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Germ-Line Mutations in the von Hippel–Lindau Tumor-Suppressor Gene Are Similar to Somatic von Hippel–Lindau Aberrations in Sporadic Renal Cell Carcinoma

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Summary

von Hippel–Lindau (VHL) disease is a hereditary tumor syndrome predisposing to multifocal bilateral renal cell carcinomas (RCCs), pheochromocytomas, and pancreatic tumors, as well as angiomas and hemangioblastomas of the CNS. A candidate gene for VHL was recently identified, which led to the isolation of a partial cDNA clone with extended open reading frame, without significant homology to known genes or obvious functional motifs, except for an acidic pentamer repeat domain. To further characterize the functional domains of the VHL gene and assess its involvement in hereditary and nonhereditary tumors, we performed mutation analyses and studied its expression in normal and tumor tissue. We identified germline mutations in 39% of VHL disease families. Moreover, 33% of sporadic RCCs and all (6/6) sporadic RCC cell lines analyzed showed mutations within the VHL gene. Both germ-line and somatic mutations included deletions, insertions, splice-site mutations, and missense and nonsense mutations, all of which clustered at the 3′ end of the corresponding partial VHL cDNA open reading frame, including an alternatively spliced exon 123 nt in length, suggesting functionally important domains encoded by the VHL gene in this region. Over 180 sporadic tumors of other types have shown no detectable base changes within the presumed coding sequence of the VHL gene to date. We conclude that the gene causing VHL has an important and specific role in the etiology of sporadic RCCs, acts as a recessive tumor-suppressor gene, and appears to encode important functional domains within the 3′ end of the known open reading frame.

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Introduction

von Hippel–Lindau disease (VHL) is a hereditary tumor predisposition syndrome associated with bilateral and multifocal renal cell carcinomas (RCCs), pheochromocytomas, neoplasms of the CNS, and islet cell tumors of the pancreas (Binkovitz et al. 1990), as well as cysts of the kidney, pancreas, epididymis, and various other organs (Melmon et al. 1964; Huson et al. 1986). Retinal angiomas and hemangioblastomas of the cerebellum and spinal cord are the common CNS manifestations (Huson et al. 1986). RCCs are the most common cause of death in VHL patients. VHL is a relatively rare syndrome with an incidence of 1/36,000–39,000 (Maher et al. 1991; Neumann and Weistler 1991) and follows an autosomal dominant pattern of inheritance with high penetrance but variable expressivity (Lamiell et al. 1989; Maher et al. 1990*b*; Neumann and Wiestler 1991). An individual with the VHL phenotype appears to be susceptible to any of its lesions in any combination and sequence of appearance, with a highly variable age at onset, usually between 15 and 50 years (Lamiell et al. 1989). The genetic basis of VHL is thought to be a germ-line mutation in a tumor-suppressor gene (Seizinger et al. 1988; Tory et al. 1989; Maher et al. 1990*a*). Genetic linkage analysis first placed the gene on the short arm of chromosome 3 (Seizinger et al. 1988), and subsequent genetic and physical mapping refined the locus to a small chromosomal region within 3p25-p26, flanked by the markers c-raf and D3S719 (Seizinger et al. 1991) and c-raf and D3S18 (Hosoe et al. 1990). Finally, physical cloning approaches led to the identification of a VHL candidate gene and the isolation of a partial cDNA with extended open reading frame (Latif et al. 1993; Richards et al. 1993*b*). Nucleotide and amino acid sequence analysis and database comparison revealed no significant homology to any other known gene and no obvious functional motifs.

Although RCCs are associated with VHL disease, they also do occur frequently as sporadic tumors in the general

population. Studies of chromosomal abnormalities and allele loss in sporadic RCC (Yoshida et al. 1986; Zbar et al. 1987; Kovacs et al. 1988; Bergerheim et al. 1989; Yano et al. 1989; Morita et al. 1991; Presti et al. 1991; Yamakawa et al. 1991; Foster et al. 1994) have implicated three distinct regions on chromosome 3p in the etiology of this tumor type: 3p13-p14.3, 3p21.3, and 3p25-p26, the region where the VHL locus resides. Constitutional chromosomal translocations involving the region 3p13-p21 have been found in families with pure familial RCC (Cohen et al. 1979; Kovacs et al. 1989), strongly implicating this region as one that contains a tumor-suppressor gene involved in the development of RCC. Loss of the chromosomal region 3p25-p26 is usually coincident with loss of one or more of these other chromosome 3p regions in sporadic RCC (Bergerheim et al. 1989; Yamakawa et al. 1991; Foster et al. 1994), and thus the role of the VHL locus in the development of sporadic RCC has been unclear. To assess the relative importance of the VHL gene in the etiology of sporadic RCC, to further characterize this gene with regard to its mutational status in VHL and its association with human cancers in general, and to identify important functional domains, we have screened 61 VHL pedigrees and >200 sporadic tumors for the presence of mutations in this gene, by PCR/SSCP analysis and direct sequence analysis of variant conformers. In addition, we have studied the expression of this gene both in normal tissue and in tumor cell lines by northern blot, reverse transcription (RT), and PCR analyses.

Our results show that the VHL gene is expressed as alternatively spliced transcripts in a variety of normal tissues and is mutated not only in the hereditary tumors, but also in a large proportion of sporadic RCCs, the most common malignancy of the adult kidney. Concurrent allele loss in these tumors confirms that the VHL gene acts as a recessive tumor-suppressor gene, consistent with a two-hit mechanism of tumorigenesis (Knudson 1971). The types and location of mutations that occur somatically are quite similar to those seen in the germ line of VHL patients, and all mutations cluster to one region of the cloned partial cDNA, implicating this region as important for the tumor-suppressor function of the VHL protein.

Material and Methods

Human Tissue Samples, Cell Lines, and Nucleic Acid Extraction

Blood and tissue samples from 61 families affected with VHL were collected with the assistance of many genetic counselors and physicians (see Acknowledgments). Affected individuals displayed typical VHL lesions upon clinical examination, according to the diagnostic criteria of Melmon and Rosen (1964). Blood and tissue samples were processed for DNA isolation using standard methods (Sambrook et al. 1989). Lymphoblastoid cell lines were generated from blood samples using established protocols (Anderson et al. 1984), and DNA was extracted from these lines as described elsewhere (Sambrook et al. 1989). RNA was extracted from normal and tumor tissues according to the method of Chomczynski and Sacchi (1987). In some cases total RNA and northern blots were obtained commercially (Clontech). Sporadic RCC cell lines (A-498, A-704, Caki-1, Caki-2, ACHN, 786-O, 769-P, and HS 758T) were obtained from, and maintained as recommended by, the American Type Culture Collection. In some cases tumor material was obtained in paraffin section. DNA was obtained from these sections by extracting samples twice in octane, washing in ethanol, drying, and boiling in a 5% dispersion of Chelex-100 resin (BioRad). Samples were then phenol/chloroform extracted, precipitated, resuspended in 10 mM Tris, 1 mM EDTA (TE), and used in PCR as described below.

RT-PCR

RT was performed using total cellular RNA (1 μg) obtained from human tissues (kidney, adrenal gland, brain, retina, bladder, lung, cerebellum, and lymphoblastoid cell lines). cDNA was generated utilizing Superscript (Gibco/BRL) reverse transcriptase and VHL gene-specific primers, based on the available 1.8-kb partial cDNA sequence (Genbank L15409). The first nucleotide in the reported Genbank sequence is referred to as *nt 1,* and the predicted 284-amino-acid protein starts at nt 1 of the reported sequence. A schematic diagram of the VHL partial cDNA showing the location of the primers used for RT-PCR is shown in figure 1*a*. The region of the VHL cDNA transcribed and amplified included the entire open reading frame (855 nt) and 99 nt of the 3′ untranslated sequence. Following first-strand cDNA synthesis, two 30-cycle rounds of PCR were performed using nested primers. PCR was performed in a total volume of 50 μl containing 1–3 μl first-strand cDNA, 20 pmol of each primer, 100 μM deoxynucleotide triphosphates, 0.5–1.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 50 mM KCl, 0.1% gelatin, and 0.5 U *Taq* polymerase (Perkin Elmer Cetus). Initial denaturation took place at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The primer sequences used are as follows: for initial priming of RNA, primer 7 (5′-actttgaaactaaggaaggaaccag-3′) was used. For first-round PCR, primers 1 (5′-cctcgcctccgttacaacagc-3′) and 11 (5′-gccatacgggcagacgacgcgcgg-3′), and primers 6 (5′-acggcggggaggagtcgg-3′) and 2 (5′-gatcaagactcatcagtacc-3′) were used. Secondary rounds of PCR were performed with nested primers to 1/11; these are primers 1E (5′-cctcgcctccgttacaacagcctac-3′) and 6R (5′-cccgactcctccccgccgtcttctt-3′). Secondary rounds of PCR were also performed using primers 15 (5′gaactcgcgcgagccctcccaggt-3′) and 3 (5′-ggctccggacaacctggaggcatcg-3′); and using primers 4 (5′-gccatctctcaatgttgacggac-3′) and 2 (see above). Secondary rounds of PCR were also performed using primers 6

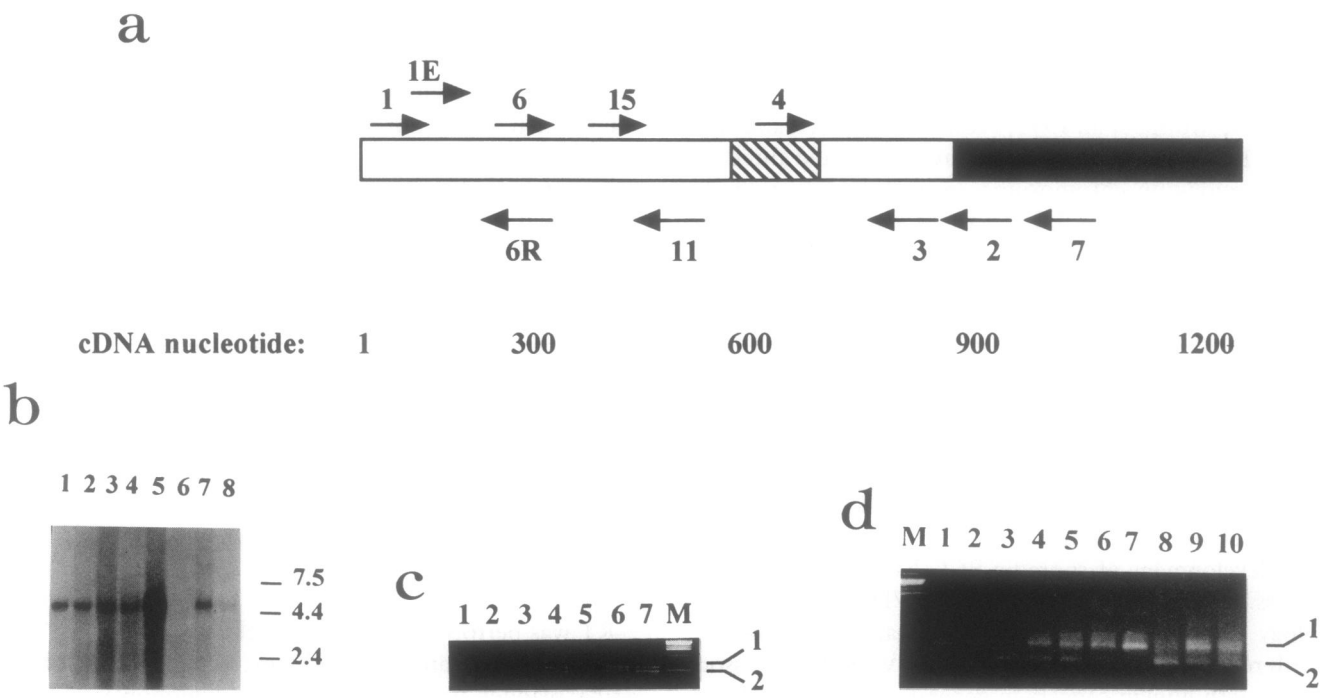


Figure 1 Expression of the VHL gene in normal tissues and in RCC cell lines. *a*, Schematic drawing of the first 1,200 nt of the published partial VHL cDNA and the location of the primers used in RT-PCR. Nt 1–855 represent the open reading frame of the published sequence. The blackened region indicates the 3′ untranslated region, and the hatched region indicates the region corresponding to the alternatively spliced cassette exon 2. *b*, Human multitissue northern blot (2 μg poly-A RNA/lane) hybridized with a RT-PCR product spanning nt 624–954 of the published human VHL cDNA sequence (see Material and Methods). Size markers in kilobases are indicated on the right of the panel. The origins of the tissues are as follows: lane 1, pancreas; lane 2, kidney; lane 3, skeletal muscle; lane 4, liver; lane 5, lung; lane 6, placenta; lane 7, brain; lane 8, heart. *c*, RT-PCR analysis of human VHL mRNA derived from normal tissues. Total RNA was primed with primer 7 and PCR-amplified with primers 6 and 2 in two 30-cycle rounds of amplification. The origins of the tissues are as follows: lane 1, kidney; lane 2, adrenal gland; lane 3, retina; lane 4, pancreas; lane 5, bladder; lane 6, lung; lane 7, cerebellum; lane M, marker lane (1-kb ladder; Gibco/BRL). Band 1 = 622 bp; band 2 = 499 bp. *d*, RT-PCR analysis of VHL mRNA derived from eight sporadic RCC cell lines and normal kidney. RNA was primed and amplified as in *b*, except that the second round of PCR amplification was done with primers 15 and 3. The cell lines are as follows: lane 1, A-498; lane 2, A-704; lane 3, Caki-1; lane 4, Caki-2; lane 5, ACHN; lane 6, 786-O; lane 7, 769-P; lane 8, HS 758T; lanes 9 and 10, normal kidney; lane M, marker lane, as in *b*. Band 1 = 307 bp; band 2 = 184 bp.

and 2. The fragments generated by RT-PCR were named for the primers used, i.e., 4/7, 6/7, 6/2, and so forth. Analysis of the amplification products was performed by agarose gel electrophoresis. RT-PCR products were subcloned into the pCRII vector using the TA Cloning kit (Invitrogen). The nucleotide sequence of these clones was determined using the dideoxy-termination method with *Taq* polymerase and dye-terminator chemistry, on an Applied Biosystems model 373A DNA sequencer.

Northern Blot Analysis

Multitissue northern blots containing poly-A RNA (2 μg/lane) from a variety of human tissues were obtained from Clontech. RT-PCR products spanning nt 1–954, nt 624–954, and a cDNA clone spanning nt 184–1800 (J. M. Whaley, unpublished data) of the available sequence, were used as probes. Probes were radioactively labeled by the random hexamer method (Feinberg and Vogelstein 1983), and used at an activity of $1–2 \times 10^6$-cpm probe per ml buffer. Filters were independently hybridized with each of these probes using conditions recommended by the manufacturer and were washed under stringent conditions (Sambrook et al. 1989). Filters were exposed to film for 18 h with intensifying screens, at −80°C.

Southern Blotting and Hybridization

DNA samples (8 μg) were digested overnight with the restriction enzymes *Eco*RI or *Pst*I (Sambrook et al. 1989). Digested DNA fragments were resolved by electrophoresis in 0.8% agarose/1X Tris-borate-EDTA (TBE) (Sambrook et al. 1989) gels for 16 h and were transferred overnight to Hybond N+ membranes (Amersham) using 0.5 M NaOH and 1.5 M NaCl. Prehybridization and hybridization were performed in 10% PEG, 7% SDS, and 100 μg/ml human placental DNA at 65°C. An RT-PCR product spanning nt 1–918 and a cDNA clone spanning nt 184–1800 of the available sequence were used as probes in the hybridization. Probes were radioactively labeled (Feinberg and Vogelstein 1983) and used at an activity of $1–2 \times 10^6$ cpm per ml buffer. Washes were performed at room temperature in

0.1 × SSC, 0.1% SDS, with a final wash at 60°C. Filters were exposed to film for 12–18 h with intensifying screens at −80°C.

Genomic PCR and SSCP Analysis

RT-PCR analysis of alternatively spliced transcripts indicated the presence of at least two introns in the region of the VHL gene spanning the published cDNA sequence (Latif et al. 1993). Subsequently, we noted the exon-intron structure as reported by Crossey et al. (1993) and obtained their primer sequences for the flanking intron regions. PCR amplification from genomic DNA was performed for all three known exons, for each DNA sample, using a combination of primers derived from the cDNA sequence (see above) and primers that flank the exon-intron boundaries (Crossey et al. 1993). PCR amplification was performed as described above, using 100–300 ng total genomic DNA in the reaction, with the following exceptions: (*a*) the initial denaturation was 5 min at 94°C and (*b*) amplification of exon 1 was performed in 2% dimethylsulfoxide at an annealing temperature of 60°C.

SSCP analysis was performed essentially as described by Orita et al. (Orita et al. 1989*a*, 1989*b*). The conditions for PCR were the same as described above, except that the dCTP concentration in the reaction mixture was lowered to 10 μM, and 1 μCi of alpha ^{32}P-dCTP was added to the mixture. Following PCR, the samples were diluted sevenfold using 10 mM EDTA and 0.1% SDS, and these diluted samples were mixed with an equal volume of loading buffer containing 95% formamide (United States Biochemical). Samples were denatured for 2 min at 95°C, and radiolabeled DNA fragments were resolved by electrophoresis in 0.5 × Hydrolink Mutation Detection Enhancement (MDE) gels (J. T. Baker) in 0.6 × TBE overnight at room temperature. In most cases samples were also resolved on gels containing 5% glycerol. Gels were dried and exposed to X-ray film for 18 h at room temperature. Variant conformers detected by SSCP were excised from the gel, eluted in water, and reamplified for sequence analysis. All nucleotide sequencing was performed as described above.

Results

Identification of an Alternatively Spliced VHL-Gene Transcript

Expression analysis of the VHL gene in a variety of normal tissues by northern blot analyses, using probes spanning the entire published sequence, all revealed a single strongly hybridizing band of ~4.7 kb. An example of one such blot is shown in figure 1*b*. Repeated northern blot analyses of mRNA derived from a variety of tissues with probes spanning the entire open reading frame revealed no other hybridizing species (data not shown). The gene is expressed in many human tissues, and at especially high levels in lung (fig. 1*b*). RT-PCR analysis using human RNA from normal tissues (kidney, adrenal gland, brain, lung, and cerebellum), as well as RNA from lymphoblastoid cell lines (data not shown) revealed the presence of two mRNA species. Two distinct PCR products were observed on agarose gel analysis following RT-PCR (fig. 1*c*). These PCR products were subcloned and sequenced, and the larger of the two products, derived from transcript isoform I, was found to be identical in sequence to the reported VHL cDNA. The smaller fragment derived from a distinct transcript, isoform II, which lacked a region of 123 nt between nt 553 and nt 676, as compared with transcript isoform I. When translated, the sequence in isoform II corresponds to an in-frame 41-amino-acid deletion within the 284-amino-acid open reading frame, between amino acids 184 and 226. Thus, transcript isoform II is generated by the alternative use of a 123-nt cassette exon, consistent with the gene structure as recently reported by Crossey et al. (1993) and Gnarra et al. (1994). Both transcript isoforms are detected in all tissues examined, except bladder (fig. 1*c*), in which only transcript isoform II was detected. It is possible that lymphocytic infiltration could potentially account for the presence of both isoforms in the other whole tissues. However, we also detected both isoforms in five established RCC cell lines, while in the remaining three RCC cell lines only isoform I was detected (fig. 1*d*).

Characterization of Mutations in the VHL Gene

Germ-Line mutations.—Sixty-one VHL families were analyzed for the presence of SSCP variant conformers, using the genomic amplification strategy described in Material and Methods. Of these, 22 showed variant conformers within the reported VHL gene open reading frame (Latif et al. 1993). Inheritance of such variant conformers in affected individuals was demonstrated in three families (pedigrees 1, 2, and 3). A branch of pedigree 3 and the observed inherited SSCP alleles are shown in figure 2. A summary of the mutations identified within such variants among 61 VHL families is given in table 1. All mutations mapped to the region downstream of nt 375, which is downstream of the sequence in the VHL cDNA previously suggested to encode an acidic pentamer repeat (Latif et al. 1993). Mutations mapped to all three defined exons, at a frequency of 8/22 for exon 1, 6/22 for exon 2, and 8/22 for exon 3. Upstream of nt 375, one variant that was detected was present both in VHL patients and in 17/34 normal individuals (data not shown) and therefore represents a polymorphism. Sequence analysis revealed this polymorphism to be an A-to-G transition at nt 19. No other variants were detected in VHL patients upstream of nt 375. In addition to the VHL pedigree mutations described in table 1, two additional VHL pedigrees showed altered *Eco*RI fragments on Southern blot, using the 4/7 RT-PCR probe derived from the coding sequence (data not shown). These are unlikely to be polymorphisms, since

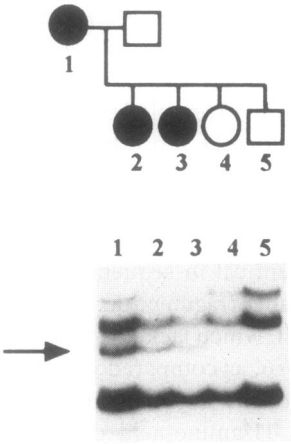


Figure 2 SSCP analysis of exon 3 of the VHL gene in a branch of VHL pedigree 3. The upper panel shows the family relationship, with the blackened figures representing VHL-affected individuals and the unblackened figures representing individuals currently unaffected (ages 26 and 25 years, for individuals 4 and 5, respectively). The bottom panel shows the respective SSCP pattern from blood-derived DNA from these individuals, the arrow indicating the variant bands that were shown by sequence analysis to contain the inherited mutation (C to T at nucleotide 712).

altered *Eco*RI fragments were not observed among >100 normal individuals screened with the VHL cDNA clone (Latif et al. 1993) nor among 20 unrelated normal individuals screened with the 4/7 probe (data not shown). Thus, of 61 VHL families analyzed, 24 (39%) show mutations within the reported open reading frame of the VHL gene.

One notable clinical feature of VHL is the presence or absence of pheochromocytoma in association with other tumor types, such as hemangioblastoma and RCC, within certain families. The heritable predisposition to pheochromocytomas in association with VHL suggested that specific mutant alleles of the gene may account for this phenotype (Glenn et al. 1991; Neumann and Wiestler 1991; Neumann et al. 1993). Since many of the pedigrees we have examined are small, and some have been lost to follow-up, we cannot at this time draw any definitive conclusion regarding the association of specific mutations with the predisposition (or lack thereof) to develop pheochromocytoma. We note, however, that pedigrees 1 and 3—two large and well-characterized kindreds displaying widely differing degrees of predisposition to pheochromocytoma in association with retinal, cerebellar, spinal, and renal manifestations of VHL (Go et al. 1984; Green et al. 1986; Lamiell et al. 1989)—both have sustained missense mutations affecting codons within exon 3 (table 1).

In combination with the analysis for germ-line mutations, tumor material derived from VHL patients was also analyzed in order to screen for the loss or mutation of the remaining normal allele. The degree of contamination of tumor tissues with normal tissue made this analysis difficult. However, in one VHL patient from pedigree 3 we could detect loss of heterozygosity for a *Pst*I polymorphism within the gene (Richards et al. 1993*a*) in a pheochromocytoma, in which the germ-line mutation present in the variant SSCP conformer is retained (fig. 3*a*). Since the mutation itself has not removed a *Pst*I restriction site, and the probes used in the hybridization cover the entire known sequence of the VHL gene, we can infer that it is the wild-type allele that has been lost in this tumor. This observation indicates that loss of the remaining normal allele within the tumor is likely to be a critical step in the development of VHL-associated tumors and is consistent with a two-hit mechanism of tumorigenesis (Knudson 1971) in this syndrome.

Mutations in sporadic cancers.—Thirty sporadic RCC samples with corresponding normal tissue and six sporadic RCC cell lines were screened by genomic PCR/SSCP analysis for the presence of variants in the VHL gene. Ten (33%) of the sporadic tumors and all six RCC cell lines show mutations within the gene (figs. 3 and 4). A summary of these mutations is given in table 1. Mutations mapped to all three defined exons with a frequency of 6/16 for exon 1, 4/16 for exon 2, and 6/16 for exon 3, within the set of tumors examined. The polymorphism at nt 19 was also detected in these samples, as was a different polymorphism in one sporadic RCC patient (fig. 4), a G-to-A transition at nt 345. As with the hereditary tumors, many of the sporadic RCCs analyzed did have ≤60% normal tissue contamination, and it was therefore not possible in most cases to assess loss of the remaining allele. However, for one RCC informative for a *Pst*I polymorphism within the gene (Richards et al. 1993*a*), loss of one allele could be demonstrated (fig. 3*b*), concurrent with the detection of a variant SSCP conformer which, when sequenced, was found to contain a mutation (C to G at nt 666). In addition, clear loss of the normal allele is seen in four RCC cell lines (fig. 3*c*). These observations are consistent with the hypothesis that the mechanism of tumor formation in at least a substantial proportion of sporadic RCCs involves the loss or inactivation of both copies the VHL tumor-suppressor gene (Knudson 1971).

In addition to sporadic RCCs, we have screened >180 sporadic tumors and tumor cell lines of other types, listed in table 2, and have thus far detected no SSCP variants within the reported open reading frame of the VHL gene. In these analyses we did detect the nt 19 polymorphism, but no other variants in any other portion of the coding region. Figure 5 summarizes the location of all the mutations detected in this study within the known VHL open reading frame in VHL patients and in sporadic RCCs.

Discussion

The study of hereditary tumor syndromes has been a fruitful avenue for the identification of genes involved not only in the development of hereditary tumors, but also in

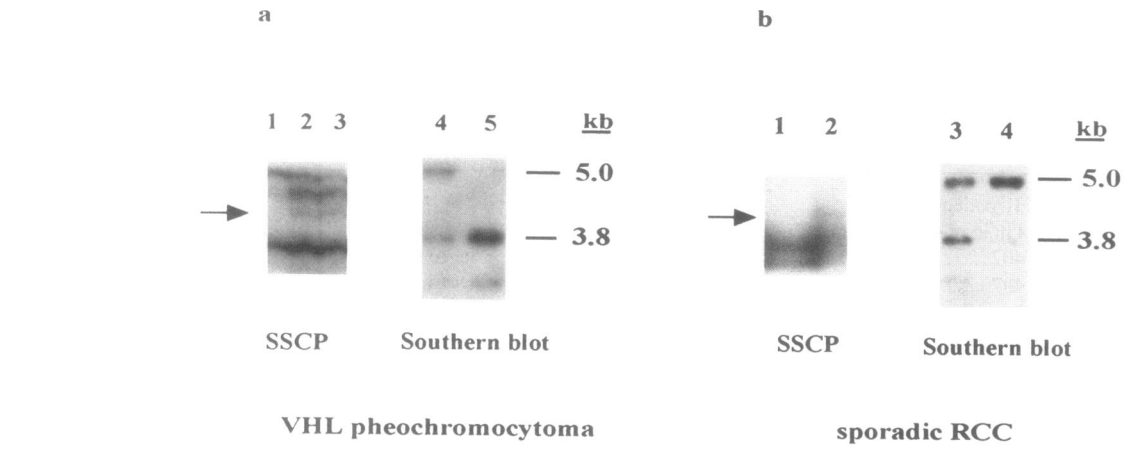
Table 1

Summary of Mutations in the VHL Gene Detected by PCR/SSCP in VHL Patients, Sporadic RCC, and Sporadic RCC Cell Lines

Mutation	Pedigree No.	Exon	Predicted Effect[a]
Detected in VHL patients:			
Deletion of 4 nt at nt 402–405	19	1	Frameshift to stop codon at aa 136
C to A at nt 407	12	1	Ser to stop, aa 136
C to A at nt 407	13	1	Ser to stop, aa 136
Deletion of 3 nt at nt 437–439	20	1	In-frame deletion of Phe at aa 147
Deletion of 3 nt at nt 437–439	17	1	In-frame deletion of Phe at aa 147
Deletion of G at nt 490	16	1	228-aa protein, altered from aa 164
T to C at nt 505	14	1	Tyr to His, aa 169
T to C at nt 505	15	1	Tyr to His, aa 169
A to G at nt 575	21	2	Asp to Gly, aa 192
T to A at nt 617	8	2	Leu to stop, aa 206
T to G at nt 620	10	2	Phe to Cys, aa 207
Deletion of GC at nt 648–49	9	2	242-aa protein, altered from aa 215
Insertion of A at nt 665	11	2	201-aa protein, altered from aa 222
C to T at nt 674	18	2	Pro to Leu, aa 225
Insertion of T at nt 685	6	3	243-aa protein, altered from aa 229
T to C at nt 686	1	3	Leu to Pro, aa 229
T to C at nt 686	2	3	Leu to Pro, aa 229
C to T at nt 712	3	3	Arg to Trp, aa 238
G to A at nt 713	5	3	Arg to Gln, aa 238
C to A at nt 761	22	3	Ser to stop, aa 254
T to G at nt 806	7	3	Leu to Arg, aa 269
A to C at 5′ splice acceptor site	4	3	Incomplete or altered splicing
	Tumor No.		
Detected in primary sporadic RCC:			
13-nt deletion at nt 379–391	22	1	132-aa protein, altered from aa 127
C to T at nt 469	53	1	Pro to Ser, aa 157
G to T at nt 476	9	1	Trp to Leu, aa 159
A to G at nt 544	19	1	Ser to Gly, aa 182
2 nt deletion at nt 608–609	57	2	Truncated protein at aa 203
C to G at nt 666	11	2	Ile to Met, aa 222
G to T at 3′ splice donor site	51	2	Presumed stop in intron 2
G to T at nt 691	23	3	Glu to stop, aa 231
C to G at nt 768	12	3	Tyr to stop, aa 256
2-nt deletion at nt 792–793	4	3	324-aa protein, altered from aa 265
	Cell Line		
Detected in sporadic RCC cell lines:			
C to T at nt 457	ACHN	1	Arg to Cys at aa 153
1-nt deletion at nt 523	786-O	1	229-aa protein, altered from aa 175
4-nt deletion at nt 639–642	A-498	2	226-aa protein, altered from aa 213
A to T at nt 742	Caki-2	3	Arg to stop, aa 248
T to A at nt 752	A-704	3	Ile to Asn, aa 251
T to A at nt 752	769-P	3	Ile to Asn, aa 251

[a] Amino acid (aa) nos. refer to those reported by Latif et al. (1993).

the etiology of sporadic human cancers, e.g., retinoblastoma, neurofibromatosis 2, and familial polyposis coli (Knudson 1993). For VHL, the positional cloning approach successfully led to the recent identification of a candidate gene (Latif et al. 1993) on the basis of the previous chromosomal localization of the defect to 3p25-p26 (Seizinger et al. 1988). The cloned partial cDNA sequence shows no homology to any other known gene, and no obvious functional motifs are encoded by the reported open reading frame, except an acidic amino acid pentamer repeat sequence (Latif et al. 1993) that shares homology to an acidic repeat domain in a protein from *Trypanosoma*



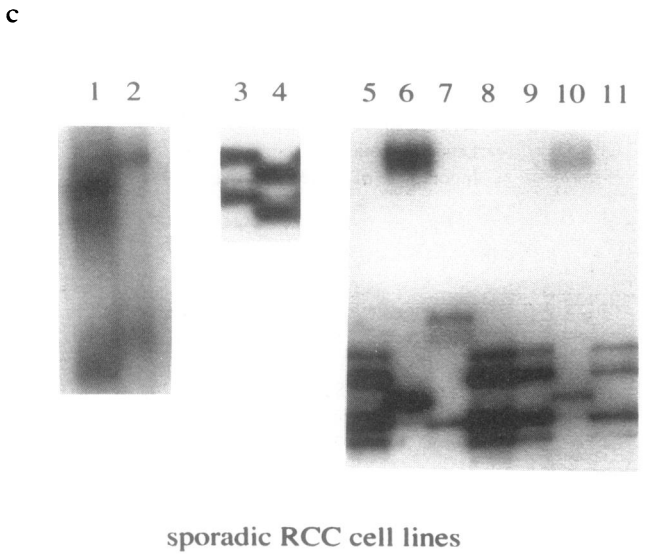


Figure 3 VHL gene allele loss in hereditary and sporadic tumors with mutations in the VHL gene. *a*, SSCP and Southern blot analysis of normal and pheochromocytoma DNA derived from a VHL patient from pedigree 3, whose germ-line mutation is known (see fig. 2 and table 1). Lane 1 shows the SSCP conformers derived from exon 3 in DNA from a normal individual; lanes 2 and 3 show the conformers of this exon in the DNA from normal tissue and a pheochromocytoma, respectively, from a VHL patient from pedigree 3. The arrow indicates the novel conformer containing the mutation, which is present in both normal and tumor tissue from this patient. Lanes 4 and 5 show normal and pheochromocytoma tissue DNA, respectively, from this patient, digested with *Pst*I and analyzed by Southern blot using as probes two cDNA fragments spanning the entire published partial VHL cDNA, as described in Material and Methods. Loss of the 5.0-kb allele of the VHL gene in the tumor is shown. *b*, SSCP and Southern blot analysis of normal and tumor DNA, derived from a patient with sporadic RCCs, which has sustained a mutation in the VHL gene (tumor no. 11 in table 1). Lanes 1 and 2 show the conformers derived from amplification of VHL exon 2 in DNA derived from normal and RCC tissue, respectively, from this patient. The arrow indicates the presence of a novel conformer in the tumor (lane 2) which, when sequenced, was found to contain the mutation. Lanes 3 and 4 show the normal and RCC DNA, respectively, from this patient, analyzed by Southern blot, as described in Material and Methods. Loss of the 3.8-kb *Pst*I allele of the VHL gene in the tumor is shown. *c*, SSCP analysis of exon 1 (nt 296–553) of the VHL gene in normal tissue (lane 1) and in 786-O cells (lane 2), of exon 2 in normal tissue (lane 3) and in A-498 cells (lane 4), of exon 3 in normal tissue (lanes 5, 8, 9, and 11) and in A-704 cells (lane 6), in Caki-2 cells (lane 7), and in 769-P cells (lane 10).

brucei. In order to assess what regions within this reported open reading frame are functionally important and in order to determine the importance of this gene in the etiology of sporadic RCC, we have characterized the location and types of mutations that occur in the VHL gene in VHL-associated hereditary and sporadic cancers. In addition, we have screened seemingly VHL-unrelated tumor types for the presence of mutations in this gene. Our analysis shows that mutations in the VHL gene are not only associated with hereditary tumors but also with a large proportion of sporadic RCCs, consistent with that observed by Gnarra et al. (1994) and confirming the important role of the VHL locus in the genesis of this tumor type. Mutations in tumors and tumor cell lines were found to be accompanied by loss of the remaining normal allele, suggesting that the VHL gene acts as a recessive tumor-suppressor gene in conformity with Knudson's two-hit model of tumorigenesis (1971). Our observation that expression of the wild type human VHL gene sequence in RCC cell lines carrying endogenous VHL gene mutations results in growth suppression (J. Naglich, J. M. Whaley, V. A. Tuomari, J. Gao, B. R. Seizinger, and N. Kley, unpublished data) further supports this hypothesis.

The region of the VHL gene that was screened for mutations in human tumors included the entire region of the large open reading frame present in the cloned partial

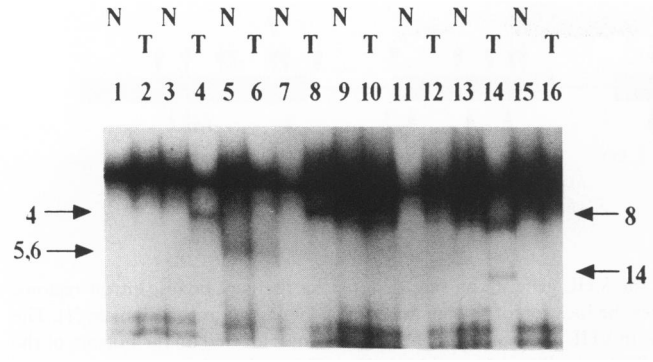


Figure 4 SSCP analysis of exon 1 (nt 296–553) of the VHL gene in eight sporadic RCCs with matched normal tissue. Odd-numbered lanes are normal (N) tissue samples; even-numbered lanes are the corresponding tumor (T) samples. Arrows labeled with lane numbers indicate the variant conformers in the tumors not present in the corresponding normal tissue. Arrow corresponding to lanes 5 and 6 indicates the presence of a polymorphism in exon 1 at nucleotide 345.

cDNA (1.8 kb) as reported by Latif et al. (1993). This region appears to be contained within three exons of the VHL gene (Crossey et al. 1993), which is comparable to the genomic structure of the mouse VHL gene homologue (J. Gao, personal communication). This open reading frame is likely to encompass the majority of the coding region of the human VHL gene, since it contains all coding sequences that are conserved in a full-length mouse VHL gene homologue we have recently cloned and characterized (J. Gao, personal communication). RT-PCR analysis of mRNA isolated from normal human tissues and from lymphoblastoid and RCC cell lines indicates that the VHL gene is alternatively spliced and that alternative use of the cassette exon 2 leads to the generation of two transcripts, termed *isoform I* and *isoform II*. Transcript isoform II lacks the 123 nt derived from exon 2, predicting an in-frame deletion of 41 amino acids in the encoded VHL protein isoform with respect to that encoded by the originally reported transcript isoform I. These observations are consistent with those of Gnarra et al. (1994), in which two VHL transcript isoforms were detected in RNA derived from normal kidney and from two RCC cell lines. Both transcript isoforms seem to be expressed in various tissues except bladder, in which only transcript isoform II was detected. While we cannot rule out lymphocyte contamination as the reason for the presence of both transcript isoforms in other whole tissues, the presence of both isoforms in five established RCC cell lines suggests that these isoforms are normally expressed in kidney tissue. Both transcripts appear to be derived from a widely expressed mRNA of ~4.7 kb (±123 nt) in size, as determined by independent northern blot analyses, using probes that are identical to, and span the entire length of, the reported sequence. This mRNA species differs in size by nearly 2 kb from the species reported by Latif et al. (1993), using probes that cover the entire known VHL gene sequence. We can only surmise that the initial determination of the VHL gene mRNA species size may have been inaccurate.

Analysis of blood or lymphoblastoid cell line DNA from VHL patients revealed that germ-line mutations in the VHL gene are distributed among all three exons containing the reported open reading frame. These include deletions, insertions, splice-site mutations, and missense and nonsense mutations (see table 1). The missense mutations we have detected are unlikely to represent polymorphisms since we did not detect any of these mutations in >180

Table 2

Sporadic Tumors and Tumor Cell Lines Screened by PCR/SSCP Analysis for the Presence of Mutations in the VHL Gene

Tumor Type	No. Showing SSCP Variant Conformers/No. Screened	% Showing Variant Conformers
RCC	10/30	33
RCC cell line	6/6	100
Pancreatic adenocarcinoma	0/18	0
Pancreatic adenocarcinoma cell line	0/11	0
Breast carcinoma	0/35	0
Lung carcinoma	0/14	0
Lung carcinoma cell line	0/16	0
Colon carcinoma	0/13	0
Melanoma	0/25	0
Ovarian carcinoma	0/10	0
Cervical carcinoma cell line	0/7	0
Prostate carcinoma	0/17	0
Bladder carcinoma	0/11	0
Endometrial carcinoma	0/4	0
Total screened	217	

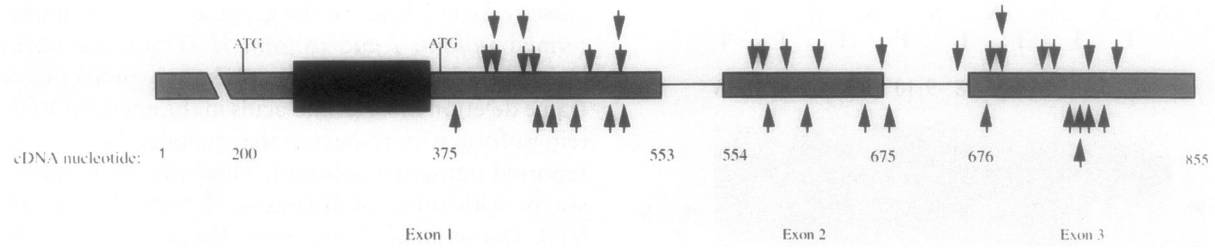


Figure 5 Map of the germ-line and somatic mutations detected in the VHL gene. Exons are denoted by the gray boxes. Intron regions, indicated by a single line, are not drawn to scale. The blackened box indicates the location of the putative Gly-X-Glu-Glu-X repeat domain (1). The arrows located on top of the map indicate the location of germ-line mutations in VHL patients (see table 1). The arrows located on the bottom of the map indicate the location of mutations detected in sporadic RCCs and sporadic RCC cell lines (see table 1). The ATG nucleotide sequences indicated in exon 1 represent the location of potential initiation codons.

tumor tissues (table 2) and 30 normal kidney tissue samples, representing material from >200 unrelated individuals (if these base changes represent rare polymorphisms, the frequency would be $<1/210$, or $<5 \times 10^{-3}$). Interestingly, nearly half of the germ-line mutations consist of missense mutations, which raises the question of whether these may be selected for because of some gain in function associated with these mutations, as appears to be the case for the p53 tumor-suppressor gene (Chen et al. 1990; Dittmer et al. 1993; Sun et al. 1993). Answers to these questions await further functional characterization of the biological activities associated with the VHL protein. Four pairs of families show the same germ-line mutations (table 1). The two families showing the same mutation at nt 686 were expected since together they represent a large VHL kindred (Lamiell et al. 1989). The other sites (nt 407, nt 437–439, and nt 505) may represent mutation "hot spots." Alternatively, these pairs of families may also be distantly related to one another, and haplotype analysis for markers flanking the VHL gene should distinguish between these two possibilities. Different germ-line mutations affecting the same codon were also found (codons 136, 229, and 238), and these sites may also represent mutational hot spots.

In our analysis of sporadic tumors we find that mutations in the VHL gene occur to a significant extent in primary sporadic RCCs, with a frequency of ≥33%. This is likely to be an underestimate due to the inherent sensitivity limits associated with PCR/SSCP analysis, estimated to be as low as 67%, depending on the length of the sequence analyzed, the sequence context and the conditions used (Hayashi 1991; Hayashi and Yandell 1993). Although the general conditions we have used for these analyses have been successfully used for the detection of point mutations by other investigators (Hayashi and Yandell 1993), we have detected mutations by PCR/SSCP at a frequency of only 36% (22/61) in VHL patients, which probably reflects both the limits of the methodology and the probability that mutations may be occurring in regions of the gene that have not yet been analyzed. All human RCC cell lines analyzed by PCR-SSCP showed mutations, and two RCC cell lines showed the same mutation (table 1). These findings, in conjunction with those reported by Gnarra et al. (1994), clearly implicate the VHL gene in the development of RCCs occurring in the general population. However, in contrast to results as discussed by Gnarra et al., we observe that the types of mutations occurring at the somatic level in sporadic RCC and in the germ line of VHL patients are similar with regard to the types and location (including exon 2) of mutations detected, suggesting a similar molecular basis for the inactivation of the VHL gene. In addition, we note that direct sequence analysis of exons 4–8 of the p53 gene in the set of sporadic RCCs examined in this study (data not shown) revealed that none of these tumors have sustained mutations within the conserved regions of the p53 gene. This finding suggests that mutations in the VHL gene occur prior to potential abnormalities occurring in the p53 tumor-suppressor gene in sporadic RCC, consistent with findings discussed by Gnarra et al. (1994) In order to establish whether mutations in the VHL gene are associated with VHL-unrelated sporadic tumors occurring in the general population, PCR/SSCP was performed on a large number of diverse human cancers (table 2). In >180 other sporadic tumors and tumor cell lines of various types we did not detect mutations in the VHL gene. Thus, continued mutational analysis will be required to establish any significant association of VHL gene mutations with tumor types other than sporadic RCCs and thus to establish whether the VHL gene indeed has a specific role in the development of sporadic VHL-associated tumors.

As indicated above, although mutations were detected in all three exons of the VHL gene that appear to span the reported open reading frame, both the germ-line and somatic mutations were located downstream of nt 375 of the corresponding cDNA (fig. 5). Interestingly, this is downstream of the sequence previously suggested to possibly encode a repeated pentamer motif (Latif et al. 1993). Thus, it is possible that the functionally important coding region of the VHL gene is actually represented by the exon

sequences downstream of this repeat sequence, including the 123-nt alternatively spliced cassette exon. The region of the open reading frame downstream of nt 375 is also the one that is highly conserved in a full length mouse VHL gene homologue we recently cloned and characterized (X. X. Gao, personal communication). Furthermore, protein analysis and expression studies indicate that indeed the predominant species of the human VHL protein that is detected in human cells and that mediates growth suppression is encoded by the region of the open reading frame that is highly conserved between mouse and human genes (J. Naglich, J. M. Whaley, V. A. Tuomari, J. Gao, B. R. Seizinger, and N. Kley, unpublished data).

The presence of an alternative transcript, transcript isoform II, which predicts a protein with a deletion of 41 amino acids in the conserved region with respect to that encoded by transcript isoform I, poses some interesting questions concerning the biological activity of this protein and associated tumor-suppressor activity. We detected missense mutations in exon 2 in three VHL families, and one sporadic RCC. Thus it appears that this encoded region is important for tumor-suppressor activity and/or that mutation in this region may affect neighboring domains or the overall structure and function of the VHL protein. Studies addressing these questions are currently in progress.

In summary, we show that mutations in the VHL gene are associated with a large proportion of sporadic RCCs in addition to the hereditary tumors, indicating a more widespread role for the VHL gene in tumorigenesis. Furthermore, while mutations in the VHL gene in hereditary and sporadic cancers occur within all three described exons of the gene, including an alternatively spliced cassette exon, these mutations are not generally distributed throughout the open reading frame, but cluster in the 3′ end of this reading frame, implicating this region as critical to the growth suppressive function of the VHL protein.

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