External Validation of a Multiplex Urinary Protein Panel for the Detection of Bladder Cancer in a Multicenter Cohort

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Running head: Multiplex Urinary Protein Panel in Bladder Cancer

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ABSTRACT

Background: Due to the faltering sensitivity and/or specificity, urine-based assays currently have a limited role in the management of patients with bladder cancer (BCa). The aim of this study was to externally validate our previously reported protein biomarker panel from multiple sites in the US and Europe.

Methods: This multicenter external validation study included a total of 320 subjects (BCa = 183). The 10 biomarkers (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SDC1 and SERPINE1) were measured using commercial ELISA assays in an external laboratory. The diagnostic performance of the biomarker panel was assessed using receiver operator curves (ROC) and descriptive statistical values.

Results: Utilizing the combination of all 10 biomarkers, the AUROC for the diagnostic panel was noted to be 0.847 [95% CI: 0.796 - 0.899], outperforming any single biomarker. The multiplex assay at optimal cutoff value achieved an overall sensitivity of 0.79, specificity of 0.79, PPV of 0.73 and NPV of 0.84 for BCa classification. Sensitivity values of the diagnostic panel for high-grade BCa, low-grade BCa, MIBC and NMIBC were 0.81, 0.90, 0.95 and 0.77, respectively.

Conclusions: Urinary levels of the biomarker panel enabled discrimination of BCa patients and controls, and the levels of biomarker subsets were associated with advancing tumor grade and stage.

Impact: If proven to be reliable, urinary diagnostic biomarker assays can detect BCa in a timely manner such that the patient can expect improvements in overall survival and quality of life.
INTRODUCTION

With an estimated 70,980 newly diagnosed cases of bladder cancer (BCa) and 14,330 deaths from BCa in 2012, cancer of the urinary bladder is the second most common genitourinary malignancy in the US and among the five most common malignancies worldwide [1, 2]. Urothelial carcinoma, the most prevalent histologic subtype, accounts for 90% of all BCa in the US [3]. When detected early (i.e., non-muscle invasive), the 5-year survival rate of BCa is > 90%, however at later stages (i.e., muscle invasive and beyond) the 5-year survival rate is < 50%. Thus, early BCa identification, both at the initial diagnosis and at recurrence can dramatically affect outcomes [4].

Urine based assays that can noninvasively detect BCa have the potential to improve the rapid diagnosis of BCa and could therefore help to avoid unnecessary and invasive cystoscopy and bladder biopsy. As such, several urine-based commercial molecular tests have been FDA-approved for BCa detection and surveillance. These tests include the measurement of soluble proteins such as bladder tumor antigen (BTA), and nuclear matrix protein 22 (NMP22), or proteins detected on fixed urothelial cells (ImmunoCyt), and chromosomal aberrations detected by fluorescent in situ hybridization (Urovysion) [5]. Because of their marginal detection performance, these urine-based assays have a limited role in the management of patients at risk for, or with BCa, and thus the search for non-invasive urine-based tests with clinical utility for BCa continues.

We have previously coupled high throughput, discovery-based technology (i.e., genomics and proteomics) with bioinformatics in order to derive diagnostic signatures that show promise for the accurate detection of BCa in voided urine samples [6-9]. Figure 1 shows the various steps in the overall phased project. Integration of data and selection based on p-value, fold change and availability of antibodies resulted in a 14-protein biomarker panel for subsequent testing and
refinement in independent cohorts. Analysis in a 127 patient cohort (64 with BCa) confirmed the promise of 10 of the biomarkers for non-invasive detection of BCa [10-14]. Recently, we reported the validation of the 10-biomarker diagnostic panel (IL8, MMP9, MMP10, SERPINA1, VEGFA, ANG, CA9, APOE, SDC1 and SERPINE1) in a large cohort of patients (n = 308; 102 BCa and 206 controls) including controls with diverse urologic conditions (e.g., urolithiasis, moderate-severe voiding symptoms, urinary tract infection and hematuria) [15]. Thus, we are the first group to extensively profile voided urine with genomics and proteomics, integrate the data and then validate the urinary multiplex BCa signature in multiple independent cohorts. In the current study, we extend the validation of the 10-biomarker assay by performing analysis of samples obtained from multiple sites in the US and in Europe in an external laboratory.

MATERIALS AND METHODS

Specimen and Data Collection

Urine samples were collected from subjects with written consent, which was approved by each of the local Institutional Review Boards (i.e., Aarhus University Hospital, Spanish National Cancer Research Center, University Hospital Duisburg-Essen, Josephine Nefkens Institute, University of Miami Miller School of Medicine, Portuguese Oncology Institute-Porto, Mayo Clinic Florida and MD Anderson Cancer Center - Orlando). At each institution, urine samples were processed and stored as previously described [6-21] with slight modifications. The tissue banks were queried for suitable specimens for analysis. Three hundred and thirty-six samples were identified. Due to inadequate volume for analysis (e.g., < 3 mL), urinary protein levels > 700 μg/mL or urinary creatinine levels < 35 mg/dL, 16 samples were excluded from analysis. Thus 320 samples (Aarhus University Hospital-89, Spanish National Cancer Research Center-53, University Hospital Duisburg-Essen-46, Erasmus MC-41, University of Miami Miller School of Medicine-40,
Portuguese Oncology Institute-Porto-37 and Mayo Clinic Florida-14) were made available for analysis. Frozen supernatant aliquots from the 320 subjects were shipped on dry ice to the laboratory of LC and KXC at the University of Central Florida for analysis.

Data are reported according to International Consensus Panel on Bladder Tumor Markers [22] and STARD criteria [23]. Of the 320 subjects, 183 were histologically confirmed to harbor active primary urothelial carcinoma, while 96 patients had benign urologic disorders (e.g., voiding symptoms, urolithiasis, urinary tract infection and microscopic hematuria) and 41 patients were healthy controls. No patient had a history of BCa. Of the 183 subjects, voided urinary cytology (VUC) results were available for 79. Cytopathologists at each institute reported VUC into one of four categories: normal, atypical/indeterminate, suspicious, or malignant.

**Urinary Enzyme-Linked Immunosorbent Assays (ELISA)**

Levels of human Interleukin 8 (IL8, Cat # ab46032 Abcam), Matrix Metalloproteinase 9 (MMP9, Cat # DMP900 R&D Systems Inc.), Plasminogen Activator Inhibitor 1 (SERPINA1, Cat # EA-0207 Signosis Inc.), Vascular Endothelial Growth Factor A (VEGFA, Cat # 100663 Abcam), Angiogenin (ANG, Cat # CK400 CellSciences), Carbonic Anhydrase 9 (CA9, Cat # DCA900 R&D Systems Inc.), Matrix Metalloproteinase 10 (MMP10, Cat # DMP1000 R&D Systems Inc.), human Apolipoprotein E (APOE, Cat # KA 1031 Abnova), human Syndecan (SDC1, Cat # ab46507 Abcam) and human A1AT (SERPINE1, Cat # ab108799, Abcam) were monitored in urine samples using commercial enzyme-linked immunosorbent assays (ELISA) as listed above. Frozen supernatants were thawed and the ELISA’s were conducted according to the manufacturer’s instructions. Calibration curves were prepared using purified standards for each protein assessed. Curve fitting was accomplished by either linear or four-parameter logistic regression following the manufacturer’s instructions. Due to the unavoidable variability of voided urine with respect to total
volume and time within the bladder, biomarkers were normalized to urinary creatinine and to urinary protein for comparison [24]. Laboratory personnel were blinded to final diagnosis.

**Data Analysis**

We investigated the diagnostic performance of the protein biomarkers for BCa detection using the logistic regression analysis with BCa status (yes vs. no) as the response variable and 10 biomarkers as the explanatory variables. The individual biomarkers were combined into a linear combination with the regression coefficients obtained in logistic regression as the weights, and the linear combination is used as a combined score for the detection of BCa. Using cutoff thresholds previously reported [15], we defined a diagnostic test that is either positive or negative when the linear combination of biomarkers is either $\geq$ or $<$ the cutoff. Then for a given cutoff threshold, we calculated the sensitivity and specificity of the test. We generated a ROC curve by plotting values for sensitivity against the false-positive rates (1-specificity) for various cutoff thresholds [25]. The relative ability of the combination of biomarkers to indicate BCa was estimated by calculating the area under the ROC curves (AUC), with a higher AUC indicating a stronger predictor. We select the optimal cutoff value defined by the Youden index [26], *i.e.*, the cutoff value that maximizes the sum of the sensitivity and the specificity. We estimated the sensitivity, specificity, positive prediction value (PPV) and negative prediction value (NPV) of the combination of biomarkers at the optimal cutoff value. Statistical significance in this study was set at $p < 0.05$ and all reported $p$ values were 2-sided. All analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC).

**RESULTS**

**Patient Characteristics**
Demographic, clinical and pathologic characteristics of the 320 subjects (183 BCa, 96 benign controls and 41 healthy volunteers) comprising the study cohort are illustrated in Table 1. Of the 183 BCa, 153 were NMIBC (Ta, Tis, T1) and 23 were MIBC (≥T2). Seventy-eight cases were low-grade and 98 were high-grade.

**Urinary Biomarker Levels**

To assess potential variability associated with urine volumes, we compared biomarker data after no normalization and normalization to urinary protein and to urinary creatinine [24]. In the current study, there was little difference between values whether biomarkers were non-normalized (AUC for combination of all 10 biomarkers 0.874), normalized to creatinine (AUC for combination of all 10 biomarkers 0.848) or normalized to protein (AUC for combination of all 10 biomarkers 0.840), so to be consistent with our previous reporting [10-15], biomarker concentrations were normalized to creatinine. Urinary concentrations of 9 of the 10 biomarkers (except MMP9) were significantly elevated in patients with BCa compared to benign controls and healthy volunteers across all sites (Table 2). To reduce skewness when comparing results from different institutes, we used the cubic-root transformation of each biomarker (Supplemental Table 1). There was some variability observed in each biomarker concentration ranges between institutions, and in comparison to our previously published data [10-15] (Supplemental Table 2), but it did not prohibit us from confirming the applicability of the previously derived prediction rules for each biomarker [15]. Furthermore applying those rules, the urinary concentrations of 8 of the 10 biomarkers (except APOE and SDC1) were significantly elevated in patients with high-grade BCa compared to low-grade BCa. Similarly, elevations in 6 of the urinary biomarkers (IL8, MMP9, SERPINA1, CA9, SERPINE1 and SDC1) were significantly associated with MIBC (Table 2).
The ability of each of the test biomarkers to predict the presence of BCa was analyzed using nonparametric ROC analyses, according to National Cancer Institute guidelines [27]. Based on our previous prediction rules [15], we were able to generate area under the ROC curve (AUROC) data and report the sum of sensitivity and specificity for each biomarker. Urinary CA9 was the most accurate single biomarker with an AUROC of 0.805 (95% CI: 0.749 - 0.861), a sensitivity of 69%, specificity of 81%, positive predictive value (PPV) of 72% and negative predictive value (NPV) of 78%. Urinary SDC1 was the second most accurate single biomarker with an AUROC of 0.802 (95% CI: 0.745 - 0.860), sensitivity of 78%, specificity of 74%, PPV of 69% and NPV of 82%, and third most accurate was VEGFA with an AUROC of 0.795 (95% CI: 0.736 - 0.854), sensitivity of 85%, specificity of 63%, PPV of 64% and NPV of 84%. Table 3 provides AUROC and corresponding sensitivity, specificity, PPV and NPV values for all biomarkers tested.

Model Development & Multivariate Analysis

Utilizing the combination of all 10 biomarkers, the AUROC (Fig. 2) for the diagnostic panel was noted to be 0.847 [95% CI: 0.796 - 0.899], outperforming any single biomarker (Table 3). The multiplex assay at optimal cutoff values defined by the Youden index calculation achieved an overall sensitivity of 0.79, specificity of 0.79, PPV of 0.73 and NPV of 0.84 for BCa classification. Sensitivity values of the combined assay (Table 4) for high-grade BCa, low-grade BCa, MIBC and NMIBC were 81%, 90%, 95% and 77%, respectively. Among the subjects with BCa that had paired results from VUC available, the diagnostic panel was significantly more sensitive than VUC (82.5% vs. 70.9%, respectively, p < 0.05). Of the control cases tested, 39 of the 41 healthy volunteers and 83 of the 96 subjects with benign urologic disorders had a negative multiplex assay, yielding an overall specificity of 85.4% (Supplemental Table 3).
DISCUSSION

At present, the gold standard for the diagnosis of BCa remains cystoscopy. While cystoscopy is highly sensitive (~73%) for the detection of BCa [28], the procedure itself is invasive, uncomfortable and costly [29-31]. Complicating matters further, cystoscopy has been associated with significant patient anxiety [32] and loss in adherence to strict follow-up set forth by current guidelines [33]. Accordingly, VUC (the microscopic evaluation of shed cancer cells in voided urine) is routinely used as a non-invasive adjunct test to cystoscopy, but the major limitation of this evaluation centers on the reported low sensitivity, particularly for the detection of low-grade, low stage tumors (~20-40%) [34, 35]. Coupled with the fact that VUC is also prone to considerable inter-observer variation [36], it is understandable that this method has not emerged as a standalone test for the detection of BCa. The limitations of cystoscopy and VUC underscore the continuing need to explore and validate novel BCa detection methods that can achieve clinically acceptable levels of sensitivity and specificity. A number of urine-based assays to detect BCa are commercially available [37-41], but they have limited accuracy most likely due to the use of only one single molecular marker. This is not surprising considering the variations between individuals, the cross-talk between molecular pathways, and the heterogeneity of solid tumors. Molecular signatures composed of multiple biomarkers are likely to be far more valuable and robust than single biomarkers. A number of molecular signature assays are now being incorporated into clinical practice, for example for the prognosis of breast cancer [42, 43]. Following that lead, we have employed a range of proteomic [6, 7] and genomic [8, 9] approaches to profile voided urine samples with the aim of identifying unique, molecular signatures that are associated with BCa. We have previously tested the diagnostic performance of the selected 10-protein biomarker panel in two independent cohorts, one comprised of 127 patients (64 with BCa, sensitivity 0.92 and specificity 0.97), and the other with 308 patients (102 with BCa, sensitivity 0.74 and specificity
In this study, we extend the evaluation to a phase II, international, multicenter external validation study. For overall classification, the 10-biomarker signature performance data included an AUC of 0.847 (95% CI 0.796 - 0.899) with a corresponding sensitivity of 79% and specificity of 79% (Fig. 2). This illustrates the reproducibility of the diagnostic protein panel in qualitative terms (i.e., the same biomarkers have similar patterns across studies) and quantitative terms (i.e., similar sensitivities and specificities have been achieved in multiple, independent cohorts).

For a biomarker assay to be effective for ruling out BCa in high-risk (e.g., smokers) or symptomatic (e.g., hematuria) patients, the assay must obtain a high NPV. The NPV of the multiplex assay was 0.84 in our cohort. Conversely, to avoid unnecessary evaluation and procedures in subjects not bearing BCa, a biomarker assay should have a high PPV. In this study, an overall PPV of 0.73 was achieved. Given the diverse, multi-site nature of the study cohort, these data are promising, and validate the concept that a multiplex panel of urinary protein biomarkers may assist the non-invasive diagnosis of BCa.

A number of the individual proteins within our 10-biomarker panel have been investigated in urine samples (VEGFA [44, 45], IL8 [46, 47], MMP9 [48, 49], CA9 [50], APOE [51] and SDC1 [52]), but a multiplex assay has the power to achieve a more accurate diagnosis of BCa across the range of bladder conditions presented clinically. We focus on a protein-based multiplex assay in this study, but a number of groups are embracing the multiplex approach and are identifying panels of nucleic acid diagnostic biomarkers for urinary BCa detection. Examples include signatures comprised of up to 14 mRNA targets [8, 9, 53, 54, 55] or multiple DNA microsatellite markers detected by PCR amplification [56]. The overlap in biomarkers between these studies and ours is not readily apparent, perhaps due to the difference in evaluating nucleic acid targets vs. proteins, however, further investigation may identify the involvement of common pathways. Though
promising, many of the reported multiplex signatures are still in early development and have not been confirmed in large studies, thus it remains to be seen whether these preliminary urinary studies can be built upon to achieve eventual success in clinical applications.

An inspection of our BCa-associated diagnostic protein panel reveals two principal ascribed functions, angiogenesis (involving IL8, VEGFA and ANG) and degradation of extracellular matrix (involving MMP9 and MMP10) (Supplemental Table 4). To a lesser extent, MMP9, MMP10 and SERPINE1 [57-59] have also been associated with angiogenesis and ANG. SERPINE1 and SERPINA1 may play a role in breaking down of the extracellular matrix. Angiogenesis, the development of new blood vessels from existing blood vessels, is essential for normal growth and development of tissues and organs. A balance of pro-angiogenic factors and anti-angiogenic factors tightly controls this process [60-62], but in a solid tumor, the balance favors angiogenesis, enabling the sustained abnormal growth of tissue [63].

Our study has several limitations. First, as part of a phased, methodical approach to biomarker discovery and validation, processed urines were retrieved from tissue banks for analysis. Prolonged storage can result in protein degradation and mute the performance of diagnostic protein tests on such samples. We are currently investigating the performance of our BCa-associated diagnostic protein panel in unprocessed freshly voided urines, which would eliminate these potentially confounding issues. Based on preliminary data of analyzing freshly voided urines, we believe that the assay performance will improve significantly. Second, urines at each institution were collected and processed with slight modifications based on the specific institution’s protocol. These modifications may account for some observed variability in urinary biomarker concentrations noted between institutions (Supplemental Table 1). Additionally, subtle differences in composition of optimal urinary diagnostic protein panels may exist in cohorts comprised of different demographic populations collected at different institutions and in different countries.
Taking this into consideration, the overall performance of the 10-protein panel is encouraging. Furthermore in this study, individual ELISA kits were used to monitor each biomarker in the BCa-associated diagnostic panel. This is obviously inefficient, being time consuming and expensive. Thus, we are in the process of incorporating the 10-biomarker measurements into a single multiplex assay utilizing a novel platform. We also realize the importance of the relevant clinical data (e.g., age, sex, race, tobacco history) and how this data may enhance the analytic potential of our BCa diagnostic assay. To address this, we are embarking on the development of a BCa diagnostic nomogram that incorporates biomarker data with relevant clinical information. Furthermore in this study we only address subjects with primary BCa, not recurrent BCa. However recently we reported that the combination of all 10 biomarkers achieved an overall sensitivity of 79% and specificity of 0.88% for detecting recurrent BCa, and significantly outperformed the Urovysion cytogenetic assay (sensitivity 42%, specificity 94%) and voided urinary cytology (sensitivity 33%, specificity 89%) [64]. Thus we believe this urinary protein multiplex BCa detection assay may be applicable to the undiagnosed BCa patient as well as the BCa patient on routine surveillance. However, we must stress that prior to utilizing this urinary protein multiplex BCa detection assay in the clinic, the above points must be addressed in addition the results must be confirmed in a large, prospective, multicenter collaborative phase III trial that compares the diagnostic utility of a BCa diagnostic panel to that of cystoscopy with bladder biopsy.

The 10-biomarker model achieved an area under the ROC of 0.847. If confirmed to be reliable in a prospective study, urinary diagnostic biomarker assays may be used to risk stratify patients, which then could be used to select who warrants a more invasive evaluation. The ultimate goal is to be able to detect BCa in a timely manner such that the patient can expect improvements in overall survival and quality of life.
REFERENCES


27. Pepe MS, Feng Z, Janes H, Bossuyt PM, Potter JD. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. J Natl Cancer


45. Eissa S, Salem AM, Zohny SF, Hegazy MG. The diagnostic efficacy of urinary TGF-beta1


Table 1 Demographic and clinical-pathologic characteristics of 320 subjects comprising study cohort

<table>
<thead>
<tr>
<th></th>
<th>Bladder Cancer[^] (n = 183)</th>
<th>Benign[^] and Healthy Controls (n = 137)</th>
<th>p-value[^]*</th>
</tr>
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<tbody>
<tr>
<td><strong>Median Age (range, y)</strong></td>
<td>69</td>
<td>65</td>
<td>0.094</td>
</tr>
<tr>
<td>(33 - 92)</td>
<td></td>
<td>(21 - 96.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Male : Female ratio</strong></td>
<td>153/29</td>
<td>99/38</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>Clinical stage &amp; grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tis high-grade</td>
<td>4 (2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta low-grade</td>
<td>59 (32%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta high-grade</td>
<td>25 (14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 low-grade</td>
<td>19 (10%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 high-grade</td>
<td>46 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;T2 high-grade</td>
<td>23 (13%)</td>
<td></td>
<td></td>
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<tr>
<td>Unknown</td>
<td>7 (4%)</td>
<td></td>
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[^]: primary BCa; no patient with a history of BCa
[^]: voiding symptoms, urinary tract infection, urolithiasis microscopic hematuria
[^]: Wilcoxon rank sum test.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Total Bladder Cancer</th>
<th>Low-Grade Bladder Cancer</th>
<th>High-Grade Bladder Cancer</th>
<th>NMIBC</th>
<th>MIBC</th>
<th>Total Controls</th>
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</thead>
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<tr>
<td></td>
<td>(57.2%) (n = 183)</td>
<td>(24.1%) (n = 77)</td>
<td>(30.6%) (n = 98)</td>
<td>(87.4%) (n = 153)</td>
<td>(12.6%) (n = 22)</td>
<td>(42.8%) (n = 137)</td>
</tr>
<tr>
<td>IL8 (pg/ml)</td>
<td>599±1765</td>
<td>269±1260</td>
<td>877±2095</td>
<td>461±1536</td>
<td>1644±2903</td>
<td>300±1355</td>
</tr>
<tr>
<td>MMP9 (ng/ml)</td>
<td>15.4±65.6</td>
<td>4.86±13.7</td>
<td>24.2±88.0</td>
<td>9.79±31.5</td>
<td>56.9±167</td>
<td>29.7±154</td>
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<tr>
<td>SERPINA1 (ng/ml)</td>
<td>26.1±100</td>
<td>21.2±105</td>
<td>30.8±101</td>
<td>21.8±96.3</td>
<td>59.6±135</td>
<td>3.76±22.3</td>
</tr>
<tr>
<td>ANG (pg/ml)</td>
<td>460±766</td>
<td>300±616</td>
<td>608±867</td>
<td>432±731</td>
<td>752±1037</td>
<td>293±633</td>
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<tr>
<td>VEGFA (pg/ml)</td>
<td>623±669</td>
<td>532±681</td>
<td>710±670</td>
<td>583±606</td>
<td>969±1014</td>
<td>345±322</td>
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<tr>
<td>CA9 (pg/ml)</td>
<td>103±562</td>
<td>51.5±171</td>
<td>150±752</td>
<td>91.5±583</td>
<td>212±513</td>
<td>7.02±42.6</td>
</tr>
<tr>
<td>MMP10 (pg/ml)</td>
<td>21.6±187</td>
<td>36.2±286</td>
<td>11.6±34.8</td>
<td>25.0±204</td>
<td>4.53±13.6</td>
<td>6.96±46.3</td>
</tr>
<tr>
<td>APOE (pg/ml)</td>
<td>0.011±0.049</td>
<td>0.005±0.017</td>
<td>0.013±0.057</td>
<td>0.007±0.020</td>
<td>0.029±0.11</td>
<td>0.003±0.007</td>
</tr>
<tr>
<td>SERPINE1 (ng/ml)</td>
<td>3866±10518</td>
<td>1773±4223</td>
<td>5621±13626</td>
<td>3478±10589</td>
<td>7061±11365</td>
<td>2487±6713</td>
</tr>
<tr>
<td>SDC1 (pg/ml)</td>
<td>141±869</td>
<td>62.9±57.9</td>
<td>207±1185</td>
<td>143±948</td>
<td>146±171</td>
<td>37.0±54.0</td>
</tr>
</tbody>
</table>

NMIBC, non-muscle invasive bladder cancer
MIBC, muscle invasive bladder cancer
*, p < 0.05 comparing Total Bladder Cancer to Total Controls
^, p < 0.05 comparing low-grade bladder cancer to high-grade bladder cancer
+, p < 0.05 comparing NMIBC to MIBC
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Area under the Curve</th>
<th>95% CI</th>
<th>No. of Correctly Predicted Events</th>
<th>No. of Correctly Predicted Nonevents</th>
<th>No. of Nonevents Predicted as Events</th>
<th>No. of Events Predicted as Nonevents</th>
<th>Sen.</th>
<th>Spec.</th>
<th>PPV</th>
<th>NPV</th>
</tr>
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<tbody>
<tr>
<td>IL8</td>
<td>0.7902</td>
<td>0.7315 - 0.8488</td>
<td>74</td>
<td>100</td>
<td>33</td>
<td>22</td>
<td>77.1%</td>
<td>75.2%</td>
<td>69.2%</td>
<td>82.0%</td>
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<tr>
<td>MMP9</td>
<td>0.7432</td>
<td>0.6781 - 0.8083</td>
<td>79</td>
<td>81</td>
<td>52</td>
<td>17</td>
<td>82.3%</td>
<td>60.9%</td>
<td>60.3%</td>
<td>82.7%</td>
</tr>
<tr>
<td>SERPINA1</td>
<td>0.7870</td>
<td>0.728 - 0.8459</td>
<td>70</td>
<td>96</td>
<td>37</td>
<td>26</td>
<td>72.9%</td>
<td>72.2%</td>
<td>65.4%</td>
<td>78.7%</td>
</tr>
<tr>
<td>ANG</td>
<td>0.7768</td>
<td>0.7173 - 0.8364</td>
<td>85</td>
<td>69</td>
<td>64</td>
<td>11</td>
<td>88.5%</td>
<td>51.9%</td>
<td>57.0%</td>
<td>86.2%</td>
</tr>
<tr>
<td>VEGFA</td>
<td>0.7947</td>
<td>0.7356 - 0.8539</td>
<td>89</td>
<td>86</td>
<td>51</td>
<td>16</td>
<td>84.8%</td>
<td>62.8%</td>
<td>63.6%</td>
<td>84.3%</td>
</tr>
<tr>
<td>CA9</td>
<td>0.8050</td>
<td>0.7488 - 0.8612</td>
<td>66</td>
<td>107</td>
<td>26</td>
<td>30</td>
<td>68.8%</td>
<td>80.5%</td>
<td>71.7%</td>
<td>78.1%</td>
</tr>
<tr>
<td>MMP10</td>
<td>0.7868</td>
<td>0.7284 - 0.8045</td>
<td>78</td>
<td>86</td>
<td>47</td>
<td>18</td>
<td>81.3%</td>
<td>64.7%</td>
<td>62.4%</td>
<td>82.7%</td>
</tr>
<tr>
<td>APOE</td>
<td>0.7549</td>
<td>0.6933 - 0.8164</td>
<td>82</td>
<td>72</td>
<td>61</td>
<td>14</td>
<td>85.4%</td>
<td>54.1%</td>
<td>57.3%</td>
<td>83.7%</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>0.7818</td>
<td>0.7230 - 0.8407</td>
<td>79</td>
<td>86</td>
<td>47</td>
<td>17</td>
<td>82.3%</td>
<td>64.7%</td>
<td>62.7%</td>
<td>83.5%</td>
</tr>
<tr>
<td>SDC1</td>
<td>0.8024</td>
<td>0.7451 - 0.8598</td>
<td>75</td>
<td>99</td>
<td>34</td>
<td>21</td>
<td>78.1%</td>
<td>74.4%</td>
<td>68.8%</td>
<td>82.5%</td>
</tr>
<tr>
<td>All 10 biomarkers</td>
<td>0.8475</td>
<td>0.7958 - 0.8992</td>
<td>76</td>
<td>105</td>
<td>28</td>
<td>20</td>
<td>79.2%</td>
<td>78.9%</td>
<td>73.1%</td>
<td>84.0%</td>
</tr>
</tbody>
</table>

CI, confidence interval
Table 4 Summary of diagnostic sensitivity of 10-biomarker panel and voided urinary cytology in bladder cancer patients

<table>
<thead>
<tr>
<th></th>
<th>Number of BCa cases predicted by biomarker panel</th>
<th>Sensitivity (%)</th>
<th>Number of BCa cases predicted by voided urinary cytology</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>151 of 183</td>
<td>82.5</td>
<td>56 of 79</td>
<td>70.9</td>
</tr>
<tr>
<td>Low-grade tumors</td>
<td>69 of 77</td>
<td>89.6</td>
<td>12 of 29</td>
<td>41.4</td>
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<tr>
<td>High-grade tumors</td>
<td>79 of 98</td>
<td>80.6</td>
<td>44 of 50</td>
<td>88.0</td>
</tr>
<tr>
<td>NMIBC</td>
<td>118 of 153</td>
<td>77.1</td>
<td>50 of 73</td>
<td>68.5</td>
</tr>
<tr>
<td>MIBC</td>
<td>21 of 22</td>
<td>95.5</td>
<td>6 of 6</td>
<td>100</td>
</tr>
</tbody>
</table>

NMIBC, non-muscle invasive bladder cancer
MIBC, muscle invasive bladder cancer
FIGURE LEGENDS

Figure 1. Flow diagram of phases project. Gene expression profiling (Affymetrix U133 Plus 2.0 arrays) followed by quantitative PCR verification and glycoprotein profiling (dual-lectin affinity chromatography and liquid chromatography/tandem mass spectrometry) followed by Western blot analysis or ELISA verification were used to discover and validate RNA and protein expression profiles associated with bladder cancer. Data integration informed the selection of a protein biomarker panel for testing in three independent cohorts using commercial ELISA assays. Gray boxes represent the work presented in the current study. (Ref 15, 16, 33-36).

Figure 2. Diagnostic performance of a 10-protein biomarker assay. ROC curve was plotted to compare performance characteristics of the 10-biomarker signature. Based on the area under the ROC curve (AUROC), Youden Index cutoff values that maximized the sum of sensitivity and specificity.
Affymetrix U133 Plus 2.0 arrays
- 92 samples (52 cancer & 40 controls)
- 45 genes identified

TaqMan Low Density Array (TLDA)
- 81 samples (44 cancer & 37 controls)
- 14 gene signature validated

Affinity chromatography and linear ion trap MS identified glycoprotein
- 100 samples (54 cancer & 46 controls)
- 9 proteins identified

Western Blot & ELISA assay
- 70 samples (35 cancer & 35 controls)
- 5 protein signature validated

Genomic and proteomic data Integrated and a panel of 14 biomarkers selected for protein-based analyses in independent cohorts

Commercial ELISA kits against 14 biomarkers
- 127 samples (64 cancer & 63 controls)
- 10 proteins confirmed

Commercial ELISA kits against 10 biomarkers
- 310 samples (208 cancer & 102 controls)
- All 10 confirmed

Current Study
- Commercial ELISA kits against 10 biomarkers
- 320 samples (183 cancer & 137 controls)
- All 10 confirmed
- AUROC = 0.848
Figure 2

Area Under the Curve = 0.8472
External Validation of a Multiplex Urinary Protein Panel for the Detection of Bladder Cancer in a Multicenter Cohort

Li-Mei Chen, Myron Chang, Yunfeng Dai, et al.

Cancer Epidemiol Biomarkers Prev  Published OnlineFirst June 11, 2014.

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