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

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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 pheochromocytoma (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 analyzed. Patients were clinically evaluated and the VHL gene was analyzed using direct sequencing, multiplex ligation-dependent probe amplification, and real-time PCR with SYBR Green chemistry.

Results: Disease-causing genetic abnormalities were identified in each of the seven VHL families and in 3 out of the 37 patients with ASP (one nonsense and six missense mutations, two 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 the Hungarian population is similar to that observed in Western, Japanese, or Chinese VHL kindreds. The presence of VHL gene mutations in 3 out 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.

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Introduction

Von Hippel–Lindau (VHL, OMIM No. 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 (1). Patients with VHL disease are at risk for the development of retinal, cerebellar, spinal and, less frequently, pancreatic, renal, pulmonary, liver, and adrenal hemangiomas/hemangiendotheliomas (2–4); clear-cell renal carcinomas, pheochromocytomas, endolymphatic sac tumors, multiple renal, epididymal and pancreatic cysts; cystadenomas of the epididymis and the broad ligament, and pancreatic islet cell tumors (1, 5, 6). VHL disease has been divided into two main groups: patients with type 1 disease have a greatly reduced risk of pheochromocytomas, but can develop all the other manifestations of the disease, whereas patients with VHL type 2 have pheochromocytomas. Type 2B 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 pheochromocytomas without other manifestations of the disease (7).

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 (1, 8, 9), and it has all the functional domains of the pVHL30 (9–11). 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.

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(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) (12). The majority of disease-causing VHL gene mutations are located in one of these two binding sites.

More than 900 different mutations of the VHL gene have been described (http://www.umd.be/) (13). The majority of 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. By contrast, VHL type 2 is usually associated with missense mutations affecting the protein-binding sites of the 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 pheochromocytomas (codons 25 and 38) or with VHL disease (codons E46X and E52K) (14–17).

VHL disease has an approximate incidence of 1/36 000 live births (18), and germline VHL gene mutations have been found in about 40% of familial or bilateral pheochromocytomas (19, 20). Screening for germline VHL gene mutations may also be recommended in patients with apparently sporadic unilateral pheochromocytomas, although a wide variation in mutation frequencies ranging from 0 to 11% in different patient cohorts has been reported (19–22). The identification of germline VHL gene mutations in patients with apparently sporadic pheochromocytomas (ASP) appears to be important, as the onset of the VHL disease may show a high variability and many patients with VHL-related pheochromocytomas 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 VHL gene in Hungarian families with VHL disease and patients with apparently sporadic unilateral pheochromocytomas.

**Subjects and methods**

**Families with VHL disease**

A total of 35 members from seven 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 24 h urinary catecholamine metabolite determination. All patients with VHL disease and family members of patients who proved to have VHL gene mutations underwent genetic counseling, and clinical as well as genetic screening was performed in all relatives who agreed to 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) hemangioblastomas, renal cysts, and retinal hemangiomas. The parents and one of the two children of the index patient had no clinical symptoms, whereas in the other child retinal hemangiomas were diagnosed at the age of 6 years.

In family B, the index patient was a young man who developed retinal hemangiomas at the age of 19 years, and cerebellar hemangioma 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 hemangioblastoma 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 hemodialysis. 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 hemangioblastoma at the age of 22 years. Multiple renal cysts and retinal detachment with vitreous hemorrhage were also observed. At the age of 26 years, pancreatic cyst, spinal cord hemangiomas, 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 vascularized 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 retinal hemangiomas and unilateral pheochromocytoma 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 (23). The index patient was a woman who was operated for pheochromocytoma and renal cell carcinoma diagnosed at the age of 34 and 40 years respectively. She also developed spinal cord, cerebellar, and medulla oblongata hemangioblastomas 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 five other family members (bilateral renal cell carcinomas in one, renal cysts in one, spinal cord, cerebellar, and medulla oblongata hemangioblastomas in three, retinal hemangioblastomas in four, and pheochromocytomas in two family members).

Patients with ASPs

The study included 37 unrelated patients with histologically confirmed, apparently sporadic unilateral pheochromocytomas evaluated at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2008. There were 17 men (age, mean ± S.D. 40.6 ± 15.4; range, 12–67 years) and 20 women (age, mean ± S.D. 37.7 ± 14.4; range, 19–63 years). Patients with bilateral and familial pheochromocytomas and those with personal or family history of syndromic disease (VHL, MEN2, neurofibromatosis type 1 (NF1), and familial paraganglioma syndromes) were excluded. Preoperative evaluation included medical history, physical examination, abdominal ultrasonography, CT or MRI, MIBG-scintigraphy, ophthalmologic examination, routine biochemical testing, and 24 h urinary catecholamine metabolite determination. Patients who proved to have VHL gene mutations underwent genetic counseling, 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). Mutation analysis of splice sites and exons 1, 2, and 3 of the VHL gene was performed by using direct sequencing of DNA samples amplified by 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 (Epipcentre Technologies, Madison, WI, USA) and an automated Li-COR IR2 sequencer (Li-COR Inc., Lincoln, NE, USA) or by direct cycle sequencing using the Big Dye Terminator Cycle

Table 1

<table>
<thead>
<tr>
<th>Family members</th>
<th>Renal cell carcinoma (age at presentation, years)</th>
<th>CNS hemangioblastoma (age at presentation, years)</th>
<th>Retina involvement (age at presentation, years)</th>
<th>Pheochromocytoma (age at presentation, years)</th>
<th>Other clinical presentation</th>
<th>VHL subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>39</td>
<td>27</td>
<td>40</td>
<td>22</td>
<td>Renal cysts, pancreatic cysts and medulla oblongata hemangioblastomas</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>35</td>
<td>21</td>
<td>40</td>
<td>22</td>
<td>Renal cysts and pancreatic cysts</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>36</td>
<td>24</td>
<td>45</td>
<td>21</td>
<td>Renal cysts, pancreatic and CNS hemangioblastomas in one family member</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>Renal cysts, pancreatic and CNS hemangioblastomas in two family members</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>39</td>
<td>33</td>
<td>42</td>
<td>27</td>
<td>Renal cysts, pancreatic and CNS hemangioblastomas in three family members</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>40</td>
<td>45</td>
<td>42</td>
<td>27</td>
<td>Renal cysts, pancreatic and CNS hemangioblastomas in four family members</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>40</td>
<td>42</td>
<td>42</td>
<td>27</td>
<td>Renal cysts, pancreatic and CNS hemangioblastomas in five family members</td>
<td>1</td>
</tr>
</tbody>
</table>

IP, index patient; IP –, genetic test was not performed.
Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), and run on an automated sequencer 310 Genetic Analyzer from Applied Biosystems.

In cases when direct sequencing failed to show VHL gene mutations, DNA samples were analyzed for germline deletion of the VHL gene. A nonsequence-specific real-time detection of PCR products was performed by 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) using highly specific primer pairs (12.5 pg each) for flanking exons of the VHL gene. Exon 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (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 (Ct) values were accepted if the s.d. was lower than 0.05. The ΔCt values (Ct(vhl_exon) – Ct(GAPDH)) were calculated for the average of each triplicate. A ΔCt of 0 indicated a ratio (R) of 1 between the target and the reference (2^(-ΔCt) = 2^0 = 1) (25).

Controls included samples from patients with known heterozygous VHL gene alterations and those from healthy subjects. The cut-off value of the ratio for submitting the absence of an exon of the VHL gene was 0.6. All primer sequences are available on request. Primers were purchased from Invitrogen Life Technologies. The specificity of all primers was checked using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Large deletions of the VHL gene were also analyzed using multiple ligation-dependent probe amplification according to the manufacturer’s instructions (MRC-Holland, Amsterdam, The Netherlands) (26). 6-FAM-labeled 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 <55% compared with controls. Control samples were obtained from individuals with known heterozygous VHL gene alterations (mutation or polymorphism).

**Results**

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 (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 retinal hemangiomas. This mutation developed *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 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 hemorrhage that is known to be closely associated with the presence of hemangiomas (27). The patient had a heterozygous deletion of exon 2 of the 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 2 bp deletion in exon 2 of the 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

<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>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>L158V</td>
<td>Exon 3</td>
<td>6/2</td>
<td>1</td>
<td>α</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>R161X</td>
<td>Exon 3</td>
<td>2/1</td>
<td>0</td>
<td>α</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>Exon 3 deletion</td>
<td>Exon 3</td>
<td>2/1</td>
<td>0</td>
<td>α and β</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>Exon 2 deletion</td>
<td>Exon 2</td>
<td>1/1</td>
<td>0</td>
<td>β</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>354_355delCT</td>
<td>Exon 2</td>
<td>1/1</td>
<td>0</td>
<td>α and β</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>R167Q</td>
<td>Exon 3</td>
<td>2/2</td>
<td>0</td>
<td>α</td>
<td>2B</td>
</tr>
<tr>
<td>G</td>
<td>S80I</td>
<td>Exon 1</td>
<td>21/7*</td>
<td>2</td>
<td>β</td>
<td>2B</td>
</tr>
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<td>L63P</td>
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<td>2</td>
<td>β</td>
<td>2C</td>
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<td>0</td>
<td>β</td>
<td>2C</td>
</tr>
<tr>
<td>ASP</td>
<td>R167G</td>
<td>Exon 3</td>
<td>4/1</td>
<td>0</td>
<td>α</td>
<td>2C</td>
</tr>
</tbody>
</table>

ASP, apparently sporadic pheochromocytomas; VBC, VHL/elongin B/elongin C complex.

*Genetic test was not performed in one clinically affected family member.

Table 2 Characteristics of the VHL gene mutation identified in patients with von Hippel–Lindau disease and in those with apparently sporadic pheochromocytomas.

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patient and in five other family members affected with VHL disease, as well as in two 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 also had the P25L variant of the VHL gene, (rs35460768; dbSNP127), but this proved to be a neutral variant (23).

Out of the 37 patients with apparently sporadic unilateral pheochromocytomas, three patients had heterozygous missense mutations of the VHL gene. The L63P mutation of the VHL gene was identified in a 50-year-old man, whose family screening showed the presence of the same mutation in two out of 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 VHL gene mutations found in our patients with apparently sporadic unilateral pheochromocytomas has 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 pheochromocytomas were also tested for large deletions, but no loss of exons of the VHL gene was found.

Discussion

In this study, we identified disease-causing genetic abnormalities in each of the seven Hungarian VHL families and in 3 out of the 37 Hungarian patients with ASPs (8.1%). The ten disease-causing genetic alterations detected in our study included one nonsense mutation (R161X), six missense mutations (L158V, R167Q, S80I, L63P, Y156C, and R167G), one small deletion (354_355delCT), and two 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 hot spot or recurrent mutations were revealed (Fig. 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 (28–30). In one of the largest series of VHL gene mutation screening, six mutation hot spots were identified and two of them (R161X and R167Q) were found in our patient cohorts (31).

Out of the ten disease-causing alterations of the VHL gene identified in our patients, all but one genetic abnormalities have already been reported in previous studies. The novel 2 bp 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 a severe impact on protein function. Large gene deletions, which occurred in about 20% of patients with VHL disease in previous studies (32), usually cause VHL type 1, but there are recent reports on Belgian and Polish ‘CNS hemangioblastoma only’ families (30, 33).

The six 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

![Figure 1](https://via-free-access.bioscientifica.com/)

**Figure 1** Localization of the VHL gene mutation identified in our study.
Western countries, but similarly to our study this mutation has been recently associated with type 2 disease in a Chinese VHL family (34, 35). Interestingly, in our VHL family, the S80I mutation co-segregated with a P25L variant of the VHL gene (23). The P25L alteration has been shown to be a benign variant (34, 35), while the S80, together with the S68, S72, and S65, is a critical residue where phosphorylation of the pVHL30 by glycogen synthase kinase 3 (GSK3) occurs. These residues play an important role in the stabilization of microtubules. The GSK3 is a component of multiple-signaling pathways that are altered in human cancer, and regulation of microtubule dynamics can be critical in pVHL tumor-suppressor events (36). Lolkema and co-workers demonstrated that an additional tumor-suppressor function is linked to phosphorylation of serine residues (S33, S38, and S43) in the N-terminal acidic domain of pVHL, which is independent of the regulation of HIF1α (37).

One interesting clinical finding in our study was the presence of polyclonubilia without evidence of any renal lesion or other underlying disorder in a young patient with VHL type 2 disease harboring 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 polycythemia (CP), a rare disease in a Chinese VHL family (34, 35). Interestingly, mutation has been recently associated with type 2 disease in a Chinese VHL family (34, 35). While the S80, together with the S68, S72, and S65, is a critical residue where phosphorylation of the pVHL30 by glycogen synthase kinase 3 (GSK3) occurs. These residues play an important role in the stabilization of microtubules. The GSK3 is a component of multiple-signaling pathways that are altered in human cancer, and regulation of microtubule dynamics can be critical in pVHL tumor-suppressor events (36). Lolkema and co-workers demonstrated that an additional tumor-suppressor function is linked to phosphorylation of serine residues (S33, S38, and S43) in the N-terminal acidic domain of pVHL, which is independent of the regulation of HIF1α (37).

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In conclusion, the spectrum of VHL gene abnormalities in the 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 VHL gene mutations can be detected in 8.1% of patients with apparently sporadic unilateral pheochromocytomas, suggesting that genetic testing is useful not only in patients with VHL disease but also in those with ASPs.

Declaration of interest
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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