Hypermethylation of Multiple Genes in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients

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ABSTRACT

Purpose: **We aimed to investigate the methylation pattern in bladder cancer and assess the diagnostic potential of such epigenetic changes in urine.**

Experimental Design: **The methylation status of 7 genes (*RAR*β, *DAPK, E-cadherin, p16, p15, GSTP1,* and *MGMT*) in 98 cases of bladder transitional cell carcinoma and 4 cases of carcinoma *in situ* was analyzed by methylation-specific PCR. Twenty-two cases had paired voided urine samples for analysis.**

Results: **In transitional cell carcinoma tumor tissues, aberrant methylation was frequently detected in *RAR*β (87.8%), *DAPK* (58.2%), *E-cadherin* (63.3%), and *p16* (26.5%), whereas methylation of *p15* (13.3%), *GSTP1* (5.1%), and *MGMT* (5.1%) is not common. No association between methylation status and grading or muscle invasiveness was demonstrated. In 22 paired voided urine samples of bladder cancer, methylation of *DAPK, RAR*β*, E-cadherin,* and *p16* could be detected in 45.5%, 68.2%, 59.1%, and 13.6% of the cases, respectively. The sensitivity of methylation analysis (90.9%) was higher than that of urine cytology (45.5%) for cancer detection. Methylation of *RAR*β (50%), *DAPK* (75%), and *E-cadherin* (50%) was also detected in carcinoma *in situ*. In 7 normal urothelium samples and 17 normal urine controls, no aberrant methylation was detected except for *RAR*β methylation in 3 normal urothelium samples (42.9%) and 4 normal urine samples (23.5%), respectively.**

Conclusions: **Our results demonstrated a distinct methylation pattern in bladder cancer with frequent methylation of *RAR*β*, DAPK, E-cadherin,* and *p16*. Detection of gene methylation in routine voided urine using selected markers appeared to be more sensitive than conventional urine cytology.**

INTRODUCTION

Silencing of genes by promoter hypermethylation is common in human cancers. The changes involving methylation of cytosine in CpG dinucleotide have been recognized as an alternative mechanism in Knudson's two-hits hypothesis where tumor suppressor genes are inactivated. (1). Although the mechanisms of these epigenetic changes are not clearly understood, the list of aberrant methylation genes in cancer is rapidly growing. Nevertheless, a certain kind of tissue-specific methylated gene has been demonstrated. For example, a high frequency of methylation of *GSTP1* has been found in prostate cancer (2) but is rare in other types of cancer. Other examples include frequent methylation of *DAPK* in lung cancer (3, 4) and retinoic acid receptor β2 (*RAR*β*2*) in both breast (5) and lung cancer (6). These findings may have potential diagnostic and therapeutic implications. Recently, these epigenetic changes have also been detected in DNA from plasma/serum (3, 7), urine (8, 9), and sputum (10), indicating that a noninvasive and early cancer detection method can be developed.

We are interested in bladder cancer, which is the sixth most common cancer in the world. The majority of bladder cancer is TCC.[2] One of the distinctive features of TCC is that multiple metachronous or synchronous cancers frequently develop. These arise from either polyclonal origin or metastasis from a single clone. Bladder cancer patients would then need to have a long-term follow-up with repeated urine cytology and cystoscopy for monitoring. Conventional urine cytology has been the standard noninvasive method for cancer detection and disease monitoring. However, the sensitivity of this method is known to be low, especially for low-grade TCC. Therefore, a more sensitive, noninvasive method for cancer detection is required. Methylation detection with appropriate markers may provide a more sensitive method for cancer detection.

We have analyzed the methylation patterns of 7 cancer-related genes including *DAPK, RAR*β*, E-cadherin, p15, p16, MGMT,* and *GSTP1* in 98 bladder TCC samples. Frequent methylation was detected in *RAR*β*, DAPK, E-cadherin,* and *p16,* and they were chosen as markers to detect DNA methylation in 22 corresponding voided urine samples. Our results show that detection of DNA methylation in voided urine is

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[2] The abbreviations used are: TCC, transitional cell carcinoma; MSP, methylation-specific PCR; IVD, *in vitro* methylated DNA.

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Table 1 Summary of clinicopathological data of tumor and normal samples

	TCC (n = 98)	Carcinoma *in situ* (n = 4)	Normal urothelium (n = 7)	Urine samples (n = 22)
Sex				
Male	73	4	3	15
Female	25	0	4	7
Age (yrs)				
Median	73	58.5	55	73
Range	39–92	48–66	43–72	51–84
Primary cases	73			
Recurrent cases	25			
Grade				
1	23			9
2	44			9
3	31			4
Non-muscle invasive	24			18
Muscle invasive	74			4

Table 2 Primer sequence, annealing temperatures, and product size for MSP

Gene	Forward primer (5′ → 3′)[a]	Reverse primer (5′ → 3′)[a]	Annealing temperature (°C)	Product size (bp)
RARβ2	M: GGTTAGTAGTTCGGGTAGGGTTTATC	M: CCGAATCCTACCCCGACG	59	235
	U: TTAGTAGTTTGGGTAGGGTTTATT	U: CCAAATCCTACCCCAACA	59	233
DAPK	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCG	60	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	60	106
E-cadherin	M: TTAGGTTAGAGGGTTATCGCGT	M: TAACTAAAAATTCACCTACCGAC	57	116
	U: TAATTTTAGGTTAGAGGGTTATTGT	U: CACAACCAATCAACAACACA	57	97
p16	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	60	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	60	151
p15	M: GCGTTCGTATTTTGCGGTT	M: CGTACAATAACCGAACGACCGA	60	148
	U: TGTGATGTGTTTGTATTTTGTGGTT	U: CCATACATAACCAAACAACCAA	60	154
MGMT	M: TTTCGACGTTCGTAGGTTTTCCG	M: GCACTCTTCCGAAAACGAAACG	59	81
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: AACTCCACACTCTTCCAAAAACAAAACA	59	93
GSTP1	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	59	91
	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	59	97

[a] M, methylated; U, unmethylated.

feasible and appears to be more sensitive than conventional urine cytology.

MATERIALS AND METHODS

Tissues Samples. Bladder tumor samples from transurethral resection specimens were obtained from 98 patients at the Prince of Wales Hospital. The samples consisted of 73 primary tumors and 25 recurrent tumors. In this study, four samples of carcinoma *in situ* and seven samples of normal urothelium from individuals without bladder cancer were also included. The clinical pathological data for all of the tissue samples are summarized in Table 1.

Urine Samples. Paired voided urine samples were collected from 22 patients (Table 1). The urine samples were spun down, and the urine sediments were subjected to subsequent analysis. The corresponding urine samples were also subjected to conventional urine cytology examination by an experienced pathologist without knowledge of the methylation results. In addition, 17 normal voided urine sediments from age- and sex-matched controls were included.

DNA Isolation. DNA was extracted from formalin-fixed, paraffin-embedded sections or voided urine sediments using a high pure PCR template preparation kit (Boehringer Mannheim, Indianapolis, IN). H&E-stained sections from each tumor sample were examined by an experienced pathologist to confirm the histological diagnosis and assess the tumor content. If tumor content was <80%, tumor content was enriched by microdissection using a fine needle under a dissection microscope as described previously (11). Microdissection was performed for all of the carcinoma *in situ* and normal urothelium cases, and 20 five-μm-thick sections were used for DNA extraction.

MSP. Extracted DNA was bisulfite-modified by using the CpGenome DNA Modification kit (Intergen, Purchase, NY). The modified DNA was subject to MSP using specific primers. Primer sequences, annealing temperatures, and the expected product size are listed in Table 2. Two μl of bisulfate-modified DNA were amplified in a total volume of 25 μl containing 1× PCR buffer II (Perkin-Elmer Corp.), 2 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 1 μM of each primer, and 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer Corp.) at 95°C for 10 min; 40 cycles of 95°C for 30 s, the specific annealing temperature for 45 s, and 72°C for 45 s; followed by a final extension at 72°C for 10 min. IVD (Intergen) was used a positive control for methylation, and water was used as negative

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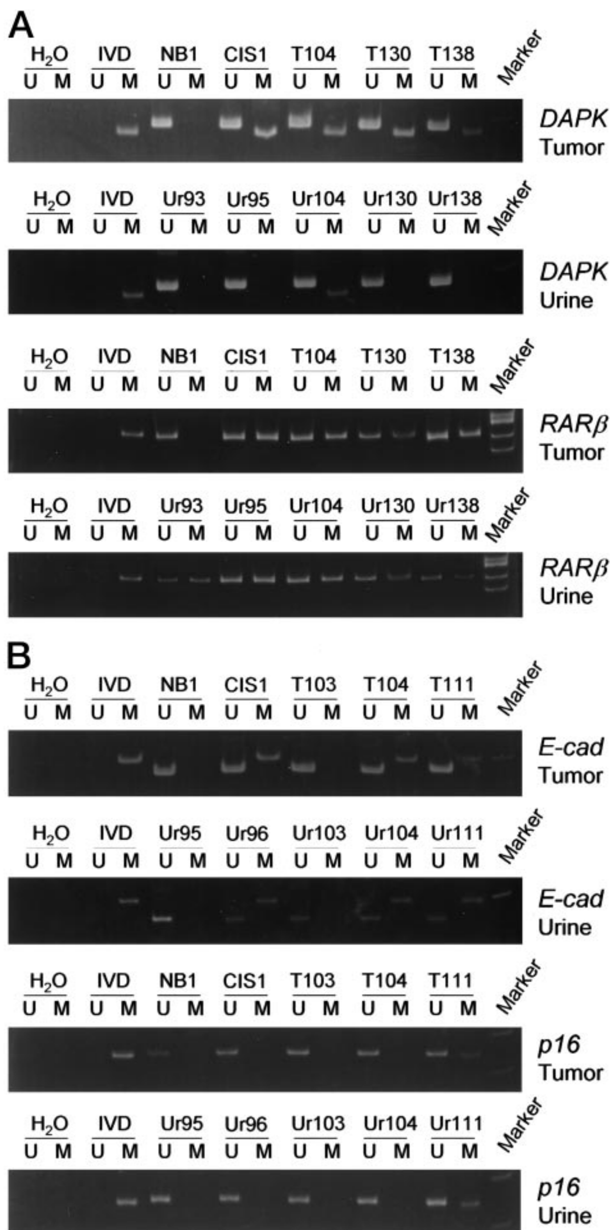

Fig. 1 Methylation analysis of *DAPK, RAR*β, *E-cadherin,* and *p16* in tumor tissues and urine samples of bladder cancer patients by MSP. *A,* results for *DAPK* and *RAR*β; *B,* results for *E-cadherin* and *p16. T,* tumor tissue; *Ur,* urine. In the tumor tissue panels, the results of normal bladder urothelium (*NB*) and carcinoma *in situ* (*CIS*) were also included. *U* indicates the presence of unmethylated genes; *M* indicates the presence of methylated genes. IVD was used as a positive control for methylation, and water (H_2O) was used as a negative control for PCR.

control. Ten μl of PCR products were loaded onto nondenaturing 10% polyacrylamide gels. The gels were then stained with ethidium bromide and visualized under UV illumination.

Statistics. All of the statistical analysis was performed with the statistical package SPSS version 10.0. Association between parameters was assessed by χ^2 or Fisher's exact test.

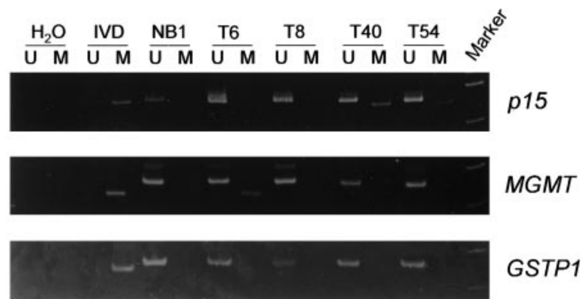

Fig. 2 Methylation analysis of *p15, MGMT,* and *GSTP1* in tumor tissues of bladder cancer patients by MSP. *U* indicates the presence of unmethylated genes; *M* indicates the presence of methylated genes. Results for normal bladder urothelium (*NB*) and carcinoma *in situ* (*CIS*) were also included. IVD was used as a positive control for methylation, and water (H_2O) was used as a negative control for PCR.

The Mann-Whitney *U* test was used to compare parameters of different groups.

RESULTS

Frequency of Methylation in Primary Bladder TCC. We have analyzed the frequency of methylation of *RAR*β, *DAPK, E-cadherin, p16, p15, MGMT,* and *GSTP1* in 98 cases of bladder TCC, 4 cases of carcinoma *in situ,* and 7 samples of normal bladder epithelium by MSP (Figs. 1 and 2; Table 3). In the tumor samples, frequent methylation was detected in *RAR*β (87.8%), *DAPK* (58.2%), *E-cadherin* (63.3%), and *p16* (26.5%). Methylation of *p15* was detected in 13.3% of cases. However, methylation of *GSTP1* (5.1%) and *MGMT* (5.1%) was rare. Methylation of one of four genes (*DAPK*, *RAR*β, *E-cadherin,* and *p16*) was found in 98 of 98 (100%) of TCC cases. We have compared the pattern of methylation of theses genes between primary and recurrent cases, and no significant difference was identified (Table 3). No statistically significant association was found between the methylation status of the genes and the clinical/pathological parameters. Methylation of *E-cadherin* has been reported to be associated with aging (12); however, no statistically significant difference in age was detected between unmethylated and methylated cases in our study (Table 4). We have also analyzed the number of genes that are methylated concurrently in the tumor (Table 5). Notably, more than three genes that are methylated concurrently in the tumor accounted for 20% of our cases. The frequency of concurrent gene methylation did not correlate with the grading of TCC or the presence or absence of muscle invasion.

Methylation of *DAPK* (75%), *E-cadherin* (50%), and *RAR*β (50%) was also detected in carcinoma *in situ* (Table 3). Normal urothelium controls did not show any aberrant hypermethylation except for *RAR*β, for which three of seven samples (42.9%) showed methylation.

Detection of Methylation in Voided Urine Samples. To assess the feasibility of detecting methylated genes in urine, we investigated the methylation frequency of *DAPK*, *RAR*β, *E-cadherin,* and *p16* in urine sediment from 22 patients (Fig. 1). Because these four genes had a higher frequency of methylation in TCC tumor samples, they were selected for urine analysis.

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Table 3 Frequency of methylation of different genes in tumor tissues and normal tissues

	*RAR*β	*DAPK*	*E-cadherin*	*p16*	*p15*	*MGMT*	*GSTP1*
Normal control ($n = 7$)	3 (42.9)[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Carcinoma *in situ* ($n = 4$)	2 (50)	3 (75)	2 (50)	0 (0)	ND[b]	ND	0 (0)
All TCC cases ($n = 98$)	86 (87.8)	57 (58.2)	62 (63.3)	26 (26.5)	13 (13.3)	5 (5.1)	5 (5.1)
Primary cases ($n = 73$)	66 (90.4)	44 (60.3)	45 (61.6)	20 (27.4)	10 (13.7)	2 (2.7)	3 (4.1)
Recurrent cases ($n = 25$)	20 (80)	13 (52)	17 (68)	6 (24)	3 (12)	3 (12)	2 (8)

[a] Numbers in parentheses are percentages.
[b] ND, not determined.

Table 4 Methylation of *E-cadherin* and age of tumor patients

E-cadherin methylation status	No. of cases	Age (yrs)	
		Mean ± SD	Median
Unmethylated	36 (36.7%)	68.31 ± 12.2	73
Methylated	62 (63.3%)	72.26 ± 9	73

Voided urine samples from 17 normal healthy individuals were included as control. MSP results are summarized in Tables 6 and 7. The results showed that gene promoter methylation could be detected in urine samples from the patients. The frequency of methylation was 45.5% for *DAPK*, 68.2% for *RAR*β, 59.1% for *E-cadherin*, and 13.6% for *p16*. Methylation of one of these four genes was found in 20 of 22 (90.9%) cases. Methylation could only be detected in those patients whose tumor tissue also showed gene methylation; in other words, no false positive was found. Besides, with the exception of *RAR*β [4 of 17 (23.5%) cases showed methylation], only unmethylated copies were detected in normal urine control. For comparison, urine cytology data were analyzed (Table 6). Only 10 cases (45.5%) were diagnosed as cancer or suspicious. The sensitivity was even lower in low-grade cases, in which only one of nine (11.1%) cases was positive (Table 7). Meanwhile, if we take any one of the four genes that showed methylation in the urine as a positive marker, the sensitivity of using a methylation marker to detect bladder TCC in urine was 90.9%, which was far greater than the sensitivity of urine cytology (Table 8). This difference was more striking when comparing low-grade cases (100% *versus* 11.1%). Methylation marker, on the other hand, has a lower specificity because of the presence of methylation copies of *RAR*β in normal urine. The sensitivity and specificity of individual gene methylation with respect to the grading of TCC is also tabulated in Table 8. For *E-cadherin, DAPK,* and *p16,* no methylated copies were detected in normal urine. Moreover, the methylation status of *E-cadherin* had a higher sensitivity in detection of bladder cancer, especially for grade 1 TCC, as compared with urine cytology. Similarly, methylation status of *DAPK* also demonstrated a higher sensitivity in detection of low-grade bladder TCC.

Sensitivity of MSP. We have assessed the sensitivity of MSP for detection of methylated alleles of *DAPK* and *RAR*β. Different amounts of IVD were mixed with 1 μg of DNA from normal bladder urothelium with unmethylated alleles of all genes before bisulfite modification. The lower limit of MSP detection was 10 ng for these two genes (Fig. 3).

DISCUSSION

We have analyzed the methylation pattern of *RAR*β, *DAPK, E-cadherin, p15, p16, MGMT,* and *GSTP1* in bladder TCC of different stages and grades. In our study, all of our samples have at least one gene methylated, and more than three genes that were methylated accounted for 20% of our cases. Thus, the epigenetic event of gene methylation was frequent in bladder cancer. However, this phenomenon did not appear to be correlated with disease grade or stage.

Reports on the methylation of various genes have been described in primary bladder cancer (12–19). Among these reports, methylation of *p16* was most commonly investigated. The frequency of *p16* methylation in bladder TCC ranged from 9–67% (13–17, 19). Our study represented the largest series and demonstrated *p16* methylation of 26.5%. Tumor suppressor gene *p16* specifically inactivates cyclin-dependent kinase 4 and cyclin-dependent kinase 6, which interact with cyclin D1 and stimulate the progression of the cell cycle from G_1 to S phase. Thus, inactivation of the *p16* gene was important for tumorigenesis in bladder cancer and other cancers (20). Another gene that is located on the same loci is $p15^{INK4b}$. Methylation of *p15* has also been detected in several tumors (20, 21) but has not been reported in bladder cancer. In our study, we find that the frequency of methylation of the *p15* gene is 13.3%, suggesting that alteration of the *p15* gene occurs in at least a subset of bladder cancers. Other mechanisms of *p15* inactivation such as deletion (22, 23) may also be involved.

In our results, we also found high methylation frequency for *DAPK, RAR*β, and *E-cadherin* in tumor tissue and carcinoma *in situ*. Methylation was demonstrated to be the mechanism of loss of expression of *DAPK* in bladder cancer cells and other cancer cells (24, 25). Recently, it has also been demonstrated that methylation of *DAPK* is associated with stage and poor prognosis in non-small cell lung cancer (26, 27). However, in our results, we do not find such a correlation. On the other hand, Esteller *et al.* (19) have demonstrated a low frequency of methylation of *DAPK* in bladder cancer using the same method. Apart from ethic or geographical factors, the differences in sample size may also account for the differences in frequency of

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Table 5 Number of genes that are concurrently methylated in different grade of TCC cases

	No. of genes methylated concurrently				
	1	2	3	4	5
All 98 cases	19[a] (19.4)[b]	26 (26.5)	34 (34.7)	14 (14.3)	5 (5.1)
Grade 1 (23 cases)	4 (17.4)	7 (30.4)	8 (34.8)	3 (13)	1 (4.3)
Grade 2 (44 cases)	10 (22.7)	11 (25)	14 (31.8)	6 (13.6)	3 (6.8)
Grade 3 (31 cases)	5 (16.1)	8 (25.8)	12 (38.7)	5 (16.1)	1 (3.2)
Non-muscle invasive (74 cases)	14 (18.9)	20 (27)	23 (31.1)	12 (16.2)	5 (6.7)
Muscle invasive (24 cases)	5 (20.8)	6 (25)	11 (45.8)	2 (8.3)	0 (0)

[a] Number of cases.
[b] Numbers in parentheses are percentages.

Table 6 Methylation of *DAPK, RAR*β, *E-cadherin*, and *p16* in tumor and urine DNA from bladder cancer patients

Case no.	Sex	Age (yrs)	Grade	MI[a]	Tumor DNA/urine DNA				Cytology
					DAPK	*RAR*β	*E-cadherin*	*p16*	
T093	F	65	3	Y	U/U	M/M	U/U	U/U	Suspicious
T095	M	78	1	N	M/U	M/M	M/U	U/U	Negative
T096	F	64	2	N	U/U	M/M	M/M	U/U	Negative
T103	M	63	2	N	U/U	M/M	U/U	U/U	Cancer
T104	M	73	2	N	M/M	M/M	M/M	U/U	Cancer
T107	M	51	1	N	M/M	M/M	U/U	U/U	Negative
T111	F	84	1	N	M/M	M/M	M/M	M/M	Negative
T123	M	64	1	N	U/U	M/U	M/M	U/U	Negative
T126	F	80	2	Y	U/U	M/M	M/M	U/U	Negative
T127	M	73	1	N	M/M	M/U	M/M	U/U	Negative
T128	M	64	1	N	M/M	M/M	M/M	U/U	Suspicious
T130	M	77	3	N	M/U	M/M	M/M	U/U	Suspicious
T134	M	71	1	N	M/M	M/U	M/U	U/U	Atypia
T135	F	80	2	N	M/M	M/U	M/M	M/U	Cancer
T138	M	72	1	N	M/U	M/M	M/M	U/U	Atypia
T141	F	75	2	N	M/U	U/U	U/U	U/U	Cancer
T142	M	67	1	N	M/M	M/M	M/U	U/U	Negative
T147	M	84	1	N	M/U	M/M	M/M	M/M	Atypia
T148	M	69	1	N	M/U	U/U	M/U	U/U	Atypia
T149	F	82	1	Y	M/M	M/U	U/U	U/U	Cancer
T150	M	79	1	Y	M/U	M/M	M/M	M/M	Cancer
T151	M	73	1	N	M/M	M/M	M/M	U/U	Cancer

[a] MI, muscle invasiveness; M, methylated; U, unmethylated.

DAPK methylation. Methylation of *RAR*β was first reported in breast cancer (5) and related to the development of retinoic acid resistance in cancer cells. A recent study has shown that methylation of *RAR*β is found in lung tumor tissue as well as in adjacent nonmalignant lung tissues (4). The presence of the methylated allele in adjacent nonmalignant tissue may represent premalignant changes. In our study, methylation of *RAR*β can also be detected in three of seven normal control urothelium samples. However, the significance of this finding needs to be further investigated. Methylation of the *E-cadherin* gene has been widely reported in different tumors as well as in bladder cancer (12, 28, 29). Recently, Bornman *et al.* (12) demonstrated a high frequency of methylation of the *E-cadherin* gene in bladder cancer tissues and in normal urothelial epithelium from elderly individuals. They suggested that methylation of *E-cadherin* in bladder epithelium was age-related. In our study, there is no difference in the median age of bladder TCC patients with methylated *E-cadherin* and those with unmethylated *E-cadherin* (Table 4). However, we cannot detect any methylation of *E-cadherin* in our normal urothelium controls. This is probably because the samples were taken from younger individuals (median age, 55 years). This result is consistent with that of Bornman *et al.* (12) because they only found methylation of *E-cadherin* in normal samples from individuals >70 years old. Methylation of *RAR*β, *DAPK,* and *E-cadherin* can also be detected in carcinoma *in situ.* The results suggest that inactivation of theses gene may be involved in both bladder TCC and carcinoma *in situ*.

We have also found a low frequency of gene methylation for *MGMT* and *GSTP1*. This finding is in keeping with those of previous studies (2, 19, 30).

Another aim of this study was to investigate whether cancer cells can be detected using methylation markers in corresponding voided urine samples of the patients. We choose *DAPK, RAR*β, *E-cadherin,* and *p16* as methylation markers because these markers have a high frequency of methylation in our tumor tissue samples. Our results showed that unmethylated allele of these four genes could be detected in all urine samples. Recent findings in prostate cancer demonstrated that methylation of the *GSTP1* gene can be detected in the urine of prostate

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Table 7 Percentage of methylation of 22 cases and corresponding urine samples

	DAPK	*RAR*β	*E-cadherin*	*p16*	Cytology[a]
Normal urine (17 cases)	0 (0)[b]	4 (23.5)	0 (0)	0 (0)	
All cases (22 cases)					
Tissue	17 (77.3)	20 (90.9)	17 (77.3)	4 (18.2)	
Urine	10 (45.5)	15 (68.2)	13 (59.1)	3 (13.6)	10 (45.5)
Grade 1 (9 cases)					
Tissue	8 (88.9)	9 (100)	8 (88.9)	2 (22.2)	
Urine	5 (55.5)	6 (66.7)	6 (66.7)	2 (22.2)	1 (11.1)
Grade 2–3 (13 cases)					
Tissue	9 (69.2)	11 (84.6)	9 (69.2)	2 (15.3)	
Urine	5 (38.4)	9 (69.2)	7 (53.8)	1 (7.6)	9 (69.2)

[a] Urine cytology was placed here for comparison; the percentages in parentheses represent those cases diagnosed as cancer or suspicious.
[b] Numbers in parentheses are percentages; numbers outside parentheses represent the number of cases.

Table 8 Comparison of sensitivity and specificity between methylation markers and cytology

	Methylation markers[a]	Cytology	*DAPK*	*RAR*β	*E-cadherin*	*p16*
Sensitivity (%)						
All cases	90.9	45.5	45.5	68.2	59.1	13.6
Grade 1	100	11.1	55.5	66.7	66.7	22.2
Grade 2–3	84.6	69.2	38.4	69.2	53.8	7.6
Specificity (%)	76.4	100	100	76.4	100	100
Positive predictive value (%)						
All cases	83.3	100	100	78.9	100	100
Grade 1	69.2	100	100	60.0	100	100
Grade 2–3	73.3	100	100	69.2	100	100
Negative predictive value (%)						
All cases	86.6	58	58.6	65.0	65.3	47.2
Grade 1	100	68	80.9	81.3	85	70.8
Grade 2–3	86.6	80.9	68.0	76.4	73.9	58.6

[a] Any one of the genes showed methylation in urine samples.
[b] Cases where either *DAPK* showed methylation or cytology diagnosed as cancer or suspicious.

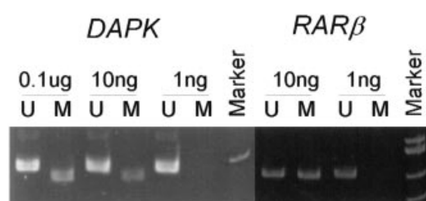

Fig. 3 Sensitivity of MSP in *DAPK* and *RAR*β. Various amounts of IVD were mixed with 1 μg of DNA from normal bladder urothelium with unmethylated alleles of all markers before bisulfite modification to assess the detection sensitivity of MSP for methylated alleles.

cancer patients (8, 9). Our results confirmed that detection of gene methylation in urine was feasible. With regard to the sensitivity of the assay, methylation of any one of the four genes could be detected in 90.9% of the urine samples, whereas urine cytology could only detect cancer cells in 45.5% of the samples. This difference is more striking in low-grade cases, where conventional urine cytology was known to have a low sensitivity. The results suggest that methylation detection has a higher sensitivity than conventional urine cytology in cancer detection in urine. Combination of methylation makers, however, had a lower specificity, which was related to the detection of *RAR*β in normal urine control. However, using a specific marker, such as *E-cadherin* or *DAPK*, could result in a higher specificity and sensitivity as compared with urine cytology, especially for low-grade cases. Thus, the diagnostic assessment could be improved by using selected methylation markers. Furthermore, a combination of conventional urine cytology and selected methylation markers may improve diagnostic accuracy, especially with regard to low-grade cases.

In conclusion, we have demonstrated a distinct methylation pattern of multiple genes in urinary bladder cancer patients. Frequent methylation of *RAR*β*, DAPK*, *E-cadherin,* and *p16* was detected. This was also the first time that methylation of *RAR*β and *p15* was reported in bladder cancer. We have also demonstrated that detection of bladder cancer in urine using methylation markers appeared to be more sensitive then conventional urine cytology. Detection of methylated genes in routinely voided urine, as a potential noninvasive diagnostic and monitoring tool, deserves further investigation.

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Hypermethylation of Multiple Genes in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients

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