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ORIGINAL INVESTIGATION

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Genotype-phenotype correlations in families with deletions in the von Hippel-Lindau (VHL) gene

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Abstract Von Hippel-Lindau (VHL) disease is a hereditary tumor syndrome characterized by predisposition for bilateral and multi-centric hemangioblastoma in the retina and central nervous system, pheochromocytoma, renal cell carcinoma, and cysts in the kidney, pancreas, and epididymis. We describe five families for which direct sequencing of the coding region of the VHL gene had failed to identify the family-specific mutation. Further molecular analysis revealed deletions involving the VHL gene in each of these families. In four families, partial deletions of one or more exons were detected by Southern blot analysis. In the fifth family, FISH analysis demonstrated the deletion of the entire VHL gene. Our results show that (quantitative) Southern blot analysis is a sensitive method for detecting germline deletions of the VHL gene and should be implemented in routine DNA diagnosis for VHL disease. Our data support the previously established observation that families with a germline deletion have a low risk for pheochromocytoma. Further unraveling of genotype-phenotype correlations in VHL disease has revealed that families with a full or partial deletion of the VHL gene exhibit a phenotype with a preponderance of central nervous system hemangioblastoma.

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Introduction

Von Hippel-Lindau (VHL) disease is a hereditary tumor syndrome characterized by predisposition for bilateral and multi-centric hemangioblastoma in the retina and central nervous system (CNS), pheochromocytoma, renal cell carcinoma, cysts in the kidney, pancreas, and epididymis, and endolymphatic sac tumors. VHL disease is a relatively rare disorder, with an estimated birth incidence of 1/36,000 (Maher et al. 1991). The basis of familial inheritance of the disease is a germline mutation in the VHL tumor suppressor gene, located in chromosome region 3p25–26 and identified in 1993 (Latif et al. 1993). The disease is inherited as an autosomal dominant trait with a high penetrance. A genotype-phenotype correlation has been described for the presence of pheochromocytoma and renal cell carcinoma, but not for other VHL-related tumors (Zbar et al. 1996).

Germline mutations are found in up to 100% (Stolle et al. 1998) of the families fulfilling the clinical VHL criteria (Maher and Kaelin 1997; Melmon and Rosen 1964). Missense, nonsense, and splice site mutations, and microdeletions and microinsertions are detected in approximately two-thirds of these families (Maher et al. 1996; Stolle et al. 1998; Zbar et al. 1996). In one-third of the VHL families, large deletions (4–380 kb) are found (Maher et al. 1996; Stolle et al. 1998; Zbar et al. 1996). Such deletions have been demonstrated by Southern blot analysis

(Stolle et al. 1998), pulsed field gel electrophoresis (Richards et al. 1993; Szymanski et al. 1993), longe range polymerase chain reaction (PCR; Cybulski et al. 1999), or fluorescent in situ hybridization (FISH; Decker et al. 1994; Pack et al. 1999; Phipps et al. 1993). The detection of germline mutations in VHL families allows diagnosis of the disease, including diagnosis at an early or presymptomatic stage. Carriers of the mutated VHL gene can be monitored closely and given the appropriate treatment, whereas in non-carriers, the inconvenience of intensive clinical surveillance can be avoided.

We describe five families for which direct sequencing of the coding region of the VHL gene had failed to identify the family specific mutation. However, further molecular analysis revealed deletions involving the VHL gene in each of these families. Evaluation of clinical features in these families suggests that VHL gene deletions result in a disease phenotype characterized by an absence of pheochromocytoma and a high incidence of CNS hemangioblastoma.

Patients and methods

Patients

The five families (A–E) described here were referred to the Department of Medical Genetics, UMC Utrecht, for germline mutation analysis in the VHL gene. The patients were clinically examined in the University Hospitals of Utrecht, Groningen, Leuven, and Gent, and in the Merwede Hospital, Dordrecht (Table 1). Clinical monitoring included annual ophthalmoscopy, yearly alternate magnetic resonance imaging (MRI) and ultrasonography of the abdomen, and (at various frequencies) MRI of the CNS (Hes and Feldberg 1999). All probands fulfilled the clinical diagnostic criteria: in the presence of a positive family history, a diagnosis of VHL disease can be made by the identification of a single retinal or cerebellar hemangioblastoma, renal cell carcinoma, or pheochromocytoma, in an at-risk individual (Maher and Kaelin 1997; Melmon and Rosen 1964).

DNA analysis

High molecular weight DNA was isolated from peripheral blood samples according to established procedures. Exons 1, 2, and 3 of the VHL gene and their immediately flanking sequences were amplified by PCR, by using oligonucleotides according to Gnarra et al. (1994). The flanking sequences included 90 nucleotides upstream of the second start codon in exon 1 and 45 nucleotides downstream of the stop codon in exon 3. Amplification products were purified and subjected to automated sequence analysis on an ABI 377 (Perkin & Elmer). The amplification primers were used as primers in the sequencing reactions.

Screening for structural rearrangements, including gross deletions, was performed by Southern blot analysis. DNA was digested with *Eco*RI alone (Latif et al. 1993) and with an *Eco*RI/*Ase*I double-digest (Stolle et al. 1998). To confirm the results, two other restriction enzymes, viz., *Hin*dIII and *Stu*I, were used (see Fig. 1 for the restriction map). After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridized with the VHL *g7*-cDNA probe (Latif et al. 1993), according to the manufacturer's instructions. The human beta globin gene was used as an internal control (Stolle et al. 1987). Additionally, exon-specific probes generated by PCR amplification of exons 1, 2, and 3 were hybridized to the same filters.

FISH analysis

FISH analysis was carried out on metaphase chromosome spreads according to established procedures (Phipps et al. 1993). The VHL cosmid-11 probe was labeled by nick translation with biotin-14-dATP. After precipitation of the labeled probe in the presence of Cot-1 DNA, pre-annealing was performed in order to block repetitive sequences. The final concentration of the probe was 15 ng/μl. Hybridization of the denatured probe onto the denatured metaphase chromosomes was carried out overnight at 37°C. Each slide was mounted with 15 μl antifade medium (Vectashield, Braunschweig) containing 4,6-diamidino-2-phenylindole. Microscopic analysis of images was performed by using a CytoVision (Applied Imaging).

Results

Clinical manifestations

No pheochromocytoma occurred in any of the 34 clinically well-monitored patients in the five families studied (Table 1). Other visceral VHL-related manifestations included: three patients with renal cell carcinoma, two with renal cysts, six with pancreatic cysts, and two with ovar-

Table 1 Genotypes and phenotypes (*Family* family with unique identification number, *Age* age of patients and range of current age in years, *Pheo* pheochromocytoma, *RCC* renal cell carcinoma, *cHAB* central nervous system hemangioblastoma, *rHAB* retinal hemangioblastoma)

Family	Age range (mean)	Origin	Deletion	Patients	Number of patients with a VHL-related manifestation				
					Pheo	RCC	cHAB	rHAB	Other
A (2)	16–37 (31)	Turkish	Exon 1+2	5	0	2	4	2	Pancreatic cysts (2 patients), bilateral renal cysts (1 patient)
B (15)	20–80 (49)	Dutch	Exon 1	20	0	1	17	12	Pancreatic cysts (2 patient), multiple ovarian cysts (1 patient)
C (23)	47–72 (57)	Belgian	Exon 1–3	5	0	0	5	0	Pancreatic cysts (1 patient), ovarian cyst and renal cyst (1 patient)
D (51)	46–? (46)	Belgian	Exon 1	2	0	0	2	1	Pancreatic cysts (1 patient)
E (61)	31–60 (46)	Dutch	Exon 1	2	0	0	0	2	Neurofibromatosis (1 patient)
Total	16–80 (47)			34	0	3	28	17	

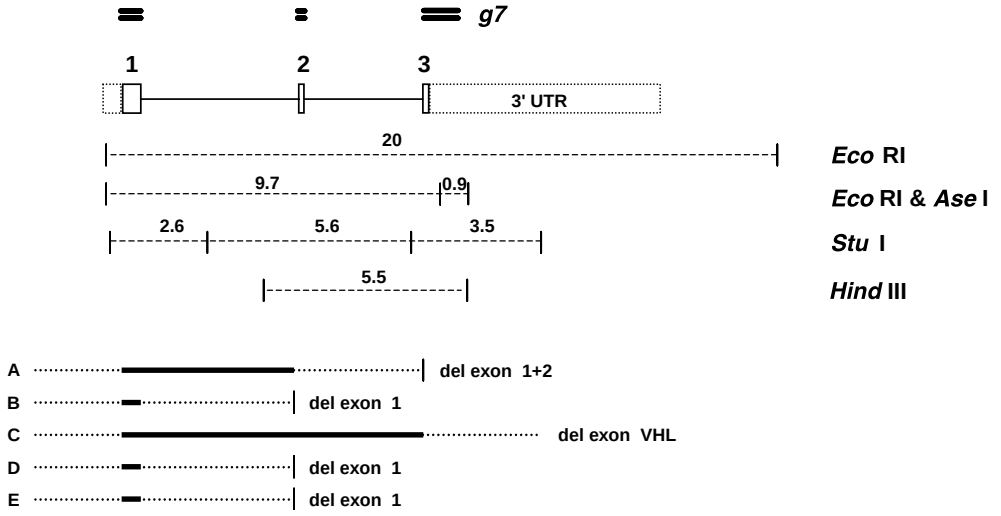


Fig. 1 Genomic organization of the VHL gene (to scale), including the 5' and 3' untranslated regions (*UTR*), the VHL *g7* probe, and a restriction map of enzymes used in this article. *Numbers* refer to the size of restriction fragments in kilobases (=1000 bp). The *solid bars below* represent genomic deletions found in families A–E, encompassing the exons indicated. The *dotted lines* characterize the possible extent of the deletions

ian cysts. Five patients had symptoms associated with an endolymphatic sac tumor (i.e., hearing loss, tinnitus, or vertigo); however, MRI did not show tumors in the posterior fossa in these patients. One patient in family E had neurofibromatosis. CNS and retinal hemangioblastoma were found in four of the five families: in the retina in 17 patients (50%), and in the CNS in 28 patients (82%).

Germline mutations in the VHL gene

In family A, direct sequencing did not reveal a germline mutation, neither was linkage analysis with highly polymorphic markers informative (data not shown). Southern blot analysis after *Eco*R1 digestion, with the *g7* probe and an internal control probe, demonstrated an extra band above the 20-kb normal fragment in the proband. To characterize the putative genetic alteration further, Southern blot analysis was repeated by using a panel of restriction enzymes, of which *Hind*III showed an aberrant fragment segregating with the disease (Fig. 2). Hybridization of the *Hin*dIII blot with radio-labeled PCR products of exons 1, 2, and 3 demonstrated a deletion of exons 1 and 2. This deletion was confirmed by digesting DNA with the enzymes *Bam*HI, *Ksp*632I, and *Bgl*II, which have restriction sites in exons 1, 2, and 3, respectively. *Bam*HI revealed a diminished intensity of the exon-1-specific band, and *Ksp* 632I demonstrated an extra band, also suggesting loss of a restriction site. Restriction with *Bgl*II gave a normal banding pattern. This finding was also confirmed with the *Stu*I restriction enzyme, which yields fragments of the three separate VHL exons. On Southern blot analysis, dimin-

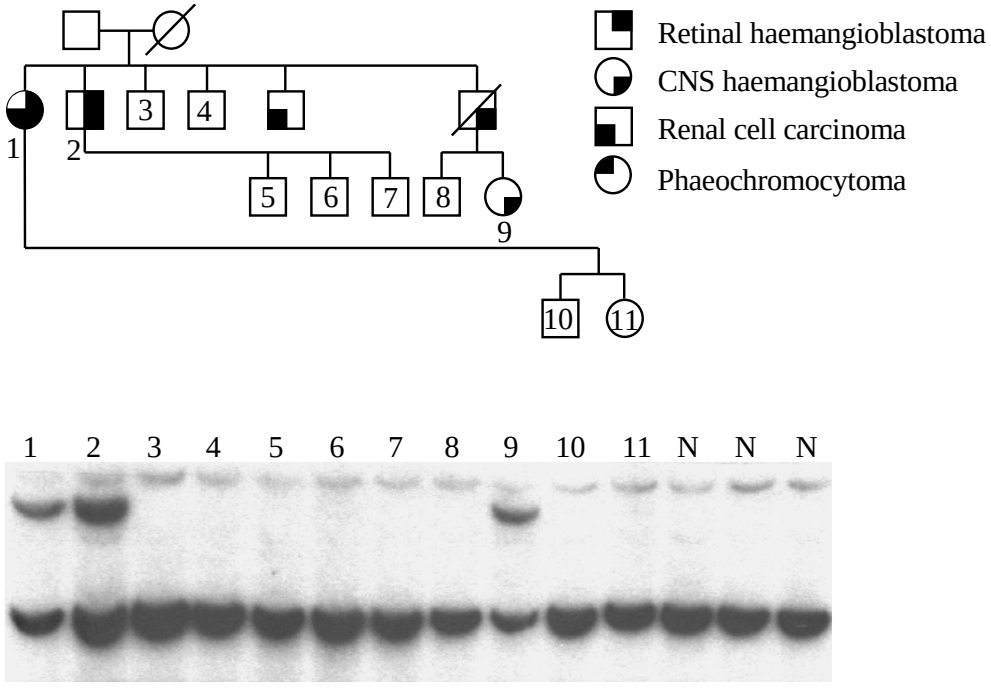


Fig. 2 Analysis of the segregation of an aberrant fragment with the disease. *Numbers* of the persons tested of family A correspond to the Southern blot analysis lanes. Genomic DNA of 11 family members and of three controls (*N*) was digested with *Hin*dIII and revealed an aberrant banding pattern in three affected family members

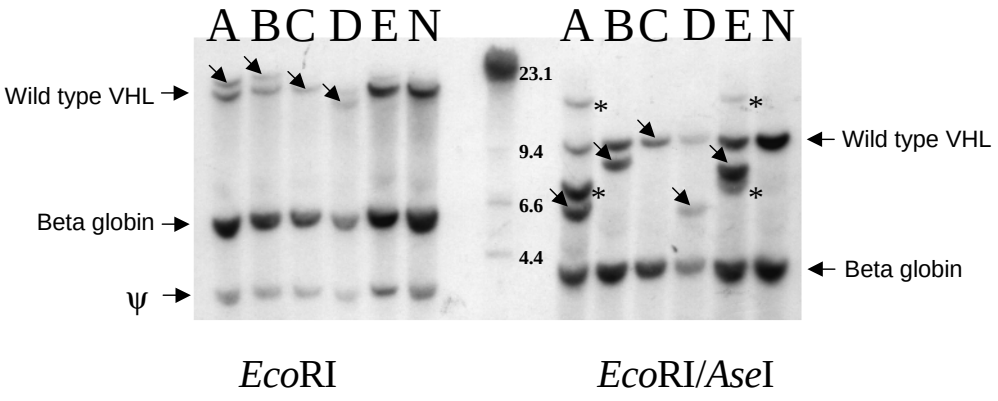


Fig. 3 Constitutional VHL gene deletions identified by Southern blot analysis in five families. Genomic DNA from families A–E and control (*N*) digested with *Eco*RI (*lanes 1–6*) and with *Eco*RI/*Ase*I (*lanes 8–13*) was hybridized with the *g7* probe and a beta-globin control probe. The lambda-x-*Hin*dIII marker (*lane 7*) shows fragment sizes in kilobases (=1000 bp). Aberrant bands are indicated with *arrows*. The bands marked with an *asterisk* might represent a restriction site polymorphism or partially digested DNA. The lower band represents a VHL pseudogene (ψ), located on chromosome 1 (Bradley and Rothberg 1999)

ished band intensity was seen for exons 1 and 2, and a normal intensity for the fragment containing exon 3 (data not shown).

Consequently, Southern blot analysis was also performed in four additional families for which no mutations in the VHL gene had been detected by direct sequencing. In families B and D, Southern blot analysis with *Eco*RI and hybridization with the *g7* probe generated an aberrant restriction fragment (Fig. 3). This aberrant restriction fragment was recognized by probes representing exons 2 and 3. However, hybridization with the exon 1 probe resulted in a normal banding pattern, indicating a deletion encompassing exon 1.

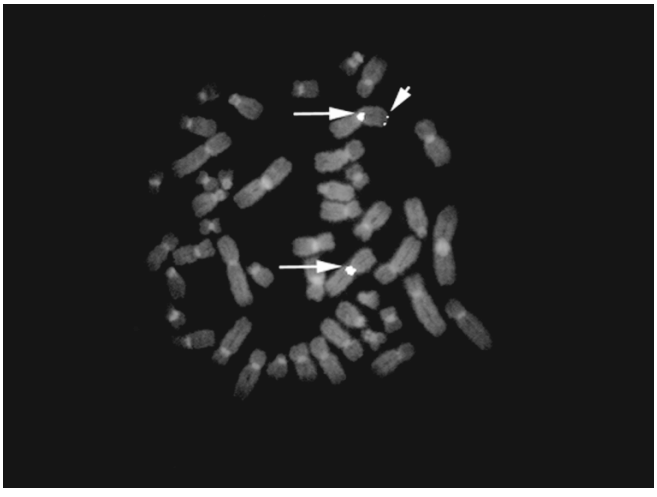


Fig. 4 Detection of a deletion encompassing the entire VHL gene by FISH analysis in family C. A loss of signal from one of the two VHL alleles (*short arrow*) was detected in a patient from family C. The *long arrows* represent the centromeric probe of chromosome 3

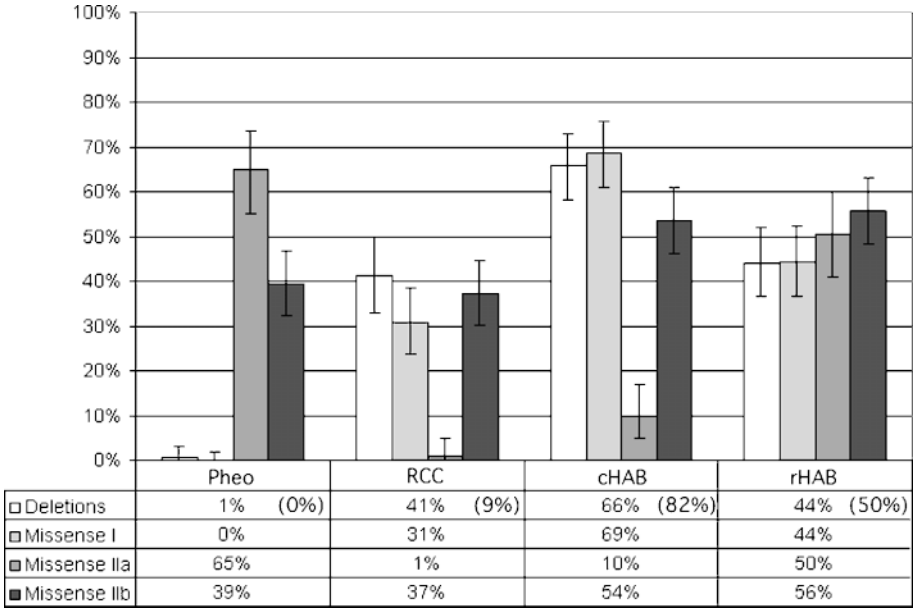
In the proband from family C, six different restriction enzymes (*Eco*RI, *Eco*RI/*Ase*I double-digestion, *Hin*dIII, *Stu*I, *Dra*I, and *Pvu*II) consistently revealed a diminished band intensity of the VHL band compared with the beta globin control probe. This indicated the presence of a deletion encompassing the entire VHL gene. Indeed, FISH analysis with the cos-11 probe demonstrated loss of signal of one of the two VHL alleles in three patients from this family, but not in an unaffected family member (Fig. 4).

In family E, we noticed that restriction with *Eco*RI, *Hin*dIII, and *Stu*I did not result in abnormalities on Southern blots. As recently described by Stolle et al. (1998), the resolution of Southern blot analysis for the VHL gene is improved by using an *Eco*RI/*Ase*I double-digestion. When we subjected all five families to the latter method, aberrations could be seen, as expected, in families A–D (Fig. 3). Surprisingly, the proband from family E showed an altered restriction fragment. Further analysis revealed a deletion of exon 1.

Discussion

Detection of VHL gene deletions

The five deletion families represent 28% of the families with a VHL germline mutation that have been identified in our department so far (unpublished data). Although, in three families (B, D, and E), the deletion involves exon 1 only, differences in the restriction fragment patterns generated by Southern blot analysis indicate that the deletions are distinct and have different breakpoints. Our results suggest that Southern blot analysis (and FISH when necessary) should be implemented in routine diagnostic screening protocols for VHL gene mutations. The *Eco*RI/*Ase*I double-digestion hybridized with *g7* cDNA and a control probe is becoming the method of choice in screening for large deletions in the VHL gene (Stolle et al. 1998). Southern blot analysis with *Eco*RI only is a less sensitive method of detecting VHL gene deletions, as illustrated in family E and two cases in the study by Stolle et al. (1998). Each of these cases had rearrangements detectable by *Eco* RI/*Ase*I digestion that were not found after *Eco*RI digestion. *Eco*RI/*Ase*I double-digestion has a high resolution because it isolates exactly the coding re-



	Pheo	RCC	cHAB	rHAB
Deletions	1% (0%)	41% (9%)	66% (82%)	44% (50%)
Missense I	0%	31%	69%	44%
Missense IIa	65%	1%	10%	50%
Missense IIb	39%	37%	54%	56%

Fig. 5 Pooled data of genotypes and phenotypes in families from the present study and in families studied by Chen et al. (1995) and Glavac et al. (1996). This figure shows the frequency of patients with four types of VHL tumors associated with their genotype. *Pheo* Pheochromocytoma, *RCC* renal cell carcinoma, *cHAB* central nervous system hemangioblastoma, *rHAB* retinal hemangioblastoma. The missense mutation T505C (Tyr98His) was the only missense mutation that we included in the missense type IIa group. More missense type IIa mutations could still be hidden in the missense IIb group. Data from the three studies were pooled, but we excluded those deletion patients from our study (frequencies between *brackets*), since they differed significantly ($P<0.001$) in their incidence of RCC compared with the two other studies

gion of the VHL gene. To delineate the molecular nature of the deletion further, the enzymes *Bam*HI, *Ksp*632I, and *Bgl*II, which have restriction sites in exons 1, 2 and 3, respectively, may be applied, in addition to hybridization with probes for the individual exons of the VHL gene.

If Southern blot analysis of the VHL gene demonstrates a diminished band intensity, FISH analysis can be used to confirm the deletion of one VHL allele. The presence of large deletions may also be revealed in studies involving highly polymorphic short-tandem repeat markers. Deletions encompassing polymorphic marker loci will result in loss of specific alleles and reduced intensities for observed alleles.

Genotype-phenotype correlations

Pheochromocytoma

The five families were affected with various VHL-related tumors, except for pheochromocytoma (Fig. 5). Families with a deletion or a mutation that predicts a truncated VHL protein are predominantly associated with a disease phenotype without pheochromocytoma (VHL type I), whereas 69% of families with pheochromocytoma (VHL type II) are associated with specific missense mutations (Stolle et al. 1998). It is hypothesized that pheochromocytoma arises from a dominant-negative effect of VHL proteins, based on the involvement of VHL in the multi-protein VCB (VHL-Elongin C-Elongin B) complex that may target proteins for degradation (via a process called ubiquitination; Stebbins et al. 1999). Structural analysis of this complex has revealed that VHL has two protein-binding sites. A mutant (type II) having a defect in only one site may exert a dominant-negative effect by sequestering key components of the ubiquitin pathway (Stebbins et al. 1999; Tyers and Willems 1999). In contrast, mutations found in families without pheochromocytoma (type I) are predicted to cause a complete unraveling of the VHL structure (Stebbins et al. 1999). Assuming that deletions of the VHL gene rule out the presence of any possible dominant-negative effect, the absence of phaeochromocytoma in our deletion patients is in good agreement with this model.

Renal cell carcinoma

Since our deletion families show a relatively low frequency of renal cell carcinoma compared with other studies, they are not included in the bar diagram of Fig. 5. Renal cell carcinoma in VHL patients occurs at a mean age of 36 years (Walther et al. 1999); the mean age (and the median age) of the VHL patients whom we studied was 47 years. The mean age of the patients in the two comparison articles was not reported. It has been hypothesized that renal lesions develop as a consequence of several structural aberrations, such as large deletions, nonsense, splice and frame shift mutations, and insertions (Glavac et al. 1996). So far, a low frequency of renal cell carcinoma has only been reported in families (VHL type IIa) with specific missense mutations (Brauch et al. 1995; Fig. 5).

The above suggests that the relationship between germline mutation and renal cell carcinoma in VHL is complex. Apart from chance, the relatively low frequencies of renal cell carcinoma reported in our clinically well-monitored families could be attributable to other factors. Like retinal hemangioblastoma in VHL patients, modifier genes (Webster et al. 1998) or external factors may contribute to a renal cell carcinoma risk (e.g., smoking is associated with a higher risk; La Vecchia et al. 1990; Muscat et al. 1995).

CNS hemangioblastoma

So far, the risks of CNS hemangioblastoma in VHL disease have not been correlated with allelic heterogeneity. Our deletion families exhibit a phenotype with a preponderance of CNS hemangioblastoma (Table 1). This prompted us to investigate whether phenotypes of families with VHL gene deletions differ from families with other VHL germline mutations (Fig. 5). With respect to the incidence of CNS hemangioblastoma, we noted that families with deletions did not significantly differ from other types of VHL germline mutations, except for families with type IIa missense mutations (Fig. 5). VHL deletion families show a significantly (Chi-square 85, $P<1\times10^{-10}$) higher incidence of CNS hemangioblastoma compared with type IIa missense mutations. Apparently, VHL IIa mutations are not only associated with a low risk for renal cell carcinoma, but also for CNS hemangioblastoma.

However, apart from considering the different functional effects of VHL mutations (i.e., null versus dominant-negative), other factors, including tissue-specific differences, may also play a role. For instance, cells could require different levels of functional VHL protein to maintain cellular homeostasis. Furthermore, the multifunctional VHL protein may be implicated in distinct cellular pathways in different tissues. Moreover, there is evidence that modifier genes play a role in the etiology of retinal hemangioblastoma (Webster et al. 1998), and this could be similar for other target tissues in VHL disease.

Interestingly, in family C, the deletion of the entire VHL gene is associated with a phenotype with a preponderance of CNS hemangioblastoma. Although complete VHL gene deletions occur in approximately 9% of VHL families (Stolle et al. 1998), no clinical details have been published for complete gene deletion families. Given that deletions of the entire VHL gene represent true null alleles, additional studies embodying carefully executed clinical analysis of patients with such deletions are required to test our observed genotype-phenotype correlation.

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