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Mutation analysis of HIF prolyl hydroxylases (PHD/EGLN) in individuals with features of phaeochromocytoma and renal cell carcinoma susceptibility

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*(D Astuti and C J Ricketts contributed equally to this work)

Abstract

Germline mutations in the von Hippel–Lindau disease (VHL) and succinate dehydrogenase subunit B (SDHB) genes can cause inherited phaeochromocytoma and/or renal cell carcinoma (RCC). Dysregulation of the hypoxia-inducible factor (HIF) transcription factors has been linked to VHL and SDHB-related RCC; both HIF dysregulation and disordered function of a prolyl hydroxylase domain isoform 3 (PHD3/EGLN3)-related pathway of neuronal apoptosis have been linked to the development of phaeochromocytoma. The 2-oxoglutarate-dependent prolyl hydroxylase enzymes PHD1 (EGLN2), PHD2 (EGLN1) and PHD3 (EGLN3) have a key role in regulating the stability of HIF-α subunits (and hence expression of the HIF-α transcription factors). A germline PHD2 mutation has been reported in association with congenital erythrocytosis and recurrent extra-adrenal phaeochromocytoma. We undertook mutation analysis of PHD1, PHD2 and PHD3 in two cohorts of patients with features of inherited phaeochromocytoma (n = 82) and inherited RCC (n = 64) and no evidence of germline mutations in known susceptibility genes. No confirmed pathogenic mutations were detected suggesting that mutations in these genes are not a frequent cause of inherited phaeochromocytoma or RCC.

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Introduction

Germline mutations in the von Hippel–Lindau (VHL) tumour suppressor gene and in the B, C and D subunits of succinate dehydrogenase (SDHB, SDHC and SDHD) are strongly linked with susceptibility to phaeochromocytoma (Latif et al. 1993, Crossey et al. 1995, Woodward et al. 1997, Baysal et al. 2000, Gimm et al. 2000, Astuti et al. 2001a,b, Neumann et al. 2002, Schiavi et al. 2005, Mannelli et al. 2007). In addition, germline mutations in VHL, SDHB and SDHD are associated with susceptibility to renal cell carcinoma (RCC), though the risk of RCC is about five times higher in VHL disease (Maher et al. 1993, Latif et al. 1993, Vanharanta et al. 2004, Ong et al. 2007, Ricketts et al. 2008, 2010). Several mechanisms have been implicated in the development of VHL-related and SDHB/D-related phaeochromocytomas. The VHL tumour suppressor gene product (pVHL) has multiple functions (see Frew & Krek 2007 references within) but its best-characterised function is the ability to regulate proteasomal degradation of hypoxia-inducible factor (HIF)-1α and HIF-2α (Maxwell et al. 1999), and overexpression of HIF-2α in a RCC cell line counteracts pVHL tumour suppressor activity (Kondo et al. 2003). Inactivation of SDHB/D has been linked to accumulation of succinate that inhibits the prolyl hydroxylases necessary for proteasomal degradation of HIF-α subunits (Selak et al. 2005); phaeochromocytomas from patients with germline VHL, SDHB and SDHD mutations demonstrate up-regulation of HIF-1α and HIF-2α and their downstream targets (Pollard et al. 2006). Although HIF dysregulation and a pseudohypoxic state are features of both VHL- and SDHB/D-associated tumours, another potential mechanism for phaeochromocytoma susceptibility in these disorders is a failure of normal prolyl hydroxylase domain isofrom 3 (PHD3) (EGLN3)-dependent developmental apoptosis of sympathetic neuronal cells causing persistence of ‘phaeochromocytoma precursor cells’ (Lee et al. 2005). This latter process has also been linked to other phaeochromocytoma susceptibility disorders (multiple endocrine neoplasia type 2 and neurofibromatosis) (Lee et al. 2005).

In normoxia, the HIF-1 and HIF-2 α-subunits are rapidly ubiquitylated and targeted for proteasomal degradation by a pVHL containing E3 ubiquitin ligase complex (Maxwell et al. 1999). The oxygen-dependent interaction of pVHL with HIF-α is determined by the hydroxylation status of key HIF-α proline residues (Pro-402 and Pro-564 in HIF-1α) (Ivan et al. 2001, Jaakkola et al. 2001, Masson et al. 2001, Yu et al. 2001); non-hydroxylated HIF-α binds to pVHL approximately a 100-fold less tightly than hydroxylated HIF-α (Chan et al. 2002). Thus in the presence of oxygen, HIF-α hydroxylation is catalysed by prolyl hydroxylases that are members of the egg-laying-defective nine (EGLN) family (Bruick & McKnight 2001, Epstein et al. 2001). Under hypoxic conditions, the rate of prolyl hydroxylation, and hence the rate of proteasomal degradation of HIF-α, slows because pVHL binds only very weakly to the HIF-α subunits leading to stabilisation of HIF-1 and HIF-2 heterodimeric transcription factors and activation of the array of genes involved in the hypoxic response. In humans, three EGLN homologues have been implicated in HIF-α modification: PHD1/EGLN2/HIFPH1, PHD2/EGLN1/HIFPH2 and PHD3/EGLN3/HIFPH3 (Bruick & McKnight 2001, Epstein et al. 2001). To date, germline mutations in PHD2 have been associated with congenital polycythaemia and, in one case/family, recurrent extra-adrenal phaeochromocytoma (Percy et al. 2006, 2007, Al-Sheikh et al. 2008, Ladroue et al. 2008). We hypothesised that patients with features of inherited susceptibility to phaeochromocytoma and/or RCC might harbour germline mutations in PHD1, PHD2 or PHD3. To test this hypothesis, we analysed two large patient cohorts without mutations in known susceptibility genes.

Materials and methods

Patients and samples

We analysed DNA from a) 82 individuals with features of inherited phaeochromocytoma (extra-adrenal tumours in 23 cases) susceptibility (i.e. familial phaeochromocytoma (n = 6), multiple tumours (n = 30) or young age at onset (diagnosed age 30 years or less, range 5–30 years, median 19 years (n = 46)) but no detectable mutations in VHL, SDHB, SDHD or RET and b) 64 patients with features of RCC susceptibility (25 probands from familial RCC kindreds, 14 individuals with bilateral or multicentric RCC and 25 individuals with isolated unilateral early-onset RCC (diagnosis aged 40 years or less; range 17–40 years, median 28 years)) but no evidence of a germline mutation in a known RCC susceptibility gene (VHL, SDHB, FH and FLCN) (most cases had clear cell RCC) (Ricketts et al. 2008, Woodward et al. 2008).

Molecular genetic analyses

Mutation analysis of HIF prolyl hydroxylases (PHD/EGLN1–3)

PHD1/EGLN2 (exons 1–5), PHD2/EGLN1 (exons 1–5) and PHD3/EGLN3 (exons 1–5) (see Figure 1) were
examined by PCR–sequencing. DNA (50 ng) was preamplified using GenomiPhi DNA amplification kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer’s instructions, and 0.2 μl of GenomiPhi amplified DNA was used for PCR amplification. The PCR conditions were as follows: 95 °C for 15 min followed by 35 cycles of 95 °C, 55–58 °C and 72 °C for 30 s each and then 72 °C for 10 min. PCR was performed using HotStarTaq DNA polymerase (Qiagen). PCR and sequencing were repeated in the original DNA if sequence variation was observed in the GenomiPhi amplified DNA. Primer sequences for exons amplification are available upon request.

CpG methylation analysis of PHD3

PHD3 CpG island methylation analysis was performed using combined bisulphite and restriction digest analysis (CoBRA). Sodium bisulphite modification of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. PHD3 CpG island was amplified by nested PCR using primers: PHD3CoF1 5′-TTTTGGTTATAAATAATTAAGGTTGGYTGYGGGTT-3′; PHD3CoR1 5′-AATAACAAAAACCCACCTCTACAACAAACATT-3′; PHD3CoF2 5′-TTYGAGGGYGGGTYGGGGYGGAGTT-3′; PHD3CoR2 5′-AAAAACATCTCCRCCAAAAATCR-3′. All amplification was done using HotStarTaq DNA polymerase (Qiagen).

Functional analyses of the PHD3-Arg8Ser mutation

Plasmid construction

The plasmid encoding wild-type PHD3/EGLN3 (pcDNA3-PHD3) has been described previously (Lee et al. 2005). Mutant PHD3/EGLN3 Arg8Ser was generated using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) with primers 5′-ACACATCATGAGCCTGGACCTGGAG-3′ (forward) and 5′-CTCCAGGTCCAGGCTCATGATGTG-3′ (reverse) and verified by DNA sequencing.

PHD3 induced apoptosis assays

Two different apoptosis assays were performed; an initial method to demonstrate differences and a second, more sensitive method. The first apoptosis assay was performed essentially as described by Lee et al. (2005). Undifferentiated rat PC12 cells were plated onto collagen-coated 6-well plates and were cotransfected the following day with 500 ng of GFP histone and either 1 μg of wild-type PHD3, PHD3-Arg8Ser, PHD3-His196Ala (catalytic dead mutant) or pcDNA3. Transfection was carried out using Lipofectamine 2000.
Scoring of apoptotic cells (characterised as having condensed or fragmented nuclei) was done 72 h after transfection. Approximately, 400 cells were scored for each sample. Samples were blinded and assays were performed in duplicate.

For the second apoptosis assay, sympathetic neurons were isolated from the superior cervical ganglia (SCG) as previously described (Palmada et al. 2002). Briefly, SCG from Sprague–Dawley rats were isolated at postnatal day 4 (P4), and sympathetic neurons were dissociated with 0.25% trypsin and 0.3% collagenase for 30 min at 37 °C. After dissociation, the neurons were then cotransfected with GFP and either wild-type PHD3, PHD3-Arg8Ser, PHD3-His196Ala or pcDNA3 by electroporation on an Amaxa Nucleofector device as described previously (Kenchappa et al. 2006). Forty-eight hours later, cells were fixed in 4% paraformaldehyde (PFA) and stained with DAPI (Vector Laboratories, Peterborough, UK). The number of GFP-positive neurons with apoptotic or non-apoptotic nuclei was counted and ~50–80 neurons were evaluated for each sample. The assay was performed in triplicate with three different electroporations using different rat litters.

Statistical analysis

Results for the apoptosis assays were compared using two-tailed Student’s t-tests.

Results

Analysis of PHD3

The five exons and flanking sequences of PHD3 were sequenced in a) 82 patients with features of non-syndromic inherited phaeochromocytoma susceptibility (i.e. familial phaeochromocytoma, multiple tumours or young age at onset) but no detectable mutations in VHL, SDHB, SDHD or RET, b) 22 sporadic phaeochromocytomas and c) 64 patients with features of RCC susceptibility. Sequence variants identified in patients were then tested for in normal controls.

A germline missense substitution, c.24G>C (p.Arg8Ser), was detected in 1 of 82 patients with features of non-syndromic inherited phaeochromocytoma susceptibility (see Fig. 2A) (but not in 222 control chromosomes). The substitution was predicted to be benign and tolerated by bioinformatic analysis with the Polyphen and SIFT programs, and occurred in the N-terminal region of the protein which, by analogy with structural and biochemical work on the catalytic domain of PHD2 (residues 181–426, tPHD2 hereafter), is unlikely to be directly involved in catalysis (McDonough et al. 2006, Flashman et al. 2008, Chowdhury et al. 2009; see Figs 1, 3 and Table 1). The female patient had bilateral adrenal phaeochromocytomas at age 22. She showed no clinical evidence of a known familial susceptibility syndrome and no family history of phaeochromocytoma. The missense substitution was not detected in her clinically unaffected father and sister, but it was not possible to determine whether it had arisen de novo because no DNA was available from her mother who had died from an unrelated cause. No novel sequence variants were detected in the 64 patients with RCC susceptibility.

Previously, wild-type PHD3 has been shown to induce apoptosis of rat phaeochromocytoma (PC12) cells and rat sympathetic neurons. To investigate whether the PHD3 p.Arg8Ser missense substitution might alter pro-apoptotic activity, PC12 cells and rat sympathetic neurons were transfected with either wild-type PHD3, p.Arg8Ser mutant PHD3, empty vector control or a catalytically inert p.His196Arg PHD3 mutant (Lee et al. 2005, Schlisio et al. 2008). For the PC12 cells, a baseline apoptosis rate of 2–4% was seen with the empty vector and the inert mutant control, but both wild-type PHD3, p.Arg8Ser mutant PHD3, empty vector control or a catalytically inert p.His196Arg PHD3 mutant (Lee et al. 2005, Schlisio et al. 2008). For the PC12 cells, a baseline apoptosis rate of 2–4% was seen with the empty vector and the inert mutant control, but both wild-type and p.Arg8Ser mutant PHD3 were associated with an increase in apoptosis.

Figure 2 Sequence chromatographs of variants of PHD1, PHD2 and PHD3. (A) Novel variants of PHD1, PHD2 and PHD3 with coding region and amino acid changes. (B) Known variants of PHD1 and PHD2 with SNPdb reference (http://www.ncbi.nlm.nih.gov/projects/SNP).
For the more sensitive rat sympathetic neuron assay, a similar baseline apoptosis rate of 3–4% was seen with the empty vector and the inert mutant control. Again wild-type and p.Arg8Ser mutant PHD3 were associated with an increase in apoptosis (30.6 and 22.8% respectively) that was highly statistically significant compared to the catalytically inert mutant (P = 0.0001 and P = 0.0003), though the p.Arg8Ser mutant demonstrated a mildly, yet statistically significant, lower level of apoptosis (P = 0.016) than that of the wild type (see Fig. 4).

We also analysed up to 22 sporadic phaeochromocytomas for somatic mutations and for de novo promoter region methylation of PHD3. No somatic mutations were detected in 22 tumours, and analysis of a PHD3 5′ CpG island (nt −345 to −160 relative to the ATG start codon; NM 022073.2 Genome Browser on Human, May 2004 assembly) by CoBRA and bisulphite sequencing in 17 sporadic phaeochromocytoma tumours demonstrated partial promoter methylation (that was not detected in matched normal (blood) tissue) in only one of 17 tumours analysed.

Analysis of PHD2

Of 82 patients with features of phaeochromocytoma susceptibility, 10 patients (12.2%) had a germline non-synonymous missense substitution, c.471G>C (p.Q157H) (see Fig. 2B), but this was also detected in 3/111 (2.7%) of normal controls (P = 0.017).

A novel missense p.Ser247Trp (c.740 C>G) substitution was detected in a patient with unilateral RCC at age 55 years and a contralateral RCC at age 56 years (see Fig. 2A). The substitution was not detected in 222 chromosomes from normal subjects and occurred in a region of the protein with no predicted function (see Fig. 1). Bioinformatic analysis with the Polyphen and SIFT programs suggests that this is possibly damaging and intolerant, and the S247 residue is conserved down to Danio rerio and Xenopus tropicalis, but not to Drosophila melanogaster or Caenorhabditis elegans (Fig. 3 and Table 1). However, there were no relatives with RCC to test segregation in the family.

Mutation analysis of 22 sporadic phaeochromocytomas revealed a novel missense variant (p.Ser289Gly; c.865A>G) in a single tumour (see Fig. 2A). Germline

**Table 1** Bioinformatic analysis of the PHD genes using the PolyPhen and SIFT programs (http://genetics.bwh.harvard.edu/pph/ and http://sift.jcvi.org/)

<table>
<thead>
<tr>
<th>Genes</th>
<th>AA change</th>
<th>PolyPhen</th>
<th>SIFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD1</td>
<td>SS8L</td>
<td>1.773</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>PHD1</td>
<td>V183M</td>
<td>1.361</td>
<td>Benign</td>
</tr>
<tr>
<td>PHD2</td>
<td>C127S</td>
<td>–</td>
<td>Benign</td>
</tr>
<tr>
<td>PHD2</td>
<td>Q157H</td>
<td>1.538</td>
<td>Possibly damaging</td>
</tr>
<tr>
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<td>S247W</td>
<td>1.529</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>PHD2</td>
<td>S289G</td>
<td>0.376</td>
<td>Benign</td>
</tr>
<tr>
<td>PHD3</td>
<td>R8S</td>
<td>1.297</td>
<td>Benign</td>
</tr>
</tbody>
</table>
Discussion

We did not find any evidence that germline mutations in PHD1, PHD2 or PHD3 are frequent causes of inherited susceptibility to phaeochromocytoma or RCC. Thus, although Ladroue et al. (2008) reported a patient with recurrent paraganglioma and a germline PHD2 missense mutation (c.1121A→G, p.H374R, with loss of the wild-type allele in paraganglioma tissue), our findings suggest that PHD2 mutations are rare in patients with features of inherited phaeochromocytoma (95% confidence interval 0–4.4%). PHD2 mutations were initially described in association with familial erythrocytosis (Percy et al. 2006, 2007, Al-Sheikh et al. 2008) and that the patient reported by Ladroue et al. (2008) had both recurrent paraganglioma and congenital erythrocytosis. Although it might be hypothesised that germline PHD2 mutations might be restricted to phaeochromocytoma patients with congenital erythrocytosis, we did not identify a PHD2 mutation in a patient with early onset phaeochromocytoma and erythrocytosis.

All three PHD isoforms regulate both HIF-1α and HIF-2α, but differ in expression patterns, specificities for the two different prolyl hydroxylation sites within the HIF-1α and 2α subunits, and a degree of selectivity between HIF-α isoforms (Appelhoff et al. 2004, Raval et al. 2005). PHD2 appears to be the major regulator of HIF-α prolyl hydroxylation in normoxia and mild hypoxia, and we postulated that PHD2 mutations (that led to HIF dysregulation) might present with inherited RCC (previously, a similar approach had revealed unsuspected germline SDHB and FLCN mutations in ∼10% of patients with apparently non-syndromic inherited RCC (Ricketts et al. 2008, Woodward et al. 2008)). Although we detected a novel PHD2 missense variant (c.840 C→G, p.S247W) in a proband with two independent RCC, the significance of this finding is uncertain. While the absence of the variant in 222 chromosomes from normal subjects and bioinformatic analysis were suggestive of pathogenicity (the S247 residue is conserved in D. rerio), we are unable to prove a loss of function effect. S247 is located in the variable region (variable with respect to PHD1, PHD2 and PHD3 sequences) between strands β2 and β3 (referred to as ‘β2ιβ3 loop’) in the published crystal structures of PHD2 (McDonough et al. 2006, Chowdhury et al. 2009). Subsequent biophysical analyses have shown that this region is flexible and involved in determining the prolyl hydroxylation site selectivity of the PHDs (Villar et al. 2007, Flashman et al. 2008, Chowdhury et al. 2009). However, a point mutation at this position (Ser289Lys) that could be

Analysis of PHD1

Mutation analysis of PHD1 in 82 patients with evidence of inherited phaeochromocytoma susceptibility revealed a novel non-synonymous p.Ser58Leu (c.173C>T) germline missense substitution (see Fig. 2B) in 5 cases (6%) and 3/116 (2.6%) normal controls (P=0.29).

A novel missense p.Val183Met (c.547 G>A) substitution was detected in a patient with familial RCC (see Fig. 2A). Both the proband and her father had been affected with RCC in the sixth decade, but germline DNA was not available from other relatives. The substitution was not detected in 222 chromosomes from normal subjects, and bioinformatic analysis gave conflicting results with Polyphen suggested that this was a benign substitution but the SIFT program reporting it to be intolerant (see Table 1). Val183 is conserved down to D. rerio, but not to D. melanogaster or C. elegans (see Fig. 3).

No potential pathogenic variants were detected in 22 sporadic phaeochromocytoma tumours.

DNA was not available, and the substitution was not detected in 222 chromosomes from normal subjects. Structurally informed bioinformatic analysis with the Polyphen and SIFT programs suggested that this substitution is likely to be a benign variant, and Ser289 residue is not conserved in rodents or in human PHDs (see Fig. 3 and Table 1). Crystallographic analyses (Chowdhury et al. 2009) reveal that Ser289 is not involved with the active site (see Figs 1 and 5).

Figure 4 Apoptosis assay of PHD3-Arg8Ser mutant variant. Rat sympathetic neurons were cotransfected with GFP and with either wild-type PHD3, PHD3-Arg8Ser, PHD3-His196Ala or pcDNA3. The number of GFP-positive neurons with apoptotic nuclei was counted, and ∼70–100 neurons were evaluated for each sample from three separate experiments. Error bars demonstrate s.d., and P values from Student’s t-tests are shown.

Discussion

We did not find any evidence that germline mutations in PHD1, PHD2 or PHD3 are frequent causes of inherited susceptibility to phaeochromocytoma or RCC. Thus, although Ladroue et al. (2008) reported a patient with recurrent paraganglioma and a germline PHD2 missense mutation (c.1121A→G, p.H374R, with loss of the wild-type allele in paraganglioma tissue), our findings suggest that PHD2 mutations are rare in patients with features of inherited phaeochromocytoma (95% confidence interval 0–4.4%). PHD2 mutations were initially described in association with familial erythrocytosis (Percy et al. 2006, 2007, Al-Sheikh et al. 2008) and that the patient reported by Ladroue et al. (2008) had both recurrent paraganglioma and congenital erythrocytosis. Although it might be hypothesised that germline PHD2 mutations might be restricted to phaeochromocytoma patients with congenital erythrocytosis, we did not identify a PHD2 mutation in a patient with early onset phaeochromocytoma and erythrocytosis.

All three PHD isoforms regulate both HIF-1α and HIF-2α, but differ in expression patterns, specificities for the two different prolyl hydroxylation sites within the HIF-1α and 2α subunits, and a degree of selectivity between HIF-α isoforms (Appelhoff et al. 2004, Raval et al. 2005). PHD2 appears to be the major regulator of HIF-α prolyl hydroxylation in normoxia and mild hypoxia, and we postulated that PHD2 mutations (that led to HIF dysregulation) might present with inherited RCC (previously, a similar approach had revealed unsuspected germline SDHB and FLCN mutations in ∼10% of patients with apparently non-syndromic inherited RCC (Ricketts et al. 2008, Woodward et al. 2008)). Although we detected a novel PHD2 missense variant (c.840 C→G, p.S247W) in a proband with two independent RCC, the significance of this finding is uncertain. While the absence of the variant in 222 chromosomes from normal subjects and bioinformatic analysis were suggestive of pathogenicity (the S247 residue is conserved in D. rerio), we are unable to prove a loss of function effect. S247 is located in the variable region (variable with respect to PHD1, PHD2 and PHD3 sequences) between strands β2 and β3 (referred to as ‘β2ιβ3 loop’) in the published crystal structures of PHD2 (McDonough et al. 2006, Chowdhury et al. 2009). Subsequent biophysical analyses have shown that this region is flexible and involved in determining the prolyl hydroxylation site selectivity of the PHDs (Villar et al. 2007, Flashman et al. 2008, Chowdhury et al. 2009). However, a point mutation at this position (Ser289Lys) that could be
produced in the soluble form was as active as wild-type PHD2 (in studies using recombinant tPHD2) with respect to hydroxylation of the peptide fragments of the N- and C-terminal oxygen-dependent degradation domains of HIF-α when these were tested either separately or simultaneously in competition revealing that (at least) under standard incubation conditions, mutations at Ser289 are unlikely to affect absolute activity or selectivity with respect to the hydroxylation sites on HIF-α (data not shown).

Distinguishing mutations from non-pathogenic sequence variants and predicting the functional effect of rare missense variants on protein function can be challenging, but while the pathogenicity of the p.S247W missense substitution cannot be unequivocally established or disproved, our results do suggest that germline PHD2 mutations are not a frequent cause of inherited RCC. Similarly, a possible PHD1 missense mutation (p.Val183Met) was detected in only one proband with features of non-syndromic inherited RCC. While this variant was not detected in >200 control chromosomes and the V183 residue is conserved in D. rerio, bioinformatic predictions of likely pathogenicity (Polyphen and SIFT programs) were equivocal and the substitution occurs outside of the prolyl hydroxylation catalytic domain (AA 189–375).

Functional studies of mutant pVHL protein associated with differing VHL disease phenotypes (e.g. with differential risks of RCC and phaeochromocytoma) suggest that whereas impairment of the ability to regulate HIF is necessary for predisposition to RCC, it is not sufficient, and impairment of non-HIF-related pVHL functions is also required (Clifford et al. 2001). Hence, mutations that lead only to dysregulation of HIF (e.g. germline mutations in PHDs, HIF-1 or HIF-2 genes) might not be sufficient to cause an inherited RCC phenotype. Interestingly, functional analysis of pVHL mutants has also suggested that some VHL mutations associated with a high risk of phaeochromocytoma do not compromise pVHL regulation of HIF, so implicating HIF-independent mechanisms in the development of VHL-associated phaeochromocytoma (Clifford et al. 2001, Hoffman et al. 2001). Consistent with this hypothesis, Lee et al. (2005) described a HIF-independent pathway linked to inherited causes of phaeochromocytoma (including those associated with germline VHL, SDHB and SDHD mutations). They suggested that inherited phaeochromocytomas originate from sympathetic neuronal precursor cells that usually undergo c-Jun-dependent apoptosis during embryogenesis when growth factors such as nerve growth factor (NGF) become limiting (Estus et al. 1994, Schlingensiepen et al. 1994, Ham et al. 1995, Xia et al. 1995). Germline VHL and SDHB mutations compromise this pathway leading to a failure of normal developmental apoptosis and survival of cells that would give rise to phaeochromocytoma. Intriguingly, PHD3 (but not PHD1 or PHD2) is a key mediator of this apoptotic pathway, and germline mutations in KIF1Bbeta (that encodes a target of PHD3/EGln3) have been associated with familial susceptibility to neural crest tumours (Schlisio et al. 2008). Furthermore, PHD3 knockout mice demonstrated increased numbers of cells in the superior cervical ganglion, adrenal medulla and carotid body (Bishop et al. 2008). However, we identified only one potential PHD3 mutation in our cohort of inherited phaeochromocytoma probands and, although the missense variant was not detected in normal controls, we did not detect any evidence that the p.Arg8Ser impaired neuronal apoptosis to a substantial degree (as compared to a catalytically inert mutant). Although the
partial loss of functional effect of the p.Arg8Ser mutant in the sensitive rat sympathetic neuron apoptosis assay suggests that the variant might contribute to phaeochromocytoma susceptibility. Germline mutations in both VHL and SDHB/D mutations are important causes of familial phaeochromocytoma; however, somatic mutations of these genes are infrequent in sporadic tumours (Eng et al. 1995, Astuti et al. 2001a,b). Hence, we concentrated our efforts on testing for germline PHD1, PHD2 and PHD3 mutations in patients with features of inherited phaeochromocytoma. In our preliminary analysis of sporadic phaeochromocytoma, we did not find any frequent evidence for somatic inactivation of PHD1, PHD2 or PHD3. In contrast to phaeochromocytoma, somatic VHL mutations are very frequent in sporadic RCC (Foster et al. 1994, Gnarra et al. 1994, Banks et al. 2006) (though somatic SDHB inactivation is rare in RCC (Morris et al. 2004)). However, large-scale resequencing of RCC samples (n = 101) did not detect mutations in PHD1, PHD2 or PHD3 (Dalgliesh et al. 2010), and we have not detected frequent evidence of up-regulation of any of the three prolyl hydroxylase genes in RCC cell lines treated with 5-azacytidine (Morris et al. 2010 and unpublished studies). The absence of germline PHD1, PHD2 or PHD3 mutations in inherited phaeochromocytoma or RCC might suggest that inactivation of one of these genes is insufficient to replicate the range of pro-oncogenic effects associated with pathogenic germline VHL or SDHB/D mutations. pVHL has been reported to have multiple functions (Frew & Krek 2007), and while inactivation of SDH has been linked to accumulation of succinate and resulting inhibition of prolyl hydroxylase enzymes that are necessary for proteasomal degradation of HIF-α subunits (Selak et al. 2005), succinate accumulation may also inhibit other 2-oxoglutarate-dependent enzyme superfamily members which may also contribute to the development of SDHB/D-associated tumours (e.g. histone demethylases, some of which are implicated in the epigenetic regulation of oncogenes and tumour suppressor genes) (Smith et al. 2008). Nevertheless, a significant fraction of patients with features of inherited phaeochromocytoma or RCC do not have a detectable mutation in a known susceptibility gene. The advent of comprehensive genomic analysis techniques (e.g. whole exome resequencing) (Kryukov et al. 2009, Summerer et al. 2010) should expedite the identification of novel phaeochromocytoma/RCC predisposition genes and so provide further insights into the mechanism of tumourigenesis in these disorders.

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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References


Bishop T, Gallagher D, Pascual A, Lygate CA, de Bono JP, Nicholls LG, Ortega-Saenz P, Oster H, Wijeyekoon B, Sutherland AI et al. 2008 Abnormal sympathoadrenal


downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes and Development* **22** 884–893. (doi:10.1101/gad.1648608)


