Germline mutations in the Von Hippel-Lindau (VHL) gene


From the Departments of Medical Genetics (FJH, RAZ, AJAV, PGFMS, PLP, RBL) and Internal Medicine (FJH, CJML), University Medical Centre Utrecht, the Netherlands; Clinical Genetics (ALWH-J, BE, DJJH, AMWO, DFM-K) University Hospital Rotterdam, the Netherlands; Clinical Genetics (MCEJ), Academic Medical Centre, Amsterdam, the Netherlands

In preparation
Abstract

**Introduction** Von Hippel-Lindau (VHL) disease is a complex, autosomal, dominant inherited disorder, variably presenting with retinal and cerebellar haemangioblastoma, renal cell carcinoma, phaeochromocytoma and endolymphatic sac tumours. Cysts and cystadenoma may develop in kidney, pancreas and epididymis. Germline mutations in the VHL tumour suppressor gene are found in most of the families fulfilling the clinical diagnostic criteria of VHL disease.

**Objective** To summarise the results of mutation analysis of the VHL gene in familial and sporadic cases of VHL disease diagnosed in the Netherlands.

**Patients and methods** Familial (n=25) and sporadic (n=7) VHL patients, as well as sporadic patients (n=2) with VHL-related tumours, not fulfilling current diagnostic criteria for VHL disease, were investigated by direct sequencing of the coding region, quantitative Southern blot analysis and Fluorescence in Situ Hybridisation (FISH) of the VHL gene.

**Results** We report 34 VHL germline mutations, including eight novel germline mutations in the open reading frame of the VHL gene. Analyses of genotype-phenotype correlations were consistent with previous reports. In nine sporadic patients with a VHL germline mutation we could identify four de novo VHL gene mutations. In four of the nine sporadic patients the parents were not available for testing. One of the nine patients shared a VHL germline mutation with a clinically unaffected parent (age 77 years) suggesting non-penetrance of VHL disease. Family histories of VHL in two other families were suggestive for reduced penetrance of VHL germline mutations.

**Conclusions** These results indicate that at least 12% of the germline mutations in the VHL gene occur de novo. Germline mutations are found in patients not fulfilling the currently accepted diagnostic criteria for VHL disease. Absence of VHL symptoms in carriers of VHL germline mutations indicate reduced penetrance and has implications for genetic counselling.
Introduction

Von Hippel-Lindau (VHL) disease is an autosomal, dominantly inherited disorder. Current estimates of the prevalence of VHL germline mutation carriers range between two and three per 100,000 persons.\cite{1-3} A germline mutation in the VHL gene predisposes carriers for haemangioblastoma in the central nervous system and retina, and for renal cell carcinoma, pheochromocytoma, endolymphatic sac tumours, and cysts and cystadenoma in kidney, pancreas and epididymis.\cite{4,5} VHL disease is characterised by multiple, richly vascularised tumours that may occur at a young age, i.e. between 20 and 40 years.\cite{6,7} The penetrance of the disease appears almost complete by the age of 60 years and the median expected survival is 49 years.\cite{2,7} However, early detection of tumours by intensive radiological and clinical screening, together with advanced operation techniques are likely to reduce both morbidity and mortality in VHL disease.\cite{5,8-10} Early identification is now facilitated by presymptomatic detection of VHL germline mutations.

With a positive family history, VHL disease can be diagnosed in a patient with at least one typical VHL-related tumour.\cite{5,11} Typical VHL-related tumours include retinal and cerebellar haemangioblastoma, renal cell carcinoma, and pheochromocytoma.\cite{5} Endolymphatic sac tumours and multiple pancreatic cysts suggest a positive carrier status (in the presence of a positive VHL family history), since they are uncommon in the general population.\cite{5,12} In contrast, renal and epididymal cysts occur more frequently in the general population and alone they are unreliable indicators of the carrier status.\cite{13} Without a family history, VHL disease can be diagnosed when two or more retinal or cerebellar haemangioblastomas or a single haemangioblastoma in combination with a typical visceral lesion are present in a sporadic patient.\cite{5}

A genetic locus for the disease was mapped to the short arm of chromosome 3 by linkage studies,\cite{14} and the VHL tumour suppressor gene was identified in 1993 by positional cloning.\cite{4} Subsequently, more than 300 VHL germline mutations have been reported world-wide.\cite{15,16} Using direct sequencing of the coding region and quantitative Southern blot analysis, a detection rate of 100% was reported in well-defined VHL families.\cite{17} VHL germline mutations of all types are scattered over the VHL gene and also include entire gene deletions.

Recently, it was demonstrated that the VHL protein (pVHL) plays a role in the degradation (via a process called ubiquitination) of hypoxia-inducible proteins, possibly including vascular endothelial growth factor (VEGF).\cite{18-20} Excessive blood vessel formation may occur when these proteins are not properly degraded.\cite{21} pVHL fulfils its function by binding to other proteins called Elongin C, Elongin B, and Cullin2.\cite{18}

Analysis of the structure of pVHL also enables the study of genotype-phenotype correlations. The disease has been divided in two phenotypes: families without (VHL type I) and with pheochromocytoma (VHL type II).\cite{15,17} Mutations in patients with VHL type I group are most commonly found in the beta domain of the pVHL and are predicted to lead to a loss of function. This beta domain probably binds the target proteins for ubiquitination.\cite{18} In contrast, most mutations in patients with VHL type II (i.e. specific missense mutations) are located in the alpha domain and allow a residual ability to bind Elongin C.\cite{18} It was suggested that specific missense mutations would have a dominant negative effect by sequestering key components of the ubiquitin
Genetic investigations 3.1

pathway. Missense mutations are present in 69% of VHL type II families and 27% of VHL type I families.\textsuperscript{17} Most VHL type II families have renal cell carcinoma (type IIB), but some do not (type IIA).\textsuperscript{22} A phaeochromocytoma-only phenotype (type IIC) is associated with specific missense mutations.\textsuperscript{23} Intrafamilial variability indicates that other genetic (‘modifier’ genes) and/or environmental factors are involved in the clinical manifestations of VHL gene germline mutations.\textsuperscript{24}

We report a survey of VHL germline mutations and their associated phenotypes found in families and sporadic patients with VHL disease, as well as sporadic patients with a VHL-related tumour (but not fulfilling the current diagnostic criteria for VHL disease) diagnosed in the Netherlands.

**Patients and methods**

**Patients**

Familial VHL patients and patients with at least one VHL-related tumour were referred for DNA testing by clinical geneticists, internists, neurologists, neurosurgeons, and ophthalmologists between January 1985 and August 1999. Detailed family histories were obtained for all carriers of a VHL germline mutation as well as clinical information from medical and pathological reports.

The patient population consisted of 25 VHL patients with well-documented family histories and seven VHL patients (4, 9, 16, 17, 21, 23 and 32) without a family history but who met the diagnostic criteria of sporadic VHL disease. We also included two patients (6 and 10) with a VHL germline mutation who do not fulfil the current VHL diagnostic criteria. The success rates of finding germline mutations in such sporadic patients with VHL-related tumours, e.g. central nervous system haemangioblastoma,\textsuperscript{25} renal cell carcinoma,\textsuperscript{26} and phaeochromocytoma\textsuperscript{27} are reported elsewhere. Most of these patients presented with a young age of onset, and/or with multicentric or bilateral manifestations.

**DNA analysis**

DNA of probands was extracted from 10 ml peripheral blood samples according to established procedures. Exons 1, 2 and 3 of the VHL gene and their flanking sequences were amplified using PCR, using oligonucleotides according to Gnarra et al.\textsuperscript{28} The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409).\textsuperscript{4} PCR products were purified and subjected to sequence analysis using an ABI 377 automated sequencer. When a missense mutation was found, DNAs of 50 non-VHL patients were used as control samples to investigate the possibility of a polymorphism. Probands with the same germline mutation were haplotyped with a panel of polymorphic markers linked to the VHL gene (D3S651, D3S656, D3S1038, D3S1304, D3S1317, and D3S1537) to study common ancestry.

Screening for structural rearrangements, including deletions, was performed by Southern blot analysis. DNA was digested with Eco RI,\textsuperscript{4} and with an Eco RI / Ase I double digest.\textsuperscript{17} After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL g7-cDNA probe.\textsuperscript{4} After detection of aberrant fragments the DNA was digested with at least two other restriction enzymes to
exclude the possibility of polymorphisms affecting an Eco RI or Ase I site. A human beta globin gene probe was used as an internal control to enable comparison of signal intensities in the case of apparently normal hybridisation patterns. Additionally, exon-specific probes generated by PCR amplification of exons 1, 2, and 3 were hybridised to the same filters, for further delineation of the abnormalities.

Deletions encompassing the entire VHL gene were confirmed by FISH analysis on lymphocytes from affected individuals. FISH analysis was carried out on metaphase chromosome spreads according to established procedures. The VHL cosmid-11 probe was labelled by nick translation with biotin-14-dATP. After precipitation of the labelled probe in the presence of Cot-1 DNA, pre-annealing was performed to block repetitive sequences. The final concentration of the probe was 15 ng/ml. Hybridisation of the denatured probe onto the denatured metaphase chromosomes was carried out overnight at 37°C. Each slide was mounted with 15 ml antifade medium (Vectashield, Brunschwig) containing DAPI. Microscopic analysis of images was performed using a CytoVision, Applied Imaging.

Fig.1 Southern blot analysis of eight patients with a partial VHL gene deletion. Numbers of the probands are depicted above the lanes and correspond with the numbers of Table 1. Genomic DNA (7 mg) was digested with an Eco RI and Ase I double digest and hybridised with 32P labelled probes specific for the VHL gene (g7 cDNA, upper band). The lower band represents a VHL pseudogene (ψ), located on chromosome 1.

Samples with a rearrangement in the VHL gene exhibit a less intense g7 band and an abnormal migrating band. The abnormal migrating band shows different lengths for all patients with a partial deletion, except for lane 2 and 9 (both members from family 26). Patient 9 is a carrier of a missense mutation and can be regarded as a normal control in this analysis.
Results

Mutation analysis of the VHL gene revealed a total of 34 variants. Sequence analysis revealed 13 missense mutations, three nonsense mutations, four microdeletions, three insertions, and one splice site mutation (Table 1). None of the missense mutations was found in a control panel of 50 non-VHL patients. Quantitative Southern blot analysis showed eight gene rearrangements (Fig. 1), and two entire VHL gene deletions (five of these ten are described in more detail in a manuscript in preparation). Since the gene rearrangements showed unique banding patterns on Southern blot analysis, no further investigation for shared haplotypes was undertaken. FISH analysis in patients with deletions encompassing the entire VHL gene showed a single fluorescent signal, whereas two signals at 3p were present on the chromosomal pairs of healthy family members.

32 of the 34 germline mutations in the VHL gene were found in patients with familial or sporadic VHL disease. The remaining mutations were identified in two sporadic patients (6 and 10) with a VHL-related tumour, but who did not meet the diagnostic criteria for VHL disease. A missense mutation (P81S) was found in a 47-year old woman (6) with a solitary cerebellar haemangioblastoma (diagnosed at 44 years). The P81S mutation was not identified in four relatives (father, son, sister and aunt; aged 77, 17, 43 and 64 years respectively), who were without signs of VHL-related lesions when clinically screened. Another missense mutation (V166A) was found in a 15-year old girl (10) with bilateral phaeochromocytoma diagnosed at age 11 years and a negative VHL family history.

In four (9, 10, 21 and 23) of the nine sporadic patients the VHL germline mutation was not identified in either of their parents; while the father of patient 6 shared the germline mutation with his daughter, but has no VHL-related tumours at the age of 77 years. The parents of four other sporadic patients were not available for DNA analysis. Patient 4 is an adopted child with bilateral retinal haemangioblastoma. Patient 16 (37 years old), has a negative family history for VHL disease; neither parents (~70 years old) nor seven sibs (25-50 years old) were reported to have VHL-related tumours. Patient 17 (59 years old) has a negative family history; his parents are deceased and he has no children. Patient 32 (29 years old) has seven relatives (brother, parents and great-parents) who underwent ophthalmological screening but were all negative for VHL-related ophthalmological lesions.

Table 1

<p>| No. | Novel germline mutation in the VHL gene; sporadic, sporadic patient and parents not tested (except for case 6, see text); de novo, sporadic patient and tested parents do not share the VHL germline mutation; *, patients not fulfilling clinical diagnostic VHL criteria; part. del, partial deletion of the VHL gene that has not been precisely characterised; na, not applicable. |</p>
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</table>

**VHL germline mutations in the Netherlands**
Clinical manifestations corresponded with VHL type I (without phaeochromocytoma) in 27 families. Different types of germline mutations were identified in these families, including eight missense mutations (30%), three nonsense mutations, three microdeletions, three insertions, one splice site mutation, seven partial deletions, and two entire VHL gene deletions. Phaeochromocytoma were present (VHL type II) in seven families, five of which had (specific) missense mutations (71%), one a microdeletion, and one a partial deletion of the VHL gene. The frequency of missense mutations was almost significantly different (Fisher’s exact test, p = 0.057) between VHL types I and II. Renal cell carcinoma occurred in six of the seven families with phaeochromocytoma (VHL type IIB). One sporadic 15-year old patient (number 10) had only phaeochromocytoma diagnosed at age 11 (VHL type IIC).

In addition to the P81S carriers, other families in this study suggest a reduced penetrance of VHL germline mutations. In family 1 with the R64P mutation, two patients had bilateral phaeochromocytoma (14 and 29 years), one renal cell carcinoma (48 years) and one a paraganglioma (29 years). Two other closely related carriers (55 and 34 years) of the R64P mutation had no symptoms after clinical screening so that the age of the first carrier would also suggest non-penetrance. Patient 4 (E70K), aged 25 years, only has retinal haemangioblastoma. Patient 5 (S80N), aged 15 years, only has retinal haemangioblastoma and his father, who died from cerebellar haemangioblastoma aged 37 years, was his only relative with a VHL-related lesion.

**Discussion**

We identified VHL germline mutations in 32 patients with familial and sporadic VHL disease and in two sporadic patients with a VHL-related tumour not fulfilling the currently accepted diagnostic VHL criteria. The mutation spectrum did not differ significantly from that reported in a comparable study (Fig. 2). We identified two unrelated families with the R167Q mutation. Codon 167 is a hotspot for mutations in the VHL gene (Fig. 3) and has a structurally central role in the functional domain of the VHL protein that binds Elongin C. In addition, we confirmed another recurrent VHL germline mutation (440 del TCT), which has been described at least ten times worldwide. Apparently, the presence of a tandem repeat (TCTTCT) renders this sequence susceptible to the deletion of a TCT triplet. Haplotype analysis with six polymorphic markers ruled out the possibility of common ancestry for both mutations.
This study identified eight novel germline mutations and 10 deletions (Table 1). Since the exact breakpoints of the deletions were not investigated in either the literature or this study, it remains obscure whether the deletions resemble reported ones. Recently, a new technique, ‘long PCR’, was developed that facilitates the detection of deletion breakpoints. The authors successfully sequenced deletion breakpoints in nine unrelated patients from VHL families, after a germline deletion was demonstrated with long PCR.

Four of the six missense mutations identified in the 5'-end of exon 1 of the VHL gene were associated with a mild phenotype or reduced penetrance (Table 1 and Fig. 3). In this study a P81S mutation presented in an apparently sporadic 44 year old patient with a solitary cerebellar haemangioblastoma. Four other closely related carriers (17, 43, 62 and 77 years) have no VHL-related tumours so far. Particularly, the cases of two older patients suggest reduced penetrance of the P81S germline mutation.

The P81S mutation has been reported four times previously: (1) in an isolated German patient with a full-blown VHL tumour spectrum (i.e. cerebellar and spinal HAB, renal cell carcinoma, and renal, pancreatic and epididymal cysts); (2) in a 34-year-old American patient with HAB-only; (3) in 35-year-old American patient with retinal haemangioblastoma and islet cell tumour of the pancreas, the father is the only other relative with a VHL-related tumour and had a phaeochromocytoma; (4) in an isolated Japanese patient with multiple HABs and a renal cell carcinoma. Confirmation of reduced penetrance will have implications for genetic counselling in these families and contrasts with the previous observation that penetrance of the disease is almost complete by the age of 60 years.

In addition, the R64P, E70K and S80N mutations possibly predispose to reduced penetrance. Since these missense mutations are present within the first part of the translated area of the VHL gene, they could have a mild effect on the VHL protein. In contrast, the two missense mutations in the Serine65 residue were associated with more severe phenotypes. Serine65 is mutated more frequently and has so far been described in seven families world-wide. This may indicate that this amino acid is located within an important functional domain of the VHL gene.

We identified four VHL germline mutations as definitely de novo in nine sporadic patients. The families of three further sporadic VHL patients were suggestive for de novo occurrence of the mutations. We estimate that de novo mutations in the VHL gene in the Netherlands therefore occur in least 12% (4/34) to 21% (7/34) of the identified cases of VHL disease. Data in the literature on de novo mutations are scarce. VHL germline mutations are detected. In 4-15% of the identified VHL germline mutations, the mutation was found in patients without a family history, and may represent de novo mutations or incomplete penetrance. Richards et al. studied 106 families with known VHL gene mutations, of whom 23 families were presumed to have a de novo mutation (22%). A de novo mutation in the VHL gene was identified in 16 sporadic patients (15%), while in the remaining seven cases the parents could not be tested. It is conceivable that only a small proportion of de novo mutations are recognised, since sporadic patients with a single VHL-related lesion are not routinely tested for VHL germline mutations.
Fig. 3 VHL germline mutations world-wide and in the Netherlands
VHL germline mutations were detected both in sporadic patients with two or more typical VHL lesions as well as in sporadic patients with a single VHL-related lesion. An alternative explanation for the occurrence of sporadic patients with a single VHL-related tumour may be that they represent mosaicism and therefore have a milder phenotype. Mosaicism has so far been described in only two VHL families. Moreover, independent somatic mutations may occur by chance and cause a single typical VHL-related lesion in a sporadic patient. The success rates of finding VHL germline mutations in sporadic patients with a single VHL-related lesion, e.g. central nervous system haemangioblastoma, phaeochromocytoma and renal cell carcinoma, are respectively 10%, 3% and 1.6%. Most of the sporadic patients with a VHL germline mutation present with a young age of onset, and/or with multicentric or bilateral manifestations.

In conclusion, we recommend genetic screening for VHL germline mutations not only for patients who meet the clinical diagnostic criteria, but also for sporadic patients with a VHL-related tumour who do not meet the classic diagnostic VHL criteria. These patients should be screened particularly when they present at a young age of onset, or with multicentric or bilateral tumours. We have presented evidence for non-penetrance of VHL germline mutations and found that a significant proportion of germline mutations in the VHL gene concern de novo mutations. Because the costs of DNA analysis are relatively low, the molecular genetic analysis of the VHL gene is readily feasible, and the vast majority of VHL gene mutations can be detected, we strongly recommend that sporadic patients should be analysed for VHL germline mutations. Especially, since each identified proband enables genetic counselling and clinical management for VHL disease of at risk family members.

Fig. 3 VHL germline mutations in the Netherlands (in black) and world-wide (deletions are not shown). The position of each mutation in the coding region is depicted by a symbol representing the specific mutation (see caption). The mutations are pooled per 10 nucleotides. Mutations that are located close to the intron-exon boundaries, for example splice site mutations, are placed in their exon of origin. A hotspot for VHL germline mutations is readily visible in the beginning of exon 3, or more specific at nucleotide 712/713 (codon 167). Note that mutations in the VHL gene are restricted to an area between nucleotide 376 (codon 55) and nucleotide 820 (codon 202). The solid bars below represent genomic deletions found in probands 25-34, the dotted lines characterise the possible extent of the deletions.

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


From the Departments of Medical Genetics (FJH, RAZ, TP, PLP, RBvdL) and Internal Medicine (FJH, CJML), University Medical Centre Utrecht, the Netherlands; Medical Genetics, (RHS, JV) and Internal Medicine (TPL), University of Groningen, the Netherlands; Human Genetics (EL, Gma), University of Leuven, Belgium; Medical Genetics (GMo), University Hospital Gent, Belgium; Ophthalmology (KvdT), Merwede Hospital, Dordrecht, the Netherlands; Ophthalmology (MLR), IJsselmeer Hospital, Emmeloord, the Netherlands.

Submitted
Abstract

Von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome characterised by predisposition for bilateral and multi-centric haemangioblastoma in the retina and central nervous system, phaeochromocytoma, renal cell carcinoma, as well as cysts in the kidney, pancreas and epididymis.

We describe five families where direct sequencing of the coding region of the VHL gene 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 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 that should be implemented in routine DNA diagnosis for VHL disease. In addition, our data support the previously established observation that families with a germline deletion have a low risk for phaeochromocytoma. Furthermore, families with a full or partial deletion of the VHL gene display a phenotype with a preponderance of central nervous system haemangioblastoma.
Introduction

Von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome characterised by predisposition for bilateral and multi-centric haemangioblastoma in the retina and central nervous system, phaeochromocytoma, renal cell carcinoma, as well as cysts in the kidney, pancreas and epididymis, and endolymphatic sac tumours. VHL disease is a relatively rare disorder, with an estimated birth incidence of 1/36,000. The basis of familial inheritance of the disease is a germline mutation in the VHL tumour suppressor gene, located in chromosome region 3p25-26 and identified in 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 phaeochromocytoma, but not for other VHL-related tumours. Germline mutations are found in up to 100% of the families fulfilling the clinical VHL criteria. Missense, nonsense, splice site mutations, and microdeletions and -insertions, are detected in approximately two-thirds of these families. In one-third of the VHL families, large deletions (4 - 380 kb) are found. Such deletions are demonstrated by Southern blot analysis, pulsed field gel electrophoresis, or fluorescent in situ hybridisation (FISH).

Detection of germline mutations in VHL families allows diagnosis of the disease, and also at an early or presymptomatic stage. Identification of carrier status avoids the inconvenience of intensive clinical surveillance of non-carriers. Carriers of the mutated VHL gene can be monitored closely and given the appropriate treatment.

We describe five families where direct sequencing of the coding region of the VHL gene failed to identify the family specific mutation. However, further molecular analysis revealed large 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 characterised by an absence of phaeochromocytoma and a high incidence of haemangioblastoma.

Patients and methods

Patients

The five families (A, B, C, D and 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. Clinical monitoring included annual ophthalmoscopy, yearly alternate Magnetic Resonance Imaging (MRI) and ultrasound of the abdomen, and - in variational frequencies - MRI of the central nervous system (CNS). All probands fulfilled the clinical diagnostic criteria; i.e. 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 haemangioblastoma, renal cell carcinoma, or phaeochromocytoma, in an at-risk individual.

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, 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. 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, and with an Eco RI / Ase I double digest. To confirm the results two other restriction enzymes, Hind III and Stu I were used (see Fig. 1 for restriction map). After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL g7-cDNA probe, according to the manufacturer’s instruction. The human beta globin gene was used as an internal control. Additionally, exon-specific probes generated by PCR amplification of exons 1, 2, and 3 were hybridised to the same filters.

Fig. 1 Genomic organisation 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 size of restriction fragments in kb (= 1000 base pairs). The solid bars below represent genomic deletions found in families A-E, encompassing the exons indicated. The dotted lines characterise the possible extent of the deletions.

FISH
FISH analysis was carried out on metaphase chromosome spreads according to established procedures. The VHL cosmid-11 probe was labelled by nick translation with biotin-14-dATP. After precipitation of the labelled probe in the presence of Cot-1 DNA, pre-annealing was performed to block repetitive sequences. The final concentration of the probe was 15 ng/ml. Hybridisation of the denatured probe onto the denatured metaphase chromosomes was carried out overnight at 37°C. Each slide was mounted with 15 ml antifade medium (Vectashield, Brunschwig) containing DAPI. Microscopic analysis of images was performed using a CytoVision, Applied Imaging.
Results

Clinical manifestations
No phaeochromocytoma 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 ovarian cysts. Five patients had symptoms associated with an endolymphatic sac tumour (i.e. hearing loss, tinnitus or vertigo), however, magnetic resonance imaging did not show tumours in the posterior fossa in these patients. One patient in family E had neurofibromatosis.

CNS as well as retinal haemangioblastoma were found in four of the five families: in the retina in 17 patients (50%), and in the central nervous system in 28 patients (82%).

Table 1. Genotypes and phenotypes

<table>
<thead>
<tr>
<th>Fam</th>
<th>Age (mean)</th>
<th>Origin</th>
<th>Deletion</th>
<th>Pts</th>
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</tr>
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</table>

Fam, family (unique identification number); Age, range of current age in years of affected family members; Deletion, germline mutation; Pts, number of clinically affected family members; Phaeo, phaeochromocytoma; RCC, renal cell carcinoma; cHAB, central nervous system haemangioblastoma; rHAB, retinal haemangioblastoma

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 further characterise the putative genetic alteration, Southern blot analysis was repeated using a panel of restriction enzymes, of which Hind III showed an aberrant fragment segregating with the disease (Fig. 2). Hybridisation of the Hind III blot with radiolabelled PCR products of exons 1, 2 and 3 demonstrated a deletion of exon 1 and 2. This deletion was confirmed by digesting DNA with the enzymes Bam HI, Ksp 632I and Bgl II, that 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. $Bgl\ II$ showed a normal banding pattern. This finding was also confirmed with $Stu\ I$ restriction enzyme that yields fragments of the three separate VHL exons. On Southern blot analysis diminished 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 where no mutations in the VHL gene had been detected by direct sequencing. In families B and D, Southern blot analysis using $Eco\ RI$ and hybridisation with the $g7$ probe, generated an aberrant restriction fragment (Fig. 3). This aberrant restriction fragment was recognised by probes representing exons 2 and 3. However, hybridisation with the exon 1 probe resulted in a normal banding pattern, indicating a deletion encompassing exon 1.

In the proband from family C, six different restriction enzymes ($Eco\ RI$, $Eco\ RI/\ Ase\ I$ double-digestion, $Hind\ III$, $Stu\ I$, $Dra\ I$ and $Pvu\ II$) consistently revealed a diminished band intensity of the VHL band compared to 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).

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**Fig. 2** Analysis of the segregation of an aberrant fragment with the disease. Numbers of the persons tested of family A correspond with the Southern blot analysis lanes. Genomic DNA of 11 family members and of three control persons was digested with $Hind\ III$ and revealed an aberrant banding pattern in three affected family members.
In family E, we noticed that Eco RI, as well as Hind III 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, B, C and D (Fig. 3). Surprisingly, the proband from family E showed an altered restriction fragment. Further analysis revealed a deletion of exon 1.

**Fig. 3** Constitutional VHL gene deletions identified by Southern blot analysis in five families. Genomic DNA from family A-E and control (N) digested with Eco RI (lane 1-6) and with Eco RI / Ase I (lane10-15) was hybridised with the g7 probe and a beta-globin control probe. The lambda-x-Hind III marker (lane 8) shows fragment sizes in kb (= 1000 base pairs). Aberrant bands are indicated with arrows. The bands marked with an asterisk (*) might represent a restriction site polymorphism or partially digested DNA.

**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.
Discussion
Detection of VHL gene deletions
We describe five kindreds with germline deletions of the VHL gene. In four families partial deletions removing one or more exons were readily detected by Southern blot analysis. In a fifth family, FISH analysis demonstrated deletion of the entire VHL gene. We found that these deletions are involved in 28% (5/18) of the VHL families that were referred to our department (unpublished data). Although in three families (B, D and E) the deletion involved exon 1 only, differences in the restriction fragment patterns generated by Southern blot analysis indicated that the deletions were distinct and had different breakpoints. Our results indicate 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 hybridised with g7 cDNA and a control probe is becoming the method of choice in screening for large deletions in the VHL gene. Southern blot analysis using 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. The Eco RI / Ase I double-digestion has a high resolution because it isolates exactly the coding region of the VHL gene. To further delineate the molecular nature of the deletion, the enzymes Bam HI, Ksp 632I and Bgl II, which have restriction sites in exons 1, 2 and 3, respectively, may be applied as well as hybridisation of Southern blot analysis with probes for the individual exons of the VHL gene.

When Southern blot analysis of the VHL gene, using Eco RI alone or the Eco RI / Ase I double-digestion, demonstrates a diminished band intensity, FISH analysis should confirm the deletion of one VHL allele. The presence of large deletions may also be revealed in studies involving highly polymorphic short-tandem repeat (STR)-markers. Deletions encompassing polymorphic marker loci will result in loss of specific alleles and reduced intensities for observed alleles.

Genotype-phenotype correlations
Phaeochromocytoma The five families were affected with various VHL-related tumours, except for phaeochromocytoma. This relationship between genotype and the VHL phenotype has been described in several studies. Families with a deletion or a mutation that predicts a truncated VHL protein are predominantly associated with a disease phenotype without phaeochromocytoma (VHL type I). Whereas, 69% of families with phaeochromocytoma (VHL type II) is associated with specific missense mutations. It is hypothesised that phaeochromocytoma may arise 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). Structural analysis of this complex 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. However, the dominant negative model would implicate that one ‘hit’ may be sufficient for the initiation of phaeochromocytoma tumourigenesis. This hit will probably concern a specific missense mutation, that occurs either in the
germline or as a somatic mutation. Since oncogenesis is a multi-step process, other genes must also be involved in tumourigenesis, otherwise every carrier of such a missense mutation would develop phaeochromocytoma. In addition, one would expect also very young patients with phaeochromocytoma.

In contrast, mutations found in families without phaeochromocytoma (type I) are predicted to cause a complete unravelling of the VHL structure.\textsuperscript{17} This genotype-phenotype correlation was confirmed in a comparison of phenotypes of our families with those reported in the literature,\textsuperscript{15,16} with deletions and missense mutations associated with VHL types I and II (Fig. 5). The absence of phaeochromocytoma in our families may be explained by the presence of deletions that are unlikely to result in dominant-negative VHL proteins.

![Fig. 5](image)

**Fig. 5** The frequency of patients with four types of VHL tumours associated with their genotype. This figure shows pooled data of genotypes and phenotypes in families from the present study, and in families studied by Glavac et al. and Chen et al.\textsuperscript{15,16} Phaeo: phaeochromocytoma; RCC: renal cell carcinoma; cHAB: central nervous system haemangioblastoma; rHAB: retinal haemangioblastoma. 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 since they differed significantly (p < 0.001) in their incidence of RCC compared to the two other studies.

**Renal cell carcinoma** Our families showed a relatively low frequency of renal cell carcinoma (9%), compared to other studies (41%).\textsuperscript{15,16} We therefore did not include our deletion patients in Fig. 5. Renal cell carcinoma in VHL patients occur at a mean age of 36 years,\textsuperscript{19} and the mean age, as well as the median age, of the VHL patients we studied was 47 years. The mean age of the patients in the two comparison articles was
not reported. It was hypothesised that renal lesions develop as a consequence of sev-
eral structural aberrations such as large deletions, nonsense, splice and frame shift
mutations, and insertions.\textsuperscript{16} So far, a low frequency of renal cell carcinoma has only
been reported in families (VHL type 2A) with specific missense mutations.\textsuperscript{20} (Fig. 5).

The above suggests that the relationship between germline mutation and renal
cell carcinoma in VHL appears to be rather complex. Apart from chance, the rela-
tively low frequencies of renal cell carcinoma reported in our clinically well-mon-
tored families could be due to other factors. Like retinal haemangioblastoma in VHL
patients, modifier genes\textsuperscript{21} or external factors may contribute to a renal cell carcinoma
risk (e.g. smoking is associated with a higher risk).\textsuperscript{22-24}

\textbf{Haemangioblastoma} So far, the risks of CNS haemangioblastoma in VHL disease
have not been correlated with allelic heterogeneity. Our deletion families exhibited a
phenotype with a preponderance of CNS haemangioblastoma (Table 1). This prompted
us to investigate whether phenotypes of families with VHL gene deletions differ from
families with other VHL gene germline mutations (Fig. 5). With respect to the inci-
dence of CNS haemangioblastoma, we noted that families with deletions did not sig-
nificantly differ from other types of VHL gene germline mutations; except for fami-
lies with missense mutations, who exhibited a low frequency of CNS haemangioblas-
toma. On a closer look, we identified that this relatively low frequency of CNS hae-
mangioblastoma in families with missense mutations was caused by a specific subset
of missense mutations, i.e. type IIa (Fig. 5). Thus, VHL deletion families show a
significantly (Chi-square 85, p < 1x10\textsuperscript{-10}) higher incidence of CNS haemangioblas-
toma compared to type IIa missense mutations. Apparently, VHL IIa mutations are
not only associated with a low risk for renal cell carcinoma, but also for CNS hae-
mangioblastoma.

In contrast to CNS haemangioblastoma, the risk for retinal haemangioblastoma
is comparable for all kinds of VHL gene germline mutations. Since retinal and CNS
haemangioblastoma are histopathologically identical and both arise from stromal
cells,\textsuperscript{25,26} one would also expect a relatively low frequency of retinal haemangioblas-
toma in families with type IIa mutations. However, this was clearly not the case. These
two findings (the high frequency of CNS haemangioblastoma in VHL deletion fami-
lies compared to type IIa missense mutations, whereas the risk of retinal haemangiob-
lastoma is similar for both groups) tempted us to speculate upon possible explana-
tions.

Assuming that type IIa missense mutations result in a high frequency of
phaeochromocytoma, the same mutations seem to have the opposite effect on the
incidence of CNS haemangioblastoma. However, apart from considering the different
functional effects of VHL mutations, it is clear that other factors, including tissue-
specific differences may also play a role. For instance, stromal cells in the retina could
require a different level of functional VHL protein to maintain cellular homeostasis
than stromal cells in the CNS. Also, the multi-functional VHL protein may be impli-
cated in different cellular pathways in the retina and the CNS. Moreover, there is
evidence that modifier genes play a role in the aetiology of retinal haemangioblas-
toma.\textsuperscript{21} 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 haemangioblastoma. Given that deletions of the entire VHL gene represent true null alleles, this family supports the manifestation of haemangioblastoma occurring when the VHL gene is in the hemizygous state. Although complete VHL gene deletions occur in approximately 9% of VHL families, no clinical details have been published for complete gene deletion families. Additional studies embodying carefully executed clinical analysis of patients with entire VHL gene deletions are required to test our hypothesis.

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Cryptic Von Hippel-Lindau disease: Germline mutations in haemangioblastoma-only patients


From the Departments of Medical Genetics (FJH, RBvdL, RAZ, PLP), Internal Medicine (FJH, CJML) and Neurology (MJBT), University Medical Centre Utrecht, the Netherlands; Regional Genetics Service (SMcK, PR, FMcD), Birmingham Women’s Hospital, Birmingham, UK; East Anglian Genetics Service (RMcM, DD, JW), Addenbrooke’s Hospital, Cambridge, UK; Clinical Genetics Centre Leiden (JJvdS), the Netherlands; Medical Genetics (ERM), The Medical School, University of Birmingham, UK.

Submitted
Abstract

Objective In addition to families with Von Hippel-Lindau (VHL) disease, sporadic patients with haemangioblastoma (HAB) in the central nervous system have also been found to carry VHL germline mutations. Carriers of such a mutation and their relatives have a risk of developing multiple tumours. We investigated the frequency of VHL germline mutations in HAB-only patients.

Patients and methods 84 patients with a single HAB (23 Dutch, 61 UK) and four with multiple HAB (two Dutch, two UK), with no clinical or radiological evidence of VHL disease, were studied by direct sequencing of the coding region and quantitative Southern blotting.

Results A VHL germline mutation was found in three of 84 (3.6%) single HAB patients. A germline VHL mutation was detected in a 44-year old woman with a solitary cerebellar HAB, as well as in four clinically unaffected close relatives, and in two single HAB cases presenting at ages 29 and 36 years. Germline VHL mutations were detected in two of four cases with multiple HAB.

Conclusions VHL gene mutation analysis should be offered to all HAB patients younger than 50 years. Further data is required to evaluate the detection rate in late-onset cases. The low detection rate in patients with multiple HAB may indicate the presence of somatic mosaicism or additional HAB susceptibility genes.
**Introduction**

Haemangioblastomas (HAB) are non-metastasising tumours of the central nervous system and account for about 2% of all intracranial tumours. HAB arise preferentially in the cerebellum (~75%), medulla and spinal cord (~25%). HAB in the cerebrum are rare. HAB are regarded as benign on their histopathological characteristics and do not normally invade the surrounding brain. However, complications may arise due to the tendency of HAB to form expanding cysts, leading to elevated or even life-threatening intracranial pressure. They are composed predominantly of vascular and stromal cells. The frequent presence of haemorrhages and cysts means the tumours vary in morphological appearance. Four types of HAB can be recognised macroscopically: 5% are cysts, 60% predominantly cystic, 26% predominantly solid, and 9% solid.

The standard treatment is complete microsurgical removal, aided if necessary by preoperative embolisation to reduce the tumour’s vascularity. Stereotactic radiosurgery shrinks or stops the growth of small- or medium-sized HAB. Adjoining cysts, however, do not respond to radiosurgery and require later and sometimes repeated evacuation.

HAB may occur in sporadic form or as a manifestation of Von Hippel-Lindau (VHL) disease. VHL disease is an autosomal dominant tumour syndrome with an estimated birth incidence of approximately 1:36,000. The disease is characterised by a predisposition to bilateral and multifocal tumours. The most common tumours in VHL disease are HAB in the central nervous system and retina, clear cell carcinoma in the kidney, phaeochromocytoma in the adrenal gland, endolymphatic sac tumours in the inner ear, as well as cysts in the kidney, pancreas and epididymis. 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 HAB, renal cell carcinoma, phaeochromocytoma, or multiple pancreatic cysts in an at-risk individual. In isolated cases of VHL disease, two or more HABs, or a single HAB in association with a visceral manifestation are required.

The basis of familial inheritance of VHL disease is a germline mutation in the VHL tumour suppressor gene, first identified in 1993 and located in chromosome region 3p25. In both VHL disease and sporadic HAB, allelic losses and mutations of the VHL tumour suppressor gene affecting stromal cells have been found, suggesting that stromal cells represent the neoplastic component of a HAB. In addition, it was demonstrated that vascular endothelial growth factor (VEGF) is upregulated in stromal cells as a consequence of mutations in the VHL gene.

VHL disease demonstrates variable expression, age-dependent penetrance, and a low but consistent new mutation rate. The diagnosis of VHL disease should be considered in all patients with a HAB, as early recognition of a predisposition to develop further HAB and other tumours (e.g. renal cell carcinoma) may reduce morbidity and mortality. The diagnosis of ‘new mutation’ VHL cases is frequently delayed because at least two typical manifestations are required, whereas molecular genetic diagnosis of VHL disease offers the potential to detect subclinical cases of VHL disease in sporadic patients with a single HAB. VHL disease demonstrates complex genotype-phenotype correlations. Most VHL gene mutations predispose to HAB,
but specific missense mutations may cause high or low risks for renal cell carcinoma or phaeochromocytoma. \(^{19-23}\) In addition, rare missense mutations may produce a phaeochromocytoma-only phenotype. \(^{24-26}\) This suggests that specific VHL gene mutations might cause a HAB-only phenotype.

To investigate the genetic epidemiology of HAB in the central nervous system, we performed an international multicentre study of patients with single HAB and multiple HAB without evidence of VHL disease (i.e. HAB-only). As the mean age of VHL patients with HAB is significantly younger than that for sporadic cases, 29 vs 48 years \(^{27}\) (or 33.5 vs 43.6 years \(^{28}\)), we directed our study towards younger patients with single HAB as these present the most difficult diagnostic problems in clinical practice.

### Patients and methods

#### Patients

We investigated two groups of patients with HAB-only in the central nervous system. Group 1 consisted of 61 UK and 23 Dutch HAB patients with a single HAB. These cases were ascertained with the help of neurosurgeons, neurologists, internists and clinical geneticists. In addition to DNA analysis, all patients underwent clinical examinations for detection of VHL associated tumours (ophthalmological examination and abdominal sonography or MRI) with negative findings. Group 2 consisted of four patients with multiple HABs, but no other evidence of VHL disease (i.e. absence of further VHL-related tumours) on clinical screening and radiological screening. All patients had histopathologically confirmed HAB at operation.

### Age at diagnosis

Between 1973 and 1996 a total of 182 HAB patients were reported to the National Dutch Pathological Archive (Palga). Figure 1 shows the age at diagnosis of Palga patients as well as studied patients. Details of age distribution of a previous population based cohort of UK HAB patients have been reported previously. \(^{27}\)

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**Fig. 1** Number of patients and age at diagnosis for:

- 182 patients with haemangioblastoma (HAB) in central nervous system (CNS) reported in the National Dutch Pathological Archive (Palga), between 1973 and 1996.
- 23 Dutch patients in this study with a single HAB, between 1996 and 1999.

The x-axis represents the age at diagnosis, the y-axis represents the number of patients with haemangioblastoma.
In 1996, guidelines were distributed via the Dutch newsletter for neurologists on screening all patients with a HAB for a VHL germline mutation. The Dutch patients in the present study were referred for DNA diagnosis from 1996 to 1999, and the mean age at diagnosis was 37.5 years (range 14-71 years). Compared to unselected cases, the age distribution in the UK as well as in the Dutch cases in this study was biased towards an earlier age at onset.

**DNA analysis**

High molecular weight DNA of the probands was isolated from peripheral blood according to established procedures. Exons 1, 2 and 3 of the VHL gene and their immediately flanking sequences were amplified using the polymerase chain reaction. The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409). PCR products were purified and subjected to sequence analysis using either an ABI automated sequencer or the dideoxy-chain termination reaction with a pUC-sequencing kit (Boehringer Mannheim, Mannheim, Germany), using g-35S dATP (600 Ci/mmol). The amplification primers were used as primers in the sequencing reactions. In the UK cases, intragenic mutations were also sought by Single-strand conformation polymorphism (SSCP).

Screening for genetic rearrangements and deletions was performed by Southern blot analysis, or a novel PCR-based deletion assay (Dow et al. in preparation). In Southern blot analysis DNA was digested with Eco RI alone or with Eco RI and Ase I double digest. After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL g7-cDNA probe, according to the manufacturer’s instruction. Quantitative Southern blotting was performed by hybridising genomic DNA with the VHL g7-cDNA probe and with a beta-globin probe, to detect deletions encompassing the entire VHL gene.

![Figure 2](image-url)  
**Fig. 2** Distribution of single HAB patients by age at diagnosis.  
dark = UK  
light = Dutch  
The x-axis represents the age at diagnosis, the y-axis represents the number of patients with haemangioblastoma.
Results

Single HAB

84 patients with single HAB in the central nervous system (23 Dutch, 61 UK) were investigated. The age distribution of the studied patients is shown in Figure 2. VHL germline mutations were identified in three patients (see Table 1). The incidence of a VHL germline mutation in single HAB patients younger than 30 years was 4.3% (1/23), 30-39 years was 4.5% (1/22), 40-49 years was 4.2% (1/24) and age 50 years or older was 0% (0/15).

Details of the three germline mutations identified were:
(a) a C to T transition at nucleotide 454 was detected in a 47-year old woman (D24) with a single cerebellar HAB, diagnosed at an age of 44 years (Fig. 3). This missense mutation leads to a change of Proline to Serine at codon 81 (P81S) in the VHL protein. DNA analysis of other close relatives revealed that four clinically unaffected first and second degree relatives (age 17-77 years) were also carriers of a P81S germline mutation;
(b) a 7 basepair frameshift mutation (del 582 GACACAC) was detected in a 29-year old patient (B1) with a single cerebellar HAB with no family history and no evidence of VHL disease on clinical and radiological screening. However, she subsequently developed pancreatic cysts at age 34 years;
(c) a large germline deletion was identified by Southern blot analysis in a 36-year old woman (B2) with a single cerebellar HAB but no other features of VHL disease.

Two patients with a single HAB developed some additional features of VHL disease during the study: one patient (B1) with a VHL gene mutation (del 582 GACACAC) developed pancreatic cysts (see above) and one patient (B3) developed renal cell carcinoma aged 44 years following a cerebellar HAB at age 40 years, but no VHL mutation was identified.

Table 1 Summary of results

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<th>Mutation</th>
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</table>

Pat, patient’s unique identification number; Age, age at diagnosis, in years; HAB, type and origin of haemangioblastoma; Clinical features, Other, further VHL associated manifestations after clinical screening; Fam Hist, family history, further VHL associated manifestations in family members of proband; Mutation, VHL germline mutation; Previously reported, whether the mutation has been published (references); n/a, not applicable.
Multiple HABs
Four patients with multiple HABs (two Dutch and two UK) and without additional VHL-related tumours were analysed for VHL germline mutations. Deletions were detected in two patients: (a) a male (B5) with one cerebellar and one medullary HAB at age 40 years; and (b) a male (B6) with multiple spinal HABs at age 44 years. Germline mutations were not identified in the two Dutch patients (D13 and D32) with both cerebellar and spinal HABs (ages 44 and 66 years).

Discussion
We found that the overall risk for finding a VHL germline mutation in a population of 84 patients with a single HAB in the central nervous system and no further features of VHL disease at the time of diagnosis was approximately 4%. In clinical studies of sporadic patients with a HAB it was suggested that a substantial proportion of HAB could be associated with VHL disease upon more detailed examination; i.e. 23% to 34.3% was found to be afflicted with VHL disease. Molecular genetic analysis of the VHL gene indicated that sporadic patients with a HAB have a risk of a VHL gene germline mutation of approximately 10%. The lower detection rate in our cases, despite use of more sensitive methods of VHL gene analysis, is presumably related to better clinical and radiological screening prior to entry into our study. The identification of a VHL germline mutation has important implications for the risk of further tumours and for the risk of VHL disease in relatives.

Statistical analysis of the age at onset of HAB in VHL disease and non-VHL cases (based on clinical criteria) is consistent with a one- and two-hit tumourigenesis model as predicted by the Knudson hypothesis. Mean age at diagnosis of cerebellar HAB in VHL disease is younger than in sporadic cases (29 years versus 48 years, respectively) so we anticipated a higher incidence of unsuspected VHL gene mutations in early onset cases. Older patients with VHL gene mutations would be more likely to manifest other evidence of VHL disease and so be excluded from our study. Thus, although our results were broadly compatible with this hypothesis, the identification of germline VHL gene mutations in two of a group of 46 patients aged 30-49 years with a single HAB and no clinical or radiological features of VHL disease suggests that molecular genetic analysis should be employed in all single HAB patients younger than 50 years. For older onset patients the frequency of germline mutations is likely to be less and larger research-based studies are required to define the risks more precisely.

A major strength of the present study was the use of recently developed techniques to detect large germline deletions. Prior to the introduction of these techniques, there was a VHL germline mutation detection rate (with Southern blotting and sequencing of the coding region) of approximately 80% in known VHL families. However, methods to detect large deletions (e.g. quantitative Southern blotting) have significantly increased the detection rate, reaching 100% in proven familial VHL disease. Therefore a conservative estimate of the mutation detection sensitivity of the strategy used in this study would be in the order of 95%. Although other studies of sporadic patients with a HAB have used less sensitive techniques, it is of interest that Oberstrass et al. detected a germline mutation in 2 of 20 patients (aged 18 and 40
years) with HAB of the central nervous system (although no data were available about a possible family history of VHL disease). Decker et al.\textsuperscript{34} reported a 29-year-old patient with recurrent spinal HAB, a negative family history of VHL disease and a \textit{de novo} frameshift VHL gene exon 2 mutation. However, this case differed from any of those in our study because a renal mass and pancreatic and renal cysts were detected on clinical screening. In a series of 18 sporadic patients with a HAB, Olschwang et al.\textsuperscript{33} found a missense mutation in two patients (42 and 56 years of age) without clinical investigations revealing any evidence of VHL disease. The latter case would only have been detected by also screening single HAB patients aged over 50 years for a VHL gene mutation.

Although specific missense VHL mutations may cause a phaeochromocytoma-only phenotype,\textsuperscript{24-26} we did not find unequivocal evidence for VHL gene mutations that would predispose to a HAB-only phenotype. However, the VHL germline mutation (P81S) that was found in a 47-year-old woman with a solitary HAB and in four clinically unaffected family members was associated with an unusually low penetrance within this family. To reduce the possibility of a genetic polymorphism, 50 non-VHL patients were sequenced. Sequence analysis of exon 1 demonstrated that all persons were homozygous for nucleotide 545C (data not shown). Phenotypic expression in VHL disease is influenced by allelic heterogeneity, stochastic events and genetic modifiers.\textsuperscript{35}
The P81S mutation has been reported four times previously: 1) in an isolated German patient with a full-blown VHL tumour spectrum (i.e. cerebellar and spinal HAB, renal cell carcinoma, and renal, pancreatic and epididymal cysts); 2) in a 34-year-old American patient with HAB-only; 3) in 35-year-old American patient with retinal haemangioblastoma and islet cell tumour of the pancreas, the father is the only other relative with a VHL-related tumour and had a phaeochromocytoma; 4) in an isolated Japanese patient with multiple HABs and a renal cell carcinoma. These findings suggest that P81S mutation carriers in the family are also at risk of renal cell carcinoma. Moreover, only one of the P81S carriers had affected family members, which may imply that this missense mutation has a low penetrance.

Interestingly, we did not find VHL germline mutations in two of the four patients with multiple HABs. As the presence of two or more retinal or cerebellar HAB satisfies the strict diagnostic criteria for VHL disease, this was an unexpected finding in the light of the high sensitivity of the mutation detection methods used, and could perhaps indicate additional HAB susceptibility gene(s). Alternative explanations would include a mutation in part of the VHL gene not analysed (e.g. regulatory domain) or somatic mosaicism. In this context it is interesting, that the two multiple HAB patients with germline mutations had the earliest age at onset and the patients with later onset may be mosaic and so have a milder phenotype or represent pheno-types (independent mutation events giving rise to various HAB could also be expected by chance). Although mosaicism has so far been described in only two VHL families, it is frequent, for example, in neurofibromatosis type 2.

We have demonstrated that VHL gene mutation analysis facilitates the management of sporadic patients with HAB and should be performed in patients younger than 50 years with a single HAB even if there is no other clinical or radiological evidence of VHL disease. Although the detection rate in older patients should be lower, we suggest that such patients need to be studied on a research basis, using the latest mutation detection strategies to define cost–benefit consequences for molecular genetic analysis of this group of patients.

Acknowledgements
The VHL g7-cDNA probe was kindly provided by I. Kuzmin, Frederick, MD, USA. The beta-globin probe was kindly provided by C. Stolle, Philadelphia, PA, USA. We thank Dr. H.P.H. Neumann, Freiburg, Germany; Dr. C. Stolle, Philadelphia, PA, USA; Dr. G. Glenn, Rockville, MD, USA; Dr. Masahiro Yao, Yokohama, Japan, for clinical data of P81S carriers.

References


Absence of VHL germline mutations in patients with phaeochromocytoma-only: implications for clinical management


From the Departments of Medical Genetics (FJH, RAZ, RBL, PLP) and Internal Medicine (FJH, CJML), University Medical Center Utrecht, the Netherlands;
Abstract

Objective Phaeochromocytoma may occur in sporadic forms or as a manifestation of Von Hippel-Lindau (VHL) disease. Germline mutations in the VHL gene are detected in virtually all well-defined families. In addition to VHL families, sporadic patients with VHL-related tumours have been found to carry germline mutations in the VHL gene. Because carriers of a VHL germline mutation and their relatives risk developing multiple tumours, we investigated the frequency of VHL germline mutations in patients with phaeochromocytoma-only.

Patients and methods Between 1996 and 1999, 24 probands (14 with solitary tumours, 7 with multiple, bilateral or recurrent tumours and 3 with familial phaeochromocytoma) were investigated. Mutation screening of the VHL gene was performed by direct sequencing of the coding region and quantitative Southern blot analysis.

Results VHL germline mutations were not found in any proband of the solitary phaeochromocytoma group (mean age at diagnosis 49 years, range 17-70 years), nor in any of the multiple phaeochromocytoma (mean age at diagnosis 37 years, range 19-64 years) or familial phaeochromocytoma groups.

Conclusions VHL germline mutations were not found in 24 probands with phaeochromocytoma, even when features suggesting a germline mutation (early onset, multiple, recurrent, bilateral or familial tumours) were present. However, the absence of VHL germline mutations in these phaeochromocytoma patients may indicate the possible occurrence of somatic mosaicism or the presence of additional phaeochromocytoma susceptibility genes. Since mutation analysis of the VHL gene detects germline mutations in virtually all well-defined VHL families, we conclude that annual clinical monitoring for further VHL-related tumours in patients with phaeochromocytoma and without a VHL germline mutation should not be recommended.
**Introduction**

Phaeochromocytoma are neuro-endocrine tumours arising from chromaffin cells in the medulla of the adrenal gland. They may also occur in the ganglia of the autonomic nervous system at extra-adrenal sites and in this case are called paraganglioma. Functioning tumours usually secrete the catecholamines norepinephrine and epinephrine, and may cause palpitations, chest discomfort, sweating attacks, hypertension or paroxysmal unstable blood pressure, and headache.

Diagnosis is based on biochemical tests and radiology. Laboratory tests may include evaluation of catecholamines in serum as well as in urine. Measurement of plasma normetanephrine and 24-hour urinary norepinephrine excretion are the most sensitive tests. Radiology testing may include ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI) and metaiodobenzylguanidine (MIBG) scintigraphy. T2-weighted MRI demonstrates high signal intensity for phaeochromocytoma in 95-100% of the cases. MIBG is sensitive for 75-95% of phaeochromocytoma and is 100% specific, but may not depict very small lesions.

Phaeochromocytoma may occur both in sporadic and in familial forms. Familial manifestation of phaeochromocytoma may occur in hereditary neoplastic syndromes including Von Hippel-Lindau (VHL) disease, multiple endocrine neoplasia type IIA and IIB (MEN IIA and MEN IIB) and neurofibromatosis type I (NF-I). In 1993, a study by Neumann showed, upon more detailed examination, that 19 of 82 patients (23%) with phaeochromocytoma had either VHL disease (19%) or MEN II (4%). Pooled data from three more recent studies indicated that 13 of 133 patients with phaeochromocytoma (10%) were familial cases. These cases could be associated with either VHL disease (n=1), MEN IIA (n=1), MEN IIB (n=5), NF-I (n=3) or genuine familial phaeochromocytoma (n=3).

VHL disease is an autosomal dominant tumour syndrome with an estimated birth incidence of 1/36,000. The disease is characterised by a predisposition for bilateral and multi-centric tumours. The most common tumours in VHL disease are haemangioblastoma in the central nervous system and retina, clear cell carcinoma in the kidney, and phaeochromocytoma in the adrenal gland. VHL patients are enrolled on an annual screening programme to enable early detection (and treatment) of these tumours. The basis of familial inheritance of VHL disease is a germline mutation in the VHL tumour suppressor gene located in chromosome region 3p25-26.

The mean age at diagnosis in VHL patients with phaeochromocytoma is approximately 28 years, with the youngest reported patient being five years old. In VHL patients, phaeochromocytoma often remain quiescent or produce few symptoms, and biochemical tests may reveal normal results. However, the behaviour of phaeochromocytoma remains unpredictable: biologically inactive lesions may suddenly become dangerous, benign phaeochromocytoma may become malignant. About 5% of VHL patients die from phaeochromocytoma-induced endogenous catecholamine intoxication, which has also caused fatal pregnancy outcome (for the mother and/or the child).

Adrenalectomy is the standard treatment for phaeochromocytoma. Satisfactory results have been reported from laparoscopic removal of adrenal tumours, and also in VHL patients. Since bilateral tumours develop in 47% of VHL patients with...
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phaeochromocytoma, most patients become steroid-dependent upon bilateral adrenalectomy.\textsuperscript{20} Enucleation rather than adrenalectomy is therefore recommended by an increasing number of surgeons.\textsuperscript{20,21} Adrenal-sparing surgery is safe, effective and can preserve adrenal function in VHL patients.

Since the identification of the VHL gene in 1993, studies of the disease have intensified and clinical studies have suggested that the number of phaeochromocytoma patients associated with a hereditary neoplastic syndrome has diminished from 23\% in 1993 to 10\% in 1998.\textsuperscript{1,5-7} We think that the putative pool of phaeochromocytoma patients possibly affected by VHL disease has decreased as the number of VHL families being identified has increased in the past few years. We therefore investigated the prevalence of VHL germline mutations in 24 probands with phaeochromocytoma-only, with the purpose of identifying new cases with VHL disease.

Patients and methods

Patients

Between 1996 and 1999, 24 patients with phaeochromocytoma-only were referred for VHL mutation analysis by endocrinologists, internists and clinical geneticists. In addition to DNA analysis, all patients underwent clinical examination for detection of VHL-associated tumours (ophthalmological examination and abdominal sonography or MRI). At the time of referral, clinical screening revealed no evidence that any of the patients had VHL disease. Cases 22, 23 and 24 had other family members with phaeochromocytoma but none of the probands’ family members exhibited other VHL-related lesions. All patients had been operated for phaeochromocytoma, later confirmed histopathologically.

![Fig. 1 Number of patients and age at diagnosis for: 395 patients with phaeochromocytoma reported in the National Dutch Pathological Archive (Palga), between 1973 and 1996. 21 sporadic patients in this study with a one or more phaeochromocytoma, between 1996 and 1999. The x-axis represents the age at diagnosis, the y-axis represents the number of patients with phaeochromocytoma](image)

The Dutch Pathological Archive (Palga)

Between 1973 and 1996, a total of 395 phaeochromocytoma patients were reported in the Dutch Pathological Archive (Fig. 1). The mean age at diagnosis of these patients was 46 years. Figure 1 demonstrates that the patients studied (mean age at diagnosis 45 years, range 17-70 years) are a representative selection of phaeochromocytoma patients by age at diagnosis.
DNA analysis
High molecular weight DNA of the probands was isolated from peripheral blood according to established procedures. Exons 1, 2 and 3 of the VHL gene and their immediately flanking sequences were amplified using the polymerase chain reaction (PCR), using oligonucleotides according to Gnarra et al.\textsuperscript{22} The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409).\textsuperscript{9} PCR products were purified and subjected to sequence analysis using an ABI 377 automated sequencer. The amplification primers were used as primers in the sequencing reactions.

Screening for genetic rearrangements and deletions was performed by Southern Blot analysis. DNA was digested with Eco RI alone or with Eco RI and Ase I double digest. After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL g7-cDNA probe,\textsuperscript{9} (kindly provided by I. Kuzmin, Frederick, MD, USA) according to the manufacturer’s instruction. Quantitative Southern blotting was performed by hybridising genomic DNA with the VHL g7-cDNA probe and with a beta-globin probe (kindly provided by C. Stolle, Philadelphia, PA, USA), to detect deletions encompassing the entire VHL gene.

Results and discussion
We found no VHL germline mutations in all the studied probands with phaeochromocytoma-only (table 1). However, our study included at least eight cases that are associated with a potentially higher risk for a hereditary tumour syndrome. These eight cases were the three patients with bilateral phaeochromocytoma (age 24, 28, 30 years), patient number 8 with a solitary tumour at an early age (17 years), patient number 21 with recurrent tumours at an early age (19 years), and three cases of familial phaeochromocytoma. VHL-associated phaeochromocytoma differ from sporadic phaeochromocytoma in having multiple or bilateral tumours and an average manifestation two decades earlier (27 years versus 46 years, respectively).\textsuperscript{1} Based on Knudson’s two-hit theory, patients with a germline mutation in a tumour suppressor gene are predisposed to multi-centric tumours that are likely to manifest at a younger age than in sporadic patients (since the first hit has already taken place).\textsuperscript{33} Therefore, familial phaeochromocytoma and also childhood cases show a tendency to multi-centricity and recurrence of tumours. Consequently, patients and at risk family members have to undergo life-long surveillance. It is important to ascertain whether a patient has an associated hereditary syndrome in order to monitor not only phaeochromocytoma, but also further lesions associated with that disease.

Six other studies have also investigated the genetic epidemiology of (familial) phaeochromocytoma (Table 2).\textsuperscript{24-29} 21 VHL germline mutations but no RET gene mutations were found in a total of 225 observations. However, we would like to comment on two of the VHL gene mutations found in one study.\textsuperscript{27} First, the P25L mutation is probably not a disease-causing mutation. So far, no VHL germline mutations have been described in VHL families upstream of the start site located at codon 54.\textsuperscript{30,31}
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Second, the R64P mutation, found in two related patients (uncle and nephew) should be considered as one case of familial phaeochromocytoma. Consequently, the presence of three patients with a VHL germline mutation in 66 sporadic patients with solitary phaeochromocytoma (i.e. 5%) is in better agreement with the other studies (table 2). Joint data demonstrate that seven out of 17 cases with familial phaeochromocytoma (41%) had a VHL germline mutation. In addition, case records have been published of VHL germline (missense) mutations in familial phaeochromocytoma. These cases suggested a distinct VHL phenotype, i.e. VHL type IIC.

Table 1 Age at diagnosis and type of phaeochromocytoma

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<td>Bilateral</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>42</td>
<td>Multiple</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>62</td>
<td>52</td>
<td>Multiple</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>64</td>
<td>Recurrent</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>37</td>
<td>19</td>
<td>Recurrent</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>9</td>
<td>64,??</td>
<td>Familial, three brothers</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>80</td>
<td>57,59</td>
<td>Familial, mother and daughter</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>31</td>
<td>23,32,44</td>
<td>Familial and bilateral, two brothers and uncle</td>
<td>-</td>
</tr>
</tbody>
</table>

Pat, patient number; Unr, unique identification number; Age, age at diagnosis; RET, germline mutation in the RET proto-oncogene.

13 of 23 probands were screened for RET mutations and were all negative (-)

Mean age at diagnosis in solitary phaeochromocytoma is 49 years; in the bilateral, multiple and recurrent group it is 37 years old.
Nearly all the mutations (20 out of 21) identified in phaeochromocytoma patients from these six reports concerned missense mutations. A hot spot was located at and around codon 167 of the VHL gene, confirming previously established genotype-phenotype correlations in VHL disease.\textsuperscript{30,31} There is evidence that the presence or absence of phaeochromocytoma is correlated with the type of VHL germline mutations.\textsuperscript{30,31} It was suggested that especially specific missense mutations would lead to phaeochromocytoma by a dominant negative effect of the mutated VHL protein.\textsuperscript{33} However, besides this clear interfamilial difference, intrafamilial differences have also been observed, suggesting that genetic or environmental modifiers play a role in the manifestation of VHL disease.\textsuperscript{34}

Table 2: Comparative studies analysing the genetic epidemiology of patients with phaeochromocytoma-only.

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>n</th>
<th>VHL</th>
<th>Mutation (age)</th>
<th>RET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar</td>
<td>sporadic</td>
<td>24</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Brauch</td>
<td>sporadic</td>
<td>62</td>
<td>2 (3%)</td>
<td>codon167 (33), splice site codon 155 (63)</td>
<td>0</td>
</tr>
<tr>
<td>Giraud</td>
<td>sporadic</td>
<td>11</td>
<td>0</td>
<td>-</td>
<td>n.t.</td>
</tr>
<tr>
<td>v/d Harst</td>
<td>sporadic</td>
<td>68</td>
<td>6 (9%)</td>
<td>P25L (38)\textsuperscript{a}, L63P (26), R64P (24)\textsuperscript{b}, G144Q (39), I147T (58)</td>
<td>n.t.</td>
</tr>
<tr>
<td>Hes</td>
<td>sporadic</td>
<td>14</td>
<td>0</td>
<td>-</td>
<td>0 (6/14 tested)</td>
</tr>
<tr>
<td>Bar</td>
<td>bilateral</td>
<td>3</td>
<td>1 (33%)</td>
<td>R161Q (13)</td>
<td>0</td>
</tr>
<tr>
<td>Giraud</td>
<td>bilateral</td>
<td>5</td>
<td>4 (80%)</td>
<td>Y98H, L129P, R167Q, V170G (m=17.5)\textsuperscript{c}</td>
<td>n.t.</td>
</tr>
<tr>
<td>Woodward</td>
<td>bilateral</td>
<td>2</td>
<td>1 (50%)</td>
<td>R167W (?)</td>
<td>0</td>
</tr>
<tr>
<td>Woodward</td>
<td>multiple\textsuperscript{d}</td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Hes</td>
<td>multiple</td>
<td>7</td>
<td>0</td>
<td>-</td>
<td>0 (5/7 tested)</td>
</tr>
<tr>
<td>Crosse\textsuperscript{e}y</td>
<td>familial</td>
<td>3</td>
<td>2 (67%)</td>
<td>V81L, R167Q</td>
<td>0</td>
</tr>
<tr>
<td>Giraud</td>
<td>familial</td>
<td>6</td>
<td>2 (33%)</td>
<td>P97L, R167Q</td>
<td>n.t.</td>
</tr>
<tr>
<td>Woodward</td>
<td>familial</td>
<td>8</td>
<td>3 (38%)</td>
<td>S80G, R161Q, R167W</td>
<td>0</td>
</tr>
<tr>
<td>Hes</td>
<td>familial</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>0 (2/3 tested)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>225</td>
<td>21</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

This table includes patients with phaeochromocytoma from our study and those reported in the literature.\textsuperscript{24-29} Patients, type and (n)umber of patients studied; VHL, number of patients with a VHL germline mutation; RET, RET proto-oncogene germline mutation (exons 10 and 11 tested and sometimes also exons 13 and 16); n.t., not tested; P25L\textsuperscript{a}, so far, no VHL germline mutations have been described in VHL families upstream of codon 54; R64P\textsuperscript{b}, found in two related (uncle and nephew) patients; (m=17.5)\textsuperscript{c}, mean age at diagnosis was 17.5 years; multiple phaeo\textsuperscript{d} were all cases of multiple extra-adrenal or adrenal phaeochromocytoma with a family history of neuro-ectodermal tumours.
The fact that we did not detect VHL mutations in our phaeochromocytoma patients was to be expected given the relatively low number investigated. VHL germline mutations are detected in virtually all well-defined VHL families.\textsuperscript{31} A conservative estimate of the mutation detection sensitivity of the strategy used in this study would therefore be in the order of 95\%. Moreover, since most VHL germline mutations in phaeochromocytoma patients concern missense mutations, these are not likely to be missed easily. This would suggest that as well as familial phaeochromocytoma associated with VHL disease (approximately 41\%), there is also room for familial cases with a distinct genetic basis. From this point of view, our case 24 is of major interest, since it shows multiple features (i.e. young age at diagnosis, bilateral and familial tumours) that would indicate a germline mutation. This type of family would be an appropriate candidate for a genome-wide screening to identify candidate genes associated with the inheritance of phaeochromocytoma. Alternative explanations would include a mutation in part of the VHL gene not analysed (e.g. regulatory domain) or somatic mosaicism. Although mosaicism has so far been described in only two VHL families.\textsuperscript{31}

We have demonstrated that sporadic patients with solitary phaeochromocytoma and even those exhibiting aspects normally regarded as caused by a hereditary mutation (early onset, multiple, recurrent, bilateral or familial tumours) could not be associated with VHL germline mutations. However, we still advise genetic screening for VHL mutations in all cases with possibly hereditary features since the costs are relatively low, the molecular genetic analysis of the VHL gene is readily feasible and the vast majority of VHL mutations can be detected. Annual clinical screening for further VHL-related tumours in patients with phaeochromocytoma-only and a negative test for VHL germline mutations seems not recommended. Since each identified proband may provide appropriate clinical management for possibly affected family members, molecular testing is likely to be cost-effective.\textsuperscript{25,35} Moreover, the absence of germline VHL gene mutations in cases showing strong hereditary features may indicate the occurrence of somatic mosaicism for VHL mutations or, alternatively, suggest that other phaeochromocytoma susceptibility loci exist.

References
Patients with phaeochromocytoma-only