

# Aberrant Promoter Methylation Profile of Bladder Cancer and Its Relationship to Clinicopathological Features<sup>1</sup>

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## Abstract

We investigated the aberrant promoter methylation profile of bladder cancers and correlated the data with clinicopathological findings. The methylation status of 10 genes was determined in 98 surgically resected bladder cancers, and we calculated the median methylation index (MI), a reflection of the methylated fraction of the genes tested. Methylation frequencies of the genes tested in bladder cancers were 36% for *CDHI*, 35% for *RASSF1A* and *APC*, 29% for *CDH13*, 16% for *FHIT*, 15% for *RARβ*, 11% for *GSTP1*, 7% for *p16<sup>INK4A</sup>*, 4% for *DAPK*, and 2% for *MGMT*. Methylation of four of the individual genes (*CDHI*, *RASSF1A*, *APC*, and *CDH13*) and the MI were significantly correlated with several parameters of poor prognosis (tumor grade, growth pattern, muscle invasion, tumor stage, and ploidy pattern). Methylation of *CDHI*, *FHIT*, and a high MI were associated with shortened survival. *CDHI* methylation positive status was independently associated with poor survival in multivariate analyses. Our results suggest that the methylation profile may be a potential new biomarker of risk prediction in bladder cancer.

## Introduction

Urinary bladder cancer is the fifth most common cancer in the Western world and is responsible for about 3% of all cancer-related deaths. Approximately 53,000 cases of bladder cancer were diagnosed in the United States in the year 2000, and ~23% of these patients will eventually die of their disease (1). DNA methylation of the promoter regions is emerging as the major mechanism of inactivation of TSGs.<sup>3</sup> DNA is methylated only at cytosines located 5' to guanosines in CpG dinucleotides and DNA methylation is a frequent epigenetic event in many human cancers (2, 3). This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, located in the promoter regions of many genes. In many cases, aberrant methylation of the CpG island genes has been correlated with a loss of gene expression, and it is proposed that DNA methylation provides an alternate pathway to gene deletion or mutation for the loss of TSG function. Markers for aberrant methylation may represent a promising avenue for monitoring the onset and progression of cancer. Aberrant promoter methylation has

been described for several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation (3, 4). We chose 10 genes (Table 1), frequently silenced by aberrant methylation in a number of tumor types, to investigate the aberrant methylation profile of bladder cancers. We correlated our findings with clinicopathological parameters of aggressive behavior.

## Materials and Methods

**Clinical Samples.** Ninety-eight fresh bladder tumor tissues were obtained by transurethral resection or from cystectomy specimens at the M. D. Anderson Cancer Center or the Affiliated Hospitals of the University of Texas Southwestern Medical Center, after obtaining Institutional Review Board-approved signed consent. The patients consisted of 58 males and 39 females. The median age of the patients was 72 years old, with a range of 40–96. The tumors were classified according to the three-tier WHO histological grading system (5), growth pattern (papillary versus nonpapillary), and DNA ploidy (6). All of the tumors were transitional cell carcinomas except for one squamous cell carcinoma. Transitional cell tumors were divided into a low-grade (grades 1 or 2) group or a high-grade (grade 3) group. The depth of invasion was recorded according to TNM staging system (7). There were 15 patients with stage 0, 6 with stage I, 8 with stage II, 18 with stage III, and 16 with stage IV. Because full staging could not be determined on tumors resected transurethraly, there were 35 cases of unknown stage. Tumors were divided into a low-stage (stage 0–II) group or a high-stage (stage III–IV) group. For DNA ploidy measurements, touch smears of tumor cells and slides containing diploid standard (human peripheral blood lymphocytes) were fixed in 100% buffered formalin and stained with the Feulgen reaction. Measurements of total nuclear DNA content in individual tumor cells were performed under visual inspection with SAMBA 4000 (Molecular Diagnostics, Inc., Chicago, IL) computer-assisted image analysis system. The frequency histograms were based on the measurements of 200–300 morphologically identifiable intact tumor nuclei. Tumors with DNA indices of major cell populations ranging from 0.9 to 1.2 were classified as diploid/near diploid; tumors in which >20% of cells formed a distinct peak with DNA index >1.2 were classified as aneuploid. No tumor with hypodiploid content (DNA index <0.9) was included in this study. Figures for clinicopathological features (tumor grade, papillary or nonpapillary histology, muscle invasion, tumor stage, and ploidy pattern) are presented in Fig. 2.

**MSP Assay.** Genomic DNA was isolated from frozen tissue by digestion with 100 μg/ml proteinase K followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation. DNA was treated with sodium bisulfite as described previously (8). Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 M sodium bisulfite (pH 5.0; 520 μl; Sigma Chemical Co.) were added, and the solution was incubated at 50°C for 16 h. Treated DNA was purified by use of Wizard DNA Purification System (Promega Corp., Madison, WI), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at –70°C until used. References for methodology, other key references, and chromosomal location of genes tested are summarized in Table 1. Two sets of primers were used to amplify each region of interest: One pair recognized a sequence in which CpG sites are unmethylated (bisulfite-modi-

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<sup>3</sup> The abbreviations used are: TSG, tumor suppressor gene; RASSF1A, RAS association domain family protein; RARβ, retinoic acid receptor β; APC, adenomatous polyposis coli; DAPK, death-associated protein kinase; MGMT, O<sup>6</sup>-methylguanine-DNA-methyltransferase; GSTP1, glutathione S-transferase P1; CDH1, E-cadherin; CDH13, H-cadherin; MSP, methylation-specific PCR; MI, methylation index.

Table 1 Summary data of genes tested

Gene abbreviation	Gene name	Chromosomal location	Reference for methodology	Other key references
<i>FHIT</i>	<i>Fragile histidine triad</i>	3p14	(26)	
<i>RASSF1A</i>	<i>RAS association domain family protein 1A</i>	3p21	(16)	(17)
<i>RARβ</i>	<i>Retinoic acid receptor β</i>	3p24	(27)	
<i>APC</i>	<i>Adenomatous polyposis coli</i>	5q21	(28)	
<i>p16<sup>INK4A</sup></i>	<i>Cyclin-dependent kinase inhibitor 2A</i>	9p21	(8)	
<i>DAPK</i>	<i>Death-associated protein kinase</i>	9q34	(29)	(30)
<i>MGMT</i>	<i>O<sup>6</sup>-methylguanine-DNA-methyltransferase</i>	10q26	(31)	
<i>GSTP1</i>	<i>Glutathione S-transferase P1</i>	11q13	(32) <sup>a</sup>	
<i>CDH1</i>	<i>E-cadherin</i>	16q22	(33)	
<i>CDH13</i>	<i>H-cadherin</i>	16q24	(22)	(23)

<sup>a</sup> We modified the annealing temperature of the methylated form from 59° C to 64° C.

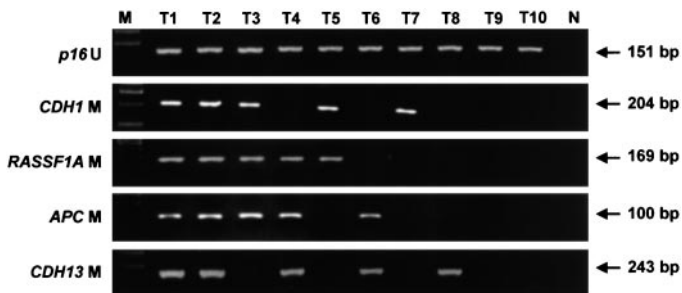


Fig. 1. Representative examples of MSP analyses of the methylated form (*M*) of four genes: *CDH1*, *RASSF1A*, *APC*, and *CDH13*. Amplification of unmethylated form of *p16<sup>INK4A</sup>* (*p16U*) was used as a control for DNA integrity. *Across top of figure*: *M*, size marker; *T*, tumor samples 1 through 10; *N*, negative control.

fied to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). Negative control samples without DNA were included for each set of PCRs. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

**Data Analysis.** A comparison of the proportion was done using  $\chi^2$  test or Fisher's exact method. To compare the extent of methylation for the panel of genes examined, we calculated the MI for each case as follows and then determined the mean for the different groups.

$$MI = \frac{\text{Total number of genes methylated}}{\text{Total number of genes analyzed}}$$

Statistical analysis of MI between two variables was performed using the Mann-Whitney *U* nonparametric test. Survival was calculated from the date of initial diagnosis until death or the date of the last follow-up (censored). The duration of the recurrence-free interval was measured from the date of the operation until the first evidence of recurrence or the last date of follow-up for patients who remained alive and recurrence free (censored). The recurrence-free interval and survival were analyzed according to the Kaplan-Meier method, and differences in their distribution were evaluated by means of the log-rank test. Cox proportional hazards models were applied for multivariate analysis. A *P* of less than 0.05 was defined as being statistically significant. All of the data were analyzed with the use of Abacus Concepts, Survival Tools for StatView (Abacus Concepts, Inc., Berkeley, CA).

## Results

**Frequency of Methylation in Bladder Cancers.** Among 98 bladder cancers, the methylation frequencies (in descending order) were as follows: 35 (36%) for *CDH1*, 34 (35%) for *RASSF1A* and *APC*, 28 (29%) for *CDH13*, 16 (16%) for *FHIT*, 15 (15%) for *RARβ*, 11 (11%) for *GSTP1*, 7 (7%) for *p16<sup>INK4A</sup>*, 4 (4%) for *DAPK*, and 2 (2%) for *MGMT*. Most tumors [76 (78%) of 98], had methylation of one or more genes, and 16 (16%) had methylation of four to seven genes. Fig. 1 illustrates representative examples of the methylation patterns of the four most frequently methylated genes. The unmethylated form of *p16<sup>INK4A</sup>*, run as a control for DNA integrity, was present in all of the samples.

**Correlation of Methylation and Risk Factors.** Fig. 2 illustrates the correlation of methylation frequencies and the mean MI with five factors of increased risk, namely high tumor grade (Fig. 2A), nonpapillary growth pattern (Fig. 2B), muscle invasion (Fig. 2C), high tumor

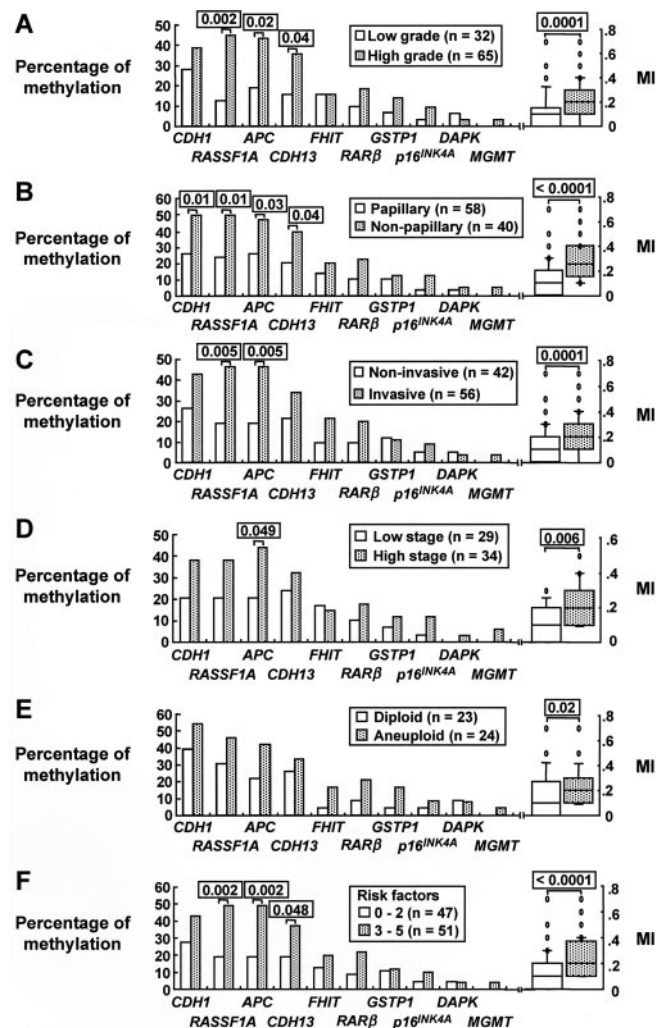


Fig. 2. Correlation of methylation findings and risk factors. For each panel, the methylation percentages are correlated with a risk factor. *Right side of each panel*, the corresponding MIs. *A*, correlation with histological grade. Transitional cell tumors were divided into a low-grade (grades 1 or 2) group or a high-grade (grade 3) group. The single squamous cell carcinoma was omitted from the analysis. *B*, correlation with growth pattern (papillary or nonpapillary). *C*, correlation with presence or absence of muscle invasion. *D*, correlation with tumor stage. Tumors were divided into a low-stage (stage 0-II) group or a high-stage (stage III-IV) group. Tumor stage status was available for only 63 of the tumors. *E*, correlation with ploidy status of the tumor (diploid or aneuploid). Ploidy status was available for only 47 of the tumors. *F*, correlation with a number of factors of increased risk. Tumors were divided into a group with zero to two factors or a group with three to five factors (*A-E*, the risk factors).

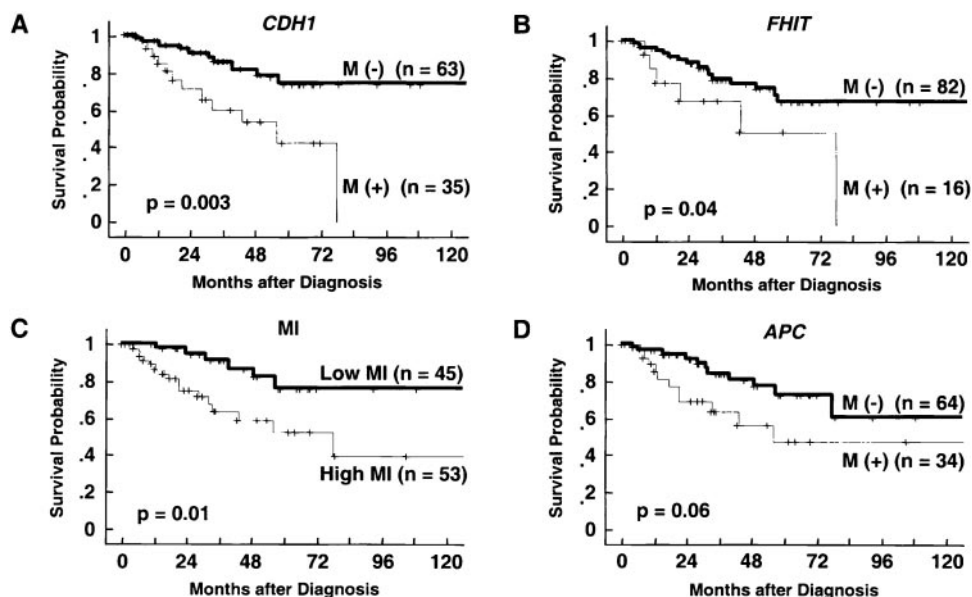


Fig. 3. Correlation of methylation status and patient survival by the Kaplan-Meier method. A, survival curves by methylation status of *CDH1*. B, survival curves by methylation status of *FHIT*. C, survival by MI (median value, 0.2). To get approximately equal numbers in each group, cases were divided into a high-MI group (MI,  $\geq 0.2$ ) and a low-MI group (MI,  $\leq 0.1$ ). D, survival curves by methylation status of *APC*.

stage (Fig. 2D), and aneuploidy (Fig. 2E; Refs. 9, 10). In addition, we correlated the methylation frequencies and MI with the number of risk factors (Fig. 2F). To get approximately equal numbers in each group, tumors were divided into a group with zero to two factors or a group with three to five factors. The MIs of tumors with any of these risk factors were significantly higher than those of tumors that were negative for the factors. Tumors having one or more risk factors (other than aneuploidy) had significantly higher frequencies of methylation of one to four genes, especially *RASSF1A* and *APC*. Ploidy data were available from only a subset of tumors, and, although the methylation frequency of any single gene was not significantly higher in the aneuploid tumor group, this group had a significantly higher MI than the diploid tumor group.

**Relationship between Methylation and Prognosis.** By Kaplan-Meier analysis, neither the individual methylation status of the 10 genes tested, nor the MI, correlated with the recurrence-free intervals. In an analysis of survival by methylation status of these genes, the survival of patients with *CDH1* or *FHIT* methylation-positive tumors was significantly shorter than that of patients with methylation-negative tumors as shown in Fig. 3, A and B. Although there were no significant survival differences between methylation-positive and -negative tumors for the other genes tested, some showed a trend toward worse survival in the methylation-positive group as illustrated for *APC* (Fig. 3D). The median value of MI was 0.2. To get approximately equal numbers in each group, we divided tumors into a low-MI group (MI values,  $\leq 0.1$ ), and a high-MI group (MI values,  $\geq 0.2$ ). The high-MI group had a significantly shorter survival than the low-MI group (Fig. 3C).

In a multivariate analysis model that included tumor grade, papillary or nonpapillary histology, muscle invasion, methylation status of the genes associated with survival (*CDH1*, *RASSF1A*, *APC*, *CDH13*, and *FHIT*), and high- and low-MI groups, *CDH1* methylation-positive status was the only independent methylation-related prognostic factor, as shown in Table 2. Tumor stage and ploidy pattern were excluded from this analysis, because data were available from only a subset of tumors.

## Discussion

Previous studies have described the importance of DNA methylation in human cancers and have focused on regions of the genome that

might have functional significance resulting from the extinction of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes (3), the methylation profiles of individual tumor types are characteristic (3, 4, 11). There is relatively modest information on the methylation profile of bladder cancers (4, 12). Genes that are reportedly methylated at frequencies greater than 20% in bladder cancers include *p16<sup>INK4A</sup>* and *PAX6*, *i.e.*, the deleted-in bladder cancer gene and the 5' end of transmembrane protein containing epidermal growth factor and follistatin domains, respectively (12, 13). Methylation is usually limited to tumor tissues and seldom involves corresponding nonmalignant bladder epithelium (12). Other than *p16<sup>INK4A</sup>*, there is scant knowledge about the role of methylation and inactivation of these genes in tumors other than bladder cancer. Of interest, a recent survey of the methylation profiles of several human tumor types failed to detect methylation of the genes tested at frequencies greater than 20% (4). We determined the methylation profile of bladder cancers testing a panel of 10 genes extensively studied in many tumor types by the widely used MSP assay. Most of the genes we examined had not previously been studied for methylation in bladder cancers. As expected, most of our patients were elderly males with transitional cell carcinoma histology. Because we mainly tested cystectomy specimens, our tumors were more advanced and had more negative prognostic features than most United States cases at initial presentation.

Frequent methylation ( $>20\%$ ) was noted for 4 of the 10 genes tested: *RASSF1A* (35%); *APC* (35%); and two members of the cadherin family,

Table 2. Multivariate statistics of survival

Variable	Hazards ratio	95% confidence interval	P
Tumor grade, high (grade 3)/low (grades 1 or 2)	1.45	0.37–5.75	0.59
Growth pattern, nonpapillary/papillary	0.80	0.25–2.52	0.70
Muscle invasion, invasive/noninvasive	0.98	0.29–3.32	0.97
<i>CDH1</i> methylation	3.30	1.20–9.11	0.02
<i>RASSF1A</i> methylation	1.12	0.36–3.48	0.85
<i>APC</i> methylation	1.95	0.62–6.15	0.25
<i>CDH13</i> methylation	1.23	0.43–3.49	0.70
<i>FHIT</i> methylation	1.66	0.55–4.99	0.37
MI, high MI/low MI	1.05	0.20–5.41	0.95

*CDH1* (36%) and *CDH13* (29%). The methylation frequency of *p16<sup>INK4A</sup>* was relatively low, a finding consistent with that of a recent survey article (4). Of the frequently methylated genes, the most studied in bladder cancer is *CDH1*. Several studies have demonstrated that decreased expression of *CDH1* in bladder cancers, as determined by immunostaining, is associated with decreased survival and with features of advanced stage and poor prognosis (14, 15). *CDH1* is inactivated by methylation in several tumor types including lung, breast, and esophageal cancers (4, 11), but its methylation status has not been described in bladder cancers. We found that methylation of *CDH1* was present in 36% of bladder cancers and correlated with nonpapillary growth pattern and poor survival. In multivariate models, *CDH1* was the only independent methylation-related prognostic factor.

Our gene panel included *RASSF1*, a recently identified putative TSG. There are two major *RASSF1* gene products, *RASSF1A* and *RASSF1C*. Selective promoter methylation of the *RASSF1A* promoter, but not of *RASSF1C*, is frequent in small cell- and non-small cell lung cancers and in breast carcinomas (16, 17). The *RASSF1A* promoter was methylated frequently in bladder cancers (35%), and methylation was associated with features of poor prognosis (high tumor grade, nonpapillary growth pattern, and muscle invasion). These findings indicate a potential role for *RASSF1A* in the pathogenesis and spread of bladder cancer.

Inactivation of the *APC* gene is frequent in colorectal and other gastrointestinal carcinomas, usually by truncating mutations (18). An alternative method of inactivation of the gene in some gastrointestinal tumors is by promoter methylation (19). Recently, we reported that selective methylation and silencing of the 1A promoter and its specific products was frequent in lung and breast cancers (20). In this report, we found methylation of the *APC* 1A promoter in 35% of bladder cancers, and the methylation correlated with high tumor grade, nonpapillary growth pattern, muscle invasion, and high tumor stage.

Several members of the cadherin gene family, including *CDH1* and *CDH13*, are located on chromosome 16q, a region of frequent allelic loss in multiple tumor types (21). Inactivation of *CDH13* by promoter methylation has been described in lung and breast cancers (22, 23). We found methylation of *CDH13* in 29% of bladder cancers, and methylation was associated with high tumor grade and nonpapillary growth pattern.

Inactivation of the *FHIT* gene is common in many forms of cancers (24). And reduced immunostaining is present in most bladder carcinomas (25). Although we found promoter methylation of *FHIT* in only 16% of bladder cancers, it was associated with poor survival. Promoter methylation of *DAPK*, *MGMT*, and the P2 promoter of *RAR $\beta$* , *GSTP1*, and *p16<sup>INK4A</sup>* genes was present in relatively few bladder cancers and was not associated with parameters of poor prognosis.

We also analyzed the MI, an index of total methylation. Tumors with features of poor prognosis (high grade, nonpapillary growth pattern, muscle invasion, high tumor stage, and aneuploidy) had a significantly higher MI than tumors without these features. High-MI tumors had a significantly shortened survival.

Our results indicated that multiple genes are methylated during the process of bladder cancer development. We found frequent methylation of four genes (*CDH1*, *RASSF1A*, *APC*, and *CDH13*). Methylation of these genes and the MI correlated with one or more parameters of worse prognosis, and with shortened survival. *CDH1* methylation-positive status was independently associated with poor survival in multivariate analyses. The methylation profile may represent a potential new biomarker of risk prediction in bladder cancer.

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