Urinary Cytokine Profile to Predict Response to Intravesical BCG with or without HS-410 Therapy in Patients with Non-muscle-invasive Bladder Cancer

Amirali Salmasi1,2, David A. Elashoff3, Rong Guo3, Alexander Upfill-Brown1, Charles J. Rosser4, Jason M. Rose5, Louise C. Giffin5, Louis E. Gonzalez5, and Karim Chamie1,2

Abstract

Background: Despite extensive research to identify biomarkers of response in patients with non–muscle-invasive bladder cancer (NMIBC), there is no biomarker to date that can serve this purpose. Herein, we report how we leveraged serial urine samples to query a panel of cytokines at varying time points in an attempt to identify predictive biomarkers of response in NMIBC.

Methods: Serial urine samples were collected from 50 patients with intermediate- or high-risk NMIBC enrolled in a phase II study, evaluating intravesical BCG ± intradural HS-410 therapy. Samples were collected at baseline, week 7, week 13, week 28, and at end of treatment. A total of 105 cytokines were analyzed in each sample. To predict outcome of time-to-event (recurrence or progression), univariate and multivariable Cox analyses were performed.

Results: Fifteen patients developed recurrence and 4 patients progressed during the follow-up period. Among clinicopathologic variables, ever-smoker versus nonsmoker status was associated with an improved response rate (HR 0.38; 95% confidence interval [CI], 0.14–0.99; P = 0.04). In the most clinically relevant model, the percent change (for 100 units) of IL18-binding protein-a (HR 1.995; 95% CI, 1.16–3.44; P = 0.01), IL23 (HR 1.12; 95% CI, 1.01–1.23; P = 0.03), IL8 (HR 0.27; 95% CI, 0.07–1.08; P = 0.06), and IFN-g-induced protein-10 (HR 0.95; 95% CI, 0.91–0.99; P = 0.04) at week 13 from baseline best predicted time to event.

Conclusions: Urinary cytokines provided additional value to clinicopathologic features to predict response to immune-modulating agents in patients with NMIBC.

Impact: This study serves as a hypothesis-generating report for future studies to evaluate the role of urine cytokines as a predictive biomarker of response to immune treatments.

Introduction

Non–muscle-invasive bladder cancer (NMIBC) includes a heterogeneous group of tumors with varying risk of recurrence and progression (1–3). Although transurethral resection is sufficient for most low-risk tumors, adjuvant intravesical treatment is recommended for intermediate- and high-risk cancers (4). In fact, intravesical bacillus Calmette-Guérin (BCG) has been utilized for more than four decades for high-risk NMIBC with favorable outcomes. However, approximately 50% of patients treated with intravesical BCG will experience disease recurrence or progression, which may have significant impact on a patient’s cancer specific outcomes and quality of life (5–7). Therefore, there is an urgent need for development of response predictive biomarkers in these patients (8). Response predictive biomarkers could readily be used to identify potentially unresponsive patients before they are exposed to treatment-related toxicities and high out-of-pocket costs with little or no benefit. Biomarkers have also the potential to increase our understanding of the mode of action of drugs, and thereby identify potential combination therapies. Currently, there are multiple ongoing or completed clinical trials for patients with NMIBC using different strategies to modulate immune system with or without intravesical BCG such as vaccines (9).

The BCG-induced immune response is complex and not fully understood (10). It involves both humoral and cell-mediated components (10, 11). Clinicopathologic features, tumor molecular biomarkers, tumor immune profile, genetic testing, and urinary cytokines have been applied as risk stratification tools to predict response to BCG treatment with mixed results (8). Currently, there is no single predictive biomarker that can be used to screen patients for BCG treatment (8, 12).

Cytokines are key mediators of immune responses that allow recruitment, activation, and differentiation of a variety of immune cells (13). It has been shown that urinary cytokine levels increase after BCG instillation (12, 14); therefore, it has been proposed that a panel of urinary cytokines could be a useful tool to assess the
BCG-induced immune response. Urinary levels of IL1, IL2, IL6, IL10, IL8, IL18, IFNγ, TNFα, and TNF-related apoptosis-inducing ligand (TRAIL) have been evaluated as predictors for treatment response after BCG (8, 12). Recently, Kamat and colleagues reported a panel of nine urinary cytokines (IL2, IL6, IL8, IL18, IL1ra, TRAIL, IL12p70, and TNFα), which predicted the likelihood of recurrence after BCG treatment with 85.5% accuracy (95% confidence interval [CI], 77.9–93.1%; ref. 15). In each study, a limited number of cytokines was analyzed, thus limiting the scope of the work. Therefore, we hypothesize that a large panel of 105 urinary cytokines measured longitudinally at varying time points during immune treatment, will provide the information needed to predict response to treatment. In this study, we sought to determine whether clinicopathologic data in addition to longitudinal urinary cytokines collected during intravesical BCG treatment with or without concomitant HS-410 vaccine are predictors of time-to-treatment failure in patients with intermediate- or high-risk NMIBC. HS-410 vaccine, Vesigenurtacel-L, was derived from a cancer cell line, and modified to express HLA-A1 protein and secrete gp96-lg fusion protein, which activates CD8+ cytotoxic T cells (16). It is postulated that concurrent administration of intravesical BCG and intradermal HS-410 might result in optimally synergistic immune activation (17).

Materials and Methods

Study population

Urine samples were collected from 50 patients with intermediate- or high-risk NMIBC enrolled in a phase II, randomized study to evaluate the safety, immune response and clinical activity of HS-410–treated individuals with NMIBC who have undergone transurethral resection of bladder tumor (TURBT) from 2013 to 2017 (clinicalTrials.gov Identifier NCT02010203). In this trial, patients with high- or intermediate-risk NMIBC received weekly intradermal injections of either 106 or 107 cells/dose of HS-410 or placebo in combination with intravesical induction BCG for 6 weeks followed by 6-weekly injection of vaccine or placebo. Patient continuing on trial received maintenance treatment consisting of 3-weekly treatments of vaccine and BCG approximately 3, 6, and 12 months after initiating induction treatment. Demographic and clinical data were collected. Midstream voided urine samples were collected from patients on study at baseline (before any treatment), week 7, 13, 28, and at end of treatment (EOT), according to prespecified time points for immunologic response assessments in the protocol. EOT evaluation was completed about 4 weeks after last dose of vaccine or placebo. Early institutional and safety measures were performed per standard-of-care (every 3 months for 2 years and then every 6 months for up to a year). Patients who were found to have an abnormality on cystoscopy or abnormal urinary cytology received standard-of-care treatment per the discretion of the investigator, which typically included cystoscopy, bladder biopsy, or TURBT. The primary endpoint of the study was time-to-recurrence and/or time-to-progression, which is based upon the pathologic interpretation of the bladder biopsy and/or TURBT samples. Progression is defined as an increase in stage from carcinoma in situ (CIS) to Ta to T1 disease, development of T2 or greater or lymph node (N+) disease or distant metastasis (M1), or an increase in grade from low to high. Recurrence is defined as presence of disease that has recurred or persisted (only for patients with CIS) to the same or a lower extent (i.e., stage/grade).

Urine biomarker measurement

Freshly voided urine samples were collected and centrifuged at 1000–1500 x g for 10 minutes. The supernatant was collected and stored at −80°C until biomarker analysis. All experiments were performed using a multiplex immunoassay-based cytokine array (R&D Systems Proteome Profiler Human XL Cytokine Array Kit) and were detected by a LI-COR Odyssey CLx imager system and quantified using the Quick Spots array analysis software by Western Vision Software. For each marker, the data are the average of analytic duplicates minus the average of duplicate negative controls. Normalization was not performed as no single urinary marker has been identified for urinary protein normalization (18).

Statistical analysis

Student t test or Wilcoxon rank-sum test was used to compare quantitative characteristics between response groups. Categorical variables were compared using χ2 or Fisher exact test. To adjust for multiple testing, the FDR approach was calculated (19). The Skillings–Mack test was used to evaluate whether cytokine levels changed over time (20). To predict outcome of time-to-event (either recurrence or progression), univariate and multivariable Cox models using clinicopathologic variables and urinary cytokines were constructed (Fig. 3). All event and late event predictive models (excluding events prior to day 120) were constructed using either baseline cytokines, week 13 cytokines, or the change in cytokines from baseline to week 13. An additional model was constructed treating cytokines as time-dependent covariates using all the time points (BL, week 7, 13, 28, and EOT). To predict early events (at first cystoscopic evaluation at 3 months), univariate and multivariable logistic regression modeling were applied to cytokine levels at baseline or week 13. For the model construction, first we created the best possible model using clinical parameters. Then, we used stepwise selection to construct the best model using the cytokines of interest (with P ≤ 0.15 in univariate Cox models). The concordance index (C-index) was used to evaluate the performance of these models (21).

As an exploratory analysis, we evaluated the predictive value of urinary cytokines at week 13 to predict treatment response (all event model) using the Naive Bayes (NB) classification technique. Naive Bayes is a classifier based on applying Bayes’ theorem, which relates a strong independence assumption between features within the classifier. Briefly, the top five cytokines with highest correlation with response status were selected using univariate logistic regression and applied to multivariable analysis. To avoid model overfitting and to test the generalizability of the results, these performance measures were assessed by applying 10-fold cross validation.

In addition, week-13 urine samples were clustered hierarchically using the complete linkage method using all cytokines or cytokines found to be associated with recurrence/progression in our statistical modeling. Cytokine levels were standardized and values limited at two SDs above and below the mean. Results were visualized on a heatmap.

All analyses were performed with Stata statistical software version 15 (StataCorp), SAS software version 9.4 (SAS Institute), R 3.3.1. [R Core Team (2016)], and Waikato Environment for
Table 1. Baseline characteristics by response status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Recurrence or progression</th>
<th>Yes (N = 19)</th>
<th>No (N = 31)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>72 (11.3)</td>
<td>69 (11.3)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Sex (%)</td>
<td>5 (26)</td>
<td>3 (10)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td>14 (74)</td>
<td>28 (90)</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>18 (95)</td>
<td>30 (97)</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (5)</td>
<td>1 (3)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>2.21 0.63</td>
<td>0.48 0.09</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Diagnosis of cancer (%)</td>
<td>8 (42)</td>
<td>19 (61)</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Recurrent</td>
<td>11 (58)</td>
<td>12 (39)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Stage (%)</td>
<td>3 (16)</td>
<td>10 (32)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>CIS only</td>
<td>9 (47)</td>
<td>8 (26)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>4 (21)</td>
<td>2 (6)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Ta + CIS</td>
<td>3 (16)</td>
<td>9 (29)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>0 (0)</td>
<td>2 (6)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Grade (%)</td>
<td>17 (89)</td>
<td>30 (97)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2 (11)</td>
<td>1 (3)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3 (17)</td>
<td>3 (10)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Previous mitomycin (%)</td>
<td>15 (83)</td>
<td>28 (90)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0 (0)</td>
<td>2 (6)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4 (21)</td>
<td>12 (39)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Previous BCG (%)</td>
<td>15 (79)</td>
<td>19 (61)</td>
<td>0.55</td>
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</table>

Knowledge Analysis (Weka). P < 0.05 were considered statistically significant.

Results

Patient demographics and Urine biomarkers

Demographic and clinical data from participants are summarized in Table 1 by treatment response. The mean age of participants was 70 years old. The majority of the cohort was white (48 patients) and male (42 patients). Twelve patients (24%) had no history of smoking. Median time of follow-up was 349 days (IQR 99–421 days). Among 23 patients (46%) who had a history of smoking, 11 patients had a positive history of smoking (31% of ever-smokers). Kaplan–Meier event-free survival curves in nonsmoker versus ever-smokers are further illustrated in Fig. 1.

A total of 105 cytokines were measured for each urine sample. Urine cytokines by response status at different time points were summarized in the Supplementary data. At FDR of 0.15, there was no significant difference in baseline cytokine levels in responders versus nonresponders. The median levels of Apolipoprotein A1, Angiopoietin-1, Chitinase-3-like 1, Complement C5-C5a, Dickkopf-related protein 1 (DKK1), growth-related protein (GRO-α), IL8, Macrophage inflammatory protein-3β (MIP-3β), IFNγ-induced protein 10 (IP10), IFNα-inducible T-cell α chemotactant (ITAC), monokine induced by IFNγ (MIG), matrix metalloproteinase 9 (MMP-9), Myeloperoxidase, Serpin E1, sex hormone-binding globulin (SHBG), VEGF significantly changed over time in patients with or without recurrence/progression (Fig. 2).

Models to predict time-to-recurrence/progression (Fig. 3)

Models using baseline and week 13 urine cytokines. All event predictive model. Simple Cox models using percent change of cytokines at week 13 from baseline were evaluated. A multivariable Cox model was constructed from smoking status and percent change of the following cytokines: insulin-like growth factor-binding protein (IGFBP), IL18-binding protein-a (IL18BPa), IP-10, IL3, platelet-derived growth factor AB/BB (PDGF-AB/BB), complement factor D, angiopoietin-1, IFNα, IL8, IL15, IL34, IL23, TNFα, and IL13. In the final selected multivariable Cox model (C-index 0.70), the percent change (for 100 units) of IL18BPa (HR 1.99; 95% CI, 1.16–3.43; P = 0.01), IL23 (HR 1.12; 95% CI, 1.012–1.23; P = 0.03), IL8 (HR 0.27; 95% CI, 0.14–0.99; P = 0.048), specifically among the patients who did not respond to the treatment, 7 patients were nonsmokers (58% of nonsmokers) and 11 patients had a positive history of smoking (31% of ever-smokers). Kaplan–Meier event-free survival curves in nonsmoker versus ever-smokers are further illustrated in Fig. 1.

Table 2. Univariate analysis to predict recurrence-free and progression-free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Age</td>
<td>1.03</td>
<td>0.98–1.07</td>
<td>0.238</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Referent</td>
<td>0.472</td>
</tr>
<tr>
<td>Male</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>Referent</td>
<td>0.38</td>
<td>0.14–0.99</td>
</tr>
<tr>
<td>Ever-smoker</td>
<td></td>
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<td></td>
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<tr>
<td>Diagnosis of cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newly diagnosed</td>
<td>Referent</td>
<td>2.21</td>
<td>0.85–5.71</td>
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<tr>
<td>Recurrent</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Previous mitomycin</td>
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<tr>
<td>No</td>
<td>Referent</td>
<td>1.29</td>
<td>0.36–4.64</td>
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<tr>
<td>Yes</td>
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<td></td>
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<tr>
<td>Previous BCG</td>
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<tr>
<td>No</td>
<td>Referent</td>
<td>0.60</td>
<td>0.20–1.85</td>
</tr>
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<td>Yes</td>
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<td></td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CIS</td>
<td>Referent</td>
<td>0.68</td>
<td>0.11–4.07</td>
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<tr>
<td>T1</td>
<td>2.80</td>
<td>0.75–10.36</td>
<td>0.124</td>
</tr>
<tr>
<td>Ta</td>
<td>2.81</td>
<td>0.63–12.60</td>
<td>0.176</td>
</tr>
<tr>
<td>Ta + CIS</td>
<td></td>
<td></td>
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</tr>
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<td>Grade</td>
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<td></td>
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</tr>
<tr>
<td>High</td>
<td>Referent</td>
<td>0.47</td>
<td>0.09–2.44</td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ethnicity</td>
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<td></td>
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<tr>
<td>White</td>
<td>Referent</td>
<td>1.12</td>
<td>0.15–8.44</td>
</tr>
<tr>
<td>Other</td>
<td></td>
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</table>

Cancer Epidemiology, Biomarkers & Prevention
Late event predictive models. To predict future events (recurrence/progression after 120 days), prognostic models were constructed using absolute values of cytokines at baseline and week 13 or percent changes of cytokines at week 13 from baseline. No marker remained significant predictor of response in multivariable Cox models.

Models using urine cytokines from all the time points. To explore the association between urine cytokine levels and event-free survival, we treated cytokine levels as time-dependent covariates in simple and multivariable Cox model. Urine levels of IL4, IL17A, Cystatin-C, IP10, ITAC, Myeloperoxidase, retinol-binding protein-4 (RBP-4), resistin, SHBG, and VEGF were significant predictors of event-free survival. In the selected multivariable Cox model (C-index 0.82), ever-smoker versus nonsmoker status (HR 0.21; 95% CI, 0.07–0.69; P = 0.05) and higher urine levels (100 units) of IP10 (HR 0.98; 95% CI, 0.96–0.99; P = 0.01) and resistin (HR 0.98; 95% CI, 0.97–0.99; P = 0.04) were associated with improved event-free survival. Higher levels (100 units) of SHBG (HR 1.10; 95% CI, 1.05–1.15; P < 0.01) were associated with worse event-free survival.

Models to predict response to treatment

Early event predictive models. Urinary cytokines at week 13 and baseline or percent change of cytokines at week 13 were used to predict recurrence or recurrence at first cystoscopic evaluation. In multivariable regression models, ever-smoker versus nonsmoker (OR 0.16; 95% CI, 0.02–1.03; P = 0.053) and percent change (100 units) of IGFBP-2 (OR 4.44; 95% CI 1.13–17.44; P = 0.033), and IL8 (OR 0.19; 95% CI, 0.03–1.34; P = 0.096) were predictors of.
early events (ROC 0.79). Additional modeling using absolute values of urinary cytokines at week 13 and baseline showed ever-smoker versus nonsmoker (OR 0.10; 95% CI, 0.01–0.84; \( P = 0.034 \)) and week 13 levels (for 100 units) of IGFBP-2 (OR 1.03; 95% CI, 1.00–1.06; \( P = 0.027 \)), monocyte-chemotactic protein 3 (MCP-3; OR 0.84; 95% CI, 0.72–0.97; \( P = 0.02 \)), and SHBG (OR 1.12; 95% CI, 0.99–1.26; \( P = 0.54 \)) are associated with response status at first cystoscopic evaluation (ROC 0.88).

To adjust for modulatory effects of cytokines on each other, we used multiple classification analysis to report the performance of urinary cytokines at week 13. We found that lower levels of ITAC, IL1b, IL2, IL16, and macrophage inflammatory protein (MIP-1a/MIP1-b) at week 13 were predictors of higher rate of recurrence or failure. Among classification modules, NB showed the most reliable results. NB allows us to analyze each marker separately if it can predict the class outcomes with high confidence (22). AUC of this model was 0.76. Figure 4 shows discriminatory features of the model using NB technique (A) and distribution of cytokines at week 13 by response status (B). Figure 5 illustrates hierarchical clustering of week 13 urine cytokines stratified by disease or smoking status [Heatmap for 105 cytokines (A) and Heatmap for predictive cytokines of our statistical modeling (B)].

**Discussion**

In this study, we evaluated the predictive value of a large panel of longitudinal urinary cytokines to predict response and disease outcome to immune treatment with intravesical BCG with or without HS-410 therapy in a cohort of patients with intermediate- or high-risk NMIBC. Our study has several important findings. First, we conclude that patients with a history of smoking respond better to immune treatment compared with nonsmokers. Second, urinary cytokine levels of ITAC and SHBG at week 13 or percent change of IP10, IL8, IL23, and IL18BP at week 13 from baseline could predict disease recurrence and/or progression. Third, urinary levels of IP10, resistin, and SHGB were associated with time-to-treatment failure. Finally, a panel of urinary cytokines (ITAC, IL1b, IL2, IL16, and MIP-1a/MIP1-b for all events and IGFBP-2, MCP-3, and SHBG for early events) at week 13 was predictor of all events and events at first cystoscopic evaluation.

Among clinical and pathologic features, only a positive history of smoking was associated with an improved response to therapy. This could be explained by an increased number of mutations and neoantigens in the tumors of smokers, which is associated with a better response to immunotherapy in lung cancer (23). Two large groups, Club Urologico Espano de Tratamiento (CUETO) and European Organization for Research of Cancer (EORTC), have reported prediction models of response to BCG treatment using clinicopathologic data (2, 3). In the CUETO study, female gender, recurrent disease, tumor multiplicity, and presence of concomitant CIS were associated with an increased risk of recurrence; high-grade tumors, T1 disease, and recurrence at 3-month cystoscopy were predictors of progression (3). The EORTC group found tumor multiplicity and grade as predictors of recurrence, and tumor grade and stage as predictors of disease progression (2). The discrepancy in our findings could be secondary to differences in sample size, study population, or treatment protocols among these studies. Moreover, Xylinas and colleagues evaluated the accuracy of these models and demonstrated a poor discrimination for disease recurrence and progression (0.597 and 0.662, and...
0.523 and 0.616, respectively, for the EORTC and CUETO models; ref. 24).

Immune response after intravesical BCG instillation involves recruitment and activation of various immune cells resulting in a cascade of cytokine secretion that favors a robust cytotoxic Th-1 (Th1) response suppressing a less favorable Th-2 (Th2) response (12, 14). Therefore, urinary cytokines levels may reflect the local immune microenvironment after BCG and have been used in multiple studies as predictors of recurrence or progression (8, 12). For example, urinary levels of IL2 and IL10 were used as indirect indicators of Th1 and Th2 responses, respectively (25).

Despite all these efforts, there is no validated urinary cytokine panel to predict response to BCG. In this study, for the first time, we evaluated the predictive value of a large panel of urinary cytokines at different time points to predict treatment failure in patients with intermediate- and high-risk NMIBC receiving immune treatment. Notably, we focused on cytokines at week 13 to assess the use of urinary cytokines to identify patients who may not benefit from further maintenance treatment.

In this cohort, we found that at week 13, the increased percent change of IL18BPa and IL23 from baseline in addition to decreased percent change of IP10 and IL8 were predictors of treatment failure. In addition, higher urinary levels of SHBG with lower levels of ITAC at week 13 were associated with worse failure-free survival. In an alternate model, urinary levels of IP10, Resistin, and SHBG as time-dependent variables were associated with treatment failure. Moreover, smoking status in addition to urinary levels of IGFBP-2, MCP-3, and SHBG at week 13 were predictors of early events in this study. These markers are directly or indirectly involved in the generation of Th1 type or innate immune responses.

IL18BPa is induced by IFNγ and has an inhibitory effect on IL18. IL18 increases expression of IL8 and plays a central role in the Th1-induced immune response (26). Likewise, IL8 participates in innate and acquired immunity. It has been shown that elevated IL8 or IL18 expression in the first hours after BCG treatment is associated with longer disease-free survival in patients with NMIBC (27). The cytokine IL23 predominantly expressed by activated dendritic cells, has a proinflammatory role. It promotes tumor development and metastases by suppressing natural or cytokine-induced innate immunity (28). Urothelial cells and endothelial cells also secrete IP10 in response to BCG, which acts as a chemoattractant for T cells, specifically for regulatory T cells (Treg; ref. 29). It has been shown that both increased and decreased urinary IP10 was associated with poor recurrence-free survival (30, 31). Further studies are needed to understand the role of IP10 in patients with bladder cancer (32). ITAC has a pivotal role in mediating effector T cells and induce Th1 type immune responses (33). Moreover, it has been shown that ITAC-modified tumor cell vaccines can enhance antitumor

**Figure 4.** Classification analysis of urinary cytokine at week 13 (ITAC, IL16, IL1β, IL2, MIP-1α/MIP-1α). A, The discriminatory features of model using Naive Bayes technique. B, Distribution of cytokines at week 13 by failure status. The negative values were imputed to zeros for these analyses. The results from dataset with and without imputation were similar.
immunity and reduce the incidence of disseminated metastasis (33). Resistin, an adipokine, has been suggested as a prognostic biomarker for breast and colorectal cancer with conflicting results (34, 35). It is secreted from monocytes and macrophages and is involved in insulin resistance, inflammation, and cell signaling. Likewise, IGFBP2, has been reported as an oncogenic marker in various cancers including bladder cancer (36). It has been suggested that blockage of IGFBP2 may increase the sensitivity of bladder cancer cells to chemotherapy (37).

SHBG modulates the bioavailability of sex hormones. There are few reports that have investigated the association between SHBG and cancers. For example, Cheng and colleagues reported that plasma levels of SHBG are significantly increased in patients with gastric cancer, whereas Huang and colleagues suggested that higher expression of SHBG in ovarian cancer is a poor prognostic factor (38, 39). Being female gender has been reported as a poor prognostic factor in patients with NMIBC (3, 40). These findings may suggest a diagnostic role for SHBG and sex hormones in patients with bladder cancer.

In an exploratory analysis, we used a classification technique (Fig. 4) to adjust for modulatory effects of cytokines on each other, and found that lower levels of ITAC, IL1b, IL2, IL16, and MIP-1a/MIP1-b at week 13 were predictors of higher recurrence rates or failure. IL2 is secreted by activated CD4⁺ T cells and stimulates growth, differentiation, and survival of cytotoxic lymphocytes among others. In multiple studies, higher levels of urine IL2 after BCG administration were an indication of longer recurrence-free survival (12, 25). Further studies are needed to investigate the role of IL1b, MIP-1a/MIP-1b, and IL16 in the immune response after BCG. Briefly, IL16 is a lymphocyte chemoattractant factor for CD4⁺ T cells, which primes these cells for IL2 responsiveness (41). Likewise, macrophage inflammatory proteins, MIP-1a and MIP-1b, attract and activate CD4⁺ and CD8⁺ T lymphocytes (42). IL1b is a proinflammatory cytokine, which is secreted by innate immune cells such as macrophages (43).

This study has several limitations. First, though prospective, it is a relatively small study with 50 patients. Second, the large number of cytokines in addition to small sample size increases the FDR. Therefore, these models need to be externally validated. Third, tumor immune profile of patients with recurrence may be different from patients who progress. Moreover, there is a complex modulatory effect between cytokines and many cytokines have multiple roles in immune response. Additional pathway analysis may be needed to find the next generation of predictive biomarkers. Fourth, there is no validated method to normalize urinary protein levels, as unlike blood there is intracellular protein levels, as unlike blood there is intracellular and interpersonal variations in urine volume and

Figure 5.
Hierarchical clustering of week 13 urine samples in patients stratified by disease or smoking status. A, Heatmap for 105 cytokines. B, Heatmap for predictive cytokines of our statistical modeling.
compositions. Perhaps, multilevel normalization methods that includes secretion, excretion, and filtration factors are needed (18). Finally, there is no analysis per treatment groups (BCG only vs. BCG + HS-410 vaccine), since the primary outcome of the trial is still pending and the authors are blinded to the treatment groups. Thus, our findings may not be applicable to patients with NMIBC receiving BCG only. Despite these limitations, this is the first study to suggest multiple models incorporating longitudinal urinary cytokines to predict treatment responses to immune-modulating agents in patients with intermediate- and high-risk NMIBC. Notably, this study serves as a hypothesis generating report for future studies to evaluate the role of urine cytokines as a predictive biomarker of response to local or systemic immune treatments.

Conclusion

Urinary cytokines provided additional value to clinicopathologic features to predict response to immune modulation in patients with intermediate- and high-risk NMIBC. Moreover, the predictive value of urinary cytokines was time-dependent. Notably, a panel of cytokines measured at week 13 can be used to identify patients who will recur, and thus, has no benefit from further maintenance treatment. Further studies are needed to validate these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Salmasi, K. Chamie
Development of methodology: A. Salmasi, J.M. Rose, K. Chamie
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Rose, J.C. Giffin, L.E. Gonzalez
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Salmasi, D.A. Elashoff, R. Guo, A. Upfill-Brown, C.J. Rosser
Writing, review, and/or revision of the manuscript: A. Salmasi, D.A. Elashoff, A. Upfill-Brown, C.J. Rosser, K. Chamie
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Chamie
Study supervision: K. Chamie

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Amirali Salmasi, David A. Elashoff, Rong Guo, et al.


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