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 MethyLight 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 MethyLight 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*, *RASSFIA*, and *TNFRSF25* showed significant increases in methylation levels when compared with nonmalignant adjacent tissue ($P \leq 0.01$). Methylation levels of *BCL2* were significantly associated with tumor staging and grading ($P \leq 0.01$), whereas methylation levels of *RASSF1A* and *ARF* were only associated with tumor stage ($P \leq 0.04$), and *TERT* methylation and *EDNRB* methylation were predictors of tumor grade ($P \leq 0.02$). 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

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

Gene	Alternate name	GenBank acession no.	Amplicon location (relative to transcription start sites)	Location of amplicon
ARF	p14	AF082338	−203/−135	Promoter
FADD	Fas (TNFRSF6)-associated via death domain	NM_003824	−138/−78	Promoter
TNFRSF21	TNF receptor superfamily, member 2	NM_014452	−426/−327	Promoter
LITAF	LPS-induced TNF-α factor	NM_004862	−238/−143	Promoter
BAX	BCL2-associated X protein, isoform β	NM_138762	+91/+169	Exon 1
TMS1	Apoptosis-associated speck-like protein (ASC)	NM_013258	+25/+105	Exon 1
DAPK	Death-associated protein kinase 1	X76104	+137/+204	Exon 1
TERT	Telomerase	AF325900	-223/-108	Promoter
BCL2	bcl-2 (B-cell lymphoma type 2)	NM_000633	+210/+293	Exon 1
EDNRB	EDNRB, endothelin receptor B, CpG island 2	NM_003991	+12/+104	Exon 1
RASSF1A	Ras association (RalGDS/AF-6) domain family 1	NM_007182	+57/+121	Exon 1
TNFRSF25	Death receptor 3	AB051850	+27/+95	Exon 1
ACTB	β-Actin			
COL2A1	Collagen type 2	L10347		

Abbreviations: TNF, tumor necrosis factor; LPS, lipopolysaccharide.

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 *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 MethyLight technique, which utilizes fluorescence-based real-time polymerase chain reaction (PCR; TaqMan) technology and is capable of detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles (26). We tested 12 genes that are associated with the apoptotic pathway for their 5′ region methylation in bladder cancer cell lines, clinical samples from patients with non–muscle-invasive and muscle-invasive tumors, and the urine sediment DNA from apparently cancer-free and bladder cancer patients. Our results indicate that methylation of the 5′ region apoptosis-related genes is a common finding in patients with bladder carcinoma. The ability to detect methylation not only in bladder tissue but also in urine suggests that methylation markers are promising tools for noninvasive detection of bladder cancers.

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).

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).

Table 1 Continued

Forward primer sequence	Reverse primer sequence	Probe sequence (5′ with 6FAM and 3′ with BHQ1)
ACGGGCGTTTTCGGTAGTT	CCGAACCTCCAAAATCTCGA	CGACTCTAAACCCTACGCACGCGAAA
CGTTCGCGGCGTCG	GCGCATACGTCCCTAACGA	CCGCAACCGTTTCCGCCCTAACTA
TCGGCGTTATTACGTGTGTTTTT	TTCCAACCACTACCAACCACC	CCTCCGCTACCCAAACTAATCGACGAA
GGTTGGGTTTAGTTTTTGCGTT	GCGACGAACCGCGAAA	CCACGCGCGATCGAACCACTCT
CGGGAGGTAGACGGGCG	CAACCCCAAACCGATAAAAAAA	AGGGCGAGTTTTTTCGTCGGTTCG
CGTTGGAGAATTTGATCGTCG	CCCGTAACCCTCGCGCAA	AGTTTAAGAAGTTTAAGTTGAAGTTGTTGTCGGTGTCG
TCGTCGTCGTTTCGGTTAGTT	TCCCTCCGAAACGCTATCG	CGACCATAAACGCCAACGCCG
GGATTCGCGGGTATAGACGTT	CGAAATCCGCGCGAAA	CCCAATCCCTCCGCCACGTAAAA
TCGTATTTCGGGATTCGGTC	AACTAAACGCAAACCCCGC	ACGACGCCGAAAACAACCGAAATCTACA
CGTTGGCGAGTTATGAGCGTT	TACCGCCCGCAACCTCT	CGCGTAAATTTGAGTTATTTTTGAGCGTGGA
ATTGAGTTGCGGGAGTTGGT	ACACGCTCCAACCGAATACG	CCCTTCCCAACGCGCCCA
GCGGAATTACGACGGGTAGA	ACTCCATAACCCTCCGACGA	CGCCCAAAAACTTCCCGACTCCGTA
TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAACACA
TCTAACAATTATAAACTCCA-ACCACCAA	GGGAAGATGGGATAGAAGGGAATAT	CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA

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 amplicon relative to the transcription start site, and the primer/probe sequences used for real-time PCR are all shown in Table 1. PCR conditions consisted of an initial denaturation for 10 minutes at 91°C followed by 50 cycles of denaturation for 15 seconds at 91°C and annealing/extension for 1 minute at 59°C. Reactions were performed for non–CpG-containing regions of two control genes, β-actin (*ACTB*) and collagen IIA (*COL2A1*). Genomic DNA (Promega, Madison, WI) treated with *Sss*I DNA methyltransferase (New England Biolab) was used as a fully methylated reference. The percentage of fully methylated DNA was expressed as the percentage of fully methylated reference (PMR) and calculated as the ratio of the methylation of a gene of interest normalized to each control gene relative to that of the *Sss*I-treated sample (26). PMR values were classified as six ranges: $<0.1\%$, 0.1% to 3.9%, 4.0% to 19.9%, 20.0% to 49.9%, 50.0% to 74.9%, and $\geq 75.0\%$. Occasionally, PMR values were $>100\%$ in cases when the *Sss*I treatment of the standard DNA was not complete or in cases of aneuploidy of the gene locus of interest.

The study sample for the analysis of 12 apoptosis-associated genes was composed of 122 consecutive cases (119 primary tumors and 3 recurrences) as well as 22 tissue bank samples (a total of 144 tumor samples). We collected data on 1,324 of 1,728 possible measurements (76.6% success rate). Seventeen of the cases (16 primary and 1 recurrence) did not yield any methylation data, reducing our analyzed sample size to 127 tumors (105 primary or recurrence and 22 tissue bank samples).

MethyLight analysis for at least one locus was also performed on 37 normal tissue samples (16 adjacent to primary tumor and 21 adjacent to tissue bank sample). In this subsample, 368 of 468 possible measurements were obtained (81% success rate).

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*, *RASSF1A*, 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′-TGACAGTTTATCATGACCGTGTTCAG-3′ (sense) and 5′-GTGCTGGATCTCCTTCAGGAT-3′ (antisense); BCL2, 5′-TGTGGATGACTGAGTACCTGAAC-3′ (sense) and 5′-AGCTTTGTTTCATGGAACATCACTGAC-3′ (antisense); TERT, 5′-GAGCTGACGTGGAAGATGAG-3′ (sense) and 5′-CTTCAAGTGCTGTCTGATTCCAATG-3′ (antisense); RASSF1A, 5′-CTCTGTGGCGACTTCATCTG-3′ (sense) and 5′-GCTTCAGCTGAACCTTGATGAAG-3′ (antisense); TNFRSF25, 5′-GACTTCCACAAGAAGATTGG-3′ (sense) and 5′-GCATGGTTGGCAGTAGAAG-3′ (antisense); and GAPDH, 5′-CAGCCGAGCCACATCG-3′ (sense) and 5′-TGAGGCTGTTGTCATACTTCTC-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 ($1 = pT_a$, $2 = pT_1$, and $3 = pT_{2-4}$) 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.[7] 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. 1*A*. Both DNAs showed positive reactions for the two control genes *COL2A1* and *ACTB,* which are not methylated and were included to quantitate the amount of DNA present (26, 29). LD137 DNA showed positive results for *TNFRSF25*, *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 *TNFRSF25* and *TERT* but negative for the other three genes, respectively.

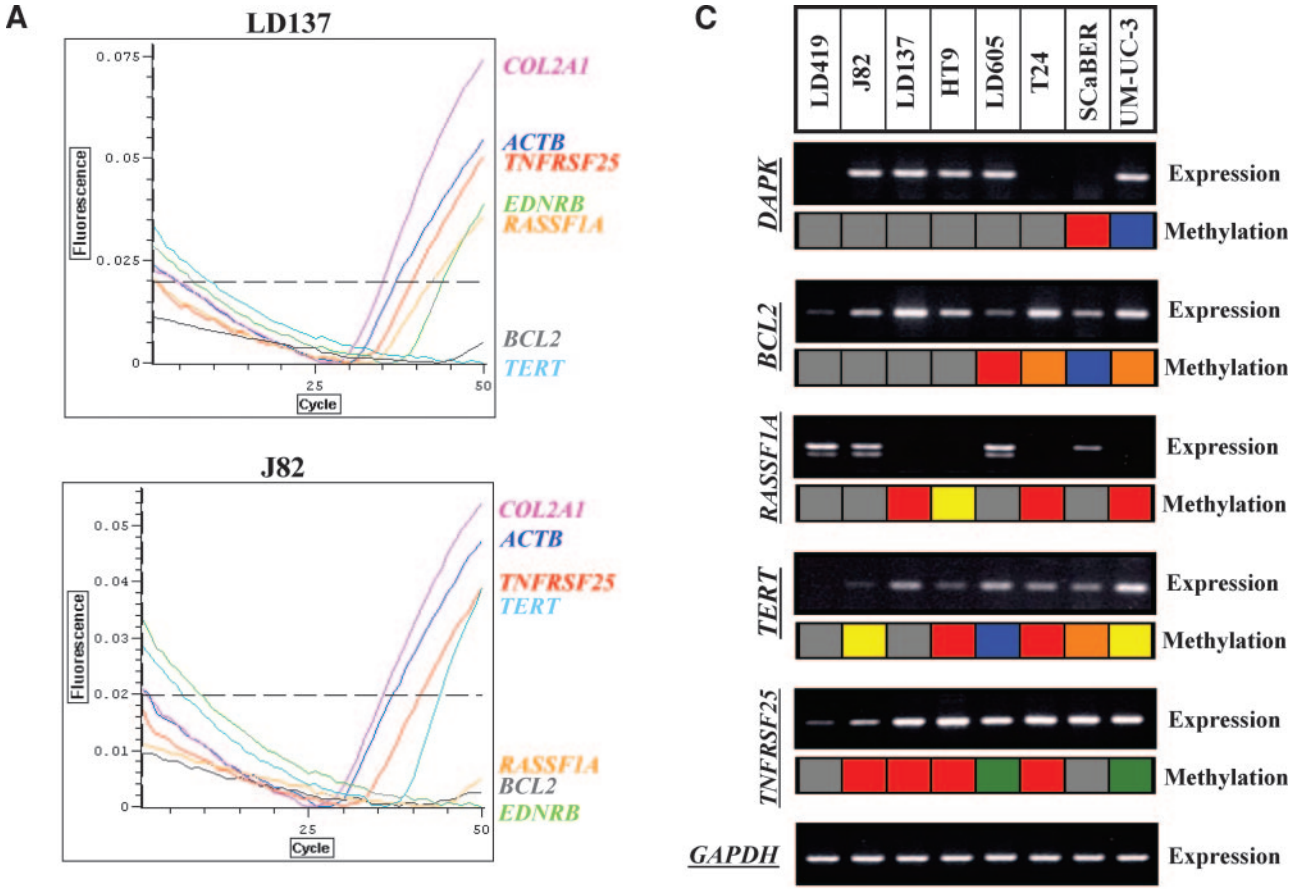
The methylation status of the 5′ regions of 12 apoptosis-associated genes was assessed by MethyLight analysis in 18 bladder cancer cell lines, as well as in normal fibroblasts (LD419) as a negative control (Fig. 1*B*). The control cell line LD419 and the RT4 cell line (derived from a low-grade tumor) 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. 1*C*). *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. 1*C*). 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. 2*A*). *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. 1*B*), 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. We next 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

[7] http://www.insightful.com/products/default.asp.



Cell Line	ARF	FADD	TNFRSF21	LITAF	BAX	TMS-1	DAPK	TERT	BCL-2	EDNRB	RASSF1A	TNFRSF25
LD 419												
RT 4	HD											
LD 627												
J 82												
LD 137												
HT 9												
LD 611	HD											
LD 71	HD											
LD 700												
LD 660												
TCC SUPP												
LD 636												
HT 1376												
LD 605	HD											
LD 600												
T24												
SCaBER	HD											
LD 692												
UM-UC-3	HD											

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 ummethylated. 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*.

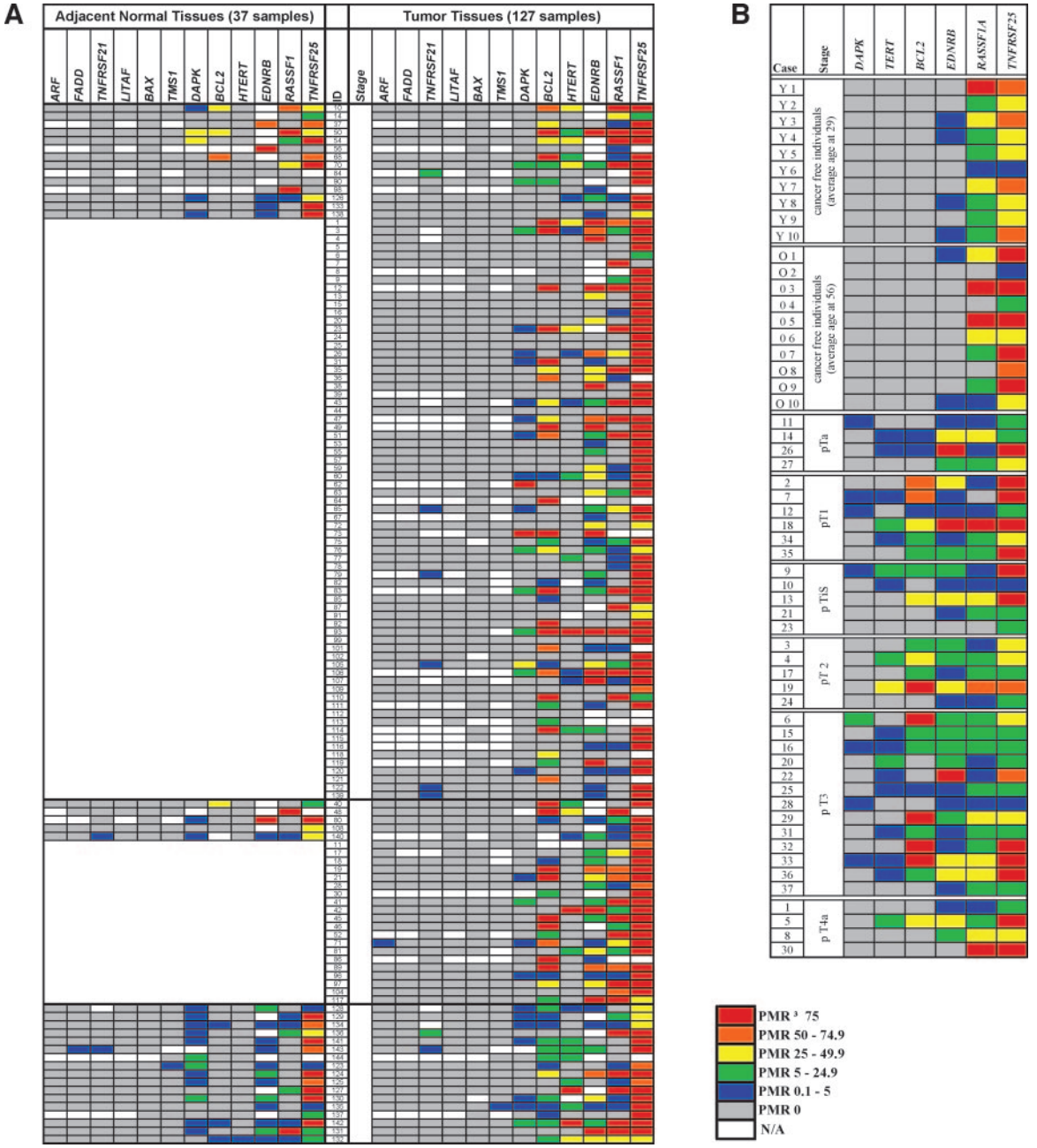


Fig. 2 *A*, methylation levels of the *ARF*, *RADD*, *TNFRSF21*, *PIG7*, *BAX*, *TMS1*, *DAPK*, *TERT*, *BCL2*, *EDNRB*, *RASSF1A*, and *TNFRSF25* genes for the 127 primary bladder tumor samples and 37 nonmalignant adjacent bladder tissue samples. The 127 tumors analyzed were categorized by tumor stage and evaluated against 37 nonmalignant adjacent tissue samples using MethyLight analysis. *B*, methylation levels of the *DAPK*, *TERT*, *BCL2*, *EDNRB*, *RASSF1A*, and *TNFRSF25* genes in urine sediment sample 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.

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*,

Table 2 Significance levels for tumor *versus* normal and stage and grade

Gene	*P*		
	Tumor *vs.* normal (n = 37)	Stage (n = 127)	Grade (n = 127)
ARF	0.33	**0.04**	0.07
FADD	0.66	0.42	0.57
TNFRSF21	0.18	0.18	0.66
LITAF	n. a	0.63	0.86
BAX	n. a	0.65	0.83
TMS-1	0.35	0.06	0.25
DAPK	**0.01**	0.58	0.07
BCL2	**<0.0001**	**0.01**	**0.001**
TERT	**<0.0001**	0.49	**0.02**
EDNRB	0.69	0.15	**0.01**
RASSF1A	**<0.0001**	**0.003**	0.07
TNFRSF25	**0.0001**	1	0.65

NOTE. Significant P values are shown in bold.

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*B*). *EDNRB*, *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. 2*B*), 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 3 Sensitivity of methylation analysis in urine samples of patients with bladder cancer

Gene	Sensitivity* No. (%)
BCL2	24/37 (64.9)
TERT	19/37 (51.4)
DAPK	8/37 (21.6)
Specificity	29/37 (78.4)

* 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*β, *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. 2*A* 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.

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