Featured Article

Detection of Methylated Apoptosis-Associated Genes in Urine Sediments of Bladder Cancer Patients

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ABSTRACT

Purpose: There is increasing evidence for a fundamental role for epigenetic silencing of apoptotic pathways in cancer. Changes in DNA methylation can be detected with a high degree of sensitivity, so we used the MethylLight assay to determine how methylation patterns of apoptosis-associated genes change during bladder carcinogenesis and whether DNA methylation could be detected in urine sediments.

Experimental Design: We analyzed the methylation status of the 5′ regions of 12 apoptosis-associated genes (ARF, FADD, TNFRSF21, BAX, LITAF, DAPK, TMS-1, BCL2, RASSF1A, TERT, TNFRSF25, and EDNRB) in 18 bladder cancer cell lines, 127 bladder cancer samples, and 37 samples of adjacent normal bladder mucosa using the quantitative MethylLight assay. We also analyzed the methylation status in urine sediments of 20 cancer-free volunteers and 37 bladder cancer patients.

Results: The 5′ regions of DAPK, BCL2, TERT, RASSF1A, and TNFRSF25 showed significant increases in methylation levels when compared with nonmalignant adjacent tissue (P < 0.01). Methylation levels of BCL2 were significantly associated with tumor staging and grading (P ≤ 0.01), whereas methylation levels of RASSF1A and ARF were only associated with tumor stage (P ≤ 0.04), and TERT methylation and EDNRB methylation were predictors of tumor grade (P ≤ 0.02). To investigate clinical usefulness for noninvasive bladder cancer detection, we further analyzed the methylation status of the markers in urine samples of patients with bladder cancer. Methylation of DAPK, BCL2, and TERT in urine sediment DNA from bladder cancer patients was detected in the majority of samples (78%), whereas they were unmethylated in the urine sediment DNA from age-matched cancer-free individuals.

Conclusions: Our results indicate that methylation of the 5′ region of apoptosis-associated genes is a common finding in patients with bladder carcinoma. The ability to detect methylation not only in bladder tissue, but also in urine sediments, suggests that methylation markers are promising tools for noninvasive detection of bladder cancers. Our results also indicate that some methylation markers, such as those in regions of RASSF1A and TNFRSF25, might be of limited use for detection because they are also methylated in normal bladder tissues.

INTRODUCTION

With more than 50,000 newly diagnosed cases in the United States each year, bladder cancer is one of the most frequent cancers. Approximately 75% to 85% of patients present with superficial disease that is confined to either the mucosa or the submucosa. Although these tumors can be removed by transurethral resection, more than 50% of them will recur (1). In addition, depending on various risk factors including tumor stage, grade, tumor size, and tumor focality, up to 40% of the patients will suffer from tumor progression, and 30% of the patients will die from initially non–muscle-invasive bladder cancers (2). In contrast to muscle-invasive carcinoma, for which treatment often consists of radical cystectomy, the development of reliable prognostic and diagnostic markers to improve strategies of disease management for patients with non–muscle-invasive cancer is crucial. Surveillance of patients with bladder cancer is performed by cystoscopy, but there is a clear need for noninvasive procedures for detection of transitional cell carcinoma of the bladder, whether at initial diagnosis or during follow-up.

Aberrant promoter methylation is commonly associated with the loss of gene function, thereby providing a selective advantage to neoplastic cells (3). Consequently, promoter methylation has been proposed to be a third pathway of carcinogenesis according to Knudson’s “two-hit hypothesis” (4). Hypermethylation of promoter regions of tumor suppressor genes is one of the most well-categorized epigenetic changes in human neoplasia, and DNA methylation defects in bladder transitional cell carcinomas have been also described. Recent work from our...
laboratory (5–8) has described progressive increases in the *de novo* methylation of CpG islands in bladder cancer cells, suggesting that epigenetic gene silencing is involved in the development and recurrence of urothelial carcinoma (9). It is, however, important to emphasize that methylation of CpG sites downstream of the transcription initiation site does not block expression (10). Therefore, the fact that a CpG island becomes abnormally methylated may yield a tumor marker but may not necessarily be associated with gene silencing.

Apoptotic pathways are targets for epigenetic silencing (11), and several apoptosis-linked genes [RASSF1A (12), CDH1 (13–15), TMS-1 (16), APAF-1 (17), p14(ARF) (18), BCL2 (19), and EDNRB (20)] that are regulated directly or indirectly by methylation have been described. It also has been reported that methylation of DAPK is associated with bladder cancer recurrence (9). In addition to these genes, other apoptosis-related genes, such as FADD (21), TNFRSF25 (22), TNFRSF21 (23), LITAF (24), and BAX (25), may also be targets for methylation-associated gene silencing because each gene harbors a CpG island in its 5′ region.

The aim of the current study was to analyze the methylation status of the 5′ regions of genes related to apoptosis and, in particular, to correlate the methylation status of certain genes with the tumor phenotype. Another goal was to investigate whether methylated genes or markers could be found in urine sediments as a possible tool for noninvasive detection of bladder cancer. Methylation status was quantitatively studied using the methylation-sensitive quantitative PCR (qPCR) technique and is capable of detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles (26). We tested 12 urethral resection samples from 119 patients undergoing transurethral resection of a primary bladder tumor at the Department of Urology at the University Hospital Hamburg (Hamburg, Germany). We also collected tumor samples from three patients who showed tumor recurrence; therefore, a total of 122 consecutive tumor samples were obtained. An additional 22 cystectomy specimens (tumor tissue and corresponding adjacent nonmalignant bladder tissue) were obtained from the tissue bank of the University of Southern California/Norris Comprehensive Cancer Center. The age of patients ranged between 33 and 86 years, with a median age of 68 years. Pathological diagnosis was established using hematoxylin and eosin (H&E)-stained sections, and the tumors were classified according to the 5th edition of the International Union Against Cancer (UICC) and the World Health Organization (28).

Microdissection was performed in samples from 122 consecutive patients undergoing transurethral resection for bladder carcinoma. One to three consecutive sections (H&E stained) were dewaxed, hydrated, and stained briefly with H&E. Tumor tissue was removed from the slides under a microscope using a sterile needle. The microdissected tissue fragments were digested with 1 mg/mL proteinase K (Sigma-Aldrich, Deisenhofen, Germany), and DNA was extracted using a commercially available kit (QiAamp DNA Mini Kit; Qiagen, Hilden, Germany). Bisulfite conversion was described previously (29).

Before DNA extraction, a H&E-stained slide was prepared for each tumor specimen collected to verify the presence of cancer cells. DNA was prepared using standard methods with a commercially available kit (QiAamp DNA Mini Kit; Qiagen).

**MATERIALS AND METHODS**

**Bladder Carcinoma Cell Lines.** Human bladder cancer cell lines were used, including those that are commercially available (T24, J82, HT1376, SCaBER, UMUC-3, TCCSUPP, and RT4; American Type Culture Collection, Manassas, VA). Cell lines derived in our laboratory (those with the prefix LD) and a cell line from normal bladder fibroblasts (LD419), which served as a negative control, have been described previously (27). Cell culture, DNA isolation, and DNA purification were performed as described previously (27).

**Tissue Collection and DNA Isolation.** We included tumor material from 119 patients undergoing transurethral resection of a primary bladder tumor at the Department of Urology at the University Hospital Hamburg (Hamburg, Germany). We also collected tumor samples from three patients who showed tumor recurrence; therefore, a total of 122 consecutive tumor samples were obtained. An additional 22 cystectomy specimens (tumor tissue and corresponding adjacent nonmalignant bladder tissue) were obtained from the tissue bank of the University of Southern California/Norris Comprehensive Cancer Center. The age of patients ranged between 33 and 86 years, with a median age of 68 years. Pathological diagnosis was established using hematoxylin and eosin (H&E)-stained sections, and the tumors were classified according to the 5th edition of the International Union Against Cancer (UICC) and the World Health Organization (28).

**Table 1 Design and location of the oligonucleotides used for quantitative methylation-sensitive real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternate name</th>
<th>GenBank accession no.</th>
<th>Amplicon location (relative to transcription start sites)</th>
<th>Location of amplicon</th>
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</thead>
<tbody>
<tr>
<td>ARF</td>
<td>p14</td>
<td>AF082338</td>
<td>−203/−135</td>
<td>Promoter</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas (TNFRSF6)-associated via death domain</td>
<td>NM_003824</td>
<td>−138/−78</td>
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<tr>
<td>TNFRSF21</td>
<td>TNF receptor superfamily, member 2</td>
<td>NM_014432</td>
<td>−426/−327</td>
<td>Promoter</td>
</tr>
<tr>
<td>LITAF</td>
<td>LPS-induced TNF receptor α factor</td>
<td>NM_004862</td>
<td>−238/−143</td>
<td>Promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein, isoform β</td>
<td>NM_138762</td>
<td>−91/+169</td>
<td>Exon 1</td>
</tr>
<tr>
<td>TMS1</td>
<td>Apoptosis-associated speck-like protein (ASC)</td>
<td>NM_013258</td>
<td>+25/+105</td>
<td>Exon 1</td>
</tr>
<tr>
<td>DAPK</td>
<td>Death-associated protein kinase 1</td>
<td>X76104</td>
<td>+137/+204</td>
<td>Exon 1</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase</td>
<td>AF325900</td>
<td>−223/−108</td>
<td>Promoter</td>
</tr>
<tr>
<td>BCL2</td>
<td>bcl-2 (B-cell lymphoma type 2)</td>
<td>NM_000633</td>
<td>+210/+293</td>
<td>Exon 1</td>
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<tr>
<td>EDNRB</td>
<td>EDNRB, endothelin receptor B, Cpg island 2</td>
<td>NM_003991</td>
<td>+12/+104</td>
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<tr>
<td>RASSF1A</td>
<td>Ras association (RalGDS/AF-6) domain family 1</td>
<td>NM_007182</td>
<td>+57/+121</td>
<td>Exon 1</td>
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<tr>
<td>TNFRSF25</td>
<td>Death receptor 3</td>
<td>AB051850</td>
<td>+27/+95</td>
<td>Exon 1</td>
</tr>
<tr>
<td>ACTB</td>
<td>β-Actin</td>
<td>L10347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL2A1</td>
<td>Collagen type 2</td>
<td></td>
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</table>

Abbreviations: TNF, tumor necrosis factor; LPS, lipopolysaccharide.
Urine Sediment Specimens. To evaluate whether DNA methylation could be detected in urine sediments, urine samples were obtained from 37 patients (average age, 63 years) undergoing radical cystectomy due to bladder carcinoma and 10 healthy volunteers with no smoking history (average age, 29 years) and another 10 healthy volunteers (average age, 56 years). Urine sediments were prepared by centrifugation of 50 mL of fresh urine at 1,500 rpm for 15 minutes. The supernatant was decanted, and DNA was purified using QIAamp DNA Mini Kit (Qiagen).

Quantitative Methylation-Sensitive Real-Time Polymerase Chain Reaction. Methylation analysis was performed with fluorescence-based real-time PCR (MJ Research, Waltham, MA) similar to the MethyLight technique as described previously (26). Each primer/probe set was designed to amplify a bisulfite-converted sequence of the promoter region overlapping at least five CpG sites (Table 1). The genes analyzed, their GenBank sequence accession numbers, the location of the PCR primer/amplification site, the transcription start site, and the primer/probe sequences used for real-time PCR are all shown in Table 1.

Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was reverse transcribed using 2 μg of RNA and random hexamers, deoxynucleotide triphosphates (Boehringer Mannheim, Mannheim, Germany), and Superscript II reverse transcriptase (Life Technologies, Inc., Palo Alto, CA) in a 50-μL reaction. The mixture was placed at room temperature for 10 minutes, 42°C for 45 minutes, and 90°C for 3 minutes and then rapidly cooled to 0°C. Complementary DNA (100 ng) was PCR amplified using the following conditions: for DAPK, BCL2, TERT, RASSFIA, and TNFRSR25 expression, 1 cycle of 95°C for 2 minutes followed by 95°C for 1 minute, 60°C for 1 second, and 72°C for 1 minute and 30 seconds for 28 to 30 cycles and 1 cycle of 72°C for 4 minutes. The PCR conditions for GAPDH were one cycle of 94°C for 2 minutes, followed by 20 cycles of 94°C for 1 minute, 58°C for 30 seconds, and 72°C for 45 seconds and 1 cycle of 72°C for 2 minutes. The PCR primers used were as follows: DAPK, 5'-T-GACAGTTTATCATGACCGTGTTTCAG-3' (sense) and 5'-G-TGCTGGATCTCTTCAAGATAG-3' (antisense); BCL2, 5'-T-TG-GAGATCAGTTAATCTGACACACTGAC-3' (sense) and 5'-AGCTTT-CATGGCTGAACCATCAGTAC-3' (antisense); TERT, 5'-GAGCTGTGAGGGAATAGTAAGTGAAGTGAAGTGTTGTCG-3' (sense) and 5'-GCTTCAAGAATTTAAAGATAGTCG-3' (antisense); RASSFIA, 5'-CCTCTTGAGGCTGTTCTCTCTCAG-3' (sense) and 5'-GCTTCAAGAATTTAAAGATAGTCG-3' (antisense); and GAPDH, 5'-CAGCCC-AGGCACATCG-3' (sense) and 5'-TGAGGCTGTTGTGTCATACTTCTC-3' (antisense).
Statistical Analysis. Differences in PMR values in paired samples of tumor and normal tissue adjacent to the tumor were tested using a paired t test. First, for each gene, the PMR values were ranked in the combined sample of tumor and normal tissues, and tied PMRs were assigned to the average rank. The differences in the ranks between paired samples of tumor and normal adjacent tissue were then analyzed. The absolute values of these differences were ranked a second time; in this case, for the differences that were negative in value, a negative rank was assigned. The signed ranks were then analyzed using a one-sample t test in which differences of zero were included.

Changes in methylation levels across categories of stage and grade were tested using linear regression. For each gene, the PMR values in the tumor tissue were ranked; the ranked PMR values were regressed on stage or grade, adjusting for the study sample. Using a linear coding for stage (1 = pT1, 2 = pT2, and 3 = pT3–4) and grade (grade 1, 2, and 3), we tested for trend across each category, and each sample was coded using an indicator variable (0 = German sample, 1 = tissue bank sample). Analyses were conducted using SPLUS version 6.1. All statistical testing was performed using a two-tailed significance level of 0.05.

RESULTS

Evaluation of Methylation and Expression in Human Bladder Cancer Cell Lines. The MethyLight primer/probe reaction for each gene was located within 500 bp on either side of the potential transcription start site (Table 1). Examples of typical MethyLight reactions using DNA from LD137 and J82 cells are shown in Fig. 1A. Both DNAs showed positive reactions for the two control genes COL2A1 and ACTB, which are not methylated and were included to quantify the amount of DNA present (26, 29). LD137 DNA showed positive results for TERT, EDNRB, and RASSF1A, indicating that those genes were methylated, whereas BCL2 and TERT were negative for methylation. On the other hand, J82 DNA was positive for methylation of TERT, but negative for the other three genes, respectively.

The methylation status of the 5′ regions of 12 apoptosis-associated genes was assessed by MethyLight analysis in 18 bladder cancer cell lines, as well as in normal fibroblasts (LD419) as a negative control (Fig. 1B). The control cell line LD419 and the RT4 cell line (derived from a low-grade tumor) did not display methylation in any of the loci examined, and none of the cell lines displayed methylation of ARF, FADD, TNFRSF21, or BAX. Methylation of LITAF was only detected in UM-UC-3 cells, whereas DAPK showed methylation in SCaBER cells (a squamous cell carcinoma cell line) and UM-UC-3 cells. TMS-1 and BCL2 showed methylation in several of the bladder cancer cell lines, and TERT, EDNRB, RASSF1A, and TNFRSF25 were highly methylated in the majority of the bladder cancer cell lines. These data show that substantial levels of methylation in the 5′ regions of apoptosis-related genes are present in human bladder cancer cell lines.

We have previously found that methylation 5′ of the transcription start site is much more likely to be associated with gene inactivity than downstream methylation (4). The MethyLight PCR amplifications of BAX, TMS-1, DAPK, BCL2, RASSF1A, EDNRB, and TNFRSF25 were located in exon 1 of each gene that is downstream of the relevant transcriptional start sites (Table 1). This was because of the experimental ease of examining the particular region and because transcriptional start sites are often reassigned in a 5′ direction as more detailed analysis of genes continues. To further understand the potential correlation between DNA methylation and expression in these studies, we performed reverse transcription-PCR to examine the expression of DAPK, BCL2, TERT, RASSF1A, and TNFRSF25, which are commonly methylated bladder tumor cell lines (Fig. 1C). EDNRB has been well studied by our laboratory (20). Our results indicated good correlations between methylation and lack of expression of DAPK and RASSF1A, but not for BCL2, TERT, and TNFRSF25. Silencing of DAPK seems to be correlated with methylation, but lack of methylation did not equate with expression in LD419 and T24 cells (Fig. 1C). The down-regulation of DAPK can be caused not only by DNA methylation but also by other transcriptional control mechanisms.

Evaluation of Methylation in Bladder Carcinoma Samples. We next analyzed the methylation status of the same 12 apoptosis-associated genes described above in 37 samples of normal adjacent bladder mucosa and 127 bladder tumor samples of differing stages and grades (Fig. 2A). ARF, FADD, TNFRSF21, LITAF, BAX, TMS-1, and TERT were unmethylated (PMR < 5) in almost all of the nonmalignant adjacent tissue as well as the tumor samples. Whereas DAPK appeared to be methylated at low levels in adjacent normal bladder mucosa, its methylation in the tumor samples was more pronounced. BCL2 was only methylated in 7 of 35 normal adjacent samples, but it was methylated in 65 of 125 tumor samples. TERT, although unmethylated in most normal tissue, showed an increase in methylation in 31 of 123 bladder tumor samples. Both EDNRB and RASSF1A displayed methylation in the normal tissue and were more highly methylated in the bladder carcinoma samples. Whereas a high proportion of the normal adjacent samples displayed methylation of TNFRSF25, this gene was almost completely methylated in the majority of bladder tumor samples analyzed (PMR > 75). These data, in agreement with the bladder cancer cell line methylation data (Fig. 1B), demonstrated that substantial methylation of apoptosis-related genes, in particular, BCL2, TERT, RASSF1A, EDNRB, and TNFRSF25, is a common finding in uncultured human bladder cancer.

Comparison of Methylation Levels from Normal and Tumor Tissue and Correlation with Tumor Staging and Tumor Grading. Next we used a statistical approach to compare the methylation levels in 37 samples of adjacent normal and tumor bladder tissue to find a possible discrimination between normal bladder samples and tumor samples. Increased methylation of DAPK, BCL2, TERT, RASSF1A, and TNFRSF25 compared with adjacent normal bladder mucosa were all statistically significant as generated by the paired t test of the signed ranks in bladder cancer patients (Table 2). PMR levels were then correlated with histopathological staging and grading. In 127 tumor samples, the methylation levels of ARF, RASSF1A, and BCL2 were significantly correlated with staging (P values of

Fig. 1. A, examples of MethyLight real-time PCR reactions in two bladder cancer cell lines. In LD137 cells, the strong fluorescence curves for TNFRSF25, EDNRB, and RASSF1A indicate the presence of DNA methylation in these genes. An absence of fluorescence signal for BCL2 and TERT shows that these genes are not methylated. In J82 cells, TNFRSF25 and TERT are methylated, whereas RASSF1A, BCL2, and EDNRB are unmethylated. In both panels, COL2A1 and ACTB were used as bisulfite-specific control reaction. Details of this method are described in Materials and Methods.

B, quantitative methylation analysis of 12 apoptosis-related genes in 18 bladder cancer cell lines and the control LD419 cell line by MethyLight analysis. The range of PMR levels that correspond to different colors in the figure is shown. HD indicates a homozygous deletion of the ARF(p14) gene. C, reverse transcription-PCR analyses of gene expression and DNA methylation status of five genes in eight bladder cell lines. The colored squares indicate the same range in PMR values as defined in B.
0.04, 0.003, and 0.01, respectively), whereas BCL2, TERT, and EDNRB were significantly correlative with tumor grading (P values of 0.001, 0.02, and 0.01, respectively; Table 2). These data suggest that the methylation of these genes in bladder cancer can be a predictor of tumor stage and/or grade and may therefore have potential clinical utility.

Detection of Methylation of DAPK, BCL2, TERT, EDNRB, RASSF1A, and TNFRSF25 in Urine Sediments.

We next determined whether methylation could be detected using the MethyLight assay in urine sediment as a possible noninvasive technique for bladder cancer detection. The methylation levels in the 5' regions of DAPK, BCL2, TERT, EDNRB, RASSF1A, and TNFRSF25 genes in urine sediment samples from patients with bladder cancer (n = 37) and controls (n = 20) using MethyLight analysis. The urine sediment samples from bladder cancer patients were subdivided by tumor stage and grade. The colored squares indicate the same range in PMR values as described in Fig. 1. White squares (N/A) indicate that data were not available for that locus.
RASSF1A, and TNFRSF25 were analyzed from urine sediments of 37 bladder cancer patients differing in stage. We also included DNA urine sediment samples from two groups of cancer-free individuals, one group with an average age of 29 years with no smoking history and another group with an average age of 56 years with unknown smoking history (Fig. 2). RASSF1A, and TNFRSF25 were methylated in the majority of the urine samples from cancer-free and bladder cancer patients, suggesting that these would not be appropriate markers for detection of bladder cancer. Because methylation of EDNRB, RASSF1A, and TNFRSF25 was found in the younger cancer-free group with no smoking history, this methylation cannot be directly related to smoking. Interestingly, it appeared that some enhanced methylation was present in the sediments of the older group of volunteers, but additional studies will be needed to investigate this. Methylation of DAPK, TERT, and BCL2, although almost completely undetectable in urine sediments from 20 cancer-free individuals, was hypermethylated in the urine sediments from bladder cancer patients. Methylation was detected in 22% (8 of 37), 51% (18 of 37), and 65% (24 of 37) of the samples, respectively (Fig. 2B), suggesting that DAPK, TERT, and BCL2 may be promising markers for bladder cancer detection. Combining the frequencies of methylation of DAPK, TERT, and BCL2 in the 37 urine samples from bladder cancer patients, an overall detection level of 78% (29 of 37) was achieved compared with pathology reports (Table 3), suggesting that the combined methylation analyses of the three genes provided a high sensitivity for a noninvasive detection of bladder cancer.

**DISCUSSION**

Herein we present the first comprehensive quantitative analysis of the methylation status of 12 apoptosis-associated genes in bladder cancer. Methylation was detected for eight apoptosis-associated genes, including ARF, TMS-1, DAPK, BCL2, TERT, EDNRB, RASSF1A, and TNFRSF25, which are also methylated in other cancer entities (9, 16, 19, 20, 22, 30–32).

Two different groups of genes were identified by analysis of methylation patterns in cell lines. Methylation levels of the first group of apoptotic-related genes, ARF, FADD, TNFRSF21, LITAF, BAX, and TMS-1, did not discriminate between normal and tumor tissue, and only ARF methylation was significantly correlated with tumor staging. Therefore, none of the genes in either group appeared to be a useful marker for bladder cancer diagnosis. Because non–muscle-invasive cancer has a recurrence rate of 30% to 50%, it seems implausible that any gene of these groups might be predictive for disease recurrence.

The second group consisted of DAPK, TERT, BCL2, EDNRB, RASSF1A, and TNFRSF25. All six genes, except EDNRB, had significantly higher methylation levels in tumor samples when compared with adjacent noncancerous tissue. We found that the 5’ region of BCL2 (downstream of the transcription start site, from +210 to +293) was methylated in tumor specimens, showing a high discrimination between normal and tumor tissue. This finding is interesting because DNA hypermethylation is often associated with decreased gene expression, and in the case of BCL2, this would be expected to promote apoptosis rather than tumor growth (33). However, our results with cell lines indicate that BCL2 can still be expressed even though its 5’ region is methylated, and similar results were also found for TERT and TNFRSF25. Indeed, methylation of the TERT promoter region does not correlate with silencing as others have reported (32). This might be due to the existence of multiple promoters, or this region might not be the true promoter region. The relationship between DNA methylation and BCL2 mRNA expression and other apoptosis-associated genes will be the focus of future studies. Whereas these changes are unlikely to be associated with inhibition of gene expression, they nevertheless represent valuable tumor markers.

In addition, we showed that BCL2 methylation was significantly associated with tumor staging and grading, whereas TERT and EDNRB methylation were associated with tumor grade, and RASSF1A methylation was statistically correlated with tumor staging. The 5’ region of RASSF1A has been shown to be methylated in 48% of bladder tumors (12). In our study, we detected RASSF1A promoter methylation in 60% of the tumor samples and in 42% of the samples from normal mucosa. The finding that RASSF1A was methylated not only in tumor samples but also in nonmalignant specimens limits its clinical value of detection of methylation, even though the methylation levels were significantly higher in the tumors than in corresponding normal samples from the same individual (Table 2). Similar results were also found for TNFRSF25 and EDNRB methylation.

To maintain high statistical power to detect associations, all statistical testing was conducted at the 0.05 (5%) level. However,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sensitivity* No. (%)</th>
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<tbody>
<tr>
<td>BCL2</td>
<td>24/37 (64.9)</td>
</tr>
<tr>
<td>TERT</td>
<td>19/37 (51.4)</td>
</tr>
<tr>
<td>DAPK</td>
<td>8/37 (21.6)</td>
</tr>
<tr>
<td>Specificity</td>
<td>29/37 (78.4)</td>
</tr>
</tbody>
</table>

* Sensitivity was defined as the percentage of methylated samples among urine samples from tumor patients (n = 37).
because we analyzed 12 genes, there is a probability that some of our results are not real associations but rather are due to natural fluctuations in the data. If we correct for multiple comparisons by controlling the false discovery rate at 5\%, we still find statistically significant results. Using the multiple comparison adjustment proposed by Benjamini et al. (34), we found that four of the five genes differentially methylated in normal and tumor tissue achieve statistical significance (RASSF1A, BCL2, TERT, and TNFRSF25; Table 2). Furthermore, the associations between RASSF1A and tumor stage and between BCL2 and tumor grade are also statistically significant after controlling the false discovery rate. We therefore have reason to believe our results suggest using DNA methylation in characterizing bladder cancer.

There is increasing interest in the clinical utility of methylation markers (35), and it is well established that DNA alterations can be detected in body fluid specimens such as urine (31, 36–38). An increasing number of reports show that DNA methylation markers can be used for the detection of cancer in various body fluids including plasma, serum, urine, ductal lavage, saliva, sputum, and bronchioalveolar lavage (35). Chan et al. (31) described a sensitivity for detection of methylation of DAPK, RAR\beta, CDH1, and p16 in 46\%, 68\%, 59\%, and 14\% of bladder cancer cases, respectively. The same group reported a sensitivity of 50\% for the methylation status of the RASSF1A gene (12). Recently, Dulaimi et al. (39) also showed a high correlation (87\%) between methylation patterns in urine sediments and primary tumors.

Urine sediment samples from control individuals displayed methylation of EDNRB, RASSF1A, and TNFRSF25, and because these were seen in individuals who were nonsmokers, they cannot be caused by smoking. Methylation of TERT, DAPK, and BCL2 was detected in 78\% of the urine samples from bladder cancer patients (Table 3). It is interesting that whereas none of the control urine samples displayed methylation of DAPK, BCL2, and TERT, these genes were shown to be hypermethylated in nonmalignant bladder mucosa from bladder cancer samples (Fig. 2A and B). It seems reasonable that this methylated subfraction can originate from contamination of tumor DNA. Otherwise, this may be caused by premalignant alteration of the adjacent mucosa in bladder cancer patients. Nonetheless, our results confirm the ability to detect methylation in urine samples as a diagnostic tool for bladder cancer detection. Using a panel of three markers (DAPK, TERT, and BCL2), we could detect methylation in nearly 80\% of the cases. Our results also indicated that some methylation markers such as EDNRB, RASSF1A, and TNFRSF25 might be of no use for detection of bladder cancer because these regions are also methylated in cancer-free individuals. It seems reasonable that an extension of the marker panel might result in a higher sensitivity with methylation analysis in the urine, therefore making this a promising tool for noninvasive bladder cancer detection. This finding and the identification of other methylation markers in bladder cancer will be the focus of future studies.

REFERENCES


Detection of Methylated Apoptosis-Associated Genes in Urine Sediments of Bladder Cancer Patients
