Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas

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Malignant pheochromocytoma (PCC) and paraganglioma (PGL) are mostly caused by germline mutations of SDHB, encoding a subunit of succinate dehydrogenase. Using whole-exome sequencing, we recently identified a mutation in the FH gene encoding fumarate hydratase, in a PCC with an ‘SDH-like’ molecular phenotype. Here, we investigated the role of FH in PCC/PGL predisposition, by screening for germline FH mutations in a large international cohort of patients. We screened 598 patients with PCC/PGL without mutations in known PCC/PGL susceptibility genes. We searched for FH germline mutations and large deletions, by direct sequencing and multiplex ligation-dependent probe amplification methods. Global alterations in DNA methylation and protein succination were assessed by immunohistochemical staining for 5-hydroxymethylcytosine (5-hmC) and S-(2-succinyl) cysteine (2SC), respectively. We identified five pathogenic germline FH mutations (four missense and one splice mutation) in five patients. Somatic inactivation of the second allele, resulting in a loss of fumarate hydratase activity, was demonstrated in tumors with FH mutations. Low tumor levels of 5-hmC, resembling those in SDHB-deficient tumors, and positive 2SC staining were detected in tumors with FH mutations. Clinically, metastatic phenotype (P = 0.007) and multiple tumors (P = 0.02) were significantly more frequent in patients with FH

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mutations than those without such mutations. This study reveals a new role for FH in susceptibility to malignant and/or multiple PCC/PGL. Remarkably, FH-deficient PCC/PGLs display the same pattern of epigenetic deregulation as SDHB-mutated malignant PCC/PGL. Therefore, we propose that mutation screening for FH should be included in PCC/PGL genetic testing, at least for tumors with malignant behavior.

INTRODUCTION

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare neuroendocrine tumors derived from chromaffin cells of the adrenal medulla and paraganglia from the autonomic nervous system, respectively. Overall, 10–20% of PCC/PGL are malignant, and malignancy remains difficult to predict and is defined only as the presence of metastases in a non-chromaffin tissue (1).

PCC/PGL are hereditary in ~40% of cases, and predisposing germline mutations have been found in one oncogene (RET) and 10 tumor suppressor genes (SDHB, SDHC, SDHD, SDHA (SDHx), SDHAF2, VHL, TMEM127, MAX and NF1) (2). The identification of PCC/PGL susceptibility genes has made a crucial contribution to our understanding of the molecular basis of this disease, and to the definition of markers of poor prognosis useful for clinical follow-up. Indeed, large genotype–phenotype studies have shown that the presence of a germline SDHB mutation is associated with a high risk of malignancy and reduced survival (3,4).

The SDHB gene encodes the catalytic subunit of a tricarboxylic acid (TCA) cycle enzyme, succinate dehydrogenase (SDH). SDH inactivation leads to the accumulation of its substrate, succinate, which acts as an oncometabolite (5) by inhibiting 2-oxoglutarate-dependent dioxygenases such as HIF prolyl-hydroxylases (6,7) and TET enzymes (8), promoting pseudo-hypoxic signaling (9) and DNA hypermethylation (10), respectively. The inhibition of these molecular pathways explains the extensive vascularization of SDH-related tumors and may contribute to metastatic dissemination, by driving epithelial-to-mesenchymal transition in SDHB-deficient tumors (10,11).

Comprehensive integrative genomics studies have improved the classification of PCC/PGL (12–14). Two main clusters of mutations have been identified in transcriptome analyses. Cluster 1 includes SDHx- (cluster 1A) and VHL-related tumors (cluster 1B) and is characterized by the pseudohypoxic signature. Cluster 2 comprises all NF1-, RET-, MAX- and TMEM127-related tumors and is associated with the activation of MAP kinase/AKT/mTOR pathways. We previously reported a tumor classified as a cluster 1A tumor (14) with a hypermethylator phenotype (10) but no SDHx gene mutation. By whole-exome sequencing, we detected a germline mutation, associated with a second somatic mutation in the FH gene, which encodes another tricarboxylic cycle enzyme, fumarate hydratase (10). Germline mutations of FH have previously been reported in cases of hereditary leiomyomatosis and renal cell cancer (HLRCC) (15) but had never been described in PCC/PGL.

Here, we identify five pathogenic germline FH mutations in five patients with PCC/PGL and without mutations in any of the known susceptibility genes for this disease. These patients present clinical characteristics compatible with malignancy. Furthermore, we determine the functional consequences of FH inactivation by examining the enzyme activity and loss of heterozygosity (LOH) in available tumors. Notably, we indicate that FH inactivation drives epigenetic dysregulation similar to SDHB deficiency, as well as widespread alterations in protein succination, both of which are accurately detected by immunohistochemistry (IHC). Therefore, we conclude that FH is a second major susceptibility gene to malignant PCC/PGL.

RESULTS

A large international series of 598 patients, collected in France (n = 359) and Spain (n = 239), diagnosed with PCC/PGL without mutations in any of the 11 known PCC/PGL susceptibility genes were analyzed for germline mutations of FH (Table 1). This population consisted of 151 patients (25%) diagnosed with PCC/PGL before the age of 35 years, 85 patients (14%) with multiple PCC/PGL and 59 patients (10%) with metastatic PCC/PGL.

Forty-one different heterozygous variants covering the entire length of the FH gene were identified (Fig. 1; Supplementary Material, Table S1). We detected 18 single nucleotide polymorphisms (SNPs): 12 with frequencies similar to those reported in the general population, and 6 for which no frequency value was available. We also identified 23 other variants: 14 (61%) were intronic, 6 (26%) were missense and 3 (13%) were synonymous. The presence of copy number variations was excluded by multiplex ligation-dependent probe amplification (MLPA). Five of the identified variants were classified as pathogenic, either because they had already been described as causal variants, or on the basis of in silico prediction (Supplementary Material, Table S2). None of these variants was found in public SNP databases.

We previously described the patient carrying the missense mutation (c.349G>C; p.Ala117Pro) in exon 3 (10), which had already been identified as pathogenic by two independent groups in patients with a diagnosis of multiple cutaneous and uterine leiomyomatosis (MCUL) (15). A second variant affecting an essential splicing site (c.268-2A>G; p.? ) had previously been found in a patient with FH deficiency and reported to be pathogenic in the TCA Cycle Gene Mutation Database (16). The remaining three FH missense variants identified in this study are previously unknown mutations, located within exon 8 (c.1142C>T; p.Thr381Ile), exon 7 (c.986A>G; p.Asn329Ser) and exon 5 (c.580G>A; p.Ala194Thr).

The clinical and genetic features of individuals carrying FH mutations are presented in Table 2. Three of these patients had metastatic PCC/PGL and three developed multiple tumors. The first patient, described in a previous study (10), presented metastatic PCC at the age of 63 years and died at 73 years old. Subsequent analysis of her clinical record revealed that she had a history of hysterectomy at the age of 35 years for hemorrhagic fibromas highly suggestive of uterine leiomyoma. The second patient was diagnosed with left adrenal PCC at the age of 68 years and died at 73 years old. She had no family history of any sporadic or hereditary tumor and multiple congenital anomalies (MCU). The third patient, described in a previous study (10), presented metastatic PCC/PGL at the age of 63 years and died at 73 years old. She had a history of hysterectomy at the age of 35 years for hemorrhagic fibromas highly suggestive of uterine leiomyoma. The fourth patient, described in a previous study (10), presented metastatic PCC/PGL at the age of 63 years and died at 73 years old. She had a history of hysterectomy at the age of 35 years for hemorrhagic fibromas highly suggestive of uterine leiomyoma. The fifth patient, described in a previous study (10), presented metastatic PCC/PGL at the age of 63 years and died at 73 years old. She had a history of hysterectomy at the age of 35 years for hemorrhagic fibromas highly suggestive of uterine leiomyoma.
of 20 years and with abdominal PGL 4 years later. Patient #3 had hypertension since childhood and was diagnosed at the age of 28 years with multiple lumbar-aortic PGLs, with evidence of bone, liver and lymph node metastases. The fourth patient, who had undergone surgery for Zuckerkandl PGL, presented a relapse 9 years later, with bilateral PCC and evidence of bone metastases. Normetanephrine concentrations were high for these four patients at the time of PCC or PGL diagnosis. Only one of these five patients presented a single, non-secreting, benign tumor, located in the carotid body, which was treated by external radiotherapy. For the entire cohort, the proportion of FH mutation carriers displaying either metastases ($P = 0.007$) or multiple PCC/PGL ($P = 0.02$) was significantly higher than that in patients not carrying FH mutations. In contrast, first PCC/PGL diagnosis before the age of 35 years was not indicative of FH mutation ($P = 0.6045$).

Table 1. Clinical and tumor characteristics of PCC/PGL patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total patients</th>
<th>n = 598</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>247 (41.3%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>351 (58.7%)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>46.1 (6–87)</td>
<td></td>
</tr>
<tr>
<td>&lt;35 years</td>
<td>151 (25.3%)</td>
<td></td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCC</td>
<td>410 (68.6%)</td>
<td></td>
</tr>
<tr>
<td>PGL</td>
<td>206 (34.4%)</td>
<td></td>
</tr>
<tr>
<td>H&amp;N</td>
<td>96 (16.6%)</td>
<td></td>
</tr>
<tr>
<td>TAP</td>
<td>114 (55.3%)</td>
<td></td>
</tr>
<tr>
<td>H&amp;N + TAP</td>
<td>4 (1.94%)</td>
<td></td>
</tr>
<tr>
<td>PCC + PGL</td>
<td>18 (3.01%)</td>
<td></td>
</tr>
<tr>
<td>Tumor characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>513 (85.8%)</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
<td>85 (14.2%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic</td>
<td>59 (9.8%)</td>
<td></td>
</tr>
</tbody>
</table>

PCC, pheochromocytoma; PGL, paraganglioma; H&N, head and neck; TAP, thoracic/abdominal/pelvic.

We had already reported that the tumor from patient #1 carrying the variant in exon 3 (c.349G>C, p.Ala117Pro) also presented a second somatic inactivating mutation in exon 7 (c.1043G>C, p.Gly348Ala) (10). We checked for LOH in two other FH mutations carriers, by directly sequencing the FH gene from the available tumor DNA (patients #2 and #3). The mutation in exon 8 (c.1142C>T; p.Thr381Ile) was homozygous in patient #3 (Fig. 2A), consistent with classical two-hit gene activation. This inactivation was confirmed by a complete loss of fumarase activity in the tumor (Fig. 2B). LOH was also observed for the mutation of patient #2 (c.268-2A>G), in DNA extracted from both her paraffin-embedded PCC and PGL tumors (Fig. 2C).

We have recently shown that SDH-related tumor cells and the first FH-deficient PGL (patient #1) identified have low 5-hydroxymethylcytosine (5-hmC), due to inhibition of TET enzymes by succinate and fumarate. Moreover, it has been shown that fumarate reacts spontaneously with cysteine sulfhydryl groups to generate a stable chemical modification of proteins, S-(2-succinyl) cysteine (2SC) (17), which can be evaluated by IHC (18). We thus studied the impact of FH inactivation on both DNA methylation and protein succination, using antibodies directed against 5-hmC and 2SC, respectively. These experiments were performed in the three FH-mutated samples for which paraffin-embedded tissues were available as well as in 18 other PGL/PCC for which the genetic screening of FH was performed and was negative. Among them, there were seven harboring germline or somatic mutations in PGL/PCC predisposing genes (two NF1, two RET, one TMEM127, one VHL and one MAX).

The evaluation of 5hmC levels by IHC confirmed that FH-mutated PCC and PGL displayed the same epigenetic changes as SDH-related tumors. In addition, we observed a specific 2SC-positive staining in FH-deficient samples, which was absent in tumors without FH mutations (Fig. 3, Table 2, Supplementary Material, Table S3). Thus, the combination of low 5hmC levels and positive immunostaining for 2SC can be used to predict or validate FH-variants in PCC/PGL.

Figure 1. Graphical representation of pathogenic FH mutations identified in this study and the residues of the protein affected. The variant already reported is shown in red, and variants affecting a highly conserved amino-acid residue are shown in blue. Sequence alignments of corresponding residues in different species.
DISCUSSION

TCA cycle enzymes are increasingly being reported to play a role in the predisposition to inherited tumor syndromes and in the pathogenesis of sporadic forms of cancer (19). Mutations in genes encoding SDH subunits (SDHA, SDHB, SDHC and SDHD) were the first to be shown to confer predisposition to PGL and PCC, but such mutations are also responsible for gastrointestinal stromal tumors (20) and kidney cancers (21). Germline FH mutations have been identified in MCUL and HLRCC (15) syndromes, characterized by benign smooth muscle tumors, uterine leiomyoma and a highly malignant form of papillary and collecting duct renal cancer. Finally, somatic mutations in the genes encoding the mitochondrial (IDH2) and cytosolic (IDH1) forms of isocitrate dehydrogenase have been found in gliomas (22), acute myelogenous leukemia (23), in central chondrosarcomas (24) and cholangiocarcinomas (25). Following our recent identification of the first patient with a germline FH mutation responsible for PGL, we report here four additional cases of predisposition to PCC and/or PGL caused by FH mutation, revealing a new role for this mitochondrial enzyme in cancer and demonstrating the functional overlap between TCA cycle enzymes in cancer predisposition.

We searched for FH mutations in germline DNA from 598 patients with PCC and/or PGL with no mutations in known susceptibility genes. We found that germline mutations of FH accounted for 0.83% of cases, a frequency similar to that reported for TMEM127 (0.9%) (26) and MAX (1.12%) (27) mutations. FH mutations should therefore be considered a new, albeit rare, source of predisposition to PCC/PGLs.

According to current data from the FH section of the TCA Cycle Gene Mutation Database (16), 268 mutations (154 unique) of this gene have been reported in total, most being found in patients with diagnoses of MCUL (112; 46%), HLRCC (47;19%) and FH deficiency (42; 17%). Variants of exon 7 (53; 19%), exon 5 (43; 16%), exon 3 (36; 13%) and exon 8 (25; 9%) are overrepresented, suggesting that some of the sequences of the gene may be more prone to mutational events than others. Consistent with these data, two of the potentially pathogenic variants found in this study concerned exon 3.

Table 2. Genetic, clinical and immunohistological characteristics of FH mutation carriers

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor type</th>
<th>Multiple</th>
<th>Metastases</th>
<th>cDNA mutation</th>
<th>Protein alteration</th>
<th>LOH</th>
<th>5hmC</th>
<th>2SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>F</td>
<td>63</td>
<td>PCC</td>
<td>No</td>
<td>Yes</td>
<td>c.349G&gt;C</td>
<td>p.Ala117Pro</td>
<td>Yes</td>
<td>Low</td>
<td>Positive</td>
</tr>
<tr>
<td>#2</td>
<td>F</td>
<td>20</td>
<td>PCC+PGL (TAP)</td>
<td>Yes</td>
<td>No</td>
<td>c.268-2A&gt;G</td>
<td>p.?</td>
<td>Yes</td>
<td>Low</td>
<td>Positive</td>
</tr>
<tr>
<td>#3</td>
<td>M</td>
<td>28</td>
<td>PGL (TAP)</td>
<td>Yes</td>
<td>Yes</td>
<td>c.1142G&gt;T</td>
<td>p.Thr381Ile</td>
<td>Yes</td>
<td>Low</td>
<td>Positive</td>
</tr>
<tr>
<td>#4</td>
<td>F</td>
<td>54</td>
<td>PCC+PGL (TAP)</td>
<td>Yes</td>
<td>Yes</td>
<td>c.580G&gt;A</td>
<td>p.Ala194Thr</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>#5</td>
<td>M</td>
<td>70</td>
<td>PGL (H&amp;N)</td>
<td>No</td>
<td>No</td>
<td>c.986A&gt;G</td>
<td>p.Asn329Scr</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Age is the age at diagnosis. The letters and numbers in the protein alteration column indicate the amino acid from the wild-type sequence (first) and the amino acid with which it is replaced in the mutated sequence (second). The number between the letters denotes the codon position. Amino acid abbreviations: Ala, alanine; Pro, proline; Thr, threonine; Ile, isoleucine; Asn, asparagine; Ser, serine; PCC, pheochromocytoma; PGL, paraganglioma; H&N, head and neck; TAP, thoracic/abdominal/pelvic; LOH, loss of heterozygosity; NA, not available.
whereas the other three missense mutations were located in exons 5, 7 and 8. All these variants affected highly conserved residues of the protein and were pathogenic, according to the bioinformatic tools.

Three of the five mutation carriers identified had either PCC + PGL or multiple PGL, but, to the best of our knowledge, none had kidney cancer and only one had had uterine fibromas, 25 years before PCC diagnosis. The reasons for this difference in the spectrum of tumor development remain unclear and will need to be addressed in future studies. Nevertheless, it could be suggested that patients harboring FH mutations should be screened for the presence of PCC or PGL. Accordingly, renal cancers and leiomyomas should be searched for in PCC/PGL patients carrying FH variants. Three patients presented evidence of metastatic disease, suggesting that FH inactivation may have a role similar to that of SDHB mutations in driving the malignant phenotype. The younger patient with apparently benign PCC and PGL underwent surgery 9 and 5 years ago, and we therefore cannot exclude the possibility of recurrence or a metastatic phenotype in the future. Given that succinate and fumarate are generated by sequential enzymatic reactions in the same metabolic signaling pathway, SDH- and FH-deficient cells are likely to have, at least to some extent, a common tumorigenic mechanism. We and others have recently demonstrated that the accumulation of succinate and fumarate is responsible for the development of a hypermethylation phenotype, mediated by the inhibition of DNA demethylases of the TET family involved in the hydroxylation of 5-methylcytosine to generate 5-hmC. Consistent with these findings, we found that FH-deficient tumors had the same altered immunostaining pattern for 5-hmC observed for SDHB-deficient tumors, indicating a common pathophysiological mechanism, involving the specific induction of genome-wide alterations in DNA methylation. These findings validate the newly identified mutations as functional. In addition, we show for the first time that the accumulation of fumarate in PCC/PGL also leads to global changes in protein succination, as revealed by 2SC immunostaining. The specificity of 2SC staining in FH-mutated PCC/PGL is further supported by a previous study, which found no 2SC immunolabeling in 44 PCC/PGL samples (17). Together, these findings suggest that fumarate also plays a key role as an oncometabolite in tumor initiation and metastasis in these neuroendocrine tumors. These results suggest that the application of a combination of these two immunohistochemical assays to tumor tissues from patients may be useful for the identification of FH-related PCC/PGL.

In conclusion, the results of this study strongly suggest that, like SDHB, FH is one of the causal genes of malignant PCC/PGL. Tumoral 5-hmC and 2SC immunostaining would be a cost-effective approach to the detection of patients at risk. FH should thus be added to the list of PCC/PGL susceptibility genes and should be considered in mutation screening, to assess the risk of malignant disease.

MATERIALS AND METHODS

Clinical samples

We analyzed 598 unrelated patients diagnosed with PCC and/or PGL with no mutation in a known PCC/PGL susceptibility gene (RET, NF1, VHL, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127 or MAX). The characteristics of the tumors and the patients are presented in Table 1. The relatively high proportion of young patients in this cohort may be explained by the fact that both departments involved is this study are referral centers for...
these rare tumors. Blood and tumor samples were collected by the French ‘Cortico et Médullosurrénale: les Tumeurs Endocrines’ (COMETE) network in Paris (France) and by public hospitals and the Spanish National Tumor Bank Network in Madrid (Spain). Each patient gave written informed consent for the analysis of germline and somatic DNA. The study was approved by the local institutional review boards (CPP Ile de France II, June, 2012 and Comité de bioética y bienestar animal del Instituto de Salud Carlos III).

Germline DNA was extracted from leukocytes according to the standard protocols. Frozen tumor samples were ground to a powder in liquid nitrogen and 30–50 mg of the sample was used for DNA extraction with the AllPrep kit (Qiagen). DNA was extracted from paraffin-embedded slides according to the standard protocols. DNA was quantified and its purity assessed with a NanoDrop ND-1000 spectrophotometer (Labtech).

Molecular genetic analyses

Direct Sanger sequencing of the 10 exons of the FH gene, including the exon–intron boundaries, was performed. For LOH analysis on tumor DNA extracted from paraffin sections, specific primers were designed, for the generation of smaller amplicons. The primer sequences and PCR conditions are available upon request. Sequencing reactions were performed with the BigDye Terminator v3.1 kit (Life Technologies), in an ABI Prism 3730XL DNA Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). For the detection of large FH deletions, MLPA was performed with the SALSA MLPA P198-A2 kit and the fumarase deficiency probe mix (MRC-Holland), according to the manufacturer’s recommendations.

Analysis of FH variants

We assessed the potential pathogenicity of variants with Alamut®-Mutation Interpretation Software version 2.3.2 (http://www.interactive-biosoftware.com/software.html). We checked the frequencies of the mutations and variants in the TCA Cycle Gene Mutation Database (16), the Exome Sequencing Project and 1000 Genomes database.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor sections (6 μm) were used for 5-hmC and S-(2-succinyl) cysteine (2SC) staining. Briefly, slides were deparaffinized in xylene and rehydrated in alcohol. Antigen retrieval was performed with a citrate-based antigen-unmasking solution (8.2 mM sodium citrate, 1.8 mM citric acid 1.8 mM, pH 6). The following antibodies and respective specifications were used: polyclonal rabbit 5-hmC (Active motif catalog; Ref. 39769, 1:1200) and polyclonal rabbit 2SC (28), 1:5000). Biotinylated secondary antibodies (Vector Laboratories) were used at a dilution of 1:400. Tumor tissues without FH mutations were used as controls and slides were scored in a blinded manner. 5-hmC IHC was analyzed by comparing the level of nuclear staining between tumor cells and adjacent endothelial cells as internal controls. Tumors were quoted as ‘low’ when tumor staining was inferior to that of endothelial cells. 2SC was scored as either positive or negative.

Measurement of FH enzyme activity

FH enzyme activity in tumor tissues was measured by spectrophotometry, as previously described (10). Briefly, this assay monitors the change in absorbance due to the forward (fumarate to malate) and backward (malate to fumarate) conversions. The enzyme activity is measured at 37°C. Reagents were obtained from Sigma-Aldrich (France).

Statistical analyses

Statistical analysis was carried out with GraphPad software. Differences between patients carrying and not carrying mutations were analyzed for the frequency of multiple tumors, PCC plus PGL, early onset of disease and malignancy, in two-tailed Fisher’s exact tests. A P-value < 0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. P.J.P. was a co-applicant on the filing for a patent application [USC-268-P(849)] covering immunohistochemical screening with a 2SC antibody for determination of FH mutations.

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