CLINICAL STUDY

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/hemangioblastomas (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 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 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

(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.

Table 1 Clinical manifestations of affected family members with von Hippel–Lindau disease.

Families	Family members	Renal cell carcinoma (age at presentation, years)	CNS hemangioblastoma (age at presentation, years)	Pheochromocytoma (age at presentation, years)	Retina involvement (age at presentation, years)	Other clinical presentation	VHL subtype
A	IP	–	39	–	22	Renal cysts	1
	Son	–	–	–	6	–	1
B	IP	–	27	–	19	Renal cysts, pancreatic cysts, and polyglobulia	1
C	IP	35, Bilateral	33	–	–	Renal cysts and pancreatic cyst	1
D	IP	–	22	–	22	Renal cysts, pancreatic cyst, and accessory spleen	1
E	IP	21	–	–	–	Renal cysts, renal cell carcinoma, and CNS hemangioblastomas in family members	1
F	IP	–	–	27	27	Horseshoe kidney	2B
G[a]	Mother	49	–	–	–	–	2B
	IP	40, Bilateral	42	34	–	–	
	Aunt 1[b]	45, Bilateral	–	–	–	–	
	Cousin 1	–	37	–	18	–	
	Cousin 2	–	38	39	29	–	
	Grand cousin 1	–	22	–	23	Renal cysts	
	Grand cousin 2	–	–	10, Bilateral	26	–	

IP, index patient.
[a]Previously reported.
[b]Genetic test was not performed.

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 (Epicentre 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

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 (C_t) values were accepted if the S.D. was lower than 0.05. The ΔC_t values ($C_t(\text{vhl\_exon}) - C_t(\text{GAPDH})$) were calculated for the average of each triplicate. A ΔC_t of 0 indicated a ratio (R) of 1 between the target and the reference ($2^{-(\Delta C_t)} = 2^0 = 1$) (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 2 Characteristics of the *VHL* gene mutation identified in patients with von Hippel–Lindau disease and in those with apparently sporadic pheochromocytomas.

Families	*VHL* genotype	Location within the *VHL* gene	Number of genetically tested/number of mutation carriers	Number of clinically asymptomatic mutation carriers	Domain of VHL protein	VHL phenotype
A	L158V	Exon 3	6/2	1	α	1
B	R161X	Exon 3	2/1	0	α	1
C	Exon 3 deletion	Exon 3	2/1	0	α and β	1
D	Exon 2 deletion	Exon 2	1/1	0	β	1
E	354_355delCT	Exon 2	1/1	0	α and β	1
F	R167Q	Exon 3	2/2	0	α	2B
G	S80I	Exon 1	21/7[a]	2	β	2B
ASP	L63P	Exon 1	5/3	2	β	2C
ASP	Y156C	Exon 3	2/1	0	β	2C
ASP	R167G	Exon 3	4/1	0	α	2C

ASP, apparently sporadic pheochromocytoma; VBC, VHL/elongin B/elongin C complex.
[a]Genetic test was not performed in one clinically affected family member.

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 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 *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
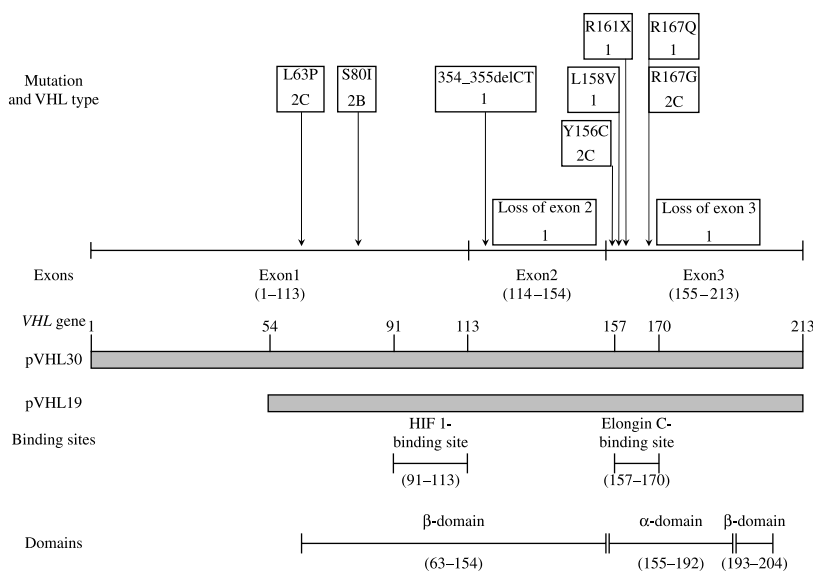

Figure 1 Localization of the *VHL* gene mutation identified in our study.

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 polyglobulia 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 congenital disorder without VHL-associated tumors (24, 38–43). 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 (5). 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 harbor germline homozygous (or compound heterozygous) mutations 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 (44, 45).

Another interesting clinical finding was the presence of horseshoe kidney in a patient with VHL type 1 harboring the R167Q mutation. Horseshoe kidney has been extremely rarely detected in patients with VHL disease (46). 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 (47).

The usual frequency of pheochromocytomas in VHL patient cohorts is about 30% (48), which appears to be similar to our findings (3 out of the 7 VHL families and 5 out of the 18 clinically affected family members). The most common genetic causes of pheochromocytomas include abnormalities of the *VHL* (VHL syndrome), *RET*

(multiplex endocrine neoplasia type 2), *NF1*, and *SDH* genes (familial paraganglioma/pheochromocytoma syndromes). Genetic alterations causing these hereditary syndromes may be detected in 20–30% of unselected pheochromocytoma cases (49). However, different prevalence of the *VHL* gene mutations ranging from 0 to 11% has been documented in patients with sporadic pheochromocytomas (14, 19, 50–52), and the relatively high frequency found in some studies (53) was considered a consequence of a founder effect (22). In our study, we showed that 3 out of the 37 (8.1%) unrelated patients with histologically confirmed, sporadic unilateral pheochromocytomas had germline mutations of the *VHL* gene. Each patient had different mutations (L63P, Y156C, and R167G) that 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 (54), while the Y156 is a critical linker between α- and β-domains (12). Missense R167 mutations have also been frequently associated with pheochromocytomas in VHL families (6), and mutation in this residue was present not only in a patient with sporadic pheochromocytoma (R167G) but also in a member of our VHL type 2 family (R167Q).

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

Funding

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