

Quantitative Detection of Promoter Hypermethylation of Multiple Genes in the Tumor, Urine, and Serum DNA of Patients with Renal Cancer

Mohammad Obaidul Hoque,¹ Shahnaz Begum,² Ozlem Topaloglu,¹ Carmen Jeronimo,¹ Elizabeth Mambo,¹ William H. Westra,² J. A. Califano,¹ and David Sidransky¹

¹Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins School of Medicine, Baltimore, Maryland, and ²Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland

ABSTRACT

Aberrant promoter hypermethylation of several known or putative tumor suppressor genes occurs frequently during the pathogenesis of human cancers and is a promising marker for cancer detection. We investigated the feasibility of detecting aberrant DNA methylation in the urine and serum samples of renal cancer patients. We examined the tumor and the matched urine and serum DNA for aberrant methylation of nine gene promoters (*CDH1*, *APC*, *MGMT*, *RASSF1A*, *GSTP1*, *p16*, *RAR-β2*, and *ARF*) from 17 patients with primary kidney cancer by quantitative fluorogenic real-time PCR. An additional 9 urine samples (total, 26) and 1 serum sample (total, 18) also were tested from renal cancer patients. Urine from 91 patients without genitourinary cancer and serum from 30 age-matched noncancer individuals were used as controls. Promoter hypermethylation of at least two of the genes studied was detected in 16 (94%) of 17 primary tumors. Aberrant methylation in urine and serum DNA generally was accompanied by methylation in the matched tumor samples. Urine samples from 91 control subjects without evidence of genitourinary cancer revealed no methylation of the *MGMT*, *GSTP1*, *p16*, and *ARF* genes, whereas methylation of *RAR-β2*, *RASSF1A*, *CDH1*, *APC*, and *TIMP3* was detected at low levels in a few control subjects. Overall, 23 (88%) of 26 urine samples and 12 (67%) of 18 serum samples from cancer patients were methylation positive for at least one of the genes tested. By combination of urine or serum analysis of renal cancer patients, hypermethylation was detected in 16 of 17 patients (94% sensitivity) with high specificity. Our findings suggest that promoter hypermethylation in urine or serum can be detected in the majority of renal cancer patients. This noninvasive high-throughput approach needs to be evaluated in large studies to assess its value in the early detection and surveillance of renal cancer.

INTRODUCTION

Approximately 30,000 new cases of renal cancer are reported each year in the United States, with ~12,000 individuals dying annually from this disease (1). Identification of patients with organ-confined renal carcinoma may be of importance for long-term disease-free survival after radical or partial nephrectomy (2). The specific clinical signs and symptoms of malignant renal disease are not usually helpful in making an early diagnosis. The classic triad of pain, hematuria, and

a palpable flank mass is encountered in only 10% of patients and is usually associated with the presence of advanced disease (3). Unlike other solid malignancies in which established serum or urinary biomarkers are available for early detection, relatively few diagnostic tools are available for the early detection of renal tumors. Although the increased use of radiographic imaging modalities, such as computed tomography and ultrasound, has aided in disease diagnosis, an ideal tumor marker with high sensitivity and specificity offers the ideal opportunity for early detection of renal carcinoma.

Epigenetic alterations, including changes in the status of DNA methylation, are one of the most common molecular alterations in human neoplasia (3–7), including renal cancer (8–13). Cytosine methylation occurs after DNA synthesis by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine. Cytosines are methylated in the human genome almost exclusively when located 5' to a guanosine. Regions with a high G:C content (so-called CpG islands) are mostly unmethylated in normal tissue but may be methylated to varying degrees in human cancers, thus representing tumor-specific alterations (14, 15). The presence of abnormally high DNA concentrations in the serum and urine of patients with various malignant diseases has been confirmed during the past decade (16–18). Some studies recently have reported DNA in the serum and urine of renal cancer patients at diagnosis (19, 20). We have reported the presence of methylated DNA in the bodily fluids of patients with various types of malignancies and the absence of methylated DNA in normal control patients (21–23). To date, most studies detecting hypermethylation rely on conventional methylation-specific PCR (MSP), a sensitive but not quantitative assay. The major advantage of using quantitative methylation-specific PCR (QMSP) is based on the ability to define a cutoff point between cancer and control groups.

We evaluated the diagnostic potential of DNA methylation-based markers in pretherapeutic urine and serum DNA from renal cancer patients. Using QMSP, we analyzed the promoter hypermethylation pattern of 9 cancer-related genes in 17 renal cell carcinomas with matched urine and serum DNA. Nine additional (total, 26 urine sediments) urine sediments and 1 serum sample (total, 18 serum samples) from renal cancer patients without matched tumor tissue also were examined. Ninety-one urine samples from patients without genitourinary cancer and 30 serum samples from patients without cancer served as controls.

MATERIALS AND METHODS

Sample Collection and DNA Preparation. After we obtained written informed consent from 26 patients with a renal lesion, 18 samples of peripheral blood and 26 urine samples were collected before surgical intervention. Overall, we collected 17 urine and serum DNA samples with matched primary tumor tissue, and 9 additional urine sediment samples and 1 serum sample from these patients also were used to determine the clinical sensitivity of the QMSP assay. The Institutional Review Board of The Johns Hopkins University School of Medicine approved the study. Neoplastic kidney tissue was obtained immediately after surgical resection and stored at -80°C . We examined urine from 91 age-matched control subjects (median age, 56.5 years; range, 28–84

Received 3/4/04; revised 5/19/04; accepted 5/25/04.

Grant support: The abstract for this article was awarded the AACR-AstraZeneca Scholar-in-Training Award at the 95th Annual Meeting of the American Association for Cancer Research, 2004. Under a licensing agreement between Oncomethylome Sciences, SA, and the Johns Hopkins University, D. Sidransky is entitled to a share of royalty received by the University on sales of products described in this article. D. Sidransky owns Oncomethylome Sciences, SA, stock, which is subject to certain restrictions under University policy. Dr. Sidransky is a paid consultant to Oncomethylome Sciences, SA, and is a paid member of the company's Scientific Advisory Board. The Johns Hopkins University in accordance with its conflict of interest policies is managing the terms of this agreement.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: M. O. Hoque and S. Begum contributed equally to this study; Supplementary data for this article can be found at Cancer Research Online (<http://cancerres.aacrjournals.org>).

Requests for reprints: David Sidransky, Division of Head and Neck Cancer Research, The Johns Hopkins School of Medicine, 720 Rutland Avenue, Baltimore, MD 21287. Phone: 410-502-5153; Fax: 410-614-1411; E-mail: dsidrns@jhmi.edu.

Table 1 Samples showing methylation in the tumor, urine, and serum

No.	Pathology ^a	Age (y)	Sex	pTNM ^b	Grade ^c	Symptoms/history	Methylation tumor/urine/serum ^d
1	RCC, clear cell	70	M	T1NXMX	I-II	None	2/3/0
2	RCC, clear cell	33	M	T2NXMX	I	Hematuria/pain	4/1/1
3	RCC, clear cell	59	M	T2NXMX	I	None	2/1/1
4	RCC, clear cell	58	M	T2NXMX	II/IV	CIS of glans, pain, choleliathesis	5/5/2
5	RCC, clear cell	74	F	T2NXMX	II	Glomerulosclerosis	3/2/1
6	RCC, clear cell	61	F	T2NOMX	II	None, renal pelvis involved	2/0/1
7	RCC, clear cell	65	M	T3aNXMX	II	Discomport	3/1/0
8	RCC, papillary	70	M	T2NXMX	III	None, collecting duct involved	5/1/0
9	RCC, clear cell	45	M	T2NXMX	I	None	4/4/2
10	RCC, clear cell	72	M	T3aNXMX	III	None	3/1/0
11	RCC, clear cell	46	F	T2NXMX	II-III	None	0/0/0
12	RCC, clear cell	65	M	T3bN0M1	III	Metastasis (lung, subcutaneous)	4/1/0
13	RCC, clear cell	60	M	T2MONX	II	None	3/2/1
14	RCC, chromoprobe	52	M	T2NOMX	II-III	Microscopic hematuria	5/4/2
15	RCC, clear cell	75	M	T2NXMX	II	Recurrent UTI, hematuria	8/6/1
16	RCC, clear cell	61	M	T2NXMX	II	Hematuria	6/6/2
17	RCC, clear cell	51	M	T2NOMX	II-III	Hematuria	5/5/2
18	RCC, clear cell	60	F	T1N0MX	I-II	Discomfort	ND/3/1
19	RCC, clear cell	69	F	NA	II/IV	Hematuria	ND/4/ND
20	RCC, clear cell	55	M	pT2, Nx, MX	I-II/IV	Pain	ND/4/ND
21	Collecting duct carcinoma	61	F	pT3 N1 MX	NA	Lyme disease	ND/2/ND
22	RCC, clear cell	63	F	pT2, Nx, MX	NA	Hematuria	ND/8/ND
23	RCC, clear cell	68	M	NA	III	Pain and microscopic hamaturia	ND/6/ND
24	RCC, clear cell	65	F	T3b Nx MX	IV/IV	Recurrent UTI, hematuria pain	ND/5/ND
25	RCC, clear cell	81	M	PT3NXMX	III/IV	Nocturia	ND/0/ND
26	RCC, clear cell	54	F	NA	NA	NA	ND/5/ND

^a RCC, renal cell carcinoma; pTNM: p, pathologic stage; T, tumor size; N, node status; M, metastatic status; NA, not available; CIS, carcinoma *in situ*; UTI, urinary tract infection; ND, not done.

^b American Joint Committee on Cancer staging.

^c American Joint Committee on Cancer.

^d Number under methylation columns indicates positive methylated genes in tumor, urine, and serum DNA. In total, nine genes were tested by quantitative methylation-specific PCR in each clinical sample. All samples are from patients with malignant tumors.

years) for all of the nine genes. Of these 91 subjects, 9 patients were diagnosed with benign prostate hyperplasia; 10 patients harbored atypical cells by urine cytology examination; 5 had cancer other than of the genitourinary system (1 non-small cell carcinoma of lung, 1 basal cell carcinoma of skin, 1 malignant melanoma of leg, 1 Kaposi's sarcoma of leg, and 1 infiltrating ductal carcinoma of the breast); 1 had fibroepithelial polyp of the bladder; 3 had tubular adenomas of the colon; 1 had organizing thrombus in the vagina; 25 visited the hospital for routine physical examination; 20 had either macroscopic or microscopic hematuria; and 17 had vague urologic symptoms but no malignant condition was detected. Among the 91 patients, 66 were male and 25 were

female. Thirty serum samples (15 from smokers and 15 from nonsmokers without any history of cancer) from age-matched individuals were collected as controls. Seventeen primary tumors were later collected, and tumor tissues were microdissected as described previously (24). DNA was obtained from serum, urine, and tumor samples by digestion with 50 μ g/ml proteinase K (Boehringer, Mannheim, Germany) in the presence of 1% SDS at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. Detailed information on these patients is summarized in Table 1.

Bisulfite Treatment. DNA from urine sediment was subjected to bisulfite treatment as described previously (25). Briefly, 2 μ g of genomic DNA were

No.	APC			ARF			CDH1			GSTP1			MGMT			P16			RAR-B2			Rassf1A			TIMP3		
	T	U	S	T	U	S	T	U	S	T	U	S	T	U	S	T	U	S	T	U	S	T	U	S			
1	■	■	■				■												■			■					
2							■												■			■					
3							■												■			■					
4	■	■	■				■												■			■					
5							■												■			■					
6							■												■			■					
7	■						■												■			■					
8							■												■			■					
9	■	■	■				■												■			■					
10	■	■	■				■												■			■					
11							■												■			■					
12							■												■			■					
13							■												■			■					
14	■	■	■				■												■			■					
15	■	■	■				■												■			■					
16							■												■			■					
17							■												■			■					

Fig. 1. Summary of methylation states of *GSTP1*, *ARF*, *P16*, *MGMT*, *RARβ2*, *TIMP3*, *CDH1*, *APC*, and *RASSF1A* in 17 primary tumors (T) and matched urine (U) and serum (S) samples. Black boxes represent samples that are methylated; white boxes represent samples without methylation.

Table 2 Frequency of methylation based on different cutoff points and median values in clinical samples

Markers	Methylation positive % (number of methylation positive/number of total cases)						Cutoff values	Median				
	Tumor	Urine (cancer)	Serum (cancer)	Urine (control)	Serum (control)	Tumor		Urine (cancer)	Serum (cancer)	Serum (control)	Urine (control)	
<i>APC</i>	29% (5/17)	38% (10/26)	6% (1/18)	4% (4/91)	3% (1/30)	4.5	2.89	2.95	0	0	0	
<i>ARF</i>	24% (4/17)	31% (8/26)	6% (1/18)	0% (0/91)	3% (1/30)	0	0	0	0	0	0	
<i>CDH1</i>	59% (10/17)	38% (10/26)	33% (6/18)	5% (5/91)	7% (2/30)	0.3	0.28	0.23	0	0	0	
<i>GSTP1</i>	12% (2/17)	15% (4/26)	6% (1/18)	0% (0/91)	0% (0/30)	0	0	0	0	0	0	
<i>MGMT</i>	6% (1/17)	8% (2/26)	0% (0/18)	0% (0/91)	3% (1/30)	0	0	0	0	0	0	
<i>p16</i>	35% (6/17)	35% (9/26)	22% (4/18)	0% (0/91)	0% (0/30)	0	0	0	0	0	0	
<i>RAR-β2</i>	53% (9/17)	31% (8/26)	6% (1/18)	9% (8/91)	0% (0/30)	0.1	0	0	0	0	0	
<i>RASSF1A</i>	88% (15/17)	65% (17/26)	11% (2/18)	11% (10/91)	3% (1/30)	0.1	10.9	9	0	0	0	
<i>TIMP3</i>	71% (12/17)	46% (12/26)	17% (3/18)	9% (8/91)	0% (0/30)	1	1.92	0.02	0	0	0	

denatured in 0.2 M NaOH for 20 min at 50°C. The denatured DNA was diluted in 500 µl of a freshly prepared solution of 10 mM hydroquinone and 3 M sodium bisulfite and was incubated for 3 h at 70°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System; Promega, Madison, WI), treated with 0.3 M NaOH for 10 min at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 120 µl of H₂O and stored at -80°C.

Methylation Analysis. The bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR (Taqman) as described previously (26). In brief, primers and probes were designed to specifically amplify the bisulfite-converted promoter of the gene of interest (23, 26–28). The ratios between the values of the gene of interest and the internal reference gene, β-actin, obtained by Taqman analysis were used as a measure for representing the relative level of methylation in the particular sample (gene of interest/reference gene × 1000) as described previously (28, 29). Fluorogenic PCRs were carried out in a reaction volume of 20 µl consisting of 600 nM of each primer; 200 nM probe; 0.75 units of platinum Taq polymerase (Invitrogen, Carlsbad, CA); 200 µM each of dATP, dCTP, dGTP, and dTTP; 16.6 mM ammonium sulfate; 67 mM Trizma; 6.7 mM MgCl₂ (2.5 mM for *p16*); 10 mM mercaptoethanol; and 0.1% DMSO. Three µl of treated DNA solution were used in each real-time MSP reaction. Amplifications were carried out in 384-well plates in a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each plate consisted of patient samples and multiple water blanks and positive and negative controls. Leukocytes from a healthy individual were methylated *in vitro* with excess SssI methyltransferase (New England Biolabs, Beverly, MA) to generate completely methylated DNA, and serial dilutions of this DNA were used for constructing the calibration curves on each plate.

Statistical Analysis. All of the statistical tests were performed using Excel software (Microsoft, Redmond, WA). The sensitivity of QMSP-based detection of hypermethylation in urine and serum was calculated as number of positive tests/number of cancer cases. The specificity was calculated as number of negative tests/number of cases without genitourinary cancer for urine (and absence of any cancer for serum).

RESULTS

Frequency of Methylation in Primary Kidney Tumors. We examined nine genes of diverse function, including cell cycle regulation, metastatic suppression, tumor suppression, and DNA repair in paired urine and serum specimens from patients with cancer and control subjects by QMSP. Aberrant promoter hypermethylation of at least two of the genes investigated was detected in 16 of 17 (94%) with malignant tumors of the kidney, and 13 of 17 (76%) were positive for at least three genes simultaneously (Table 1; Fig. 1). Interestingly, in one patient (Patient 11), no methylation was detected in any gene promoter. The frequency of aberrant methylation in all of the types of samples and median methylation values (gene/β-actin × 1000) for each gene in tumor, urine, serum, and control DNA are shown in Table 2. In the supplementary data, we also have displayed methylation frequencies for the five genes (*APC*, *CDH1*, *RAR-β2*, *RASSF1A*, and *TIMP3*) in Table 2 with cutoff points >0. In the tumor samples, frequent methylation was detected in *RASSF1A* (88%), *TIMP3* (71%), *CDH1* (59%), *RAR-β2* (53%), *p16* (35%), *ARF*

(24%), and *APC* (29%). Methylation of *GSTP1* and *MGMT* was much less common, 12% and 6%, respectively. Aberrant methylation in primary kidney tumors had no correlation with patient demographic data, including age and gender, histologic subtype, and staging of the tumor (data not shown).

Methylation in Urine and Serum DNA. The matching 17 urine and serum samples from these kidney cancer patients then were tested for methylation. An additional nine urine samples and one serum sample from renal cancer patients (without matched primary tumor) also were included in this study. The analytical and clinical sensitivity of individual genes is shown in Table 3. Overall, 23 of 26 (88%) cancer patients were methylation positive in urine sediment DNA for at least one of the nine genes tested (Table 1; Fig. 1). Urine DNA was negative in all of the 91 control subjects with no history of genitourinary neoplasm in four genes examined (*p16*, *MGMT*, *GSTP1*, and *ARF*). *CDH1*, *RASSF1A*, *TIMP3*, *RAR-β2*, and *APC* showed varying levels of methylation in some of the control urine sediment samples. For these five genes, we set the optimal cutoff value (Table 3; Fig. 2) to obtain the highest sensitivity and specificity. The analytical and clinical sensitivities of each gene with defined cutoff values are detailed in Table 3. Three urine samples harbored methylated *TIMP3* in the absence of methylation in the matched primary tissue. In nearly

Table 3 Sensitive detection of cancer in urine sediment and serum DNA of RCC^a patients using DNA methylation markers

Analytical sensitivity defined as the fraction of cases in which methylation of a marker is found in urine or serum for cases with confirmed methylation of the same marker in the associated tumor [e.g., in Table 2, the frequency of *APC* methylation in primary tumors is 29% (5/17); of these 5 methylated cases, methylation was detected in the urine of 4 patients; therefore, the analytical sensitivity is 80% (4/5)]. Clinical sensitivity is defined as the fraction of confirmed cases of disease, in which methylation of a marker is found in urine or serum, regardless of whether methylation of that marker is present in the associated tumor or regardless of whether the associated tumor has been analyzed for the presence of the marker. Cases in which urine or serum is not analyzed are excluded from both sensitivity calculations. Specificity is defined as the fraction of controls without the disease that show a lack of detectable methylation in urine or serum.

Disease	DNA source	Markers	Analytical sensitivity (%)	Clinical sensitivity (%)	Specificity (%)	Cutoff values
Renal cancer	Urine	<i>APC</i>	4/5 (80)	10/26 (38)	96	4.5
		<i>ARF</i>	3/4 (75)	8/26 (31)	100	0
		<i>CDH1</i>	6/10 (60)	10/26 (38)	95	0.3
		<i>GSTP1</i>	1/2 (50)	4/26 (15)	100	0
		<i>MGMT</i>	0/1 (0)	2/26 (8)	100	0
		<i>p16</i>	4/6 (67)	9/26 (35)	100	0
		<i>RAR-β2</i>	4/9 (44)	8/26 (31)	91	0.1
		<i>RASSF1A</i>	11/15 (73)	17/26 (65)	89	0.1
		<i>TIMP3</i>	6/12 (50)	12/26 (46)	91	1
	Serum	<i>APC</i>	1/5 (20)	1/18 (6)	97	4.5
		<i>ARF</i>	1/4 (25)	1/18 (6)	97	0
		<i>CDH1</i>	6/10 (60)	6/18 (33)	93	0.3
		<i>GSTP1</i>	0/2 (0)	1/18 (6)	100	0
		<i>MGMT</i>	0/1 (0)	0/18 (0)	97	0
		<i>p16</i>	3/6 (50)	4/18 (22)	100	0
		<i>RAR-β2</i>	1/9 (11)	1/18 (6)	100	0.1
		<i>RASSF1A</i>	2/15 (13)	2/18 (11)	97	0.1
<i>TIMP3</i>	2/12 (17)	3/18 (17)	100	1		

^a RCC, renal cell carcinoma.

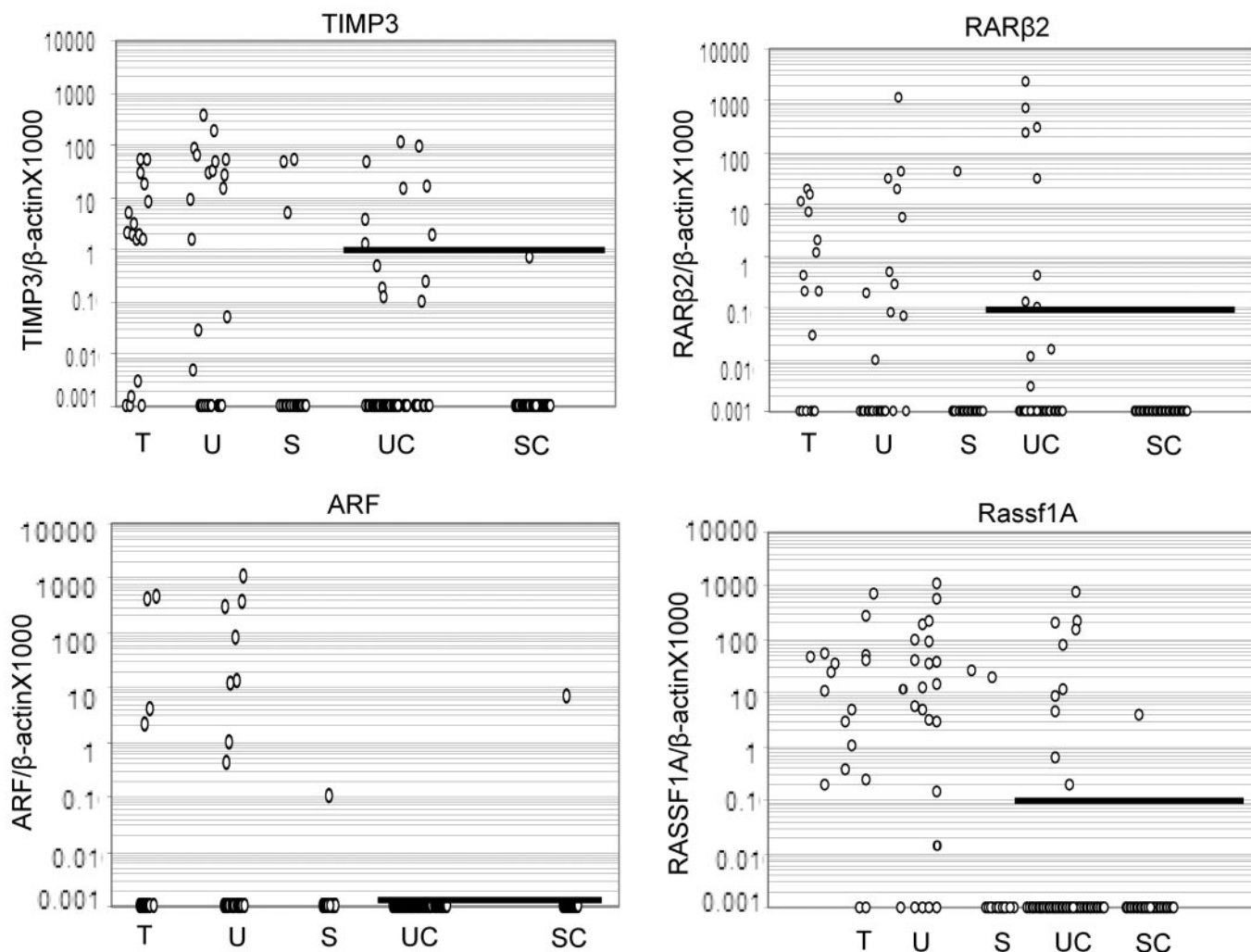


Fig. 2. Methylation levels of *TIMP3*, *RARβ2*, *ARF*, *RASS1A*, *APC*, *CDH1*, *GSTP1*, *p16*, and *MGMT* in renal cancer tissue (T), urine (U), and serum (S) samples from renal cancer patients and 91 urine (UC) and 30 serum (SC) samples from control individuals without genitourinary cancer. Calculation of the target gene:β-actin ratios were based on the fluorescence emission intensity values for the gene of interest and β-actin obtained by quantitative real-time PCR analysis. The relative amount of methylated promoter DNA is much higher in matched urine sediment and serum DNA samples from cancer patients than in the few control urine and serum samples with methylation. Black bar represent the cutoff value for each gene. Values designated as 0.001 are zero values, which cannot be plotted correctly on a log scale.

all cases, however, the identical methylation pattern was found between primary tumor and matched urine DNA samples as shown in Fig. 1. We found no correlation between the methylation index (total number of genes methylated/total number of genes analyzed) and any of the clinicopathologic characteristics (*i.e.*, tumor type, grade, and stage in urine sediment samples; data not shown).

In serum DNA, 12 of 18 (67%) patients were methylation positive for at least one of the genes tested (Fig. 1; Table 1). The frequency of aberrant promoter methylation detected in matched serum for each marker was 20% (1 of 5) for *APC*, 25% (1 of 4) for *ARF*, 60% (6 of 10) for *CDH1*, 0% (0 of 2) for *GSTP1*, 0% (0 of 1) for *MGMT*, 50% (3 of 6) for *p16*, 11% (1 of 9) for *RAR-β2*, 13% (2 of 15) for *RASS1A*, and 17% (2 of 12) for *TIMP3*. Methylation again was detected in one serum sample for *TIMP3* without evidence of methylation in the primary tumor. None of the 30 controls displayed aberrant promoter methylation in four genes examined (*p16*, *RAR-β2*, *TIMP3*, and *GSTP1*) in serum. Two of the control sera displayed methylation of *CDH1* at low levels (3.1 and 3.6; cutoff value for *CDH1* was 0.3). However, *MGMT*, *APC*, *RASS1A*, and *ARF* displayed methylation in one sample each at reasonably high level. Interestingly, all of the six control patients who displayed serum methylation above the cutoff values were smokers. No serum meth-

ylation was detected in the nonsmoker control group. The specificity, clinical sensitivity, analytical sensitivity, and cutoff points are summarized in Table 3.

DISCUSSION

Advances in basic research have shed light on key alterations that contribute to the development of renal neoplasia. Detailed studies of pathology have underscored the morphologic heterogeneity of renal cancers (30). Genetic and epigenetic studies using a variety of technologies have shown that renal cancers are characterized by specific genetic and epigenetic alterations (*e.g.*, loss of heterozygosity at the *VHL* locus; Ref. 31) and hypermethylation of *RASS1A*, *TIMP3*, *p16*, *GSTP1*, and *CDH1* (8–10, 12, 13, 32). However, these advances in basic research have not yet translated into the development of reliable diagnostic markers for renal cancer.

In a previous study using the same set of samples, we demonstrated that microsatellite analysis of urine DNA could detect the presence of malignancy in patients with clinically organ-confined renal cancer (19). In the present study, 94% of primary kidney tumors harbored CpG island hypermethylation of at least two of nine cancer-related genes. Eighty-eight percent of patients with aberrant methylation in

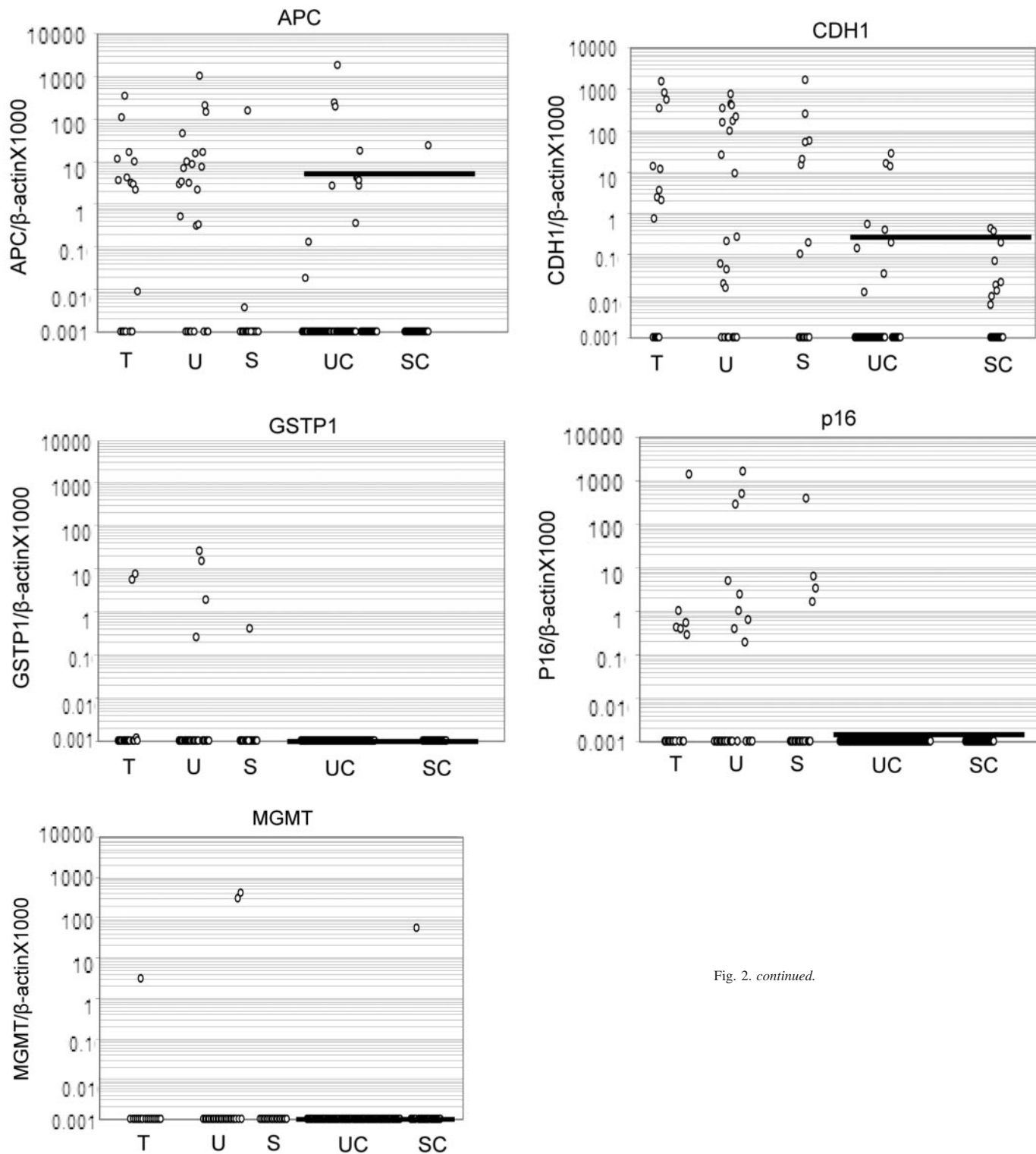


Fig. 2. continued.

primary tumors also exhibited hypermethylation in urine DNA. Because there were some false-positive results for *TIMP3*, we found a 76% sensitivity using only the remaining eight genes. Heterogeneity of neoplastic cells in urine and tumor foci may contribute to this discrepancy. Conversely, *TIMP3* methylation may be a feature of non-neoplastic tissues excluding its value as a diagnostic marker.

Excluding *TIMP3*, it is noteworthy that detection of promoter methylation in the urine of renal cancer patients was a specific event: (a) overall aberrant methylation was not detected in any of the 91

age-matched control urine samples with the exception of low levels in five genes; and (b) the identical methylation profiles were found in the corresponding tumor; aberrant methylation was not detected in the urine of kidney cancer patients without methylation in the corresponding tumor.

The development of real-time PCR has simplified the study of genes inactivated by promoter hypermethylation in human cancer. It is a highly sensitive assay that is capable of detecting methylated alleles in the presence of a 1000-fold excess of unmethylated alleles.

QMSP may be more sensitive than conventional MSP but varies based on the tested promoter, primers, and PCR conditions. On the basis of conventional MSP, methylated *p16* alleles in the primary renal cell carcinoma were detected from 20–32% (8). In the present study, *p16* was methylated in 35% of primary tumors and in 67% and 50% of matched urine and serum samples, respectively.

Several studies using different approaches have demonstrated promoter hypermethylation of *CDH1* (67%), *RASSF1A* (44–91%), *p16* (20–32%), *GSTP1* (20%), and *TIMP3* (78%) in primary renal tumor tissue (8–13, 32). We also observed a similar frequency of methylation for all of these genes, including *RASSF1A* (88%), *CDH1* (59%), *TIMP3* (71%), and *GSTP1* (12%) in primary kidney tumors. To our knowledge, methylation of *MGMT*, *RAR-β2*, *APC*, and *ARF* was not tested in renal cancer. The promoter of the latter three genes harbored frequent methylation in primary tumors, but *MGMT* (6% methylation) may not represent a good marker for kidney cancer. Thus, it is likely that an optimal panel of methylation marker can be chosen with high sensitivity and specificity. Moreover, multimarker methylation approaches no longer represent a technical barrier with new high-throughput platforms.

The detection of tumor molecular signatures in body fluids has implications for the identification of high-risk subjects, patients with preinvasive or early stage lesions, and for monitoring residual disease. Molecular approaches characterized by a high specificity have variable sensitivity, perhaps because of the presence of low tumor DNA quantities in urine or serum or because of a high level of contamination with normal DNA. Several approaches to improve assay sensitivity have been applied to tumor tissue, plasma, sputum, stool, and bronchoalveolar lavage samples. Sensitivity has been improved over conventional MSP by performing a seminested MSP after a DNA preamplification step (33) or a nested two-stage PCR with a concomitant reduction in specificity and lack of quantitation (34). We believe the sensitivity and specificity of QMSP can likely be improved by: (a) isolation of neoplastic cells or DNA from the urine by antibody or oligo-based magnetic bead technology before DNA extraction; and (b) increasing the number of renal cancer-specific markers. Regardless, more sensitive assays almost always result in imperfect specificity and must be validated in clinical samples.

We did not uncover methylation in any of the nine genes tested in two patients (Patients 11 and 25). Eventual identification of new renal cancer-specific tumor suppressor genes and their genetic and epigenetic studies may provide additional markers for such patients. Interestingly, in one of these cases (Patient 11; pT2, grade II–III) we previously found loss of heterozygosity only in one microsatellite marker in the tumor, and no loss of heterozygosity or microsatellite instability was detected in the matched urine and serum samples. Thus, it is possible that some kidney tumors do not generate or contribute sufficient DNA into the urine for this analysis.

During the preparation of this manuscript, Battagli *et al.* (35) published the results of testing five of the nine genes analyzed in our study, including *APC*, *ARF*, *p16*, *RASSF1A*, and *TIMP3*, in primary kidney tumors and matched urinary sediment. Although conventional MSP is not comparable with QMSP, our results principally confirm the results presented in their study for these particular genes. In contrast to their reported 100% specificity for *RASSF1A* and *TIMP3*, we found reduced specificity probably because of the detection of low levels of methylated allele by quantitation. Importantly, the addition of the *VHL* marker from their panel would likely improve the sensitivity of our QMSP assay. The QMSP assay provides several distinct advantages over conventional MSP: (a) omission of all of the post-amplification steps reduces the risk of contamination and increases the throughput of the system; (b) the assay is more stringent and more specific because in addition to the two PCR primers, the fluorescent-

labeled hybridization probe has to anneal correctly between the two primers; (c) the assay is quantitative, automated, and readily adaptable to clinical setting and screening studies; and (d) the assay is amenable to multiplex amplification for the analysis of panels in clinical samples. At present, we can use four different dyes for the amplification of four distinct markers, but further developments in dye chemistry will improve the multimarker diagnostic approach (presently, we can use four different dyes in Taqman technology) from nanogram quantities of low molecular weight DNA. These advances are unlikely to follow in conventional MSP.

Although the sensitivity of current cytologic analysis is low, it is routinely performed for bladder cancer. Diagnostic tools that would provide high specificity and sensitivity would clearly be of enormous benefit to patients, particularly if the specimens could be obtained by noninvasive means. To this end, the detection of aberrant methylation in urine sediment or serum DNA may offer a promising approach for the noninvasive diagnosis of renal cancer. This method is highly specific and correlates with tumor methylation status. Apart from early detection, it would be interesting to see whether the detection of aberrant methylation in the urine or serum DNA could be used to monitor disease progress after curative surgery. If methylated DNA disappears shortly in urine or serum after curative surgery, the reappearance of these markers may suggest recurrence of disease that may require more intensive screening and aggressive treatment. Additional studies are necessary to elucidate the role of detecting aberrant methylation in urine or serum as a tool for early detection and surveillance of renal cancer. If our results are confirmed in larger studies, the panel easily could be expanded in the future to simultaneously provide molecular staging and prognostic information in addition to detection. We hope that our findings reported here will provide a stimulus for such future studies.

REFERENCES

- Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 2000;50:7–33.
- Pantuck AJ, Zisman A, Rauch MK, Belldegrun A. Incidental renal tumors. *Urology* 2000;56:190–6.
- Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998;72:141–96.
- Bird A. The essentials of DNA methylation. *Cell* 1992;70:5–8.
- Merlo A, Herman JG, Mao L, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995;1:686–92.
- Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998;95:6870–5.
- Esteller M, Levine R, Baylin SB, Ellenson LH, Herman JG. MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene* 1998;17:2413–7.
- Romanenko A, Morell-Quadreny L, Lopez-Guerrero JA, et al. P16INK4A and p15INK4B gene alteration associated with oxidative stress in renal cell carcinomas after the Chernobyl accident (pilot study). *Diagn Mol Pathol* 2002;11:163–9.
- Bachman KE, Herman JG, Corn PG, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* 1999;59:798–802.
- Nojima D, Nakajima K, Li LC, et al. CpG methylation of promoter region inactivates E-cadherin gene in renal cell carcinoma. *Mol Carcinog* 2001;32:19–27.
- Kawakami T, Okamoto K, Ogawa O, Okada Y. Multipoint methylation and expression analysis of tumor suppressor genes in human renal cancer cells. *Urology* 2003;61:226–30.
- Wagner KJ, Cooper WN, Grundy RG, et al. Frequent RASSF1A tumour suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. *Oncogene* 2002;21:7277–82.
- Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB, Herman JG. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res* 1998;58:4515–8.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–28.
- Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003;3:253–66.
- Ngan RK, Lau WH, Yip TT, et al. Remarkable application of serum EBV EBER-1 in monitoring response of nasopharyngeal cancer patients to salvage chemotherapy. *Ann N Y Acad Sci* 2001;945:73–9.

17. Lo YM. Prognostic implication of pretreatment plasma/serum concentration of Epstein-Barr virus DNA in nasopharyngeal carcinoma. *Biomed Pharmacother* 2001;55:362–5.
18. Sidransky D, Von Eschenbach A, Tsai YC, et al. Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 1991;252:706–9.
19. Eisenberger CF, Schoenberg M, Enger C, et al. Diagnosis of renal cancer by molecular urinalysis. *J Natl Cancer Inst* 1999;91:2028–32.
20. Gonzalgo ML, Eisenberger CF, Lee SM, et al. Prognostic significance of preoperative molecular serum analysis in renal cancer. *Clin Cancer Res* 2002;8:1878–81.
21. Sanchez-Cespedes M, Esteller M, Wu L, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 2000;60:892–5.
22. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;59:67–70.
23. Topaloglu O, Hoque MO, Tokumaru Y, et al. Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. *Clin Cancer Res* 2004;10:2284–8.
24. Hoque MO, Lee CC, Cairns P, Schoenberg M, Sidransky D. Genome-wide genetic characterization of bladder cancer: a comparison of high-density single-nucleotide polymorphism arrays and PCR-based microsatellite analysis. *Cancer Res* 2003;63:2216–22.
25. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;93:9821–6.
26. Harden SV, Tokumaru Y, Westra WH, et al. Gene promoter hypermethylation in tumors and lymph nodes of stage I lung cancer patients. *Clin Cancer Res* 2003;9:1370–5.
27. Eads CA, Lord RV, Wickramasinghe K, et al. Epigenetic patterns in the progression of esophageal adenocarcinoma. *Cancer Res* 2001;61:3410–8.
28. Eads CA, Danenberg KD, Kawakami K, et al. MethylLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 2000;28:E32.
29. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* 1999;59:2302–6.
30. Thoenes W, Storkel S, Rumpelt HJ. Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. *Pathol Res Pract* 1986;181:125–43.
31. Gnarr JR, Tory K, Weng Y, et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 1994;7:85–90.
32. Dreijerink K, Braga E, Kuzmin I, et al. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. *Proc Natl Acad Sci USA* 2001;98:7504–9.
33. Kersting M, Friedl C, Kraus A, Behn M, Pankow W, Schuermann M. Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers. *J Clin Oncol* 2000;18:3221–9.
34. Palmisano WA, Divine KK, Saccomanno G, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res* 2000;60:5954–8.
35. Battagli C, Uzzo RG, Dulaimi E, et al. Promoter hypermethylation of tumor suppressor genes in urine from kidney cancer patients. *Cancer Res* 2003;63:8695–9.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Quantitative Detection of Promoter Hypermethylation of Multiple Genes in the Tumor, Urine, and Serum DNA of Patients with Renal Cancer

Mohammad Obaidul Hoque, Shahnaz Begum, Ozlem Topaloglu, et al.

Cancer Res 2004;64:5511-5517.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/64/15/5511
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2004/08/24/64.15.5511.DC1

Cited articles	This article cites 35 articles, 17 of which you can access for free at: http://cancerres.aacrjournals.org/content/64/15/5511.full#ref-list-1
Citing articles	This article has been cited by 26 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/64/15/5511.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/64/15/5511 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.