ONLINE MUTATION REPORT

High frequency of novel germline mutations in the *VHL* gene in the heterogeneous population of Brazil

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J Med Genet 2003;**40**:e31(http://www.jmedgenet.com/cgi/content/full/40/3/e31)

Von Hippel-Lindau disease (VHL) (MIM 193300) is a heritable autosomal dominant disease characterised by predisposition to the development of a combination of benign and malignant tumours affecting multiple organs. The main features of the disease include retinal angiomas (RA), haemangioblastomas of the central nervous system (HB), clear cell renal carcinomas (RC), phaeochromocytomas (PH), multiple renal and pancreatic cysts, adenomas and carcinomas of the pancreas, neuroendocrine tumours, endolymphatic sac tumours, and papillary cystadenomas of the epididymis and broad ligaments.[1–5] Reported birth incidence ranges from 1:36 000 to 1:45 000.[6–8] Penetrance is essentially complete at 65 years of age and subjects at risk may develop a combination of clinical manifestations during their lifetime.[6]

Usually the disease presents with a family history, but de novo mutations have been reported in as many as 23% of VHL patients.[9] In general, the incidence of HB, AR, RC, and PH are approximately 60.2%, 41%, 25.3%, and 14.5%, respectively.[7] The diagnostic criteria for VHL are based on the presence of RA, HB, visceral lesions (RC, PH, or multiple pancreatic cysts) and a family history of similar lesions. If a family history of VHL disease is present, only one RA, HB, or visceral lesion is required to make the diagnosis of VHL. However, the presence of two or more RA/HB or one RA/HB and a visceral lesion is required in cases without a family history.[6]

The *VHL* gene was mapped to 3p25-26[10] and cloned in 1993 by an international cooperative study.[11] The gene was shown to be mutated in the germline of VHL patients and to be inactivated in most sporadic tumours associated with VHL.[12–21] The coding sequence of the *VHL* gene spans three exons and has two transcriptional start codons that result in protein products (pVHL) of 213 and 160 amino acid residues, respectively.

pVHL is multifunctional and is associated with the inhibition of angiogenesis, cell cycle exit, extracellular fibronectin matrix assembly, and proteolysis.[22 23] It forms a multimeric complex that contains, at minimum, elongin B, elongin C, Cul2 and Rbx1 (VCB), and which controls the stability and degradation of the α and β subunits of hypoxia inducible transcription factor 1 (HIF-1) in response to available oxygen levels.[24] Wild type pVHL polyubiquitinates HIF-1 subunits when oxygen is available, targeting them for degradation by the 26S proteasome. Mutation in the *VHL* gene results in constitutive expression of many HIF-1 inducible genes, including vascular endothelial growth factor (*VEGF*), erythropoietin (*EPO*), and platelet derived growth factor B chain (*PDGF-B*).[25–27] In addition, it has recently been shown that pVHL plays a crucial role in cytoskeletal organisation, focal adhesion formation, and motility.[28]

Several groups in North America, Europe, and Japan have collectively reported more than 224 distinct intragenic germline mutations (HGMD online), although the frequency of mutation detection has varied considerably among studies.[12 17 28–32] However, the recent improvement in mutation detection methodologies has raised this rate to virtually 100% in confirmed VHL patients.[33]

Phenotypes vary among families and reflect genotypic differences.[31 32 34 35] Currently, VHL is subclassified into type 1 and type 2 according to the low or high risk of developing PH, respectively. Type 2 VHL is subclassified into type 2A (low risk for RC and high risk for HB), type 2B (high risk for RC and HB), and type 2C (with familial PH).[3 25] As long as VHL type 2 is associated with missense mutations in more than 90%, most type 1 VHL families present large deletions or protein truncating mutations.[12 29 30 36]

Most deleterious *VHL* germline mutations damage the pVHL-elongin C binding α domain or affect the HIF-1 binding site at the pVHL β domain.[37] Mutations associated with RC cause complete HIF-1 deregulation and loss of p220 (fibronectin) binding, whereas the products of such type 2C VHL alleles retain the ability to downregulate HIF-1 but are defective for promotion of fibronectin matrix assembly, suggesting that loss of other pVHL functions are necessary for PH susceptibility.[38–40]

Key points

- Von Hippel-Lindau (VHL) disease (MIM 193300) is an autosomal dominant disorder caused by germline mutations in the *VHL* gene that predisposes to development of retinal and central nervous system haemangioblastomas, renal cell carcinomas, pancreatic tumours, phaeochromocytoma, as well as multiple cysts of the kidneys and pancreas.
- We report here the analysis of 20 patients with VHL, mainly from Brazil (17 familial and three without a family history), in whom we detected germline *VHL* gene mutations in every case using a combination of direct sequencing and quantitative Southern blotting.
- The mutations consisted of 16 point mutations (nine missense, three frameshift, one in frame deletion, two splice defects, and one nonsense mutation), three partial deletions, and one complete deletion of the *VHL* gene. As observed in other populations, the presence of PH (type 2 phenotype) was associated with the presence of a full length but mutated protein, whereas VHL without PH (type 1 phenotype) resulted from a truncated protein. Point mutations tended to concentrate within exons 1 and 2 (87.5%), a high proportion of which were novel (50%).
- The molecular analyses of germline mutations allowed presymptomatic identification of affected relatives, the early diagnosis of VHL in cases without complete clinical criteria, the characterisation of de novo cases, and the individualisation of risks and screening programmes based on the associated phenotype.
- This is the first study of VHL in Brazil and has contributed eight novel germline mutations in the *VHL* gene. We suggest that the Brazilian population (mostly of Portuguese origin) may also have unique features in respect to *VHL* mutations and risk of malignancies.

Here, we report the nature of *VHL* gene mutations in 20 consecutive VHL patients belonging to distinct families, mainly from Brazil. Mutations were detected in all VHL patients using a combination of direct sequencing and quantitative Southern blotting. To our knowledge, this is the first study of molecular diagnosis of VHL in Latin America. The study contributes eight novel germline *VHL* mutations and discusses whether increased risk of RC observed within these families may be attributable to mutations affecting the HIF-1 binding domain of the pVHL and/or the VCB complex.

SUBJECTS AND METHODS

Patients and clinical evaluation

Seventeen affected probands with a family history of VHL (15 from Brazil, one from Portugal, and one from Ecuador) and three Brazilian VHL patients without a family history (defined by clinical criteria) were included in this study. Fifteen of these were clinically evaluated at the Department of Oncogenetics of the Hospital do Câncer - Sao Paulo, Brazil, and the others were referred by other centres that were following the families. All families received appropriated pre- and post-test genetic counselling.

Blood samples from the affected probands were collected following receipt of signed informed consent. Clinical status was determined by physical examinations (including ophthalmoscopy), radiological evaluations, and laboratory testing according to methods described elsewhere.[3 5] Previous medical records of affected members and follow up were reviewed and registered in a database.

Amplification and sequencing of the *VHL* gene

Genomic DNA was extracted from 10 ml of peripheral blood using phenol-chloroform by standard procedures after separation of mononuclear cells and stored at 4°C. The three exons of the *VHL* gene were amplified by PCR in a total volume of 25 µl containing 50 ng of template DNA, 1 × PCR buffer (20 mmol/l Tris-HCl, 50 mmol/l KCl), 200 µmol/l each dNTP (Promega, USA), 1.5 mmol/l $MgCl_2$, 0.25 units of *Taq* DNA polymerase (Gibco-BRL Life Technologies, USA), and 0.5 µmol/l each primer set. Primers used for the amplification of the coding region of the *VHL* gene were: 1F 5′ CCATCCTCTACCGAGCGCGCG 3′ and 1R 5′ GGGCTTCAGACCGTGCTATCG 3′ for exon 1, yielding a fragment of 522 bp; 2F 5′ TGCCCAGCCACCGGTGTG 3′ and 2R 5′ GTCTATCCTGTACTTACCACAACA 3′ for exon 2, 232 bp; and 3F 5′ CACACTGCCACATACATGCACTC 3′ and 3R 5′ ACTCATCAGTACCATCAAAAGCTG 3′ for exon 3, 383 bp. Reactions were incubated at 95°C for five minutes and amplified for 35 cycles of 95°C for one minute, 58°C for one minute (exons 2 and 3), or 68°C (exon 1) for one minute, and 72°C for one minute, with a final extension cycle of 72°C for five minutes. PCR products were sequenced in both strands with Big Dye terminators (Applied Biosystems) and sequences were collected and analysed on an ABI Prism 377 (Applied Biosystems). The same PCR primers were used in the sequencing reactions, with the exception of exon 3, which was sequenced in the sense direction with an internal primer 3I 5′ CGTTCCTTGTACTGAGACCCT 3′.

Southern blot analysis

Germline rearrangements involving part or the entire *VHL* gene were detected by quantitative Southern blotting using g7 cDNA[11] and a β globin probe, as control, as previously described.[33] In brief, 10 µg of genomic DNA were digested with the endonucleases *Eco*RI and *Ase*I and DNA fragments were separated by electrophoresis. The DNA was transferred to a nylon membrane and hybridised to random primer labelled probe specific for the *VHL* gene (g7) and for the β globin gene. Filters were exposed to *x* ray film (Kodak X-AR, USA) in a film cassette with two intensifying screens at −70°C for one to three days. Quantification was possible by comparing the intensity between the two bands. DNA from normal subjects and from patients with known rearrangements in the *VHL* gene (kindly provided by Dr Catherine Stolle, University of Pennsylvania, USA) was used as negative and positive controls, respectively. In normal subjects, a single 9.7 kb *Eco*RI and *Ase*I fragment is detected by hybridisation with the g7 probe, whereas in subjects with partial deletions of the *VHL* gene, a slower or faster migrating fragment was detected in addition to the normal sized fragment. Complete deletion of the *VHL* gene produced a single 9.7 kb band with 50% relative intensity after normalisation, which was confirmed by densitometry of the bands.

RESULTS

Twenty VHL probands and their families were evaluated in this study, of whom 17 presented with a family history (15 from Brazil, one from Portugal, and one from Ecuador). Fifteen familial VHL and two VHL patients were classified as having type 1 VHL (without PH) and two familial VHL and one VHL patient were classified as having type 2 VHL (with PH). Ten families were large enough (four or more affected subjects from at least two generations) for full phenotype analysis (table 1).

We were able to define germline *VHL* gene mutations in all probands who fulfilled the clinical criteria for VHL. In 16 cases (80%), these were point mutations involving either the coding region or exon boundaries of the *VHL* gene (nine missense, three frameshift, one in frame deletion, two splice defect, one nonsense), while four (20%) were large deletions of which three were partial deletions and one a complete deletion of the *VHL* gene (table 1). Eight of the 16 point mutations (50%) have not been previously described and thus represent novel mutations. The point mutations were not distributed uniformly along the *VHL* gene and most were concentrated within exons 1 and 2 (87.5%). The 13 point mutations associated with type 1 VHL occurred in this region in contrast to the findings of others.[29 31 42] We were able to analyse the germline mutation linkage to disease in nine families (families 2, 8, 9, 12, 14, 15, 16, 18, and 20). Four of these families carried known point mutations described before by others, two presented large deletions, and three had novel point mutations. The novel mutations 238A>C, 344delA, and IVS1+7G>A were detected in five, three, and two affected family members, respectively. The pathogenic nature of the IVS1+7G>A remains to be confirmed at the mRNA level; however, the fact that this alteration was not detected in one unaffected first degree relative (after clinical and radiological screening) suggests that it may be associated with the disease. Additionally, none of the novel mutations were represented in 120 alleles from normal subjects in the Brazilian population suggesting that these base changes are not common polymorphisms.

Linkage analyses were not possible in eight of the VHL families owing to the absence of live family members (families 1, 3, 4, 5, and 7) or relatives alive but not assessable (families 11, 17, and 19). Only in family 1 was genetic testing performed in a non-symptomatic boy, since no relatives were alive for testing. Although many members of this family had already presented with HB, the diagnosis of VHL was confirmed after necropsy of the proband's father, who died at 37 years from complications following emergency surgery for cerebellar HB. The necropsy showed many occult lesions related to VHL, including RC, multiple pancreatic and renal cysts, and cystadenoma of the epididymis.

From the eight families in which linkage to disease could not be established, four carried mutations previously described as having a deleterious effect (table 1), two had partial deletions of the *VHL* gene, and two had novel missense mutations. Although these two missense mutations have not been previously described, they affect amino acid residues for which

Table 1 Clinical manifestation of 20 consecutive families with von Hippel-Lindau disease (VHL). Details of germline mutations found in VHL families without (type 1) and with (type 2) phaeochromocytoma

VHL Family	VHL type	Family history	No	HB	RA	RC	PC	PH	Mutation at nt level of ORF[f]	Exon	Consequence to pVHL[f]	Type of mutation	Reference
1	1	Yes	5	4	–	2	–	–	226-228 del TCT	1	delPhe76	In frame del	41
2	1	Yes	10	7	3	3	–	–	238A>C	1	Ser80Arg	Missense	Novel[i]
3	1	Yes	2	1	1	–	–	–	262T>C	1	Trp88Arg	Missense	Novel[i]
4	1	Yes	3	2	3	–	–	–	280G>T	1	Glu94Stop	Nonsense	12
5	1	Yes	2	2	–	–	–	–	302T>G	1	Leu101Arg	Missesnse	31
6	1	No	1	1	–	1	–[d]	–	305 del C	1	Frameshift at aa 102[g]	Frameshift	Novel
7	1	Yes	5	4	3	2	–	–[e]	320G>C	1	Arg107Pro	Missense	33
8	1	Yes	2	2	–	–	–	–	IVS1+7G>A	Intron 1	–	Splicing defect	Novel
9	1	Yes	3	1	1	1	–	–	344 del A	2	Frameshift at aa 115[h]	Frameshift	Novel
10	2	No	1	–	1	–	–	1	371C>T	2	Thr124Ile	Missense	Novel
11	1	Yes	14	7	2	1	–[d]	–	388G>T	2	Val130Phe	Missense	Novel
12	1	Yes	8	7	1	2[b]	–	–	407T>C	2	Phe136Ser	Missense	12
13	1	No[a]	1	1	1	1[b]	–	–	436 del C	2	Frameshift at aa 146[h]	Frameshift	Novel
14	1	Yes	5	3	1	1	–[d]	–	IVS2+1G>A	Intron 2	–	Splicing defect	32
15	2	Yes	2	1	–	–[c]	–	2	499C>T	3	Arg167Trp	Missense	12
16	2	Yes	4	2	–	–[c]	–	3	500G>A	3	Arg167Gln	Missense	12
17	1	Yes	10	10	1	5[b]	–	–	Partial deletion	–		Rearrangement	–
18	1	Yes	5	2	1	–	–[d]	–	Partial deletion	–	–	Rearrangement	–
19	1	Yes	3	3	–	–	–	–	Partial deletion	–	–	Rearrangement	–
20	1	Yes	6	4	–	3[b]	1	–	Complete deletion	–	–	Rearrangement	–
9, 17	–	–	–	–	–	–	–	–	183C>G	1	Synonym	Polymorphism	1.16%[j]
16	–	–	–	–	–	–	–	–	IVS2 –95T>A	Intron 2	Apparently no effect	Polymorphism	1.69%[j]

[a]Further investigation showed that the mother of the patient was an asymptomatic carrier of the mutation. [b]One patient per family with renal tumour <3 cm under follow up detected by imaging. [c]Although we did not observe RC, the germline mutations are described as associated with the VHL type 2B phenotype. [d]Pancreatic cystadenoma. [e]The proband presented with one tumour which biopsy showed to be a non-functional paraganglioma. [f]Nomenclature according to Antonarakis *et al* (1998). [g]Protein stops at residue 142. [h]Protein stops at residue 157. [i]New at nucleotide level, but already described at aa level. [j]Allele frequency in normal Brazilian population.
No = number of affected members in each VHL family; HB = haemangioblastoma of CNS; RA = retinal angioma; RC = renal carcinoma; PC = pancreatic carcinoma; PH = phaeochromocytoma; nt = nucleotide; ORF = open reading frame; aa = amino acid; new site = novel germline mutation.

other different mutations have already been described.[28 29 31 43] Additionally, these mutations (Trp88Arg and Val130Phe) affect the HIF-1α binding site within the β domain of pVHL and the hydrophobic core of pVHL responsible for the structural integrity of the β sheets, respectively.[37] Thus, in both cases these novel mutations are predicted to result in significant alteration of pVHL function.

Three novel germline mutations were detected in all VHL patients with no family history: frameshift mutation 305delC, frameshift mutation 436delC, and missense mutation Thr124Ile. Both novel frameshift mutations introduce a premature stop codon leading to a truncated protein and the missense mutation was not detected in normal subjects from the Brazilian population. Following parental testing, two VHL patients were confirmed as having de novo VHL (families 6 and 10), since their mutation was not found in their parents (although no test to confirm the paternity was performed). However, in family 13, analysis of the proband's parents showed that the mutation 436delC was present in two independent samples from his asymptomatic mother and thus it cannot be considered a de novo mutation. On complete clinical investigation, however, the proband's mother was shown to have two occult HBs (one cerebellar and one spinal) as well as multiple pancreatic cysts.

In addition to the mutations described above, two variants that apparently do not interfere with the protein structure were found in three families: the synonymous mutation Pro61Pro (in families 9 and 17), previously reported as a known polymorphism, and the variant IVS2-94T>A (in family 16) that has not been previously reported. In all the families, except family 15, the variants did not segregate with the related *VHL* gene mutation. The allele frequency of each of these variants was shown to be at least 1% in normal DNA samples from the Brazilian population (a minimum of 120 alleles evaluated) (table 1). In contrast, none of the novel point mutations described in the present study were detected in normal subjects.

Genotype-phenotype correlations were consistent with those reported in other studies. Most of the mutations found in the 17 families classified as type 1 VHL resulted in a truncated protein (66%), whereas all three mutations found in families whose phenotype included PH (type 2 VHL) predicted a full length protein. There was no correlation between the type of germline mutation and the occurrence of HB or RA, since these manifestations were present in almost all families analysed. Two families harboured distinct mutations in codon 167, a VHL hotspot associated with high risk of RC, HB, and PH (type 2B). However, RC was not observed in these families, and we considered that a larger number of affected members and a longer period of follow up will be necessary for full phenotypic characterisation in these families. Eleven out of 17 type 1VHL families had a RC phenotype (table 1). Almost all mutations detected in these families affect residues in the HIF-1 binding site domain (residues 67-117), residues in the hydrophobic core important for VCB complex assembly, or are associated with the presence of a truncated form of pVHL. Interestingly, we have observed in large VHL families with a RC phenotype that although the principle cause of mortality in previous generations was HB, in younger generations this is now mostly the result of RC (data not shown).

DISCUSSION

We have studied von Hippel-Lindau disease in Brazil since 1998, and have identified 20 families with VHL. Many studies in Japanese and western populations have highlighted differences in the spectrum of *VHL* germline mutations around the world.[28 31 42] The heterogeneity of the Brazilian population, consisting mostly of descendants of Portuguese immigrants, African, and Brazilian Indians raises the possibility of a different spectrum of *VHL* germline mutations and risks of VHL associated lesions. The VHL families included in this study are representative of our population, since they came from many different regions of the country, including the south east, north east, and the central region. Also, one family from Ecuador and another from Portugal were included in our study. To our knowledge this is the first study in Portuguese speaking families and the first in Latin America.

The spectrum of known mutations in the *VHL* gene is enormous and includes missense and nonsense mutations, deletions or insertions of one to several nucleotides, splice site mutations, and rearrangements consisting of partial or complete deletion of the gene. Here, we found that by complete sequencing of the coding regions of the *VHL* gene, followed by quantitative Southern blotting when a sequence mutation could not be detected, we were able to reproduce the results obtained by Stolle *et al*,[33] in that we detected germline mutations in all 20 distinct VHL families analysed. Because of the relatively short length of the *VHL* gene, no screening method based on single stranded DNA conformation, such as SSCP or CDGE, was used before sequencing. Since not all investigators achieved the same rate of mutation detection, we consider that both methodology and patient selection are important for achieving a high efficiency of mutation detection in VHL. We believe that the high rate of mutation detection achieved in this work can also be explained by the stringent clinical criteria used for family selection. All patients selected for the study had the diagnosis of VHL confirmed by clinical, pathological, or radiological evidence.

We found that molecular screening was particularly invaluable in situations where the diagnosis of VHL could not be achieved by clinical criteria alone, such as where family data were not available, or where patients had a negative family history. In addition, the genetic test was important to determine the status of the parents in the apparently de novo VHL patients. In one case, it was possible to detect the same mutation in the mother of the patient, with implications for the risk assessment and screening of other family members. Indeed, we emphasise here that the study of the patient's parents is essential for the characterisation of de novo mutations, and for risk assessment and counselling of other relatives. Recent studies have shown that about 23% of probands have parents and sibs with no history of the disease at the time of the diagnosis.[9]

We have contributed eight novel germline mutations, including four missense mutations, three frameshift mutations, and one splice site mutation (table 1). Five novel mutations occurred with a familial pattern. However, two of them could not be analysed further in other family members: the missense mutation Trp88Arg, because the mother of the proband had died and was adopted, and the missense mutation Val130Phe, because there were no available relatives at the time of the study. Although the mutation IVS1+7G>A does not affect the invariant 3′ AG splicing donor site, segregation and population analysis presented in this work suggests it can be a pathogenic mutation. Splicing mutations outside the invariant receptor or donor site have been reported in many cancer susceptibility genes, including *VHL*, *TP53*, *BRCA1*, and *hMLH1*, and they represent as many as 27% of the splice sites mutations reported in these genes (HGMD online). None of the novel point mutations described in this study were represented in the normal population, excluding the possibility that they might be common polymorphisms.

Germline mutations in the *VHL* gene are distributed throughout the coding region of the gene. Although other groups have found that the germline mutations of the *VHL* gene tended to concentrate in exon 1 and exon 3, we observed in our series that most point mutations (87.5%) occurred in exon 1 (43.7%), exon 2 (31.3%), and the boundaries of these exons (12.5%). We found large deletions of the *VHL* gene in four of our families (20%), including three partial deletions and one complete deletion. Previous studies showed a frequency of partial *VHL* gene deletion of 25% and complete VHL gene deletion in 9% in the VHL families.[33]

In general, genotype-phenotype correlations observed in this study were consistent with those reported by others.[12 29 30 36 44] It is known that distinct phenotypes can result from almost identical mutations.[45] Here we found that the mutation Ser80Arg detected in a large family conferred a high risk for RC, HB, and RA, but no apparent risk for PH. A similar missense mutation in this same codon, Ser80Gln, has been reported to be associated with type 2C VHL (only PH).[46] It should be emphasised that new genotype-phenotype correlations are emerging for VHL and further studies such as the one presented here are necessary. Recently functional studies have shown that phenotypes could be related to loss of specific pVHL functions.

Here, we observed an increased risk of RC in most of our families, based on the family phenotype or the presence of high risk mutations. Most of these mutations directly affected the HIF-1 binding site of the pVHL and/or the proper assembly of the VCB complex. Functional studies strengthen the notion that HIF-1 deregulation plays a causal role in HB and RC and contributes to RC pathogenesis in the setting of VHL disease.[38 47 48]

Although the phenotype in VHL may vary among families, intrafamilial variability can also be observed,[34 35] suggesting that other factors could be involved, such as environmental exposure, modifier genes, or single nucleotide polymorphisms (SNPs).[49] All these factors could influence the clinical spectrum of VHL in different populations, and so far there is no satisfactory explanation for the clinical heterogeneity observed in VHL. Recently, the cyclin D1 genotype was investigated as a modifier gene in VHL, and it was found that the presence of the nucleotide G at position 870 was associated with an increased risk of RA and HB.[50 51] More molecular and epidemiological studies will be necessary for complete understanding of the phenotype associated with VHL.

The improvement of life expectancy of VHL patients in recent years is the result of the refinement of image based diagnostic methods and the development of screening programmes. We argue that this trend is now likely to be strengthened by the incorporation of accurate mutational analysis, risk assessment, and genetic counselling. Here, we have confirmed that it is now reasonable to expect to find *VHL* mutations in all affected subjects, but that the spectrum of mutations is highly population dependent. Furthermore, the phenotypic implications of specific mutations are also likely to be influenced by the genetic background of the population. Thus, we have contributed eight novel germline mutations in the *VHL* gene found in the Brazilian families whom we suspect may have unique features in respect to risk of malignancies. Continued, careful study of these and other VHL families in Latin America will be required to confirm this altered disease profile, which would have significant implications for genetic counselling and screening programmes of VHL disease on this continent.

ACKNOWLEDGEMENTS

Database information. The accession number and URL for data in this article are as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for VHL (MIM 193300)]), Human Gene Mutation Database - Cardiff, http://www.uwcm.ac.uk/uwcm/mg/search/120488.html

We are very grateful to Dr Alessandra Murgia (University of Padua, Italy) for the probes used in Southern blotting experiments and to Anna Christina Matos Salim and Valéria Paixão for dedicated technical support. We wish to extend our special thanks to Joyce Graff (VHLFA) for her outstanding support, and to all genetic centres in Brazil that collaborated with this study. JCCR is a PhD. student supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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REFERENCES

1 **Melbon KL**, Rosen SW. Lindau's disease. Review of the literature and study of a large kindred. *Am J Med* 1964;**36**:595-617.
2 **Glenn GM**, Choyke PL, Zbar B, Linehan W. von Hippel-Lindau disease: clinical review and molecular genetics. *Probl Urol* 1990;**4**:312-30.
3 **Choyke PL**, Glenn GM, Walther MM, Patronas NJ, Linehan WM, Zbar B. von Hippel-Lindau disease: genetic, clinical, and imaging features. *Radiology* 1995;**194**:629-42.
4 **Choyke PL**, Glenn GM, Wagner JP, Lubensky IA, Thakore K, Zbar B, Linehan WM, Walther MM. Epididymal cystadenomas in von Hippel-Lindau disease. *Urology* 1997;**49**:926-31.
5 **Manski TJ**, Heffner DK, Glenn GM, Patronas NJ, Pikus AT, Katz D, Lebovics R, Sledjeski K, Choyke PL, Zbar B, Linehan WM, Oldfield EH. Endolymphatic sac tumors. A source of morbid hearing loss in von Hippel-Lindau disease. *JAMA* 1997;**277**:1461-6.
6 **Maher ER**, Iselius L, Yates JR, Littler M, Benjamin C, Harris R, Sampson J, Williams A, Ferguson-Smith MA, Morton N. von Hippel-Lindau disease: a genetic study. *J Med Genet* 1991;**28**:443-7.
7 **Neumann HP**, Wiestler OD. Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. *Lancet* 1991;**337**:1052-4.
8 **Maddock IR**, Moran A, Maher ER, Teare MD, Norman A, Payne SJ, Whitehouse R, Dodd C, Lavin M, Hartley N, Super M, Evans DG. A genetic register for von Hippel-Lindau disease. *J Med Genet* 1996;**33**:120-7.
9 **Sgambati MT**, Stolle C, Choyke PL, Walther MM, Zbar B, Linehan WM, Glenn GM. Mosaicism in von Hippel-Lindau disease: lessons from kindreds with germline mutations identified in offspring with mosaic parents. *Am J Hum Genet* 2000;**66**:84-91.
10 **Seizinger BR**, Rouleau GA, Ozelius LJ, Lane AH, Farmer GE, Lamiell JM, Haines J, Yuen JW, Collins D, Majoor-Krakauer D, Bonner T, Marrew C, Rubenstein A, Halperin J, McConkie-Rosell A, Green JS, Trofatter JA, Ponder BA, Eierman L, Bowner MI, Schmike R, Oostra B, Aronin N, Smith DI, Drabkin H, Wazari MW, Hobbs WJ, Marruza RL, Conneally PM, Hsia YE, Gusella JF. von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. *Nature* 1988;**332**:268-9.
11 **Latif F**, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, Schmidt L, Zhou F, Li H, Wei MH, Chen F, Glenn G, Choyke P, Walther MM, Weng Y, Duan DSR, Dean M, Glavac D, Richards FM, Crossey PA, Ferguson-Smith MA, Le Paslier D, Chumakov I, Cohen D, Chinaut AC, Maher ER, Linehan WM, Zbar B, Lerman MI. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993;**260**:1317-20.
12 **Crossey PA**, Richards FM, Foster K, Green JS, Prowse A, Latif F, Lerman MI, Zbar B, Affara NA, Ferguson-Smith MA, Maher ER. Identification of intragenic mutations in the von Hippel-Lindau disease tumour suppressor gene and correlation with disease phenotype. *Hum Mol Genet* 1994;**3**:1303-8.
13 **Foster K**, Prowse A, van den Berg A, Fleming S, Hulsbeek MM, Crossey PA, Richards FM, Cairns P, Affara NA, Ferguson-Smith MA. Somatic mutations of the von Hippel-Lindau disease tumour suppressor gene in non-familial clear cell renal carcinoma. *Hum Mol Genet* 1994;**3**:2169-73.
14 **Gnarra JR**, Tory K, Weng Y, Schmidt L, Wei MH, Li H, Latif F, Liu S, Chen F, Duh FM, Lubensky I, Duan DR, Florence C, Pozzatti R, Walther MM, Bander NH, Grossman HB, Brauch H, Pomer S, Brooks JD, Isaacs WB, Lerman MI, Zbar B, Linehan WM. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 1994;**7**:85-90.
15 **Kanno H**, Kondo K, Ito S, Yamamoto I, Fujii S, Torigoe S, Sakai N, Hosaka M, Shuin T, Yao M. Somatic mutations of the von Hippel-Lindau tumor suppressor gene in sporadic central nervous system hemangioblastomas. *Cancer Res* 1994;**54**:4845-7.
16 **Shuin T**, Kondo K, Torigoe S, Kishida T, Kubota Y, Hosaka M, Nagashima Y, Kitamura H, Latif F, Zbar B, *et al*. Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. *Cancer Res* 1994;**54**:2852-5.
17 **Whaley JM**, Naglich J, Gelbert L, Hsia YE, Lamiell JM, Green JS, Collins D, Neumann HP, Laidlaw J, Li FP, Klein-Szanto AJP, Seizinger BR, Kley N. Germ-line mutations in the von Hippel-Lindau tumor-suppressor gene are similar to somatic von Hippel-Lindau aberrations in sporadic renal cell carcinoma. *Am J Hum Genet* 1994;**55**:1092-102.
18 **Gilcrease MZ**, Schmidt L, Zbar B, Truong L, Rutledge M, Wheeler TM. Somatic von Hippel-Lindau mutation in clear cell papillary cystadenoma of the epididymis. *Hum Pathol* 1995;**26**:1341-6.
19 **Olschwang S**, Richard S, Boisson C, Giraud S, Laurent-Puig P, Resche F, Thomas G. Germline mutation profile of the VHL gene in von Hippel-Lindau disease and in sporadic hemangioblastoma. *Hum Mutat* 1998;**12**:424-30.
20 **Kawahara N**, Kume H, Ueki K, Mishima K, Sasaki T, Kirino T. VHL gene inactivation in an endolymphatic sac tumor associated with von Hippel-Lindau disease. *Neurology* 1999;**53**:208-10.
21 **Vortmeyer AO**, Huang SC, Koch CA, Governale L, Dickerman RD, McKeever PE, Oldfield EH, Zhuang Z. Somatic von Hippel-Lindau gene mutations detected in sporadic endolymphatic sac tumors. *Cancer Res* 2000;**60**:5963-5.
22 **Ohh M**, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, Gavin BJ, Kley N, Kaelin WG Jr, Iliopoulos O. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* 1998;**1**:959-68.
23 **Pause A**, Lee S, Lonergan KM, Klausner RD. The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc Natl Acad Sci USA* 1998;**95**:993-8.
24 **Maxwell PH**, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;**399**:271-5.
25 **Gnarra JR**, Zhou S, Merrill MJ, Wagner JR, Krumm A, Papavassiliou E, Oldfield EH, Klausner RD, Linehan WM. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. *Proc Natl Acad Sci USA* 1996;**93**:10589-94.
26 **Iliopoulos O**, Levy AP, Jiang C, Kaelin WG Jr, Goldberg MA. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci USA* 1996;**93**:10595-9.
27 **Siemeister G**, Weindel K, Mohrs K, Barleon B, Martiny-Baron G, Marme D. Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. *Cancer Res* 1996;**56**:2299-301.
28 **Clinical Research Group for VHL in Japan**. Germline mutations in the von Hippel-Lindau disease (VHL) gene in Japanese VHL. *Hum Mol Genet* 1995;**4**:2233-7.
29 **Kamada M**, Suzuki K, Kato Y, Okuda H, Shuin T. von Hippel-Lindau protein promotes the assembly of actin and vinculin and inhibits cell motility. *Cancer Res* 2001;**6**:4184-9.
30 **Chen F**, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarra JR, Orcutt ML, Duh FM, Glenn G, Green J, Hsia YE, Lamiell J, Li H, Wei MH, Schmidt L, Tory K, Kuzmin I, Stackhouse T, Latif F, Linehan WM, Lerman MI, Zbar B. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 1995;**5**:66-75.
31 **Maher ER**, Webster AR, Richards FM, Green JS, Crossey PA, Payne SJ, Moore AT. Phenotypic expression in von Hippel-Lindau disease: correlations with germline VHL gene mutations. *J Med Genet* 1996;**33**:328-32.
32 **Zbar B**, Kishida T, Chen F, Schmidt L, Maher ER, Richards FM, Crossey PA, Webster AR, Affara NA, Ferguson-Smith MA, Brauch H, Glavac D, Neumann HP, Tisherman S, Mulvihill JJ, Gross DJ, Shuin T, Whaley J, Seizinger B, Kley N, Olschwang S, Boisson C, Richard S, Lips CH, Lerman M. Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 1996;**8**:348-57.
33 **Glavac D**, Neumann HP, Wittke C, Jaenig H, Masek O, Streicher T, Pausch F, Engelhardt D, Plate KH, Hofler H, Chen F, Zbar B, Brauch H. Mutations in the VHL tumor suppressor gene and associated lesions in families with von Hippel-Lindau disease from central Europe. *Hum Genet* 1996;**98**:271-80.
34 **Stolle C**, Glenn G, Zbar B, Humphrey JS, Choyke P, Walther M, Pack S, Hurley K, Andrey C, Klausner R, Linehan WM. Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 1998;**12**:417-23.
35 **Glenn GM**, Daniel LN, Choyke P, Linehan WM, Oldfield E, Gorin MB, Hosoe S, Latif F, Weiss G, Walther M, Lerman MI, Zbar B. von Hippel-Lindau (VHL) disease: distinct phenotypes suggest more than one mutant allele at the VHL locus. *Hum Genet* 1991;**87**:207-10.
36 **Neumann HP**, Bender BU. Genotype-phenotype correlations in von Hippel-Lindau disease. *J Intern Med* 1998;**243**:541-5.
37 **Zbar B**. von Hippel-Lindau disease and sporadic renal cell carcinoma. *Cancer Surv* 1995;**25**:219-32.
38 **Stebbins CE**, Kaelin WG Jr, Pavletich NP. Structure of the VHL-elonginC-elonginB complex: implications for VHL tumor suppressor function. *Science* 1999;**284**:455-61.
39 **Clifford SC**, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, Ratcliffe PJ, Maher ER. Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. *Hum Mol Genet* 2001;**10**:1029-38.
40 **Hoffman MA**, Ohh M, Yang H, Klco JM, Ivan M, Kaelin WG Jr. von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* 2001;**10**:1019-27.
41 **Hansen WJ**, Ohh M, Moslehi J, Kondo K, Kaelin WG, Welch WJ. Diverse effects of mutations in exon II of the von Hippel-Lindau (VHL) tumor suppressor gene on the interaction of pVHL with the cytosolic chaperonin and pVHL-dependent ubiquitin ligase activity. *Mol Cell Biol* 2002;**22**:1947-60.
42 **Patel RJ**, Appukuttan B, Ott S, Wang X, Stout JT. DNA-based diagnosis of the von Hippel-Lindau syndrome. *Am J Ophthalmol* 2000;**129**:258-60.
43 **Cybulski C**, Krzystolik K, Murgia A, Gorski B, Debniak T, Jakubowska A, Martella M, Kurzawski G, Prost M, Kojder I, Limon J, Nowacki P, Sagan L, Bialas B, Kaluza J, Zdunek M, Omulecka A, Jaskolski D, Kostyk E, Koraszewska-Matuszewska B, Haus O, Janiszewska H, Pecold K, Starzycka M, Slomski R, Cwirko M, Sikorski A, Gliniewicz B, Cyrylowski L, Fiszer-Maliszewska L, Gronwald J, Toloczko-Grabarek A, Zajaczek S, Lubinski J. Germline mutations in the von Hippel-Lindau (VHL) gene in patients from Poland: disease presentation in patients with deletions of the entire VHL gene. *J Med Genet* 2002;**39**:e38.
44 **Mattocks C**, Tarpey P, Bobrow M, Whittaker J. Comparative sequence analysis (CSA): a new sequence-based method for the identification and characterization of mutations in DNA. *Hum Mutat* 2000;**16**:437-43.
45 **Hes F**, Zewald R, Peeters T, Sijmons R, Links T, Verheij J, Matthijs G, Leguis E, Mortier G, van der Torren K, Rosman M, Lips C, Pearson P, van der Luijt R. Genotype-phenotype correlations in families with deletions in the von Hippel-Lindau (VHL) gene. *Hum Genet* 2000;**106**:425-31.

46 **Bradley JF**, Collins DL, Schimke RN, Parrott HN, Rothberg PG. Two distinct phenotypes caused by two different missense mutations in the same codon of the VHL gene. *Am J Med Genet* 1999;**87**:163-7.
47 **Woodward ER**, Eng C, McMahon R, Voutilainen R, Affara NA, Ponder BA, Maher ER. Genetic predisposition to phaeochromocytoma: analysis of candidate genes GDNF, RET and VHL. *Hum Mol Genet* 1997;**6**:1051-6.
48 **Kondo K**, Klco J, Nakamura E, Lechpammer M, Kaelin WG Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. *Cancer Cell* 2002;**1**:237-24.
49 **Maranchie JK**, Vasselli JR, Riss J, Bonifacino JS, Linehan WM, Klausner RD. The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. *Cancer Cell* 2002;**1**:247-55.
50 **Webster AR**, Richards FM, MacRonald FE, Moore AT, Maher ER. An analysis of phenotypic variation in the familial cancer syndrome von Hippel-Lindau disease: evidence for modifier effects. *Am J Hum Genet* 1998;**63**:1025-35.
51 **Zatyka M**, da Silva NF, Clifford SC, Morris MR, Wiesener MS, Eckardt KU, Houlston RS, Richards FM, Latif F, Maher ER. Identification of cyclin D1 and other novel targets for the von Hippel-Lindau tumor suppressor gene by expression array analysis and investigation of cyclin D1 genotype as a modifier in von Hippel-Lindau disease. *Cancer Res* 2002;**62**:3803-11.