Detection of Bladder Cancer Using Novel DNA Methylation Biomarkers in Urine Sediments

Woonbok Chung1, Jolanta Bondaruk2, Jaroslav Jelinek1, Yair Lotan4, Shoudan Liang3, Bogdan Czerniak2, and Jean-Pierre J. Issa1

Abstract

**Background:** Bladder cancer (BCa) remains a lethal malignancy that can be cured if detected early. DNA hypermethylation is a common epigenetic abnormality in cancer that may serve as a marker of disease activity.

**Methods:** We selected 10 novel candidate genes from the most frequently hypermethylated genes detected by DNA microarray and bisulfite pyrosequencing of bladder cancers and applied them to detect bladder cancer in urine sediments. We analyzed DNA methylation in the candidate genes by quantitative methylation-specific real-time PCR (qMSP) to detect bladder cancer in urine sediments from 128 bladder cancer patients and 110 age-matched control subjects.

**Results:** Based on a multigene predictive model, we discovered 6 methylation markers (MYO3A, CA10, SOX11, NKX6-2, PENK, and DBC1) as most promising for detecting bladder cancer. A panel of 4 genes (MYO3A, CA10, NKX6-2, and DBC1 or SOX11) had 81% sensitivity and 97% specificity, whereas a panel of 5 genes (MYO3A, CA10, NKX6-2, DBC1, and SOX11 or PENK) had 85% sensitivity and 95% specificity for detection of bladder cancer (area under curve = 0.939). By analyzing the data by cancer invasiveness, detection rate was 47 of 58 (81%) in non-muscle invasive tumors (pTa, Tis, and pT1) and 62 of 70 (90%) in muscle invasive tumors (T2, T3, and T4).

**Conclusions:** This biomarker panel analyzed by qMSP may help the early detection of bladder tumors in urine sediments with high accuracy.

**Impact:** The panel of biomarker deserves validation in a large well-controlled prospectively collected sample set. *Cancer Epidemiol Biomarkers Prev; 20(7); 1483–91. ©2011 AACR.*

Introduction

Bladder cancer is the 5th most common cancer in the United States and causes approximately 3% of all cancer-related deaths (1). More than 90% of urothelial cancers are transitional cell carcinomas (TCC). Most (75%–85%) bladder cancers are non-muscle invasive tumors (pTa, Tis, and pT1) at first diagnosis (2). Generally, the prognosis of noninvasive tumors is good, although up to 80% of cases will recur after complete transurethral resection and up to 45% of cases will progress to invasive cancer in 5 years (2–5). The gold standard for bladder tumor diagnosis is cystoscopy along with biopsy of suspicious lesions. However, this approach can miss 10% to 30% of malignancies and the procedure is invasive and uncomfortable (6–8). Voided urine cytology is the most common noninvasive method for detecting bladder tumors in symptomatic patients and population screening (9) and it was reported to have 34% to 35% median sensitivity and 94% to 99% median specificity by meta-analyses (10, 11).

Other methods have been developed to improve the detection of bladder cancer based on monoclonal antibodies or fluorescence in situ hybridization (FISH) with probes associated with abnormalities in urinary sediment cells. These include UroVysion (sensitivity 72% and specificity 83%), ImmunoCyt (sensitivity 67%–86% and specificity 75%–79%), BTAsstat (sensitivity 58%–71% and specificity 73%), BTAtrack (sensitivity 69%–71% and specificity 66%–90%), NMP22 (sensitivity 71%–73% and specificity 73%–80%), and Urinary fibrinogen degradation products (sensitivity 54%–77% and specificity 61%–87%; refs. 10–12).

Recently, other molecular markers with high sensitivities and specificity like AURKA gene amplification (sensitivity 87% and specificity 97%), microsatellite analysis

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(sensitivity 72%–97% and specificity 80%–100%), detection of telomerase (sensitivity 70%–100% and specificity of 60%–96%), MMP-9/TIMP-2 (sensitivity 97% and specificity 88%), HYAL1 (sensitivity 83%–94% and specificity 77%–93%), survivin (sensitivity 64%–94% and specificity 93%–100%), and combination of HYAL1 and survivin RNA (sensitivity 98% and specificity 100%) were also reported (13–18). These urinary markers are promising but randomized multicenter trials are needed to test their usefulness.

DNA hypermethylation of CpG islands in the promoter of tumor-associated genes and their consequent silencing is a common epigenetic abnormality in cancer and may serve as a useful marker to clone new cancer-related genes as well as a marker of disease activity (19, 20). Several reports indicate that body fluids, including urine (21–25), gastric juice (26), plasma/serum (27), and sputum (28, 29), can be used for noninvasive detection of cancer by hypermethylation of DNA. Previous studies in paired samples indicated that methylation in urine sediment DNA was almost always accompanied by methylation of bladder tumor DNA (22–24).

Here, we report that a group of genes (DBC1, MYO3A, SOX11, NPTX2, NXX6-2, A2BP1, PENK, and CA10) highly methylated in primary bladder tumor samples can be used to detect bladder cancer in urine sediment.

Materials and Methods

Samples analyzed
We used 6 human bladder cancer cell lines [UM-UC-2 (T24), UM-UC-3, UM-UC-6, UM-UC-9, UM-UC-13, and UM-UC-14] which were established and authenticated by Dr. Grossman (30) and 26 fresh cystectomy specimens (11 cases of papillary, 13 cases of nonpapillary, and 2 cases of squamous cell carcinoma) from patients who underwent surgery at The University of Texas MD Anderson Cancer Center (Houston, TX). Normal urothelial cells were prepared from ureters of nephrectomy specimens and used as a control. First-voided urine was collected from 128 bladder cancer patients with cystoscopically evident bladder cancer before they underwent surgery at The University of Texas MD Anderson Cancer Center. Among 128 cases, primary cancers were 88 cases and recurrent cancers were 40 cases. There were no age, gender, ethnic, or cancer stage restrictions on recruitment. Age-matched controls (n = 110) were recruited in medicine and urology clinics at The University of Texas Southwestern Medical Center (Dallas, TX). Controls consisted of 71 patients with benign urologic disorder without cystoscopically visible bladder cancer and 39 unaffected healthy individuals. The majority of controls visited the hospital for annual medical checkup. A total of 50 mL of the urine were centrifuged for 15 minutes at 200 g, and the resulting pellet material was washed twice with PBS and stored at −70°C until study. DNA from urine sediments was extracted by QIAamp DNA Mini Kit (Qiagen). All samples were collected from consenting patients according to institutional guidelines at The University of Texas MD Anderson Cancer Center and The University of Texas Southwestern Medical Center.

CpG methylation analysis by bisulfite pyrosequencing and qMSP
Bisulfite conversion of extracted DNA was done by EpiTect Bisulfite Kits (Qiagen). We previously used methylated CpG island amplification and microarray (MCAM), a high-throughput genome-wide methylation assay (31, 32), to investigate methylation changes in the promoters of about 6,600 genes in 85 primary bladder tumors and 12 bladder cancer cell lines. Based on the MCAM results, we selected 10 genes (A2BP1, NPTX2, SOX11, PENK, NXX6-2, DBC1, MYO3A, HSPB9, NPY2R, and CA10) from the most frequently hypermethylated genes and studied them by bisulfite pyrosequencing to analyze the quantitative methylation status (Supplementary Fig. S1 and Supplementary Table S1) in 6 bladder cancer cell lines and 26 primary bladder tumors. We also analyzed DNA methylation of normal leukocytes (NL) and a mixture of normal bladder (NB) DNA from 3 persons (2 men and 1 woman) as a control. Bisulfite-treated DNA was amplified by hot start and a 2-step PCR to reduce the contamination in products due to the amplification of unexpected primer binding sites. A universal primer sequence tag was added to the reverse primer in the nested PCR step, as previously described (33). For pyrosequencing, biotin-labeled DNA strands were prepared and analyzed by PSQ HS 96 Pyrosequencing System (Biotage AB). We used M. SssI methylase (New England Biolabs) treated NLs DNA as a positive control for methylation studies. Bisulfite pyrosequencing in primary bladder tumors was conducted twice and averaged. To call a cancer as methylation positive, we required that its methylation status was at least 10% higher than that seen in a mixture of NB DNA.

For quantitative methylation-specific real-time PCR (qMSP), we selected 8 genes that had hypermethylation in bladder cancer and low levels of methylation in NLs. All primers were designed to have the same annealing temperature. Quantity of human DNA in urine was determined after bisulfite conversion by C-LESS DNA (chr20:19,199,387-19,199,455, UCSC Blat 2006 Mar. version), a unique sequence that does not contain cytosines (34). Supplementary Table S2 lists primers and TaqMan probes (Applied Biosystems) for mC-LESS and the 8 genes examined. For quality control, we adjusted diluted bisulfite-treated DNA to 100 µL throughout. We used the same volume (3 µL) of bisulfate-treated DNA as a template for every qMSP (total reaction volume, 20 µL). Each qMSP reaction batch was checked with positive (M. SssI methylase–treated DNA) and negative (NL DNA) controls, and multiple blanks with no DNA. Each plate was amplified with mC-LESS (internal control) and the tested genes together to avoid interassay variation. We also used as validation criteria an intra-assay variation of ΔCt < 1 at
a duplicated sample and $r^2 \geq 0.99$ for at least 4 relevant $C_t$ points. NL DNA was methylated in vitro with twice excess M. Sss1 methylase and 5-fold serial dilutions (100–0.032 ng) of this DNA were used to construct a calibration curve. When we compared the $C_t$ of mC-LESS with those of other test genes, the correlation was $r^2 > 0.99$. All qMSP reactions were done in duplicate in a blinded manner and averaged. All samples were within the assay’s range of sensitivity and reproducibility based on amplification of internal control [threshold cycle ($C_t$) value for mC-LESS of less than 40].

**Statistical analysis**

The relative level of methylated DNA for each gene in each sample was determined as a Δ$C_t$ of qMSP-amplified gene to mC-LESS (internal control). The samples were categorized as unmethylated or methylated on the basis of the criterion values and coordinates of the receiver operating characteristics (ROC) curve of the assay.

The predictive accuracy of biomarkers was evaluated by calculating the area under the curve (AUC) of ROC. The ROC curve of both specificity and sensitivity of single or combined biomarkers sets consisting of up to 8 methylation biomarkers was constructed.

Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, Inc.) and SPSS statistical software V11.0 (SPSS, Inc.). All $P$ values were 2-sided and $P < 0.05$ was considered statistically significant. When multiple tests were conducted, Bonferroni corrections were applied to the $P$ values.

**Results**

Bisulfite pyrosequencing results of 10 selected genes in 6 bladder cancer cell lines and 26 primary bladder tumors are shown in Figure 1. Eight genes (ABBP1, NPTX2, SOX11, PENK, NXkx6-2, DBC1, MYO3A, and CA10) were highly methylated in bladder tumors and had very low levels of methylation in NLs. Their methylation frequencies in 26 primary bladder cancer were 62%, 88%, 77%, 92%, 69%, 69%, 65%, and 85%, respectively. HSPB9 was highly methylated in both bladder tumors and NL DNA. NPYR2 showed lower methylation frequency (54%) than the other genes in bladder tumors.

To apply this gene panel to early detection, we first analyzed DNA methylation by bisulfite pyrosequencing in urine sediments for 2 genes, SOX11 and HSPB9 as examples. SOX11 showed increased methylation in urine from bladder cancer patients compared with control but the differences were small, in part, due to the relatively high background of pyrosequencing (5%). HSPB9 was highly methylated in the urine sediment of controls, and similarly methylated (though more variable) in the urine sediment of patients (Supplementary Fig. S2). These results are consistent with the fact that urine sediment DNA contains a high proportion of leukocyte-derived DNA (even in patients with cancer) and detection of cancer would require more sensitive and clear cutoff point methods to detect a low frequency of tumor-derived DNA. We therefore applied the qMSP method to overcome these problems and analyzed the 8 genes (DBC1, MYO3A, SOX11, NPTX2, NXkx6-2, A2BP1, PENK, and CA10) which had low levels of methylation in NLs.

Overall, we studied urine sediments from 128 bladder cancer patients (median age 69) and 110 control subjects (median age 67; Table 1 and Supplementary Table S3). The bladder cancer patients consisted of 58 cases of non-muscle invasive tumors (30 cases of pTa, 5 cases of Tis, and 23 cases of T1) and 70 cases of muscle invasive tumors (62 cases of T2, 6 cases of T3, and 2 cases of T4). Most (87%) of them were of TCC type. Control subjects consisted of 71 cases of benign urologic symptoms and 39 normal controls including 5 healthy volunteers (Supplementary Table S3). The distribution of qMSP results of each gene in urine sediments is shown in Figure 2. It is obvious that all 8 genes show substantially and significantly more methylation in tumor cases than controls.

We evaluated the power of each methylation marker by calculating the area under curve (AUC) of ROC using total data set of 128 tumors and 110 controls. A random marker unrelated to bladder cancer is expected to have an AUC value of 0.5. The AUC values for the 8 methylation markers we selected in the order from high to low are MYO3A (AUC = 0.841, $P < 0.0001$), CA10 (AUC = 0.835, $P < 0.0001$), NXkx6-2 (AUC = 0.823, $P < 0.0001$), PENK (AUC = 0.802, $P < 0.0001$), SOX11 (AUC = 0.797, $P < 0.0001$), DBC1 (AUC = 0.774, $P < 0.0001$), NPTX2 (AUC = 0.747, $P < 0.0001$), and A2BP1 (AUC = 0.710, $P < 0.0001$). We also performed a correlation analysis for all pairs of markers (Table 2). All pairs of methylation level of genes were correlated with statistical significance ($P < 0.0001$).

To develop a multigene predictive model, we used a combinatorial analysis of methylation of 8 biomarkers. In this analysis, a model including 4 genes, MYO3A + CA10 + NXkx6-2 + DBC1 or MYO3A + CA10 + NXkx6-2 + SOX11, yielded an AUC of 0.939 (95% CI = 0.901–0.966, $P < 0.0001$) for the set [tumor patients urine (TU) = 128 and controls urine (NU) = 110]. The models including 5 genes, MYO3A + CA10 + NXkx6-2 + DBC1 + SOX11 or MYO3A + CA10 + NXkx6-2 + DBC1 + PENK, yielded the same AUC of 0.939 (95% CI = 0.901–0.966, $P < 0.0001$).

The performances of single and combined qMSP markers for detection of bladder cancer in urine sediments are shown in Table 3. Comparison of ROC curve of the panel of combined markers is shown in Figure 3A. In the panel of 3 genes (MYO3A + CA10 + NXkx6-2), if a urine sample has 2 or 3 genes methylation, the sensitivity was 86% (95% CI = 78.7–91.4, $P < 0.0001$) and specificity 93% (95% CI = 86.2–96.8, $P < 0.0001$) for detection of bladder tumors and an AUC of 0.939 (95% CI = 0.894–0.962, $P < 0.0001$). In the models of 4-gene panel (MYO3A + CA10 + NXkx6-2 + DBC1 or MYO3A + CA10 + NXkx6-2 + SOX11), if a urine sample has 3 or 4 genes methylation, the sensitivity was 81% (95% CI = 73.4–87.6, $P < 0.0001$) and specificity 97% (95% CI = 92.2–99.4, $P < 0.0001$).
Figure 1. Scatter plot of bisulfite pyrosequencing results of candidate genes in NBs, NLs, 6 BTC lines, and 26 primary bladder tumors (BCa). We used a mixture of normal bladder DNA from 3 persons (2 men and 1 woman) as a control (NB). We also analyzed leukocytes because urine sediment DNA contains a high proportion of leukocyte-derived DNA.
Table 1. Demographic and clinical characteristics of the bladder cancer patients (n = 128)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of subjects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological cell type</td>
<td></td>
</tr>
<tr>
<td>TCC</td>
<td>110 (86.0)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>8 (6.3)</td>
</tr>
<tr>
<td>Mixed</td>
<td>5 (3.9)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Small-cell carcinoma</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Pleomorphic sarcomatoid</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>114 (89.1)</td>
</tr>
<tr>
<td>African American</td>
<td>10 (7.8)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Latin American</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;41</td>
<td>9 (7.0)</td>
</tr>
<tr>
<td>41–50</td>
<td>4 (3.1)</td>
</tr>
<tr>
<td>51–60</td>
<td>20 (15.6)</td>
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<tr>
<td>61–70</td>
<td>40 (31.3)</td>
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<tr>
<td>71–80</td>
<td>42 (32.8)</td>
</tr>
<tr>
<td>&gt;80</td>
<td>13 (10.2)</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>101 (78.9)</td>
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<tr>
<td>Female</td>
<td>27 (21.1)</td>
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<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7 (5.5)</td>
</tr>
<tr>
<td>2</td>
<td>13 (10.2)</td>
</tr>
<tr>
<td>3</td>
<td>108 (84.4)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>30 (23.4)</td>
</tr>
<tr>
<td>Tis</td>
<td>5 (3.9)</td>
</tr>
<tr>
<td>T1</td>
<td>23 (18.0)</td>
</tr>
<tr>
<td>T2</td>
<td>62 (48.4)</td>
</tr>
<tr>
<td>T3</td>
<td>6 (4.7)</td>
</tr>
<tr>
<td>T4</td>
<td>2 (1.6)</td>
</tr>
</tbody>
</table>

![Graph of qMSP results of each gene in urine sediments of bladder cancer patients (n = 128) and controls (n = 110). The relative level of methylated DNA is depicted as 40-dCt [Ct of specific gene – Ct of mC-LESS (internal control)]. A higher 40-dCt represents more methylation of the target biomarker. All 8 genes show significantly more methylation in tumor cases than controls (P < 0.0001).](image)

Analyzing by the stage of bladder cancer using the 5-gene panel, detection rate based on 3-gene methylation or the models of 5-gene panel (MYO3A + CA10 + NKKX6-2 + DBC1 + SOX11 or MYO3A + CA10 + NKKX6-2 + DBC1 + PENK), if a sample has 3 or more than 3 gene methylation, the sensitivity was 85% (95% CI = 77.8–90.8, P < 0.0001) and specificity 95% (95% CI = 88.5–98.0, P < 0.0001). Panels of 4 or 5 selected methylation markers had the same AUC of 0.939 and showed the best accuracy of detection of bladder cancer in urine sediments (Table 3).

Bladder cancer (T2–T3–T4 stages; Fig. 3C). When classified by grade, detection rate by the 5-gene panel was 95 of 111 (86%) in grade 3, 8 of 13 (61%) in grade 2, and 1 of 3 (33%) in grade 1. The 4-gene panel had a sensitivity or specificity of 76% or 97% (AUC = 0.913, P < 0.0001) in Tis–pTa–T1 stages and 86% or 97% (AUC = 0.961, P < 0.0001) in T2–T3–T4 stages. By analyzing the 2 control groups using the 5-gene panel, the benign urological group (n = 71) had 3 false positive cases and the nonurologic control group (n = 39) had 3 false positive cases. There were no significant differences between the benign urological group and the normal control group. The detection rate of primary and recurrent cancers using the 5-gene panel was identical [75 of 88 (85%) and 34 of 40 (85%), respectively].

### Discussion

Early detection of cancer can result in improved clinical outcomes. Early and noninvasive detection methods for bladder cancer screening and diagnosis of recurrence will be useful in high-risk populations. As the bladder is the exclusive reserve organ for urine, urine sediments can provide a source of detection of exfoliated bladder tumor cells (BTC; ref. 35). These noninvasive tests include cytology, FISH analysis, and detection of mutations in urine. Overall, the tests have reported sensitivities of 54% to 86% and specificities of 61% to 90% (10–12); thus, there is a room for improvement, and development of novel biomarkers and combinations of different biomarkers may be most helpful for this purpose (13–18).

DNA hypermethylation occurs at a high frequency in both non-muscle invasive and invasive bladder cancers. Previous studies suggested that methylation in urine could be a useful diagnostic test (21–25) but were limited...
by selection of markers on the basis of methylation in other cancers, complicated algorithms, nonquantitative methods, and/or inappropriate controls (e.g., non-age matched). Here, starting from an unbiased DNA methylation analysis screen, we identify a panel of the least number of biomarkers that has high sensitivity and specificity with a simple algorithm for detection of bladder cancer. With a sensitivity of 85% and a specificity of 95%, the positive predictive value (PPV) of this test would be 52% based on 5.9% of prevalence in a high-risk population that had history of smoking or symptoms of hematuria and dysuria (36). This is 1.2 to 8.7 times

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>NPTX2</th>
<th>A2BP1</th>
<th>SOX11</th>
<th>MYO3A</th>
<th>NNX6-2</th>
<th>PENK</th>
<th>CA10</th>
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<tbody>
<tr>
<td>DBC1</td>
<td>0.55</td>
<td>0.57</td>
<td>0.71</td>
<td>0.56</td>
<td>0.49</td>
<td>0.62</td>
<td>0.63</td>
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<td>NPTX2</td>
<td>0.70</td>
<td>0.59</td>
<td>0.55</td>
<td>0.63</td>
<td>0.74</td>
<td>0.63</td>
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<td>A2BP1</td>
<td>0.59</td>
<td>0.56</td>
<td>0.52</td>
<td>0.71</td>
<td>0.64</td>
<td></td>
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<tr>
<td>SOX11</td>
<td>0.70</td>
<td>0.57</td>
<td>0.72</td>
<td>0.66</td>
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<tr>
<td>MYO3A</td>
<td></td>
<td>0.61</td>
<td>0.66</td>
<td>0.68</td>
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<tr>
<td>NNX6-2</td>
<td></td>
<td></td>
<td>0.64</td>
<td>0.60</td>
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<td>PENK</td>
<td></td>
<td></td>
<td></td>
<td>0.71</td>
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</table>

NOTE: Each gene methylation level was 40-dCt (qMSP) of the gene. All correlations were statistically significant ($P < 0.0001$).

Table 3. Diagnostic information of single or combined qMSP markers for detection of bladder cancer in urine sediments

<table>
<thead>
<tr>
<th>Biomarkers (no. of combined biomarkers)</th>
<th>Cutoffa</th>
<th>TP/FN</th>
<th>% sensitivity</th>
<th>TN/FP</th>
<th>% specificity</th>
<th>AUC</th>
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<tr>
<td>MYO3A, NNX6-2 (2)</td>
<td>&gt;0</td>
<td>99/29</td>
<td>77.3</td>
<td>100/10</td>
<td>90.9</td>
<td>0.841</td>
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<tr>
<td>CA10, NNX6-2 (2)</td>
<td>&gt;0</td>
<td>109/19</td>
<td>85.2</td>
<td>90/20</td>
<td>81.8</td>
<td>0.835</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, PEnK (4)</td>
<td>&gt;2</td>
<td>104/24</td>
<td>81.3</td>
<td>87/23</td>
<td>79.1</td>
<td>0.802</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, SOX11 (4)</td>
<td>&gt;2</td>
<td>104/24</td>
<td>81.3</td>
<td>102/8</td>
<td>92.7</td>
<td>0.933</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, DBC1 (4)</td>
<td>&gt;2</td>
<td>104/24</td>
<td>81.3</td>
<td>107/3</td>
<td>97.3</td>
<td>0.936</td>
</tr>
<tr>
<td>MYO3A, CA10, NNX6-2, NPTX2 (4)</td>
<td>&gt;2</td>
<td>100/28</td>
<td>78.1</td>
<td>105/5</td>
<td>95.5</td>
<td>0.934</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, PENK, SOX11 (5)</td>
<td>&gt;2</td>
<td>107/21</td>
<td>83.6</td>
<td>103/7</td>
<td>93.6</td>
<td>0.935</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, DBC1, SOX11 (5)</td>
<td>&gt;2</td>
<td>109/19</td>
<td>85.2</td>
<td>104/6</td>
<td>94.5</td>
<td>0.939b</td>
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<td>MYO3A, CA10, NNX6-2, DBC1, PENK (5)</td>
<td>&gt;2</td>
<td>109/19</td>
<td>85.2</td>
<td>106/6</td>
<td>94.5</td>
<td>0.939b</td>
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<tr>
<td>MYO3A, CA10, NNX6-2, PENK, SOX11, DBC1 (6)</td>
<td>&gt;3</td>
<td>105/23</td>
<td>82.0</td>
<td>108/2</td>
<td>98.2</td>
<td>0.937</td>
</tr>
<tr>
<td>MYO3A, CA10, NNX6-2, PENK, SOX11, DBC1, A2BP (7)</td>
<td>&gt;4</td>
<td>105/23</td>
<td>82.0</td>
<td>104/6</td>
<td>94.5</td>
<td>0.936</td>
</tr>
<tr>
<td>MYO3A, CA10, NNX6-2, PENK, SOX11, DBC1, NPTX2 (8)</td>
<td>&gt;4</td>
<td>105/23</td>
<td>82.0</td>
<td>108/2</td>
<td>98.2</td>
<td>0.930</td>
</tr>
</tbody>
</table>

aWhen the number of methylated biomarkers of the case passed cutoff value, we considered it as a positive case.

bThe best combinations of biomarker with the highest area under the ROC curve (AUC) value were shown by bold character ($P < 0.0001$). The other biomarker combinations did not improve the AUC.

Abbreviations: TP, true positive; FN, false negative; FP, false positive; TN, true negative.
superior to what can be achieved by PSA (prostate-specific antigen) testing (PPV = 30%–43%), mammography (PPV = 9%–19%), or fecal occult blood screening (PPV = 6%–11%; refs. 37–44).

The panel of genes found by MCAM and validation by bisulfite pyrosequencing was methylated in 62%–92% of 26 tested primary bladder tumors and most of bladder cancer cell lines analyzed. These are higher methylation frequencies than genes previously tested for bladder cancer detection (19), which explains the improved performance of our assay with a smaller number of the biomarkers. Our 5-gene panel showed higher sensitivity with preserved specificity than a previous report (19) of a panel of 9 genes. One limitation of this analysis is the focus on promoter CpG islands. It is possible that other sequences such as exonic CpG islands may provide even more sensitive markers for cancer detection.

The final selected biomarker genes were PENK, MYO3A, SOX11, CA10, DBC1, and NKX6-2. The NKX6-2 gene is located at 10q26.3. Loss of heterozygosity (LOH) of chromosome 10q was previously linked to muscle-invasive TCC (45). LOH in the 10q26 region has also been observed in various malignant brain tumors (46). These results may point to NKX6-2 as a candidate tumor suppressor gene for brain and bladder tumors. In the case of SOX11, it was previously reported that silencing of the gene correlates with promoter methylation and regulates tumor growth in hematopoietic malignancies (47, 48). PENK is on chromosome 8q12.1 and was reported to be downregulated in prostate cancer (49). It was previously found that PENK is frequently methylated in pancreatic cancer (50). The deleted in bladder cancer 1 (DBC1) gene is located at 9q33 and a candidate tumor suppressor gene within a
region of frequent LOH in bladder cancer (51). Hypermethylation of DBC1 has been suggested as one of the earliest events in the development of TCC (52). MYO3A is a myosin family gene that is expressed primarily in the retina and cochlea, and is functionally involved in hearing. CA10 is carbonic anhydrase X, a gene primarily expressed in the brain.

Although our study is promising for bladder cancer detection, there are limitations that need to be considered. The data need to be validated in larger studies, including a high-risk population that has a history of heavy smoking or symptoms of hematuria, dysuria, urgent urination, and frequent urinary tract infections. There is also a need to validate these markers in early-stage low-grade tumors because the present study did not test enough G1-grade tumors. The representativeness of our case patients and controls should be tested in further studies. Generally, most cancers have DNA hypermethylation although different genetic background and environmental exposures could affect the results. For example, Schistosoma-associated bladder cancers showed higher DNA methylation abnormalities than non-Schistosoma-associated bladder cancer (53). Therefore, other populations may need a different cutoff of positive/negative values for each methylation biomarker. The use of our markers to follow patients longitudinally post resection also needs to be tested. In non-muscle invasive tumors (pTa, Tis, and pT1), our markers provided higher sensitivity or specificity (81% or 95%) than most reported urine markers (10–12). Therefore, our novel DNA methylation biomarker panel could help in the early detection of bladder cancer, although this needs to be clarified further in future studies. It would therefore be useful to directly compare accuracy of this assay with commercially available kits to detect bladder cancer in urine sediments.

One of the issues to consider in future studies is marker selection. A frequency-based approach may yield false positives due to age-related methylation (54) or field effects (52, 55, 56). It might be productive to study whether progression markers in bladder cancer might prove better (i.e., more sensitive or specific) at detecting the invasive malignancies that pose a greater threat of ultimate metastasis and death. Conceivably, these progression markers might even be useful to predict prognosis and therapeutic response to chemotherapy and/or surgery.

In summary, we have identified a novel DNA methylation biomarker panel that will help the noninvasive detection with high accuracy. In addition, to screening, this noninvasive early detection method may reduce the cystoscopy frequency. The panel of biomarker deserves validation in a large well-controlled prospectively collected sample set.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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