Predictive Biomarkers and Personalized Medicine

A Panel of Three Markers Hyper- and Hypomethylated in Urine Sediments Accurately Predicts Bladder Cancer Recurrence

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Abstract

Purpose: The high risk of recurrence after transurethral resection of bladder tumor of nonmuscle invasive disease requires lifelong treatment and surveillance. Changes in DNA methylation are chemically stable, occur early during tumorigenesis, and can be quantified in bladder tumors and in cells shed into the urine. Some urine markers have been used to help detect bladder tumors; however, their use in longitudinal tumor recurrence surveillance has yet to be established.

Experimental Design: We analyzed the DNA methylation levels of six markers in 368 urine sediment samples serially collected from 90 patients with noninvasive urothelial carcinoma (Tis, Ta, T1; grade low-high). The optimum marker combination was identified using logistic regression with 5-fold cross-validation, and validated in separate samples.

Results: A panel of three markers discriminated between patients with and without recurrence with the area under the curve of 0.90 [95% confidence interval (CI), 0.86–0.92] and 0.95 (95% CI, 0.90–1.00), sensitivity and specificity of 86%/89% (95% CI, 74%–99% and 81%–97%) and 80%/97% (95% CI, 60%–96% and 91%–100%) in the testing and validation sets, respectively. The three-marker DNA methylation test reliably predicted tumor recurrence in 80% of patients superior to cytology (35%) and cystoscopy (15%) while accurately forecasting no recurrence in 74% of patients that scored negative in the test.

Conclusions: Given their superior sensitivity and specificity in urine sediments, a combination of hyper- and hypomethylated markers may help avoid unnecessary invasive exams and reveal the importance of DNA methylation in bladder tumorigenesis. Clin Cancer Res; 20(7); 1978–89. ©2014 AACR.

Introduction

Bladder cancer was one of the 10 most prevalent malignancies in males in 2011 ranking fourth and eighth in terms of deaths and new cases, respectively (1, 2). Nonmuscle invasive bladder cancer (NMIBC) accounts for 80% of all the cases, and can be further classified as mucosa only (Ta), carcinoma in situ (T1), and lamina propria invading (T1) lesions (3, 4). The primary treatment for NMIBC is transurethral resection of bladder tumor (TURBT) with or without intravesical chemo or immunotherapy; however, more than 50% of patients recur after the TURBT procedure, with the highest rate of recurrence occurring in patients with high-risk disease (5, 6). As a result, patients require frequent and lifelong monitoring following TURBT, making bladder cancer one of the most costly types of cancer to manage.

The current gold standard for monitoring of bladder cancer recurrence involves the use of cystoscopy and cytology (2, 3). Disease surveillance is cumbersome because of the invasive nature of cystoscopic examination and the low sensitivity of urinary cytology in the detection of low-grade tumors (7). Recently, efforts have been devoted to find better markers of disease diagnosis and prognosis in samples collected by noninvasive methods, such as urine sediments (8). The addition of nuclear matrix protein 22 (NMP-22), bladder tumor antigen, or UroVysion FISH has shown to help increase the sensitivity of cytology (9). However, due to their inconsistent performance in terms of specificity or sensitivity, the markers proposed to date have not been widely adopted in routine clinical practice (10). Therefore, there is a need to find reliable markers to monitor recurrence in TURBT patients, which in turn, may improve disease management.

Epigenetic changes, namely changes in chromatin structure that regulate gene expression, occur during tumorigenesis.
Translational Relevance

Nonmuscle invasive bladder cancer, characterized by a high rate of recurrence, is a relatively high-cost disease in cancer management. Urinary DNA methylation changes have shown their stable, reliable, and early appearance in bladder carcinogenesis. We longitudinally analyze DNA methylation changes in urine sediments serially collected from patients that underwent bladder tumor resections at the time of follow-up visits. Our results show that the combination of a transcription factor (SOX1), a specific LINE-1 element, and a key epigenetic driver gene interleukin-1 receptor-associated kinase 3 (IRAK3) provides better resolution than cytology and cystoscopy in the detection of early recurrence. Therefore, our markers may help avoid unnecessary invasive exams during clinical tumor surveillance in a cost-effective manner. We provide new insights into the value of incorporating both hyper- and hypo-DNA methylation markers into the screening of urine sediments for personalized following and monitoring of tumor recurrence in transurethral resection of bladder tumor (TURBT) patients.

Materials and Methods

Patients and sample collection

The study population includes patients under surveillance for tumor recurrence following TURBT for noninvasive urothelial carcinoma (Tis, Ta, T1; grade low-high). Urine samples were obtained from 90 such patients with NMIBC at each available clinical follow-up visit. Patient’s age ranged from 41 to 96 years, with a median age of 69 years. Urine collection at follow-up visits was performed at the Department of Urology, USC Norris Comprehensive Cancer Center (Los Angeles, CA) from 2004 to 2011 according to the institutional guidelines, in compliance with Institutional Review Board-approved protocols. Patients at high risk of recurrence (Tis, high-grade Ta/T1 disease) had received prior intravesical therapy with Bacillus Calmette-Guerin (BCG) or mitomycin C at the discretion of the treating physician. A total of 368 samples were collected under patient informed consent at follow-up visits over a period ranging from 5 to 89 months (Fig. 1A). The baseline clinicopathological characteristics of the patients showed no significant differences between the study groups (Table 1).

Tumor recurrence was defined as biopsy-proven bladder cancer occurring subsequent to complete resection of the visible primary tumor. Severe atypia concomitant with papillary lesions detected by cytology and cystoscopy was recorded as recurrence only when the biopsy results were absent. Over the collection period, 34 patients had tumor recurrence, whereas 56 patients were not diagnosed with recurrence through the last follow-up visit. The clinical characteristics of 34 recurrent tumors are summarized in Table 2. Out of the 34 patients with recurrence, 31 provided a urine sample at the time of diagnosis. Tumors were characterized according to the criteria of the American Joint Committee on Cancer (World Health Organization/International Society of Urological Pathology (ISUP); ref. 26) and staging was based on the tumornodemetastasis classification (International Union Against Cancer; ref. 4) across the entire study period.

DNA extraction from urine sediments and DNA methylation analysis by pyrosequencing

Urine specimens (~50 mL) included samples from both “urine” and “bladder wash.” The bladder wash was
collected at the time of cystoscopy by the nurses or urologist. The same sample underwent cytology and DNA methylation analysis in a double-blinded fashion. The samples that underwent DNA methylation analysis were stored at 4°C until cells were pelleted by centrifugation for 10 minutes at 1,500 g. DNA was then extracted from urine sediments as previously described and stored at 4°C (21). DNA was bisulfite converted using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s instructions. Six DNA methylation markers were selected from our previous study (12, 13); the regions of interest were PCR amplified using biotin-labeled primers (Supplementary Table S2) and analyzed by pyrosequencing, a high-throughput and quantitative tool for DNA sequence detection. The percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines was measured using PSQ HS96 (Qiagen) as previously described (13).

Table 1. The clinicopathological characteristics of 90 TURBT patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No recurrence N = 56</th>
<th>Recurrence N = 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Median 71</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Range 42–96</td>
<td>41–87</td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td>Male 48 (86)</td>
<td>27 (79)</td>
</tr>
<tr>
<td></td>
<td>Female 8 (14)</td>
<td>7 (21)</td>
</tr>
<tr>
<td>Histology TCC, no. (%)</td>
<td>56 (100)</td>
<td>34 (100)</td>
</tr>
<tr>
<td>Number of tumors, no. (%)</td>
<td>Unifoci 19 (34)</td>
<td>18 (53)</td>
</tr>
<tr>
<td></td>
<td>Multifoci 16 (29)</td>
<td>12 (35)</td>
</tr>
<tr>
<td></td>
<td>Missing 21 (37)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>T Stage, no. (%)</td>
<td>Tis 2 (4)</td>
<td>1 (3)</td>
</tr>
<tr>
<td></td>
<td>Ta 37 (66)</td>
<td>19 (56)</td>
</tr>
<tr>
<td></td>
<td>T1 17 (30)</td>
<td>14 (41)</td>
</tr>
<tr>
<td>Tumor grade*, no. (%)</td>
<td>Low 26 (46)</td>
<td>17 (50)</td>
</tr>
<tr>
<td></td>
<td>High 30 (54)</td>
<td>17 (50)</td>
</tr>
<tr>
<td>Concomitant CIS, no. (%)</td>
<td>11 (20)</td>
<td>7 (21)</td>
</tr>
<tr>
<td>Treatment, no. (%)</td>
<td>Adjuvant BCG 36 (64)</td>
<td>20 (59)</td>
</tr>
<tr>
<td></td>
<td>Adjuvant chemotherapy instillation 11 (19)</td>
<td>12 (35)</td>
</tr>
<tr>
<td>Follow-up time since TURBT, y</td>
<td>5.5 (0.6–9.7)</td>
<td>4.7 (0.4–26)</td>
</tr>
<tr>
<td>Study follow-up time, y</td>
<td>3.5 (0.4–7.1)</td>
<td>3.6 (0.5–7.4)</td>
</tr>
<tr>
<td>Total urines analyzed, no.</td>
<td>208</td>
<td>160</td>
</tr>
<tr>
<td>Urines analyzed/patient, no.</td>
<td>Mean (±SD) 3.7 ± 1.8</td>
<td>4.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Range 2–9</td>
<td>2–10</td>
</tr>
</tbody>
</table>

Abbreviations: TCC, transitional cell carcinoma; CIS, carcinoma in situ; no., number; (%), percentage.

*Grade 1 and 2 are reported as low grade. Grade 3 and more are reported as high grade.

Statistical analysis

Receiver operating characteristic (ROC) curves summarize the accuracy of DNA markers in urine sediment from 87 independent samples, selected at the time of the last follow-up visit (nonrecurrent patients), or at the time of first recurrence (patients with recurrence). A subset of 83 patients with complete data on all markers was used to build a multivariable predictor model. We used stepwise
Table 2. The clinical characteristics of 34 patients with recurrence bladder cancer

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Baseline</th>
<th>Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 34</td>
<td></td>
</tr>
<tr>
<td>Histology TCC, no. (%)</td>
<td>34 (100)</td>
<td>30 (88)</td>
</tr>
<tr>
<td>Number of tumors, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unifoci</td>
<td>18 (53)</td>
<td>19 (56)</td>
</tr>
<tr>
<td>Multifoci</td>
<td>12 (35)</td>
<td>12 (35)</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (12)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>T Stage, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tis</td>
<td>1 (3)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>Ta</td>
<td>19 (56)</td>
<td>20 (59)</td>
</tr>
<tr>
<td>T1</td>
<td>14 (41)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>T2</td>
<td>0</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Tumor gradea, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>17 (50)</td>
<td>16 (47)</td>
</tr>
<tr>
<td>High</td>
<td>17 (50)</td>
<td>13 (38)</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>5 (15)</td>
</tr>
<tr>
<td>Treatment, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant BCG</td>
<td>20 (59)</td>
<td>15 (44)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy instillation</td>
<td>12 (35)</td>
<td>5 (15)</td>
</tr>
</tbody>
</table>

Baseline | Recurrence
---|---
Patient number | Number of tumors | T stage | Grade | Number of tumors | T stage | Grade
4843 | Multifoci | T1 | High | Multifoci | T1 | High
5137 | Unifoci | Ta | Low | Unifoci | Ta | Low
6664 | Multifoci | T1 | High | Unifoci | Missing | Low
6675 | Unifoci | Ta | Low | Multifoci | Ta | Low
6762 | Missing | Ta | Low | Unifoci | Ta | Low
6804 | Multifoci | T1 | High | Unifoci | Missing | Low
6851 | Unifoci | T1 | High | Unifoci | Ta | Low
7145 | Multifoci | T1 | High | Multifoci | CIS | High
7258 | Unifoci | T1 | High | Unifoci | CIS | High
7346 | Unifoci | Ta | High | Unifoci | Ta | High
7397 | Unifoci | T1 | Low | Multifoci | Ta | Low
7592 | Unifoci | Ta | High | Unifoci | Ta | High
7662 | Missing | Ta | Low | Multifoci | Ta | High
7716 | Multifoci | Ta | High | Multifoci | Ta | High
7718 | Unifoci | T1 | Low | Unifoci | T1 | High
7728 | Unifoci | T1 | Low | Multifoci | Missing | NA
7743 | Unifoci | CIS | CIS | Unifoci | Ta | Low
7774 | Unifoci | CIS | CIS | Unifoci | Ta | Low
7792 | Multifoci | Ta | Low | Missing | Missing | NA
7809 | Multifoci | Ta | Low | Unifoci | CIS | High
7810 | Unifoci | Ta | Low | Multifoci | Ta | Low
7817 | Multifoci | Ta | Low | Multifoci | Ta | Low
7859 | Multifoci | T1 | High | Unifoci | Ta | High
7873 | Multifoci | Ta | Low | Unifoci | Missing | NA
7891 | Missing | T1 | High | Unifoci | CIS | High
7896 | Unifoci | Ta | Low | Unifoci | Ta | Low
8659 | Unifoci | T1 | High | Missing | Missing | NA
8792 | Unifoci | T1 | High | Unifoci | T2 | High

(Continued on the following page)
logistic regression, selecting variables to add or subtract based on the Akaike Information Criterion (AIC). The risk score was obtained using logistic regression, and represents the probability of a positive result (recurrence) on the log-odds scale. On this scale, a score of 0 represents a probability of 0.5 (50% chance) for a patient having recurrence. This suggests that the best cutoff of the risk score to predict recurrence is 0, with scores > 0 having a more than 50% chance of being from a recurrent patient, and scores < 0 having a less than 50% chance of being from a recurrence patient. The risk score was computed using 83 patients with complete data on all markers (29 samples taken at the time of first recurrence after TURBT, and 54 samples from patients who were recurrence free at the last time of urine collection). The three-marker panel was selected using the forward and backward stepwise variable selection procedure. The AIC is the optimality criterion used for model selection. When comparing two models, the model with the lowest AIC is preferred. We compare the AIC of the model with no variables to the AIC of all 1-variable models, and add the variable reducing the AIC the most. This is repeated, by adding the next variable that further reduces the AIC. This forward step is repeated once more, with the addition of a backward step that evaluates the possibility of removing one of the variables already in the model. For each new step, the addition/removal of a variable is considered, providing a means of ‘stepping’ through models with different combinations of variables, to search for the best predictive model. The procedure ends when the model with the lowest AIC is found.

Sensitivity and specificity were estimated using 5-fold cross-validation, repeating the model selection for each subdivision of the data. Five-fold cross-validation was used to obtain the reported (less biased) estimates of sensitivity and specificity. Model selection was performed using forward and backward stepwise selection on four fifths of the dataset, and the predictive ability assessed on the fifth that was not used for variable selection, an independent data subset. This was repeated five times, each time holding a separate fifth of the dataset out for validation, and performing a new model selection on the remaining four fifths. The five data subsets consisted of four groups of 17 (6 recurrences/11 nonrecurrences) and one group of 15 (3 recurrences/10 nonrecurrences). The final model was then evaluated on the remaining samples from our dataset to evaluate the performance of the markers providing the best fit to our training data. Control samples (n = 134) included visits before the last follow-up visit where the patient was not diagnosed with bladder cancer; case samples (n = 25) included recurrences occurring after the first recurrence and samples at the initial clinic visit when the patient presented with bladder cancer.

Results

DNA methylation analysis in urine sediments

To evaluate whether hypermethylation of HOXA9, SOX1, NPY, IRAK3, and ZO2, and hypomethylation of L1-MET could be detected in urine sediments, we analyzed urine samples collected from patients with bladder tumors (n = 20) and from age-matched cancer-free controls (n = 20) using pyrosequencing. The results show that DNA methylation of HOXA9 (P < 0.0001), SOX1 (P = 0.0017), NPY (P = 0.005), IRAK3 (P < 0.0001), and ZO2 (P < 0.0001) was significantly increased, whereas methylation of L1-MET (P < 0.0001) was significantly decreased in urine sediments from patients with cancer compared with healthy donors, indicating that the DNA methylation status in urine sediments mirror that of the tumor (Supplementary Fig. S1).

Longitudinal study of DNA methylation changes in urine sediments collected from TURBT patients at the time of follow-up visits

To examine whether aberrant DNA methylation of five hypermethylated and one hypomethylated markers in urine sediments is associated with tumor recurrence, we analyzed their DNA methylation status in 368 urine sediments collected in follow-up visits followed under standard care amongst patients that had undergone prior tumor resections. Figure 1A shows the representative time-dependent methylation analysis. Patients without recurrence had longer median follow-up time than the recurrence group (Table 1). The Spearman correlation of

Table 2. The clinical characteristics of 34 patients with recurrence bladder cancer (Cont’d)

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Baseline</th>
<th>Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of tumors</td>
<td>T stage</td>
</tr>
<tr>
<td>8928</td>
<td>Missing</td>
<td>T1</td>
</tr>
<tr>
<td>9216</td>
<td>Multifoci</td>
<td>Ta</td>
</tr>
<tr>
<td>9532</td>
<td>Unifoci</td>
<td>Ta</td>
</tr>
<tr>
<td>9536</td>
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<td>Ta</td>
</tr>
<tr>
<td>9626</td>
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<td>9627</td>
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</tr>
</tbody>
</table>

Abbreviations: TCC, transitional cell carcinoma; CIS, carcinoma in situ; no., number; (%), percentage.

aGrade 1 and 2 are reported as low grade. Grade 3 and more are reported as high grade.
DNA methylation level for each marker was then calculated (Supplementary Fig. S2). Individual DNA methylation marker success rates averaged 98.9% across all samples (94.9–100%). Next, the DNA methylation levels of these markers in 31 urine sediments from patients collected at the time of first recurrence was compared with that of 56 samples from the last follow-up visit of patients who did not recur within the study period (Supplementary Table S1). Our results show that the six markers individually displayed high sensitivity and specificity in recurrence detection as evidenced by the ROC curves and area under the curve (AUC) values [0.93–0.95, 95% confidence interval (CI) shown in Fig. 1B]. In the group of patients without bladder tumor recurrence, urine sediment samples showed consistent DNA methylation levels throughout the duration of surveillance; markers methylated in tumors decreased in methylation levels, whereas the marker hypomethylated in tumors (L1-MET) increased and maintained high methylation levels after tumor resection (Fig. 1C; patients 7,873 and 7,844). In contrast, patients who had bladder tumor recurrence displayed changes in the DNA methylation status of all six markers at the time of clinically defined recurrence. DNA methylation levels of hypermethylated markers continued to increase until recurrence was confirmed with a positive cystoscopy and biopsy 19 and 33 months after the first urine sample was obtained. The elevated methylation levels decreased following resection surgery (Fig. 1D; patients 7,258 and 7,145). Our results demonstrate that the methylation levels of these markers displayed a clear trend in the samples obtained at follow-up visits leading to the confirmation of recurrence and showed not only a significant correlation with recurrence ($P < 0.0001$), but also a possible predictive value as methylation changes could be detected before clinical evidence of recurrence.

### A three-marker panel

To determine the combination of markers capable of detecting tumor recurrence in urine sediments with the highest sensitivity and specificity, we built a model of multiple markers by logistic regression, using 29 samples taken at the time of first recurrence and 54 samples from patients who were recurrence free at the last time of urine collection. Three markers SOX1, IRAK3, and L1-MET were found to provide the best possible marker combination (risk score = $-0.37608 + 0.17095 \times \text{SOX1} + 0.21604 \times \text{IRAK3} - 0.09887 \times \text{L1-MET}$). Scores above zero predict recurrence. Ninety-four percent of patients with no recurrence showed negative scores (95% CI, 88%–100%) and 93% of patients with recurrence showed positive scores (95% CI, 84%–100%; Fig. 2A). The 5-fold cross-validation analysis estimated an AUC of 0.90 (95% CI, 0.86–0.92) with sensitivity of 86% (95% CI, 74%–99%) and specificity of 89% (95% CI, 81%–97%). C, this three-marker model was validated in a separate urine sediment samples that included urine sediments from recurrence-free patients before the last follow-up visit (CU) and urine sediments of patients with known urothelial carcinoma (TU) with the sensitivity of 80% (95% CI, 60%–96%) and specificity of 97% (95% CI, 91%–100%). Risk scores above the cutoff value (red dashed line) denote positive scores, whereas those below signify negative scores.

**Figure 2.** A three-marker signature showed high sensitivity and specificity in detecting tumor recurrence. A, the risk score of $-0.37608 + 0.17095 \times \text{SOX1} + 0.21604 \times \text{IRAK3} - 0.09887 \times \text{L1-MET}$ was calculated in the urine sediments of TURBT patients with no recurrence at the last follow-up and with recurrence. B, 5-fold cross-validation showed a sensitivity of 86% (95% CI, 74%–99%) and specificity of 89% (95% CI, 81%–97%). C, this three-marker model was validated in a separate urine sediment samples that included urine sediments from recurrence-free patients before the last follow-up visit (CU) and urine sediments of patients with known urothelial carcinoma (TU) with the sensitivity of 80% (95% CI, 60%–96%) and specificity of 97% (95% CI, 91%–100%). Risk scores above the cutoff value (red dashed line) denote positive scores, whereas those below signify negative scores.
DNA methylation scores within the no recurrence and recurrence groups showed no correlation with any of the primary tumor characteristics (Supplementary Table S3). However, a positive correlation was found between DNA methylation status and grade of the primary tumor in the recurrence group as well as stage (Ta vs. T1) at recurrence ($P<0.05$; Supplementary Table S4). These results demonstrate that the combination of a tumor-specific hypermethylated marker, SOX1, an epigenetic driver, IRAK3, and a field defect-associated hypomethylated marker, L1-MET, can detect disease recurrence with high sensitivity and specificity.

**Power of prediction of recurrence**

To evaluate whether methylation of the three-marker model predicts recurrence in our longitudinal study samples, we screened DNA methylation and calculated risk scores given by the combination of SOX1, IRAK3, and L1-MET in every urine sample obtained at follow-up visits from 90 TURBT patients. Risk scores over follow-up fall primarily below the cutoff in samples from patients without recurrence (Fig. 3A) and often above the cutoff in samples from patients with recurrence (Fig. 3B). Positive DNA methylation scores were found in 90% of the samples (34/38) at the time of recurrence diagnosis, exceeding the sensitivity of both cytology (16%) and cystoscopy (8%) for the same visits to the clinic (Fig. 4A). To quantify the prediction value of the three markers, we analyzed risk scores in patients/samples in the period before recurrence (Fig. 4B and Supplementary Fig. S4A) or at any time visits (Fig. 4C and Supplementary Fig. S4B). Eighty percent of patients (16/20) whose urine samples showed a history of positive DNA methylation scores developed recurrence later (95% CI, 62%–98%). Out of the 70 patients who did not have a history of positive DNA methylation scores, 52 (74%) did not recur (95% CI, 64%–85%; Fig. 4B). Our results indicate that the three-marker signature detected in urine sediments of follow-up visits can reliably predict recurrence in 80% of patients, which is superior to the 35% (95% CI, 14%–56%) and 15% (95% CI, 0%–31%) predicted by cytology and cystoscopy, respectively (Fig. 4B). Sample-level charts report the percentage of samples by DNA methylation score from patients with or without recurrence. The results demonstrate that the three-marker model can successfully detect current and subsequent recurrence in 90% (64/71) of DNA methylation-positive samples (95% CI, 83%–97%), whereas these same samples showed 30% (95% CI, 19%–40%) suspicious plus positive in cytology and 44% (95% CI, 32%–55%) suspicious plus positive in cystoscopy (Supplementary Fig. S4B).

**Discussion**

Markers that can be detected in urine sediments provide a noninvasive method to test for the presence of bladder tumor cells and premalignant cell populations in the urinary tract (22). Some U.S. Food and Drug Administration-approved tests, such as NMP-22, ImmunoCyt, and UroVysis, have been used for the surveillance of bladder cancer, and have shown a higher degree of sensitivity than cytology. However, the following situations make them less than ideal for comprehensive utilization and general adoption into the clinical practice: (i) these markers have a lower specificity than cytology, (ii) the specificity of NMP-22 and ImmunoCyt are influenced by other urinary benign conditions, (iii) they are not meant to replace urinary cytology and cystoscopy, but to complement those surveillance methods, and (iv) they are expensive, labor intensive, and provide marginal improvement in disease detection (3, 10, 27, 28). Although some of these markers are currently used to predict the responses to intravesical therapies like BCG, further studies in a larger population and consistent performance assessment are still needed (29). In addition, some new investigational urine markers such as microsatellite alterations and gene mutations (e.g.,
fibroblast growth factor receptor 3; FGFR3) have not been widely deployed as a routine screening method for recurrence (30–32).

Changes in DNA methylation are chemically stable, occur early during tumorigenesis, and can be quantified on high-throughput platforms, which make them potentially good tumor markers. Many studies have shown that aberrant DNA methylation of a single or a combination of markers in urine sediments of patients carrying bladder cancer stably reflects their methylation status in bladder tumors independently of the presence of hematuria, bladder infections, or other bladder benign conditions.

Figure 4. Three DNA methylation markers help predict the risk of recurrence of bladder tumors in 80% of patients. A, percentage of urine sediments that had positive scores (DNA methylation score calculated to be higher than cutoff values) at the time of recurrence (38 samples, 29 patients). B and C, pie charts summarize all patients in the period before recurrence (B), or at anytime (C) and the comparison with cytology and cystoscopy reports in these same groups of patients. A patient-level positive score represents a history of positive DNA methylation scores at any eligible visits. Patient-level charts report the percentage of recurrence-free patients in those without a history of positive samples (negative predictive value, NPV) and percentage of patients with recurrence in those with a history of DNA methylation positive samples (positive predictive value, PPV).
thereby establishing DNA methylation screening of urine sediments as a promising noninvasive approach for bladder cancer detection (10, 19, 21, 33–37). However, most studies have focused on finding correlations between the methylation status of markers present in the primary tumor or urine sediments at the time of diagnosis (before TURBT) and recurrence (38). Although some of such markers showed positive correlations with the number, size, grade, and stage of primary tumors and prior recurrence history, others did not, likely due to the variation of the study population or the sample collection conditions (39–43). The variety of methods used to detect methylation, the fact that only one sample was evaluated by patient, and the reduced number of control samples used in the different studies, have made it difficult to accurately predict recurrence.

More recently, it has been proposed that longitudinal collection and testing of urine sediments may help assess the prognostic and recurrence predictive value of markers (43, 44). Several studies undertook this approach by using DNA methylation analysis, microsatellite markers, and a FGFR3 mutation assay (45, 46). Although these markers were highly sensitive, they displayed low specificity in some cases comparable with that of cytology or a high rate of false-positive results (47, 48). The three-marker model proposed in this study may circumvent the specificity problem. As far as we know, we are the first group using multiple DNA methylation markers to directly test risk value and monitor recurrence in serial urine samples from patients with a history of noninvasive urothelial carcinoma. SOX1, IRAK3, and L1-MET had a recurrence predictive power far superior to that of cytology and cystoscopy (80% vs. 35% vs. 15% accuracy), and therefore, they could supplement visits that reveal cytologically or cystoscopically atypical or suspicious results. NPVs of the three-marker panel were slightly lower than those obtained by cytology and cystoscopy, largely due to the definition of recurrence in our study. Patients with “no recurrence” displayed negative cytologic or cystoscopic results.

In addition, the three markers we identified here may also contribute to functional changes during tumorigenesis. For example, IRAK3 shows significantly decreased expression and promoter methylation in various cancer types, and our laboratory identified IRAK3 as a key driver for cancer cell survival through the activation of survivin (25). Some of the limitations of our study are that the mechanisms underlying bladder tumor recurrence are still unclear and that the samples of the validation set are different from those used to derive the statistical model. Undoubtedly, validation of these urine markers in a larger, independent patient cohort with appropriate follow-up visits is needed. However, the length of the follow-up time for each individual patient could be a difficult point for such a long-term study.

In conclusion, our study provides new insights into the value of a combination of hypermethylated and hypomethylated tumor-specific markers to screen urine sediments from patients following bladder tumor resections. To our knowledge, this is the first study to analyze multiple urine sediment samples collected over the course of many years by DNA methylation markers for bladder tumor recurrence. This study provides evidence that a marker panel may help minimize the frequency of cystoscopy for patients with a negative score. We suggest that patients with a positive urinary methylation test but no clinical evidence of bladder cancer disease should still be closely monitored because they carry a high risk of recurrence.

Disclosure of Potential Conflicts of Interest

P.A. Jones is a consultant/advisory board member of Astex, Lilly, and Zymo. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Ravi Agarwal for carefully reading and revising the manuscript; Dr. Sue Ellen Martin and Moli Chen for urine sample processing; the members of the Cytopathology Laboratory of the Keck Medical Center of USC for their assistance with this project; and Hui Shen and Drs. Terry Kelly and Jueng Soo You for the helpful discussion.

Grant Support

This work was supported by NCI (RO1 CA083867-PAJ, RO1 CA 124518-GL, and RO1 CA097346-KDS) and part of P30CA014089-tissue procurement.

Received September 27, 2013; revised December 19, 2013; accepted January 9, 2014; published online April 1, 2014.

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