Featured Article

Promoter Hypermethylation Profile of Kidney Cancer

Essel Dulaimi,[1] Inmaculada Ibanez de Caceres,[1] Robert G. Uzzo,[1] Tahseen Al-Saleem,[2] Richard E. Greenberg,[1] Thomas J. Polascik,[4] James S. Babb,[3] William E. Grizzle,[5] and Paul Cairns[1,2]

Departments of [1]Surgical Oncology, [2]Pathology, and [3]Biostatistics, Fox Chase Cancer Center, Philadelphia, Pennsylvania; [4]Division of Urology Duke University Medical Center, Durham, North Carolina; and [5]Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama

ABSTRACT

Purpose: **Promoter hypermethylation is an important mechanism of inactivation of tumor suppressor genes in cancer cells. Kidney tumors are heterogeneous in their histology, genetics, and clinical behavior. To gain insight into the role of epigenetic silencing of tumor suppressor and cancer genes in kidney tumorigenesis, we determined a hypermethylation profile of kidney cancer.**

Experimental Design: **We examined the promoter methylation status of 10 biologically significant tumor suppressor and cancer genes in 100 kidney tumors (50 clear cell, 20 papillary, 6 chromophobe, 5 collecting duct, 5 renal cell unclassified, 7 oncocytoma, 6 transitional cell carcinomas of the renal pelvis, and 1 Wilms' tumor) by methylation-specific PCR. The hypermethylation profile was examined with regard to clinicopathological characteristics of the kidney cancer patients.**

Results: **Hypermethylation of one or more genes was found in 93 (93%) of 100 tumors. A total of 33% of kidney tumors had one gene, 35% two genes, 14% three genes, and 11% four or more genes hypermethylated. The frequency of hypermethylation of the 10 genes in the 100 tumor DNAs was *VHL* 8% (all clear cell), $p16^{INK4a}$ 10%, $p14^{ARF}$ 17%, *APC* 14%, *MGMT* 7%, *GSTP1* 12%, *RARβ2* 12%, *RASSF1A* 45%, *E-cadherin* 11%, and *Timp-3* 58%. Hypermethylation was observed in all of the histological cell types and grades and stages examined. No hypermethylation was observed in specimens of normal kidney or ureteral tissue from 15 patients. Hypermethylation of *VHL* was specific to clear cell tumors. *RASSF1A* methylation was detected at a significantly higher frequency in papillary renal cell tumors and in high-grade tumors of all cell types. *MGMT* methylation was more frequent in nonsmokers. Simultaneous methylation of five or more genes was observed in 3 (3%) of 100 tumors and may indicate a methylator phenotype in kidney cancer. In addition, the CpG island in the promoter of the *fumarate hydratase* (*FH*) tumor suppressor gene was bisulfite sequenced and was found to be unmethylated in 15 papillary renal tumors.**

Conclusions: **Promoter hypermethylation is common, can occur relatively early, may disrupt critical pathways, and, thus, likely plays an important role in kidney tumorigenesis. A hypermethylation profile may be useful in predicting a patient's clinical outcome and provide molecular markers for diagnostic and prognostic approaches to kidney cancer.**

INTRODUCTION

Alterations in DNA methylation, an epigenetic process present in mammalian cells, are a hallmark of human cancer. The CpG dinucleotide is not underrepresented in the promoter region of many genes, particularly "housekeeping" genes, as it is underrepresented in the remainder of the genome. These regions are termed "CpG islands" and with the exception of genes on the inactive X chromosome in females and imprinted genes, CpG islands are generally protected from methylation in normal cells. This protection is critical because methylation of CpG islands is associated with loss of expression of that particular gene. In addition to loss of heterozygosity and point mutation, alleles of tumor suppressor and cancer genes can be silenced by promoter hypermethylation. Silencing of tumor suppressor genes, such as $p16^{INK4a}$, the mismatch repair gene *hMLH1*, and *BRCA1* have established hypermethylation as a common mechanism for tumor suppressor inactivation in human cancer (1, 2). In the types of cancer analyzed to date, promoter hypermethylation has been found to be frequent, to occur in tumor suppressor and cancer genes involved in many different signaling pathways and to be present in a different pattern in different tumor types (3–9).

Cancer of the kidney, specifically renal cell carcinomas (RCCs) and transitional cell carcinomas of the renal pelvis, accounts 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 (10). Hereditary clear cell RCC arises from an inherited mutation in the *VHL* tumor suppressor gene located on chromosome 3p. In the sporadic clear cell type of RCC, chromosome 3p deletion and inactivation of the *VHL* suppressor gene is reported to be the most common genetic alteration (11). That the inactivation of *VHL* is common in sporadic disease and that it is also the predisposing factor to familial clear cell RCC both suggest that alteration of *VHL* may be the initiating event in sporadic clear cell cancer. The *MET* oncogene and the *fumarate hydratase* (*FH*) tumor suppressor gene have been identified as predisposition genes for hereditary papillary renal cancer (12, 13), and

Received 1/28/04; revised 3/23/04; accepted 3/29/04.
Grant support: Supported by The Early Detection Research Network through the National Cancer Institute Grant U01. P. Cairns is a recipient of a Flight Attendants Medical Research Institute (FAMRI) Young Clinical Scientist Award.
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.
Requests for reprints: Paul Cairns, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. E-mail: Paul.Cairns@fccc.edu.

the *Birt-Hogg-Dube* (*BHD*) suppressor gene has been identified as a predisposition gene for oncocytomas and chromophobe renal cancer (14). Adult sporadic cancers arise through the clonal accumulation of multiple genetic alterations, often in a general temporal order (15). Relatively little is known about the secondary and later genetic alterations that drive progression, or about the importance of different pathways in the development and progression of renal cancer. The *p53*, *Rb*, *p16*, and *PTEN* tumor suppressor genes are infrequently inactivated in RCC, and the target suppressor gene(s) on several of the most frequently deleted chromosomal arms, implicated by cytogenetic, allelotype, and comparative genomic hybridization studies in RCC, has not yet been identified (11).

DNA methylation is known to occur in kidney tumorigenesis. For example, the *VHL* gene is inactivated by hypermethylation in a subset of clear cell renal cancers (16), and $p16^{INK4a}$ (17) and *RASSF1A* (18) can be hypermethylated in clear cell and other histological subtypes. However, for the most part, hypermethylation of a single gene has been examined only in limited types and numbers of kidney tumors (4, 18). To gain insight into the biological background and significance of hypermethylation in kidney tumorigenesis, we selected 10 tumor suppressor or cancer genes known to be hypermethylated with associated loss of expression in human cancer. These included the *von Hippel-Lindau* (*VHL*), $p16^{INK4a}$, $p14^{ARF}$, and *adenomatous polyposis coli* (*APC*) tumor suppressor genes; the DNA repair gene O^6-*methyl-guanine-DNA-methyltransferase* (*MGMT*); the detoxifying gene *glutathione S-transferase* π*1* (*GSTP1*); the putative suppressor genes *retinoic acid receptor* β*2* (*RAR*β*2*) and *RAS association domain family protein 1A* (*RASSF1A*); and the invasion and metastasis genes *E-cadherin* and *tissue inhibitor of metalloproteinase-3* (*Timp-3*); as well as the recently described *fumarate hydratase* (*FH*) tumor suppressor gene, inherited mutation of which can predispose to familial papillary renal carcinoma (13). Our study profiled promoter hypermethylation status by methylation-specific PCR (MSP) in a series of 100 tumors representative of all major types of kidney cancer. The gene hypermethylation status was correlated to clinicopathological features of the kidney cancer patients.

MATERIALS AND METHODS

Specimen Collection and DNA Extraction. After approval from the Fox Chase Institutional Review Board, tumor tissue was obtained from the Fox Chase Cancer Center Tumor Bank Facility from 100 patients, ages 30–80 years, who underwent nephrectomy or nephro-ureterectomy for enhancing renal masses. Tumors were graded according to the American Joint Committee on Cancer (19) and staged according to the 1997 tumor-node-metastasis system (Ref. 20; Fig. 1). Specimens of normal kidney distant to the tumor were examined from 10 of the RCC patients as controls. In addition, specimens of histologically confirmed normal ureteral urothelium were collected from five patients with RCC to provide DNA from normal transitional cells. Tumor tissue was obtained immediately after surgical resection and was imbedded in optimum cold temperature medium (OCT). A H&E-stained section was examined by a pathologist (T. A-S.), and the area of highest neoplastic cell content was selected and manually microdissected. DNA was extracted from tumor or normal tissue using a standard technique of digestion with proteinase K in the presence of SDS at 37°C overnight, followed by phenol/chloroform extraction and precipitation with 100% ethanol (21).

Methylation-Specific PCR. Specimen DNA (0.5–1 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 profile were *VHL* (22), $p16^{INK4a}$ (22), $p14^{ARF}$ (23), *APC* (24), *GSTP1* (25), *MGMT* (26), *RASSF1A* (27), *RAR*β*2* (28), *E-Cadherin* (29), and *Timp-3* (30). MSP analysis of 6 of the genes in 50 tumors was previously reported (31). 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 positions 13–15 on the reverse primer, as described previously (27). The primer sequences for *RAR*β*2* are as described previously (28) but with the addition of 2 bp to the 5′ end of both primers for the unmethylated product to aid in discrimination between the unmethylated and methylated *RAR*β*2* PCR products. PCR amplification of template DNA was performed for 31–35 cycles at 95°C denaturing, 58–66°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 kidney tissue DNA as 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 (*VHL*, *APC*, *E-cadherin*) 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 and analyzed using a 6% nondenaturing acrylamide gel with appropriate size markers.

***FH* Promoter CpG Island Analysis.** The *FH* gene promoter sequence data were obtained from GenBank Database accession no. NM_000143. We selected the CpG island situated between −181 upstream and +166 bases downstream from the ATG codon. This region fulfilled the original CpG island definition criteria of Gardiner-Garden and Frommer (32) and the modifications suggested by Takai and Jones (33). No *Alu* repetitive elements were detected by REPEAT-MASKER mail server (University of Washington Genome Center, Seattle, WA).[6] Papillary renal tumor DNAs were modified with sodium bisulfite as described above and were used as template for PCR amplification of the *FH* promoter CpG island region containing 35 CpG sites with the following primers; Forward: 5′-GAAGGTTT-TATATTTTATATTATTAT-3′ positioned at −207 and −181, Reverse: 5′-AACAAAAAAACTAAAAATC-3′ positioned at

[6] Internet address: http:/ftp.genome.Washington.edu/cgi-bin/RepeatMasker.

No.	Age	Cell Type	Size	Grade	TNM	SH	*Timp3*	*RASSF*	*p14*	*APC*	*GSTP1*	*RARB*	*ECAD*	*p16*	*VHL*	*MGMT*
1	66M	Clear Cell	2	I	T1aN0MX	N	M	M	U	U	U	U	M	U	U	U
2	43M	Clear Cell	3	I	T1aNOMX	Y	M	U	M	U	U	M	U	U	U	U
3	56M	Clear Cell	3.5	I	T1aN0MX	Y	M	U	U	U	U	U	M	U	U	U
4	62M	Clear Cell	3.5	I-II	T1aN0MX	Y	M	U	U	U	M	U	U	U	U	U
5	61M	Clear Cell	2	II	T1aN0MX	Y	M	U	U	U	U	U	U	U	M	U
6	57M	Clear Cell	2.2	II	T1aN0MX	Y	U	U	M	U	U	U	U	U	U	U
7	60F	Clear Cell	3.5	II	T1aN0MX	N	M	U	U	U	U	U	U	U	U	U
8	67M	Clear Cell	4	II	T1aNOMX	Y	M	U	U	U	U	U	U	U	M	U
9	72F	Clear Cell	4	II	T1aN0MX	N	M	U	M	U	U	U	M	M	U	U
10	52M	Clear Cell	2.5	II	T1aN0MX	N	M	U	M	U	U	M	U	M	U	U
11	48F	Clear Cell	4	II	T1aNOMX	Y	U	U	U	U	U	U	U	U	U	U
12	69M	Clear Cell	3	III	T1aNOMX	N	M	M	U	M	U	U	U	U	M	U
13	59M	Clear Cell	3.5	III	T1aN0MX	Y	U	M	U	U	U	U	U	U	U	U
14	42M	Clear Cell	4	III	T1aN0MX	Y	M	U	U	U	U	U	U	U	U	U
15	59M	Clear Cell	4	III-IV	T1aN0MX	Y	M	U	U	U	U	U	U	U	U	U
16	78M	Clear Cell	2.7	IV	T1aNOMX	Y	U	M	U	U	M	U	M	U	U	U
17	52M	Clear Cell	4	IV	T1aNOMX	Y	U	M	U	U	U	U	U	U	U	U
18	62M	Clear Cell	6	I-II	T1bN0MX	Y	M	U	U	U	U	U	U	U	U	U
19	74F	Clear Cell	4.4	II	T1bN0MX	N	U	M	U	M	U	U	U	U	U	U
20	70M	Clear Cell	4.5	II	T1bN0MX	N	U	M	U	M	U	U	U	M	M	U
21	56F	Clear Cell	4.5	II	T1bN0MX	N	M	U	U	U	U	U	U	U	U	M
22	34F	Clear Cell	5	II	T1bN0MX	Y	M	U	U	U	U	U	U	U	U	U
23	57M	Clear Cell	5.5	II	T1bN0MX	Y	U	U	U	U	U	U	U	U	M	U
24	68M	Clear Cell	5.5	II	T1bN0MX	Y	M	U	U	U	U	U	U	U	U	U
25	61F	Clear Cell	6.5	II	T1bN0MX	Y	M	M	U	M	U	U	U	U	U	U
26	52M	Clear Cell	2.6, 5.5	II	T1bN0MX	N	M	U	U	U	M	U	U	U	U	U
27	52F	Clear Cell	4.5	II	T1bN0MX	N	M	M	U	U	U	U	U	U	U	M
28	43M	Clear Cell	6	II-III	T1bN0MX	N	M	U	U	U	U	U	U	U	U	U
29	64F	Clear Cell	5	III	T1bN0MX	N	U	M	U	U	U	U	U	U	U	U
30	60M	Clear Cell	5.5	IV	T1bN0MX	N	U	M	U	U	U	U	U	U	U	M
31	59M	Clear Cell	4.5	IV	T1bN0MX	Y	U	M	U	U	U	U	M	U	U	U
32	61M	Clear Cell	15	I	T2N0MX	Y	M	U	U	U	U	U	U	U	U	U
33	80M	Clear Cell	8.5	I-II	T2N0MX	Y	U	M	U	U	U	U	U	U	U	U
34	49M	Clear Cell	9	IV	T2N0MX	Y	M	M	U	U	U	U	U	U	U	U
35	57F	Clear Cell	3	II	T3aN0MX	N	U	M	U	U	U	U	U	U	U	U
36	59M	Clear Cell	13	II	T3aN0MX	Y	U	U	M	U	U	U	U	U	M	U
37	54F	Clear Cell	7	III	T3aN0MX	Y	M	M	U	U	U	U	U	U	U	U
38	59M	Clear Cell	3.5	III	T3aNOMX	Y	M	M	U	U	M	U	U	U	U	U
39	52F	Clear Cell	1.4, 5.2	III	T3aN0MX	Y	M	M	U	U	U	U	M	U	U	U
40	64M	Clear Cell	4	II	T3bNXMX	Y	M	M	U	U	U	U	U	U	U	U
41	54M	Clear Cell	6	II	T3bNOMX	Y	U	U	U	U	U	U	U	U	U	U
42	63M	Clear Cell	5.5	III	T3bN0MX	Y	U	M	U	U	U	U	U	U	U	U
43	62F	Clear Cell	7.3	III	T3bN0MX	Y	M	U	U	U	U	U	U	U	U	U
44	56M	Clear Cell	11	IV	T3bN0MX	Y	U	U	U	U	U	U	M	U	M	U
45	52M	Clear Cell	8.2	IV	T3bN0MX	Y	M	U	M	U	U	M	U	U	U	U
46	56M	Clear Cell	10	NK	T3bN0MX	Y	M	M	U	M	U	U	U	M	U	U
47	78M	Clear Cell	5.5	II-III	T3bN2MX	N	U	U	U	U	M	U	U	U	U	U
48	63M	Clear Cell	8.7	III	T3bN2MX	Y	U	M	U	U	U	M	U	U	U	U
49	55M	Clear Cell	15	III	T4NXMX	Y	M	U	U	U	U	U	U	U	M	U
50	78F	Clear Cell	9.5	IV	T3N2MX	Y	U	M	U	U	U	U	U	U	U	U
51	67F	Papillary	1.2	I	T1aN0MX	Y	U	U	U	U	U	U	U	U	U	U
52	73F	Papillary	2.5	I	T1aN0MX	Y	U	M	M	M	U	M	M	M	U	M
53	73M	Papillary	3	I	T1aN0MX	Y	U	M	U	U	U	U	U	U	U	U
54	65F	Papillary	4	I	T1aN0MX	Y	M	U	U	U	U	U	U	M	U	U
55	57F	Papillary	0.8, 2	II	T1aN0MX	Y	U	M	U	U	M	U	U	U	U	U
56	75F	Papillary	3	II	T1aNXMX	NK	M	M	M	U	M	M	U	U	U	U
57	74F	Papillary	3	II	T1aN0MX	Y	M	U	M	U	M	U	U	U	U	U
58	30F	Papillary	4	III	T1aNOMX	N	M	M	U	M	U	U	U	U	U	M
59	63M	Papillary	2.5	III	T1aNOMX	Y	M	M	U	U	U	U	U	U	U	U
60	47M	Papillary	5.5	I	T1bN0MX	Y	U	U	U	U	U	U	M	U	U	U
61	64F	Papillary	4.5	II	T1bN0MX	Y	U	M	U	M	U	U	U	U	U	U
62	34M	Papillary	7.5	II	T2NOMX	N	M	M	U	U	U	U	U	U	U	U
63	42M	Papillary	8	II	T2N0MX	N	U	U	U	U	U	U	U	U	U	U
64	74M	Papillary	12.5	II	T2N0MX	Y	M	M	U	U	U	U	M	M	U	U
65	53F	Papillary	9.5	IV	T2N0MX	Y	M	M	U	U	U	U	U	U	U	U
66	75F	Papillary	12.5	II	T3aN0MX	N	U	M	U	U	M	U	U	U	U	U
67	42M	Papillary	5.5	II-III	T3aN0MX	Y	U	M	U	U	U	M	U	U	U	U
68	69M	Papillary	3	III	T3aN0MX	N	U	M	U	U	U	U	U	U	U	U
69	76M	Papillary	5.7	III	T3aN0MX	N	M	U	U	U	U	U	U	U	U	U
70	39F	Papillary	8.5	IV	T3bNOMX	Y	U	M	M	M	U	M	U	M	U	U

Fig. 1

71	66M	Chromophobe	3.5	I	T1aN0MX	N	M	U	M	U	U	U	U	U	U	U
72	70M	Chromophobe	2.5,3.5	I	T1aN0MX	Y	M	U	U	U	U	U	U	U	U	U
73	65F	Chromophobe	2	I	T1aN0MX	Y	M	U	M	U	U	M	U	U	U	U
74	69F	Chromophobe	4.5	I	T1bN0MX	N	M	U	U	U	U	U	U	U	U	U
75	75M	Chromophobe	5	I	T1bN0MX	Y	M	U	U	U	U	U	U	U	U	U
76	52M	Chromophobe	11.5	II-III	T3aNXMX	NK	M	M	U	U	U	U	U	U	U	U
77	70M	Oncocytoma	2			Y	U	U	U	U	U	U	U	M	U	U
78	59F	Oncocytoma	6			N	M	U	M	U	U	U	U	U	U	U
79	73M	Oncocytoma	2.5			N	M	U	U	M	U	U	U	U	U	U
80	73F	Oncocytoma	2.5			N	U	U	U	U	U	U	U	U	U	U
81	73F	Oncocytoma	1.5			Y	U	U	U	U	M	U	U	U	U	M
82	73M	Oncocytoma	5			NK	U	M	U	U	U	U	U	U	U	U
83	69M	Oncocytoma*	4.1			Y	U	U	U	M	U	U	U	U	U	U
84	63M	Collecting Duct	5.5	II	T1bN0MX	Y	M	M	U	U	U	M	U	U	U	U
85	58M	Collecting Duct	7.5	II	T2N0MX	Y	U	U	M	U	U	U	U	U	U	M
86	61F	Collecting Duct	8	IV	T3N1MX	NK	U	U	U	U	U	U	U	U	U	U
87	56M	Collecting Duct	"large"	IV	T3cN1MX	Y	M	M	U	M	U	U	U	U	U	U
88	70M	Collecting Duct	5.5	IV	T3aN2MX	Y	U	M	U	U	U	U	U	U	U	U
89	68M	RCC Unclassified	16	III	T3aN1MX	Y	M	U	U	U	M	U	U	U	U	U
90	55F	RCC Unclassified	9	IV	T3aN0MX	Y	U	U	M	M	U	U	U	U	U	U
91	53M	RCC Unclassified	10.2	IV	T3aNXMX	NK	U	U	U	U	U	U	U	U	U	U
92	71F	RCC Unclassified	11	III	T3bN0MX	Y	M	U	M	U	U	M	U	U	U	U
93	78M	RCC Unclassified	10	IV	T3bN0MX	Y	M	M	U	U	U	U	M	U	U	U
94	80F	Transitional Cell	3.5	II	TaN0MX	Y	M	U	U	U	U	U	U	U	U	U
95	66M	Transitional Cell	2.5	II	T1N0MX	Y	M	U	U	U	U	U	U	U	U	U
96	37M	Transitional Cell	0.4	II	T1N0MX	Y	M	U	U	U	U	U	U	U	U	U
97	56M	Transitional Cell	3	II	T1N0MX	Y	U	U	M	M	U	M	U	M	U	U
98	70M	Transitional Cell	8	III	T3N0MX	N	M	M	U	U	U	U	U	U	U	U
99	66M	Transitional Cell	4	IV	T3N1MX	N	M	M	U	U	U	U	U	U	U	U
100	57M	Wilms' tumor	30			Y	M	U	U	U	M	U	U	U	U	U

Fig. 1 Clinicopathological data and hypermethylation profile of 10 genes in kidney cancer. *U*, unmethylated; *M*, methylated. Number 1–50, clear cell (conventional) tumors; 51–100, other kidney tumors. *Age*, years and gender (*M*, male; *F*, female). *Size*, cm; *Grade*, per American Joint Committee on Cancer. *TNM*, pathological stage; *T*, tumor size; *N*, node status; *M*, metastatic status. Oncocytomas are not graded or staged; all were confined to the kidney; ∗, patient 83 had multiple oncocytomas and a 2-mm focus of chromophobe carcinoma. *SH*, smoking history; *Y*, yes; *N*, never; *NK*, not known. Methylation-specific PCR analysis of six genes in 50 tumors was reported previously (31). *ECAD, E-cadherin gene.*

+166 and +185 from the ATG codon. The resulting 392-bp PCR product was cycle-sequenced.

Statistical Analysis. Binary logistic regression was conducted to assess whether patient age was associated with the hypermethylation of each individual gene. Fisher's exact test was used to explore the pairwise association between genes with respect to hypermethylation and whether the hypermethylation of a given gene was related to tumor stage, grade, or cell type or with patient gender and smoking status (smoker *versus* nonsmoker). Results were declared statistically significant at the two-sided 5% comparison-wise significance level (*i.e.*, without correction for multiple comparisons). Kaplan-Meier analysis was used to examine any association between gene hypermethylation and time to recurrence as well as overall survival.

RESULTS

We examined the hypermethylation status of a panel of 10 normally unmethylated tumor suppressor or cancer genes: *VHL*, *p16^{INK4a}*, *p14^{ARF}*, *APC*, *MGMT*, *GSTP1*, *RAR*β*2*, *RASSF1A*, *E-cadherin*, and *Timp-3* in 100 kidney tumor (50 clear cell, 20 papillary, 6 chromophobe, 5 collecting duct, 5 unclassified RCC, 7 oncocytoma, 6 transitional cell of renal pelvis, and 1 Wilms' tumor) DNAs using the MSP assay (Fig. 1). The fre-

Table 1 Summary of gene hypermethylation in kidney cancer

	VHL	*RASSF1A*	*p16*	*p14*	*APC*	*MGMT*	*GSTP1*	*RAR*β*2*	*E-cad*[a]	*Timp-3*
Clear cell	16% 8/50	46% 23/50	8% 4/50	12% 6/50	10% 5/50	6% 3/50	10% 5/50	8% 4/50	14% 7/50	60% 30/50
Papillary	0% 0/20	70% 14/20	20% 4/20	20% 4/20	20% 4/20	10% 2/20	20% 4/20	20% 4/20	15% 3/20	45% 9/20
Chromophobe	0% 0/6	17% 1/6	0% 0/6	33% 2/6	0% 0/6	0% 0/6	0% 0/6	17% 1/6	0% 0/6	100% 6/6
Oncocytoma	0% 0/7	14% 1/7	14% 1/7	14% 1/7	29% 2/7	14% 1/7	14% 1/7	0% 0/7	0% 0/7	29% 2/7
Collecting duct	0% 0/5	60% 3/5	0% 0/5	20% 1/5	20% 1/5	20% 1/5	0% 0/5	20% 1/5	0% 0/5	40% 2/5
RCC unclassified	0% 0/5	20% 1/5	0% 0/5	40% 2/5	20% 1/5	0% 0/5	20% 1/5	20% 1/5	20% 1/5	60% 3/5
TCC renal pelvis	0% 0/6	33% 2/6	17% 1/6	17% 1/6	17% 1/6	0% 0/6	0% 0/6	17% 1/6	0% 0/6	83% 5/6
Wilms' tumor	0% 0/1	0% 0/1	0% 0/1	0% 0/1	0% 0/1	0% 0/1	100% 1/1	0% 0/1	0% 0/1	100% 1/1
Kidney cancer	8% 8/100	45% 45/100	10% 10/100	17% 17/100	14% 14/100	7% 7/100	12% 12/100	12% 12/100	11% 11/100	58% 58/100

[a] E-cad, E-cadherin; RCC, renal cell carcinoma; TCC, transitional cell carcinoma.

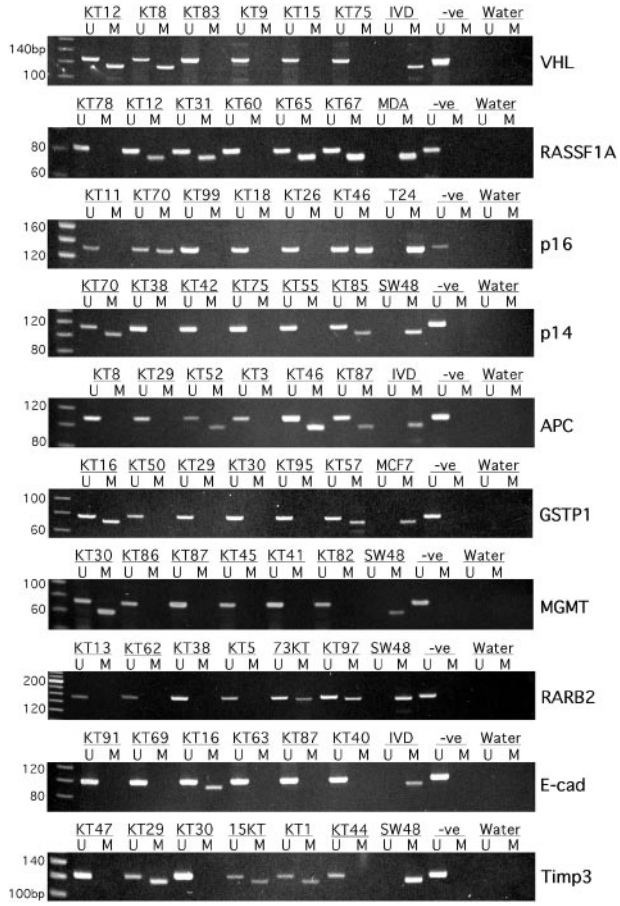

Fig. 2 Methylation-specific PCR of tumor suppressor genes in kidney tumor DNAs. *In each gel panel*, six kidney tumor DNAs (*KT*) with *numbers* corresponding to the patient numbers in Fig. 1. The presence of a PCR product in the methylated lane (*M*) indicates that the tumor DNA has methylated alleles, *e.g., KT12* and *KT8 in the VHL gel panel*. 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 that is retained in the cell but that is unmethylated. Also shown: tumor cell lines MDA231 (*RASSF1A* gene), *T24* (*p16* gene), *SW48* DNA (*p14, MGMT, RAR*β, and *Timp-3* genes), *MCF7* (*GSTP1* gene) and *in vitro* methylated DNA (*IVD*) for *VHL, APC,* and *E-cadherin* (*E-cad*) genes as a positive control; normal lymphocyte DNA as a negative control (*-ve*); a no template control for contamination in the PCR reaction (*Water*). Twenty-bp molecular ruler as a molecular weight marker (*unlabled lane, far left*) are also shown.

quency of promoter hypermethylation of the tumor suppressor gene loci included in the panel was *VHL* 8%, $p16^{INK4a}$ 10%, $p14^{ARF}$ 17%, *APC* 14%, *MGMT* 7% *GSTP1* 12%, *RAR*β*2* 12%, *RASSF1A* 45%, *E-cadherin* 11%, and *Timp-3* 58% of the 100 tumors (Table 1). Fig. 2 shows representative examples of MSP analysis of each gene. Hypermethylation was observed in all of the histological cell types, grades, and stages of kidney cancer examined and in patients of all ages. Ninety-three tumors showed methylation of at least 1 gene, and 7 tumors showed no methylation of any of the 10 genes. A total of 33% of kidney tumors had one gene, 34% two genes, 15% three genes, 8% four genes, 2% five genes, and 1% seven genes hypermethylated (Fig. 1). The mean number of genes hypermethylated in each tumor was 1.94 (SD, 1.229), and the median number was 2. No methylation was observed in 10 normal renal or 5 normal ureteral tissue DNAs. We did not find any evidence of methylation of the CpG island in the promoter of the *FH* gene after bisulfite sequencing of 15 papillary renal tumors (Fig. 3). We, therefore, did not perform further analysis of the *FH* gene.

Using statistical analysis, we examined methylation with regard to the kidney cancer patient clinicopathological parameters of age, gender, cell type, grade, stage, size (T_{1a} *versus* T_{1b}), and smoking history (yes or never) and with regard to associations between methylation of one particular gene and methylation of another gene in a particular tumor. *VHL* methylation was only found in clear cell tumors ($P = 0.006$) and only in males ($P = 0.048$). Hypermethylation of *RASSF1A* was significantly more frequent in papillary renal tumors ($P = 0.011$) and in higher grade tumors of all cell types ($P = 0.003$). *MGMT* methylation was more frequent in women ($P = 0.049$) and also in never-smokers of both sexes ($P = 0.045$). *MGMT* methylation also showed a trend to associate with high stage but not at a statistically significant level ($P = 0.0868$). *GSTP1* methylation was associated with older patients ($P = 0.029$). Hypermethylation of several individual genes was negatively associated with hypermethylation of other individual genes (all, $P < 0.05$), specifically, $p14^{ARF}$ with *RASSF1A*, *RAR*β*2* or $p16^{INK4a}$; $p16^{INK4a}$ with *APC* or *RAR*β*2*; and *RASSF1A* with *APC*. There was no association between gene hypermethylation and survival.

DISCUSSION

The growing number of tumor suppressor and other cancer genes reported to be hypermethylated with associated transcriptional silencing provides an opportunity for the examination of the pattern of epigenetic alteration in kidney cancer cells (1, 2, 4). The majority of kidney cancers (80–85%) are RCCs originating from the renal parenchyma. The remaining 15–20% are mainly transitional cell carcinomas of the renal pelvis. The classification of RCC includes several histological subtypes with different genetic backgrounds and natural histories. Conventional (clear cell) carcinoma (70%) and papillary carcinoma (10–15%) account for the majority of RCCs. The remaining types include chromophobe carcinoma (5%), the benign tumor oncocytoma (5–10%), and rarer forms such as collecting duct carcinoma (<1%) and RCC unclassified (≤5%; Ref. 34). The 100 tumors studied here included all major cell types and are representative of grade and stage at presentation of kidney cancer. We examined the methylation status of 10 tumor suppressor or cancer genes selected on the basis of both the biological significance of the gene and the methylation associated with loss of expression being well described for the gene (16, 17, 23–30).

The frequency of methylation of the genes examined varied from 58% for *Timp-3* and 45% for *RASSF1A* to 7% for *MGMT*. The gene methylation frequencies observed in our study were broadly similar to previous reports on primary kidney tumors (4, 18) but lower compared with kidney tumor cell lines (17, 35). Previous studies had examined the methylation status of either a single gene only in kidney cancer or several genes in a smaller

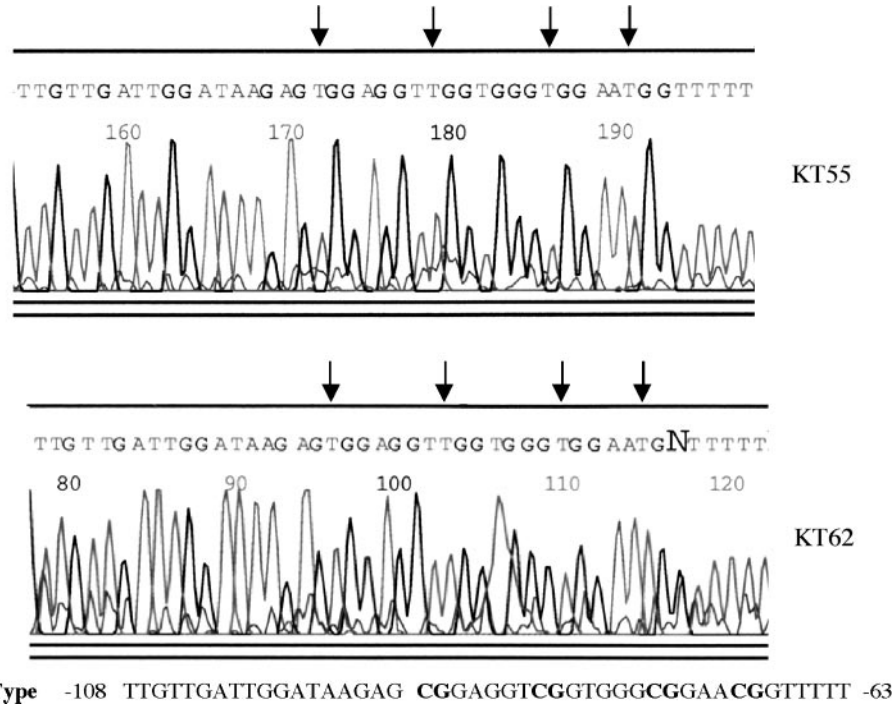

Fig. 3 Bisulfite sequencing of the *FH* promoter region. Sequencing of two papillary renal tumor DNAs (*KT55* and *KT62*) after bisulfite modification are shown. Unmethylated cytosines (*C*) are converted to uracil (*T*). The absence of C preceding G in the four CpG sites (*indicated by arrows*) demonstrates that these cytosines were unmethylated in the wild-type tumor DNA. All 35 CpG sites in the 392-bp PCR product were unmethylated in all 15 papillary renal tumors examined. *At the bottom of the figure,* the wild-type sequence is shown.

number of kidney tumors (4, 18, 36). Although the genes examined in our study have been reported to be unmethylated in normal tissue (22–30), we confirmed that the methylation was specific to cancer cells because our analysis of 15 nonmalignant renal or upper-tract tissue specimens found all 10 genes to be unmethylated (Ref. 31; data not shown). The finding of hypermethylation in kidney tumors of the lowest pathological stage (T_{1a}) and grade (I), as well as tumors <3 cm in diameter, indicates that suppressor gene methylation can be a relatively early event in kidney tumorigenesis. There is also evidence in cancers from other organ sites that methylation is an early event, *e.g.*, in breast (37), colorectal (24), and lung tumorigenesis (38). Hypermethylation was found in all of the pathways examined including the p16/Rb and p53/p14 tumor suppressor pathways (39) and the Wnt signaling pathway (40) emphasizing the widespread role of epigenetic silencing in kidney tumorigenesis.

We also examined whether promoter methylation of the recently identified *FH* tumor suppressor gene (13), inherited mutation of which can predispose to papillary renal cancer, occurs in sporadic papillary renal tumors. We examined the *FH* promoter region in 15 papillary renal cell tumors by bisulfite sequencing and found all to be unmethylated. Point mutation of *FH* has also been reported to be absent or rare in sporadic papillary renal cell tumors (41). The *p53* and *PTEN* tumor suppressor genes, known to be inactivated in kidney cancer by point mutation and deletion, have been found to be unmethylated in human cancer cells and were, therefore, not examined for promoter methylation (2). The *Rb* suppressor gene appears to be methylated at an appreciable frequency in retinoblastoma only (2, 42), and, because we have found no case of any urological tumor to be methylated (43), this gene was also not examined. The recently identified *BHD* gene has been reported to be methylated (44), but another study found no evidence of methylation in renal cancer (45).

The comparison of the clinicopathological data and methylation data revealed that hypermethylation of the *VHL* gene was specific for clear cell renal cancer, as expected (16). We also noted that hypermethylation of *RASSF1A* was significantly more frequent in papillary RCC compared with other cell types. *RASSF1A* methylation was also significantly associated with high-grade tumors. Although hypermethylation of $p14^{ARF}$ or *APC* was more common in non-clear cell cancers, the difference in frequency was not statistically significant in the present sample size. Analysis of larger numbers of specimens will determine whether this tendency is significant. *MGMT* hypermethylation was more frequent in tumors from nonsmokers. A similar finding has been reported in lung tumors from nonsmokers (46). It is important to note that, because of the statistical analyses performed, some significant associations could potentially be type I errors. For example, there is no obvious biological rationale for differences in gene methylation frequencies between genders. The statistical tests to identify patient characteristics associated with hypermethylation were conducted without explicit multiple comparison correction (*e.g.*, Bonferroni) to the significance level of the individual tests. That is, in order for the tests to have reasonable statistical power, no formal effort was made to control the family-wise type I error rate. Therefore, until further validation, the statistical data should be considered as preliminary.

We found no significant correlation between methylation and pathological stage, which is the most important determinant of survival for kidney cancer patients. Several profiles of tumors from other organ sites have also failed to

display a clear association between gene methylation and tumor grade or stage (5, 6, 8, 9). Survival data were available for 86 patients with a median follow-up period of 29 months. Kaplan-Meier analysis indicated that there was no association between methylation of any gene and survival or time to recurrence. A more lengthy follow-up will likely be necessary to determine whether gene hypermethylation can predict survival. Another issue to be considered is that present knowledge provides, at best, a partial picture of gene methylation in cancer. It has been estimated that several hundred, as yet unidentified, genes are hypermethylated in human cancer (33, 47); therefore, many more genes remain to be discovered in kidney cancer. The majority of the known hypermethylated genes have been identified through a candidate gene approach (1, 2, 4). In the future, global analysis (48–50) will be of increasing importance in the identification of novel hypermethylated genes in kidney cancer.

We recently reported on the detection of tumor suppressor gene hypermethylation in urine DNA from kidney cancer patients (31), which provided further impetus to profile gene hypermethylation in kidney tumorigenesis. For a potential diagnostic test to have maximal utility, it should be able to detect all types of kidney cancer and provide as much information as possible. Simultaneous differential diagnosis and molecular prognosis might be possible with an optimal panel of hypermethylated genes.

Our profile has demonstrated that aberrant promoter hypermethylation of tumor suppressor and cancer genes is frequent, widespread in terms of cell type, and can occur relatively early and in many cancer pathways in kidney tumorigenesis. The associations of *VHL*, *RASSF1A*, and *MGMT* hypermethylation with cell type, grade, and stage, respectively, if confirmed in larger studies, outline the potential as markers for the prediction of patient clinical outcome and for simultaneous differential diagnosis and molecular prognosis in urine-based screening.

REFERENCES

1. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa J-PJ. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–96.

2. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999;21:163–7.

3. Melki JR, Vincent PC, Clark SJ. Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res 1999;59:3730–40.

4. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225–9.

5. Ueki T, Toyota M, Sohn T, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000;60:1835–9.

6. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 2001;61:249–55.

7. Dong SM, Kim HS, Rha SH, Sidransky D. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. Clin Cancer Res 2001;7:1982–6.

8. Maruyama R, Toyooka S, Toyooka KO, et al. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res 2001;61:8659–63.

9. Kwong J, Lo KW, To KF, Teo PM, Johnson PJ, Huang DP. Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin Cancer Res 2002;8:131–7.

10. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer Statistics, 2003. CA Cancer J Clin 2003;53:5–26.

11. Zambrano NR, Lubensky IA, Merino MJ, Linehan WM, Walther MM. Histopathology and molecular genetics of renal tumors: toward unification of a classification system. J Urol 1999;162:1246–58.

12. Schmidt L, Duh FM, Chen F, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet 1997;16:68–73.

13. Tomlinson IP, Alam NA, Rowan AJ, et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet 2002;30:406–10.

14. Nickerson ML, Warren MB, Toro JR, et al. Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dube syndrome. Cancer Cell 2002;2:157–64.

15. Bishop JM. Molecular themes in oncogenesis. Cell 1991;64:235–48.

16. Herman JG, Latif F, Weng Y, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA 1994;91:9700–4.

17. Herman JG, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995;55:4525–30.

18. Morrissey C, Martinez A, Zatyka M, et al. Epigenetic inactivation of the *RASSF1A* 3p21.3 tumor suppressor gene in both clear cell and papillary renal cell carcinoma. Cancer Res 2001;61:7277–81.

19. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. Am J Surg Pathol 1982;6:655–63.

20. UICC TNM Classification of malignant tumors. New York: Wiley-Liss, 1997.

21. Sambrook J, Russell DW. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2001.

22. Herman JG, Graff JR, Myöhänen SBDN, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands Proc Natl Acad Sci USA 1996;93:9821–6.

23. Esteller M, Tortola S, Toyota M, et al. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 2000;60:129–33.

24. Esteller M, Sparks A, Toyota M, et al. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 2000;60:4366–71.

25. 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.

26. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 1999;59:793–7.

27. Dammann R, Li C, Yoon J-H, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 2000;25:315–9.

28. Cote S, Sinnett D, Momparler RL. Demethylation by 5-aza-2′-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743–50.

29. Corn PG, Smith BD, Ruckdeschel ES, Douglas D, Baylin SB, Herman JG. E-Cadherin expression is silenced by 5′ CpG island methylation in acute leukemia. Clin Cancer Res 2000;6:4243–8.

30. Bachman KE, Herman JG, Corn PG, et al. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggests a

suppressor role in kidney, brain, and other human cancers. Cancer Res 1999;59:798–802.

31. 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.

32. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987;196:261–82.

33. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 2002;99: 3740–5.

34. Storkel S, Eble JN, Adlakha K, et al. Classification of renal cell carcinoma: Workgroup No. 1. Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Cancer (Phila) 1997;80:987–9.

35. 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.

36. Morris MR, Hesson LB, Wagner KJ, et al. Multigene methylation analysis of Wilms' tumour and adult renal cell carcinoma. Oncogene 2003;22:6794–801.

37. Holst CR, Nuovo GJ, Esteller M, et al. Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res 2003;63:1596–601.

38. Belinsky SA, Nikula KJ, Palmisano, WA, et al. Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci USA 1998;95: 11891–6.

39. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103–12.

40. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature (Lond) 2001;411:349–54.

41. Kiuru M, Lehtonen R, Arola J, et al. Few FH mutations in sporadic counterparts of tumor types observed in hereditary leiomyomatosis and renal cell cancer families. Cancer Res 2002;62:4554–7.

42. Ohtani-Fujita N, Fujita T, Aoike A, Osifchin NE, Robbins PD, Sakai T. CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene 1993; 8:1063–7.

43. Dulaimi E, Uzzo RG, Greenberg RE, Al-Saleem T, Cairns P. Detection of bladder cancer in urine by a tumor suppressor gene hypermethylation panel. Clinical Cancer Res 2004;10:887–93.

44. Khoo SK, Kahnoski K, Sugimura J, et al. Inactivation of BHD in sporadic renal tumors. Cancer Res 2003;63:4583–7.

45. da Silva NF, Gentle D, Hesson LB, Morton DG, Latif F, Maher ER. Analysis of the Birt-Hogg-Dube (BHD) tumour suppressor gene in sporadic renal cell carcinoma and colorectal cancer. J Med Genet 2003;40:820–4.

46. Pulling LC, Divine KK, Klinge DM, et al. Promoter hypermethylation of the O6-methylguanine-DNA methyltransferase gene: more common in lung adenocarcinomas from never-smokers than smokers and associated with tumor progression. Cancer Res 2003;63:4842–8.

47. Costello JF, Frühwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type–specific patterns. Nat Genet 2000;25:132–8.

48. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–9.

49. Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002;2:485–95.

50. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. Hum Mol Genet 1999;8:459–70.

Clinical Cancer Research

Promoter Hypermethylation Profile of Kidney Cancer

Essel Dulaimi, Inmaculada Ibanez de Caceres, Robert G. Uzzo, et al.

Clin Cancer Res 2004;10:3972-3979.