

*Featured Article***Detection of Bladder Cancer in Urine by a Tumor Suppressor Gene Hypermethylation Panel**

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Abstract

Purpose: Bladder cancer is potentially curable in the majority of cases; however, the prognosis for patients with advanced disease at presentation remains poor. Current noninvasive tests such as cytology lack sufficient sensitivity to detect low-grade, low-stage tumors. Silencing of tumor suppressor genes, such as *p16^{INK4a}*, *VHL*, and the mismatch repair gene *hMLH1*, has established promoter hypermethylation as a common mechanism for tumor suppressor inactivation in human cancers. It is also a promising new target for molecular detection in bodily fluids including urine, a readily accessible fluid known to contain bladder cancer cells. Methylation-specific PCR (MSP) can determine the presence or absence of methylation of a gene locus at a sensitivity level of up to 1 methylated allele in 1000 unmethylated alleles, appropriate for identifying cancer cell DNA in a bodily fluid.

Experimental Design: We first determined the frequency of hypermethylation of the *Rb* tumor suppressor gene by bisulfite sequencing and of the *p16^{INK4a}*, *p14^{ARF}*, *APC*, and *RASSF1A* tumor suppressor genes by MSP in 45 bladder cancers. We then designed a panel optimal for diagnostic coverage composed of the *APC*, *RASSF1A*, and *p14^{ARF}* tumor suppressor genes. This panel was tested for detection of hypermethylation in matched sediment DNA from urine specimens obtained before surgery from the same 45 bladder cancer patients (2 Tis, 16 Ta, 10 T1, and 17 T2–4) as well as normal and benign control DNAs.

Results: Hypermethylation of at least one of three suppressor genes (*APC*, *RASSF1A*, and *p14^{ARF}*) was found in all 45 tumor DNAs (100% diagnostic coverage). We detected gene hypermethylation in the matched urine DNA from 39 of 45 patients (87% sensitivity), including 16 cases that had negative cytology. No hypermethylation of *APC*, *RASSF1A*, or *p14^{ARF}* was observed in normal transitional cell DNAs or

in urine DNAs from normal healthy individuals and patients with inflammatory urinary disease (cystitis). Furthermore, an unmethylated gene in the tumor DNA was always found to be unmethylated in the matched urine DNA (100% specificity).

Conclusions: Promoter hypermethylation of tumor suppressor genes is common in bladder cancer and was found in all grades and stages of tumors examined. Hypermethylation was detected in the urine DNA from 39 of 45 (87%) patients, including cases of early-stage disease amenable to cure. MSP may enhance early detection of bladder cancer using a noninvasive urine test.

Introduction

Bladder cancer is the fourth most common male cancer in the Western world. There will be more than 60,000 new cases of cancer in the bladder, ureters, or renal pelvis in the United States this year (1). Although up to 75–80% of new cases present as noninvasive (Ta), superficially invasive (T1), or carcinoma *in situ* (Tis) disease, the remaining 20–25% of tumors present as muscle-invasive (T2–4) or more advanced disease with a poor prognosis. Furthermore, although approximately 20% of Ta and T1 tumors are cured, after initial removal 60–70% recur at least once in 5 years, and 10–20% progress to muscle-invasive cancer (\geq T2). The established association between tobacco or certain occupational exposures and bladder cancer has identified high-risk populations. The most common symptom of bladder cancer is hematuria. However, hematuria often appears late in individuals with bladder cancer, and the majority of individuals with hematuria do not have bladder cancer. Currently, the most sensitive diagnostic test for bladder cancer is invasive (cystoscopy). Noninvasive tests such as cytology and other urinary markers have significant limitations, particularly in detecting low-grade, low-stage disease. New approaches to screen for bladder cancer are imperative. A noninvasive sensitive and specific test could prescreen patients with clinical symptoms or those at high risk before cystoscopy and would also be useful in monitoring patients for recurrence. Furthermore, because early detection may successfully identify potentially lethal lesions (T1 or Tis) before they become muscle invasive, such a test could significantly impact the morbidity and mortality of the disease (2, 3).

Recent advances in the understanding of cancer as a genetic disease have allowed the identification of clonal genetic alterations, the accumulation of which drives progression. These cancer-specific alterations that arise during tumorigenesis can serve as targets for the detection of neoplastic cells in clinical specimens such as readily accessible bodily fluids (4). The use of genetic and epigenetic alterations for the early detection of bladder cancer has the potential advantage that because some events will occur early in the disease process, molecular diagnosis, *e.g.*, in urine, may allow detection before clinical or overt radiographic manifestations. An understanding of the genetic

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and epigenetic events responsible for the malignant transformation of bladder epithelium is rapidly emerging. This includes inactivation of at least five identified tumor suppressor genes (*p53*, *Rb*, *p16^{INK4a}*, *p14^{ARF}*, and *PTEN*) by the genetic alterations of deletion and point mutation (5). In cancer cells, several tumor suppressor genes such as *p16^{INK4a}*, *VHL*, *hMLH1*, and *BRCA1* have been found to have another alteration, aberrant methylation of normally unmethylated CpG islands within the promoter region. The hypermethylation is associated with transcriptional silencing of the gene and therefore acts as an alternative mechanism of inactivation of a tumor suppressor gene allele (6, 7). Genes identified as hypermethylated in cancer cells include tumor suppressor genes of clear biological significance already known to be involved in bladder tumorigenesis, such as *Rb*, *p16^{INK4a}*, and *p14^{ARF}* (8–11); tumor suppressors implicated in other types of cancer, *e.g.*, *APC* (12); and putative tumor suppressor genes such as *RASSF1A* (13). Hypermethylation can be analyzed by the sensitive methylation-specific PCR (MSP) technique, which can identify up to 1 methylated allele in 1000 unmethylated alleles (14), appropriate for the detection of neoplastic cells in a background of normal cells. MSP also allows rapid analysis of multiple gene loci, does not require prior knowledge of epigenetic alteration, and can provide a “yes or no” answer (14, 15).

We therefore determined an optimal panel of tumor suppressor genes for the detection of bladder cancer and examined the hypermethylation status of *APC*, *RASSF1A*, and *p14^{ARF}* in paired bladder tumor and urine DNA samples as well as normal and benign disease controls. Promoter hypermethylation identified in the tumor DNA was used as a molecular target for cancer detection in the corresponding urine DNA. We found each of the 45 tumors to have hypermethylation of at least one gene from the panel, thereby providing a target that we successfully detected in 39 (87%) of the matched urine DNAs with 100% specificity.

Materials and Methods

Specimen Collection and DNA Extraction. After approval from the Institutional Review Board, we obtained tumor tissue via the Fox Chase Cancer Center Tumor Bank Facility and 10–100 ml of preoperative urine from 45 patients (age, 37–85 years) who underwent transurethral resection, cystectomy, or nephro-ureterectomy for cancer of the bladder, ureter, or renal pelvis. All cases were transitional cell carcinomas. Tumors were graded according to American Joint Committee on Cancer and staged according to American Joint Committee on Cancer-Union Internationale Contre le Cancer tumor-node-metastasis (TNM) classifications (Ref. 16; Table 1). Urine specimens from 12 normal, healthy individuals and 9 patients with inflammatory urinary disease (cystitis) of the bladder were obtained as controls. Specimens of histologically confirmed normal ureteral urothelium were collected from five patients with renal cell carcinoma to provide normal (nonneoplastic) transitional cell DNA. Tumor tissue was obtained immediately after surgical resection and subsequently microdissected with the assistance of a pathologist (T. A-S.). DNA was extracted from tissue and fluid using a standard technique of digestion with proteinase K in

the presence of SDS at 37°C overnight, followed by phenol/chloroform extraction (17). Tissue specimen DNA was spooled out after precipitation with 100% ethanol. Urine DNA was precipitated with 0.1 volume of 10 M ammonium acetate, 2 μ l of glycogen (Roche Diagnostics Corp., Indianapolis, IN), and 2.5 volumes of 100% ethanol, followed by incubation at –20°C and centrifugation at top speed (16,000 relative centrifugal force). All urinary cytology was reviewed by an experienced oncologic cytopathologist.

Bisulfite Sequencing of the *Rb* Promoter CpG Island.

A 289-bp fragment of the *Rb* promoter CpG island was amplified from bisulfite-modified DNA with sense primer Rb11 and antisense primer Rb12 as described previously (18) and then cycle sequenced for 35 cycles. Ten bladder tumor cell lines (T24, J82, RT4, ScaBer, TCCSUP, UM-UC-3, HT-1197, HT-1376, 5637, and SV-HUC) and seven primary bladder tumors previously found to be negative for RB protein expression by immunohistochemistry (19) were examined.

MSP. Specimen DNA (0.25–1 μ g) was modified with sodium bisulfite, converting all unmethylated (but not methylated) cytosines to uracil, followed by amplification with primers specific for methylated *versus* unmethylated DNA (14). The genes used in the bladder cancer detection panel were *APC* (12), *RASSF1A* (13), and *p14^{ARF}* (11). The primer sequences used have all been reported previously and can be found in the report referenced after each gene. The primers for *RASSF1A* include CpG site positions 7–9 on the forward primer and 13–15 on the reverse primer as described previously (13). PCR was performed for 31–35 cycles at 95°C denaturing, 58°C–62°C annealing, and 72°C extension with a final extension step of 5 min. Cycle number and annealing temperature depended on the primer set to be used, each of which had been previously optimized for the PCR technology in our laboratory. For each set of DNA modification and PCR, a cell line or tumor with known hypermethylation as a positive control, normal lymphocyte or normal transitional cell tissue DNA as a negative control, and water with no DNA template as a control for contamination were included. If no tumor cell line or primary tumor with known hypermethylation of a particular gene was available, normal human lymphocyte DNA *in vitro* methylated with *SssI* methylase according to the manufacturer’s instructions (New England Biolabs, Beverly, MA) was used as a positive control. After PCR, samples were run on a 6% nondenaturing acrylamide gel with appropriate size markers and analyzed. PCR was repeated for each positive sample.

Statistical Analysis. The sensitivity of MSP-based detection of hypermethylation in urine was calculated as the number of positive tests/number of cancer cases. The specificity was calculated as the number of negative tests/number of cases without cancer and in a second, distinct approach as the number of negative tests/number of cases without hypermethylation of a particular gene. The ability of hypermethylation *versus* cytology to detect cancer was compared using McNemar’s test. The association of tumor grade or stage with positive detection of hypermethylation in urine was assessed using Fisher’s exact test. Results were considered statistically significant if the two-sided *P* was ≤ 0.05 .

Table 1 Clinicopathological and hypermethylation detection data from 45 bladder cancer patients

No.	Age/sex	pTNM ^a	Stage	Grade	Cytology	APC	RASSF1A	p14
1	59/F	Ta	0a	II–III	+	M/U	U/U	M/M
4	72/F	Ta	0a	III	+	U/U	U/U	M/M
8	77/M	Ta	0a	II	+	M/M	M/M	M/M
13	68/M	Ta	0a	II	+	U/U	M/M	U/U
22	72/M	Ta	0a	II	+	M/M	M/M	U/U
39	78/F	Ta	0a	III	–	U/U	M/M	U/U
40	80/M	Ta	0a	II	–	M/M	M/M	U/U
43	57/F	Ta	0a	II	–	M/M	U/U	U/U
55	65/M	Ta	0a	I	N/A	M/M	U/U	M/U
69	79/M	Ta	0a	II	–	U/U	M/M	U/U
82	85/F	Ta	0a	II	+	M/M	M/M	U/U
72	56/M	Ta	0a	I	–	M/M	M/M	U/U
101	67/M	Ta	0a	II	–	U/U	M/M	U/U
103	59/M	Ta	0a	II	–	M/M	U/U	M/M
104	84/M	Ta	0a	I	–	M/U	U/U	M/U
105	56/M	Ta	0a	II	–	U/U	U/U	M/M
48	84/F	T1	Ia	II	–	U/U	U/U	M/M
6	66/F	T1	I	II	–	U/U	M/U	M/M
24	37/M	T1	I	II	–	M/M	U/U	U/U
27	75/M	T1	I	II	–	M/M	M/M	U/U
35	61/M	T1	I	III	+	M/M	U/U	U/U
37	57/M	T1	I	III	+	M/M	M/M	U/U
53	69/M	T1	I	II	–	M/U	M/M	U/U
63	76/M	T1	I	II–III	+	M/M	M/U	U/U
85	56/M	T1	I	II	–	M/M	U/U	M/U
96	77/F	T1	I	III	+	U/U	U/U	M/M
15	64/M	Tis	0is	III	+	U/U	M/U	U/U
34	72/F	Tis	0is	I	+	M/M	U/U	U/U
5	74/M	T2	II	III	+	M/M	U/U	U/U
44	43/M	T2	III	II	–	M/M	M/M	M/U
28	74/F	T3	III	III	N/A	M/M	U/U	U/U
42	62/M	T3	III	III	N/A	M/U	U/U	U/U
51	73/F	T3	III	III	N/A	M/M	U/U	M/M
71	71/M	T3	III	III	N/A	M/M	M/M	U/U
98	72/F	T3	III	III	N/A	M/U	U/U	U/U
99	67/M	T3	III	III	+	U/U	M/M	M/M
29	70/M	T3	III	III	–	U/U	M/M	U/U
25	82/M	T4	III	III	N/A	M/M	M/U	U/U
17	66/M	T3N1	IV	III	N/A	M/M	M/M	U/U
45	66/M	T3N1	IV	III	+	U/U	M/U	U/U
66	67/M	T3N1	IV	III	N/A	M/U	U/U	M/U
74	63/M	T4	IV	III	+	U/U	M/M	U/U
76	61/M	T4	IV	III	N/A	M/M	U/U	M/M
59	70/M	T4N1	IV	III	N/A	M/M	U/U	U/U
75	44/M	T4N2	IV	III	+	M/M	U/U	U/U

^a p, pathologic stage; T, tumor size; N, node status; M, metastatic status; stage, American Joint Committee on Cancer stage grouping; grade, American Joint Committee on Cancer; cytology positive, positive and 1 suspicious case (no. 22); negative, negative or mild/rare atypia and 1 degenerated case (no. 39); N/A, not available; M/M, tumor DNA methylated/urine DNA methylated; U/U, tumor DNA unmethylated/urine DNA unmethylated; M/U, tumor DNA methylated/urine DNA unmethylated. No cases of U/M (tumor DNA unmethylated/urine DNA methylated) were identified.

Results

The frequency of promoter hypermethylation of the *Rb* tumor suppressor gene was examined by bisulfite sequencing, and the frequency of promoter hypermethylation of the *p16^{INK4a}*, *p14^{ARF}*, *APC*, and *RASSF1A* tumor suppressor genes was examined by MSP in a series of bladder tumors to establish an optimal panel of genes for detection of bladder cancer in urine DNA. The *Rb* promoter CpG island was found to be unmethylated by bisulfite sequencing in 10 bladder cancer cell lines and 7 primary bladder tumors analyzed (data not shown). We observed hypermethylation of *p16^{INK4a}* in 3 of 45 (7%) bladder tumors and hypermethylation of *p14^{ARF}* in 16 of 45 (35%) bladder tumors by MSP. The frequency of promoter

hypermethylation of *APC* was 31 of 45 (69%) by MSP, and that of *RASSF1A* was 23 of 45 (51%) by MSP. Analysis of the data revealed that one or more of the three suppressor genes (*APC*, *RASSF1A*, and *p14^{ARF}*) was hypermethylated in each of the 45 tumor DNAs (Table 1). The diagnostic coverage (whether a hypermethylated gene was available as a target in each case) of this small three-gene panel was therefore 100%. Hypermethylation was found in all pathological grades and stages of bladder cancer including 16 Ta and 2 Tis tumors (Table 1), which indicated that hypermethylation can be a relatively early event in bladder tumorigenesis. Hypermethylation was found in patients of all ages (Table 1).

The pattern of hypermethylation status of the three-gene

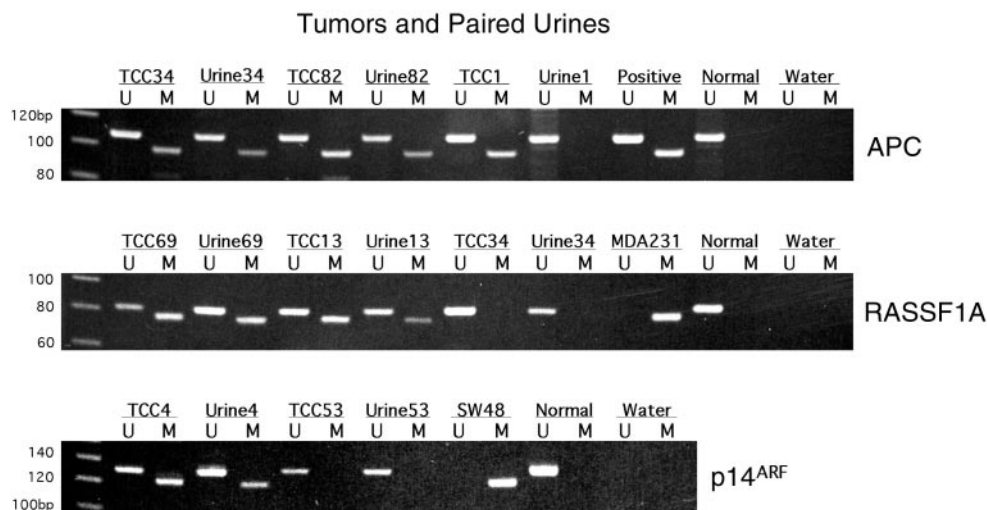


Fig. 1 Methylation-specific PCR of *APC*, *RASSF1A*, and *p14^{ARF}* genes in bladder tumor and urine DNAs. In the *APC* gel panel viewed from left to right, the first (*TCC34*) and second patient's tumor (*TCC82*) DNA is hypermethylated (*M*) and positively detected in the corresponding urine DNA (*M*), whereas the third patient's tumor (*TCC1*) DNA shows hypermethylation that was not detected in the matched urine DNA. In the *RASSF1A* gel panel, the first (*TCC69*) and second patient's tumor (*TCC13*) DNA and urine DNA show hypermethylation. The third patient's tumor (*TCC34*) DNA is not methylated, and the corresponding urine DNA also shows no hypermethylation (*M*). In the *p14^{ARF}* gel panel, the first patient's tumor (*TCC4*) DNA is hypermethylated, which is detected in the urine. The second patient's tumor (*TCC53*) DNA is not hypermethylated, and the corresponding urine DNA also shows no hypermethylation (*M*). The PCR product in the unmethylated lane (*U*) from all tumor DNAs arises from normal cell contamination of the tumor specimen or from an unmethylated allele. A primary tumor DNA with methylated alleles of *APC*, tumor cell line MDA231 (*RASSF1A*) and SW48 DNA (*p14^{ARF}*) as a positive control, normal lymphocyte DNA as a negative control, a water control for contamination in the PCR reaction (*right*), and a 20-bp molecular ruler as a molecular weight marker (*far left*) are also shown.

panel in the paired urine DNAs from the same 45 bladder cancer patients was then determined by the sensitive MSP assay, which can detect 0.1% cancer cell DNA from a heterogeneous cell population (14), and compared with the hypermethylation pattern in the corresponding tumor DNAs. We detected gene hypermethylation in 39 of 45 (87%) matched urine DNAs (Table 1). For confirmation, MSP was repeated with matched tumor and urine-paired DNAs loaded side by side on gels (Fig. 1). The urine-positive cases (designated M/M in Table 1) included 15 of 16 cases of Ta tumors (*e.g.*, patient 69 in Fig. 1), 10 of 10 T1 tumors, and 1 of 2 Tis lesions. No hypermethylation was detected in the urine DNA from six patients (patients 15, 42, 45, 66, 98, and 104 in Table 1). Urine cytology results were available for 34 cases. MSP detected hypermethylation in 31 of 34 (91%) cases, whereas cytology detected hypermethylation in 17 of 34 (50%) cases overall.

Most significantly, hypermethylation was detected in urine DNA from eight of nine Ta tumors and six of six T1 tumors with negative cytology. Urine specimens from both patients with Tis disease had positive cytology, but hypermethylation was detected in the urine DNA of only one case. One additional urine from a patient with T3 disease (patient 45) had positive cytology but was negative for hypermethylation. There was no statistical association between the stage (superficial *versus* muscle-invasive disease, $P = 0.18$, Fisher's exact test) or grade (low-grade *versus* high-grade disease, $P = 0.19$, Fisher's exact test) of the 45 tumors and positive detection in urine. Hypermethylation was positive in a statistically significantly ($P = 0.001$, McNemar's test) greater number of urines (31 of 34) than was cytology analysis (17 of 34).

In contrast, no hypermethylation of *APC*, *RASSF1A*, or *p14^{ARF}* was observed in urine DNA from 12 normal, healthy, age-matched controls and 9 patients with inflammatory urinary disease (cystitis) or in 5 normal urothelium specimens (Table 2; Fig. 2). Furthermore, a gene negative for hypermethylation in the tumor DNA was always negative in the matched urine DNA (Table 1), for example, tumor 53 and urine 53 in the *p14^{ARF}* gel panel shown in Fig. 1. The specificity of the test was therefore 100%.

Discussion

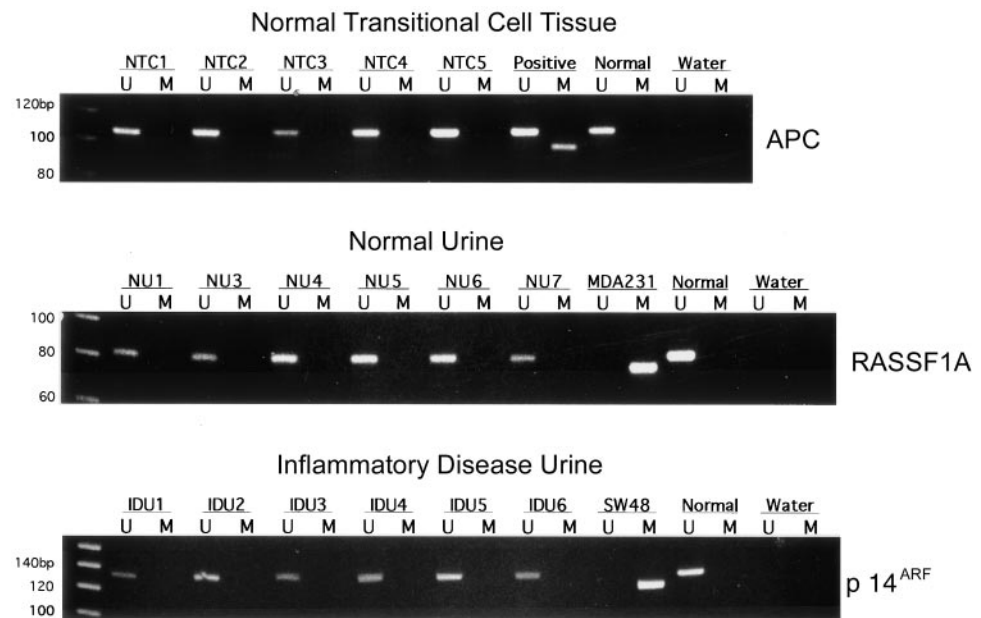
We assembled an optimal panel of tumor suppressor genes with which to screen urine for promoter hypermethylation in bladder cancer cell DNA. Our criteria were that a normally unmethylated gene must have robust cancer-specific hypermethylation, must be frequently hypermethylated in bladder cancer, and preferably have known biological significance. We first considered the five known tumor suppressor gene loci in

Table 2 Summary of gene hypermethylation in controls

	APC	RASSF1A	DAP-K ^a
Normal urines	0/12	0/12	0/12
Non-neoplastic bladder urines	0/9	0/9	0/9
Normal urothelium	0/5	0/5	0/5
Non-neoplastic kidney urines	0/12	0/12	0/12
Non-neoplastic prostate urines	0/5	0/5	0/5
MSP+ urine/MSP- tumor	0/14	0/22	0/29

^a DAP-K, death-associated protein kinase.

Fig. 2 Methylation-specific PCR of *APC*, *RASSF1A*, and *p14^{ARF}* genes in normal and inflammatory disease control DNAs. The absence of a PCR product in the methylated lane (*M*) of *APC* in normal transitional cell (*NTC*) tissue DNAs 1–5, of *RASSF1A* in normal urine (*NU*) DNAs 1–5, and of *p14^{ARF}* in urine DNAs from patients with inflammatory disease (*IDU*) 1–5 indicates that these specimen DNAs have unmethylated alleles only (*U*). A primary tumor DNA with methylated alleles of *APC*, tumor cell line MDA231 (*RASSF1A*) and SW48 DNA (*p14^{ARF}*) as a positive control, normal lymphocyte DNA as a negative control, a water control for contamination in the PCR reaction (*right*), and a 20-bp molecular ruler as a molecular weight marker (*far left*) are also shown.



bladder cancer (5). Because the *p53* gene has not been found to be hypermethylated in human cancer (7), and the *PTEN* gene was found to be unmethylated in bladder cancer (20), we examined the *Rb*, *p16^{INK4a}*, and *p14^{ARF}* tumor suppressor genes only. The *Rb* promoter CpG island region was found to be unmethylated in all bladder tumor cell lines and primary tumors examined. Thus, hypermethylation of *Rb* is likely infrequent or absent in bladder carcinoma. The *p16^{INK4a}* suppressor gene was hypermethylated at a low frequency (7%) in the bladder tumors examined and therefore was not included in our present panel. However, the *p14^{ARF}* gene was hypermethylated in 35% of bladder tumors and was included. Two additional genes reported to be frequently hypermethylated in cancer cells, *APC* and *RASSF1A*, were also examined (21–23). The *APC* tumor suppressor gene is of known biological significance but was not previously known to be involved in bladder tumorigenesis (5). The *RASSF1A* gene is a putative tumor suppressor because it is commonly methylated in human tumors, and this hypermethylation is associated with loss of expression (13), but inactivation of alleles by point mutation has not been found (24, 25). We found *APC* (69%) and *RASSF1A* (51%) to be frequently hypermethylated in the series of 45 bladder tumors, and both genes were included in the panel to screen the 45 paired urine DNAs.

The frequency of hypermethylation of the three genes observed in our study was broadly in line with previous reports (21–23). We obtained 100% diagnostic coverage in 45 transitional cell carcinomas representative of all grades and stages. We have demonstrated that promoter hypermethylation of three tumor suppressor genes is common in bladder cancer and can be readily detected in a specific manner in urine DNA. In this initial feasibility study, hypermethylation was positive in a statistically significantly ($P = 0.001$, McNemar's test) greater number of urines (31 of 34) than was cytology analysis (17 of 34). MSP was particularly successful in detecting superficial

tumors (Ta, T1, and Tis) in our study, a finding reported recently in another study (26).

Hypermethylation was not detected in six urine DNAs (from one Ta, one Tis, and four muscle-invasive cancer patients), most likely because in these urine samples neoplastic DNA was present in an amount lower than can currently be detected by conventional MSP. As is routine in PCR methodology, we chose to limit PCR to a maximum number of cycles ($n = 35$) because it is known that specificity can decrease in MSP, as in other PCR protocols, with increased cycle number (27). The nondetection by MSP of urine DNAs from two cytology-positive cases (patients 15 and 45) might be explained by the different time points of urine sampling. In eight cases (patients 1, 6, 25, 44, 53, 55, 63, and 85) where more than one gene was hypermethylated in the tumor DNA, not all of the methylated genes was detected in the paired urine DNA. This is likely due to differences in PCR amplification efficiencies between primer sets but will require further study. We believe that the sensitivity level of hypermethylation-based detection can be improved upon by the study of optimal urine collection techniques, enrichment of neoplastic cells or DNA from the urine by antibody or oligo-based magnetic bead technology, and improvements in PCR technology.

Although we only included in the hypermethylation panel genes reported to be unmethylated in normal cells, we also performed several controls for specificity (Table 2). First, we examined the urine DNA for the methylation status of a gene known to be unmethylated in the tumor DNA. This approach has been validated in previous MSP-based detection studies (28–30). Tumor 53 in Fig. 1 did not have *p14^{ARF}* hypermethylation, and the matched urine DNA was also negative. There was no case where a urine DNA gave a positive methylation result in the absence of methylation in the corresponding tumor (potential false positive). Moreover, we did not observe *APC*,

RASSF1A, or *p14^{ARF}* hypermethylation in urine DNA from 12 normal, healthy, age-matched controls and 9 patients with the benign disease cystitis (Fig. 2). We also examined urethral tissue containing nonneoplastic transitional cells from five patients with a single discrete RCC (*i.e.*, nontransitional cell cancer). We found no *APC*, *RASSF1A*, or *p14^{ARF}* hypermethylation in these normal urothelial cells and thus observed absolute specificity for cancer, albeit in a limited study. Furthermore, no hypermethylation was observed in urine DNAs from 5 patients with benign prostatic hyperplasia or prostatitis and 12 patients with nonneoplastic renal disease, *i.e.*, cysts or stones (31).

Promoter hypermethylation of a gene is not necessarily specific to only one type of cancer and, like other mechanisms of inactivation of suppressor genes, deletion and point mutation, can be found in different types of cancer (21). The three genes in our panel are known to be hypermethylated in other types of urological cancer with access to the urine (21–23, 32). Two genes that may be useful for differential diagnosis are *GSTP1*, which is hypermethylated in 90% of prostate cancer (33) but in only 5% of bladder and renal cancer (21, 22), and *VHL*, hypermethylation of which is found only in classical clear cell renal tumors (21). It is likely that genes hypermethylated exclusively or more frequently in bladder cancer will be identified in the near future. Inclusion of such genes in a bladder cancer detection panel would provide greater specificity for this cancer and allow differential diagnosis from renal or prostate cancer. Ultimately, all types of urological cancer might be screened for in urine with a larger panel of hypermethylated genes.

As the timing of hypermethylation of certain genes is found to be associated with a defined biological event or pathological stage of bladder cancer, the panel could be easily extended in the future to simultaneously provide molecular staging and prognostic information. It may also be possible to predict the behavior of individual tumors within a particular pathological stage. The heterogeneity of genetic alterations in cancer, for example, which tumor suppressor gene pathways are abrogated in an individual tumor, is likely one underlying cause of differences in tumor behavior. For example, the panel used here contained the *p14^{ARF}* gene involved in the *p53/p14^{ARF}* tumor suppressor gene pathway (34) and the *APC* gene involved in the Wnt signaling pathway (35). Inclusion of *p16^{INK4a}* in a methylation panel would allow the status of the *Rb/p16^{INK4a}* pathway to be determined in some tumors (34).

In previous studies of molecular detection of bladder cancer, microsatellite analysis of loss of heterozygosity and new alleles revealed alterations matching the tumor in 95% of urine specimens from patients (36) and, in a second pilot study, identified 10 of 11 recurrences (37). Point mutations of the *p53* tumor suppressor gene, more common in high-grade and high-stage bladder cancers (5), and mitochondrial DNA have also been used as targets for molecular detection of bladder cancer in urine (38, 39). However, MSP-based detection has several advantages over microsatellite or point mutation-based detection of bladder cancer in urine. These include (a) the greater sensitivity of MSP, which will be important for detection of early, small, or possible precursor lesions; (b) the fact that, unlike point mutation, no prior knowledge of the gene status is needed; and (c) the fact that a normal blood sample is not needed to verify heterozygosity or that a base alteration is a somatic

mutation and not a polymorphism. Fluorescence *in situ* hybridization has also been used to detect chromosome copy number changes in urine from bladder cancer patients (40), but there may not be an obvious target of detection, *e.g.*, trisomies, in early bladder cancer (2, 5).

A recent study of methylation in urine DNAs from bladder cancer patients reported a diagnostic coverage of 100% using a panel of four genes (*death-associated protein kinase*, *RARβ2*, *E-cadherin*, and *p16^{INK4a}*) in bladder cancer and positive detection of hypermethylation in urine specimens from 20 of 22 (91%) bladder carcinoma patients (26). However, it should be noted that *RARβ2* was found to be methylated in several normal urothelium specimens, and the frequency of hypermethylation of the other genes examined was much higher than that in original reports or a recent hypermethylation profile in bladder cancer (21, 22). In addition, methylation of *E-cadherin* has been associated with aging in normal urothelial tissue (41). The detection of hypermethylation in tumors and matched urine from patients with early-stage bladder cancer suggests that hypermethylation is both frequent and a relatively early event in bladder tumorigenesis and is therefore a suitable target for early detection of bladder cancer. However, both our present study and that of Chan *et al.* (26) examined only transitional cell carcinoma of the bladder. The ability of hypermethylation to detect squamous cell carcinoma and adenocarcinoma, which represent <10% of bladder cancers, will need to be determined.

The gene hypermethylation panel tested here provided 100% diagnostic coverage of 45 bladder cancers, representative of grade and stage at presentation, and is certainly manageable in terms of time and economy in view of recent microarray and high-throughput technology. In this study, we have demonstrated the feasibility of sensitive (87%) and 100% specific (no false positives) detection of hypermethylation in urine from bladder cancer patients including 16 of 18 patients with noninvasive (Ta or Tis) disease. If these results are confirmed in larger studies, promoter hypermethylation may have useful clinical application in bladder cancer diagnosis and management.

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