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Somatic Inactivation of the VHL Gene in Von Hippel–Lindau Disease Tumors

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Summary

Von Hippel–Lindau (VHL) disease is a dominantly inherited disorder predisposing to retinal and CNS hemangioblastomas, renal cell carcinoma (RCC), pheochromocytoma, and pancreatic tumors. Interfamilial differences in predisposition to pheochromocytoma reflect allelic heterogeneity such that there is a strong association between missense mutations and risk of pheochromocytoma. We investigated the mechanism of tumorigenesis in VHL disease tumors to determine whether there were differences between tumor types or classes of germ-line mutations. Fifty-three tumors (30 RCCs, 15 hemangioblastomas, 5 pheochromocytomas, and 3 pancreatic tumors) from 33 patients (27 kindreds) with VHL disease were analyzed. Overall, 51% of 45 informative tumors showed loss of heterozygosity (LOH) at the VHL locus. In 11 cases it was possible to distinguish between loss of the wild-type and mutant alleles, and in each case the wild-type allele was lost. LOH was detected in all tumor types and occurred in the presence of both germ-line missense mutations and other types of germ-line mutation associated with a low risk of pheochromocytoma. Intragenic somatic mutations were detected in three tumors (all hemangioblastomas) and in two of these could be shown to occur in the wild-type allele. This provides the first example of homozygous inactivation of the VHL by small intragenic mutations in this type of tumor. Hypermethylation of the VHL gene was detected in 33% (6/18) of tumors without LOH, including 2 RCCs and 4 hemangioblastomas. Although hypermethylation of the VHL gene has been reported previously in nonfamilial RCC and although methylation of tumor-suppressor genes has been implicated in the pathogenesis of other sporadic cancers, this is the first report of somatic methylation in a familial cancer syndrome.

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Introduction

Familial cancer syndromes account for only a small proportion of all human cancers, but, as exemplified by retinoblastoma, molecular-genetic analysis of inherited cancer susceptibility can provide important insights into the mechanism of tumorigenesis in both familial and sporadic cancers. Many familial cancer syndromes are caused by inactivating mutations in tumor-suppressor genes, and they exhibit a tumorigenesis mechanism similar to that in retinoblastoma, such that inactivation of both alleles is an early event in tumor development. The mutational events in both inherited and sporadic tumors are similar, with the "first hit" (germ-line or somatic mutation) being typically a localized intragenic mutation (e.g., point mutation, microdeletion, etc.) and with the "second hit" frequently being a large deletion or mitotic recombination event resulting in loss of heterozygosity (LOH) at polymorphic markers within or close to the tumor-suppressor gene (Marshall 1991). In addition, hypermethylation has been reported as a cause of tumor-suppressor-gene inactivation (including RB1) in sporadic retinoblastoma and in brain, lung, and kidney cancers (Laird and Jaenisch 1994). However, it is not yet known whether similar mechanisms can operate in inherited cancer.

Von Hippel–Lindau (VHL) disease is an autosomal dominantly inherited familial cancer syndrome predisposing to a variety of benign and malignant neoplasms (Maher et al. 1990). The most common of these are retinal and CNS hemangioblastomas, renal cell carcinoma (RCC), pheochromocytoma, and pancreatic tumors. The VHL disease gene, which maps to chromosome 3p25, was isolated by a positional cloning approach, and the VHL protein is thought to down-regulate transcriptional elongation by binding to components of the elongin complex (Latif et al. 1993; Aso et al. 1995; Kibel et al. 1995). The demonstration of LOH at chromosome 3p25 markers in tumors from VHL patients, as well as the detection of somatic VHL gene mutations in sporadic RCCs and hemangioblastomas, are similar to what has been seen in inherited and noninherited retinoblastoma (Crossey et al. 1994; Foster et al. 1994; Gnarra et al. 1994; Kanno et

al. 1994; Shuin et al. 1994). However, the mechanism of tumorigenesis in VHL disease may be more complex than that in inherited retinoblastoma. Large germ-line deletions, frameshifts, and nonsense mutations in the VHL gene are associated with susceptibility to retinal and CNS hemangioblastomas and RCC but with a low risk of pheochromocytoma (Crossey et al. 1994*b*; Chen et al. 1995). Missense mutations may produce a similar phenotype, but, in addition, in some cases there is also a high risk of pheochromocytoma, and specific missense mutations have been reported to cause susceptibility to either hemangioblastoma and pheochromocytoma with a low risk of RCC or familial pheochromocytoma only (Brauch et al. 1995; Crossey et al. 1995; Neumann et al. 1995). These complex genotype-phenotype relationships are not easily explicable by simple loss-of-function mutations and suggest that VHL protein functions may be complex and tissue specific (Maher et al. 1996). Furthermore, although somatic VHL gene mutations are frequent in sporadic hemangioblastomas and RCCs, they are uncommon in sporadic pheochromocytomas (Eng et al. 1995). Since we published our previous study of LOH in VHL tumors (Crossey et al. 1994*a*), the identification of the VHL gene has provided the opportunity to further investigate the mechanism of tumorigenesis in VHL disease by (i) using intragenic polymorphisms to detect small VHL deletions, (ii) screening for the presence of somatic VHL point mutations, and (iii) investigating whether a second hit is required in patients with missense germ-line mutations. In addition, Herman et al. (1994) have reported VHL gene methylation in 19% of sporadic RCC cell lines, so we investigated whether de novo VHL gene methylation also occurs in VHL tumors.

Patients, Material, and Methods

Patients and Tumors

A diagnosis of VHL disease was made on the basis of conventional criteria (Maher et al. 1990). Fifty-three tumors (30 RCCs, 15 cerebellar hemangioblastomas, 5 pheochromocytomas, and 3 pancreatic tumors) from 33 patients from 27 kindreds were analyzed. The underlying VHL germ-line mutation had been determined in 26 kindreds while in one patient (patient 47/1) it has remained undetermined (see table 1). All tumor samples were snap frozen in liquid nitrogen and were stored at −70°C until analyzed. The results of LOH analysis in 24 tumors by use of chromosomes 3p, 5q, 11p, 13q, 17p, 17q, and 22q markers have been reported elsewhere (Crossey et al. 1994*a*).

Molecular-Genetic Analysis

High-molecular-weight DNA was isolated from peripheral blood and frozen tissue samples by conventional methods as described elsewhere. Tumors were analyzed for LOH at the VHL locus by different methods, according to the nature of the germ-line VHL mutation: (i) tumors from kindreds with large germ-line deletions (kindreds 1, 2, 13, 15, and 132) were informative by Southern analysis; (ii) for deletions or insertions of 3–9 bp (kindreds 35, 46 and 48), primers flanking the mutation were designed and the wild-type and mutant VHL alleles were separated; (iii) when the intragenic mutation created or obliterated a restriction-endonuclease site, tumor DNA was amplified with the appropriate primers and was digested with the informative restriction enzyme (*Msp*I, *Hph*I, *Sst*II, and *Mae*I), according to the manufacturer's guidelines; and (iv) other tumors were analyzed by use of the diallelic nucleotide polymorphisms within the 5′ and 3′ regions of the VHL gene (Payne et al. 1994; Sekido et al. 1994). Alleles were separated on an 8% acrylamide gel containing 5% glycerol and were visualized by silver staining. Tumors with no evidence of LOH at the VHL locus were screened for the presence of somatic intragenic mutations and VHL-gene hypermethylation. Intragenic VHL mutations were sought by SSCP analysis as described elsewhere (Crossey et al. 1994*b*). To increase the clarity of sequencing, the abnormal band was excised from the acrylamide gel and was incubated overnight at 37°C with 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. The DNA was then reextracted from the supernatant, and the PCR was repeated. The amplified exon was then sequenced by use of dye-terminator chemistry (ABI AmpliTaq DNA polymerase, FS™) on an ABI 373™ automated sequencer, according to the manufacturer's guidelines. For methylation analysis, we developed a PCR-based technique to detect hypermethylation of the *Not*I site within exon 1 (Herman et al. 1994). Two hundred nanograms of DNA was digested with 5 U of *Not*I (Boehringer Mannheim), and 100 ng of digested product was used in a 30-μl multiplex PCR reaction. One set of primers (F-CGGAGGGCGGAGAACTGG and R-GAGGGCTCGCGCGAGTTC) flanked the exon 1 *Not*I site, and the second set (F-CACCGGTGTGGCTCTTTAACAA and R-ACATCAGGCAAAAATTGAGAACTGG) amplified exon 2 as a control for failure of the PCR reaction. Fragments were PCR amplified in a Perkin Elmer Cetus DNA thermal cycler for 30 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C. The 30-μl reaction volumes contained 1 mM Mg, 0.2 mM dNTPs, 0.5 U of *Taq* polymerase (New Brunswick Scientific), and 1 μM primers. In the presence of VHL methylation a 208-bp fragment from exon 1 was visualized, in addition to the 262-bp fragment from exon 2. To assess whether *Not*I would cut within the myc gene in samples in which the VHL gene was considered to be hypermethylated, 200 ng of DNA was digested with 5 U of *Not*I (Boehringer Mannheim). In addition, a separate reaction, containing

Table 1

LOH and Intragenic Mutations of 53 Tumors from 33 Patients of 27 Different Kindreds

Kindred/ Patient	Mutation Exon(s) or Nucleotide(s)	Codon	Type[a]	Tumor[b]	Somatic Mutation[c]	LOH[d]
1/1	Deletion of exon 3		del	RCC1	ND	No
1/1	Deletion of exon 3		del	RCC2	ND	No
2/1	Deletion of exons 1 and 2		del	PT	NT	Yes
2/1	Deletion of exons 1 and 2		del	HB	ND	No
13/1	Deletion of exon 3		del	RCC	NT	Yes
15/1	Deletion of exon 1		del	HB	ND	No
15/2	Deletion of exon 1		del	HB	NT	Yes
15/3	Deletion of exon 1		del	RCC	NT	Yes
21/1	796C→T	Gln195Ter	ns	RCC	NT	NI
21/2	796C→T	Gln195Ter	ns	RCC	NT	NI
26/1	721G→T	Val170Phe	ms	HB	Deletion of 13 bp, nt 789	No
35/1	456del, 9 bp	82–84 del Arg-Val-Val	ms	RCC	NT	Yes
38/1	430C→T	Gln73Ter	ns	RCC	ND	No
43/1	409delG	Val66Ter	ns	RCC	ND	No
43/1	409delG	Val66Ter	ns	PT	ND	No
46/1	427insGCCC	72 Frameshift	fs	HB	ND	No
47/1	Unknown			HB	Insertion of A, nt 737	NI
48/1	440delTCT	Deletion of Phe 76	ms	RCC	NT	Yes
49/1	674C→T	Pro154Leu	ms	RCC	NT	Yes
49/2	674C→T	Pro154Leu	ms	RCC	NT	Yes
54/1	741delG	176 Frameshift	fs	HB	NT	Yes
54/2	741delG	176 Frameshift	fs	HB	NT	Yes
56/1	388delC	59 Frameshift	fs	RCC	NT	Yes
56/1	388delC	59 Frameshift	fs	Pheo	ND	No
58/1	Deletion of exons 1–3		del	HB	Deletion of C, nt 752	NI
86/1	441insC	76 Frameshift	fs	RCC1	ND	No
86/1	441insC	76 Frameshift	fs	RCC2	ND	No
95/1	712C→T	Arg167Trp	ms	Pheo	NT	Yes
96/1	694C→T	Arg161Ter	ns	RCC	NT	Yes
132/1	Deletion of exon 1		del	RCC1	ND	No
132/1	Deletion of exon 1		del	RCC2	ND	No
132/1	Deletion of exon 1		del	RCC3	ND	No
132/1	Deletion of exon 1		del	RCC4	ND	No
132/1	Deletion of exon 1		del	RCC5	ND	No
132/1	Deletion of exon 1		del	RCC6	ND	No
143/1	677-1G→T	Splice	sp	RCC1	NT	Yes
143/1	677-1G→T	Splice	sp	RCC2	NT	Yes
143/1	677-1G→T	Splice	sp	RCC3	NT	Yes
143/1	677-1G→T	Splice	sp	RCC4	NT	Yes
143/1	677-1G→T	Splice	sp	RCC5	NT	Yes
143/1	677-1G→T	Splice	sp	RCC6	NT	Yes
143/1	677-1G→T	Splice	sp	RCC7	NT	Yes
150/1	449G→C	Arg79Pro	ms	HB	ND	No
152/1	713G→A	Arg167Gln	ms	PT	NT	Yes
P1/1	712C→T	Arg167Trp	ms	Pheo	ND	No
P1/1	712C→T	Arg167Trp	ms	HB	ND	No
P1/2	712C→T	Arg167Trp	ms	HB1	ND	No
P1/2	712C→T	Arg167Trp	ms	HB2	ND	No
P30/1	712C→T	Arg167Trp	ms	Pheo	ND	No
P51/1	del C	191 Frameshift	fs	RCC	NT	Yes
P51/1	del C	191 Frameshift	fs	Pheo	NT	Yes
P3/1	694C→T	Arg161Ter	ns	HB1	NT	NI
P3/1	694C→T	Arg161Ter	ns	HB2	NT	NI

[a] del = large deletion; ns = nonsense; ms = missense; fs = frameshift; and sp = splice site.
[b] PT = pancreatic tumor; and Pheo = pheochromocytoma;
[c] ND = not detected; NT = not tested.
[d] NI = not informative.

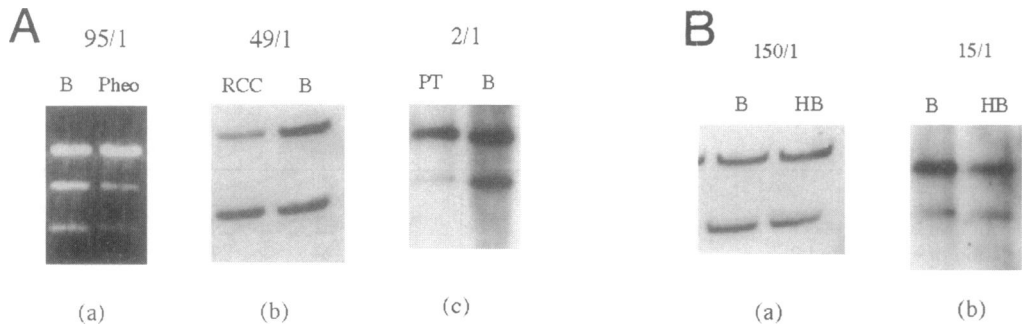

Figure 1 *A*, VHL tumors with LOH. Tumors were analyzed for LOH by different methods, according to the germ-line mutation: (*a*), patient 95/1, analyzed by *Msp*I digestion; (*b*), patient 49/1, analyzed by VHL nucleotide 1149 polymorphism; and (*c*), patient 2/1, analyzed by Southern analysis. B = blood; pheo = pheochromocytoma; and PT = pancreatic tumor. *B*, VHL tumors without LOH. Tumors were analyzed for LOH by different methods, according to the germ-line mutation: (*a*), patient 150/1, analyzed by VHL nucleotide 1149 *Acc*I polymorphism; and (*b*), patient 15/1, analyzed by Southern blotting. B = blood; and HB = hemangioblastoma.

restriction-enzyme buffer but without enzyme, was set up, and this was used as a control for failure of PCR. One hundred nanograms of DNA was used in a PCR amplification using primers that flanked a *Not*I site within the myc gene. Fragments of 398 bp were PCR amplified in a Perkin Elmer Cetus DNA cycler for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The 20-μl reactions contained 0.5 mM Mg, 0.2 mM dNTPs, 0.5 μM primers, and 0.5 U of *Taq* polymerase.

Results

Forty-seven of the 53 tumors investigated were informative for LOH analysis at the VHL locus, and LOH was detected in 23 (49%) of the tumors (see table 1). In 11 of the 23 tumors with LOH it was possible to distinguish between the germ-line mutant and the wild-type allele, and in all 11 cases the wild-type allele was lost (see fig. 1).

The frequency of LOH was assessed according to (i) tumor type and (ii) class of germ-line mutation. LOH was detected in all four tumor types analyzed: RCC, 57% (16/28); hemangioblastomas, 27% (3/11); pheochromocytomas, 40% (2/5); and pancreatic tumors, 66% (2/3). Second, 7/14 (50%) tumors from patients with nonsense and frameshift mutations and 4/15 (27%) tumors from patients with germ-line deletions had LOH, but this did not differ significantly from the frequency of LOH in tumors with missense mutations (8/15 [53%]). This included 2/8 tumors from patients with germ-line codon 167 mutations, which are associated with a high risk of pheochromocytoma (Maher et al. 1996). A single patient (patient 143/1) with a splice-site germ-line mutation demonstrated VHL LOH at all six primary RCCs. Another patient (patient 132/1), who had an exon 1 germ-line deletion, showed no evidence of LOH at six primary RCCs.

Twenty-two tumors from 13 patients without LOH were investigated to determine whether the second hit in these tumors was (i) an intragenic point mutation or microdeletion/insertion or (ii) due to hypermethylation. Three (14%) of the 22 tumors studied by SSCP analysis of the whole coding region showed an abnormal band shift not evident in the corresponding blood DNA (fig. 2). All three tumors were hemangioblastomas, and all three band shifts occurred in exon 3. In two tumors, the mutation could be shown to be on the wild-type allele, since in one patient (patient 58/1) germ-line mutation was known to be due to a deletion of the whole VHL

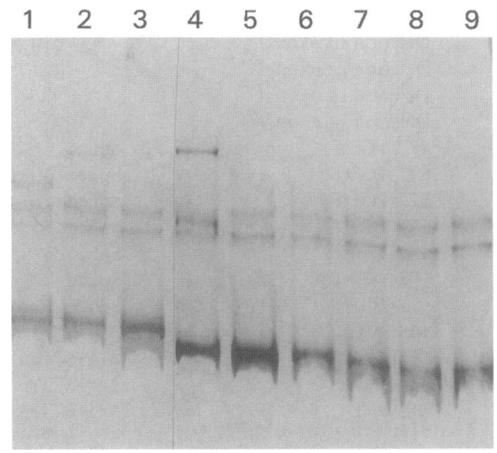

Figure 2 SSCP analysis of tumor DNA. Lanes 5–9 show normal single-strand conformation bands and a double-stranded DNA band for the VHL exon 3. Lanes 1 and 3 show tumor DNA and blood DNA, respectively, from patient 58/1, showing both an SSCP band shift and a heteroduplex dsDNA band shift, in lane 1, corresponding to a somatic point mutation (fig. 3). Lanes 2 and 4 show blood and tumor DNA, respectively, from patient 26/1, showing both an SSCP band shift in lanes 2 and 4, corresponding to the germ-line mutation, and a further band-shift in lane 4, corresponding to a somatic point mutation (deletion of 13 nucleotides).

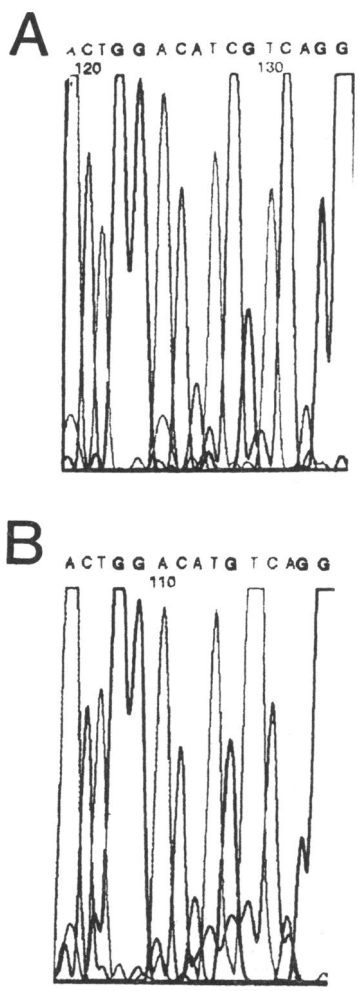

Figure 3 Automated sequencing analysis of the sense strand of exon 3 in patient 58/1. *A*, Blood DNA showing normal sequence (–ACTGGACATCGTCAGG–). *B*, DNA derived from the excised SSCP abnormal band (fig. 2, lane 1), showing deletion of 1 C nucleotide (–ACTGGACAT(delC)GTCAGG–), corresponding to a somatic VHL mutation.

region and in another patient (patient 26/1) the germline mutation was known to be an exon 3 missense mutation causing a specific band shift separate from that caused by the somatic mutation in the same exon (fig. 2). The mutations were all small deletions causing a frameshift (table 1 and fig. 3). Six of 18 tumors with no LOH had methylation of VHL exon 1, demonstrating amplification of both exon 1 fragments and exon 2 fragments (see fig. 4). No hypermethylation was detected in the corresponding blood DNA or in DNA extracted from normal kidney tissue, both of which consistently amplified only the 262-bp product from exon 2. Also, blood DNA from a patient with a characterized germline mutation involving a nucleotide substitution at the *Not*I recognition site consistently amplified both 262-bp and 208-bp fragments, from exons 2 and 1, respectively (fig. 4). One hypermethylated hemangioblastoma was from a patient with a germ-line deletion of exon 1, demonstrating that the hypermethylation must involve the wild-type allele. To confirm that the failure of *Not*I to digest tumor DNA from these six tumors was caused by hypermethylation and not by a mutation within the *Not*I recognition site, the exon 1 fragment was sequenced in each case; no abnormality was detected. In addition, all tumor DNA with a hypermethylated VHL allele was shown to cut normally at a *Not*I site in the myc proto-oncogene (see Patients, Material, and Methods).

Discussion

We found evidence of somatic inactivation of the wild-type VHL allele in most VHL tumors, which was consistent with a "two hit" model of tumorigenesis. The most frequent mechanisms of somatic inactivation were loss or hypermethylation of the wild type-allele (although some point mutations might not be detected by SSCP). Chromosome 3p allele loss is frequent in both VHL and related sporadic tumors, such as RCC, and also is frequent in unrelated tumors, including breast, lung, and gonadal tumors (Seizinger et al. 1991). There is no evidence that VHL mutations are involved in the pathogenesis of these non–VHL-related sporadic tumors, but, rather, there is evidence that the target tumor-suppressor genes in these tumors are contained within chromosome 3p12-p21 (Sekido et al. 1994; Foster et al. 1995). Since chromosome 3p allele loss in sporadic RCC frequently extends into these regions, it had been suggested that loss of chromosome 3p tumor-suppressor genes, in addition to VHL mutation, is required for tumorigenesis. However, in some VHL tumors LOH may be limited to 3p25 and may not involve large regions of 3p (Crossey et al. 1994*a*; Decker et al. 1994). Analysis of genotype-phenotype relationships in VHL patients

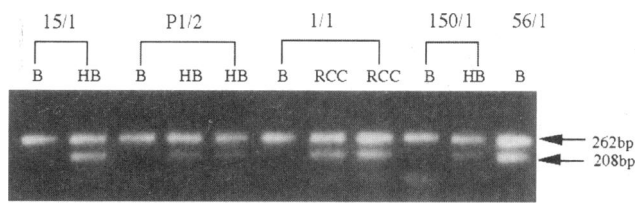

Figure 4 Analysis of VHL hypermethylation, by use of multiplex PCR after *Not*I digestion. Products at 208 bp are from primers that flank the *Not*I site and will only amplify if cytosines in the *Not*I recognition site are methylated (exon 1). Products at 262 bp (exon 2) show that the PCR reaction was successful. B = blood DNA; and HB = hemangioblastoma. There are six separate tumors from four patients (patients 15/1 [HB], P1/2 [two HB1 and HB2], 1/1 [RCC1 and RCC2], and 150/1 [HB]). Blood DNA from patient 56/1, whose germline mutation abolishes the *Not*I site, was used as a positive control. Primer-dimers are visible under the 208-bp band.

has suggested a complex model of inherited tumor predisposition such that large germ-line deletions and protein-truncating mutations are associated with a low risk of pheochromocytoma, but specific missense mutations can cause (i) full VHL phenotype with retinal and CNS hemangioblastoma, RCC, and pheochromocytoma; (ii) incomplete phenotype with pheochromocytoma and hemangioblastomas but without RCC; and (iii) pheochromocytoma only (Crossey et al. 1994*b*; Brauch et al. 1995, Chen et al. 1995, Crossey et al. 1995, Neumann et al. 1995, Maher et al. 1996). These findings might suggest that the VHL gene product has different tissue-specific functions (Maher et al. 1996); and it is of interest that the "Black Forest" mutation (Tyr96His), which predisposes to pheochromocytoma and hemangioblastomas but not to RCC, does not appear to interfere with binding of the VHL protein to the elongin subunits (Duan et al. 1995). Therefore, the mechanism of tumorigenesis might be different in the presence of a missense germ-line mutation or in the various tumor types. However, we found no evidence for either of these hypotheses, and inactivation of the wild-type VHL allele occurred in all VHL tumor types and with all types of germ-line mutations. Somatic intragenic mutations were detected in 3 of 22 VHL tumors without LOH, and all of these were cerebellar hemangioblastomas. The mutation-detection techniques employed would not detect all intragenic mutations and would not detect intronic mutations or deletions of single exons. Although somatic mutations have been detected in sporadic hemangioblastoma (Kanno et al. 1994), this is the first demonstration of two hits by homozygous inactivation of both VHL alleles by small intragenic mutations. Together with our hypermethylation findings, this suggests that inactivation of both VHL alleles is necessary for the development of hemangioblastomas, and it argues against other mechanisms of tumorigenesis, such as heterozygous inactivation causing a reduced gene dosage.

Hypermethylation of tumor-suppressor genes is being implicated increasingly in the pathogenesis of human cancers (Laird and Jaenisch 1994; Merlo et al. 1995). Herman et al. (1994) reported VHL gene methylation in 19% (5/26) of sporadic RCC cell lines (4 of which had no detectable VHL gene mutation) but not in normal renal tissue. In addition, they found that hypermethylation of a VHL allele in sporadic kidney-cancer cell lines was associated with absent mRNA expression but that demethylation led to reexpression of VHL mRNA. Our findings further support the concept that hypermethylation of VHL is a primary event in renal carcinogenesis, and it also represents both the first report of somatic hypermethylation in an inherited cancer and the first case of hypermethylation in hemangioblastoma. The similarities between the mechanisms of somatic inactivation of the VHL gene in VHL and in sporadic RCC suggests that models of VHL disease might provide a suitable system to investigate novel approaches to the treatment of clear-cell kidney cancer.

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