Genetic predisposition to phaeochromocytoma: analysis of candidate genes GDNF, RET and VHL

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Inherited predisposition to phaeochromocytoma (MIM No 171300) occurs in multiple endocrine neoplasia type 2 (MEN 2) (MIM No 171400), von Hippel-Lindau (VHL) disease (MIM No 199300), and neurofibromatosis type 1 (NF1) (MIM No 162200). In addition, familial phaeochromocytoma alone has also been reported and we and others have identified germline VHL mutations in five of six kindreds analysed previously. Germline mutations in the RET proto-oncogene, which encodes a receptor tyrosine kinase, and in the VHL tumour suppressor gene cause MEN 2 and VHL disease, respectively. To further investigate the genetics of phaeochromocytoma predisposition, we analysed three groups of patients with no evidence of VHL disease, MEN 2 or NF1: Group A, eight kindreds with familial phaeochromocytoma; Group B, two patients with isolated bilateral phaeochromocytoma; and Group C, six cases of multiple extra-adrenal phaeochromocytoma or adrenal phaeochromocytoma with a family history of neuroectodermal tumours. Germline missense VHL mutations were identified in three of eight kindreds with familial phaeochromocytoma. A germline VHL mutation was also characterised in one of the two patients with bilateral phaeochromocytoma. No VHL or RET mutations were detected in the final group of patients with multiple extra-adrenal phaeochromocytoma or adrenal phaeochromocytoma with a family history of neuroectodermal tumours. Germline mutations in the RET proto-oncogene, which encodes a receptor tyrosine kinase, and in the VHL tumour suppressor gene cause MEN 2 and VHL disease, respectively. To further investigate the genetics of phaeochromocytoma predisposition, we analysed three groups of patients with no evidence of VHL disease, MEN 2 or NF1: Group A, eight kindreds with familial phaeochromocytoma; Group B, two patients with isolated bilateral phaeochromocytoma; and Group C, six cases of multiple extra-adrenal phaeochromocytoma or adrenal phaeochromocytoma with a family history of neuroectodermal tumours. Germline missense VHL mutations were identified in three of eight kindreds with familial phaeochromocytoma. A germline VHL mutation was also characterised in one of the two patients with bilateral phaeochromocytoma. No VHL or RET mutations were detected in the final group of patients with multiple extra-adrenal phaeochromocytoma or adrenal phaeochromocytoma with a family history of neuroectodermal tumours. The absence of germline VHL and RET gene mutations in many of these families suggested that other phaeochromocytoma susceptibility loci may exist. Glial cell line-derived neurotrophic factor (GDNF) has been recently identified as a natural ligand for RET. Thus, it seems plausible that GDNF is a good candidate gene to play a role in phaeochromocytoma susceptibility. We searched for germline mutations in GDNF in 16 cases of familial phaeochromocytoma (groups A, B and C) and looked for evidence of somatic change in GDNF in 28 sporadic phaeochromocytomas, 12 MEN 2 phaeochromocytomas and five VHL phaeochromocytomas. No GDNF mutations were identified in patients with familial phaeochromocytoma disease, but a c277C→T (R93W) sequence variant was identified in one of 28 sporadic tumours. This candidate mutation was identified in the germline and tumour tissue but was not present in 104 control GDNF alleles. GDNF sequence variants including R93W have been suggested previously to represent low penetrance susceptibility mutations for Hirschsprung disease and the R93W was not identified in 376 control alleles studied by others. These findings suggest that although GDNF mutations do not appear to have a major role in the pathogenesis of familial or sporadic phaeochromocytomas, allelic variation at the GDNF locus may modify phaeochromocytoma susceptibility.

INTRODUCTION

Phaeochromocytoma is a tumour of chromaffin-staining cells which are derived from the embryonic neural crest. Most phaeochromocytomas arise within the adrenal medulla but extra-adrenal phaeochromocytomas (paraganglioma) account for ~10% of cases. It has been estimated that 10% of patients with phaeochromocytoma have a genetic susceptibility (1). Predisposition to phaeochromocytoma occurs in three familial cancer syndromes: (i) multiple endocrine neoplasia type 2 (MEN 2) (MIM No 171400) (2), (ii) von Hippel-Lindau (VHL) disease

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VHL disease is caused by mutations in the \textit{VHL} tumour suppressor gene and certain germline missense mutations have been associated with a high risk of phaeochromocytoma in addition to other classical features of VHL disease such as retinal and cerebellar haemangioblastomas and renal cell carcinoma (3,6–8). The three clinical variants of MEN 2 [MEN 2A, MEN 2B and familial medullary thyroid cancer (FMTC)] are caused by gain of function mutations of the \textit{RET} proto-oncogene which encodes a receptor tyrosine kinase (9–16). Most cases of MEN 2A and FMTC have amino acid substitutions at one of five cysteine residues in the extracellular domain (exons 10 and 11) (17) and MEN 2B patients usually have missense mutations in the methionine of codon 918 in the intracellular tyrosine kinase domain (11–13). Germline \textit{RET} mutations may also cause Hirschsprung disease (HSCR) (18–20). Recently, glial cell line-derived neurotrophic factor (GDNF) has been shown to be the functional \textit{RET} ligand (21–24) and \textit{GDNF} mutations have been implicated in the pathogenesis of HSCR (25–27). Therefore point mutations in \textit{GDNF} which alter GDNF function in terms of \textit{RET} binding capacity could be involved in the genesis of MEN 2 related tumours.

Recently, we and others have demonstrated that a proportion of familial phaeochromocytoma is allelic with VHL disease. To date, five of six kindreds with a diagnosis of familial phaeochromocytoma only have been shown to have missense \textit{VHL} gene mutations (28–30). In contrast, somatic \textit{RET} and \textit{VHL} gene mutations are infrequent in sporadic phaeochromocytomas (31–32). To further investigate the genetic epidemiology and pathogenesis of phaeochromocytoma, we have (i) analysed patients with familial phaeochromocytoma for germline mutations in \textit{RET}, \textit{VHL} and \textit{GDNF} and (ii) screened for somatic \textit{GDNF} mutations in sporadic, MEN 2 and VHL phaeochromocytomas. Our results suggest that although germline \textit{VHL} mutations may manifest as familial phaeochromocytoma, germline mutations in \textit{VHL} and \textit{RET} are unlikely to account for all cases of phaeochromocytoma susceptibility. In addition, although germline and somatic \textit{GDNF} mutations do not appear to have a major role in phaeochromocytoma tumorigenesis, they may modify phaeochromocytoma susceptibility.

**RESULTS**

**Identification of germline \textit{VHL} gene mutations in familial phaeochromocytoma**

SSCP analysis revealed variants in: three of the eight families in group A; one of the two individuals in group B; and none of the six families in group C. Genomic sequencing confirmed a germline \textit{VHL} missense mutation in each of the four cases with an SSCP bandshift: c695G→A, R161Q mutation was identified in two unrelated kindreds and a c451A→G, S80G; and a c712C→T, R167W in the other two families (Fig. 1). To further increase the efficiency of \textit{VHL} mutation analysis, sequencing analysis of both strands of the coding region was then performed for all cases in Group A (adrenal phaeochromocytoma kindreds) and of the forward strand in the remaining patients (groups B and C). However this did not reveal any further variants.
To try and exclude linkage to \textit{VHL}, we performed linkage studies using linked markers (D3S1038 and D3S1317) in five families negative for \textit{VHL} gene mutations. However, family structures were not optimal and these studies were not informative.

Mutation analysis (SSCP in 28 cases and direct sequencing of the complete coding sequence in 14) of 28 sporadic phaeochromocytomas did not reveal evidence of somatic or germline \textit{VHL} gene mutations.

**Analysis of GDNF**

SSCP analysis of \textit{GDNF} was performed in (i) tumour DNA from 28 sporadic phaeochromocytomas, 12 MEN 2 phaeochromocytomas, and five \textit{VHL} phaeochromocytomas, (ii) germline DNA from 16 cases of familial phaeochromocytoma (groups A, B and C) described previously and (iii) five cases of familial phaeochromocytoma described previously \cite{28,31}, four of which have a germline \textit{VHL} mutation. No abnormality was detected in the cases of familial phaeochromocytoma or MEN 2 and \textit{VHL} associated phaeochromocytoma tumour tissue. However one of 28 sporadic phaeochromocytomas demonstrated an SSCP band shift and direct sequencing revealed a C to T transition at nt 277 (Fig. 2) which is predicted to produce a non-conservative change, Arg to Trp, at codon 93. Germline DNA from the same individual demonstrated the same sequence variant. The c277C→T sequence change results in the abolition of a \textit{HinII} restriction site and 104 \textit{GDNF} chromosones from normal control individuals were analysed to determine the frequency of this change. None of the 104 chromosomes analysed had evidence of this change. To assess the sensitivity of \textit{GDNF} SSCP analysis, we sequenced the \textit{GDNF} coding region in all 16 cases of familial phaeochromocytoma but did not identify any sequence variants.

**Analysis of RET**

Germline mutations in exons 10 and 11 were searched for in all 16 kindreds with familial phaeochromocytoma and no sequence changes were identified. In addition three cases (two from Group C and one from Group A) were screened for \textit{RET} mutations in exons 2, 3 and 5–20 and no abnormality detected. All the results for \textit{VHL}, \textit{GDNF} and \textit{RET} are summarized in Table 1.

**DISCUSSION**

The identification of germline \textit{VHL} mutations in familial phaeochromocytoma kindreds demonstrates the importance of molecular genetic testing for clinical management. Although none of the kindreds investigated had clinical evidence of \textit{VHL} disease, three of the four kindreds found to have a \textit{VHL} gene mutation had a mutation (c695G→A, R161Q and c712C→T, R167W) reported previously in classical \textit{VHL} disease with phaeochromocytoma \cite{3,33,34}. Therefore, the identification of these \textit{VHL} gene mutations enables presymptomatic screening for the onset of \textit{VHL} disease related tumours. The absence of the other clinical features of \textit{VHL} disease in these patients may reflect mutation-specific variations in phenotype, variable expression or modifier effects. Allele specific variation in predisposition to phaeochromocytoma has been reported in \textit{VHL} disease such that missense mutations, most notably at codon 167 (Arg to Trp and Arg to Gln), are more frequent in \textit{VHL} disease with phaeochromocytoma whereas large deletions, frameshift mutations, and intragenic mutations predicted to produce a truncated protein are less frequent \cite{3,33,34}. The identification of novel (i.e. not found in classical \textit{VHL} families) \textit{VHL} mutations in familial phaeochromocytoma kindreds suggests that phaeochromocytoma specific mutations do exist. In support of this view is the third mutation we describe, c451A→G, present in a Polish mother and daughter with bilateral phaeochromocytoma. Further support for this concept comes from our group’s previous finding of a novel mutation, V84L, (c463G→T) in a Welsh kindred \cite{28} and from the finding of a missense mutation (c775G→G, L188V) in two apparently unrelated German families with familial phaeochromocytoma \cite{29}. These findings suggest that the \textit{VHL} protein (pVHL) has tissue-specific functions which may be differentially affected by specific missense mutations. Comparison of missense mutations in phaeochromocytoma only families and \textit{VHL} with phaeochromocytoma kindreds does not, however, demonstrate any clear differences in the sites of mutation. The mutation detection methods used in this study can identify a germline \textit{VHL} mutation in excess of 80% of \textit{VHL} families \cite{3,33}. Together with our previous study \cite{28}, our \textit{VHL} gene mutation detection rate in familial phaeochromocytoma group A kindreds is 45% (five of 11) and 0% (none of six) in group C patients. Therefore, the involvement of other genes in phaeochromocytoma susceptibility is most likely, particularly in patients with extra-adrenal phaeochromocytoma.

Allele specific variation in predisposition to phaeochromocytoma has also been reported in MEN 2A such that missense mutations at codon 634 are associated with the presence of phaeochromocytoma and hyperparathyroidism \cite{14,17}. The analysis of \textit{RET} in our familial phaeochromocytoma kindreds failed to reveal any mutations in exons 10 and 11. This may be explained on several counts. Firstly, our analysis was restricted to the regions mutated in MEN 2 and mutations could occur outside this region (although one Group A and two Group C cases without \textit{VHL} mutations were screened for mutations in exons 2, 3 and 5–20 of \textit{RET} and no abnormality was detected). In FMTC, several families with a codon 768 (exon 13) mutation have been described \cite{17}. However gain of function mutations occur at specific sites and the studies we performed should detect mutations in at least 95% of MEN 2 kindreds \cite{17} whereas loss of function \textit{RET} mutations as described in Hirschsprung disease occur throughout the gene (18–20). Extra-adrenal phaeochromocytoma has rarely, if ever, been reported in MEN 2, and it may not be considered surprising that no \textit{RET} mutations were detected in our group C individuals.

**Figure 2.** Sequence variant in germline \textit{GDNF} of a sporadic phaeochromocytoma. There is a C to T transition at nt 277 (arrowed).
Table 1. Summary of phaeochromocytoma mutation results from the present and previous studies (28,29,31)

<table>
<thead>
<tr>
<th>Clinical subtype of familial phaeo</th>
<th>Germline VHL mutation detected</th>
<th>Germline RET mutation detected</th>
<th>Germline GDNF mutation detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6/12</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>B</td>
<td>2/3</td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>C</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Phaeo tumours</td>
<td>Somatic VHL mutation detected</td>
<td>Somatic RET mutation detected</td>
<td>GDNF mutation detected</td>
</tr>
<tr>
<td>Unilateral sporadic phaeo</td>
<td>1/46</td>
<td>1/46</td>
<td>1/28*</td>
</tr>
<tr>
<td>MEN 2 phaeo</td>
<td>0/17</td>
<td>0/17</td>
<td>0/12</td>
</tr>
<tr>
<td>VHL phaeo</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

phae: phaeochromocytoma.

*This mutation was subsequently proven germline.

A, kindreds with familial phaeochromocytoma; B, individuals with bilateral phaeochromocytoma; C, individuals with either bilateral phaeochromocytoma or adrenal phaeochromocytoma and a family history of neuroectodermal tumours.

Table 2. Details of GDNF exon 2 sequencing primers

<table>
<thead>
<tr>
<th>Position of primer (nucleotide no.)</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>387–408</td>
<td>F: 5' CTTGGGTCCTGGGCTATGAAACC 3'</td>
</tr>
<tr>
<td>286–262</td>
<td>R: 5' CTGCAGTACCTAAAAATCAGTTCC 3'</td>
</tr>
</tbody>
</table>

The infrequency of somatic VHL and RET mutations in sporadic phaeochromocytomas suggests that additional genes are involved in the genesis of this tumour (31). We then proceeded to analyse glial cell line-derived neurotrophic factor (GDNF) as a candidate gene for familial and sporadic phaeochromocytoma. GDNF has been identified recently as a ligand for RET (21–24). Germline GDNF mutation analysis in an isolated case of HSCR. Our results suggest that GDNF alleles such as R93W could also function as low-penetration susceptibility mutations for phaeochromocytoma. The suggestion that a single GDNF mutation may predispose to both phaeochromocytoma and HSCR susceptibility is intriguing. The spectrum of RET mutations in MEN 2 and HSCR individuals differ. However rarely, HSCR and MEN 2 may be found in the same family suggesting a shared susceptibility factor. Our results suggest that additional phaeochromocytoma susceptibility genes remain to be identified. Candidate genes would include GDNGR-α. Loss of heterozygosity (LOH) studies of phaeochromocytomas from sporadic and syndromic cases have indicated loss of 1p, suggesting that this region may harbour a tumour suppressor gene or genes whose inactivation may be important in the development of this tumour (34,35).

This study represents the largest molecular genetic investigation into the genetic susceptibility to phaeochromocytoma. We conclude that: in some cases familial phaeochromocytoma is allelic with VHL disease; MEN 2 associated RET mutations do not account for cases of familial phaeochromocytoma; and GDNF does not have a major role in the genetic susceptibility to phaeochromocytoma; however GDNF allelic variants may influence phaeochromocytoma susceptibility. Nevertheless, our results suggest that further susceptibility loci remain to be identified.

MATERIALS AND METHODS

Patients

VHL analysis. All subjects analysed had no clinical evidence or family history of MEN 2, VHL, or NF1. The patients were divided into three groups according to clinical criteria: group A, eight kindreds with familial phaeochromocytoma; group B, two individuals with bilateral phaeochromocytoma; and group C, six individuals with either multiple extra-adrenal phaeochromocytoma or adrenal phaeochromocytoma and a family history of neuroectodermal tumours.

GDNF analysis. Germline GDNF was analysed in patients from the above three groups in whom no germline VHL mutations were detected. In addition, tumour DNA samples from 28 sporadic phaeochromocytomas, 12 MEN 2 phaeochromocytomas and five...
VHL phaeochromocytomas were screened for somatic mutations in GDNF (Table 2).

Molecular analysis

Investigation of germline VHL mutations in familial phaeochromocytoma. The method is as described in Crossey et al. (33) but with some modifications. The nucleotide sequence 677–1036 was amplified using one primer pair (annealing temperature 63°C) with the following sequence: F: 5′-CACACCTGCCACA-TACATGCACCTC 3′; and R: 5′-TGCCCCAACTAT-CACAAGGCTCA 3′. Each reaction required 0.8 µM of primer and a ‘hot’ start. The Mg2+ concentration was 1.5 mM and the Taq polymerase ‘Supertaq’. Primer set 3 required an annealing temperature of 63°C and each fragment was examined initially for point mutations by SSCP analysis.

The Applied Biosystems ABI model 373 automated sequencer was then used to: characterise the nature of the SSCP bandshifts detected; sequence both strands of the VHL coding sequence in adrenal phaeochromocytoma kindreds (group A); and to sequence the forward strand of the VHL coding region in the remaining patients (groups B and C).

The PCR products remaining after SSCP analysis and representing the VHL coding region were purified using Promega Wizard™ PCR purification columns. The purified products were ethanol precipitated, redissolved in 4.4 µl H2O and added to 1.6 pmol of primer and 4.0 µl of Prism™ ready reaction Dye Deoxy™ Terminator Cycle Sequencing Kit in a total volume of 10 µl. The reaction mixture was overlaid with mineral oil and cycle sequencing was performed on a Perkin Elmer Cetus Thermal Cycler for 35 cycles: 96°C 30 s, 54°C 30 s, and 60°C 4 min. Dideoxy terminated DNA fragments were ethanol precipitated before analysis using the ABI automated sequencer.

Family linkage studies with microsatellite markers linked to VHL (D3S1038 and D3S1317) were performed when a mutation could not be detected.

Analysis of GDNF. Phaeochromocytoma tumour DNA and germline DNA from familial phaeochromocytoma patients was analysed for mutations in GDNF.

636 bp of coding sequence containing two exons were amplified using the following two sets of primer sequences (F: 5′-ATGAAGTTATGGAATCGTGGGC 3′; R: 5′-AGCAGCTGTCAGCCGGAAGG 3′ and F: 5′-AAATGCGCCAGTATCCTGA 3′; R: 5′-CAGATACATCAGACCTTTTACG 3′). Each fragment was amplified using PCR and analysed for point mutations according to Crossey et al. (33) with some minor changes; the SSCP analysis was performed at 4°C and the gels for exon 2 analysis were electrophoresed for 24 h.

The nature of the exon 2 SSCP bandshift detected was characterised using a Thermo Sequenase radiolabeled terminator cycle sequencing kit supplied by Amersham Life Science and appropriate primer sequences (F: 5′-CTTGGGTTCTGGGCTAT-GAAAACC 3′ and R: 5′-CTGCAGTACCTAAAAATCAGTTCC 3′).

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