Aberrant Promoter Methylation of Multiple Genes during Pathogenesis of Bladder Cancer

Mariana Brait,[1,3] Shahnaz Begum,[3] André L. Carvalho,[1] Santanu Dasgupta,[1] André L. Vettore,[3] Bogdan Czerniak,[4] Otávia L. Caballero,[5] William H. Westra,[1,3] David Sidransky,[1] and Mohammad Obaidul Hoque[1]

[1]Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins University School of Medicine; [2]Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland; [3]Ludwig Institute for Cancer Research/Fundação Antonio Prudente, São Paulo, São Paulo, Brazil; [4]Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; and [5]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, New York

Abstract

Purpose: **The aims of our study were to elucidate the role of methylation of a large panel of genes during multistage pathogenesis of bladder cancer and to correlate our findings with patient age and other clinicopathologic features.** Experimental Design: **We studied the methylation status of 21 genes by quantitative methylation-specific PCR in an evaluation set of 25 tumor and 5 normal samples. Based on methylation frequency in tumors and normals in gene evaluation set, we selected 7 candidate genes and tested an independent set of 93 tumors and 26 normals. The presence or absence of methylation was evaluated for an association with cancer using cross-tabulations and χ^2 or Fisher's exact tests as appropriate. All statistical tests were two-sided.** Results: **Most primary tumors (89 of 93, 96%) had methylation of one or more genes of independent set; 53 (57%) *CCNA1*, 29 (31%) *MINT1*, 36 (39%) *CRBP*, 53 (57%) *CCND2*, 66 (71%) *PGP9.5*, 60 (65%) *CALCA*, and 78 (84%) *AIM1*. Normal uroepithelium samples from 26 controls revealed no methylation of the *CCNA1* and *MINT1* genes, whereas methylation of *CRBP*, *CCND2*, *PGP9.5*, and *CALCA* was detected at low levels. All the 7 genes in independent set were tightly correlated with each other and 3 of these genes showed increased methylation frequencies in bladder cancer with increasing age. *PGP9.5* and *AIM1* methylation correlated with primary tumor invasion.** Conclusion: **Our results indicate that the methylation profile of novel genes in bladder cancers correlates with clinicopathologic features of poor prognosis and is an age-related phenomenon.** (Cancer Epidemiol Biomarkers Prev 2008;17(10):2786–94)

Introduction

In the United States, approximately 68,810 newly diagnosed bladder cancer cases and 14,100 deaths are expected in 2008 (1). More than 90% of bladder carcinomas have a transitional cell origin (2). Approximately 75% of these neoplasms are superficial at first presentation and are curatively treated by transurethral resection alone, although 30% to 85% will recur and 10% to 30% will progress to a muscle-invasive disease with a poorer prognosis (3). Like many other neoplastic diseases, the survival rate of bladder cancer patients is critically influenced by the progression of the tumor. Whereas the 5-year survival rate for patients with a localized tumor is 92%, it decreases to 45% and 6% for regional and distant disease, respectively (1). There is a need for improved clinical stratification methods that can identify patients with early-stage disease and identify those with high risk of recurrence. Conventional methods, including computed tomography, urine cytology, histopathology, or tumor-node-metastasis classification, have thus far failed to overcome limitations in early detection and risk assessment. Conversely, a variety of novel molecular methods, such as detection of activating mutation of FGFR3 (4), p53 mutation status (5), loss of heterozygosity (5, 6), and methylation profiling (7, 8), have shown promising results. Despite growing research efforts, the molecular mechanisms underlying bladder cancer development and progression remain to be fully dissected. A better understanding of the molecular alterations associated with bladder cancer is likely to contribute to improved diagnosis, clinical management, and outcome prediction.

Epigenetic modifications are defined as all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself. Methylation of the C^5 positions of cytosine residues in DNA has long been recognized as an epigenetic silencing mechanism of fundamental importance (9). DNA methylation alters chromosome structure, inhibits the binding of proteins such as CTCF (a candidate tumor suppressor protein that binds to highly divergent DNA sequences), and defines regions of transcriptional regulation (10). DNA

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Requests for reprints: Mohammad Obaidul Hoque, Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins University School of Medicine, 1550 Orleans Street, CRB II, 5M, Baltimore, MD 21231. Phone: 410-502-8778; Fax: 410-614-1411. E-mail: mhoque1@jhmi.edu

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methylation can also promote the binding of proteins, such as MECP2, MBD1, MBD2, MBD3, and MBD4, which induce histone modification (11).

CpG dinucleotides are found at increased frequency in the promoter region of many genes, and methylation in the promoter region is frequently associated with ''gene silencing''; the gene is not expressed in the presence of methylation but is expressed in its absence (12). Both global hypomethylation and gene-specific promoter hypermethylation are associated with malignancy (13, 14), and studies in animals and in humans have shown that these epigenetic changes are an early event in carcinogenesis and are present in the precursor lesions of a variety of cancers including lung (15), head and neck (16), and colon (17). This epigenetic alteration provides an alternative pathway to gene silencing in addition to gene mutation or deletion. Moreover, the finding of promoter methylation of several genes in small biopsies and bodily fluids of cancer patients has proven to be useful as a molecular tool for cancer detection (7, 18).

In bladder cancer, several gene promoters were found to be hypermethylated by using conventional or quantitative methylation-specific PCR (QMSP; refs. 7, 19, 20). Indeed, the methylation of individual genes was found to correlate with clinicopathologic indicators of poor prognosis (19, 20). Conventional MSP is of limited usefulness for specific cancer detection because benign lesions can be weakly positive and cannot be distinguished from cancer cases. This limitation can be overcome by the development of quantitative assays such as QMSP.

In this study, we sought to characterize and quantitate the promoter methylation status of 21 genes derived from previously tested genes in several human cancers and novel genes we identified recently by our pharmacologic unmasking strategy (21). We tested the methylation status of these 21 genes (*CTNNB1, CALCA, hMLH1, PGP9.5, DAPK, CCND2, HIC1, AIM1, DCC, MINT1, MINT31, FANC-F, TGF-β, ATM, CRBP, THBS1, 14-3-3σ, CCNA1, ESR, FHIT*, and *MT1G*) first in an evaluation set consisting of 25 bladder tumor samples and 5 bladder normal samples. Based on the frequency of methylation observed for these genes in the evaluation set, 7 genes were selected (*CALCA, PGP9.5, CCND2, AIM1, MINT1, CRBP*, and *CCNA1*) for further analysis in an independent set of 93 tumor samples and 26 normal controls. Relationships between methylation levels and clinicopathologic indicators were further assessed.

Materials and Methods

Study Cohort. The bladder cancer samples were retrospectively collected from our tissue archive. To be included in the cohort, an eligible patient had to have a confirmed diagnosis of bladder cancer and a sufficient amount of archived tumor material to allow for DNA extraction (tissue preserved in sectioned blocks; >50% of tumor cells). Cases of bladder cancers with mixed histology, metastatic tumors to the bladder, and those of indeterminate clinical stage were excluded. Ninety-four percent (111 of 118) of our samples were urothelial cell carcinomas. Demographic and clinical information, including survival information, was obtained from the computerized tumor registry at The Johns Hopkins Healthcare System and M. D. Anderson Cancer Center. The total number of patient samples consisted of 93 males and 25 females. The tumors were classified according to the three-tier WHO histologic grading system (22), growth pattern (papillary versus nonpapillary), and DNA ploidy (23). Urothelial cell carcinomas were divided into a low-grade (grade 1 or 2) group or a high-grade (grade 3) group. The depth of invasion was recorded according to tumor-node-metastasis staging system (24). Tumors were divided into a superficial (T_a, T_{is}, and T_1) group or a muscle-invasive (T_2-T_4) group. The independent test set consisted of 30 patients with clinical stage 1 (T_a and T_{is}), 32 with clinical stage 2 (T_1), and 31 with clinical stage 3 (T_2-T_4). A detailed summary of the patient populations of the evaluation and test set are available in Table 1. The normal uroepithelium was obtained from archived autopsy samples (n = 16) or any benign condition of the uroepithelium (n = 10). The median age of the controls was 60.5 years (range, 8-99). Approval for research on human subjects was obtained from The Johns Hopkins University institutional review boards. This study qualified for exemption under the U.S. Department of Health and Human Services policy for protection of human subjects [45 CFR 46.101(b)].

Gene Selection. A total of 21 genes were selected for QMSP-based examination of methylation abnormalities.

Table 1. Demographic and clinical characteristics of bladder cancer patients (evaluation set n = 25, independent test set n = 93)

Characteristics	No. (%) patients	
	Evaluation set	Independent test set
Gender		
Male	18 (72)	75 (80.7)
Female	7 (28)	18 (19.3)
Age (y)		
Median (range)	66 (34–84)	67 (39–83)
≥65	13 (52)	61 (51.3)
<65	12 (48)	32 (48.7)
Stage		
Superficial	5 (20)	62 (66.7)
T_a	1 (4)	17 (18.3)
T_{is}	2 (8)	13 (14.0)
T_1	2 (8)	32 (34.4)
Muscle invasive	20 (80)	31 (33.3)
T_2	8 (32)	7 (7.5)
T_3 and T_4	12 (48)	24 (25.8)
Clinical T stage		
I (T_a and T_{is})	3 (12)	30 (32.3)
II (T_1)	2 (8)	32 (34.4)
III (T_2-T_4)	20 (80)	31 (33.3)
Grade		
High (grades 1 and 2)	24 (96)	82 (88.2)
Low (grade 3)	1 (4)	11 (11.8)
Disease recurrence		
No	22 (88)	38 (40.9)
Yes	3 (12)	31 (33.3)
Unknown	0	24 (25.8)
Metastasis presence		
No	14 (56)	61 (65.6)
Yes	11 (44)	14 (15.0)
Unknown	0	18 (19.4)
Current or former smoker		
No	5 (20)	7 (7.5)
Yes	13 (52)	33 (35.5)
Unknown	7 (28)	53 (57.0)

The gene profile included *CTNNB1, CALCA, hMLH1, PGP9.5, DAPK, CCND2, HIC1, AIM1, DCC, MINT1, MINT31, FANC-F, TGF-β, ATM, CRBP, THBS1, 14-3-3σ, CCNA1, ESR, FHIT*, and *MT1G.* The panel included genes reported as targets for epigenetic silencing in human cancer. *ATM* was included in this panel given the recent reports of hypermethylation in head and neck cancers (25), also a tobacco-related malignancy. *hMLH1* is involved in DNA repair, the inactivation of which results in increased mutagenicity. *DAPK* gene is involved in apoptosis and *CCNA1* and *CCND2* affect cell cycle and are frequently inactivated in human cancers. The function of *DCC, AIM1*, and *PGP9.5* are not yet well understood. Detailed summary of these genes including their proposed function is available in Supplementary Table S1.

DNA Extraction. After initial patient de-identification, all original histologic slides from the bladder cancer specimens were reviewed to reconfirm the diagnosis by a senior pathologist (W.H.W.). A representative block was retrieved for DNA extraction. Microdissected normal bladder epithelium was isolated from autopsy material or any noncancerous condition. Histologic slides from the formalin-fixed, paraffin-embedded tissue were taken. Slides were microdissected to obtain >70% neoplastic cells. DNA was extracted using standard protocols as described previously (26).

Bisulfite Treatment. DNA extracted from primary tumors and normal uroepithelium was subjected to bisulfite treatment, which converts unmethylated cytosine residues to uracil residues, as described previously (27) with minor modification. Briefly, 2 μg genomic DNA from each sample was denatured with NaOH (final concentration, 0.2 mol/L) in a total volume of 20 μL for 20 min at 50°C. The denatured DNA was diluted in 500 μL of a freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite and incubated for 3 h at 70°C. Bisulfite-modified DNA was purified using a Wizard DNA Clean-Up System (Promega), treated with 0.3 mol/L NaOH for 10 min at room temperature, precipitated with ethanol, resuspended in 120 μL LoTE [2.5 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8)], and stored at −80°C.

Methylation Analysis. Bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR as described previously (28). Amplification reactions were carried out in triplicate in a final volume of 20 μL that contained 3 μL bisulfite-modified DNA; 600 nmol/L concentrations of forward and reverse primers; 200 nmol/L probe; 0.6 units platinum Taq polymerase (Invitrogen); 200 μmol/L concentrations each of dATP, dCTP, dGTP, and dTTP; and 6.7 mmol/L $MgCl_2$. Primers and probes were designed to specifically amplify the promoters of the 21 genes of interest and the promoter of a reference gene, *ACTB*; primer and probe sequences and annealing temperatures are provided in Supplementary Table S2. Amplifications were carried out using the following profile: 95°C for 3 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplification reactions were carried out in 384-well plates in a 7900 sequence detector (Perkin-Elmer Applied Biosystems) and analyzed by a sequence detector system (SDS 2.2.1; Applied Biosystems). Each plate included patient DNA samples, positive (*in vitro* methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls, and multiple water blanks. Leukocyte DNA from a healthy individual was methylated *in vitro* with excess *Sss*I methyltransferase (New England Biolabs) to generate completely methylated DNA, and serial dilutions (90-0.009 ng) of this DNA were used to construct a calibration curve for each plate. All samples were within the assay's range of sensitivity and reproducibility based on amplification of internal reference standard [threshold cycle (C_T) value for *ACTB* of 40]. The relative level of methylated DNA for each gene in each sample was determined as a ratio of MSP-amplified gene to *ACTB* (reference gene) and then multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest divided by the average value of triplicates of *ACTB* × 1,000). The samples were categorized as unmethylated or methylated based on the sensitivity of the assay. The threshold of the assay of individual genes was determined by maximizing sensitivity and specificity.

Statistical Analysis. We used Fisher's exact method to test the methylation frequency differences in gene evaluation set. In the test set, the major statistical endpoints in this study involved comparing normal and cancer methylation levels of seven genes thought to be associated with cancer. Methylation values were visually compared using boxplots (29) of the log-transformed values. In these plots, the length of the box is the interquartile range of the data and depicts the spread of the middle 50% of the observations. The median is displayed with a horizontal line inside of this box. The lines extending out from the box extend from the upper and lower quartiles to values defined as adjacent values. The adjacent values are the upper quartile plus 1.5 × interquartile range and the lower quartile minus 1.5 × interquartile range. Any value lying outside of this range is displayed with an open circle and can be considered an outlier.

The presence or absence of methylation was evaluated for an association with cancer using cross-tabulations and χ^2 or Fisher's exact tests as appropriate. Continuous methylation levels were evaluated using logistic regression (30). Internal validation of the logistic regression models was done using an approximation to the leave-one-out jackknife procedure provided by the SAS classification table option (31).

Methylation index was calculated as the total number of genes having any methylation divided by the total number of genes examined. Mean methylation index was compared across age, stage, and grade categories with ANOVA models. Correlations of the methylation levels of genes were calculated with Spearman correlation coefficients.

The presence or absence of methylation was also evaluated for an association with survival. Event time distributions were estimated with the method of Kaplan and Meier (32) and compared using the log-rank statistic (33) or the proportional hazards regression model (34). All *P* values are two-sided. Computations were done using the Statistical Analysis System (31, 35).

Results

Clinical and Pathologic Data. We tested tissue samples from a total of 118 patients (evaluation and test

set) with bladder cancer and a total of 31 normals (evaluation and test set) from autopsies and benign diseases of uroepithelium. The clinicopathologic and demographic characteristics of these patients (evaluation and independent test set) are depicted in Table 1.

Determination of Appropriate Genes in a Gene Evaluation Set by QMSP. We initially tested 21 genes in DNA from 25 primary bladder tumor tissues and 5 normal uroepithelium samples from the deceased archive or uroepithelium of any benign condition. An overview of the frequency of methylation in the investigated samples is given in Table 2. In general, methylation levels were higher in tumors compared with normal samples (Fig. 1). The most appropriate genes for our additional analyses were determined to be those that met one of the following criteria: (*a*) unmethylated in normal samples from controls and ≥48% methylated in primary bladder cancer tissues or (*b*) ≤40% methylated in normal controls and >72% methylated in primary bladder cancer tissues. A total of seven genes, *CALCA, PGP9.5, CCND2, AIM1, MINT1, CRBP*, and *CCNA1*, met at least one of these criteria (Table 2).

Frequency of Methylation in Bladder Tissues in Independent Set by QMSP. We then tested these 7 genes in the independent set of cancer and control tissues. The frequencies of methylation of the 7 genes in 93 bladder cancers samples and 26 nonmalignant tissues are detailed in Table 2. The differences of methylation values between tumor tissue and normal were all statistically significant (Fisher's exact test) (Table 2). The sensitivity and specificity of each gene vary from 31% to 84% and 73% to 100%, respectively (Table 2). There was no methylation of *CCNA1* or *MINT1* in normal bladder tissues. Most cancers (89 of 93, 95.6%) had methylation of one or more genes. Thirteen (14%) had methylation of 2 genes, 8 (9%) had methylation of 3 genes, 12 (13%) had methylation of 4 genes, 11 (12%) had methylation of 5 genes, 21 (23%) had methylation of 6 genes, and 12 (13%) had methylation of 7 genes. There was no methylation of any gene in 4 (4%) cancer cases. By contrast, 50% of nonmalignant tissue (13 of 26) lacked methylation of any gene tested, 7 had methylation of 1 gene, 3 had methylation of 2 genes, 3 had methylation of 3 genes, and none had methylation of ≥4 genes.

The methylation values differ among normal and tumor and within the tumor and normal samples. The boxplots in Fig. 1 show the distribution of relative methylation values for each of the 7 genes of interest versus *ACTB* obtained by QMSP in cancer patients versus normal samples. Methylation markers have been categorized as methylated for any value greater than zero.

Correlation of Primary Tumor Hypermethylation Profiles with Clinicopathologic Variables. There were 93 cancer samples and 26 normal samples in the independent set of genes of this study. Age, gender, and race were compared between groups. Race was not significantly different, and the median age in the cancer group was 67 compared to 60.5 years in the normal group.

Several clinicopathologic and demographic variables (age, gender, histologic type, tumor stage, tumor grade, metastasis, recurrence, smoking, and overall patient survival) were compared with the methylation patterns observed in the tumor DNA. Logistic regression analysis was done to determine whether the frequency of QMSP markers or the combination thereof correlated with these variables associated with bladder cancer prognosis. In a

Table 2. Promoter methylation frequency for the 21 genes analyzed in the evaluation set of samples and 7 genes in the independent test set

Gene	Evaluation set			Independent set			Sensitivity (%)	Specificity (%)
	Methylation positive % (no. methylation positive/no. total cases)			Methylation positive % (no. methylation positive/no. total cases)				
	Tumor	Normal	*P**	Tumor	Normal	*P**		
CCNA1	56 (14/25)	0 (0/5)	0.045	57 (53/93)	0 (0/26)	< 0.0001	57	100
MINT1	56 (14/25)	0 (0/5)	0.045	31 (29/93)	0 (0/26)	0.0004	31	100
CRBP	48 (12/25)	0 (0/5)	0.128	39 (36/93)	4 (1/26)	0.0005	39	96
CCND2	72 (18/25)	20 (1/5)	0.047	57 (53/93)	19 (5/26)	0.0007	57	81
PGP9.5	92 (23/25)	40 (2/5)	0.022	71 (66/93)	19 (5/26)	< 0.0001	71	81
CALCA	88 (22/25)	40 (2/5)	0.041	65 (60/93)	15 (4/26)	< 0.0001	65	85
AIM1	72 (18/25)	40 (2/5)	0.3	84 (78/93)	27 (7/26)	< 0.0001	83	73
DAPK	28 (7/25)	0 (0/5)	0.304	ND	ND	ND		
DCC	24 (6/25)	0 (0/5)	0.553	ND	ND	ND		
MINT 31	20 (5/25)	0 (0/5)	0.556	ND	ND	ND		
FHIT	12 (3/25)	0 (0/5)	1	ND	ND	ND		
ATM	4 (1/25)	0 (0/5)	1	ND	ND	ND		
FANC-F	0 (0/25)	0 (0/5)	1	ND	ND	ND		
TGFβR2	0 (0/25)	0 (0/5)	1	ND	ND	ND		
hMLH1	0 (0/25)	0 (0/5)	1	ND	ND	ND		
ESR	44 (11/25)	20 (1/5)	0.622	ND	ND	ND		
MT1G	20 (5/25)	20 (1/5)	1	ND	ND	ND		
THBS1	4 (1/25)	20 (1/5)	0.31	ND	ND	ND		
CTNNB1	0 (0/25)	20 (1/5)	0.2	ND	ND	ND		
HIC1	100 (25/25)	60 (3/5)	0.023	ND	ND	ND		
14-3-3σ	100 (25/25)	100 (5/5)	1	ND	ND	ND		

Abbreviation: ND, not done.
*Fisher's exact test.

univariate analysis, age, tumor-node-metastasis stage, and invasion were associated with methylation of at least one of the seven gene promoters. Patients ages ≥65 years were significantly more likely to have *CCNA1* ($P = 0.004$), *PGP9.5* ($P = 0.01$), or *CCND2* ($P = 0.02$) methylation. *PGP9.5* ($P = 0.04$) and *AIM1* ($P = 0.02$) were associated with invasive tumors. *CALCA* methylation was associated with late-stage (≥pT_2) tumors ($P = 0.0001$).

Mean methylation index was significantly greater in patients ages ≥65 years, 3.67 [95% confidence interval (95% CI), 3.16-4.18] compared with the younger group, 2.56 (95% CI, 2.0-3.1; $P = 0.004$). Stage III mean methylation index, 4.4 (95% CI, 3.8-5.0), was also significantly greater than that in stage I, 3.4 (95% CI, 2.7-4.2; $P = 0.05$), or stage II, 3.5 (95% CI, 2.7-4.2; $P = 0.05$). The mean methylation index is significantly higher in the muscle-invasive stage then in the superficial stages, 4.4 (95% CI, 3.8-5.0) and 3.4 (95% CI, 2.9-4.0), respectively ($P < 0.0001$; Fig. 2). Mean methylation index was the lowest in the normal samples, 0.85 (95% CI, 0.4-1.3). Low-grade and high-grade mean indexes were similar, low-grade mean, 3.06 (95% CI, 1.9-4.2) and high-grade mean, 3.9 (95% CI, 3.4-4.3; $P = 0.2$). For all markers, higher methylation level was significantly associated with an increased probability of cancer. The boxplots in Fig. 3 show the distribution of relative promoter methylation levels for four representative markers (*CCND2, PGP9.5, CCNA1*, and *CALCA*) in the bladder cancer patient samples in noninvasive stages (<pT_2) and muscle-invasive stage (≥pT_2). The median of normalized methylation value and the frequency for *CCND2, PGP9.5, CCNA1*, and *CALCA* genes are higher in ≥pT_2 tumors (Fig. 3). Aberrant methylation in primary bladder cancer had no correlation with disease metastasis, smoking history, recurrence, tumor grade, and survival of the patients (data not shown).

Finally, we performed a correlation analysis for all pairs of markers (Table 3). Promoter methylation of every pair of genes was statistically significantly correlated (all $P < 0.0014$). The strongest correlations were between *CCND2* and *PGP9.5, CRBP*, and *MINT1* ($r \geq 0.59$). In addition, promoter methylation of *CALCA* was highly correlated with MINT1 ($r = 0.53$).

Discussion

There is growing evidence that DNA methylation is an important mechanism of gene inactivation in cancer and its potential as a molecular marker. Considerable variations exist in promoter methylation profiles of different cancers, such as individual tumor types have characteristic methylation profiles (36, 37). Some methylation markers have been shown to be promising in the

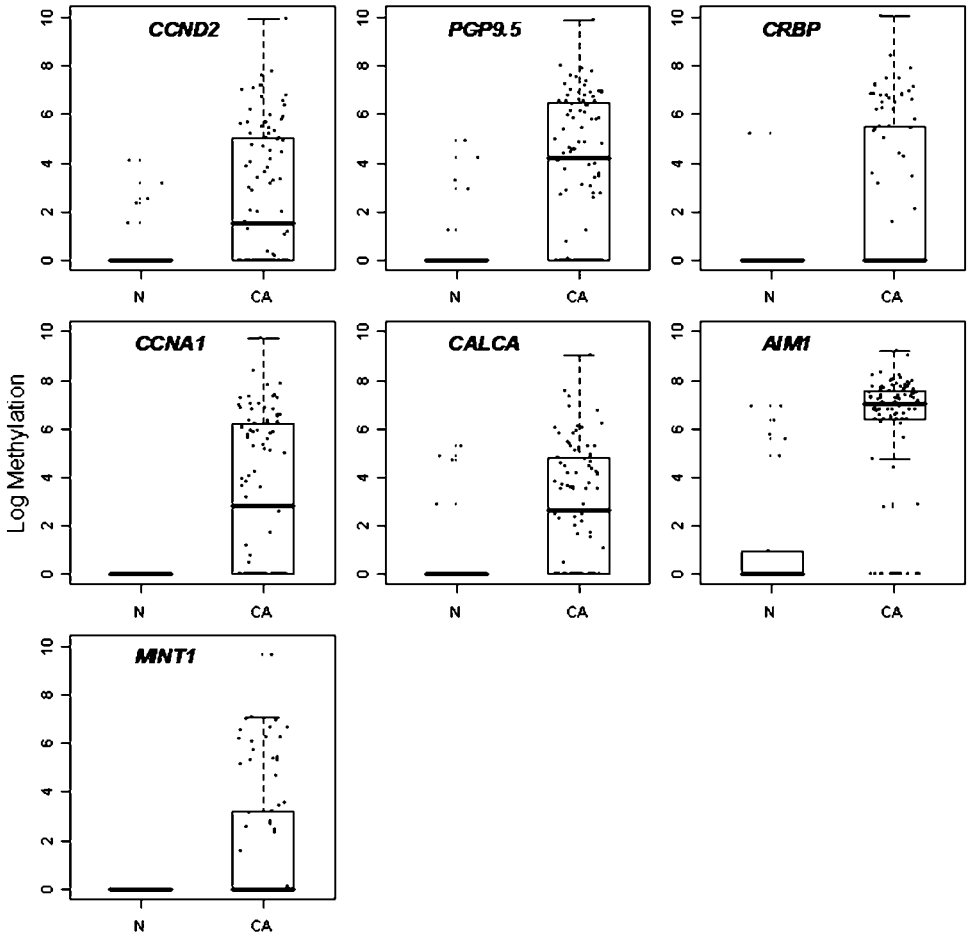

Figure 1. Promoter methylation levels for the seven markers in the bladder cancer patient samples (*CA*; $n = 93$) and normal bladder tissues (*N*; $n = 26$). The quantity of each methylated gene promoter was expressed as the ratio of the amount of PCR products amplified from the methylated gene to the amount amplified with the reference gene β-actin multiplied by 1,000. Boxplots show the middle 50% of data, the line is the median, and the bars extend the median by 1.5 times the interquartile range.

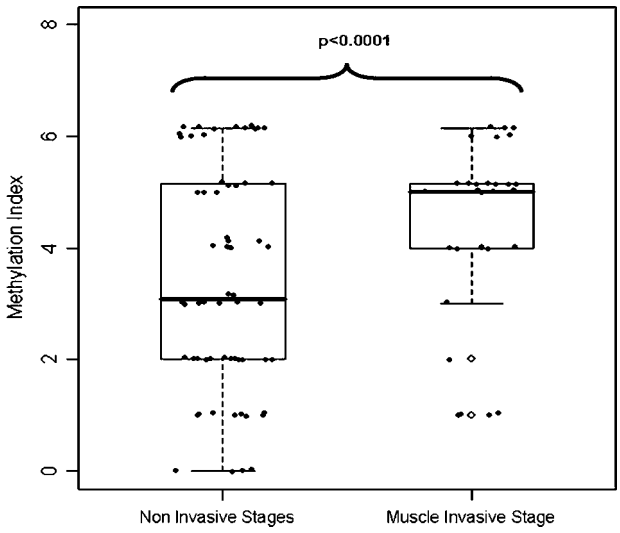

Figure 2. Mean methylation index levels markers in bladder cancer samples compared across noninvasive stages and muscle-invasive stage. Methylation index was calculated as the total number of genes having any methylation divided by the total number of genes examined. The median of methylation index in noninvasive stages and muscle-invasive stage is 3.1 and 5.0, respectively. Boxplots show the middle 50% of data, the line is the median, and the bars extend the median by 1.5 times the interquartile range.

noninvasive diagnosis of bladder cancer and predicting patient survival and tumor progression (7, 8, 38). Based on our experience and literature review, it is evident that methylation can occur in a tissue-specific or cancer-specific manner. Cancer-specific methylation is usually limited to tumor tissues and seldom observed in progenitor tissues (21, 39).

We determined the methylation profile of bladder cancers testing a panel of 21 genes, which include genes extensively studied in other tumor types (*CTNNB1, CALCA, hMLH1, PGP9.5, DAPK, CCND2, HIC1, DCC, MINT1, MINT31, FANC-F, TGF-β, ATM, CRBP, THBS1, 14-3-3σ, CCNA1, ESR, FHIT*, and *MT1G*), genes not yet reported (*AIM1*), and genes reported to be methylated in bladder cancer by the widely used conventional MSP assay (*hMLH1, CCND2, DAPK, HIC1*, and *FHIT*). In addition to the 7 genes in the independent set, further studies of the remaining genes in a larger number of samples will elucidate the role of these genes in bladder cancer stratification. Most of the genes that we examined have not been studied previously for methylation in bladder cancers and we identified 8 methylated genes, which are bladder cancer specific (methylation in tumor not in normal). Although some genes seemed to have strong individual associations with different clinicopathologic variables, we were keen to develop a panel of loci that would be specific to all urothelial cell carcinoma and represent the extent of global epigenetic disruption. These findings also corroborate the use of candidate gene approach for the characterization of organ-specific methylated genes.

In this study, the frequency of *CCND2* promoter methylation was higher than reported previously (40). Besides possible differences in the patient population, it is noteworthy that conventional MSP was used previously, whereas we used a quantitative methodology with different PCR conditions that might be more sensitive than conventional MSP. Interestingly, divergent results were also obtained by QMSP and conventional MSP for the frequency of *APC* methylation in the same small-cell lung cancer cell lines: 58% by QMSP versus 26% by conventional MSP (41, 42). Yet, we found low level of methylation in normals, in contrasting to previous reports (40). This discrepancy is likely due to the high sensitivity of QMSP and its ability to detect rare methylated cells in normal urothelium. Hence, precautions should be taken when comparing results of different studies concerning aberrant promoter methylation in the same tumor type, because different methodologies and use of different primers may yield varied results. The major advantage of QMSP is the ability to quantitatively compare samples and to more accurately segregate varied pathologic covariates based on appropriate cutoffs. For example, we analyzed 5 genes using optimal cutoffs; as shown, by compromising sensitivity, specificity was increased to almost 90% for all 5 genes combined (data not shown).

In several human cancer cell lines, aberrant promoter methylation of the majority of genes examined in this study has been shown to abrogate transcription (43), and reactivation was observed in the nonexpressing cell lines after treatment with demethylating agents (21, 44, 45). Moreover, these genes are involved in important molecular pathways of carcinogenesis such as cell cycle regulation, DNA repair, apoptosis, and signal transduction (13, 46, 47). Two of the seven genes tested (*CCNA1* and *MINT1*) displayed high frequency of methylation in bladder cancer and no methylation in normal uroepithelium. For the remaining 5 genes (*CRBP, CALCA, PGP9.5, CCND2*, and *AIM1*), we found significantly more methylation in bladder cancer cases than normal epithelium. Although we found a low frequency of methylation in normal uroepithelium, the differences in methylation levels of these genes between neoplastic and normal controls are even more striking than the differences in methylation frequency. Indeed, the isolated presence of methylation is not necessarily specific for malignant disease, whereas quantitation of methylation allows for accurate discrimination between bladder carcinoma and uroepithelium, confirming our previous observations for different cancer types and various genes (7, 48). By establishing an empiric cutoff value, we calculated the frequency of methylation in the independent set, which

Table 3. Spearman correlation matrix of promoter methylation levels for all genes in bladder cancer samples

Genes	*CCND2*	PGP9.5	CRBP	CCNA1	CALCA	AIM1	MINT1
CCND2	1	0.64	0.62	0.39	0.41	0.25	0.59
PGP9.5		1	0.52	0.36	0.42	0.08	0.5
CRBP			1	0.35	0.46	0.12	0.47
CCNA1				1	0.46	0.18	0.42
CALCA					1	0.34	0.53
AIM1						1	0.26
MINT1							1

NOTE: All correlations were statistically significant (all $P < 0.0014$).

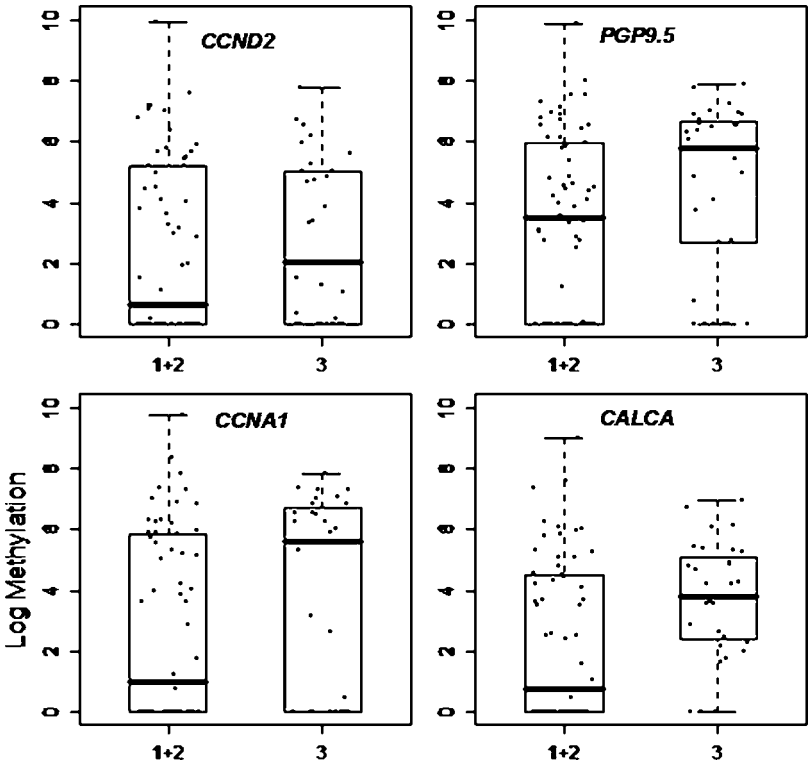

Figure 3. Promoter methylation levels for four representative markers in bladder cancer samples for noninvasive stages (1 + 2) and muscle-invasive stage (3). The quantity of methylated allele of each gene was expressed as the ratio of the amount of PCR products amplified from the methylated gene to the amount amplified with the reference gene β-actin multiplied by 1,000. Boxplots show the middle 50% of data, the line is the median, and the bars extend the median by 1.5 times the interquartile range.

shows an increased specificity but decreased sensitivity (Table 4).

We also showed a correlation between methylation levels of individual or panel of genes and standard clinicopathologic conditions (tumor grade, invasiveness, recurrence, and stage). *CCNA1* was reported previously to be associated with invasive cervical cancer (43). We found an increased methylation frequency in muscle-invasive tumors. These findings indicate a potential role of *CCNA1* in the pathogenesis and spread of bladder cancer. Cancer-specific promoter hypermethylation of *PGP9.5* was reported in different cancer types by our group and others (45, 49, 50); however, cancer-specific hypomethylation (*PGP9.5*) was also reported in gall bladder cancer (51). We found a high frequency of bladder cancer-specific methylation of *PGP9.5* in the present study and the median methylation values are significantly higher in invasive tumors (odds ratio, 2.68; 95% CI, 1.05-6.82; $P = 0.04$). Furthermore, we found that patients with higher *PGP9.5* methylation ratios in the primary tumor showed poorer 5-year survival rates than those without *PGP9.5* methylation in esophageal cancer (45). Hypermethylation of the cell cycle gene *CCND2* was also associated with advanced stage of the disease in ovarian cancer (52), which is consistent with our findings here in bladder cancer. The significant association of several methylated genes were also observed in higher stages of tumor in bladder cancer using urine sediment of bladder cancer patients (7). These findings may have clinical implications for the management of bladder cancer. Because methylation levels were found to correlate with tumor aggressiveness, these molecular assays could be included in predictive models for preoperative bladder cancer staging. If confirmed, carcinomas found to harbor high levels of promoter methylation in bladder biopsy may be more likely to benefit from appropriate therapeutic intervention.

Table 4. Promoter methylation frequency based on an empiric cut off value for the 7 genes in the independent test set

Gene	Methylation positive % (no. methylation positive/no. total cases)			*P**	Specificity (%)	Sensitivity (%)
	Tumor	Normal	Empiric cutoff			
CCNA1	57 (53/93)	0 (0/26)	0	<0.0001	100	57
MINT1	31 (29/93)	0 (0/26)	0	0.0004	100	31
CRBP	39 (36/93)	4 (1/26)	0	0.0005	96	39
CCND2	33 (32/93)	0 (0/26)	60	0.0001	100	33
PGP9.5	41 (38/93)	0 (0/26)	131	<0.0001	100	41
CALCA	28 (26/93)	12 (3/26)	100	0.1205	88	28
AIM1	54 (50/93)	0 (0/26)	1040	<0.0001	100	54

*Fisher's exact test.

A correlation between the methylation index (the proportion of methylated genes) and markers of poor outcome in bladder cancer has been reported by our group and others (19, 38). However, in this study, we did not find any statistically significant association between the methylation status of a single gene or methylation index as a marker of clinical outcome. Longer follow-up of this cohort of patients is needed to clarify any association of any methylated gene with clinical outcome.

There are differences in the pattern of gene methylation in tumors of different organs. Methylated genes can be divided into those that occur generally in most cancers (such as *E-cadherin* and *RASSF1a*), those that occur commonly in a few cancers (e.g., *APC* in bladder and colonic cancer), and those that are mostly specific to one organ (e.g., *GSTP1* in prostate cancer or *VHL* in renal cancer; ref. 36). Although the loci and extent of genes affected by hypermethylation vary between tumors, their loss reflects global changes in epigenetic control. By selecting loci for various tumors, one could devise organ-specific gene panels that reveal events across the entire epigenome. This knowledge is useful as epigenetic alterations affect the molecular mechanisms within a tumor and thus the subsequent clinical phenotype. For example, high levels of methylation are found in sporadic cancers with microsatellite instability due to *hMLH1* methylation (53). Tumors with this form of genomic instability are relatively chemoresistant and behave more indolently than matched tumors without microsatellite changes (54). Similarly, *MGMT* methylation alters sensitivity to temozolomide and could eventually be used to stratify patient treatment (55).

The present study constitutes one of the most comprehensive methylation profile reports available for bladder cancer. We have identified groups of tumor-specific genes as well as genes that are frequently methylated in normal urothelium. We have confirmed and extended the observations of others that methylation of some genes are tightly correlated in tumors as well as genes that show age-related methylation. Organ-specific panels of loci could be developed that reflect global epigenetic events and would be small enough to be practical for routine application. Further multi-institutional studies are required to validate these markers and their general applicability in the management of patients with urothelial cancer.

Disclosure of Potential Conflicts of Interest

D. Sidransky: Oncomethylome Sciences, commercial research grant and ownership interest. The other authors disclosed no potential conflicts of interest.

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