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

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Abstract

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

**Methods:** This multicenter external validation study included a total of 320 subjects (bladder cancer = 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 area under the ROC for the diagnostic panel was noted to be 0.847 (95% confidence interval, 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, positive prediction value of 0.73, and negative prediction value of 0.84 for bladder cancer classification. Sensitivity values of the diagnostic panel for high-grade bladder cancer, low-grade bladder cancer, muscle invasive bladder cancer, and non-muscle invasive bladder cancer were 0.81, 0.90, 0.95, and 0.77, respectively.

**Conclusions:** Urinary levels of the biomarker panel enabled discrimination of patients with bladder cancer 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 bladder cancer in a timely manner such that the patient can expect improvements in overall survival and quality of life. Cancer Epidemiol Biomarkers Prev; 23(9); 1804–12. ©2014 AACR.

Introduction

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

Urine-based assays that can noninvasively detect bladder cancer have the potential to improve the rapid diagnosis of bladder cancer 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 bladder cancer detection.
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; ref. 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 bladder cancer, and thus the search for noninvasive urine-based tests with clinical utility for bladder cancer 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 bladder cancer 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 bladder cancer) confirmed the promise of 10 of the biomarkers for noninvasive detection of bladder cancer (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 bladder cancer and 206 controls), including

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 3 independent cohorts using commercial ELISA assays.
controls with diverse urologic conditions (e.g., urethra, moderate-severe voiding symptoms, urinary tract infection and hematuria; ref. 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 bladder cancer signature in multiple independent cohorts. In this study, we extend the validation of the 10-biomarker assay by performing analysis of samples obtained from multiple sites in the United States 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. Because of inadequate volume for analysis (e.g., <3 mL), urinary protein levels >700 mg/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 L.-M. Chen and K.X. Chai 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, whereas 96 patients had benign urologic disorders (e.g., voiding symptoms, urethra, urinary tract infection, and microscopic hematuria) and 41 patients were healthy controls. No patient had a history of bladder cancer. Of the 183 subjects, voided urinary cytology (VUC) results were available for 79. Cytopathologists at each institute reported VUC into 1 of 4 categories: normal, atypical/indeterminate, suspicious, or malignant.

Urinary enzyme-linked immunosorbent assays

Levels of human interleukin 8 (IL8; cat. no. ab46032, Abcam), matrix metalloproteinase 9 (MMP9; cat. no. DMP900, R&D Systems Inc.), plasminogen activator inhibitor 1 (SERPINA1; Cat. no. EA-0207, Signosis Inc.), vascular endothelial growth factor A (VEGFA; Cat. no. 100663, Abcam), angiogenin (ANG; Cat. no. CK400, Cell-Sciences), carbonic anhydrase 9 (CA9; Cat. no. DCA900, R&D Systems Inc.), matrix metalloproteinase 10 (MMP10; Cat. no. DMP1000, R&D Systems Inc.), human apolipoprotein E (APOE; Cat. no. KA 1031, Abnova), human Syndecan (SDC1; Cat. no. ab46507, Abcam), and human A1AT (SERPINE1; Cat. no. 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 4-parameter logistic regression following the manufacturer’s instructions. Because of 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 bladder cancer detection using the logistic regression analysis with bladder cancer 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 bladder cancer. 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 ≥ or < the cutoff. Then for a given cutoff threshold, we calculated the sensitivity and specificity of the test. We generated an 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 bladder cancer was estimated by calculating the area under the ROC curves (AUROC), with a higher AUC indicating a stronger predictor. We select the optimal cutoff value defined by the Youden index (26), that is 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.).

Results

Patient characteristics

Demographic, clinical, and pathologic characteristics of the 320 subjects (183 bladder cancer, 96 benign controls, and 41 healthy volunteers) comprising the study cohort are illustrated in Table 1. Of the 183 bladder cancer, 153 were non-muscle invasive bladder cancer (NMIBC; Ta,
Tis, T1) and 23 were muscle invasive bladder cancer (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 this study, there was little difference between values whether biomarkers were nonnormalized (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 bladder cancer compared with 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 (Supplementary Table S1). There was some variability observed in each biomarker concentration ranges between institutions, and in comparison to our previously published data (refs. 10–15; Supplementary Table S2), 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 bladder cancer compared with low-grade bladder cancer. 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 bladder cancer 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 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% confidence interval (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 and 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 0.69, and NPV of 0.82, 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.

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

<table>
<thead>
<tr>
<th></th>
<th>Bladder cancer&lt;sup&gt;a&lt;/sup&gt; (n = 183)</th>
<th>Benign&lt;sup&gt;b&lt;/sup&gt; and healthy controls (n = 137)</th>
<th>P value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range, y)</td>
<td>69 (33–92)</td>
<td>65 (21–96.5)</td>
<td>0.094</td>
</tr>
<tr>
<td>Male : female ratio</td>
<td>153/29</td>
<td>99/38</td>
<td>0.010</td>
</tr>
<tr>
<td>Clinical stage and grade</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>≥T2 high grade</td>
<td>23 (13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Primary bladder cancer; no patient with a history of bladder cancer.

<sup>b</sup VOID symptoms, urinary tract infection, urolithiasis microscopic hematuria.

<sup>c</sup>Wilcoxon rank sum test.
Table 2. Mean urinary (SD) levels of 10 biomarkers assessed by ELISA in cohort of 320 subjects

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Low-grade bladder cancer</th>
<th>Total bladder cancer</th>
<th>High-grade bladder cancer</th>
<th>NMIBC (87.4%)</th>
<th>MIBC (12.6%)</th>
<th>Total controls (42.8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 27</td>
<td>n = 77</td>
<td>n = 88</td>
<td>n = 153</td>
<td>n = 22</td>
<td>n = 137</td>
</tr>
<tr>
<td>MMP9 (ng/mL)</td>
<td>19.3 ± 8.5</td>
<td>26.2 ± 16.8</td>
<td>19.9 ± 9.6</td>
<td>23 ± 13.5</td>
<td>13.7 ± 6.2</td>
<td>19.1 ± 9.6</td>
</tr>
<tr>
<td>SERPINA1 (ng/mL)</td>
<td>2.0 ± 1.5</td>
<td>4.0 ± 2.1</td>
<td>2.1 ± 1.2</td>
<td>4.0 ± 2.1</td>
<td>2.0 ± 1.0</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>ANG (pg/mL)</td>
<td>340 ± 140</td>
<td>736 ± 313</td>
<td>320 ± 133</td>
<td>520 ± 260</td>
<td>210 ± 90</td>
<td>420 ± 180</td>
</tr>
<tr>
<td>VEGFA (pg/mL)</td>
<td>623 ± 303</td>
<td>1,094 ± 547</td>
<td>623 ± 303</td>
<td>1,094 ± 547</td>
<td>623 ± 303</td>
<td>1,094 ± 547</td>
</tr>
<tr>
<td>CA9 (pg/mL)</td>
<td>103 ± 50</td>
<td>206 ± 103</td>
<td>103 ± 50</td>
<td>206 ± 103</td>
<td>103 ± 50</td>
<td>206 ± 103</td>
</tr>
<tr>
<td>MMP10 (pg/mL)</td>
<td>21.8 ± 4.2</td>
<td>34.8 ± 6.4</td>
<td>21.8 ± 4.2</td>
<td>34.8 ± 6.4</td>
<td>21.8 ± 4.2</td>
<td>34.8 ± 6.4</td>
</tr>
<tr>
<td>APOE (pg/mL)</td>
<td>0.011 ± 0.005</td>
<td>0.016 ± 0.009</td>
<td>0.011 ± 0.005</td>
<td>0.016 ± 0.009</td>
<td>0.011 ± 0.005</td>
<td>0.016 ± 0.009</td>
</tr>
<tr>
<td>SERPINE1 (ng/mL)</td>
<td>1,765 ± 269</td>
<td>3,260 ± 514</td>
<td>1,765 ± 269</td>
<td>3,260 ± 514</td>
<td>1,765 ± 269</td>
<td>3,260 ± 514</td>
</tr>
<tr>
<td>SDC1 (pg/mL)</td>
<td>141 ± 45</td>
<td>286 ± 71</td>
<td>141 ± 45</td>
<td>286 ± 71</td>
<td>141 ± 45</td>
<td>286 ± 71</td>
</tr>
</tbody>
</table>

Note: <P < 0.05 comparing total bladder cancer to total controls.

Discussion

At present, the gold standard for the diagnosis of bladder cancer remains cystoscopy. Although cystoscopy is highly sensitive (~73%) for the detection of bladder cancer (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 noninvasive 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%; refs. 34 and 35). Coupled with the fact that VUC is also prone to considerable interobserver variation (36), it is understandable that this method has not emerged as a standalone test for the detection of bladder cancer. The limitations of cystoscopy and VUC underscore the continuing need to explore and validate novel bladder cancer detection methods that can achieve clinically acceptable levels of sensitivity and specificity. A number of urine-based assays to detect bladder cancer are commercially available (37–41), but they have limited accuracy most likely because of the use of only 1 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 used 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 bladder cancer. We have previously tested the diagnostic performance of the selected 10-protein biomarker panel in 2 independent cohorts, one comprised of 127 patients (64 with bladder cancer, sensitivity 0.92 and specificity 0.97), and the other with 308 patients (102 with bladder cancer, sensitivity 0.74 and specificity 0.90; refs. 10–15). 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 bladder cancer 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...
Conversely, to avoid unnecessary evaluation and procedures in subjects not bearing bladder cancer, a biomarker assay should have a high PPV. In this study, an overall PPV of 0.73 was achieved. Given the diverse, multisite nature of the study cohort, these data are promising, and validate the concept that a multiplex panel of urinary protein biomarkers may assist the noninvasive diagnosis of bladder cancer.

A number of the individual proteins within our 10-biomarker panel have been investigated in urine samples [VEGFA (44 and 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 bladder cancer 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 bladder cancer detection. Examples include signatures comprised of up to 14 mRNA targets (8, 9, 53–55) or multiple DNA microsatellite markers detected by PCR amplification (56). The overlap in biomarkers between these studies and ours is not readily apparent, perhaps because of the difference in evaluating nucleic acid targets versus proteins; however, further investigation may identify the involvement of common pathways. Although 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.

### Table 3. Individual and combined biomarker performance data for bladder cancer detection

<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 events predicted as nonevents</th>
<th>No. of nonevents predicted as events</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>0.7902</td>
<td>0.7315–0.8488</td>
<td>74</td>
<td>33</td>
<td>100</td>
<td>22</td>
<td>74.9</td>
<td>77.1</td>
<td>84.8</td>
<td>77.1</td>
</tr>
<tr>
<td>MMP9</td>
<td>0.7670</td>
<td>0.728–0.8083</td>
<td>79</td>
<td>52</td>
<td>81</td>
<td>17</td>
<td>82.3</td>
<td>82.3</td>
<td>84.0</td>
<td>75.2</td>
</tr>
<tr>
<td>SERPIN1</td>
<td>0.7978</td>
<td>0.756–0.8454</td>
<td>79</td>
<td>37</td>
<td>98</td>
<td>25</td>
<td>82.9</td>
<td>79.9</td>
<td>84.0</td>
<td>75.2</td>
</tr>
<tr>
<td>ANG</td>
<td>0.7840</td>
<td>0.7406–0.8384</td>
<td>80</td>
<td>64</td>
<td>69</td>
<td>11</td>
<td>88.5</td>
<td>82.8</td>
<td>82.3</td>
<td>75.2</td>
</tr>
<tr>
<td>VEGFA</td>
<td>0.7947</td>
<td>0.7566–0.8454</td>
<td>89</td>
<td>51</td>
<td>64</td>
<td>15</td>
<td>84.8</td>
<td>77.1</td>
<td>84.0</td>
<td>75.2</td>
</tr>
<tr>
<td>CA9</td>
<td>0.8050</td>
<td>0.763–0.8451</td>
<td>89</td>
<td>26</td>
<td>26</td>
<td>8</td>
<td>84.8</td>
<td>82.8</td>
<td>84.0</td>
<td>75.2</td>
</tr>
<tr>
<td>MMP10</td>
<td>0.7868</td>
<td>0.7468–0.8312</td>
<td>78</td>
<td>42</td>
<td>78</td>
<td>18</td>
<td>81.3</td>
<td>82.6</td>
<td>82.3</td>
<td>75.2</td>
</tr>
<tr>
<td>APOE</td>
<td>0.7960</td>
<td>0.7566–0.8454</td>
<td>79</td>
<td>47</td>
<td>72</td>
<td>14</td>
<td>82.6</td>
<td>82.6</td>
<td>82.3</td>
<td>75.2</td>
</tr>
<tr>
<td>SDC1</td>
<td>0.8100</td>
<td>0.763–0.8451</td>
<td>79</td>
<td>36</td>
<td>79</td>
<td>17</td>
<td>78.1</td>
<td>79.8</td>
<td>82.3</td>
<td>75.2</td>
</tr>
<tr>
<td>All 10 biomarkers</td>
<td>0.8475</td>
<td>0.8043–0.8927</td>
<td>76</td>
<td>100</td>
<td>28</td>
<td>20</td>
<td>79.2</td>
<td>79.2</td>
<td>79.2</td>
<td>79.2</td>
</tr>
</tbody>
</table>

**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 AUROC, Youden index cutoff values that maximized the sum of sensitivity and specificity.
An inspection of our bladder cancer–associated diagnostic protein panel reveals 2 principal ascribed functions, angiogenesis (involving IL8, VEGFA, and ANG) and degradation of extracellular matrix (involving MMP9 and MMP10; Supplementary Table S4). To a lesser extent, MMP9, MMP10, and SERPINE1 (57–59) have also been associated with angiogenesis and ANG, SERPIN1, 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 bladder cancer–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 (Supplementary Table S1). In addition, 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 bladder cancer–associated diagnostic panel. This is obviously inefficient, 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, and tobacco history) and how these data may enhance the analytic potential of our bladder cancer diagnostic assay. To address this, we are embarking on the development of a bladder cancer diagnostic nomogram that incorporates biomarker data with relevant clinical information. Furthermore, in this study we only address subjects with primary bladder cancer, not recurrent bladder cancer. 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 bladder cancer, and significantly outperformed the UroVysion cytogenetic assay (sensitivity 42%, specificity 94%) and voided urinary cytology (sensitivity 33%, specificity 89%; ref. 64). Thus, we believe this urinary protein multiplex bladder cancer detection assay may be applicable to the undiagnosed patient with bladder cancer as well as the patient with bladder cancer on routine surveillance. However, we must stress that before utilizing this urinary protein multiplex bladder cancer 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 bladder cancer diagnostic panel to that of cystoscopy with bladder biopsy.

The 10-biomarker model achieved an AUROC 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 bladder cancer in a timely manner such that the patient can expect improvements in overall survival and quality of life.

**Disclosure of Potential Conflicts of Interest**

V. Urquidi is an employee of Nonagen Bioscience Corporation. S. Goodison is CEO of Nonagen Biosciences Corporation and has ownership interest (including patents) in Nonagen Biosciences Corporation. C.J. Rosser is President of Nonagen Biosciences Corporation and has ownership interest (including patents on bladder signature) in...
Nonagen Biosciences Corporation. No potential conflicts of interest were disclosed by the other authors.

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**Grant Support**

This work was supported by research grants from Florida Department of Health James and Esther King Team Science Award 10KT-01 (to C.J. Rosser) and NCI ROI CA16161 (to S. Goodison).

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Received January 15, 2014; revised April 28, 2014; accepted May 16, 2014; published OnlineFirst June 11, 2014.

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