Head and neck paragangliomas: genetic spectrum and clinical variability in 79 consecutive patients

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Abstract

Head and neck paragangliomas (HNPGLs) are neural crest-derived tumors. In comparison with paragangliomas located in the abdomen and the chest, which are generally catecholamine secreting (sPGLs) and sympathetic in origin, HNPGLs are, in fact, parasympathetic in origin and are generally nonsecreting. Overall, 79 consecutive patients with HNPGL were examined for mutations in $SDHA$, $SDHB$, $SDHC$, $SDHD$, $SDHAF2$, $VHL$, $MAX$, and $TMEM127$ genes by PCR/sequencing. According to a detailed family history (FH) and clinical, laboratory (including metanephrines), and instrumental examinations, patients were divided into three groups: a) patients with a positive FH for HNPGL (index cases only), b) patients with a negative FH and multiple HNPGLs (synchronous or metachronous) or HNPGL associated with an sPGL, and c) patients with negative FH and single HNPGL. The ten patients in group a) proved to be $SDHD$ mutation carriers. The 16 patients in group b) proved to be $SDHD$ mutation carriers. Among the 53 patients in group c), ten presented with germ-line mutations (three $SDHB$, three $SDHD$, two $VHL$, and two $SDHAF2$). An sPGL was found at diagnosis or followed up in five patients (6.3%), all were $SDHD$ mutation carriers. No $SDHC$, $SDHA$, $MAX$, and $TMEM127$ mutations were found. In $SDHD$ mutation carriers, none of the patients affected by HNPGL associated with sPGL presented missense mutations. In conclusion, a positive FH or the presence of multiple HNPGLs is a strong predictor for germ-line mutations, which are also present in 18.8% of patients carefully classified as sporadic. The most frequently mutated gene so far is $SDHD$ but others, including $SDHB$, $SDHAF2$, and $VHL$, may also be affected.

Introduction

Paragangliomas of the head and neck region (HNPGLs) are hypervascular tumors arising from the neural crest cells. They are mostly found at the bifurcation of the common carotid artery where the carotid body is located, but they may also arise from...
the jugular, tympanic, vagal, or laryngeal paraganglia (Lack 2004).

With HNPGLs generally being parasympathetic in origin and nonsecreting, they are consequently diagnosed for symptoms caused by compression of the surrounding cervical nerves, or they are incidentally detected during radiological (mainly ultrasonographic) examination of the thyroid or neck vessels.


When hereditary, they may sometimes occur in association with sympathetic catecholamine-secreting paragangliomas (sPGLs), located in the abdomen or in the chest, and/or they may occur as multiple tumors (Burnichon et al. 2009, Mannelli et al. 2009, Neumann et al. 2009, Ricketts et al. 2010).

Gene mutations responsible for HNPGL occur mostly in genes encoding the subunits of the succinate dehydrogenase or mitochondrial complex II. These genes include SDHD, located on 11q23 (Baysal et al. 2000); SDHB, located on 1p36 (Astuti et al. 2001); SDHC, located on 1q21 (Niemann & Mueller 2000); and SDHAF2, located on 11q12, which is responsible for SDHA flavination (Hao et al. 2009).

Heterozygous mutation of the SDHA gene has been found in one patient affected by a catecholamine-secreting abdominal PGL (Burnichon et al. 2010); whether it may also cause HNPGL is unknown.

Very rarely, HNPGLs have also been described in VHL gene mutation carriers (Ercolino et al. 2008, Boedeker et al. 2009, Gaal et al. 2009).

Although with a very low frequency, HNPGLs have also been recently reported in patients affected by germ-line mutations in TMEM127 gene (Neumann et al. 2011).

The occurrence of HNPGLs in carriers of RET and NF1 mutations is extremely rare (Boedeker et al. 2009), and in these syndromes, HNPGLs have never been reported as first lesions, while the frequency of HNPGLs in patients affected by a germ-line mutation in MAX, the last PGL susceptibility gene so far discovered (Comino-Méndez et al. 2011), is at present unknown.

Here, we report the results of genetic analysis and the clinical picture in 79 consecutive patients affected by HNPGL.

Materials and methods

The study protocol was approved by the institutional review boards of all participating centers, and each participant provided written informed consent. Unless otherwise stated, all commercial products mentioned were used according to the manufacturers’ instructions.

Patients

This study consisted of consecutive patients (52 females and 27 males; mean age, 45.7±16.8 years; age range, 14–82 years) affected with HNPGL, evaluated between January 1, 2003 and March 30, 2011. Thirty-three patients, enrolled between January 1, 2003 and December 31, 2007 had been included in a previous study evaluating patients affected by sPGL and/or HNPGL (Mannelli et al. 2009). In this study, the previous genetic analysis of these patients was completed by SDHAF2, SDHA, MAX, and TMEM127 sequencing.

HNPGL tumors were diagnosed by imaging (presence in the region of a highly vascular mass on CT or MRI) and, when possible, confirmed by histology.

Upon enrollment, each participant was evaluated according to a well-established protocol that included complete personal and family histories (FH), clinical evaluation, and measurement of urinary metanephrines (MNs) to assess the presence, if any, of sPGL. In patients showing pathological concentration of urinary MNs, the presence of sPGL was confirmed by radiological, scintigraphic, or surgical findings. Patients presenting with fractionated urinary MNs in the normal range were considered as not affected by an sPGL. In these patients, at the first examination, a thorax CT scan was performed to diagnose nonsecreting PGL located in this site.

Measurement of urinary MNs and imaging of the head and neck region were performed annually in each mutation carrier. Follow-up duration, as calculated from the date of the first diagnosis, ranges from 11 to 349 months (mean ± s.d., 104 ± 91).

On the basis of the results of this workup, patients were subsequently divided into three main groups: a) patients with a positive FH for HNPGL, b) patients with a negative FH and multiple HNPGLs (synchronous or metachronous) or HNPGL associated with sPGL, and c) patients with negative FH and a single HNPGL.

Mutation analysis

All germ-line mutations were documented by the results of genetic testing performed in Florence according to standardized protocols. DNA was extracted from the peripheral blood leukocytes of each patient using the NucleoSpin Blood L kit
(Macherey-Nagel, Düren, Germany) and analyzed for germ-line mutations of SDHA (all exons), SDHB (all exons), SDHC (all exons), SDHD (all exons), SDHAF2 (all exons), MAX (all exons), TMEM127 (all exons), and VHL (all exons). For each gene, coding regions and exon–intron boundaries were amplified by PCR as described previously (Astuti et al. 2001). PCR products, purified using a commercial kit (PCR purification kit, Qiagen), were subjected to 2% agarose gel electrophoresis with ethidium bromide staining and subsequently sequenced with a genetic analyzer (ABI PRISM 310; Applied Biosystems, Milan, Italy).

**Multiplex ligation-dependent probe amplification reactions**

Patients younger than 50 years, whose DNA sequencing was wild type for SDHB, SDHC, SDHD, SDHAF2, and VHL, underwent analysis for genomic rearrangements in these genes. For this purpose, we used commercial kits for multiplex ligation-dependent probe amplification (MLPA)-based assays (SALSA MLPA P016B VHL and SALSA MLPA P226 SDHD; MRC-Holland, Amsterdam, The Netherlands) following the manufacturers’ instructions.

Amplification products were diluted in HiDi formamide containing 500TAMRA internal size standards (MRC-Holland) and then separated by size using an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Electropherograms were analyzed using Coffalyser MLPA DAT software (MRC-Holland).

**Statistical analysis**

Statistical analysis was based on $\chi^2$ and $t$-tests. $P$ values <0.05 were considered statistically significant.

**Results**

Table 1 summarizes the characteristics of the patient groups defined on the basis of FH and clinical presentations.

Group a (HNPGL with positive FH): consisted of ten patients who were index cases of their corresponding families. Three presented with a single HNPGL, four with multiple or recurrent HNPGLs, one with HNPGL associated with an sPGL, and two with HNPGL associated with a nonsecreting thoracic PGL.

All the ten patients were affected by SDHD mutation.

Group b (multiple HNPGL with negative FH): consisted of 16 patients presenting with multiple HNPGLs (ten patients) or HNPGL associated with sPGL (four patients) or HNPGL associated with nonsecreting thoracic PGL (two patients). All patients were found to be SDHD mutation carriers.

![Electrophoresis patterns of the two SDHAF2 mutations. cDNA mutations are indicated by small arrow.](image-url)
Group c (single HNPGL with negative FH) consisted of 53 patients. Among these, ten patients were found to be mutation carriers (three in SDHD, two in VHL, three in SDHB, and two in SDHAF2) (Fig. 1).

The germ-line mutation rate in these groups was 18.8%.

Mean age (mean ± s.d.) at tumor presentation was significantly lower \((P < 0.01)\) in 36 mutation carriers (39.7 ± 14.9 years, range 14–66 years) than in 43 wild-type patients (50.8 ± 16.8 years, range 18–82 years).

The different types of germ-line mutations and the clinical characteristics of mutation carriers are reported in Table 2.

Overall, we diagnosed 114 HNPGLs in 79 patients (Table 3).

SDHD mutations were so far the most frequent (29/36, 80.5%), and all the different types of mutation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Mutation cDNA</th>
<th>Mutation amino acid change</th>
<th>Mutation type</th>
<th>Clinical presentation</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHAF2</td>
<td>2</td>
<td>c.232 G&gt;C</td>
<td>p.Gly78Arg</td>
<td>1</td>
<td>Right tympanic</td>
<td>c</td>
</tr>
<tr>
<td>SDHAF2</td>
<td>4</td>
<td>c.357_358insT</td>
<td>p.Tyr119LeufsX7b</td>
<td>2</td>
<td>RCB</td>
<td>c</td>
</tr>
<tr>
<td>SDHB</td>
<td>1</td>
<td>c.3G&gt;A</td>
<td>p.Met1Ile</td>
<td>1</td>
<td>Left tympanic</td>
<td>c</td>
</tr>
<tr>
<td>SDHB</td>
<td>6</td>
<td>c.572G&gt;A</td>
<td>p.Cys191Tyr</td>
<td>1</td>
<td>LCB</td>
<td>c</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB + abdominal PGL</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB + thoracic ns c PGL</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB + left jugular</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>Right jugular + RCB + bilateral vagal + laryngeal</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB + thoracic PGL</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB + left jugular + left vagal</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.325C&gt;T</td>
<td>p.Gln109X</td>
<td>3</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.317G&gt;A</td>
<td>p.Gly106Asp</td>
<td>1</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.317G&gt;A</td>
<td>p.Gly106Asp</td>
<td>1</td>
<td>BCB + left jugular + right vagal</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>Right vagal + thoracic ns c PGL</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>BCB</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.170-1G&gt;T</td>
<td>c.IVS2-1G&gt;T</td>
<td>5</td>
<td>LCB</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.341A&gt;G</td>
<td>p.Thr114Cys</td>
<td>1</td>
<td>Left tympanic</td>
<td>c</td>
</tr>
<tr>
<td>SDHD</td>
<td>1, 2, 3, 4</td>
<td>1-7_480+7del</td>
<td>DEL1-4</td>
<td>4</td>
<td>LCB</td>
<td>c</td>
</tr>
<tr>
<td>SDHD</td>
<td>2</td>
<td>c.64C&gt;T</td>
<td>p.Arg22X</td>
<td>3</td>
<td>BCB + thoracic ns c PGL</td>
<td>a</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.445_448dupATCT</td>
<td>p.Cys150TyrfsX42</td>
<td>2</td>
<td>BCB + bilateral jugular + abdominal PGL</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.445_448dupATCT</td>
<td>p.Cys150TyrfsX42</td>
<td>2</td>
<td>Left tympanic + LCB + left jugular</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.387_388dupGG</td>
<td>p.Ala130GlyfsX5b</td>
<td>2</td>
<td>Right tympanic + left jugular</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.467T&gt;C</td>
<td>p.Leu156Pro b</td>
<td>1</td>
<td>LCB</td>
<td>c</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.264_273delCTCT</td>
<td>p.Cys88TrpfsX22b</td>
<td>2</td>
<td>BCB + pheochromocytoma</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>3</td>
<td>c.304C&gt;T</td>
<td>p.His102Tyr b</td>
<td>1</td>
<td>BCB + thoracic ns c PGL</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>1</td>
<td>c.2T&gt;A</td>
<td>p.Met1Lys b</td>
<td>1</td>
<td>BCB + left jugular</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>c.445_448dupATCT</td>
<td>p.Cys150TyrfsX42</td>
<td>2</td>
<td>BCB + left tympanic</td>
<td>b</td>
</tr>
<tr>
<td>SDHD</td>
<td>1, 2, 3</td>
<td>1-7_314+7del</td>
<td>DEL1-3</td>
<td>4</td>
<td>BCB + left vagal + left jugular + thoracic PGL</td>
<td>a</td>
</tr>
<tr>
<td>VHL</td>
<td>3</td>
<td>c.455C&gt;T</td>
<td>p.Thr152Ile</td>
<td>1</td>
<td>LCB</td>
<td>c</td>
</tr>
<tr>
<td>VHL</td>
<td>1</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>1</td>
<td>RCB</td>
<td>c</td>
</tr>
</tbody>
</table>

RCB, right carotid body; LCB, left carotid body; BCB, Bilateral carotid body.

a1, missense; 2, frameshift; 3, nonsense; 4, large deletion; 5, splice.

bMutations not reported in the TCA Cycle Gene Mutation Database (formerly SDH Complex database).

cns, nonsecreting paraganglioma.

dGroup to which patients belong on clinical grounds.

Table 2 Summary of identified germ-line mutations with clinical presentation of patients

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Table 3 Sites of tumors in wild-type patients and mutation carrier patients

<table>
<thead>
<tr>
<th>Type of HNPGL</th>
<th>Wild type</th>
<th>Mutation carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tympacic</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Jugular</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Carotid body</td>
<td>29</td>
<td>49</td>
</tr>
<tr>
<td>Vagal</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Laryngeal</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>71</td>
</tr>
</tbody>
</table>

(missense, nonsense, splicing, frameshift, and large deletion) were represented, but no missense mutations were found in the five patients affected by HNPGL associated with sPGL. The VHL and SDHB mutations were missense while the two SDHAF2 carriers showed a missense and a frameshift mutation respectively. Large deletions were found in two SDHD mutation carriers (2.6%) (Table 4).

We found five novel mutations, three missense and two frameshift (Table 2). Four of them were in the SDHA gene and one in the SDHAF2 gene.

No mutations were found in SDHA, MAX, and TMEM127 genes.

Discussion

Clinical and genetic characteristics of patients presenting with HNPGL have been reported in other studies that generally also included patients affected by sPGL (Burnichon et al. 2009, Neumann et al. 2009, Ricketts et al. 2010, Waguespack et al. 2010). Only a few papers have been focused on patients primarily presenting with HNPGL (Baysal et al. 2002, Lima et al. 2007, Neumann et al. 2009, 2011, Hensen et al. 2011) and, to our knowledge, none has conducted a genetic screening of these patients including all the genes potentially susceptible for HNPGL so far known.

In the present series, a positive FH, the presence of multiple HNPGLs, and the association of HNPGL with sPGL were invariably characterized by a germ-line mutation.

In patients clinically classified as sporadic, a germ-line mutation was found in 18.8%, a percentage similar to that reported by a French study, 16.7% (Burnichon et al. 2009), by our group, 14.3% (Mannelli et al. 2009), and only slightly lower than that reported in a smaller Spanish series, 22.2% (Lima et al. 2007).

As a whole, 45.6% of our patients presented with a germ-line mutation, in close agreement with the findings of the French study at 55.2% (Burnichon et al. 2009).

As expected, and in agreement with all the papers so far published (Burnichon et al. 2009, Mannelli et al. 2009), age at presentation was significantly lower in mutation carriers than in sporadic cases, and this difference is still present when excluding patients with a positive FH, which might have played a role in anticipating the detection of the tumor.

In our series, genders are differently represented, with females being almost double the males (P < 0.03). The rate of mutations does not differ among females (23/52, 44.2%) and males (13/27, 48.1%).

PGLs of the carotid body were so far the most widely represented tumors. They were diagnosed in 60 patients, isolated in 39 patients, bilateral in six patients, and in association with other types of PGL in 15 patients.

Among patients with a single carotid body PGL, 29 resulted wild type and ten mutated and, as a whole, there were significantly more females than males (28 F/11 M, P < 0.001). A significant increase in the number of females (20 F/9 M) (P < 0.05) was also found in the nonmutation carriers, thus confirming that, similarly to the French (Burnichon et al. 2009), Italian women also seem to have their HNPGLs detected more easily than men.

Among the 16 patients affected by a tympanic tumor, five were mutation carriers: three had isolated tumors and two had tumors associated with other HNPGLs. Therefore, at least in our experience, and in comparison with the French series (Burnichon et al. 2009), patients presenting with a tympanic PGL should also be considered for genetic analysis.

In our series, SDHD mutation was so far the most represented (80.5%), although, in comparison with other series (Lima et al. 2007, Neumann et al. 2009, Ricketts et al. 2010), the percentage of SDHB mutations turned out to be lower.

Among the SDHD mutations, p.Gln109X and p.Pro81Leu were found in eight and six patients respectively. While p.Glu109X variant is due to a deletion, p.Pro81Leu was found in eight patients.

Table 4 Genotype/phenotype correlation in SDHD carriers according to severity of mutation

<table>
<thead>
<tr>
<th>Types of mutations</th>
<th>Single HNPGL</th>
<th>HNPGL plus sPGL</th>
<th>Multiple synchronous or metachronous HNPGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Nonsense</td>
<td>0</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Splicing</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Frameshift</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Large deletion</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>

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founder effect (Simi et al. 2005), an haplotype study in patients with p.Pro81Leu mutation has not been performed.

Surprisingly, we found no SDHC mutations, and SDHB mutations showed similar incidences as VHL and SDHAF2 mutations. The presence of HNPGL in VHL mutation carriers has already been reported in the literature (Ercolino et al. 2008, Boedeker et al. 2009, Gaal et al. 2009, Waguespack et al. 2010). Of note, our two VHL patients presented a single carotid body PGL not associated with other syndromic lesions, suggesting that isolated HNPGL might be included in the VHL clinical picture, although with an atypical presentation.

Only few SDHAF2 mutations have been reported in the literature (Mariman et al. 1995, Bayley et al. 2010, Hensen et al. 2011) and almost all seem to be recurrent mutations. Of our two SDHAF2 mutation carriers, one is a 14-year-old boy carrying a missense mutation (Fig. 1) affecting the same nucleotide as in the Dutch (Mariman et al. 1995) and Spanish (Bayley et al. 2010) families, while the other carrier is a 44-year-old woman presenting with a novel frameshift mutation (Fig. 1).

No malignant HNPGLs were present in our study, in comparison with other studies.

This and other differences, like the different gene mutation frequencies, may be explained by genetic differences among series collected in different countries, thus suggesting that the recommended sequential genetic testing might vary according to the different national experiences.

In our series, no missense mutations were found in patients presenting with sPGL associated with HNPGL, which, in fact, presented all the other more severe genetic mutations. Should this finding be confirmed in other more extended studies, the difference might have clinical relevance, suggesting a lower risk of developing sympathetic sPGL in SDHD missense mutation carriers.

In conclusion, our study, which is the first to include the genetic analysis of all the PGL susceptibility genes so far discovered, confirms that, in the presence of HNPGL, SDHD is the first gene to screen, especially in the presence of multiple HNPGLs and when HNPGL is associated with an sPGL. It also suggests that any type of HNPGL may be familiar, including the symatcnic ones, and that, at least in Italy, HNPGL due to SDHAF2 mutations does not seem to be rarer than mutations in SDHB, SDHC, or VHL while the occurrence of HNPGL in SDHA, TMEM127, and MAX mutation carriers is, if any, extremely low.

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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