[CANCER RESEARCH 63, 8695–8699, December 15, 2003]

Promoter Hypermethylation of Tumor Suppressor Genes in Urine from Kidney Cancer Patients

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ABSTRACT

Kidney cancer confined by the renal capsule can be surgically cured in the majority of cases, whereas the prognosis for patients with advanced disease at presentation remains poor. Novel strategies for early detection are therefore needed. Molecular DNA-based tests have successfully used the genetic alterations that initiate and drive tumorigenesis as targets for the early detection of several types of cancer in bodily fluids, including urine. Using sensitive methylation-specific PCR, we screened matched tumor DNA and sediment DNA from preoperative urine specimens obtained in 50 patients with kidney tumors, representing all major histological types, for hypermethylation status of a panel of six normally unmethylated tumor suppressor genes *VHL*, *p16/CDKN2a*, *p14ARF*, *APC*, *RASSF1A*, and *Timp-3*. Hypermethylation of at least one gene was found in all 50 tumor DNAs (100% diagnostic coverage) and an identical pattern of gene hypermethylation found in the matched urine DNA from 44 of 50 patients (88% sensitivity), including 27/30 cases of stage I disease. In contrast, hypermethylation of the genes in the panel was not observed in normal kidney tissue or in urine from normal healthy individuals and patients with benign kidney disease (100% specificity). Hypermethylation of *VHL* was found only in clear cell, whereas hypermethylation of *p14ARF*, *APC*, or *RASSF1A* was more frequent in nonclear cell tumors, which suggested that the panel might facilitate differential diagnosis. We conclude that promoter hypermethylation is a common and early event in kidney tumorigenesis and can be detected in the urine DNA from patients with organ-confined renal cancers of all histological types. Methylation-specific PCR may enhance early detection of renal cancer using a noninvasive urine test.

INTRODUCTION

Renal cell carcinomas (RCCs) and tumors of the renal pelvis account for ~3% of all solid neoplasms with an incidence (estimated at 31,900 cases in the United States in 2003) roughly equal to that of all forms of leukemia combined (1). Between 25 and 40% of patients with RCC present with locally advanced or metastatic disease. Early clinical manifestations of RCC are diverse and may give rise to a spectrum of nonspecific and often misattributed symptoms. Indeed, a majority of RCCs are now discovered in patients not suspected of harboring a genitourinary malignancy. Unlike with other solid malignancies in which established serum or urinary biomarkers are available for early detection, diagnosis of RCC is confounded by the lack of cancer-specific diagnostic techniques. Because RCC is curable if detected when still confined to the renal capsule, the development of novel diagnostic noninvasive approaches for the early detection of kidney cancer is imperative (2, 3).

Silencing of tumor suppressor genes such as *p16*, *VHL*, *BRCA1*, and the mismatch repair gene *human homologue of MutL gene 1* have established promoter hypermethylation as a common mechanism for tumor suppressor inactivation in human cancer and as a promising new target for molecular detection (4, 5). Several cancer genes, including *p16* and von Hippel-Lindau gene (*VHL*), have been found to have hypermethylation of normally unmethylated CpG islands within the promoter regions in kidney cancer cells (6–8). Hypermethylation can be analyzed by the sensitive methylation-specific PCR (MSP) technique, which can identify 1 methylated allele in 1000 unmethylated alleles (9), appropriate for the detection of few neoplastic cells in a background of normal cells.

Bodily fluids that surround or drain the organ of interest from patients with various solid malignancies have been successfully used for MSP-based detection. These include detection of lung cancer in serum (10), sputum (11) and bronchial lavage (12), head and neck cancer (13) in serum, breast cancer in ductal lavage (14), and prostate cancer in urine (15). However, kidney cancer has not yet been tested. As most renal tumors arise from the tubular epithelium with potential access to urine, we hypothesized that urine from patients with kidney tumors could contain aberrant promoter hypermethylation of tumor suppressor genes in cancer cells or free DNA from apoptotic or necrotic cancer cells amenable to MSP analysis. We therefore screened paired kidney tumor and urine DNAs and normal and benign disease controls for hypermethylation of a panel of tumor suppressor genes.

MATERIALS AND METHODS

Specimen Collection and DNA Extraction. After approval from the Institutional Review Board, we obtained matched renal tumor and normal kidney tissue via the Fox Chase Cancer Center Tumor Bank Facility and 10–100 ml of preoperative urine from 50 patients, ages 30–80 years, who underwent nephrectomy or nephroureterectomy for enhancing renal masses. Tumors were graded according to American Joint Committee on Cancer (16) and staged after the 1997 tumor-node-metastasis system (Ref. 17; Table 1). Urine specimens from 12 normal, healthy individuals, 9 patients with nephrolithiasis (renal stones), and 3 patients with benign renal cysts were obtained as controls. Specimens of histologically confirmed normal ureteral urothelium were collected from 5 patients with RCC to provide normal transitional cell DNA. Tumor tissue was obtained immediately after surgical resection and subsequently microdissected with the assistance of a pathologist (T. A-S.). The urine specimen was centrifuged for 20 min at 5000 relative centrifugal force, and the supernatant decanted, except for ~200–500 μl surrounding the sediment pellet. 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 (18). Tissue specimen DNA was simply spooled out after precipitation with 100% ethanol. Urine DNA was precipitated with one-tenth volume of 10 M ammonium acetate, 2 μl of glycogen (Roche Diagnostics Corporation, 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).

Methylation-Specific PCR. Specimen DNA (0.25–1 μg) was modified with sodium bisulfite, converting all unmethylated but not methylated cytosine to uracil followed by amplification with primers specific for methylated *versus* unmethylated DNA. The genes used in the renal cancer detection panel were *VHL* (9), *p16* (9), *p14* (19), *adenomatous polyposis coli* [(*APC*); Ref. 20], *RAS association domain family protein 1A* [(*RASSF1A*); Ref. 21], and *tissue inhibitor of metalloproteinase-3* [(*Timp-3*); Ref. 7]. The primer sequences used have all been reported previously and can be found in the report referenced after

Received 6/5/03; revised 8/20/03; accepted 9/23/03.

Grant support: The Early Detection Research Network through the National Cancer Institute Grant U01. P. C. is a recipient of a Flight Attendants Medical Research Institute Young Clinical Scientist Award.

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Table 1 *Clinicopathological and hypermethylation detection data of 50 kidney cancer patients*

Age (years); grade, American Joint Committee on Cancer; pTNM:p, pathologic stage; T, tumor size; N, node status; M, metastatic status; stage, American Joint Committee on Cancer stage grouping, oncocytomas are not graded or staged, and all were confined to the kidney.

No.	Age/Sex	Cell Type	Size (cm)	Grade	TNM	Stage	VHL	RASSF1A	p16	p14	APC	Timp-3
1	43M	Clear cell	3	I	T1aNOMX	I	U/U	U/U	U/U	M/M	U/U	M/M
56	56M	Clear cell	3.5	I	T1aN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
57	62M	Clear cell	2.5	I–II	T1aN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
62	61M	Clear cell	2	II	T1aN0MX	I	M/M	U/U	U/U	U/U	U/U	M/M
18	70M	Clear cell	2.8	II	T1aN0MX	I	M/M	M/M	M/M	U/U	M/M	U/U
46	72F	Clear cell	4	II	T1aN0MX	I	U/U	U/U	M/M	M/M	U/U	M/M
53	60F	Clear cell	3.5	II	T1aN0MX	I	U/U	U/U	U/U	U/U	U/U	M/U
54	57M	Clear cell	2.2	II	T1aN0MX	I	U/U	U/U	U/U	M/M	U/U	U/U
13	67M	Clear cell	4	II	T1aNOMX	I	M/M	U/U	U/U	U/U	U/U	M/M
3	59M	Clear cell	3.5	III	T1aN0MX	I	U/U	M/M	U/U	U/U	U/U	U/U
37	42M	Clear cell	4	III	T1aN0MX	I	U/U	U/U	U/U	U/U	U/U	M/U
10	69M	Clear cell	3	III	T1aNOMX	I	M/M	M/M	U/U	U/U	M/M	M/M
7	78M	Clear cell	2.5	IV	T1aNOMX	I	U/U	M/M	U/U	U/U	U/U	U/U
11	52M	Clear cell	4	IV	T1aNOMX	I	U/U	M/M	U/U	U/U	U/U	U/U
48	62M	Clear cell	6	I–II	T1bN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
6	74F	Clear cell	4.4	II	T1bN0MX	I	U/U	M/M	U/U	U/U	M/M	U/U
5	56F	Clear cell	4.5	II	T1bN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
8	34F	Clear cell	5	II	T1bN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
49	57M	Clear cell	5.5	II	T1bN0MX	I	M/M	U/U	U/U	U/U	U/U	U/U
50	68M	Clear cell	5.5	II	T1bN0MX	I	U/U	U/U	U/U	U/U	U/U	M/U
38	61F	Clear cell	6.5	II	T1bN0MX	I	U/U	M/M	U/U	U/U	M/M	M/M
55	43M	Clear cell	6	II–III	T1bN0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
23	64F	Clear cell	5	III	T1bN0MX	I	U/U	M/M	U/U	U/U	U/U	U/U
34	60M	Clear cell	5.5	IV	T1bN0MX	I	U/U	M/M	U/U	U/U	U/U	U/U
45	61M	Clear cell	15	I	T2N0MX	II	U/U	U/U	U/U	U/U	U/U	M/M
59	80M	Clear cell	8.5	I–II	T2N0MX	II	U/U	M/M	U/U	U/U	U/U	U/U
32	52F	Clear cell	4.5	II	T2N0MX	II	U/U	M/M	U/U	U/U	U/U	M/M
27	49M	Clear cell	9	IV	T2N0MX	II	U/U	M/M	U/U	U/U	U/U	M/M
2	57F	Clear cell	3	II	T3aN0MX	III	U/U	M/M	U/U	U/U	U/U	U/U
41	59M	Clear cell	13	II	T3aN0MX	III	M/M	U/U	U/U	M/M	U/U	U/U
30	59M	Clear cell	3.5	III	T3aNOMX	III	U/U	M/M	U/U	U/U	U/U	M/M
4	54F	Clear cell	7	III	T3aN0MX	III	U/U	M/M	U/U	U/U	U/U	M/M
52	63M	Clear cell	5.5	III	T3bN0MX	III	U/U	M/M	U/U	U/U	U/U	U/U
19	78M	Clear cell	5.5	II–III	T3bN2MX	IV	U/U	U/U	M/U	U/U	U/U	U/U
21	78F	Clear cell	9.5	IV	T2N2MX	IV	U/U	M/M	U/U	U/U	U/U	U/U
58	78M	RCC unclassified	10	IV	T3bN0MX	III	U/U	M/M	U/U	U/U	U/U	M/M
33	73F	Papillary	2.5	I	T1aN0MX	I	U/U	M/M	M/M	M/M	M/M	U/U
25	63M	Papillary	3	III	T1aNOMX	I	U/U	M/M	U/U	U/U	U/U	M/M
15	30F	Papillary	4	III	T1aNOMX	I	U/U	M/M	U/U	U/U	M/M	M/M
20	34M	Papillary	7.5	II	T2NOMX	II	U/U	M/M	U/U	U/U	U/U	M/M
31	69M	Papillary	3	III	T3aN0MX	III	U/U	M/U	U/U	U/U	U/U	U/U
28	39F	Papillary	8.5	IV	T3bNOMX	III	U/U	M/M	M/M	M/M	M/M	U/U
78	65F	Chromophobe	2	I	T1aN0MX	I	U/U	U/U	U/U	M/M	U/U	M/M
73	66M	Chromophobe	3.5	I	T1aN0MX	I	U/U	U/U	U/U	M/M	U/U	M/M
24	73M	Oncocytoma	2.5				U/U	U/U	U/U	U/U	M/U	M/U
40	69M	Oncocytoma[a]	4.1				U/U	U/U	U/U	U/U	M/M	U/U
9	59F	Oncocytoma	6				U/U	U/U	U/U	M/M	U/U	M/M
16	70M	Collecting duct	5.5	IV	T3aN2MX	IV	U/U	M/M	U/U	U/U	U/U	U/U
44	66M	TCC renal pelvis	2.5	II	T1N0MX	I	U/U	U/U	U/U	U/U	U/U	M/M
29	70M	TCC renal pelvis	8	III	T3N0MX	III	U/U	M/M	U/U	U/U	U/U	M/M

[a] Patient 40 had multiple oncocytomas and a 2-mm focus of chromophobe carcinoma; 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.

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 (21). PCR amplification of template DNA was performed for 31–36 cycles at 95°C denaturing, 58°C–66°C annealing, and 72°C extension with a final extension step of 5 min. Cycle number and annealing temperature depended upon 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 kidney tissue DNA because a negative control and water with no DNA template as a control for contamination were included. If no tumor cell line with known hypermethylation of a particular gene (*APC*) was available, normal human lymphocyte DNA *in vitro* methylated with *Sss*I methylase according to the manufacturers 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.

Statistical Analysis. The sensitivity of MSP-based detection of hypermethylation in urine was calculated as number of positive tests/number of cancer cases. The specificity was calculated as number of negative tests/number of cases without cancer and in a second distinct approach as number of negative tests/number of cases without hypermethylation of a particular gene. The association of tumor stage with positive detection of hypermethylation in urine and the association of frequency of hypermethylation of a particular gene in different histological cell types were compared using Fisher's exact test. Results were considered statistically significant if the two-sided P was ≤ 0.05.

RESULTS

We examined the hypermethylation status of a panel of six normally unmethylated cancer genes (the tumor suppressor genes *VHL*, *p16*, *p14*, *APC* and the putative suppressor genes *RASSF1A* and *Timp-3*) in 50 kidney tumor (35 clear cell, 6 papillary, 3 oncocytoma, 2 chromophobe, 2 transitional cell, 1 collecting duct, and 1 unclassified RCC) and matched urine DNAs using the sensitive MSP assay, which can detect 0.1% cancer cell DNA from a heterogeneous cell population (9). The frequency of promoter hypermethylation of the tumor suppressor gene loci included in the panel was *VHL* 6 of 50 (12%), *p16* 5 of 50 (10%), *p14* 9 of 50 (18%), *APC* 9 of 50 (18%), *RASSF1A* 26 of 50 (52%), and *Timp-3* 30 of 50 (60%) tumors. Each of the 50 tumor DNAs showed hypermethylation of at least one gene from the panel (Table 1). The diagnostic coverage (whether a hyper-

methylated gene was available as a target in each case) of our panel was therefore 100%. Hypermethylation was therefore found in all histological cell types examined. Hypermethylation of the *VHL* gene was observed only in clear cell renal cancer (6 of 35, 17%) as expected (22), whereas hypermethylation of *p14* or *APC* appeared to be more common in nonclear cell cancers but not at a statistically significant level ($P = 0.10$ and $P = 0.20$, Fisher's exact test). *RASSF1A* was hypermethylated in 6 of 6 (100%) of papillary renal tumors and 19 of 43 (44%) of nonpapillary tumors (excluding 1 case of unclassified RCC). The association of *RASSF1A* hypermethylation and papillary tumors was statistically significant ($P = 0.022$, Fisher's exact test). Hypermethylation was observed in all pathological stages of kidney cancer, including 30 stage I tumors. Moreover, 19 of the 30 (63%) stage I lesions were subclassified as stage T1a (≤4 cm; Table 1), which indicated that promoter hypermethylation of the tumor suppressor genes in the panel can be a relatively early event in renal tumorigenesis. Hypermethylation was found in patients of all ages (Table 1).

We compared the hypermethylation status of the six genes in the panel in the urine DNAs to the corresponding tumor DNAs. We detected an identical pattern of gene hypermethylation in 44 of 50 (88%) matched urine DNAs (Fig. 1 and Table 1). The urine-positive cases (designated M/M in Table 1) included 17 of 19 cases of T1a (≤4 cm) and 32 of 35 organ-confined (stages I and II) kidney tumors, as well as 2 of 3 oncocytomas. No hypermethylation was detected in urine DNA from 6 patients (nos. 53, 37, 50, 19, 31, and 24 designated M/U in Table 1). MSP of tumor and urine DNAs from patients 19 and 24 are shown in the *p16* and *APC* gel panels, respectively, in Fig. 1. There was no statistical association ($P = 0.51$, Fisher's exact test) between pathological stage of the 50 tumors and positive detection in urine (29 of 33 stage I, including the 3 oncocytomas, 5 of 5 stage II, 8 of 9 stage III, and 2 of 3 stage IV).

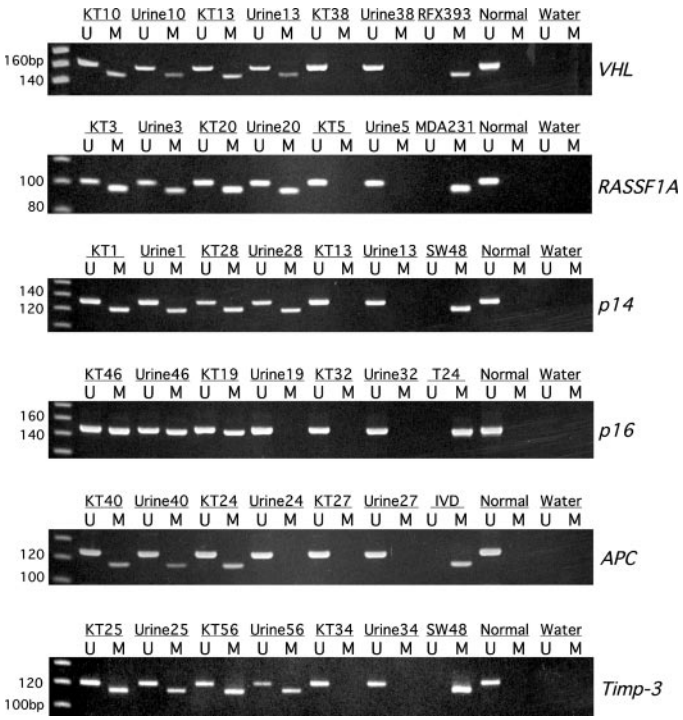

Fig. 1. MSP of *VHL*, *RASSF1A*, *p14*, *p16*, *APC*, and *Timp-3* genes in kidney tumor and urine DNAs. Viewed from left to right, 3 patients are shown in each gel. In the *VHL*, *RASSF1A*, *p14*, and *Timp-3* gel panels, the first and second patient's kidney tumor (KT) DNA is hypermethylated (M) and positively detected in the corresponding urine DNA (M). In the *p16* and *APC* gel panels, the first patient's tumor DNA and urine DNA show hypermethylation, whereas the second patient's tumor shows hypermethylation that was not detected in the matched urine DNA. In all six panels, the third patient's tumor DNA is not methylated 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, *e.g.*, point mutation inactivates a *VHL* allele, which is retained in the cell but is unmethylated. Tumor cell line RFX398 (*VHL*), MDA231 (*RASSF1A*), T24 (*p16*), SW48 DNA (*p14* and *Timp-3*), and *in vitro* methylated DNA (IVD) for *APC* 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.

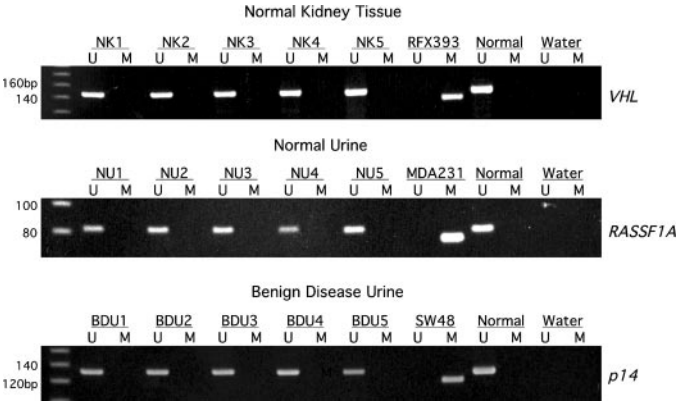

Fig. 2. MSP of *VHL*, *RASSF1A*, and *p14* genes in normal and benign disease control DNAs The absence of a PCR product in the methylated lane (M) of *VHL* in normal kidney (NK) tissue DNAs 1–5, *RASSF1A* in normal urine (NU) DNAs 1–5, and *p14* in urine DNAs from patients with benign disease (BDU) 1–5 indicates that these specimen DNAs have unmethylated alleles only (U). Tumor cell lines RFX398 (*VHL*), MDA231 (*RASSF1A*), and SW48 DNAs (*p14*) as a positive control for methylation, 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.

In contrast, we did not observe hypermethylation of the gene panel in urine DNA from 12 normal, healthy controls and 12 patients with nonneoplastic kidney disease (renal stones or benign cysts) or in 10 paired normal kidney tissue DNAs from the renal cancer patients (Fig. 2) and 5 normal urothelium specimens. Furthermore, a gene negative for hypermethylation in the tumor DNA was always negative in the matched urine DNA, *e.g.*, example, tumor and urine 38 in the *VHL* gel panel shown in Fig. 1. The specificity of the test was therefore 100%.

DISCUSSION

The use of DNA-based methods for the early detection of renal cancer has several potential advantages. Because some genetic and epigenetic events will occur early in the disease process, molecular diagnosis may allow detection before symptomatic or overt radiographic manifestations. In addition, through screening bodily fluids such as the urine, it may ultimately provide a truly noninvasive diagnostic modality, thereby limiting the need for current imaging techniques that provide anatomical detail without definitive pathological correlation. Gene alterations at the DNA level such as aberrant promoter hypermethylation can be detected at sensitive levels by PCR (1 in 1000) and, perhaps most importantly because the alteration is a qualitative change, can provide a "yes or no" answer (23) and are thus potentially very specific.

The majority of kidney cancers (80–85%) are RCCs originating from the renal parenchyma. The remaining 15–20% are mainly transitional cell carcinomas (TCCs) of the renal pelvis. The classification of RCC comprises several histological subtypes with different genetic backgrounds and natural histories. Clear cell carcinoma (70%) and papillary carcinoma (10–15%) account for the majority. The remaining types include chromophobe carcinoma (5%), the benign tumor oncocytoma (5–10%), rarer forms such as collecting duct carcinoma (<1%) and RCC unclassified (≤5%; Ref. 24). TCC of the renal pelvis

involves similar genetic alterations to TCC of the bladder (25). The heterogeneity of genetic alterations found in distinct histological types of kidney cancer (26) and indeed within the same histological type dictated the use of a panel of genes. Indeed, no single gene is known to be hypermethylated in more than a proportion of renal tumors. For example, *RASSF1A* has been reported to be hypermethylated in up to 56% (27) but *p14* in only 13% (6) of primary kidney tumors. The genes included in the panel were selected on the basis of having been previously reported (7, 9, 19, 20, 27, 28) and confirmed by ourselves to be hypermethylated in kidney cancer but not normal cells. It will likely be necessary to use a panel of genes to maximize detection of any type of adult sporadic cancer, analogous to the need for analysis of several genes for the diagnosis of familial breast cancer or hereditary nonpolyposis colorectal cancer. Analysis of a panel of six genes does not present a technical barrier particularly when current advances in array and high throughput technology are considered.

Using a panel of six tumor suppressor genes, we have demonstrated that promoter hypermethylation is common in kidney cancer and can be readily detected in a specific manner in urine DNA, including urines from 17 of 19 patients with kidney tumors of the lowest pathological stage (T1a). However, it should be noted that although T1a lesions are indeed the smallest tumor and have the best prognosis under the current staging system, a minority of small RCCs can still be biologically advanced. In our study, we examined two cases of TCC (nos. 29 and 44) where urinary cytology is standard clinical practice. In both cases, traditional cytology was negative for cancer, whereas MSP was positive for hypermethylation. In this initial feasibility study, we observed a sensitivity of 88%. Hypermethylation was not detected in six (12%) urine DNAs. In these urine samples, neoplastic DNA may have been 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 ($n = 36$) because it is known that specificity can decrease in MSP (29), as in other PCR protocols, with increased cycle number. It is possible that a higher number of cycles or a two-stage (nested) MSP approach (30) would have resulted in the positive detection of hypermethylation in the six negative urine DNAs. We observed no significant difference in detection frequency between different pathological stages which suggested that tumor stage was not the main determinant of positive detection in urine. We believe the sensitivity of this assay can likely be improved 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, as well as improvements in PCR technology.

For a feasibility study of detection, it is important that the target genetic alteration is cancer specific and not present in normal or benign cells. Although we only included in the hypermethylation panel genes reported to be unmethylated in normal cells (7, 9, 19, 20, 27, 28), we still performed several controls to determine specificity. First, we tested and did not observe gene hypermethylation in urine DNA from 12 normal, healthy controls and 12 patients with nonneoplastic kidney disease (Fig. 2). Furthermore, no hypermethylation was observed in urine DNAs from 5 patients with BPH or prostatitis and 9 patients with inflammatory disease of the bladder, *e.g.*, cystitis (data not shown). Second, 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 (10, 13, 15). A particular gene that is unmethylated in tumor DNA should always be unmethylated in the corresponding bodily fluid DNA. For example, tumor 38 in Fig. 1 did not have *VHL* hypermethylation, and the matched urine 38 DNA was also negative. Additional representative examples can be seen in the gel panels shown in Fig. 1. 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; Table 1). Third, we examined 10 paired normal kidney tissue DNAs from the renal cancer patients and observed no hypermethylation at our routine PCR amplification sensitivity (Fig. 2). The possibility that histologically normal tissue taken from a neoplastic kidney may contain occult neoplastic cells with gene promoter hypermethylation should be noted. Similarly, the possible field effect of transitional cell carcinoma suggested that a normal urothelium specimen from a patient with TCC of the kidney might contain neoplastic cells with hypermethylated alleles. We therefore obtained 5 ureter tissue specimens containing transitional cells from patients with a single discrete renal cell cancer. No gene hypermethylation was found in the transitional cells. These findings indicate that urine hypermethylation is highly specific for cancer. Future studies could use sufficient controls to address larger issues beyond this exploratory study.

In our study, hypermethylation of the *VHL* gene was specific for clear cell renal cancer as expected (22). We also noted that hypermethylation of *RASSF1A* was significantly more frequent in papillary RCC compared with other kidney tumors. Although hypermethylation of *p14* or *APC* was more common in nonclear cell cancers, the difference in frequency was not statistically significant in the current sample size. Analysis of larger numbers of specimens will determine whether this tendency is significant. Thus, MSP-based detection also has the potential for differential diagnosis of renal cancer based on the pattern of gene methylation found. Promoter hypermethylation, as with other mechanisms of inactivation of suppressor genes, deletion, and point mutation, can be found in different types of cancer (6). However, tissue-specific patterns of hypermethylation have been previously reported (6, 30), and it has been estimated that several hundred as yet unidentified genes are hypermethylated in human cancer (31). Moreover, the tissue specificity of genes predisposing to the familial forms of different histological forms of renal cancer and the fact that genetic alterations have aided in the classification of kidney cancer (26) suggest that it is likely genes hypermethylated exclusively or more frequently in renal cancer will be identified in the near future. Inclusion of such genes in a renal cancer detection panel would provide greater specificity for kidney cancer and minimize the potential confounding variables of bladder or prostate cancer. Algorithms could be developed to score the specificity of a particular gene hypermethylation panel for the detection of renal cancer compared with other cancer types.

In addition to early detection and differential diagnosis of renal cancer, if the timing of hypermethylation of certain genes was found to be associated with a defined pathological stage, the panel could be extended in the future to simultaneously provide molecular staging and prognostic information. For example, inactivation of *VHL* is an early event (26), whereas inactivation of *p16* is believed to be a late event (32) in renal tumorigenesis, although additional work is required for more precise timing of hypermethylation of *p16* and other genes. The overall number of genes and which genes are hypermethylated could form a basis for molecular staging. Furthermore, molecular staging might eventually extend to the prediction of the behavior of individual tumors within a particular pathological stage. The heterogeneity of genetic alterations between tumors, *e.g.*, which tumor suppressor gene pathways are abrogated, is likely one underlying cause of differences in individual tumor behavior and response. The panel used here contained genes of clear biological significance such as the *p16*, *p14*, and *APC* genes involved in the *p16/Rb* and *p53/p14* tumor suppressor gene pathways and the Wnt signaling pathway (33). As new genes are found to be hypermethylated in kidney cancer, future studies of the gene hypermethylation profile in large, representative series of renal cancers will determine both the number of

genes and which genes to be screened to obtain optimal diagnostic coverage and information.

Molecular detection by microsatellite loss of heterozygosity analysis has been reported in 19 of 25 (76%) urine and 15 of 25 (60%) serum specimen DNAs from renal cancer patients (34) and, in another study, in 65% of plasma DNAs from clear cell renal cancer patients (35). Other potential targets for detection in urine might include point mutation of *VHL* or mitochondrial DNA (36). However, MSP-based detection has several advantages over microsatellite or point mutation-based detection of renal cancer in urine. These include (*a*) the greater sensitivity of MSP, which will be important for detection of early, small, or 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.

The hypermethylation panel of six genes tested here provided 100% diagnostic coverage of 50 kidney cancers, including all major histological cell types and pathological stages, and is certainly manageable in terms of time and economy in view of recent chip, array, and high-throughput technology. We believe that an optimal hypermethylation panel could provide simultaneous early detection, differential diagnosis, and molecular prognosis and prediction of behavior of kidney cancer. In this study, we have demonstrated for the first time the feasibility of hypermethylation-based sensitive (88%) and 100% specific (no false positives) noninvasive detection of renal cancer in urine from patients with early-stage as well as advanced carcinoma. If these results are confirmed in larger studies, promoter hypermethylation may have useful clinical application in kidney cancer diagnosis and management.

REFERENCES

1. Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E., and Thun, M. J. Cancer Statistics, 2003. CA - Cancer J. Clin., *53:* 5–26, 2003.
2. Motzer, R. J., Bander, N. H., and Nanus, D. M. Renal-cell carcinoma. N. Engl. J. Med., *335:* 865–875, 1996.
3. Jones, P. A., Vogelzang, N. J., and Gomez, J. Report of the Kidney/Bladder Cancer Progress Review Group. Bethesda, MD: National Cancer Institute, 2002.
4. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J-P. J. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv. Cancer Res., *72:* 141–196, 1998.
5. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.
6. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. A gene hypermethylation profile of human cancer. Cancer Res., *61:* 3225–3229, 2001.
7. Bachman, K. E., Herman, J. G., Corn, P. G., Merlo, A., Costello, J. F., Cavenee, W. K., Baylin, S. B., and Graff, J. R. Methylation-associated silencing of the tissue inhibitor of *metalloproteinase-3* gene suggests a suppressor role in kidney, brain, and other human cancers. Cancer Res., *59:* 798–802, 1999.
8. Morrissey, C., Martinez, A., Zatyka, M., Agathanggelou, A., Honorio, S., Astuti, D., Morgan, N. V., Moch, H., Richards, F. M., Kishida, T., Yao, M., Schraml, P., Latif, F., and Maher, E. R. Epigenetic inactivation of the *RASSF1A* 3p21.3 tumor suppressor gene in both clear cell and papillary renal cell carcinoma. Cancer Res., *61:* 7277–7281, 2001.
9. Herman, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
10. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res., *59:* 67–70, 1999.
11. Belinsky, S. A., Nikula, K. J., W. A., P., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S. B., and Herman, J. G. Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc. Natl. Acad. Sci. USA, *95:* 11891–11896, 1998.
12. Ahrendt, S. A., Chow, J. T., Xu, L-H., Yang, S. C., Eisenberger, C. F., Esteller, M., Herman, J. G., Wu, L., Decker, P. A., Jen, J., and Sidransky, D. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J. Natl. Cancer Inst. (Bethesda), *91:* 332–339, 1999.
13. Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., Jen, J., Herman, J. G., and Sidransky, D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res., *60:* 892–895, 2000.
14. Evron, E., Dooley, W. C., Umbricht, C. B., Rosenthal, D., Sacchi, N., Gabrielson, E., Soito, A. B., Hung, D. T., Ljung, B-M., Davidson, N. E., and Sukumar, S. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet, *357:* 1335–1336, 2001.
15. Cairns, P., Esteller, M., Herman, J. G., Schoenberg, M., Jeronimo, C., Sanchez-Cespedes, M., Chow, N-H., Grasso, M., Wu, L., Westra, W. B., and Sidransky, D. Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin. Cancer Res., *7:* 2727–2730, 2001.
16. Fuhrman, S. A., Lasky, L. C., and Limas, C. Prognostic significance of morphologic parameters in renal cell carcinoma. Am. J. Surg. Pathol., *6:* 655–663, 1982.
17. Union Internationale Contre le Cancer. TNM Classification of Malignant Tumors. New York: Wiley-Liss, 1997.
18. Sambrook, J., and Russell, D. W. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.
19. Esteller, M., Tortola, S., Toyota, M., Capella, G., Peinado, M. A., Baylin, S. B., and Herman, J. G. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res., *60:* 129–133, 2000.
20. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., Baylin, S. B., and Herman, J. G. Analysis of *adenomatous polyposis coli* promoter hypermethylation in human cancer. Cancer Res., *60:* 4366–4371, 2000.
21. Dammann, R., Li, C., Yoon, J-H., Chin, P. L., Bates, S., and Pfeifer, G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat. Genet., *25:* 315–319, 2000.
22. Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D-S. R., Gnarra, J. R., Linehan, W. M., and Baylin, S. B. Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. Proc. Natl. Acad. Sci. USA, *91:* 9700–9704, 1994.
23. Baylin, S. B., Belinsky, S. A., and Herman, J. G. Aberrant methylation of gene promoters in cancer: concepts, misconcepts, and promise. J. Natl. Cancer Inst. (Bethesda), *92:* 1460–1461, 2000.
24. Storkel, S., Eble, J. N., Adlakha, K., Amin, M., Blute, M. L., Bostwick, D. G., Darson, M., Delahunt, B., and Iczkowski, K. Classification of renal cell carcinoma: Workgroup No. 1. Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Cancer (Phila.), *80:* 987–989, 1997.
25. Cairns, P., and Sidransky, D. Bladder cancer. *In:* B. Vogelstein and K. W. Kinzler (eds.), The Genetic Basis of Human Cancer, 2nd Edition, Chp. 44, pp. 697–702. New York: McGraw-Hill, 2002.
26. Zambrano, N. R., Lubensky, I. A., Merino, M. J., Linehan, W. M., and Walther, M. M. Histopathology and molecular genetics of renal tumors: toward unification of a classification system. J. Urol., *162:* 1246–1258, 1999.
27. Yoon, J. H., Dammann, R., and Pfeifer, G. P. Hypermethylation of the CpG island of the *RASSF1A* gene in ovarian and renal cell carcinomas. Int. J. Cancer, *94:* 212–217, 2001.
28. Esteller, M., Corn, P. G., Urena, J. M., Gabrielson, E., Baylin, S. B., and Herman, J. G. Inactivation of glutathione *S*-transferase *P1* gene by promoter hypermethylation in human neoplasia. Cancer Res., *58:* 4515–4518, 1998.
29. Corn, P. G., Smith, B. D., Ruckdeschel, E. S., Douglas, D., Baylin, S. B., and Herman, J. G. E-Cadherin expression is silenced by 5′ CpG island methylation in acute leukemia. Clin. Cancer Res., *6:* 4243–4248, 2000.
30. Palmisano, W. A., Divine, K. K., Saccomanno, G., Gilliland, F. D., Baylin, S. B., Herman, J. G., and Belinsky, S. A. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res., *60:* 5954–5958, 2000.
31. Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomäki, P., Lang, J. C., Schuller, D. E., Yu, L., Bloomfield, C. D., Caligiuri, M. A., Yates, A., Nishikawa, R., Huang, H-J. S., Petrelli, N. J., Zhang, X., O'Dorisio, M. S., Held, W. A., Cavenee, W. K., and Plass, C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat. Genet., *25:* 132–138, 2000.
32. Cairns, P., Tokino, K., Eby, Y., and Sidransky, D. Localization of tumor suppressor loci on chromosome 9 in primary human renal cell carcinomas. Cancer Res., *55:* 224–227, 1995.
33. Sherr, C. J., and McCormick, F. The RB and p53 pathways in cancer. Cancer Cell, *2:* 103–112, 2002.
34. Eisenberger, C. F., Schoenberg, M., Enger, C., Hortopan, S., Shah, S., Chow, N-H., Marshall, F. F., and Sidransky, D. Diagnosis of renal cancer by molecular urinalysis. J. Natl. Cancer Inst. (Bethesda), *91:* 2028–2032, 1999.
35. Goessl, C., Heicappell, R., Munker, R., Anker, P., Stroun, M., Krause, H., Muller, M., and Miller, K. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. Cancer Res., *58:* 4728–4732, 1998.
36. Fliss, M. S., Usadel, H., Caballero, O. L., Wu, L., Buta, M. R., Eleff, S. M., Jen, J., and Sidransky, D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science (Wash. DC), *287:* 2017–2019, 2000.

Promoter Hypermethylation of Tumor Suppressor Genes in Urine from Kidney Cancer Patients

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Cancer Res 2003;63:8695-8699.