p = 0.001</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>ELISA</td>
<td>Jackson 1998 [56]</td>
<td>34 BCa</td>
<td>BCG</td>
<td>Levels significantly different between patients with good and poor short-term therapeutic outcome, p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>IFN-α</td>
<td>ELISA</td>
<td>Saint 2001 [57]</td>
<td>19 Ta/T1 NMIBC</td>
<td>BCG</td>
<td>Decreasing levels in patients associated with non-recurrence, p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>IFN-α, IL-1ra, IL-2, IL-6, IL-8, IL-12[p70], IL-18, TNF-α, TRAIL</td>
<td>Luminex</td>
<td>Kamat 2016 [24]</td>
<td>130 with intermediate and high-risk NMIBC</td>
<td>BCG</td>
<td>Panel and nomogram results predict likelihood of recurrence (Accuracy: 0.855% (95% CI 77.9-93.1%))</td>
</tr>
<tr>
<td>IL-2</td>
<td>ELISA</td>
<td>Saint 2001 [57]</td>
<td>19 Ta/T1 NMIBC</td>
<td>BCG</td>
<td>Increasing levels in patients associated with BCG response, p = 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Saint 2002 [58]</td>
<td>37 Ta/T1 NMIBC, 13 healthy</td>
<td>BCG</td>
<td>Patients with levels less than 27 pg./micromol. creatinine more likely to have recurrence, p = 0.0009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Saint 2003 [59]</td>
<td>39 NMIBC or CIS</td>
<td>BCG</td>
<td>Failure to detect during first BCG induction and extended induction cycle correlated with time to recurrence, p = 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA, RIA</td>
<td>Watanabe 2003 [60]</td>
<td>20 CIS, 8 OUC</td>
<td>BCG</td>
<td>High levels correlated with BCG treatment efficacy (p &lt; 0.01) as well as tumor recurrence after treatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Sanchez-Carbayo 2001 [61]</td>
<td>121 patients, including BCa and OUC</td>
<td>Intravesical therapy</td>
<td>Peaks in IL-2 associated with BCG response, p = 0.041</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>de Reijke 1996 [62]</td>
<td>23 TCC</td>
<td>BCG</td>
<td>Presence in urine correlated with tumor recurrence, p = 0.003</td>
<td></td>
</tr>
</tbody>
</table>

TURBT = transurethral resection of bladder tumor. Italics indicate a sensitivity ≥ 90%, and an underline indicates specificity ≥ 90%. Note: For the FDA-approved tests, ranges are given and medians are included in parentheses.
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<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Type</th>
<th>Study</th>
<th>Tumor Type</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>ELISA</td>
<td>de Reijke 1996 [62]</td>
<td>23 TCC</td>
<td>BCG</td>
<td>Correlated with early (&lt; 6 months) tumor recurrence, <em>p</em> = 0.040</td>
</tr>
<tr>
<td>IL-6</td>
<td>ELISA, RIA</td>
<td>Watanabe 2003 [60]</td>
<td>20 CIS, 8 OUC</td>
<td>BCG</td>
<td>High levels correlated with BCG treatment efficacy (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>IL-8</td>
<td>ELISA</td>
<td>Sagnak 2009 [63]</td>
<td>41 NMIBC</td>
<td>BCG</td>
<td>Change in levels before first BCG and at 2 hours after BCG correlates with recurrence, cutoff is 12 pg/mL (<em>p</em> = 0.047)</td>
</tr>
<tr>
<td>IL-8</td>
<td>ELISA</td>
<td>Thalmann 2000 [64]</td>
<td>28 NMIBC</td>
<td>BCG</td>
<td>Levels &gt; 4,000 ng. after BCG correlated to higher chance of remaining disease-free (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>IL-8</td>
<td>ELISA</td>
<td>Kumar 2002 [65]</td>
<td>26 NMIBC</td>
<td>BCG</td>
<td>Levels higher in responders than in nonresponders at 4 hours after BCG (<em>p</em> = 0.001)</td>
</tr>
<tr>
<td>IL-8</td>
<td>ELISA, RIA</td>
<td>Watanabe 2003 [60]</td>
<td>20 CIS, 8 OUC</td>
<td>BCG</td>
<td>High levels correlated with BCG treatment efficacy (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>IL-8</td>
<td>ELISA</td>
<td>Thalmann 1997 [66]</td>
<td>20 BCa</td>
<td>BCG</td>
<td>Levels &lt; 4,000 ng. during first 6 hours after BCG correlated to higher risk of recurrence and progression (<em>p</em> &lt; 0.0002)</td>
</tr>
<tr>
<td>IL-10</td>
<td>ELISA, RIA</td>
<td>Watanabe 2003 [60]</td>
<td>20 CIS, 8 OUC</td>
<td>BCG</td>
<td>High levels correlated with BCG treatment efficacy (<em>p</em> &lt; 0.01)</td>
</tr>
<tr>
<td>IL-18</td>
<td>ELISA</td>
<td>Thalmann 2000 [64]</td>
<td>17 NMIBC</td>
<td>BCG</td>
<td>Elevated expression correlated to longer disease-free survival (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>Survivin</td>
<td>CLA</td>
<td>Hausladen 2003 [68]</td>
<td>25 NMIBC</td>
<td>BCG (23), mitomycin C (4)</td>
<td>Posttreatment presence indicates high likelihood of recurrence</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ELISA</td>
<td>Shintani 2007 [69]</td>
<td>28 BCa</td>
<td>BCG</td>
<td>Higher levels in non-recurrent group than in recurrent group (<em>p</em> = 0.07)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ELISA, RIA</td>
<td>Watanabe 2003 [60]</td>
<td>20 CIS, 8 OUC</td>
<td>BCG</td>
<td>High levels correlated with BCG treatment efficacy (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>TRAIL</td>
<td>ELISA</td>
<td>Ludwig 2004 [70]</td>
<td>17 NMIBC</td>
<td>BCG</td>
<td>Higher levels present in responders (&gt; 12 months tumor free) than non-responders (<em>p</em> &lt; 0.05)</td>
</tr>
</tbody>
</table>

BCG = Bacillus Calmette-Guerin immunotherapy, NMIBC = non-muscle-invasive bladder cancer, CIS = carcinoma in-situ, TCC = transitional cell carcinoma, OUC = other urinary conditions. Bolded font indicates a biomarker that has been independently validated by 2+ studies.
Discussion

A hierarchy of phases of biomarker validation, from discovery to incorporation into clinical practice, have been defined, with a special emphasis on bladder cancer [26-28]. This hierarchy consists of four phases: assay development and exploratory studies (phase I), independent validation of accuracy using large cohorts (phase II), external validation studies across multiple institutions and prospective clinical trials (phase III), and post-approval reports (phase IV). While it should be noted that there are several markers that are already commercially available with no intention to seek FDA-approval, this scale provides a useful yardstick for the assessment of biomarker studies and their progress. The 3 different types of BCa marker studies listed in Tables 1-3 are reviewed below in this context.

Table 1 first lists the validation metrics of several FDA-approved biomarkers for comparison to potential candidates for BCa detection. Assessed by BladderChek and ELISA tests, NMP22 was found to have a sensitivity of 52-59% and a specificity of 87-89% in a meta-analysis of 19 studies (Table 1). Using the BTA STAT test, BTA demonstrated a sensitivity of 64-69% and a specificity of 73-77% in a meta-analysis of 13 studies (Table 1). The BTA TRAK test showed similar sensitivity of 62-71% but a notably lower specificity of 45-81% in a meta-analysis of 5 studies (Table 1). Not included in the table are the UroVysion (sensitivity 57.1%, specificity 87.5%) and uCYT+ (sensitivity 67-100%, specificity 62-84%) FDA-cleared tests, which use fluorescence in situ hybridization and fluorescence immunodetection respectively [27, 28]. A laboratory-developed BCa detection test that meets the regulatory standards of the Clinical Laboratory Improvement Amendments (CLIA) is the Cxbladder Monitor (sensitivity 82%, specificity 85%), which measures the expression of five genes [30]. Additionally, the commercially available UBC Rapid Test (an immunochromatographic method that measures fragments of cytokeratin 8 and 18) has demonstrated a sensitivity of 59% and a specificity of 76% [31]. In general, these phase III-IV markers suffer from subpar sensitivities. Hence, the field would clearly benefit from newer urine-based tests with higher sensitivities and specificities for the detection of BCa.

All urine candidate biomarkers reviewed in Table 1 demonstrate sensitivities that surpass those of the FDA-approved biomarkers, and many have specificities that are comparable or better. Notably, Apo-A1 has been independently validated in at least three studies, yielding a sensitivity range of 89-100% and a specificity range of 85-92% (Table 1). Apo-A1 is the primary protein component of high-density lipoprotein, and is often used as a biomarker of cardiovascular disease. Lipoproteins are theorized to play a role in facilitating tumor survival through kinase activation or in the development of tumor angiogenesis, but the association between lipoproteins and BCa progression is still not well-understood [19, 20]. Further research into this association would help confirm the value of Apo-A1 and other lipoproteins as biomarkers of BCa. Another promising biomarker included in this review is BLCA-4, which Cai et al. found to demonstrate a sensitivity and specificity of 93% and 97% across nine studies. BLCA-4 is a nuclear transcription factor found in bladder tumors in early stages of disease: its association with BCa is clear, but its value as a marker for BCa detection still requires further validation. Hyaluronidase is a third independently validated biomarker that showed promise, as demonstrated by Pham et al. and Eissa et al. Its sensitivity and specificity ranged from 87-100% and 89-98%, respectively. Hyaluronidases catalyze the breakdown of hyaluronic acid, which functions to facilitate cellular proliferation and motility [20]. Besides these three proteins, which represent phase II-III biomarkers, many other proteins have been evaluated as potential biomarkers for the detection of BCa as shown in Table 1; although several exhibit promising performance metrics, these await independent validation by other groups.

Head-to-head comparisons of novel urine biomarker against the FDA-approved tests for BCa detection were conducted in some of the reviewed studies. Goodison et al. found that an eight-biomarker panel and a three-biomarker panel produced a sensitivity of 92% and 90%, and a specificity of 97% and 97%, respectively, while the BTA TRAK ELISA test achieved a sensitivity of 78% and a specificity of 83% in the same cohort for BCa detection [20]. Urquidi et al. compared results from a CCL18 assay to the BTA TRAK test. Urine CCL18 exhibited a sensitivity of 88% and a specificity
of 86%, while BTA TRAK exhibited a sensitivity of 80% and a specificity of 84% for BCa detection [22]. These head-to-head comparisons add significant validity to these studies by comparing the proposed biomarkers against the FDA-approved test in the same patient cohort, hence removing possible confounding factors.

One potential use for these detection biomarkers may be for healthy population screening of BCa, not unlike the population screening that is conducted for breast, cervical, and colon cancer. Early detection (i.e., the detection of BCa at an earlier asymptomatic stage) is vital in BCa as it leads to improved survival [17]. However, population-wide screening has generally been deemed impractical as the general prevalence of BCa is low. Instead, screening specific groups with a high risk of BCa may offer improved cost-benefit yield [17]. For example, Steiner et al. used urine dipstick, NMP22, cytology, and UroVysion to screen 183 at-risk patients who had a greater than 40 pack-per-year smoking history [18]. Out of the 75 patients with at least one positive test result, three were diagnosed with non-muscle invasive BCa. Lotan et al. screened 1502 high risk subjects aged over 50 and a greater than ten-year smoking history or a significant occupational exposure to carcinogens such as dyes, petroleum, or chemicals. The NMP22 BladderChek test was used for screening, and 85 patients had a positive test with two being diagnosed with non-muscle invasive BCa [31]. A high-risk population screening study using any of the novel biomarker candidates listed in Table 1 has yet to be conducted. Promising evidence from such studies would help transition these early-phase markers into the clinical environment.

Another potential risk-factor based evaluation approach would be to test these markers in patients with gross or asymptomatic microscopic hematuria. However, asymptomatic microscopic hematuria is also seen in many patients without BCa, and practitioners must decide which patients need to undergo a complete evaluation for BCa. The current standard evaluation procedure for patients with hematuria is cystoscopy, which has high sensitivity. Because false negative rates can be high with cystoscopy, cytology is often used as an adjunct test to aid in the detection of bladder cancer. However, cytology is only recommended for use in patients with persistent asymptomatic microscopic hematuria or risk factors such as irritative voiding symptoms, tobacco use, and chemical exposure. Therefore, there is a need for a sensitive urine-based marker that can help physicians determine which patients truly need BCa evaluation. Lotan et al. conducted a prospective multi-center study of patients referred for hematuria evaluation, using a nomogram based on age, gender, smoking status, ethnicity, hematuria, and NMP22 BladderChek results to predict whether a patient would develop BCa. In that study, 23 (6%) of 381 patients were found to have BCa, resulting in a predictive accuracy of 0.79 for the model [35]. Further independent evaluation is needed to assess if any of the biomarkers in Table 1 could outperform current tools for BCa detection in hematuria patients, in terms of their predictive potential.

Candidate biomarkers that have been evaluated for BCa surveillance (to predict recurrence of NMIBC after initial treatment) are fewer than candidates evaluated for BCa detection. A couple of FDA-approved markers have been extensively evaluated for their surveillance potential. A review of multiple studies reported a median sensitivity of 50% and 64% and a median specificity of 91% and 78% for NMP22 BladderChek (14 studies) and NMP22 ELISA (15 studies), respectively (Table 2). BTA exhibits a median sensitivity of 57% and 61% and a median specificity of 86% and 82% when assessed with BTA STAT (12 studies) and BTA TRAK (six studies), respectively (Table 2). The FDA-approved ImmunoCyt (fluorescence immunohistochemistry) and UroVysion (FISH) tests exhibit median sensitivities of 74% and 60%, and median specificities of 73% and 90% when assessed over 10 studies and 17 studies, respectively [26]. From the same review surveying two studies assessing the CLIA-approved Cxbladder test and five studies assessing the commercial UBC (bladder cancer) test, median sensitivities of 92% and 61% were reported, and a median specificity of 87% was found for the UBC test. With the exception of Cxbladder, for which only two studies were surveyed, sensitivities tended to be consistently low, and although some tests had median specificities above 90%, others have substantial room for improvement. Among the novel urine proteins interrogated, bladder tumor fibronectin (BTF), a tumor-derived glyco-
protein that binds extracellular matrix components, appears to be a promising singular biomarker that exhibits high sensitivity and comparable specificity in comparison to the FDA-approved and commercially available biomarkers. BTF produced a sensitivity and specificity of 91% and 88% for BCa surveillance, respectively (Table 2). Until it is validated by independent groups, however, BTF remains a phase I-II marker for BCa surveillance.

Although several urine protein biomarkers for the detection and surveillance of BCa have demonstrated high sensitivity and specificity relative to FDA-approved and other commercial urinary biomarker tests, none have been validated sufficiently to be considered viable alternatives or additions to current laboratory measures. Potential biomarkers for the detection of BCa, three of which are in phase II-III (as discussed above), are closer to this goal than are biomarker candidates for the surveillance of BCa. Until the improved diagnostic metrics of these potential urine protein markers are validated further, combinations of cystoscopy, cytology, and FDA-approved urinary biomarker tests remain the most reliable BCa detection methods.

Many biomarkers have also been tested for their potential in predicting response to treatment. Kamat et al. tested the FDA-approved UroVysion FISH and found that patients with positive FISH results during BCG therapy were more likely to develop recurrent tumors and suffer disease progression (p < 0.01) [34]. Other FDA-approved and commercial tests have yet to be extensively researched for their potential to predict treatment response. Among the newer urine protein treatment response biomarkers evaluated, IL-2, IL-6, IL-8 and TNF-α have been validated independently as promising predictors of response to intravesical BCG therapy, and can be considered phase II-III biomarkers. All have functions associated with immune response and inflammation, and are likely to play key functional roles in disease and response to treatment. Clearly, further validation across multiple institutions and in prospective clinical trials are warranted, in order to transition these promising markers to phase III and IV.

Kamat et al. tested a panel of nine biomarkers to assess BCa response to BCG, and concluded that due to the multifactorial nature of the immune response in BCG antitumor activity, the use of multiple biomarkers may be required to reliably predict patient response to intravesical immunotherapy [24]. This conclusion has tentative support from the other studies presented in this review based on the observation that the group’s panel, which produced a high AUC of 0.855, included all of the independently validated biomarkers listed above. This suggests the possibility of increased statistical power when combined biomarkers are used to gauge treatment response, although further independent validation of such multi-marker panels are warranted. Finally, the majority of the markers evaluated in this context assessed BCG response, highlighting the lack of studies testing biomarkers’ ability to predict response to chemotherapy, radiotherapy, or other forms of intravesical therapy such as mitomycin or epirubicin.

Several intrinsic aspects pertaining to biomarkers and biomarker studies impose limitations. Considerable variation in biomarker levels is often observed due to differences in subjects (ethnicity, age groups, gender, and comorbidities), detection platforms, and sample collection methods. Few of the studies reviewed here have attempted to control for these variables. Even the potential biomarkers that have been validated by independent groups in this study have yet to be tested over a wider spectrum of cohorts and sample collection techniques. Additionally, it is often the case that only studies with positive results are reported, artificially making certain biomarkers appear more promising than they actually are. The reverse could also happen if biomarkers are not tested with a sufficiently sensitive platform; they may be discarded because they are below the detection limits of the particular platform or kit used. Furthermore, many studies are inherently biased by pre-selecting specific molecules to study. In contrast, a transcriptome-wide or proteome-wide approach is a comprehensive and exhaustive method by which to identify candidate molecules that have yet to be studied as potential biomarkers of BCa. Targeted proteomics using antibodies or aptamers as ligands and high sensitivity mass spectrometry may be especially useful in identifying low-abundance but clinically significant protein biomarkers, in a more comprehensive fashion [25].
In conclusion, urine biomarkers of BCa hold great promise, but would necessitate concerted efforts to screen for markers more comprehensively (using various OMICs platforms), standardization of ELISA kits used, controlling for patient demographics, and extensive multi-institution validation. Given that comprehensive OMICs-based screens for urine biomarkers have yet to be reported for BCa, it is likely that the best BCa biomarkers with the highest clinical utility are yet to be discovered. Finally, the utility of biomarker panels composed of leads from proteomic studies and genomic insights warrant investigation.

**Disclosure of conflict of interest**

None.

**Abbreviations**

BCa, bladder cancer; BCG, Bacillus Calmette-Guerin; BTF, bladder tumor fibronectin; CIS, carcinoma in situ; CLIA, Clinical Laboratory Improvement Amendments; ELISA, enzyme-linked immunosorbent assay; NPV, negative predictive value; NMIBC, non-muscle invasive bladder cancer; NMP22, Nuclear matrix protein 22; PPV, positive predictive value.

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**References**


Urine biomarkers of bladder cancer


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