VHL mutation analysis in patients with isolated central nervous system haemangioblastoma

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Haemangioblastomas of the CNS are a cardinal feature of von Hippel–Lindau (VHL) disease, a dominantly inherited multisystem familial cancer syndrome caused by germline mutation of the VHL tumour suppressor gene. We investigated the frequency of VHL mutations in 188 patients presenting with a single haemangioblastoma, no family history of VHL disease and no evidence of retinal or abdominal manifestations of the disease at the time of diagnosis. We found that ~4% of patients had a detectable VHL mutation and all of these cases presented age 40 years or less. Although the identification of a germline VHL mutation has important consequences for the patient (e.g. risk of further CNS and extra-CNS tumours) and their relatives, four patients had germline VHL missense mutations [C162Y, D179N and R200W (two patients)] that may represent haemangioblastoma-only and/or low penetrance mutations. Approximately 5% of patients without a detectable VHL mutation subsequently developed a further ‘VHL type tumour’ (in most cases a further CNS haemangioblastoma). These findings suggest that a subset of patients with apparently sporadic CNS haemangioblastoma will have a germline VHL mutation but may not be at risk for developing classical VHL disease and a further group may be mosaic for a germline VHL mutation that cannot be detected in blood cells.

Keywords: genetics; haemangioblastoma; mutation; VHL

Abbreviations: CCRCC = clear cell renal cell carcinoma; CHB = cerebellar haemangioblastoma; CNS = central nervous system; CT = computerised tomography; ELST = endolymphatic sac tumour; FDR = first degree relative; h = hour; HB = haemangioblastoma; HIF = hypoxia-inducible factor; m = months; MLPA = multiplex ligation-dependent probe amplification; MRI = magnetic resonance imaging; Phaeo = phaeochromocytoma; RA = retinal angioma; RCC = renal cell carcinoma; USS = ultrasound scan; VEGF = vascular endothelial growth factor; VHL = von Hippel-Lindau; y = years

VHL disease. However, ~20% of patients with VHL disease do not have a family history and in such cases two haemangioblastomas or a haemangioblastoma and another VHL-type tumour are required for diagnosis. The identification of the VHL tumour suppressor gene in 1993 has facilitated the diagnosis of VHL disease, particularly in patients who do not meet the clinical diagnostic criteria (Latif et al., 1993). In patients with classical VHL disease, mutation analysis by direct sequencing and deletion analysis (e.g. by MLPA) reveals a germline mutation in almost all cases (Stolle et al., 1998). However, somatic mosaicism may reduce mutation detection rates (Sgambati et al., 2000). Complex genotype–phenotype correlations have been described in VHL disease with surface missense mutations associated with a high risk of phaeochromocytoma and germline deletions and truncating mutations associated with a low risk of phaeochromocytoma (Crossey et al., 1994; Zbar et al., 1996; Maher et al., 1996; Ong et al., 2006). In addition, heterozygous germline missense VHL mutations may cause familial phaeochromocytoma without other features of VHL disease (Crossey et al., 1995; Neumann et al., 1995; Woodward et al., 1997) and homozygous missense mutations may be associated with recessively inherited polycythaemia (Ang et al., 2002; Pastore et al., 2003a).

Although the link between haemangioblastomas and VHL disease is well recognized, clinical and radiological investigation at the time of presentation of the haemangioblastoma may not reveal evidence of VHL disease. Hence, VHL mutation analysis can be used to isolate patients with no family history and no other features of VHL disease at presentation. Previously, the frequency of germline VHL mutations in patients presenting with an apparently isolated CNS haemangioblastoma has been reported to range from 4 to 14% (Olschwang et al., 1998; Glasker et al., 1999; Hes et al., 2000; Catapano et al., 2005). In the current study we report the largest cohort of patients (n = 188) with a single isolated haemangioblastoma and define the risk of VHL-related complications in patients who test negative for a germline VHL mutation.

**Material and methods**

**Patient ascertainment and assessment**

VHL mutation analysis was undertaken in 188 patients presenting with a single CNS haemangioblastoma. Information on previous medical history and family history was collected and all patients were evaluated for retinal (by indirect ophthalmological examination) and abdominal (by USS, CT or MRI scans) VHL lesions. Patients with a family history of VHL disease, multiple haemangioblastomas or evidence of a retinal haemangioblastoma (angioma), and renal, adrenal or pancreatic tumours or cysts were excluded from the study. Follow-up information was also provided by the referring centre. Forty-seven UK patients were also included in a previous UK/Netherlands study (Hes et al., 2000).

**Molecular genetic analysis**

Following informed consent and extraction of DNA from whole blood, VHL mutation analysis of the pVHL1a coding sequence and flanking intronic sequences was performed by direct sequencing (primer details and conditions available on request). Quantitative Southern blotting and multiplex ligation-dependent probe amplification (MLPA) studies were performed to detect large genomic rearrangements. Nucleotides are numbered according to Latif et al. (1993).

**Results**

**Study cohort**

188 individuals (99 males, 89 females) presenting with a single isolated haemangioblastoma and no evidence of VHL disease were investigated. One hundred and fifty-eight (84.0%) had a cerebellar haemangioblastoma, 27 had a spinal haemangioblastoma (14.4%) and 3 (1.6%) had a brainstem haemangioblastoma. The mean age at diagnosis was 37.7 years (range 11–70 years) (Fig. 1).

**VHL mutation analysis and clinical follow-up**

Seven patients demonstrated an abnormality on VHL mutation analysis: one patient had a VHL gene rearrangement on Southern blotting; one a germline frameshift mutation; and five an intragenic missense substitution (Table 1). All seven patients with a putative VHL mutation presented with a haemangioblastoma at 40 years or less and mean age at diagnosis was 33 years (P = 0.05 compared to those without a mutation).

Four of the five potential VHL missense mutations have been reported previously (see later) but the D179N substitution is, to our knowledge, a novel finding. This substitution was not detected in 200 control chromosomes, but was present in the patient’s father (who was clinically unaffected age 63 years).

One hundred and eighty-one individuals (96.3%) (mean age 37.9 years) showed no evidence of a germline VHL...
Ten of these 181 individuals subsequently developed possible features of VHL disease (Table 2). For these 10 patients, the mean age at diagnosis was 29.3 years (range 11–41 years) (Fig. 1) and mean time to the second event was 12.3 years. The clinical features of these individuals are presented in Table 2. Eight developed a further CNS haemangioblastoma separate from the initial presenting lesion (one of these also developed a pancreatic cystadenocarcinoma) and one an RCC age 55 years. In one case it was not clear if the cerebellar haemangioblastoma occurring 18 years after the initial cerebellar haemangioblastoma was a recurrence or a separate tumour. In seven of the ten cases the potential manifestations of underlying VHL disease were restricted to the CNS. To allow for

<table>
<thead>
<tr>
<th>Patient</th>
<th>HB type</th>
<th>Age at diagnosis (years)</th>
<th>VHL mutation</th>
<th>Novel</th>
<th>Follow-up</th>
<th>Clinical features post HB</th>
<th>Family history</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Spinal</td>
<td>28</td>
<td>c.582_589delGACACAC</td>
<td>N/A</td>
<td>5 y 10 m</td>
<td>Renal cyst 32 y</td>
<td>No testing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pancreatic cyst 32 y</td>
<td>No testing</td>
</tr>
<tr>
<td>B2</td>
<td>CHB</td>
<td>36</td>
<td>Abnormal Southern blot</td>
<td>N/A</td>
<td>5 m</td>
<td>Nil</td>
<td>No features VHL disease</td>
</tr>
<tr>
<td>B3</td>
<td>CHB</td>
<td>40</td>
<td>c.713G→A, R167Q</td>
<td>No</td>
<td>1 y 11 m</td>
<td>Phaeo 41 y</td>
<td>No features VHL disease</td>
</tr>
<tr>
<td>B4</td>
<td>CHB</td>
<td>36</td>
<td>c.811C→T, R200W</td>
<td>No</td>
<td>3 y 1 m</td>
<td>Nil</td>
<td>No features VHL disease</td>
</tr>
<tr>
<td>B5</td>
<td>CHB</td>
<td>39</td>
<td>c.811C→T, R200W</td>
<td>No</td>
<td>6 y 3 m</td>
<td>Nil</td>
<td>No features VHL disease</td>
</tr>
<tr>
<td>B6</td>
<td>CHB</td>
<td>22</td>
<td>c.698G→A, C162Y</td>
<td>No</td>
<td>2 y 7 m</td>
<td>Nil</td>
<td>No features VHL disease</td>
</tr>
<tr>
<td>B7</td>
<td>CHB</td>
<td>30</td>
<td>c.748G→A, D179N</td>
<td>Yes</td>
<td>8 y</td>
<td>Nil</td>
<td>No features VHL disease</td>
</tr>
</tbody>
</table>

HB, haemangioblastoma; CHB, cerebellar haemangioblastoma; RA, retinal angioma; y, years; m, months; FDR, first degree relative; Phaeo, phaeochromocytoma.

Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>HB type</th>
<th>Age at diagnosis (years)</th>
<th>Follow-up</th>
<th>Clinical features post HB</th>
<th>Age at which further feature developed (years)</th>
<th>Time to diagnosis of further feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>CHB</td>
<td>34</td>
<td>6 y 5 m</td>
<td>Spinal HB</td>
<td>40</td>
<td>6 y 5 m</td>
</tr>
<tr>
<td>A2</td>
<td>CHB</td>
<td>41</td>
<td>14 y</td>
<td>CCRCC</td>
<td>55</td>
<td>14 y</td>
</tr>
<tr>
<td>A3</td>
<td>CHB</td>
<td>23</td>
<td>14 y</td>
<td>CHB</td>
<td>36</td>
<td>13 y</td>
</tr>
<tr>
<td>A4</td>
<td>CHB</td>
<td>38</td>
<td>27 y</td>
<td>Bilateral renal cysts and pancreatic mucinocystadenocarcinoma</td>
<td>59</td>
<td>22 y</td>
</tr>
<tr>
<td>A5</td>
<td>CHB</td>
<td>38</td>
<td>7 y</td>
<td>Spinal HB</td>
<td>42</td>
<td>4 y</td>
</tr>
<tr>
<td>A6</td>
<td>CHB</td>
<td>17</td>
<td>11 y 10 m</td>
<td>Cerebral HB</td>
<td>28</td>
<td>10 y 11 m</td>
</tr>
<tr>
<td>A7</td>
<td>CHB</td>
<td>17</td>
<td>18 y</td>
<td>CHB</td>
<td>35</td>
<td>18 y</td>
</tr>
<tr>
<td>A8</td>
<td>Conus</td>
<td>11</td>
<td>7 y 5 m</td>
<td>Multiple spinal HBs</td>
<td>13</td>
<td>16 m</td>
</tr>
<tr>
<td>A9</td>
<td>CHB</td>
<td>38</td>
<td>2 y</td>
<td>Multiple CHBs</td>
<td>13</td>
<td>16 m</td>
</tr>
<tr>
<td>A10</td>
<td>CHB</td>
<td>36</td>
<td>18 y</td>
<td>Cauda equina HB</td>
<td>46</td>
<td>10 y 7 m</td>
</tr>
</tbody>
</table>

HB, haemangioblastoma; CHB, cerebellar haemangioblastoma; CCRCC, clear cell renal cell carcinoma; y, years; m, months.
variable periods of follow up we calculated the risk of developing a 'VHL type' tumour by Kaplan–Meier analysis for 'VHL-mutation negative cases'. At 10 years post haemangioblastoma diagnosis there was a 5.0% risk of developing a 'VHL type' tumour (Fig. 2A). A further six patients developed renal cysts on follow up. In no case were these multiple and bilateral. The mean age at diagnosis of haemangioblastoma in these cases was 49 years and the actuarial risk of developing a simple renal cyst post haemangioblastoma diagnosis was 6.6% at 10 years (Fig. 2B).

Discussion

We detected a putative VHL mutation in seven of 188 individuals presenting with a single haemangioblastoma, no family history of VHL disease and no evidence of retinal or abdominal manifestations of VHL disease at the time of diagnosis of the haemangioblastoma. To our knowledge, this study is by far the largest cohort of patients with apparently isolated haemangioblastoma in which VHL mutation analysis has been reported. Thus we previously detected a germline mutation in three of 84 patients with isolated haemangioblastomas (Hes et al., 2000), Olschwang et al. (1998) and Catapano et al. (2005) have also reported results of VHL mutation studies in patients with CNS haemangioblastomas and have described higher detection rates (germline VHL mutations were detected in two of 18, nine of 66 and two of 14 patients, respectively). However Olschwang et al. (1998) and Catapano et al. (2005) studied only a small number of patients. Glasker et al. (1999) studied approximately a third of the number reported by us, but the higher detection rate in their study is likely to reflect differences in inclusion criteria. Thus we rigorously excluded patients with evidence of VHL disease at the time of diagnosis of the haemangioblastoma, whereas Glasker et al. (1999) reported that 6 of the 9 patients in whom a germline VHL mutation was detected were found to have extra-CNS VHL associated lesions on screening and three had a positive family for VHL disease. The identification of a germline VHL mutation in an individual with a single isolated haemangioblastoma and no other evidence of VHL disease has important implications for that individual and their family. In three patients we identified mutations that have previously been associated with classical VHL disease [e.g. a frameshift mutation in B1, a germline VHL gene rearrangement in B2 (both also in our previous study; Hes et al., 2000), and a R167Q missense mutation in B3] and at the time of latest follow-up two of these patients had gone on to develop non-CNS features of VHL disease. In a further four patients a heterozygous germline missense substitution was detected [R200W in two patients (B4 and B5) and C162Y (B6) and D179N (B7)]. All of these patients presented before age 40 years and the missense substitutions were not detected in at least 160 control chromosomes. At the time of latest follow-up (range 2.6–8 years) none had developed other features of VHL disease. Previously, we reported a germline P81S missense substitution in a 44-year-old Dutch woman with a single haemangioblastoma. The P81S substitution was also detected in four of her clinically unaffected first and second degree relatives (aged 17–77 years) raising the possibility that this mutation might represent a low penetrance/haemangioblastoma-only mutation (Hes et al., 2000). Although rare pVHL missense mutations may cause
a phaeochromocytoma-only phenotype (Type 2C VHL disease) (Crossey et al., 1995; Neumann et al., 1995; Woodward et al., 1997), the P81S mutation had also been reported four times previously and was not always associated with a haemangioblastoma-only phenotype: (i) in an isolated German patient with a full-blown VHL tumour spectrum (cerebellar and spinal haemangioblastoma, renal cell carcinoma, and renal, pancreatic and epididymal cysts); (ii) in a 34-year-old American patient with haemangioblastoma only; (iii) in a 35-year-old American patient with retinal haemangioblastoma and islet cell tumour of the pancreas (the father was the only other relative with a VHL-related tumour and had a phaeochromocytoma); and (iv) in an isolated Japanese patient with multiple haemangioblastomas and a renal cell carcinoma (Zbar et al., 1996; Stolle et al., 1998; Clinical Research Group for VHL in Japan, 1995). Nevertheless in the current study we identified four carriers of VHL missense mutations without other features of VHL disease and we considered whether any of these might represent a haemangioblastoma-only/low penetrance VHL mutation. A C162Y substitution was detected in a 22-year-old female with a cerebellar haemangioblastoma. The early age at onset is consistent with an inherited susceptibility and this mutation was previously reported in a Japanese kindred in which 4/4 C162Y heterozygotes developed CNS haemangioblastomas but none developed retinal angiomas or RCC (Clinical Research Group for VHL in Japan, 1995). Hence the suggestion that C162Y is a haemangioblastoma-only mutation cannot be dismissed. A novel D179N missense substitution was detected in a 30-year-old male. Evidence that this is a pathogenic mutation rather than a polymorphic missense substitution include (i) it was not present in 200 control chromosomes (ii) D179 is conserved in mouse and rat pVHL orthologues and (iii) polymorphic non-pathogenic missense substitutions distal to codon 54 have not yet been described in pVHL. Nevertheless the presence of the substitution in an older unaffected relative would suggest that, if it is a pathogenic mutation, there is incomplete penetrance. We identified two unrelated patients with a heterozygous R200W missense mutation. This mutation is well-characterized and homozygotes develop Chuvash polycythaemia. However although Chuvashian R200W homozygotes and heterozygotes have not been reported to have an increased risk of VHL-associated tumours, the R200W mutation was initially reported (in a heterozygous state) in a French family with Type 1 VHL disease (Zbar et al., 1996) suggesting that in certain circumstances it may be tumourigenic. We note that, in addition to allelic heterogeneity, modifier genes have been reported to influence phenotypic expression in VHL disease (Webster et al., 1998; Zatyka et al., 2002).

Neither of our two patients was of Chuvash origin and although the frequency of R200W heterozygotes in Chuvashia is ~1 in nine persons, in non-Chuvash controls the frequency is <1 in 300 (this study; Pastore et al., 2003; Liu et al., 2004). Thus the finding that 2 of 189 patients with apparently isolated haemangioblastoma were R200W heterozygotes suggests that heterozygosity for this mutation may not be benign and it may, in some cases, be a low penetrance VHL disease allele.

The VHL gene product has multiple functions, but the best characterized is as a key component of an E3 ubiquitin ligase complex that targets the α-subunits of the HIF (hypoxia-inducible factor) transcription factors for ubiquitination and proteasomal degradation (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000). VHL mutations associated with classical VHL disease disrupt pVHL targeting of HIF-α subunits leading to stabilization of the HIF-1 and HIF-2 heterodimeric transcription factors and activation of downstream hypoxia-inducible target genes such as VEGF and other angiogenic growth factors (Jiang et al., 2003; Wykoff et al., 2004; Maina et al., 2005). However pVHL mutations associated with familial phaeochromocytoma-only (Type 2C VHL disease) may not disrupt the ability of pVHL to regulate HIF-α subunits (Clifford et al., 2001; Hoffman et al., 2001). Although VHL inactivation and HIF dysregulation alone appears to be insufficient for haemangioblastoma development (Vortmeyer et al., 2006), genotype–phenotype correlations and in vitro functional analysis do suggest a strong association between the risk of haemangioblastoma development and the ability of a germline VHL mutation to impair HIF regulation (Clifford et al., 2001). Functional analysis of the R200W mutant pVHL revealed a reduction in ubiquitination and hence degradation of HIF1α under non-hypoxic conditions leading to increased expression of downstream hypoxia-inducible targets (Ang et al., 2002). Of note the four missense substitutions detected in this study (R200W, C162Y, D179N) affect surface residues of pVHL, and might therefore not be expected to disrupt completely pVHL function. In vitro functional studies of candidate low penetrance/haemangioblastoma missense mutations would provide insights into the significance of the missense substitution for pVHL regulation of HIF degradation. However, given the variable phenotype of some pVHL missense substitutions (e.g. P81S and R200W) it would seem prudent to follow-up patients with such mutations (and a single haemangioblastoma and no family history) as though they are at risk for other VHL-related tumours, whilst informing them that they may have a ‘mild non-classical form of VHL disease’.

VHL mutation analysis is very sensitive but during follow-up 10 patients (~5%) with an apparently isolated haemangioblastoma and no detectable VHL mutation subsequently developed tumours within the VHL disease spectrum (Table 2). This corresponded to an actuarial risk of ~5% at 10 years post-diagnosis of a haemangioblastoma. Possible explanations for this observation include (i) they represent a phenocopy; (ii) there is a mutation outside the examined coding sequence; (iii) they represent a previously
undescribed condition characterized by the predisposition to the development of central nervous system haemangioblastomas only; or (iv) they are mosaic for a VHL mutation. Mosaicism in VHL disease has been described and we note that 25–30% of individuals with neurofibromatosis type 2 in whom there is no family history of the condition have been shown to be mosaic for the disease causing mutation (Kluwe et al., 2003; Moyhuddin et al., 2003). Most of the 10 patients who subsequently developed VHL-type tumours developed a further CNS lesion and none had an affected relative—all of which would be consistent with variable tissue mosaicism for a VHL mutation. Nevertheless some, particularly older, patients with a single renal cyst are likely to represent a phenocopy.

Our experience of the largest cohort of patients presenting with isolated haemangioblastoma leads us to recommend that VHL mutation analysis should be performed in all cases. In addition, because of the possibility of false-negative mutation analysis (e.g. from mosaicism), baseline investigations for the presence of abdominal (abdominal USS or MRI and 24 h urine for catecholamines) and retinal (direct and indirect ophthalmoscopy and fluorescein angiography if necessary) features of VHL disease should be undertaken. For older patients (>50 years) with negative investigations the risk of familial VHL disease appears small. In view of the association between VHL disease and young onset haemangioblastomas, there may be a case for keeping patients with early onset tumours under surveillance even if mutation analysis is negative. The detection of a germline VHL mutation should lead to long-term surveillance and testing and screening of at risk relatives. However, clinicians should be aware that allelic heterogeneity in VHL disease is associated with complex genotype–phenotype correlations and some VHL mutations may be best considered as low penetrance alleles. This interpretation has important implications for the management of these families and heterozygote carriers should be aware that they will not necessarily develop classical VHL disease. Further clinical and functional investigation of candidate ‘low penetrance VHL mutations’ will enhance the management of haemangioblastoma patients and provide further insights into the relationship between impairment of specific pVHL functions and the development of haemangioblastomas and RCC in VHL disease.

Acknowledgements

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References


