

## A Novel Set of DNA Methylation Markers in Urine Sediments for Sensitive/Specific Detection of Bladder Cancer

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**Abstract Purpose:** This study aims to provide a better set of DNA methylation markers in urine sediments for sensitive and specific detection of bladder cancer.

**Experimental Design:** Fifty-nine tumor-associated genes were profiled in three bladder cancer cell lines, a small cohort of cancer biopsies and urine sediments by methylation-specific PCR. Twenty-one candidate genes were then profiled in urine sediments from 132 bladder cancer patients (8 cases for stage 0a; 68 cases for stage I; 50 cases for stage II; 4 cases for stages III; and 2 cases for stage IV), 23 age-matched patients with noncancerous urinary lesions, 6 neurologic diseases, and 7 healthy volunteers.

**Results:** Despite six incidences of four genes reported in 3 of 23 noncancerous urinary lesion patients analyzed, cancer-specific hypermethylation in urine sediments were reported for 15 genes ( $P < 0.05$ ). Methylation assessment of an 11-gene set (*SALL3*, *CFTR*, *ABCC6*, *HPR1*, *RASSF1A*, *MT1A*, *RUNX3*, *ITGA4*, *BCL2*, *ALX4*, *MYOD1*, *DRM*, *CDH13*, *BMP3B*, *CCNA1*, *RPRM*, *MINT1*, and *BRCA1*) confirmed the existing diagnosis of 121 among 132 bladder cancer cases (sensitivity, 91.7%) with 87% accuracy. Significantly, more than 75% of stage 0a and 88% of stage I disease were detected, indicating its value in the early diagnosis of bladder cancer. Interestingly, the cluster of reported methylation markers used in the U.S. bladder cancers is distinctly different from that identified in this study, suggesting a possible epigenetic disparity between the American and Chinese cases.

**Conclusions:** Methylation profiling of an 11-gene set in urine sediments provides a sensitive and specific detection of bladder cancer.

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Received 4/12/07; revised 7/17/07; accepted 7/30/07.

**Grant support:** Shanghai Science Foundation grants 04DZ14006 and 05DZ19318 (J. Zhu), National Science Foundation grants 30570850 and 10574134 (J. Zhu), National Research Program for Basic Research of China grants 2004CB518804 (J. Zhu) and 2002CB713700 (X. Yao), National Research Program for High Technology grants 2006AA02Z320 and 2006AA02Z197 (J. Zhu), European 6th program grant LSHB-CT-2005-019067 (J. Zhu), and a Georgia Cancer Coalition Research Project Grant (X. Yao). X. Yao is a Georgia Cancer Coalition Eminent Scholar. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi:10.1158/1078-0432.CCR-07-0861

Bladder cancer is a common cancer in the United States (1) and is increasingly being diagnosed in China, where industrialization has taken place rapidly (2). More than 70% patients suffering from superficial disease can be successfully treated by surgery (3), whereas 30% patients face devastating outcome upon diagnosis (4). The tumors at the same grade and stage have variable behaviors (5), creating the need for better detection and molecular staging of bladder cancer.

The gold standard for its detection is cystoscopy/biopsy of suspicious lesions. However, 10% to 40% of malignancies were undetected by this procedure (6–8). Urine cytology is known for its good specificity and poor sensitivity for T<sub>a</sub>, G<sub>1</sub>, and T<sub>1</sub> stage disease (9). Genetic alterations (10, 11) have also been explored in urine sediment DNA for bladder cancer detection.<sup>9</sup> The use of a number of the overexpressed proteins in cancer tissues and/or urine identified by proteomic profiling, including the nuclear matrix protein 22 in urine (12–14), has also been tested in bladder cancer screening (15, 16).

Epigenetic perturbation of gene regulation is pathologically important to tumorigenesis (17, 18). Both local hypermethylation of tumor-suppressor genes (19) and global hypomethylation of genomic DNA (20–22) frequently occur in human cancers (17, 23). Therefore, it has been intensively attempted to

<sup>9</sup> <http://www.urovysion.com>

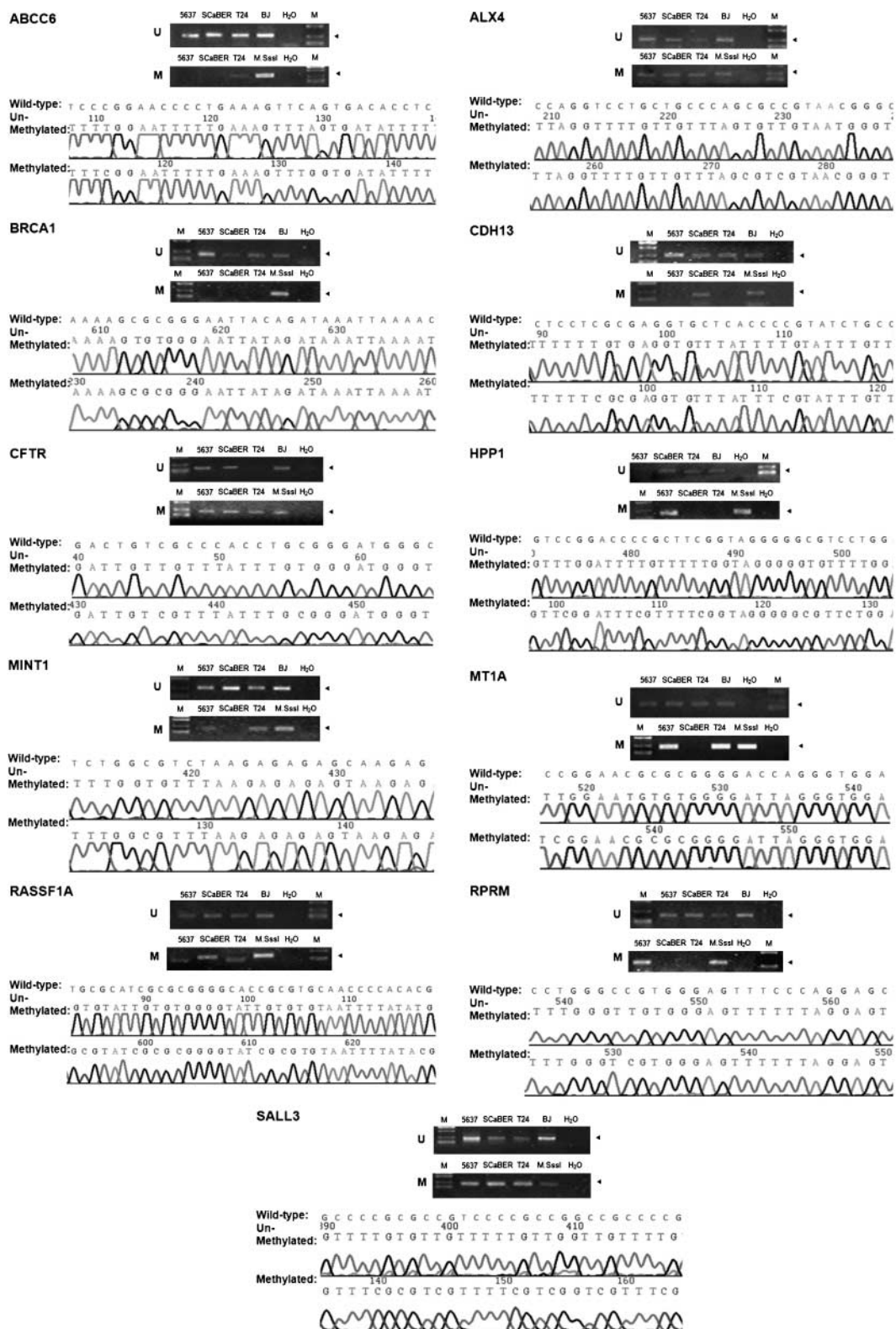


Fig. 1. The MSP profile and sequencing verification. The MSP profile and sequencing verification of both methylated and unmethylated targeted regions of the 11 informative genes in each of three bladder cancer cell lines. Both the electrophoretic patterns of the representative MSP data and the sequencing verification are shown. Cell lines, 5637, SCaBER, and T24. M. Sss I, the positive control with the DNA of the normal liver tissue *in vitro* methylation modified. BJ, the bisulfate DNA from BJ, a normal fibroblast cell line as the positive control for the unmethylated template. The target identity of each panel is indicated. The wild-type sequence is aligned with the sequence made from the T-vector cloned with the representative PCR product.

exploit the tumor-associated pattern of DNA methylation for better cancer detection and staging. Using methylation-specific PCR (MSP) of bisulfite-treated DNA to distinguish cytosine from the methylated cytosine residues (24), as few as 1 to 10 tumor cells among  $10^4$  normal cells in clinical samples can be detected (25), forming a logical base for the detection of cancer by DNA methylation in bodily fluids, including bronchoalveolar lavage fluid (26), stool (27, 28), serum/plasma (29), and the urine sediments (30–35). For example, hypermethylated *DAPK*, *BCL2*, or *HTERT* genes were detected in urine sediment DNA from 78% of bladder cancer patients

(34). Recently, at least one among *CDK2A*, *ARF*, *MGMT*, and *GSTP1* were found to be hypermethylated in urine sediments of 69% of bladder cancer patients tested (32). Both reports highlight the great potential of DNA methylation markers in urine sediments for bladder cancer detection. Epigenetic components such as DNA methylation respond to changing environments more readily than their genetic counterparts. It is, therefore, desirable to establish a better set of DNA methylation markers to detect bladder cancer in China, which may be significantly different from the sets informative for the U.S. patients (32, 34).

**Table 1.** The MSP profiles of the genes in the initial screening

Gene no.	Gene symbol	Cell lines (3)			Urine sediments		Bladder tissue	
		Gene (n = 59)			Gene (n = 45)		Gene (n = 32)	
		5637	SCaBER	T24	Cancer (11)	Glandularis cystitis (3)	Cancer (15)	Normal (3)
1	<i>CFTR</i>	2	2	3	90.9	0	53.3	0
2	<i>SALL3</i>	2	2	2	81.8	0	60	0
3	<i>ALX4</i>	2	2	2	45.5	0	40	0
4	<i>DRM</i>	2	1	2	45.5	0	26.7	0
5	<i>BCL2</i>	1	1	2	36.4	0	20	0
6	<i>HPP1</i>	2	1	1	36.4	0	80	0
7	<i>ITGA4</i>	2	1	2	36.4	0	73.3	0
8	<i>MINT1</i>	2	1	2	36.4	0	20	0
9	<i>RASSF1A</i>	1	3	1	36.4	0	26.7	0
10	<i>MT1A</i>	2	1	2	27.3	0	53.3	0
11	<i>RUNX3</i>	2	1	2	27.3	0	53.3	0
12	<i>CCNA1</i>	2	1	1	18.2	0	13.3	0
13	<i>MYOD1</i>	2	1	2	18.2	0	40	0
14	<i>ABCC6</i>	1	1	2	18.2	0	60	0
15	<i>BMP3B</i>	1	1	2	18.2	0	53.3	0
16	<i>RPRM</i>	2	1	1	18.2	0	46.7	0
17	<i>GSTP1</i>	2	2	2	9.1	0	6.7	0
18	<i>PTCHD2</i>	1	1	2	9.1	0	0	0
19	<i>BRCA1</i>	1	1	1	18.2	0	26.7	0
20	<i>CDH13</i>	1	2	1	27.2	0	33.3	0
21	<i>TMS1</i>	3	3	1	0	0	0	0
22	<i>CDH1</i>	2	1	2	0	0	0	0
23	<i>p14<sup>ARF</sup></i>	2	1	2	0	0	0	0
24	<i>p16<sup>INK4a</sup></i>	2	1	2	0	0	0	0
25	<i>FADD</i>	1	1	1	0	0	0	0
26	<i>LITAF</i>	1	1	1	0	0	0	0
27	<i>MGMT</i>	1	1	1	0	0	0	0
28	<i>TNFRSF21</i>	1	1	1	0	0	0	0
29	<i>ABCC13</i>	2	1	2	0	0	ND	0
30	<i>TNFRSF10C</i>	2	1	2	0	0	ND	0
31	<i>CDKN1C</i>	2	1	1	0	0	ND	0
32	<i>DAPK1</i>	2	1	2	0	0	ND	0
33	<i>HTERT</i>	2	1	1	0	0	ND	0
34	<i>OCN</i>	2	1	1	0	0	ND	0
35	<i>STAT1</i>	1	2	1	0	0	ND	0
36	<i>LAMA3</i>	3	1	1	9.1	0	ND	0
37	<i>ABCC8</i>	1	1	2	0	0	ND	0
38	<i>BRCA2</i>	2	1	2	0	0	ND	0
39	<i>ICAM1</i>	2	1	1	9.1	0	ND	0
40	<i>GALC</i>	2	1	2	9.1	0	ND	0
41	<i>MAGEA1</i>	2	2	2	90.9	100	ND	0
42	<i>ENDRB</i>	3	3	3	100	100	100	100
43	<i>MINT2</i>	2	1	2	100	100	100	100
44	<i>SERPINB5</i>	2	1	1	100	100	100	100
45	<i>TNFRSF10D</i>	2	2	2	100	100	100	100

NOTE: 1, in the white background: the unmethylated in both alleles; 2, in gray background: one allele methylated and the other unmethylated; and 3, in the dark background: methylated in both alleles. The case number studied is indicated in bracket. Abbreviation: ND, not determined.

## Materials and Methods

**Sample collection and DNA isolation.** With the informed consent and approval of the ethics committee, 15 primary bladder cancer tissues (seven stage I and eight stage II) were collected from Guangxi Cancer Hospital, China. Three normal bladder tissues were obtained from healthy organ donors. DNA of morning urine samples were collected from the bladder cancer patients at Guangxi Cancer Hospital (40 cases) and Zhongshan Hospital, Shanghai China (92 cases). Seventy-nine paired postsurgery urine samples were also collected from Shanghai Zhongshan Hospital. The controls were 23 cases of noncancerous urinary disease patients (eight cystitis glandularis, four prostatic hyperplasia, three calculus of bladder, five calculus of kidney, and three mass in adrenal gland), six cases of neurologic patients, and seven healthy volunteers. The urine cytologic analysis (9) and tumor-node-metastasis staging/classification were done according to the WHO classification (3) and American Joint Committee on Cancer guidelines (36). DNA preparation from frozen tissues or urine sediments were made using the conventional proteinase K/organic extraction method as previously described (37, 38).

**Bisulfite treatment and MSP analysis.** The primer pairs for PCR detection of the methylated and unmethylated allele of 59 genes were either directly taken from the literature or designed using the relevant software<sup>10,11</sup> (Supplementary Table S1).

The bisulfate conversion and PCR analyses were done as described previously (37, 38). The PCR products were cloned and verified by sequencing (Fig. 1 and data not shown). The *in vitro* methylated DNA from normal liver tissue by M. Sss I was used as a positive control (37, 38).

**Statistics.** The association between gene methylation and clinical-pathologic variables was statically tested.<sup>12</sup> The hypermethylation frequency with a 95% confidence interval (95% CI;  $\alpha = 0.05$ ) was calculated (Supplementary Table S2) using R package Hmisc.<sup>13</sup> The hypermethylated incidence in bladder cancer (132 cases) versus the noncancerous urinary lesion patients (23 cases) was computed by a  $2 \times 2$  Fisher's exact test and tested by the R statistical package.<sup>14</sup> The receiver operating characteristics of both specificity and sensitivity of the sets consisting of 2 to 11 genes was constructed (Table 4; Fig. 3).

## Results

**Identification of an informative set of DNA methylation markers for a sensitive/specific detection of bladder cancer.** The promoter of 59 tumor-associated genes (Supplementary Table S1) for methylation profiling consisted of those hypermethylated in bladder cancer, *CDKN2A*, *ARF*, *MGMT*, *GSTP1*, *BCL2*, *DAPK*, and *TERT* (32, 34), which we have previously methylation profiled in five types of common human cancer (37–43) and which have been bioinformatically classified as being tumor associated (39, 42). Figure 1 shows the MSP results of both methylated and unmethylated regions of 11 genes derived from three bladder cancer cell lines and their sequencing verification. These markers are novel except for the *GSPT1* that was reported previously (42).

Established cancer cell lines are expected to share genetic as well as epigenetic features of cancer cells *in vivo*. Hence, MSP

analysis of 59 genes was done in T24, SCaBER, and 5637 cells for the candidates. Forty-one genes found to be hypermethylated in at least one allele of one cell line (Table 1) were further profiled in urine sediment DNA from 11 bladder cancer and three glandularis cystitis patients. Although being unmethylated in both alleles, *FADD*, *LITAF*, *MGMT*, and *TNFRSF21* genes were kept for the next stage of analysis for the favorite claims of relevance (32, 34). The 14 genes in the same methylation profile taken out for the further analysis are *APC*, *BCAR3*, *BNIP3*, *CBR1*, *CBR3*, *COX2*, *DRG1*, *HNFB3B*, *MDR1*, *MTSS1*, *SLC29A1*, *TIMP3*, *TNFRSF10A*, and *WVWX*. The methylated targets of 45 genes were MSP profiled in urine sediments of 11 bladder cancer and 3 healthy control individuals. Twenty-one genes were found to be hypermethylated in 11 bladder cancer samples (incidence ranging from 9% to 90.1%) but not in the normal controls. Reactivation of *MAGEA1* transcription concomitant with promoter hypomethylation frequently occurs in human cancer, such as liver cancer (37, 39, 44). However, its hypomethylation rates in cancer tissues and urine sediments of this assay were too low for further evaluation (Table 1). Although the methylation pattern of *ENDRB*, *MINT2*, *SERPINB5*, and *TNFRSF10D* genes differed among cell lines, the hypermethylated targets were detected in all the tissue and urine samples, showing no tumor specificity and were, therefore, excluded from further analysis. *LAMA3*, *ICAM1*, and *GALC* genes were also excluded from the next stage of analysis, as they were barely methylated in the bladder cancer samples.

To assess the association of the DNA methylation in cancer tissues and urine sediments, nine pairs of samples were compared (Supplementary Fig. S1). Eighty-six methylated events of 99 total detected (87%) were found in both the cancer tissues and urine sediments. Eleven (11%) were found only in cancer tissues and two (2%) methylated events were detected only in the urine sediments. In view of a low but significant discordance (13%) of the methylated targets detected in urine sediments versus the cancer tissues, we have also included *PTCHD2* that was hypermethylated only in urine sediments for the next stage of analysis. Owing to its favorable claim for prostate cancer detection (32), *TMS1* was tested further despite its unmethylated state observed thus far (Table 1).

**MSP profiling of the promoter CpG islands of 21 genes in urine sediments of bladder cancer and control groups.** We then categorized MSP-profiled DNA of bladder cancer urine sediments (132 cases) with one of three groups as a control: (a) neurologic disease (6 cases); (b) healthy volunteers (7 cases); and (c) nontumorous urinary lesion (23 cases): eight cystitis glandularis, four prostatic hyperplasia, three calculus of bladder, and five calculus of kidney. The average age of the bladder cancer cohort was 63.4 years (34–88 years), matching well to that for nontumorous urinary lesion, 55.7 years (16–83 years), and for the neurologic diseases, 64.1 years (46–78 years; Table 2).

Although no genes were hypermethylated in the healthy volunteers and the neurologic disease patient controls, four genes were found to be hypermethylated six times in total in three cases of the nontumorous urinary lesion patients: *RASSF1A* (2 of 23), *MT1A* (2 of 23), *RUNX3* (1 of 23), and *ITGA4* (1 of 23; Fig. 2; Table 3). Such "false positive" incidents were considered statistically to determine the cancer specificity of the hypermethylated state of each of this set of genes (Fig. 2; Table 3). The top four informative genes were *SALL3* (58.3%;

<sup>10</sup> <http://www.ebi.ac.uk/emboss/cpgplot/index.html>

<sup>11</sup> <http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.www.cgi>

<sup>12</sup> <http://www.r-project.org>

<sup>13</sup> <http://cran.r-project.org/src/contrib/Descriptions/Hmisc.html>

<sup>14</sup> <http://www.r-project.org/>

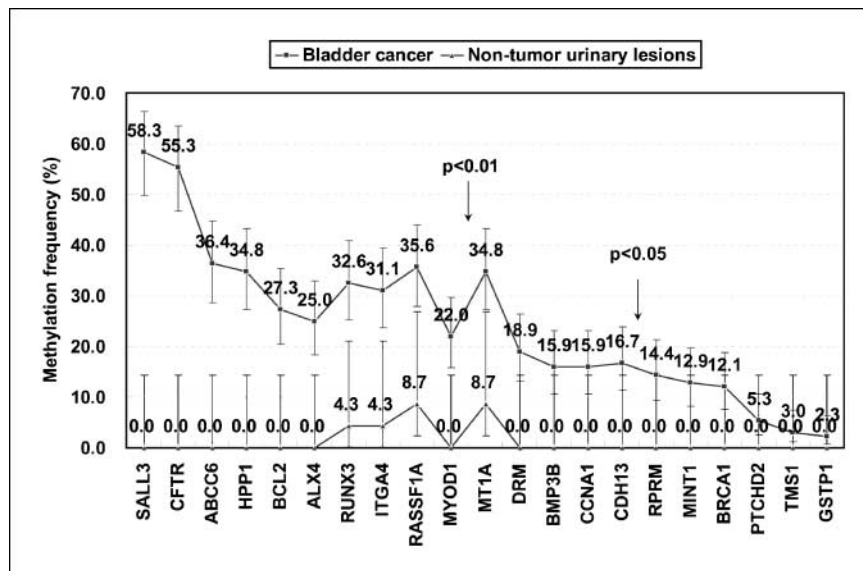
**Table 2.** Clinical profile of the bladder cancer patients and controls

	Bladder cancer (n = 132)	Nontumor urinary lesions (n = 23)	Neurologic diseases (n = 6)	Healthy control (n = 7)
Gender				
F	25	6	2	4
M	107	17	4	3
Age (y)				
19-30	0	2		6
31-40	5	2		1
41-50	22	4	1	
51-60	24	7		
61-	81	8	5	
Range	34-88	16-83	46-78	23-34
Median	63.4	55.7	64.1	25.7
Stage				
0a	8			
I	68			
II	50			
III	4			
IV	2			
Primary cases	99			
Recurrent cases	33			

95% CI, 49.8-66.4%), *CFTR* (55.3%; 95% CI, 46.8-63.5%), *ABCC6* (36.4%; 95% CI, 28.7-44.8%), and *HPP1* (34.8%; 95% CI, 27.3-43.3%). The following six genes remained in the highly valuable category ( $P < 0.01$ ): *BCL2* (27.3%; 95% CI, 20.4-35.4%), *ALX4* (25.0%; 95% CI, 18.4-33.0%), *RUNX3* (32.6%; 95% CI, 25.2-41.0%), *ITGA4* (31.1%; 95% CI, 23.8-39.4%), *RASSF1A* (35.6%; 95% CI, 28.0-44.1%), and *MYOD1* (22.0%; 95% CI, 15.8-29.8%). The genes regarded as useful markers ( $P < 0.05$ ) were *MT1A* (34.8%; 95% CI, 27.3-43.3%), *DRM* (18.9%; 95% CI, 13.2-26.5%), *BMP3B* (15.9%; 95% CI, 10.6-23.1%), *CCNA1* (15.9%; 95% CI, 10.6-23.1%), and *CDH13* (16.7%; 95% CI, 11.3-23.9%). The genes hypermethylated in >12.1% of bladder cancer cases ( $P < 0.131$  or below), *RPRM*, *MINT1*, and *BRCA1*, may also have some value. It was surprising that both *TMS1* ( $P = 1$ ) and *GSTP1* ( $P = 1$ ) were hypermethylated in no more than two cases (1.5%; 2 of

132) of bladder cancer patients, contradicting the reported informative value of *GSTP1* for bladder cancer (32) and *TMS1* for prostate cancer (45). By taking any hypermethylated gene in a list (excluding *PTCHD2*, *TMS1*, and *GSTP1*) of indicator for bladder cancer, 121 of 132 (91.7%) patients tested exhibited hypermethylation: 6 of 8 stage 0a (sensitivity 75%), 60 of 68 stage I (88.2%), 49 of 50 stage II (98.2%), 4 of 4 stage III (100%), and 2 of 2 stage IV (100%) bladder cancer (Supplementary Table S2). This assay has a superior sensitivity to urine cytology (Supplementary Table S3).

No stage association of the hypermethylated gene in this list was evident statistically (Supplementary Table S3). To stringently test the association between the tumor lesion and the hypermethylated genes in urine sediments, the methylation profiles of the urine samples of postsurgery (79 cases) was compared with that of their presurgery counterparts (132



**Fig. 2.** The occurrence of the methylated genes in bladder cancer and nontumor urinary lesion control. The methylation frequency (%) (Y axis) of each gene (X axis) in urine sediments of bladder cancer patients (column 2, Table 3) and nontumor urinary lesion cases (column 3, Table 3) was plotted. 95% CI for each gene is presented as vertical line in the plot. The positions of  $P$  values <0.01 and <0.05 in the plot are indicated.

cases). Although the methylation rate of *MYOD1* and *MINT1* genes decreased from 22.2% and 12.9%, respectively, before to 0% after surgery, the methylation rate of all the other genes tested was also greatly reduced ( $P < 0.01$ ; Supplementary Table S4). The incidence of methylation that was seen in the postsurgery urine sediments is likely attributed to the incomplete surgical removal of the cancer lesions. Therefore, the DNA methylation pattern in the postsurgery urine sediments could be a valuable indicator for how well the tumors have been surgically removed. No significant difference was found in the occurrence ( $P > 0.05$ ) of the altered DNA methylation pattern between the primary (99) and recurrent cases (33) of bladder cancer (Supplementary Table S5). A negative conclusion was drawn for the concordant methylation of any pair of genes ( $P > 0.05$ , not shown), between hypermethylated gene and clinical profiles, including sex and age; and between the patients from Shanghai (92 cases), one of the most industrialized cities in China, and from Guangxi (40 cases), a less developed region in the Southern part of China.

**Methylation profiling of an 11-gene set in urine sediments sensitively detected bladder cancer with remarkable specificity.** Assaying a single gene detected no more than 58.3% bladder cancer (Fig. 2). Methylation profiling of multiple genes that were hypermethylated in the bladder cancer at statistically significantly higher frequency than the noncancerous controls (10 genes with  $P < 0.01$  and another 5 genes with  $P < 0.05$ ; Fig. 2) should detect more diseases without compromising specificity. Four genes were also infrequently methylated in the noncancerous urinary lesion cohort: *RUNX* and *ITGA4* (one case, 4.3%), and *RASSF1A* and *MT1A* (two cases, 8.7%). "True positive" (TP) is defined as the bladder cancer case with at least one gene methylated, whereas "false negative" (FN) is the bladder cancer case with no gene methylated (column 2, Table 4). "False positive" (FP) is defined as the noncancerous urinary lesion case with at least one gene methylated, whereas

"true negative" (TN) is the noncancerous urinary lesion case with no genes methylated (column 3, Table 4); both sensitivity (%), TP / (TP + FN) (column 4, Table 4), and specificity (%), TN / (TN + FP) (column 5, Table 4) of each gene sets were calculated. The receiver operating characteristics of both specificity and sensitivity was constructed for the sets consisting of 2 to 11 genes (Fig. 3). *SALL4*, *CFTR*, *ABCC6*, and *HPP1* were the top four most frequently methylated in the patient group only (columns 2 and 3, Table 4). Therefore, methylation profiling these four genes together should detect more bladder cancer in 100% specificity than when fewer genes were tested. The sensitivity was 58.3% (77 of 132) for *SALL3* alone; 74.2% (98 of 132) for *SALL3* and *CFTR*; 80.3% (106 of 132) for *SALL3*, *CFTR*, and *ABCC6*; and 82.6% (109 of 132) for *SALL3*, *CFTR*, *ABCC6*, and *HPP1* together (Table 4, columns 4 and 5). An inclusion of *RASSF1A* that was found in 2 (8.7%) of 23 cases in the noncancerous urinary group (2 false positives and 21 true negatives; Table 4, column 3), in the five-gene set improves the sensitivity to 85.6%, but compromises specificity to 91.3% (Table 4, columns 4 and 5). Addition of *MT1A* into the six-gene set slightly improved the sensitivity to 86.4% and reduced specificity to 87%, as it was methylated in another noncancerous urinary lesion case (3 accumulated false positives and 20 true negatives; Table 4, column 3). Inclusion of the gene *RUNX*, *ITGA4*, or *BCL2* failed to improve the sensitivity, which should not be considered. The sensitivity continues to improve with the addition of *ALX4* in 7-gene (sensitivity 87.1%), of *CDH13* in 8-gene (88.6%), of *RPRM* in 9-gene (90.2%), of *MINT* in 10-gene (90.9%), and of *BRCA1* in 11-gene (91.7%) sets with 87.6% specificity remaining.

## Discussion

**MSP profiling of an 11-gene set in urine sediments detected 91.7% bladder cancer with remarkable specificity.** There is

**Table 3.** MSP profiles in urine sediments of bladder cancer and controls

Genes	Bladder cancer cases/frequency (%), n = 132	Noncancerous urinary lesions case(s)/frequency (%), n = 23	95% CI, lower-upper (P; significance as markers)	Neurologic diseases case/frequency (%), n = 6	Healthy control case/frequency (%), n = 7
<i>SALL3</i>	77/58.3	0/0.0	49.8-66.4 (0.000)	0/0.0	0/0.0
<i>CFTR</i>	73/55.3	0/0.0	46.8-63.5 (0.000)	0/0.0	0/0.0
<i>ABCC6</i>	48/36.4	0/0.0	28.7-44.8 (0.000)	0/0.0	0/0.0
<i>HPP1</i>	46/34.8	0/0.0	27.3-43.3 (0.000)	0/0.0	0/0.0
<i>BCL2</i>	36/27.3	0/0.0	20.4-35.4 (0.002)	0/0.0	0/0.0
<i>ALX4</i>	33/25.0	0/0.0	18.4-33.0 (0.004)	0/0.0	0/0.0
<i>RUNX3</i>	43/32.6	1/4.3	25.2-41.0 (0.005)	0/0.0	0/0.0
<i>ITGA4</i>	41/31.1	1/4.3	23.8-39.4 (0.005)	0/0.0	0/0.0
<i>RASSF1A</i>	47/35.6	2/8.7	28.0-44.1 (0.008)	0/0.0	0/0.0
<i>MYOD1</i>	29/22.0	0/0.0	15.8-29.8 (0.008)	0/0.0	0/0.0
<i>MT1A</i>	46/34.8	2/8.7	27.3-43.3 (0.013)	0/0.0	0/0.0
<i>DRM</i>	25/18.9	0/0.0	13.2-26.5 (0.027)	0/0.0	0/0.0
<i>BMP3B</i>	21/15.9	0/0.0	10.6-23.1 (0.045)	0/0.0	0/0.0
<i>CCNA1</i>	21/15.9	0/0.0	10.6-23.1 (0.045)	0/0.0	0/0.0
<i>CDH13</i>	22/16.7	0/0.0	11.3-23.9 (0.046)	0/0.0	0/0.0
<i>RPRM</i>	19/14.4	0/0.0	9.4-21.4 (0.078)	0/0.0	0/0.0
<i>MINT1</i>	17/12.9	0/0.0	8.2-19.7 (0.078)	0/0.0	0/0.0
<i>BRCA1</i>	16/12.1	0/0.0	7.6-18.8 (0.131)	0/0.0	0/0.0
<i>PTCHD2</i>	7/5.3	0/0.0	2.6-10.5 (0.595)	0/0.0	0/0.0
<i>TMS1</i>	4/3.0	0/0.0	1.2-7.5 (1.000)	0/0.0	0/0.0
<i>GSTP1</i>	3/2.3	0/0.0	0.8-6.5 (1.000)	0/0.0	0/0.0

strong biological/clinical rationale for exploiting the well-characterized DNA methylation pattern in urine sediments to detect bladder cancer. We have screened 59 genes in three bladder cancer cell lines, small groups of urine sediments and tissues from bladder cancer patients, and the normal controls (Table 1). The methylated state of 21 genes was further characterized in urine sediments from bladder cancer (132 cases) and the controls (Fig. 2). The observation of six hypermethylated incidents of four genes in three patients from the total 483 (21 targets × 23 cases) tested for the noncancerous urinary lesion group (Fig. 2) is consistent with the notion that the aberrant DNA methylation can take place at the preneoplastic stage during tumorigenesis (17, 46). The finding that the total loss or drastic reduction of DNA methylation in the urine sediments of bladder cancer has established for the first time a strong correlation between the tumor lesions in bladder and the methylated state of this set of genes in urine sediments (Supplementary Table S4), suggesting that the DNA methylation marker in urine sediment could be a valuable reporter for

whether the tumor lesions have been completely removed in surgery. The ongoing efforts to monitor the disease recurrence in the postsurgical group with no methylation detected versus those with some methylation retained should provide a critical assessment of the practical value of this assay in a clinical setting. Although no evident disease staging value was found, methylation profiling in urine sediments can effectively detect early disease, as 75% (6 of 8) stage 0a and 88.2% (60 of 68) stage I disease were spotted (Supplementary Tables S2-S4). Finally, MSP profiling of the top four frequently methylated genes together can detect 82.6% (113 of 132) with 100% specificity. For the maximum sensitivity (91.7%) with decent specificity (87.6%), an 11-gene set (*SALL3*, *CFTR*, *ABCC6*, *HPP1*, *RASSF1A*, *MT1A*, *ALX4*, *CDH13*, *RPRM*, *MINT1*, and *BRCA1*) should be profiled (Table 2; Fig. 3).

*Distinct DNA methylation profiling for bladder cancer detection between Chinese and American populations may reflect disease disparity.* Bladder cancer occurs prevalently in developed countries, such as the United States (1). A unique profile

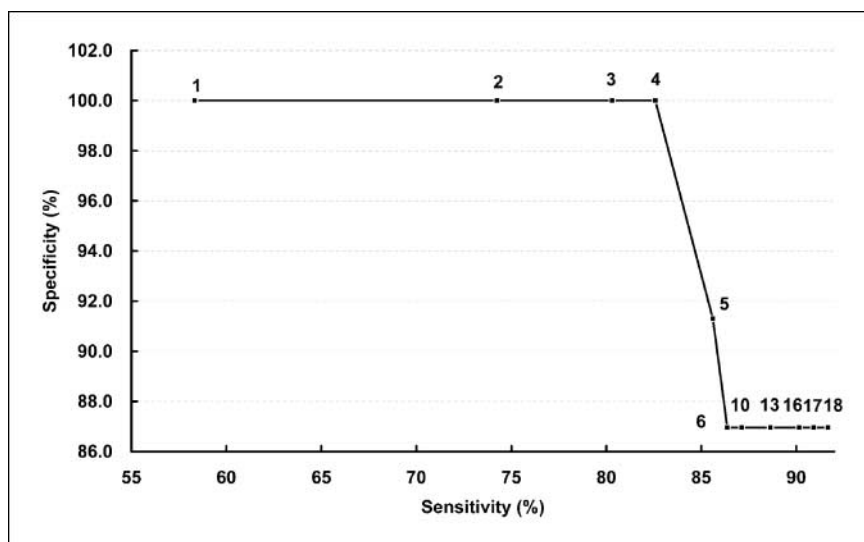
**Table 4.** Receiver operating characteristics of the informative sets for bladder cancer detection

No.	Genes	TP/FN	FP/TN	Sensitivity (%) TP / (TP + FN)	Specificity (%) TN / (TN + FP)	Statistical significance
1	<i>SALL3</i>	77/55	0/23	58.3	100.0	3.551E-08
2	<i>SALL3</i> , <i>CFTR</i>	98/34	0/23	74.2	100.0	3.205E-12
3	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i>	106/26	0/23	80.3	100.0	3.522E-14
4	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i>	109/23	0/23	82.6	100.0	4.970E-15
5	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i>	113/19	2/21	85.6	91.3	5.274E-13
6	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i>	114/18	3/20	86.4	87.0	5.392E-12
(6)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> ( <i>RUNX3</i> )	114/18	3/20	86.4	87.0	5.392E-12
(6)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> ( <i>RUNX3</i> , <i>ITGA4</i> )	114/18	3/20	86.4	87.0	5.392E-12
(6)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> ( <i>RUNX3</i> , <i>ITGA4</i> , <i>BCL2</i> )	114/18	3/20	86.4	87.0	5.392E-12
7	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i>	115/17	3/20	87.1	87.0	2.617E-12
(7)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> ( <i>MYOD1</i> )	115/17	3/20	87.1	87.0	2.617E-12
(7)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> ( <i>MYOD1</i> . <i>DRM</i> )	115/17	3/20	87.1	87.0	2.617E-12
8	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i>	117/15	3/20	88.6	87.0	5.607E-13
(8)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i> ( <i>BMP3B</i> )	117/15	3/20	88.6	87.0	5.607E-13
(8)	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i> ( <i>BMP3B</i> , <i>CCNA1</i> )	117/15	3/20	88.6	87.0	5.607E-13
9	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i> , <i>RPRM</i>	119/13	3/20	90.2	87.0	1.037E-13
10	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i> , <i>RPRM</i> , <i>MINT1</i>	120/12	3/20	90.9	87.0	4.184E-14
11	<i>SALL3</i> , <i>CFTR</i> , <i>ABCC6</i> , <i>HPP1</i> , <i>RASSF1A</i> , <i>MT1A</i> , <i>ALX4</i> , <i>CDH13</i> , <i>RPRM</i> , <i>MINT1</i> , <i>BRCA1</i>	121/11	3/20	91.7	87.0	1.606E-14
	Bladder cancer (123)	Nontumor control (23)				
Methylated	TP (121)		FP (3)			
Unmethylated	FN (11)		TP (20)			

NOTE: The first column is the list of each gene sets. The gene in brackets was considered redundant as its inclusion did not improve the sensitivity. The second is the true positive (the bladder cancer case having at least one gene methylated) and false negative (the bladder cancer case having no gene methylated) events in the bladder cancer cohort. The third is the false positive (the noncancerous urinary lesion case having at least one gene methylated) and true negative (the noncancerous urinary lesion case having no gene methylated) events in the noncancerous urinary lesion cohort. The fourth is sensitivity in percentage derived from the equation  $[TP/(TP+FN)]$ . The fifth is specificity in percentage from the equation  $(TN+FP)$ .

Abbreviations: TP, true positive; FN, false negative; FP, false positive; TN, true negative.

**Fig. 3.** Receiver operating characteristics of the informative sets for bladder cancer detection. Both sensitivity (%), TP / (TP + FN) (column 4, Table 4), and specificity (%), TN / (TN + FP) (column 5, Table 4) of each gene sets were calculated and plotted. TP, true positive; FN, false negative; FP, false positive; TN, true negative.



of both environmental and genetic factors in the United States may contribute to such a geographically biased disease pattern (36). Besides the set of genes that are hypermethylated in other types of cancer in China (37–43) or bioinformatically associated with cancer formation, we have also MSP profiled all of the “informative” genes for bladder cancer detection in the United States (32–35, 47, 48). Two regions in the promoter CpG island of *BCL2*, *HTERT*, and *DAPK1* genes were profiled. Hypermethylation of these three genes in combination was found in 24 of 37 cases of bladder cancer (64.9%; ref. 34). Neither *HTERT* nor *DAPK1* was hypermethylated in a single case of urine sediments (132 cases) and tissues (15 cases) of bladder cancer (Table 1). Although *BCL2* was found to be hypermethylated in 36 of 132 cases (27.3%, Supplementary Table S6), its incidence was 2-fold lower than that previously reported (34). We also conducted a MSP profiling of the promoter CpG island of *CDKN2A* (p16<sup>INK4a</sup>), *ARF* (p14<sup>ARF</sup>), *MGMT* and *GSTP1* genes, another informative set of DNA methylation markers (32). Unexpectedly, methylation of

*GSPT1* in two cases (1.4%) of bladder cancer was the only positive readout (Fig. 2). Because we have previously used the same MSP procedure and primers to detect tumor-associated hypermethylated genes in liver cancer (*GSPT1*; refs. 37–39, 42), glioma and colorectal cancer (p14<sup>ARF</sup> and *MGMT*; refs. 40, 41), lung cancer, and ovarian cancer,<sup>15</sup> failure to show the informativeness of this set of DNA methylation markers in this study cannot be technical (Supplementary Table S6). To conclude whether a disease disparity does indeed exist between China and U.S. bladder cancer cases, a forthcoming methylation profiling effort in larger patient cohorts of these two countries is needed.

### Acknowledgments

We thank Jianren Gu and Dangsheng Li for comments on the manuscript.

<sup>15</sup> Unpublished results.

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*Clin Cancer Res* 2007;13:7296-7304.

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