Germline VHL gene mutations in Hungarian families with von Hippel-Lindau disease and patients with apparently sporadic unilateral phaeochromocytomas

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Running title: Germline VHL gene mutations in Hungarian families

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Abstract

Objective: Von Hippel-Lindau (VHL) disease is a hereditary tumor syndrome caused by mutations or deletions of the VHL tumor-suppressor gene. Germline VHL gene alterations may be also present in patients with apparently sporadic phaeochromocytoma (ASP), although a wide variation in mutation frequencies has been reported in different patient cohorts.

Design: Herein we report the analysis of the VHL gene in Hungarian families with VHL disease and in those with ASP.

Methods: Seven families (35 members) with VHL disease and 37 unrelated patients with unilateral ASP were analysed. Patients were clinically evaluated and the VHL gene was analysed using direct sequencing, multiplex ligation probe amplification and real-time polymerase chain reaction with SYBRGreen chemistry.

Results: Disease-causing genetic abnormalities were identified in each of the 7 VHL families and in 3 of the 37 patients with ASP (one nonsense and 6 missense mutations, 2 large gene deletions and one novel 2 bp deletion). Large gene deletions and other genetic alterations resulting in truncated VHL protein were found only in families with VHL type 1, whereas missense mutations were associated mainly, although not exclusively with VHL type 2B and type 2C.

Conclusions: The spectrum of VHL gene abnormalities in Hungarian population is similar to that observed in Western, Japanese or Chinese VHL kindreds. The presence of VHL gene mutations in 3 of the 37 patients with ASP suggests that genetic testing is useful not only in patients with VHL disease but also in those with ASP.
Introduction

Von Hippel-Lindau (VHL, OMIM Nr. 19330)) disease is a rare dominantly inherited multisystem family tumor syndrome caused by mutations or deletions of the VHL tumor-suppressor gene mapped to chromosome locus 3p25-26. Patients with VHL disease are at risk for the development of retinal, cerebellar, spinal and, less frequently, pancreatic, renal, pulmonary, liver and adrenal haemangiomas/haemangioblastomas, clear-cell renal carcinomas, phaeochromocytomas, endolymphatic sac tumors, multiple renal, epididymal and pancreatic cysts; cystadenomas of the epididymis and the broad ligament, and pancreatic islet cell tumors. VHL disease has been divided into two main groups; patients with type 1 disease have a greatly reduced risk of phaeochromocytomas, but can develop all the other manifestations of the disease, whereas patients with VHL type 2 have phaeochromocytomas. Type 2 families may have either a low (type 2A) or high risk (type 2B) for renal cell carcinoma. VHL type 2C is a separate entity in which affected patients have phaeochromocytomas without other manifestations of the disease.

The VHL gene encodes two different protein isoforms due to the use of two translation initiation sites; the larger protein (pVHL30) consists of 213 amino acids, whereas the smaller pVHL19 contains residues 54-213 of the pVHL30. The pVHL19 shows a high homology among human, dog, mouse and rat and it has all functional domains of the pVHL30. The protein model of pVHL19 published in 1999 by Stebbins et al. contains two functional subdomains; a smaller, helical α-domain (residues 155-192) which consists of three helices (H1, H2, and H3) and a larger β-domain (residues 63-154 and residues 193-204) which forms a seven-stranded β sandwich and an α helix (H4). For VHL protein function two important sites have been identified; one of them represents the Elongin C binding site (amino acid residues 157-170) and the other is the hypoxia inducible factor 1α (HIF1α) binding site.
(amino acid residues 91-113 in β-domain)\textsuperscript{12}. The majority of disease-causing \textit{VHL} gene mutations are located in one of these two binding sites.

More than 900 different mutations of the \textit{VHL} gene have been described (http://www.umd.be:2020/)\textsuperscript{13}. The majority of \textit{VHL} type 1 cases are caused by missense mutations affecting the hydrophobic core of the protein, by mutations resulting in truncated protein, and by partial gene deletions which cause a complete defect of protein function. In contrast, \textit{VHL} type 2 is usually associated with missense mutations affecting the protein binding sites of the \textit{VHL} protein. To date, only a few mutations upstream of the internal start codon 54 have been described (codons 25, 38, 46 and 52), and these mutations have been associated either with phaeochromocytomas (codons 25, 38) or with \textit{VHL} disease (codons E46X and E52K)\textsuperscript{14-17}.

\textit{VHL} disease has an approximate incidence of 1/36000 live births\textsuperscript{18} and germline \textit{VHL} gene mutations have been found in about 40\% of familial or bilateral phaeochromocytomas\textsuperscript{19, 20}. Screening for germline \textit{VHL} gene mutations may be also recommended in patients with apparently sporadic unilateral phaeochromocytomas, although a wide variation in mutation frequencies ranging from 0 to 11\% in different patient cohorts has been reported\textsuperscript{19-22}. The identification of germline \textit{VHL} gene mutations in patients with apparently sporadic phaeochromocytomas appears to be important, as the onset of the \textit{VHL} disease may show a high variability and many patients with \textit{VHL}-related phaeochromocytomas may carry a high risk for developing other manifestations of the disease.

In this study we present the results of germline mutation analysis of the \textit{VHL} gene in Hungarian families with \textit{VHL} disease and patients with apparently sporadic unilateral phaeochromocytomas.

\textbf{Subjects and methods}
Families with VHL disease

A total of 35 members from 7 unrelated families with VHL disease were evaluated. Initial screening included medical history, physical examination, abdominal ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI), brain and spinal cord MRI, ophthalmologic examination, and laboratory tests which included routine biochemical testing and 24h urinary catecholamine metabolite determination. All patients with VHL disease and family members of patients who proved to have VHL gene mutations underwent genetic counselling, and clinical as well as genetic screening was performed in all relatives who agreed the screening tests. After genetic screening all index patients were reinvestigated for VHL-related tumors, but no newly-detected tumors were found. Clinical findings are summarized in Table 1.

In family A, the index patient was a woman who had multiple (occipital, parietal and cerebellar) haemangioblastomas, renal cysts and retina haemangiomas. The parents and one of the two children of the index patient had no clinical symptoms whereas in the other child retina haemangiomas were diagnosed at the age of 6 years.

In family B, the index patient was a young man who developed retina haemangiomas at the age of 19 years, and cerebellar haemangioma, as well as renal and pancreatic cysts at the age of 27 years. A moderate erythrocytosis with normal white blood cell and platelet count was also observed. The parents of the patient rejected clinical and genetic screening, whereas clinical screening in a younger brother of the index patient revealed no abnormality.

In family C, the index patient was a man. He was operated for cerebellar haemangioblastoma at the age of 33 years. Preoperative clinical screening also showed renal cysts. Two years later a pancreatic cyst and bilateral renal cell carcinomas were found and the patients underwent bilateral nephrectomy with subsequent chronic haemodialysis. Family
members were not available for clinical and genetic screening except a brother whose screening revealed no abnormality.

In family D, the index patient was a young woman who underwent surgery for cerebellar haemangioblastoma at the age of 22 years. Multiple renal cysts and retinal detachment with vitreous haemorrhage were also observed. At the age of 26 years pancreatic cyst, spinal cord haemangiomas, and recurrence of the cerebellar lesion were found. Family members were not available for clinical and genetic screening but family history revealed that the patient’s father died of metastatic renal cell carcinoma.

In family E, the index patient was a 21-year-old man, who had bilateral highly vascularised renal masses and a large number of renal cysts. Family history indicated a high suspicion for VHL disease, as his mother was operated for renal cell carcinoma, his uncle had spinal cord tumor and his grandmother underwent operation for renal cell carcinoma. However, detailed clinical evaluation could not be performed in family members.

In family F, the index patient was a man who developed retina haemangiomas and unilateral phaeochromocytoma at the age of 27 years. Abdominal CT showed also horseshoe kidneys. His mother underwent operation for renal cell carcinoma at the age of 49 years, but other manifestations of the VHL disease were absent during her regular follow up.

Family G was a large VHL family spanning five generations. The index patient was a woman who was operated for phaeochromocytoma and renal cell carcinoma diagnosed at the age of 34 years and 40 years, respectively. She also developed spinal cord, cerebellar and oblong medulla haemangioblastomas at the age of 42 years. Her parents showed no clinical, biochemical or radiological evidence of the disease, but VHL-associated tumors were detected in 5 other family members (bilateral renal cell carcinomas in one, renal cysts in one, spinal cord, cerebellar and oblong medulla haemangioblastomas in 3, retina haemangioblastomas in 4, and phaeochromocytomas in 2 family members).
Patients with apparently sporadic phaeochromocytomas

The study included 37 unrelated patients with histologically confirmed apparently sporadic unilateral phaeochromocytomas evaluated at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998-2008. There were 17 men (age, mean±SD 40.6±15.4; range, 12-67 years) and 20 women (age, mean±SD 37.7±14.4; range, 19-63 years). Patients with bilateral and familial phaeochromocytomas and those with personal or family history of syndromic disease (VHL, multiple endocrine neoplasia type 2, neurofibromatosis type 1, familial paraganglioma syndromes) were excluded. Preoperative evaluation included medical history, physical examination, abdominal ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI), MIBG-scintigraphy, ophthalmologic examination, routine biochemical testing and 24h urinary catecholamine metabolite determination. Patients who proved to have VHL gene mutations underwent genetic counselling, and clinical screening for other VHL-associated tumors was performed. Genetic and clinical screening for VHL-associated tumors was also offered for relatives of mutation positive patients.

Genetic screening for VHL gene mutations

Written informed consent was obtained from all patients and family members who participated in the study. Genomic DNA was extracted from peripheral blood leukocytes using DNA isolation kit for mammalian blood (Roche Diagnostics GmbH, Mannheim, Germany). Mutation analysis of splice sites and exons 1, 2 and 3 of the VHL gene was performed using direct sequencing of DNA samples amplified by polymerase chain reaction (PCR). PCR amplifications were carried out using modified oligonucleotide primers 24, which
covered the exon-intron boundaries. Sequence analysis was performed by direct cycle-sequencing using the SequiTherm Excel II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI) and an automated LiCOR IR2 sequencer (Li-COR Inc., Lincoln, NE) or by direct cycle-sequencing using the Big Dye Terminator Cycle-Sequencing kit v3.1 (Applied Biosystems, Foster City, CA), and run on an automated sequencer 310 Genetic Analyser from Applied Biosystems.

In cases when direct sequencing failed to show \textit{VHL} gene mutations, DNA samples were analysed for germline deletion of the \textit{VHL} gene. A non-sequence-specific real-time detection of PCR products was performed using the DNA binding dye SYBR Green I, as described by Ponchel et al. In a final volume of 25 µl, 120 ng DNA was amplified with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using highly specific primer pairs (12.5 pg each) for flanking exons of the \textit{VHL} gene. Exon 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (\textit{GAPDH}, 12p13) was used as a reference in simultaneous co-amplification. The Applied Biosystems 7500 Fast Real-Time PCR System was used for absolute quantitation. All reactions were performed in triplicates, and comparative threshold (\(C_t\)) values were accepted if the standard deviation was lower than 0.05. The \(\Delta C_t\) values \([C_t(\text{vhl exon})-C_t(\text{GAPDH})]\) were calculated for the average of each triplicate. A \(\Delta C_t\) of 0 indicated a ratio (R) of 1 between the target and the reference \(2^{-(\Delta C_t)} = 2^0 = 1\). Controls included samples from patients with known heterozygous \textit{VHL} gene alterations and those from healthy subjects. The cut-off value of the ratio for submitting the absence of an exon of the \textit{VHL} gene was 0.6. All primer sequences are available on request. Primers were purchased from Invitrogen Life Technologies (Glasgow, United Kingdom). The specificity of all primers was checked using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).
Large deletions of the \textit{VHL} gene was also analysed using multiple ligation-dependent probe amplification (MLPA) according to the manufacturer’s instructions (MRC-Holland, Amsterdam, The Netherlands) \textsuperscript{26}. 6-FAM-labelled fragments were separated using capillary electrophoresis (310 Genetic Analyser from Applied Biosystems). The relative peak areas were compared according to the manufacturer’s instructions. Deletions were established if reductions in relative peak areas were less than 55% compared to controls. Control samples were obtained from individuals with known heterozygous \textit{VHL} gene alterations (mutation or polymorphism).

\textbf{Results}

\textit{VHL} gene mutations or deletions were identified in all index patients with VHL disease as well as in several other members of our VHL families (\textbf{Table 2}). In family A, a previously described disease-causing heterozygous L158V mutation was found in the index patient and in one of the two children who had retina haemangiomas. This mutation developed \textit{de novo} in the index patient, as it was absent in the two parents. Repeat clinical evaluation of the affected son of the index patient revealed no other VHL-related tumors. The index patient of family B proved to have a heterozygous R161X mutation, which has been already published in VHL families. This mutation was absent in one clinically healthy brother of the index patient, while other members of this family were not available for genetic screening. The index patient in family C proved to have a heterozygous deletion of exon 3 of the \textit{VHL} gene, which was absent in a clinically healthy brother of the index patient. In family D the index patient had several manifestations of the disease, including retinal detachment with vitreous haemorrhage that is known to be closely associated with the presence of haemangiomas \textsuperscript{27}. The patient had a heterozygous deletion of exon 2 of the \textit{VHL} gene; genetic screening could not be performed in other members of this family. The index patient was also the only member available for
genetic screening in family E, who had a previously unpublished heterozygous 2bp deletion in exon 2 of the \textit{VHL} gene (354_355delCT). In family F genetic analysis showed the presence of a previously described disease-causing heterozygous R167Q mutation in the index patient and in his mother, both clinically affected with VHL disease. In family G, a previously described disease-causing heterozygous S80I was identified in the index patient, in 5 other family members affected with VHL disease as well as in 2 clinically healthy family members. Repeat clinical evaluation of family members who carried the mutation showed no newly-developed VHL-related tumors. Some members of this family had also the P25L variant of the \textit{VHL} gene, (rs35460768; dbSNP127), but this proved to be a neutral variant$^{23}$. 

Of the 37 patients with apparently sporadic unilateral phaeochromocytomas, 3 patients had heterozygous missense mutations of the \textit{VHL} gene. The L63P mutation of the \textit{VHL} gene was identified in a 50-year-old man, whose family screening showed the presence of the same mutation in two of the three clinically healthy brothers, whereas the mutation was absent in a clinically healthy son. The Y156C mutation was found in a 55-year-old woman, but the mutation was absent in her clinically healthy son. Finally, the R167G mutation was detected in a 20-year-old woman, whose family screening indicated the absence of mutation in her mother and two brothers. Each of these three \textit{VHL gene} mutations found in our patients with apparently sporadic unilateral phaeochromocytomas have been previously described as disease-causing mutations. Clinical evaluation of the three patients indicated the absence of other VHL-related tumors.

All patients with apparently sporadic unilateral phaeochromocytomas were also tested for large deletions but no loss of exons of the \textit{VHL} gene was found.

\textbf{Discussion}
In this study we identified disease-causing genetic abnormalities in each of the 7 Hungarian VHL families and in 3 of the 37 Hungarian patients with apparently sporadic phaeochromocytomas (8.1%). The 10 disease-causing genetic alterations detected in our study included one nonsense mutation (R161X), 6 missense mutations (L158V, R167Q, S80I, L63P, Y156C and R167G), one small deletion (354_355delCT) and 2 large deletions of the VHL gene. In addition, one neutral VHL gene variant (P25L) was found. These genetic alterations were distributed throughout the entire VHL gene, and no hotspot or recurrent mutations were revealed (Figure 1.). Mutations resulting in truncated protein and large deletions were found only in families with VHL type 1, whereas missense mutations were associated mainly, although not exclusively with VHL type 2B and type 2C. This mutational spectrum in Hungarian VHL patients does not differ significantly from those observed in Western, Japanese or Chinese VHL kindreds. In one of the largest series of VHL gene mutation screening 6 mutation hotspots were identified and two of them (R161X and R167Q) were found in our patient cohorts.

Of the 10 disease-causing alterations of the VHL gene identified in our patients, all but one genetic abnormalities have been already reported in previous studies. The novel 2bp deletion (354_355delCT) and the previously described R161X mutation, both found in VHL type 1 families in our study result in truncated VHL proteins and, obviously, these truncated proteins may have impaired function. The two different large gene deletions detected in two VHL probands with type 1 disease may also exert severe impact on protein function. Large gene deletions, which occurred in about 20% of patients with VHL disease in previous studies usually cause VHL type 1, but there are recent reports on Belgian and Polish “CNS haemangioblastoma only” families.

The 6 different missense mutations detected in our study have been previously described mainly in VHL patients with type 2 disease. The S80I mutation has been previously
characterized as type 1 mutation in Western countries, but similarly to our study this mutation has been recently associated with type 2 disease in a Chinese VHL family. Interestingly, in our VHL family the S80I mutation co-segregated with a P25L variant of the VHL gene. The P25L alteration has been shown to be a benign variant, while the S80, together with the S68, S72 and S65 are critical residues where phosphorylation of the pVHL30 by glycogen synthase kinase 3 (GSK-3) occurs. These residues play important role in the stabilization of microtubules. The GSK3 is a component of multiple signalling pathways that are altered in human cancer, and regulation of microtubule dynamics can be critical in pVHL tumor-suppressor events. Lolkema and co-workers demonstrated that an additional tumor suppressor function is linked to phosphorylation of serine residues (S33, S38, S43) in the N-terminal acidic domain of pVHL, which is independent of the regulation of HIF1α.

One interesting clinical finding in our study was the presence of polyglobulia without evidence of any renal lesion or other underlying disorder in a young patient with VHL type 2 disease harbouring a truncating mutation (R161X), suggesting that inappropriate erythropoietin (EPO) production might play a role in this clinical abnormality. Mutations of the VHL gene have been associated with Chuvash polycythaemia (CP), a rare congenital disorder without VHL-associated tumors. According to our knowledge, there is no systematic review on the presence of erythrocytosis in patients with VHL disease with and without possible renal EPO overproduction, however, erythrocytosis has been reported in 5-20% of patients. The fundamental genetic difference between CP and VHL disease is that the inheritance of CP is autosomal recessive, while VHL disease is inherited in an autosomal dominant manner. Thus, patients with CP harbour germline homozygous (or compound heterozygous) whereas patients with VHL disease have heterozygous VHL gene mutations. This raises questions not only on further HIF1α functions or modifier genes in CP patients but also on the significance of erythrocytosis in VHL phenotype prediction.
Another interesting clinical finding was the presence of horseshoe kidney in a patient with VHL type 1 harbouring the R167Q mutation. Horseshoe kidney has been extremely rarely detected in patients with VHL disease. It might be related to an early defect in embryonic renal development during the caudal extension of mesonephric ducts, as VHL protein has been shown to be involved in the development of the definitive metanephric kidney.

The usual frequency of phaeochromocytomas in VHL patient cohorts is about 30%, which appears to be similar to our findings (3 of the 8 VHL families, and 5 of the 18 clinically affected family members). The most common genetic causes of phaeochromocytomas include abnormalities of the VHL (VHL syndrome), RET (multiplex endocrine neoplasia type 2, MEN2), NFI (neurofibromatosis type 1) and SDH genes (familial paraganglioma/phaeochromocytoma syndromes). Genetic alterations causing these hereditary syndromes may be detected in 20-30% of unselected phaeochromocytoma cases. However, different prevalence of the VHL gene mutations ranging from 0 to 11% have been documented in patients with sporadic phaeochromocytomas, and the relatively high frequency found in some studies was considered as a consequence of a founder effect. In our study we showed that three of the 37 (8.1%) unrelated patients with histologically confirmed sporadic unilateral phaeochromocytomas had germline mutations of the VHL gene. Each patient had different mutations (L63P, Y156C, and R167G) which firmly excluded a founder effect. These mutations have been previously associated with VHL disease and have been linked to functional alterations of the VHL protein. The L63 is the first residue of the β-domain, and L63P results in a chain brake, while the Y156 is a critical linker between α and β domains. Missense R167 mutations have been also frequently associated with phaeochromocytomas in VHL families and mutation in this residue was present not only in a
patient with sporadic phaeochromocytoma (R167G) but also in one of our VHL type 2 family (R167Q).

In conclusion, the spectrum of \textit{VHL} gene abnormalities in Hungarian population is similar to that observed in Western, Japanese or Chinese VHL kindreds. Our findings confirm previous observations showing that germline abnormalities resulting in truncated VHL proteins cause VHL type 1, whereas missense mutations are associated mainly, although not exclusively with VHL type 2 disease. In addition, we found that missense \textit{VHL} gene mutations can be detected in 8.1\% of patients with apparently sporadic unilateral phaeochromocytomas suggesting that genetic testing is useful not only in patients with VHL disease but also in those with apparently sporadic phaeochromocytomas.
Acknowledgments:

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


**Figure legend**

**Figure 1.** Localisation of the *VHL* gene mutation identified in our study.
**Mutations and VHL Type**

- **Exon 1 (1-113)**
  - L63P
  - S80I
  - 354_355delCT

- **Exon 2 (114-154)**
  - L158V
  - Y156C

- **Exon 3 (155-213)**
  - R161X
  - R167Q
  - R167G

**Binding Sites**

- HIF 1 binding site (91-113)
- Elongin C binding site (157-170)

**Domains**

- β-domain (63-154)
- α-domain (155-192)
- β-domain (193-204)
### Table 1. Clinical manifestations of affected family members with von Hippel-Lindau disease

<table>
<thead>
<tr>
<th>Families</th>
<th>Family members</th>
<th>Renal cell carcinoma (age at presentation, years)</th>
<th>CNS haemangioblastoma (age at presentation, years)</th>
<th>Phaeochromocytoma (age at presentation, years)</th>
<th>Retina involvement (age at presentation, years)</th>
<th>Other clinical presentation</th>
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IP, index patient; <sup>a</sup> Previously reported; <sup>b</sup> Genetic test was not performed. CNS, central nervous system.
Table 2. Characteristics of the VHL gene mutation identified in patients with von Hippel-Lindau disease and in those with apparently sporadic phaeochromocytomas.

<table>
<thead>
<tr>
<th>Families</th>
<th>VHL genotype</th>
<th>Location within the VHL gene</th>
<th>Number of genetically tested/number of mutation carriers</th>
<th>Number of clinically asymptomatic mutation carriers</th>
<th>Domain of VHL protein</th>
<th>VHL phenotype</th>
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* Genetic test was not performed in one clinically affected family member; ASP, apparently sporadic phaeochromocytoma; VBC, VHL/Elongin B/Elongin C complex