[CANCER RESEARCH 60, 1942–1948, April 1, 2000]

VHL Alterations in Human Clear Cell Renal Cell Carcinoma: Association with Advanced Tumor Stage and a Novel Hot Spot Mutation[1]

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ABSTRACT

To elucidate the role of somatic alterations for renal cancer etiology and prognosis, we analyzed 227 sporadic renal epithelial tumors for mutations and hypermethylations in the von Hippel-Lindau tumor suppressor gene *VHL*. Tumors were classified according to the recommendations of the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Somatic *VHL* mutations were identified by PCR, single-strand conformation polymorphism analysis, and sequencing, and hypermethylations were identified by restriction enzyme digestion and Southern blotting. Frequencies of *VHL* alterations were established, and an association with tumor type or tumor type and tumor stage was evaluated. *VHL* mutations and hypermethylations were identified in 45% of clear cell renal cell carcinomas (CCRCCs) and occasionally (3 of 28) in papillary (chromophilic) renal cell carcinomas (RCCs). Lack of *VHL* mutations and hypermethylations in chromophobe RCCs and oncocytomas was statistically significant ($P = 0.0001$ and $P = 0.0004$, respectively). RCCs carrying *VHL* alterations showed, in nine cases (12%), mutations at a hot spot involving a thymine repeat (ATT.TTT) in exon 2. Tumor staging was critical to the *VHL* mutation/hypermethylation detection rate in CCRCCs shown by separate evaluation of patients from medical centers in Munich, Heidelberg, and Mainz. The spectrum of pT_1, pT_2, and pT_3 CCRCCs and the *VHL* mutation/hypermethylation detection rate varied among these three groups. Altogether, *VHL* alterations were significantly associated with pT_3 CCRCCs ($P = 0.009$). This is the first evidence of frequent somatic *VHL* mutations at a particular site within exon 2 and an association of *VHL* mutations/hypermethylations with a standard prognostic factor.

INTRODUCTION

CCRCC[3] is the most common malignant neoplasm of the kidney and belongs to the few human tumors known to evolve from mutations of a specific gene, the *VHL* tumor suppressor gene (1). Originally, *VHL* was identified in families with VHL disease, a rare hereditary multitumor syndrome (1). Molecular studies have shown that *VHL* germ-line mutations are associated with hereditary CCRCCs in *VHL* disease (2), and moreover, that somatic *VHL* mutations account for the majority of the more common sporadic CCRCCs (3). Together with the loss of the homologous chromosome 3p allele (3p LOH), *VHL* mutations are rate-limiting events in renal tumorigenesis (3, 4). Somatic *VHL* mutations were identified in 33–57% of CCRCCs from the United States, Europe, and Japan (3, 5–8), and in 20% of another subset of CCRCCs, the *VHL* gene was methylated (9). Current knowledge suggests that mutations and transcriptional silencing (3–9) of *VHL* in renal epithelial cells cause loss or modulation of cellular functions operated by the wild-type *VHL* protein (pVHL). The molecular mechanisms by which pVHL modulates the expression of target genes leading to CCRCCs are not well understood. Accumulated evidence suggests that pVHL is involved in targeted protein degradation and control of angiogenesis (10–13), and there is evidence that pVHL is implicated in regulation of extracellular pH (14), formation of extracellular matrix (15), and cell cycle control (10).

Molecular analyses revealed a broad spectrum of somatic *VHL* defects. Any nucleotide of the coding sequence downstream of a *Not*I restriction site may be affected by substitutions, deletions, or insertions (3, 5–8), and other changes may involve methylation (9). Although most of these alterations seem distributed at random, some occur more frequently. For example, somatic mutations in RCCs cluster within *VHL* exon 2 (3). Also, RCCs of patients with defined occupational exposure to a human carcinogen, *i.e.*, trichloroethylene, show frequent cytosine to thymine (C→T) transitions and/or a *VHL* hot spot mutation in exon 1 (16). With the exception of patients with known environmental exposure for which nonrandom *VHL* mutations may be linked to a specific carcinogen, the origin of frequent mutations at other sites remains elusive.

Nonrandom distribution of somatic *VHL* mutations may not only originate endogenously but also from exposure to exogenous carcinogens, which is supported by the observation of regional variations in RCC incidence. For example, for yet unknown reasons, the Bas Rhin region of France has one of the highest incidences of RCC in the world (17). Furthermore, on the molecular level, the findings of a wide spectrum of somatic *VHL* mutation frequencies in patients from the United States, Europe, and Japan are unexplained. We reasoned that comprehensive molecular and histopathological data analyses of patients from potentially high-risk areas may provide further clues to the etiology of RCCs and the meaning of somatic *VHL* alterations.

MATERIALS AND METHODS

Renal Tissue and Histopathological Classification. Normal and tumor tissue of 227 patients with renal epithelial tumors were obtained from three medical centers in Germany. Tumors were designated according to the medical center of origin, *i.e.*, MRI refers to the Institute of Pathology, Technical University Munich-Klinikum rechts der Isar, KT and KTCL to the University Hospital and German Cancer Research Center Heidelberg, and MZ to the

Received 8/24/99; accepted 2/1/00.

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[1] Supported by Grants 93.004.1-3 and 93.038.2 from Wilhelm Sander-Stiftung, Grant De356/3-3/4 from the Deutsche Forschungsgemeinschaft, Grant 519, C2 from SFB, and a grant from the Bioscientia Institute Ltd. D. G. was supported by Deutscher Akademischer Austauschdienst and Deutsche Forschungsgemeinschaft, and C. P. was supported by Deutscher Akademischer Austauschdienst.

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[3] The abbreviations used are: CCRCC, clear cell renal cell carcinoma; RCC, renal cell carcinoma; UICC, Union Internationale Contre le Cancer; AJCC, American Joint Committee on Cancer; VHL, von Hippel-Lindau; pVHL, VHL protein; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity; MRI, tumors from University Hospital Munich rechts der Isar, Technical University Munich; KT and KTCL, kidney tumors and kidney tumor cell lines from University Hospital Heidelberg; MZ, tumors from University Hospital Mainz.

University Hospital Mainz. Patients had no history of hereditary VHL disease. Tissue was stored in liquid nitrogen until DNA isolation. Peripheral blood lymphocytes served as a source of normal DNA from patients without available normal renal tissue. Histopathological evaluation (Table 1) and grading were according to Thoenes *et al.* (18) and in concordance with the recently established classification by the UICC and the AJCC (19). Readings were independently confirmed by repeated microscopic evaluation by specifically trained pathologists of participating institutes (G. W., M. R., P. S., and S. S). Tumor-Node-Metastasis stages were established according to the UICC (20). Detailed information on tumors without *VHL* alterations are available upon request. Informed consent was given by all patients. Investigations were performed retrospectively and approved by a human investigations committee.

DNA Isolation and Molecular Analysis. DNA was isolated according to standard procedures. For the detection of nucleotide substitutions, deletions and insertions at *VHL* PCR-based methods including SSCP and sequencing were conducted in two participating laboratories. Samples with SSCP bandshifts were subjected to sequencing analysis to confirm mutations. Samples without SSCP bandshifts were sequenced at random. Both samples with and without somatic *VHL* mutations were exchanged between the two laboratories and repeatedly analyzed at random in a blinded fashion. PCR oligonucleotides were FR III (5′-ACTCGGGAGCGCGCACGCA-3′) and R 30 (5′-GAGGGCTCGCGC-GAGTTCAC-3′-), VHL 28 and VHL 22 (21), MA2A and 101 (21), I5 and I3 (21), YH1A and 6b (21), and K 55 and K56 (22). The analysis of DNA hypermethylation was according to Herman *et al.* (9). Large deletions of *VHL* were not analyzed because this would require quantitative Southern blotting (23). This method cannot be used for the analysis of somatic *VHL* mutations because of variable amounts of contaminating lymphocytes in tumor tissues.

LOH was determined by comparison of normal and tumor DNA at microsatellite loci *D3S1038, D3S1530,* and *D3S1435* according to published procedures (24). For comprehensive evaluation, results from LOH studies from Brauch *et al.* (25) were included.

Statistical Analysis. An association between presence of somatic *VHL* mutations and the CCRCC phenotype compared with lack of somatic *VHL* mutations in chromophobe RCCs or oncocytomas was calculated according to Fisher's exact test (26). Two-sided *P*s were used and were considered statistically significant when <0.05. An association between the presence of a somatic *VHL* mutation and tumor staging (pT) was calculated by contingency table analysis with χ^2 testing (likelihood ratio). All statistical computations were performed with the software SPSS for Windows Rel. 9.0.1 (SSPS, Inc.).

RESULTS

All RCCs with somatic *VHL* mutations and hypermethylations are listed in Table 2. Carcinomas are consecutively arranged according to affected nucleotides from the 5′ to the 3′ direction, respectively. Data include pathological characteristics of 77 RCCs, their *VHL* alterations, *i.e.*, 64 mutations and 13 hypermethylations, as well as 3p LOH of 56 of these RCCs, respectively (Table 2).

***VHL* Mutations and Hypermethylations in Renal Tumors.** *VHL* alterations, *i.e.*, mutations and hypermethylations, affected 68 of 151 (45%) CCRCCs, 4 of 13 (31%) RCCs not further classified, a low differentiated and a dedifferentiated RCC, as well as 3 of 28 (11%) papillary (chromophilic) RCCs (Table 3). All patients were negative for germ-line mutations. *VHL* alterations were absent in chromophobe RCCs and renal oncocytomas (Table 3). Lack of *VHL* alterations in chromophobe RCCs (0 of 17) compared with the high frequency of *VHL* alterations in CCRCCs (68 of 151; 45%) was statistically significant ($P = 0.0001$). Lack of *VHL* alterations in renal oncocytoma (0 of 15) was also statistically significant when compared with CCRCCs ($P = 0.0004$).

Table 1 *Renal epithelial tumors screened for VHL alterations and an association between pathological tumor staging and the presence of VHL alterations*

	No. of tumors included in	
Histopathological classification	*VHL* alteration studies	Association study
212 RCCs[a]		
CCRCC	151	145
Papillary (chromophilic) RCC	28	
Chromophobe RCC	17	
Not further classified	13	
Low differentiated	1	
Dedifferentiated	2	
15 renal oncocytomas	15	
227 renal epithelial tumors	227	145

[a] Carcinomas were 185 primaries and 27 RCC cell lines; there was one brain metastasis and three multifocal carcinomas.

***VHL* Mutation Types and Hypermethylation.** Seventy-seven RCCs shared 60 different mutations and epigenetic changes. In particular, 64 carcinomas had mutations (83%), and 13 had hypermethylations (17%). Mutations included 32 frameshift mutations (50%), 18 missense mutations (28%), 3 in-frame deletions or insertions (5%), 4 nonsense mutations (6%), 6 splice site mutations (9%), and 1 change at the 3′ untranslated region. Mutations of the coding sequence were located in exon 1 in 17 cases (27%), in exon 2 in 27 cases (42%), and in exon 3 in 13 cases (20%). An overview of the frequencies and distribution of *VHL* alterations is given in Fig. 1.

LOH. 3p LOH was identified in 115 CCRCCs (93%) of 124 informative cases (not shown). Also, 6 of 27 (22%) papillary (chromophilic) RCCs and 5 of 11 (45%) chromophobe RCCs had 3p LOH (not shown). No LOH was observed in renal oncocytomas (0 of 15). Both *VHL* mutation/hypermethylation and 3p LOH were present in 56 of 77 (73%) RCCs (Tables 2 and 3). This refers to 51 of 68 (75%) CCRCCs, 2 of 3 (66%) papillary (chromophilic) RCCs, 1 dedifferentiated, 1 low differentiated, and 1 not further classified RCC, respectively (Table 3).

***VHL* Mutation Hot Spot.** Nine of 77 (12%) RCCs with *VHL* alterations had a nucleotide change, insertion, or deletion at a thymine cluster (ATT.TTT) within exon 2 encoding amino acids isoleucine and phenylalanine at positions 147 and 148 of pVHL (Table 3). Examples of deletion T and insertion T are given in Fig. 2, respectively. In all cases, the mutation was predicted to truncate pVHL at codon 147 or 148.

Association between *VHL* Mutations/Hypermethylations and Prognostic Factor pT in CCRCCs. We tested for an association between the presence of somatic *VHL* mutation/hypermethylation in CCRCCs and prognostic factors, *i.e.*, tumor stage (pT) and nuclear grading (G). Information on tumor stage was available for 145 patients (Table 4). Four tumors (3%) were <2.5 cm in greatest extension (pT_1), 68 (47%) were >2.5 cm in greatest extension limited to the kidney (pT_2), and 73 (50%) invaded the adrenal gland or perinephric tissue or extended into the major veins (pT_3). *VHL* mutations or hypermethylations were identified in 64 CCRCCs of all three stages pT_1, pT_2, and pT_3 (Table 4). pT_3 CCRCCs carried *VHL* mutations or hypermethylations in 64% ($n = 41$; Table 4). This association was statistically significant ($P = 0.009$). Also three papillary (chromophilic) RCCs had *VHL* hypermethylations, of which two were of advanced stage (pT_3) and one was of intermediate stage (pT_2; Table 2). Whereas most CCRCCs carrying *VHL* mutations/hypermethylations were of advanced tumor stage (pT_3), there was no significant association between presence of *VHL* mutations/hypermethylations and nuclear grades. Three papillary (chromophilic) RCCs with *VHL* hypermethylations were highly malignant tumors (G3).

Influence of Tumor Stage (pT) on the Somatic *VHL* Mutation/Hypermethylation Detection Rate. The present study included patients from three different medical centers with various numbers of CCRCCs of pT_3, pT_2, and pT_1 stages. We compared the overall *VHL* mutation/hypermethylation detection rate, first between CCRCCs recruited from different hospitals, and second between CCRCCs of different pT stages (Table 5). Mutation/hypermethylation detection rates were 56% in patients from the Technical University in Munich

Table 2 *Pathological characteristics of CCRCCs and papillary (chromophilic) RCCs with VHL mutations, hypermethylations, and 3p LOH*

Tumor[a]	Histology[b]	pTNM[c]	G[c]	Nucleotide changes[d]	Codon	Protein change	Mutation type	LOH[e]
MRI 15	CCRCC	$pT_2N_0M_x$	G1	366del13nt	51	Frameshift	Yes	
MRI 35	papillary (chrphl)	$pT_{3b}N_2M_1$	G3	385	58		Hypermethylation	Yes
MZ 69	papillary (chrphl)	$pT_2N_xM_x$	G3	385	58		Hypermethylation	No
MZ 70	papillary (chrphl)	$pT_3N_2M_x$	G3	385	58		Hypermethylation	Yes
KT 126[g]	CCRCC	$pT_{3b}N_0M_0$	G2	385	58		Hypermethylation	Yes
KT 141[g]	CCRCC	$pT_3N_0M_1$	G3	385	58		Hypermethylation	Yes
MRI 37	CCRCC	$pT_{3a}N_0M_x$	G2	385	58		Hypermethylation	Yes
MRI 10	CCRCC (multiple)	$pT_{3a}N_xM_x$	G2	385	58		Hypermethylation	Yes
MRI 17	CCRCC	$pT_{3a}N_xM_x$	G2	385	58		Hypermethylation	Yes
MZ 71	CCRCC	$pT_2N_xM_x$	G3	385	58		Hypermethylation	Yes
MZ 72	CCRCC	$pT_2N_xM_x$	G3	385	58		Hypermethylation	Yes
MZ 34	CCRCC	$pT_2N_xM_x$	G2	385	58		Hypermethylation	No
MZ 46	CCRCC	$pT_{3a}N_xM_x$	G2	385	58		Hypermethylation	Yes
MZ 58	CCRCC	$pT_{3b}N_xM_x$	G3	385	58		Hypermethylation	Yes
KTCL 48	CCRCC	NA	NA	385 C→A	58	Arg→Arg	Silent	NA
				382 del G	57	Truncation	Frameshift	
MZ 15	CCRCC	$pT_2N_0M_x$	G2	416 C→A	68	Ser→Stop	Nonsense	NA
MZ 18	CCRCC	$pT_{3b}N_3M_x$	G3	418 del 3nt	69	Truncation	Frameshift	Yes
KTCL 185	CCRCC	brain metastasis		434 T→A	74	Val→Asp	Missense	NA
KT 153[g]	CCRCC	$pT_xN_0M_0$	G1	449 GC→CA	79	Arg→Pro	Missense	Yes
MZ 66	CCRCC	$pT_2N_xM_x$	G2	453T→A	80	Ser→Arg	Missense	Yes
MRI 19	CCRCC	$pT_{3b}N_0M_x$	G2	462 delCG	83	Truncation	Frameshift	NA
KTCL 53[f]	CCRCC	$T_{3a}N_2M_1$	G3	463 delGTG.CTG	84/85	del Val.Leu	In-frame deletion	NA
MRI 43	CCRCC	$pT_{3a}N_0M_x$	G2	475 T→C	88	Trp→Arg	Missense	Yes
MRI 33	CCRCC	$pT_{3b}N_0M_x$	G2	489ins 72nt	93≫116	ins24aa (redupl)	In-frame insertion	Yes
KT 123[g]	CCRCC	$pT_{3b}N_0M_0$	G2	498 G→A	95	Pro→Pro	Silent	Yes
				499 C→T	96	Gln→Stop	Nonsense	
KT 152[g]	CCRCC	$pT_3N_0M_0$	G1	504 delC	97	Truncation	Frameshift	Yes
KTCL 54	#	$pT_2N_xM_x$	G1	505T→G	98	Tyr→Asp	Missense	NA
MZ 35	CCRCC	$pT_{3b}N_1M_x$	G3	507 C→A	98	Tyr→Stop	Nonsense	NA
KTCL 195	#	NA	NA	542 ins6nt	110	Truncation	Frameshift	NA
MZ 39	CCRCC	$pT_{3b}N_xM_x$	G3	553 delG	114	Truncation	Frameshift	Yes
KTCL 2[f]	Low differentiated	$pT_{3a}N_1M_x$	G3	554-2 a→t			Splice	Yes
KT 145[g]	CCRCC	$pT_2N_0M_0$	G2	554-1 g→t			Splice	Yes
KT 124[g]	CCRCC	$pT_{3b}N_0M_0$	G1	555 del7nt	114	Truncation	Frameshift	Yes
KTCL 140[f,g]	CCRCC	pT_{3a}	G2	556 C→T	115	His→Tyr	Missense	Yes
MZ 73	CCRCC	$pT_xN_xM_x$	G2	557 A→C	115	His→Pro	Missense	NA
MZ 53	CCRCC	$pT_2N_xM_x$	G2	559 delC	116	Truncation	Frameshift	Yes
MZ 22	CCRCC	$pT_2N_xM_x$	G1	564 delG	117	Truncation	Frameshift	Yes
MRI 2	CCRCC	$pT_{3b}N_xM_x$	G2	571 delAG	120	Truncation	Frameshift	Yes
MZ 50	CCRCC	$pT_1N_xM_x$	G2	574 ins T	121	Truncation	Frameshift	Yes
MZ 16	CCRCC	$pT_2N_xM_x$	G2	578 C→A	122	Ala→Glu	Missense	Yes
MZ 08	CCRCC	$pT_{3a}N_0M_x$	G3	578 C→A	122	Ala→Glu	Missense	NA
MRI 54	CCRCC	$pT_{3a}N_0M_x$	G2	591 delTG	126	Truncation	Frameshift	Yes
MZ 32	CCRCC	$pT_2N_xM_x$	G1	591 delT	126	Truncation	Frameshift	Yes
KT 131[g]	CCRCC	$pT_{3a}N_0M_0$	NA	593delGG	127	Truncation	Frameshift	Yes
MRI 11	CCRCC	$pT_2N_0M_x$	G2	608 del A	132	Truncation	Frameshift	NA
MRI 14	CCRCC	$pT_{3a}N_0M_x$	G1	620 T→G	136	Phe→Cys	Missense	Yes
KTCL 13[f]	CCRCC	NA	G2	642 delC	143	Truncation	Frameshift	NA
KTCL 50	#	$pT_2N_0M_1$	G1	642 C→G	143	Asp→Glu	Missense	NA
MZ 26	CCRCC	$pT_2N_xM_x$	G2	652 del16nt	147	Truncation	Frameshift	Yes
MZ 74	CCRCC	$pT_{3b}N_xM_x$	G2	653 del7nt	147	Truncation	Frameshift	Yes
MZ 05	CCRCC	$pT_3N_xM_x$	G3	651 T→C	147	Pro→Pro	Silent	NI
				652 del 5nt	147	Truncation	Frameshift	
KTCL 69	#	$pT_3N_0M_x$	G1	652 delA	147	Truncation	Frameshift	Yes
KTCL 26A[f,g]	CCRCC	$pT_{2a}N_xM_1$	G2	653–657 delT	147/148	Truncation	Frameshift	Yes
MRI 5	CCRCC	$pT_2N_0M_x$	G2	653–657 delT	147/148	Truncation	Frameshift	Yes
MZ 29	CCRCC	$pT_2N_xM_1$	G2	653–657 delT	147/148	Truncation	Frameshift	No
KT 150[g]	CCRCC	$pT_{3a}N_1M_1$	NA	653–657 delT	147/148	Truncation	Frameshift	Yes
MRI 31	CCRCC (multi focal)	$pT_{3b}N_xM_x$	G2	653–657 insT	147/148	Truncation	Frameshift	Yes
MZ 06	CCRCC	$pT_2N_xM_x$	G1	664 A→T	151	Ile→Phe	Missense	No
MRI 12	CCRCC	$pT_{3b}N_0M_x$	G3	665 T→G	151	Ile→Ser	Missense	Yes
MRI 59	CCRCC	$pT_{3b}N_0M_x$	G3	676+1 g→t			Splice	Yes
KTCL 31	CCRCC	$pT_2N_0M_x$	G1	676+2 t→a			Splice	NA
KT 127[g]	CCRCC	$pT_3N_0M_0$	NA	677-1 g→c			Splice	Yes
MRI 58	CCRCC	$pT_{3a}N_0M_x$	G2	677-1 g→a			Splice	NA
KT 149[g]	CCRCC	$pT_3N_0M_1$	G3	677 del11nt	155	Truncation	Frameshift	Yes
MZ 17	CCRCC	$pT_{3b}N_xM_x$	G2	679/681 delTA	156	Truncation	Frameshift	Yes
MRI 29	CCRCC	$pT_2N_xM_x$	G2	680 del9nt	156	Truncation	Frameshift	Yes
KT 134[g]	CCRCC	$pT_3N_0M_0$	G2	686 T→A	158	Leu→Gln	Missense	Yes
MZ 76	CCRCC	$pT_{3a}N_xM_x$	G3	686 T→C	158	Leu→Pro	Missense	Yes
MRI 60	CCRCC	$pT_{3b}N_xM_x$	G3	686 T→A	158	Leu→Gln	Missense	Yes
MRI 1	CCRCC	$pT_{3a}N_xM_x$	G2	703 C→T	164	Gln→Stop	Nonsense	Yes
MRI 52	CCRCC	$pT_1N_xM_x$	G2	709 G→T	166	Val→Phe	Missense	Yes
KT 137[g]	Dedifferentiated	$pT_2N_2M_1$	NA	713 G→A	167	Arg→Gln	Missense	Yes
MRI 41	CCRCC	$pT_{3a}N_0M_x$	G2	769 del7nt	186	Truncation	Frameshift	Yes
MRI 20	CCRCC	$pT_{3b}N_0M_x$	G2	770 del5nt	186	Truncation	Frameshift	NA
MRI 48	CCRCC	$pT_{3b}N_0M_0$	G3	773 del21nt	187	Truncation	In-frame deletion	Yes
MRI 53	CCRCC	$pT_{3b}N_2M_x$	G3	796 insT	195	Truncation	Frameshift	Yes
MZ 27	CCRCC	$pT_2N_xM_x$	G2	863 delA	UTR	Unknown		Yes
Total of 77 carcinomas				64 mutations 13 hypermethylations				56 LOH

[a] Tumors are encoded according to the medical center of origin: MRI, Klinikum rechts der Isar, University of Munich; KT and KTCL, German Cancer Research Center and University Hospital of Heidelberg; MZ, University Hospital of Mainz.

[b] chrphl, chromophilic; #, cultured tumors defined as RCC but not further classified.

[c] pTNM tumor staging and G nuclear grading. NA, not available.

[d] nt: nucleotides; del, deletion; ins, insertion. Exonic nts are capitalized and intronic nts are in lower case. Alterations in exonic sequences are indicated either by exact nt or the 5′ most nt. Alterations in intronic sequences are indicated by the distance from the closest exonic nt. *VHL* alterations are listed from 5′ to 3′ direction.

[e] NA, not available; NI, not informative.

[f] Tumors whose mutations were published previously (3).

[g] Tumors included in previous LOH studies (25).

Table 3 *VHL alterations in renal epithelial tumors: tumors with mutations and hypermethylations, tumors with a hot spot mutation, and tumors with both mutation/hypermethylation and 3p LOH*

Renal epithelial tumors[a]	No. of tumors analyzed	Tumors with *VHL* mutation/ hypermethylation	Tumors with *VHL* hot spot mutation at ATT.TTT (codon 147/148)	Tumors with *VHL* mutation/ hypermethylation and 3p LOH
CCRCC	151	68/151 (45%)	8/68 (12%)	51/68 (75%)
Papillary (chromophilic) RCC	28	3/28 (11%)	0/3	2/3 (66%)
Chromophobe RCC	17	none	none	none
RCC not further classified	13	4/13 (31%)	1/4	1/4
Low differentiated RCC	1	1	0/1	1
Dedifferentiated RCC	2	1	0/1	1
Total RCCs	212	77/212 (36%)	9/77 (12%)	56/77 (73%)
Oncocytoma	15	none	none	

[a] Renal epithelial tumors were classified according to recommendations by UICC and AJCC (19).

(MRI), 51% in patients from the University of Heidelberg (KT/KTCL), and 36% in patients from the University of Mainz (MZ). Aside from two pT_1 tumors, most mutations and hypermethylations affected pT_3 and pT_2 CCRCCs. Detection rates were 42% in pT_3 and 26% in pT_2 CCRCCs from Mainz (MZ), 60% in pT_3 and 36% in pT_2 CCRCCs from Heidelberg (KT/KTCL), and 66% in pT_3 and 40% in pT_2 in patients from Munich (MRI; Table 5). MRI and KT/KTCL patient groups consisted predominantly of pT_3 CCRCCs, whereas the MZ patient group consisted predominantly of pT_2 CCRCCs (Table 5). This distribution is reflected in the association of *VHL* alterations in pT_3 CCRCCs.

DISCUSSION

Progress has been made in the identification of cancer-specific mutations; however, the meaning of molecular data for cancer etiology on one hand, and cancer prognosis on the other hand, largely remains unclear. We present a *VHL* mutation hot spot in sporadic CCRCCs of patients recruited from three German regions and report an association between somatic *VHL* alterations and advanced tumor stage. Our observations are based on the uniform histopathological classification of a large panel of renal epithelial tumors following the refined recommendations of the UICC and AJCC (19), somatic *VHL* mutation and hypermethylation analyses of the tumors, and the statistical evaluation of an association between histopathological characteristics, molecular data, and tumor staging (pT). We analyzed patients from three medical centers in Germany, *i.e.*, the Technical University of Munich, the University of Heidelberg, and the University of Mainz, located within regions suspect to high renal cancer incidence. Although detailed epidemiological data on renal cancer incidence of these geographic regions are sparse (27), high renal cancer incidence in Germany and Northern Europe has been reported (17, 28); there should be mention of the neighboring location of two areas (Heidelberg and Mainz) to the French Bas Rhin region, which is known for one of the highest renal cancer incidences in the world (17).

Similarly to previous studies from the United States, Great Britain, Europe, and Japan (3, 5–9), we established somatic *VHL* mutations and hypermethylations in 45% of sporadic CCRCCs; however, the observation of a somatic *VHL* mutation hot spot and the association of *VHL* mutations with pT_3 CCRCCs are novel observations. Altogether, we observed 60 different genetic and epigenetic changes at *VHL*, yet 30% of all alterations affected two major sites: (*a*) 18% of *VHL*-defective carcinomas showed hypermethylation and frameshift mutation at the *Not*I restriction site of exon 1; and (*b*) 12% had somatic *VHL* mutations that clustered within the nucleotide sequence containing a pentamer thymine repeat. Whereas frequent hypermethylation has been reported previously (9), only this study revealed frequent somatic mutations at a specific sequence. The independent origin of mutations at the proposed somatic *VHL* mutation hot spot is suggested by the findings of different mutations, *i.e.*, deletion of adenine, deletion or insertion of a single thymine, and deletions of 5, 7, or 16 nucleotides, and confirmed by independent repetition of experiments. The combined data set of published somatic *VHL* mutations failed to identify this hot spot (3, 5–8). We propose that our findings of a somatic *VHL* mutation hot spot point to geographical and/or epidemiological differences in the occurrence of RCCs and CCRCCs, respectively, which is in agreement with the observed high incidence of RCCs in Europe, in particular the Bas Rhin region (17, 28).

All mutations at the observed *VHL* exon 2 mutation hot spot predict truncation of the pVHL at amino acids isoleucine at codon 147 or phenylalanine at codon 148. These residues are located within the S7 unit of the β sheet domain, and truncation will result in loss of the entire pVHL α-domain that contains the critical residues for ElonginC binding (29, 30). The structural similarity of ElonginC-VHL with the Skp1-F-box protein complex of the Skp1-Cul1-F-box protein multiprotein complex (which targets proteins for degradation) supports the role of pVHL in an analogous pathway (29). Thus, tumorigenesis of CCRCCs may be determined by loss of that particular function. Among mutations identified in the germ-line of patients with VHL disease, mutations are biased to occur within the nucleotide sequence encoding the ElonginC binding region. In contrast, somatic mutations

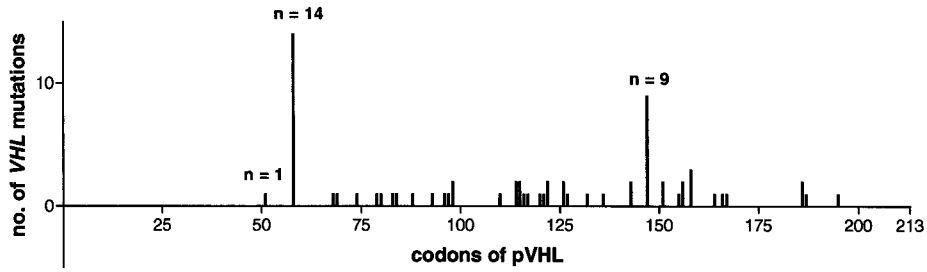

Fig. 1. Somatic mutations and hypermethylations of the *VHL* gene. Summary of data from patients with RCCs from three German medical centers (Munich, Heidelberg, and Mainz). The codons of the *VHL* gene are shown along the *X axis*. Exon 1 of the *VHL* gene encodes amino acids 1–114, exon 2 encodes amino acids 114–155, and exon 3 encodes amino acids 155–213. The numbers of mutations or hypermethylations at a particular codon are shown on the *Y axis*. *VHL* aberrations identified in RCCs are drawn as *perpendicular bars*. Most mutations were identified only once, as indicated by the *shortest length bar*. The methylation-sensitive codon 58 was affected by hypermethylation and a point mutation in 14 patients ($n = 14$). Codons 147 of 148 were affected in RCCs of nine patients ($n = 9$). Other mutations that were identified more than once affected codons 98, 114, 115, 122, 126, 143, 151, 156, 158, and 186.

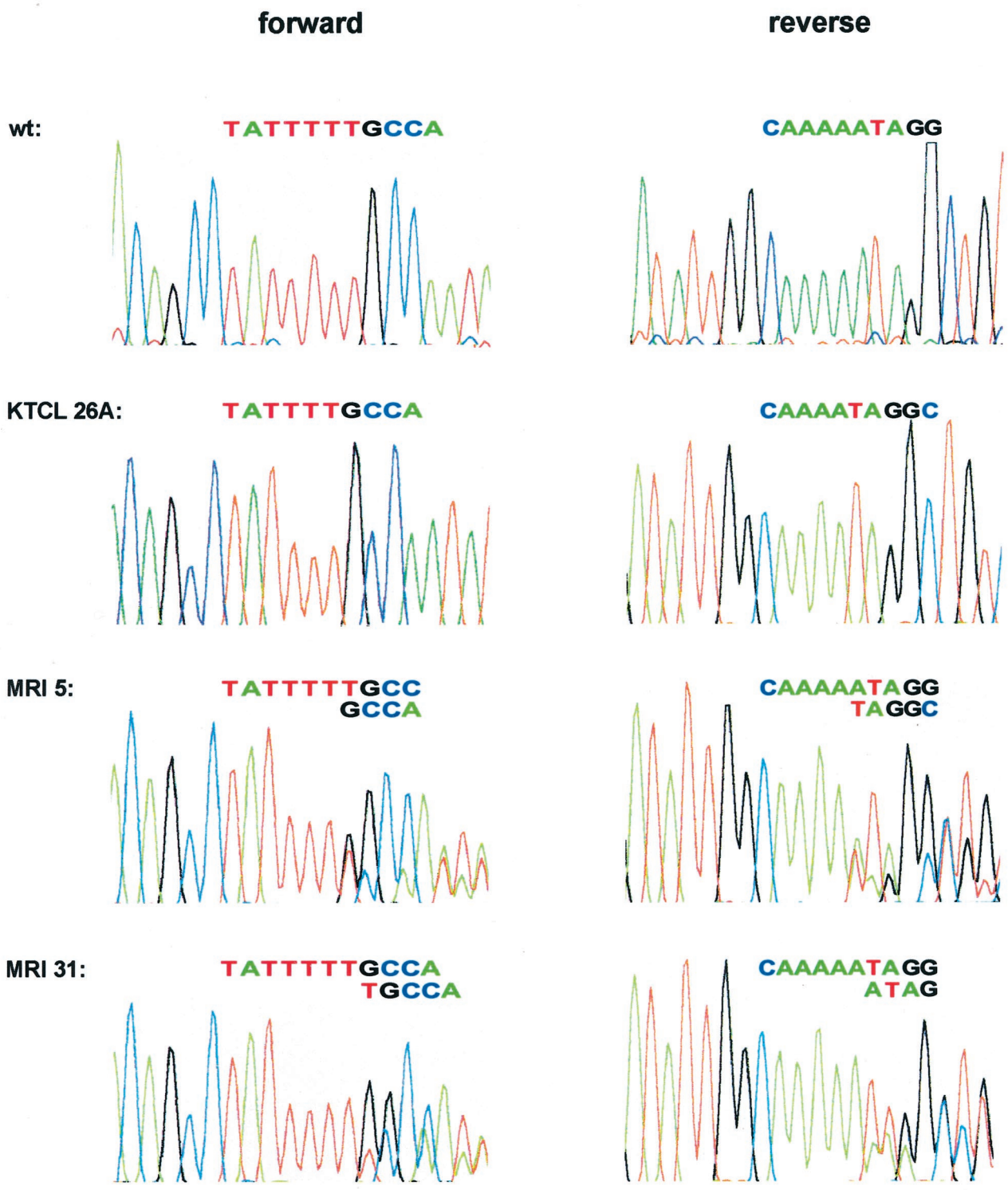

Fig. 2. Mutations in CCRCCs at a mutation hot spot sequence (ATT.TTT) in *VHL* exon 2. Sequences of CCRCC KTCL 26A, MRI 5, and MRI 31 are compared with the wild-type sequence (*wt*) in forward (*left*) and reverse (*right*) direction. The cell line KTCL 26A shows a deletion T; the sequence is homozygous because of a loss of the homozygous allele and absence of contaminating wild type. MRI 5, a primary tumor, shows the heterozygous sequence pattern of a wild-type allele and a mutated allele with a deletion T. Likewise MRI 31, a primary tumor, shows the heterozygous sequence pattern of a wild-type allele and a mutated allele with an insertion T.

mainly seem to affect this region indirectly by mutations at sites 5′ upstream within exon 2. The different mutation spectrum between the germ-line and somatic cells was assumed previously to reflect environmental carcinogenic effects (3). The herein observed frequent thymine cluster mutations within exon 2 support this view. Known mechanisms that may explain thymine mutation involve direct or indirect radiation effects (31) and interaction of DNA with alkylating agents (32). For example, the formation of cyclobutane pyrimidine dimers is a well-known mechanism to covalently link adjacent pyrimidine rings in the same polynucleotide chain upon UV radiation (33). The thymine run (ATT.TTT) in *VHL* exon 2 provides the structural basis of adjacent pyrimidine rings for dimerization to occur.

Table 4 *Association between VHL mutation/hypermethylation and tumor stage (pT) in CCRCC*

The association between tumor stage pT_3 and presence of *VHL* mutation/hypermethylation was statistically significant in χ^2 testing (likelihood coefficient; $P = 0.009$).

Number of staged (pT) CCRCC	pT_1	pT_2	pT_3	Total
With *VHL* mutation/hypermethylation	2	21	41	64
	3.1%	32.8%	64.1%	100%
Without *VHL* mutation/hypermethylation	2	47	32	81
	2.5%	58.0%	39.5%	100%
Total	4	68	73	145
	2.8%	46.9%	50.3%	100%

Likewise, alkylating agents known to be electrophilic compounds to interact with strong nucleophilic sites such as N^3-adenine may explain frequent mutations at T-rich sites (34). Although no defined carcinogen is known to be involved in RCCs of patients of this study panel, shared environmental exposures and life-style as well as variation in individual human susceptibility may contribute to a confounding effect of somatic *VHL* hot spot mutations.

VHL alterations are considered to be an early event in renal tumorigenesis (3). Our findings of mutations and hypermethylations in CCRCCs of all stages and nuclear grade are in agreement with the current view of the *VHL* gene being a gatekeeper in the development of RCCs. Although we were only able to analyze a few small (pT_1) CCRCCs, the presence of *VHL* mutations in any pT_1 tumor supports the current concept of the *VHL* gene being an early event in renal tumorigenesis. However, according to another current view, the *VHL* gene may not be the sole tumor suppressor or gatekeeper gene for CCRCCs and RCCs. This has long been suspected on the basis of LOH data as well as gene mapping efforts and mutation analyses by others (35–37). Now, detailed somatic *VHL* analysis in various tumor stages also supports this view. In light of the current understanding of the pleiotropy of *VHL* functions (10–15), these two concepts are not mutually exclusive but may rather complement each other. Whereas the *VHL* gene may be a gatekeeper for renal epithelial growth in those RCCs with *VHL* alterations, those RCCs for which *VHL* mutations have not been identified may enter the carcinogenic pathway through damage of other genes. Hence, because pVHL participates in numerous cellular control pathways, dysregulation of pVHL without any synchronously underlying *VHL* gene mutation may be critical in the tumorigenesis of these RCCs. Papillary (chromophilic) RCCs also seem to follow more than one molecular pathway, *i.e.*, mutations in the *MET* proto-oncogene (36) are responsible for the expression of a distinct morphological phenotype referred to as papillary RCC type 1 (38). We occasionally observed *VHL* hypermethylation in highly malignant papillary (chromophilic) RCCs. Our findings are in contrast to a previous report that excluded *VHL* mutations from RCCs other than nonpapillary (8), a previously used synonym for CCRCCs.

VHL mutations and hypermethylations seem not to influence malignant behavior determined by nuclear grade but rather may provide a growth advantage to mutated renal epithelial cells in their transition to CCRCCs. We predominantly observed *VHL* mutations and hypermethylations in stage pT_3 CCRCCs, which invade adrenal gland and perinephric tissue and grow beyond major veins, but we observed fewer mutations and hypermethylations in stage pT_2 tumors limited to the kidney parenchyma. To our knowledge, this is first evidence for a possible link between somatic *VHL* mutations and a standard prognostic factor. In contrast, CCRCCs associated with germ-line *VHL* mutations tend to be more indolent than their somatic counterparts (39). Growth delay in germ-line *VHL*-affected renal epithelial cells and growth advantage in somatically *VHL*-affected renal epithelial cells may point to a confounding role of differentiation during tumorigenesis of CCRCCs.

Further evidence for an association of somatic *VHL* mutations with nonfavorable prognosis comes from the observations of the lack of *VHL* mutations in chromophobe RCCs and renal oncocytomas. Renal oncocytomas are benign, and the more favorable prognosis of chromophobe RCC has been reported in the literature (40, 41). Lack of *VHL* alterations in these two classes of renal tumors may be a molecular hint for benign or less aggressive behavior. These findings are in agreement with the evolutionary model of renal tumors, according to which CCRCCs and papillary (chromophilic) RCCs develop from proximal tubular cells, whereas chromophobe RCCs and renal oncocytomas derive from intercalated cells of the cortical portion of the collecting duct (18). Our data suggest that aside from different morphological and biological characteristics, proximal and distal nephrons differ with respect to their susceptibility to acquire somatic *VHL* mutations. It follows that *VHL* mutations may be useful in the assessment of RCCs with aggressive biological behavior. The late recognition of chromophobe RCCs as a separate entity among RCCs with "light cell" appearance reflects the difficulty for inexperienced viewers to distinguish less favorable CCRCCs and more favorable chromophobe RCCs (18). However, the different biological behavior noticed by others makes this distinction clinically important (40, 41). We infer that identification of somatic *VHL* mutation in any unknown "light cell" RCC suggests the diagnosis of CCRCC with less favorable prognosis, which consequently mandates stringent clinical management.

Finally, our work provides another important novel aspect. We noticed the wide range of somatic *VHL* mutation detection rates reported by others previously, which ranged from 33 to 57% (3, 5–8). Together with hypermethylations (9), somatic *VHL* alterations accounted for up to 80% in CCRCCs. Although a 100% *VHL* mutation detection frequency was reported for germ-line mutations (23), no study of sporadic tumors showed alterations of all tumors; however, it was inferred that all sporadic CCRCCs may carry somatic *VHL* alterations. In our study, which technically matches those of other somatic *VHL* mutation and hypermethylation studies (3, 5–9), we now demonstrated that there may be true differences in somatic *VHL* mutation detection rates, reflecting the number of pT_3 tumors included in the analyzed patient samples. In these previous studies, no attention was paid to detailed histopathological parameters (3, 5–9). This study showed that the *VHL* mutation/hypermethylation detection rate was highest in those patient groups with the highest numbers of pT_3 CCRCCs. In contrast, it was lowest in the patient group with predominant pT_2 tumors. These coincidences provided us with the opportunity to establish various *VHL* mutation/hypermethylation detection frequencies in relationship to tumor staging. The interpretations of our data are based on the sensitivity of enzymatic *Not*I digestion and Southern blotting as well as SSCP and sequencing to detect most somatic *VHL* alterations. Our data support the notion that the presence of *VHL* defects may not only provide a growth advantage to affected CCRCCs but also may allow this growth advantage to be determined at all tumor stages.

Table 5 *VHL mutation detection rate in CCRCC of patients from three medical centers in relationship to tumor staging*

CCRCC pT (G1–G3)	With mut/MET[a]	Without mut/MET[a]	Rate	MRI[b] $n = 41$	KT/KTCL[b] $n = 31$	MZ[b] $n = 73$
pT_3	41	32	41/73	19/29	12/20	10/24
			56%	66%	60%	42%
pT_2	20	48	20/68	4/10	4/11	12/47
			29%	40%	36%	26%
pT_1	2	2	2/4	1/2		1/2
			50%	50%		50%
All	63	82	63/145	23/41	19/37[c]	26/73
			43%	56%	51%	36%

[a] mut/MET refers to patients with or without *VHL* mutations or hypermethylations.
[b] Abbreviations MRI, KT/KTCL, and MZ refer to patients treated at university hospitals Munich (Technical University, Klinikum rechts der Isar), Heidelberg, and Mainz (Germany); *n* is number of patients.
[c] No data on staging were available for 6 CCRCC.

ACKNOWLEDGMENTS

We acknowledge establishment of RCC cell lines (KTCL) by D. Komitowski and H. Löhrke, German Cancer Research Center, Heidelberg, and preparation of Figs. 1 and 2 by B. Borstel, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany.

REFERENCES

1. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. S. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Le Paslier, D., Chumakov, I., Cohen, D., Chinault, A. C., Maher, E. R., Linehan, W. M., Zbar, B., and Lerman, M. I. Identification of the von Hippel-Lindau disease tumor suppressor gene. Science (Washington DC), *260:* 1317–1320, 1993.
2. Zbar, B., Kishida, T., Chen, F., Schmidt, L., Maher, E. R., Richards, F. M., Crossey, P. A., Webster, A. R., Affara, N. A., Ferguson-Smith, M. A., Brauch, H., Glavac, D., Neumann, H. P. H., Tisherman, S., Mulvehill, J., Gross, D., Shuin, T., Whaley, J., Seizinger, B., Kley, N., Olschwang, S., Boisson, C., Richard, S., Lips, C. H. M., Linehan, W. M., and Lerman, M. I. Germline mutations in the von Hippel-Lindau disease gene (*VHL*) in families from North America, Europe, and Japan. Hum. Mutat., *8:* 348–357, 1996.
3. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. W., Li, H., Latif, F., Liu, S., Chen, F., Duh, F. M., Lubensky, I., Duan, D. R., Florence, C., Pozzati, R., Walther, M. M., Bander, N. H., Grossman, H. B., Brauch, H., Pomer, S., Brooks, J. D., Isaacs, W. B., Lerman, M. I., Zbar, B., and Linehan, W. M. Mutations of the *VHL* tumour suppressor gene in renal carcinoma. Nat. Genet., *7:* 85–90, 1994.
4. Zbar, B., Brauch, H., Talmadge, C., and Linehan, M. Loss of alleles of loci on the short arm of chromosome 3 in renal cell carcinoma. Nature (Lond.), *327:* 721–724, 1987.
5. Foster, K., Prowse, A., van den Bergh, A., Fleming, S., Hulsbeek, M. M. F., Crossey, P. A., Richards, F. M., Cairns, P., Affara, N. A., Ferguson-Smith, M. A., Buys, C. H. C. M., and Maher, E. R. Somatic mutations of the von Hippel-Lindau disease tumor suppressor gene in non-familial clear cell renal carcinoma. Hum. Mol. Genet., *3:* 2169–2173, 1994.
6. Shuin, T., Kondo, K., Torigoe, S., Kishida, T., Kubota, Y., Hosaka, M., Nagashima, Y., Kitamura, H., Latif, F., Zbar, B., Lerman, M. I., and Yao, M. Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinoma. Cancer Res., *54:* 2852–2855, 1994.
7. Whaley, J. M., Naglich, J., Gelbert, L., Hsia, Y. E., Lamiell, J. M., Green, J. S., Collins, D., Neumann, H. P. H., Laidlaw, J., Li, F. P., Klein-Szanto, A. J. P., Seizinger, B., and Kley, N. Germline mutations in the von Hippel-Lindau tumor suppressor gene are similar to somatic von Hippel-Lindau aberrations in sporadic renal cell carcinoma. Am. J. Hum. Genet., *55:* 1092–1102, 1994.
8. Kenck, C., Wilhelm, M., Bugert, P., Staehler, G., and Kovacs, G. Mutations of the *VHL* gene is associated exclusively with the development of non-papillary renal cell carcinomas. J. Pathol., *179:* 157–161, 1996.
9. 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. Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. Proc. Natl. Acad. Sci. USA, *91:* 9700–9704, 1994.
10. Pause, A., Lee, S., Worrell, R., Chen, D. Y. T., Burgess, W. H., Linehan, W. M., and Klausner, R. D. The von Hippel-Lindau tumor suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc. Natl. Acad. Sci. USA, *94:* 2156–2161, 1997.
11. Gorospe, M., Egan, J. M., Zbar, B., Lerman, M. I., Geil, L., Kuzmin, I., and Holbrook, N. J. Protective function of the von Hippel-Lindau protein against impaired protein processing in renal carcinoma cells. Mol. Cell. Biol., *19:* 1289–1300, 1999.
12. Siemeister, G., Weindel, K., Mohrs, K., Barleon, B., Martiny-Baron, G., and Marme, D. Reversion of deregulated expression of vascular endothelial growth factor in human renal cell carcinoma cells by von Hippel-Lindau tumor suppressor protein. Cancer Res., *56:* 2299–2301, 1996.
13. Maxwell, P. H., Wiesener, M. S., Chang, G-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (Lond.), *399:* 271–275, 1999.
14. Ivanov, S. V., Kuzmin, I., Wei, M. H., Pack, S., Geil, L., Johnson, B. E., Stanbridge, E. J., and Lerman, M. I. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc. Natl. Acad. Sci. USA, *95:* 12596–12601, 1998.
15. Ohh, M., Yauch, R. L., Lonergan, K. M., Whaley, J. M., Stemmer-Rachmamimov, A. O., Louis, D. N., Gavin, B. J., Kaelin, W. G., Jr., and Iliopoulos, O. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol. Cell, *1:* 959–968, 1998.
16. Brauch, H., Weirich, G., Hornauer, M. A., Störkel, S., Wöhl, T., and Brüning, T. Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. J. Natl. Cancer Inst., *91:* 854–861, 1998.
17. Parkin, D. M., Muir, C. S., Whelan, S. L., Gao, Y-T., Ferlay, J., and Powell, J. Cancer Incidence in Five Continents. VI. IARC Scientific Publication No. 120. Lyon, France: IARC, 1992.
18. Thoenes, W., Störkel, S., and Rumpelt, H. J. Histopathology and classification of renal cell tumors (adenomas, renal oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. Path. Res. Pract., *181:* 125–143, 1986.
19. Störkel, 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 *80:* 987–989, 1997.
20. TNM Atlas Illustrated Guide to the TNM/pTNM Classification of Malignant Tumours. Int. Union Against Cancer–Union Internationale Contre le Cancer, Ed. 3. Berlin: Springer Verlag, 1992.
21. Glavac, D., Neumann, H. P. H., Wittke, C., Jähnig, H., Rödl, H., Masek, O., Streicher, T., Pausch, F., Engelhardt, D., Plate, K., Höfler, H., Chen, F., Zbar, B., and Brauch, H. Mutations in the *VHL* tumor suppressor gene and associated lesions with von Hippel-Lindau disease from Central Europe. Hum. Genet., *98:* 271–280, 1996.
22. Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J. R., Orcutt, M. L., Duh, F. M., Glenn, G., Green, J., Hsia, E., Lamiell, J., Li, H., Wei, M. H., Schmidt, L., Tory, K., Kuzmin, I., Stackhouse, T., Latif, F., Linehan, M. W., Lerman, M. I., and Zbar, B. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. Hum. Mutat., *5:* 66–75, 1995.
23. Stolle, C., Glenn, G., Zbar, B., Humphrey, J. S., Choyke, P., Walther, M., Pack, S., Hurley, K., Andrey, C., Klausner, R., and Linehan, W. M. Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. Hum. Mutat., *12:* 417–423, 1998.
24. Brauch, H., Kishida, T., Glavač, D., Chen, F., Pausch, F., Höfler, H., Lerman, M. I., Zbar, B., and Neumann, H. P. H. Von Hippel-Lindau (VHL) disease with pheochromocytoma in the Black Forest region in Germany: evidence for a founder effect. Hum. Genet., *95:* 551–556, 1995.
25. Brauch, H., Pomer, S., Hieronymus, T., Schadt, T., Löhrke, H., and Komitowski, D. Genetic alterations in sporadic renal-cell carcinoma: molecular analyses of tumor suppressor gene harboring chromosomal regions 3p, 5q, and 17p. World J. Urol., *12:* 162–168, 1994.
26. Stokes, M. E., Davis, C. S., and Koch, G. G. Categorical Data Analysis Using the SAS System, p. 499. Cary, NC: SAS Institute, Inc., 1995.
27. Becker, N., and Wahrendorf, J. Atlas of Cancer Mortality in the Federal Republic of Germany 1981–1990, Ed. 3. (Krebsatlas der Bundesrepublik Deutschland 1981–1990, Ed. 3.) Berlin: Springer Verlag, 1998.
28. McLaughlin, J. K., and Schuman, L. M. Epidemiology of renal carcinoma. *In:* A. M. Lilienfeld (ed.), Reviews in Cancer Epidemiology, pp. 170–210. New York: Elsevier, 1983.
29. Stebbins, C. E., Kaelin, W. G., Jr., and Pavletich, N. P. Structure of the VHL-ElonginC-ElonginB complex: implications for *VHL* tumor suppressor function. Science (Washington DC), *284:* 455–461, 1999.
30. Kibel, A. Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G., Jr. Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. Science (Washington DC), *269:* 1444–1446, 1995.
31. Ward, J. F. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. Prog. Nucleic Acid Res. Mol. Biol., *35:* 95–125, 1988.
32. Singer, B. *O*-Alkyl pyrimidines in mutagenesis and carcinogenesis: occurrence and significance. Cancer Res., *46:* 4879–4885, 1986.
33. Setlow, R. B. Cyclobutane-type pyrimidine dimers in polynucleotides. Science (Washington DC), *153:* 379–386, 1966.
34. Pegg, A. E. Methylation of the O^6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. Cancer Investig., *2:* 223–231, 1984.
35. van den Berg, A., and Buys, C. H. Involvement of multiple loci on chromosome 3 in renal cell cancer development. Genes Chromosomes Cancer, *19:* 59–79, 1997.
36. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. F., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J. M., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Lipan, J., Slife, L., Brauch, H., Decker, J., Niehans, G., Hughson, M. D., Moch, H., Störkel, S., Lerman, M. I., Linehan, W. M., and Zbar, B. Germline and somatic mutations in the tyrosine kinase domain of the *MET* proto-oncogene in papillary renal carcinomas. Nat. Genet., *16:* 68–73, 1997.
37. Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H. P. H., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. Novel mutations in the *MET* proto-oncogene in papillary renal carcinomas. Oncogene, *18:* 2343–2350, 1999.
38. Lubensky, I. A., Schmidt, L., Zhuang, Z., Weirich, G., Pack, S., Zambrano, N., Walther, M. M., Choyke, P., Linehan, W. M., and Zbar, B. Hereditary and sporadic papillary renal carcinomas with c-met mutations share a distinct morphological phenotype. Am. J. Pathol., *155:* 517–526, 1999.
39. Neumann, H. P. H., Bender, B. U., Berger, D. P., Laubenberger, J., Schultze-Seemann, W., Wetterauer, U., Ferstl, F. J., Herbst, E. W., Schwarzkopf, G., Hes, F. J., Lips, C. J. M., Lamiell, J. M., Masek, O., Riegler, P., Mueller, B., Glavač, D., and Brauch, H. Prevalence, morphology and biology of renal cell carcinoma in von Hippel-Lindau disease compared to sporadic renal cell carcinoma. J. Urol., *160:* 1248–1254, 1998.
40. Thoenes, W., Störkel, S., Rumpelt, H. J., Moll, R., Baum, H. P., and Werner, S. Chromophobe cell renal carcinomas and its variants–a report on 32 cases. J. Pathol., *155:* 277–287, 1988.
41. Crotty, T. B., Farrow, G. M., and Lieber, M. M. Chromophobe cell renal carcinoma: clinicopathological features of 50 cases. J. Urol., *154:* 964–967, 1996.