Hereditary phaeochromocytomas and paragangliomas: a study of five susceptibility genes

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Non-functioning paragangliomas (Pgls), also called chemodectomas, glomus tumours, or non-chromaffin Pgls, are rare, highly vascularised tumours, originating from neural crest derived chief cells of paraganglia in the head and neck region. The main affected sites are the carotid body, a small chemoreceptive organ that senses blood oxygen level, and the jugulotympanic paraganglia. These tumours are typically slow growing and benign but local invasion and distant metastases can occur. Most of the tumours present without causing specific symptoms. Rarely, chemodectomas can secrete catecholamines. Therefore these tumours are diagnosed late on in development when their growth causes symptoms such as dysphagia, bradycardia, or hearing loss. Most chemodectomas occur sporadically whereas 10% to 50% could correspond to familial forms inherited as an autosomal dominant trait with incomplete penetrance. For one susceptibility locus named Pgl1, the tumours are transmitted through the paternal line, a fact suggestive of genomic imprinting. Recently, patients with hereditary chemodectomas were shown to harbour a germline mutation in one of the two genes coding the small (SDHD) and large (SDHC) subunits of cytochrome b or in the gene encoding the iron sulphur protein subunit of succinate dehydrogenase (SDHB). Gimm et al also identified germline mutations of SDHD in patients with isolated phaeochromocytomas (Pheao), thus showing that SDHD was a new Pgl and Pheo predisposing gene. The three subunits encoded by SDHB, SDHC, and SDHD together with the SDHA flavoprotein subunit are components of the mitochondrial complex II (succinate-coQoxidoreductase) that plays a central part in electron transport and the tricarboxylic acid cycle. Indeed, inherited Pheaos resulting from a germline SDHD mutation show a complete selective loss of complex II enzymatic activity and express higher levels of hypoxia induced factors (HIF1α and EPAS1) and VEGF than their sporadic counterparts.

Functioning Pgls, also called extra-adrenal Pheaos or sympathoadrenal Pgls, are rare catecholamine producing tumours arising in chromaffin tissue within sympathetic or other autonomic ganglia. These tumours are mostly intra-abdominal and can develop from the organ of Zuckerkandl or the bladder, for example. Pheaos are therefore a subclass of functioning Pgls affecting the chromaffin cells of the adrenal medulla. Both sympathoadrenal and parasympathetic paraganglia are part of a diffuse neuroendocrine system derived from neural crest cells, a fact which probably explains why functioning and non-functioning Pgls display common histopathological features. Patients with catecholamine secreting tumours often present with severe hypertension that may evolve to hypertensive crisis, arrhythmia, and death. Until recently, about 10% of Pheaos were considered hereditary, occurring as an autosomal dominant trait with incomplete penetrance. The three patients had polymorphisms of unknown significance. By comparison, 13 of 15 patients with familial tumours were (1) to evaluate the percentage of heritable Pheos and Pgls, and their families. Therefore, the aims of our study were (1) to evaluate the percentage of heritable Pheos and Pgls by looking for germline mutations in the five susceptibility genes already known (RET, VHL, SDHD, SDHC, SDHB) in a study of five susceptibility genes.

Key points

- Non-functioning and functioning paragangliomas (Pgls) are rare tumours of neural crest derived paraganglia. According to their localisation, 10-50% of Pgls are inherited as an autosomal dominant trait with incomplete penetrance. Five susceptibility genes for these tumours are already known: the RET proto-oncogene (causing multiple endocrine neoplasia type 2), the VHL tumour suppressor gene (causing VHL disease) and the recently described SDHB, SDHC, and SDHD genes encoding subunits of succinate dehydrogenase.
- We assessed the frequency of VHL, RET, SDHD, SDHC, and SDHB germline mutations by nucleotide sequencing in 21 patients clinically classified as having apparently sporadic Pheoa or Pgls and in 15 patients clinically classified as having familial tumours.
- Germline nucleotide variations were found in seven of 21 patients (33.3%). Four patients had clearly deleterious mutations in VHL (one patient), RET (one), SDHD (one) and SDHC (one with a novel mutation) whereas three patients had polymorphisms of unknown significance. By comparison, 13 of 15 patients with familial tumours had a germline mutation (86.7%) involving VHL in 12 whereas one patient had an SDHB nucleotide variation.
- Our data confirmed that clinical features suggesting an inherited tumour include a young age at onset (<18), a bilateral phaeochromocytoma, or multiple Pgls.
- The germline mutation rate in patients with apparently sporadic Pgls is at least 19% in western Europe. Therefore, introducing systematic germline mutation testing for the five susceptibility genes is recommended in these patients.

Abbreviations: CT, computed tomography; MEN 2, multiple endocrine neoplasia type 2; NF1, neurofibromatosis type 1; PCR, polymerase chain reaction; Pgl, paraganglioma; Pheao, phaeochromocytoma; SDHB, iron sulphur protein subunit of succinate dehydrogenase; SDHC, large subunit of cytochrome b; SDHD, small subunit of cytochrome b; VHL, von Hippel-Lindau disease.
sample of patients with non-syndromic PglS or Phaeos and (2) to define genotype-phenotype correlations in our population sample.

**MATERIALS AND METHODS**

**Patients with Pgl and Phaeo**

The patients included in this study were diagnosed with head and neck PglS or Phaeos or functioning abdominal PglS at the Department of Endocrinology, Clinique Marc Linquette or at the Department of Endocrine Surgery, Hôpital Claude Huriez, CHRU Lille, France. All the patients seen during their postsurgical follow up from 1998 to the middle of 2002 who gave their informed written consent for the genetic analysis were enrolled in the study. The aim of the genetic analysis was to identify heritable cases of PglS or Phaeos. The patients were clinically classified as having familial tumours if at least two patients of the kindred were diagnosed with PglS or Phaeos as documented by medical or surgical records, or if the subjects harboured a syndromic Phaeo (associated with a VHL disease or MEN 2). Based on these criteria, tumours of 15 patients corresponding to 13 families were classified as familial tumours. Tumours of 21 patients were classified as apparently sporadic.

**Phenotyping**

All patients included in the study underwent a careful physical examination including thyroid palpation and a search for cutaneous manifestations of neurofibromatosis type 1 (NF1). The patients’ personal and familial histories were recorded by the doctor. The routine screening protocol comprised direct ophthalmoscopy to look for retinal haemangioblastomas or Lisch nodules (associated with NF1), an abdominal computed tomography (CT) scan, and an MIBG scintigraphy (and when necessary a somatostatin receptor scintigraphy) to localise Phaeos or PglS and to detect renal or pancreatic lesions associated with VHL disease. Biological studies included measurement of serum and urinary catecholamines and metanephrines, serum chromogranin A, and basal serum calcitonin.

**Table 1** Distribution of the mutations in the patients and their phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Consequence</th>
<th>Patient’s identity</th>
<th>Initial classification and age at diagnosis</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>c.499C&gt;G</td>
<td>R167W</td>
<td>1 F</td>
<td>F - 30</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5† F</td>
<td>F - 29</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6† F</td>
<td>F - 15</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>F - 31</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>F - 13</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>F - 39</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>F - 49</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>F - 27</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>F - 43</td>
<td>Left Phaeo and right Pgl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9§</td>
<td>F - 47</td>
<td>Abdominal Pgl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10‡</td>
<td>F - 39</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18 S</td>
<td>S - 13</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
<td>RET</td>
<td>c.1901T&gt;C</td>
<td>C634R</td>
<td>17</td>
<td>S - 24</td>
<td>Bilateral Phaeo</td>
</tr>
<tr>
<td>SDHD</td>
<td>c.34G&gt;A</td>
<td>G12S</td>
<td>15 S</td>
<td>S - 19</td>
<td>Unilateral Pgl</td>
</tr>
<tr>
<td></td>
<td>c.204C&gt;T</td>
<td>S68S</td>
<td>16 S</td>
<td>S - 42</td>
<td>Unilateral Phaeo</td>
</tr>
<tr>
<td></td>
<td>c.34G&gt;A+c.204C&gt;T</td>
<td></td>
<td>11 F</td>
<td>F - 30</td>
<td>Juxtaadrenal Pgl and cervical Pgl</td>
</tr>
<tr>
<td>SDHC</td>
<td>c.473T&gt;C</td>
<td>L158P*</td>
<td>19 S</td>
<td>S - 22</td>
<td>Carotid body Pgl</td>
</tr>
<tr>
<td></td>
<td>c.129G&gt;A* (promoter)</td>
<td>Decreased expression?</td>
<td>20 S</td>
<td>S - 53</td>
<td>Inferior vena cava Pgl</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.263T&gt;C</td>
<td>L88S</td>
<td>12 S</td>
<td>F - 51</td>
<td>Bladder Pgl</td>
</tr>
</tbody>
</table>

The numbering of the nucleotides begins at the A of the first ATG denoted +1.

F, familial tumour; S, apparently sporadic tumour; Phaeo, phaeochromocytoma; Pgl, paraganglioma.

*The mutation is novel, † these patients belong to the same family.

**Genotyping**

DNA was isolated from peripheral blood using a commercial kit (QIAamp DNA Blood MaxiKit, Qiagen, France) according to the manufacturer’s protocol. SDHD intronic primers flanking each of the four exons were used for polymerase chain reaction (PCR) amplification of 300 ng DNA, using the experimental conditions described by Baysal et al.10 The PCR amplification of the eight exons of SDHB was carried out using the primer pairs and the conditions described by Astuti et al. For SDHC we designed six intronic primer pairs for exon amplification, except for primer 6R, which was located in the 3’ untranslated region (UTR) of exon 6, according to the genomic sequences.11 Primer sequences were as follows: 1F: 5′ CGTCACAATGACACCCCCAC 3′ 1R: 5′ CTCCCCAGTCCCCACTGAA 3′ (amplicon size 140 bp), 2F: 5′ TGGATTCTTTCATCCTGAGC 3′ 2R: 5′ ATCTCCAGATTTAGAATCT 3′ (amplicon size 98 bp), 3F: 5′ TCAACCTGATCTTGTAT 3′ 3R: 5′ CTGGTCCTGAGATCTCCTIC 3′ (amplicon size 154 bp), 4F: 5′ ACTCTCTACCTGAGCTTCT 3′ 4R: 5′ TGGGTAAAACACATTTATAT CATC 3′ (amplicon size 144 bp), 5F: 5′ AGCAGCGTGAGCAAGCTACT 3′ 5R: 5′ CTCCTCTTACGAGAAGATG 3′ (amplicon size 243 bp) 6F: 5′ CTTGGTTATGCTTCTTAC 3′ 6R: 5′ CCAAGGAGATCTGAAATACTA 3′ (amplicon size 293 bp). For VHL, the three exons were amplified according to the protocol of Crosse et al11 with some modifications: exon 2 primers: 2F: 5′ GTGGGCTTTTAAACACCTTGC 3′, 2r: 5′ TGGA-TACCGTGCTCTGACATCA 3′; exon 3 primers: 3F: 5′ CATTAGTCAGTGTAGTTG 3′, 3r: 5′ TACCAT-CAAAAAGGTAGATGAACAG 3′. Genomic DNA (300 ng) was amplified by PCR using Taq Gold polymerase (Applied Biosystems) and the following experimental conditions: initial denaturation at 95°C for seven minutes; then 35 cycles of 30 seconds denaturation at 95°C, 90 seconds of annealing at 52–60°C, 90 seconds of extension at 72°C; and a final extension step at 72°C for seven minutes. Exons 10, 11, and 16 of RET were studied using previously published protocols.12

The amplicons were first visualised in an ethidium bromide stained agarose gel before being purified on microcolumns (Microcon YM-50, Millipore) according to the manufacturer’s instructions. Both strands of the purified amplicons were

**Statistical analysis**

For the comparison of means, Student’s t test was used. For the comparison of frequencies Fisher’s unpaired exact test was used for small groups and for larger groups we used the standard χ² test. p values <0.05 were considered to be significant.

**RESULTS**

**Prevalence and distribution of germline mutations in patients with apparently sporadic tumours**

A total of 21 patients with apparently sporadic Phaeos (13 patients), PglS (seven patients), or both (one patient) were enrolled in the study. Molecular genetic analysis was performed as follows: direct nucleotide sequencing of VHL (three exons) and RET (exons 10, 11, and 16) to identify VHL disease or MEN 2, respectively; if negative, SDHD, SDHB, and SDHC sequencing was performed. A germline sequence variant was found in seven patients: one patient had a VHL mutation (14.3% of the mutations), another patient had a RET mutation (14.3%), three patients had germline sequence variants in SDHD (42.8%), and two patients had germline sequence variants in SDHC (28.6%, table 1, patients S).

The sequence variants of SDHD seen in three patients comprised the G12S+S68S double variation in two unrelated patients (15 and 16) and a splice acceptor site mutation IVS2–1G>T, comprised the G12S+S68S double variation in two unrelated patients (15 and 16) and a splice acceptor site mutation IVS2–1G>T, which is pathogenic as it has been found at the germinal level in one patient with a chemodectoma by Dannenberg et al. However, this change was not detected in 100 control chromosomes. The G nucleotide at -12 bp lies near two consensus binding sites for the transcription factor Sp1 (-30,-25; -18,-13) and the G>A mutation creates a cis element for the Ikaros trans acting factor (-15,-12, GGGGA), which was shown to repress the transcriptional activity of several promoters such as VPAC-1. Further functional studies are required to determine whether such a mechanism may apply to the SDHC promoter. Meanwhile, this G>A variation should be considered as not obviously deleterious. Patient 17 with a RET germline mutation was enrolled into the study after the discovery of a bilateral Phaeo operated on at the age of 24. Two months later a thyroidec- tomy was performed, on the basis of a positive pentagastrin test, showing a bilateral medullary thyroid carcinoma.

In conclusion, the rate of deleterious germline mutations in patients with apparently sporadic tumours reached 19% (four of 21 patients). The subclassification of these patients according to their phenotype showed that two patients out of 13 with Phaeos only (15%) had a germline mutation whereas two patients out of seven with PglS only (28.6%) had a germline mutation (NS).

**Prevalence and distribution of the germline mutations in patients with familial tumours**

By comparison, we identified 14 germline mutations in the 15 patients (table 1, patients F) classified as familial Phaeo (11 patients), familial Pgl (three patients), or both (one patient) after clinical investigation. The mutations affected VHL in 12 patients (86% of the mutations), SDHD in one patient (7%), and SDHB in another (7%). The SDHB L88S missense mutation was found in a 51 year old woman with a familial malignant Pgl of the urinary bladder (patient 12). A partial cystectomy was performed showing that the tumour infiltrated the vesical detrusor. Two years later an abdominal metastatic lymph node was removed because of a clinical and biological recurrence. Her father was operated on at the age of 56 for a pararenal Pgl; unfortunately, no blood or tumour DNA was available to check the presence of the L88S mutation. The 32 year old daughter of the proband did not inherit the mutation and is at present asymptomatic. This mutation was also found by Astuti et al in the germline of one patient with a sporadic Phaeo and in one of 200 control chromosomes. Therefore, the significance of this sequence variant is still uncertain. Overall, we identified 13 deleterious mutations in the 15 patients with familial tumours; the mutation rate was thus 86.7%. This high level of
germline mutations allows us to validate a posteriori our clinically based classification. Based on the results of the genetic analysis, we thus identified 17 patients with inherited Phaeos or PglS, 15 with sporadic tumours, and four with nucleotide variations of unknown relevance, whose main characteristics are shown in tables 2 and 3. Molecular analysis of the five genes was negative in one 27 year old patient who, however, probably had a familial tumour (right Pheo operated on at the age of 22, a left adrenal mass discovered in 2002, and a history of bilateral Pheos in her paternal grandfather).

**Phenotype of the patients according to their genotype**

The age at presentation of the disease did not differ significantly between patients with hereditary disease and patients without germline mutations. All the patients who presented at the age of 13 years or younger had germline mutations (of *VHL* in 100%) whereas this rate decreased to 57% among patients with an age at diagnosis younger than 18 years. No patients older than 50 years at onset had a clearly deleterious germline mutation. At diagnosis, patients with hereditary disease had multiple tumours more often (58.8%) than those without mutations (20%, p<0.05). Interestingly, patients who presented with bilateral Pheos had an inherited disease in 80% (table 3) in comparison with 28.6% of the patients with unilateral Phaeos (p<0.05). In both cases, the inherited tumours were almost always associated with a *VHL* mutation. Indeed, 15.4% of patients with a *VHL* mutation had an extra-adrenal lesion whereas this lesion was present in all the patients with a *SDH* mutation (p<0.05). In our hands, the presence of a head and neck Pgl is highly indicative of an inherited disease involving one *SDH* gene. Finally, the mutations that are not clearly deleterious are often seen in patients with an isolated abdominal Pgl.

**DISCUSSION**

The discovery of new susceptibility genes for familial cancer syndromes leads to an increase of the proportion of inherited tumours among patients presenting with an apparently sporadic tumour. Applied to the field of Phaeos and PglS, the recent description of germline mutations in three of the four genes encoding subunits of the succinate dehydrogenase, namely *SDHD*, *SDHC*, and *SDHB*, in patients with PglS or Phaeos has increased the number of susceptibility genes for familial Phaeo or Pgl (that is, *RET*, *VHL*, *SDH*, and *SDHD*) by studying the five predisposing genes, *SDHD*, *SDHC*, *SDHB*, *RET*, and *VHL*. The results presented here show that seven patients (33%) had a germline nucleotide variation. Based on the nature of this variation, patients could be classified into two groups: (1) four patients (19%) with a deleterious mutation and thus a hereditary tumour; (2) three patients (14%) with sequence variants of unknown significance (including one patient with a familial abdominal Pgl (patient 12)). Accordingly, the mutation rate is at least 19%, a result similar to that reported by Neumann et al.1 In this context, we wanted to reassess the frequency of germline mutations in a western European sample of patients with apparently sporadic Phaeos or PglS by studying the five predisposing genes, *SDHD*, *SDHC*, *SDHB*, *RET*, and *VHL*. The results presented here show that seven patients (33%) had a germline nucleotide variation. Based on the nature of this variation, patients could be classified into two groups: (1) four patients (19%) with a deleterious mutation and thus a hereditary tumour; (2) three patients (14%) with sequence variants of unknown significance (including one patient with a familial abdominal Pgl (patient 12)). Accordingly, the mutation rate is at least 19%, a result similar to that reported by Neumann et al.1 Interestingly, we report a new *SDHC* mutation identified in a patient with a Pgl of the carotid body. This mutation is the second germline variation affecting this gene besides the original one described by Niemann et al.9 It thus seems necessary to include this gene in the familial Phaeo or Pgl predisposing survey. Among the 15 patients without mutations, two had multiple tumours and one young patient had multiple tumours and a positive familial history suggesting that they could have familial tumours resulting from non-detectable mutations in the genes studied or involvement of as yet unknown susceptibility genes. Based on the 36 patients included, we also confirmed that multiple tumours are more frequent in patients with familial disease than in the sporadic patients.

Considering that up to 24% of apparently sporadic Phaeos or PglS are in fact inherited tumours, that patients with germline mutations are prone to develop multiple tumours (adrenal and extra-adrenal), and that the identification of a mutation could lead to a predictive genetic test in the kindred, it seems mandatory to perform a search for germline mutations in the

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**Table 3** Position of the tumours according to the results of the genetic analysis

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>n=36</th>
<th>n=17</th>
<th>n=15</th>
<th>n=4</th>
<th>n=13</th>
<th>n=1</th>
<th>n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position of the tumours:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single: head and neck</td>
<td>2</td>
<td>(5)</td>
<td>1</td>
<td>(6.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single: adrenal</td>
<td>14</td>
<td>(42.9)</td>
<td>9</td>
<td>(60.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single: extra-adrenal</td>
<td>7</td>
<td>(11.4)</td>
<td>3</td>
<td>(21.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple: adrenal only</td>
<td>10</td>
<td>(80)</td>
<td>7</td>
<td>(50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple: adrenal and extra-adrenal</td>
<td>10</td>
<td>(76.9)</td>
<td>1</td>
<td>(6.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple: head, neck, and extra-adrenal</td>
<td>1</td>
<td>(100)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The p values (Fisher’s test) are for the comparison between hereditary disease with sporadic disease.

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Electronic letter

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five susceptibility genes so far identified in patients with apparently sporadic Phaeos or Pgls. A reasonable approach would be to test initially young patients with a positive family history of the disease, or with multiple or extra-adrenal tumours. Taking into account (1) the repartition of the germ-line mutations; (2) the fact that an isolated Phaeo is rarely the first clinical manifestation of MEN 2; and (3) the fact that the diagnosis of NF1 is usually made based on clinical criteria, it seems to us that the genes should be screened in the following order: VHL, SDHD, and if negative SDHB, RET, and SDHC.

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