Tumoral EPAS1 (HIF2A) mutations explain sporadic pheochromocytoma and paraganglioma in the absence of erythrocytosis

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Pheochromocytomas (PCCs) and paragangliomas (PGLs) are chromaffin-cell tumors that arise from the adrenal medulla and extra-adrenal paraganglia, respectively. The dysfunction of genes involved in the cellular response to hypoxia, such as VHL, EGL nine homolog 1, and the succinate dehydrogenase (SDH) genes, leads to a direct abrogation of hypoxia inducible factor (HIF) degradation, resulting in a pseudo-hypoxic state implicated in PCC/PGL development. Recently, somatic post-zygotic mutations in EPAS1 (HIF2A) have been found in patients with multiple PGLs and congenital erythrocytosis. We assessed 41 PCCs/PGLs for mutations in EPAS1 and herein describe the clinical, molecular and genetic characteristics of the 7 patients found to carry somatic EPAS1 mutations; 4 presented with multiple PGLs (3 of them also had congenital erythrocytosis), whereas 3 were single sporadic PCC/PGL cases. Gene expression analysis of EPAS1-mutated tumors revealed similar mRNA EPAS1 levels to those found in SDH-gene- and VHL-mutated cases and a significant up-regulation of two hypoxia-induced genes (PCSK6 and GNA14). Interestingly, single nucleotide polymorphism array analysis revealed an exclusive gain of chromosome 2p in three EPAS1-mutated tumors. Furthermore, multiplex-PCR screening for small rearrangements detected a specific EPAS1 gain in another EPAS1-mutated tumor and in three non-EPAS1-mutated cases. The finding that EPAS1 is involved in the sporadic presentation of the disease not only increases the percentage of PCCs/PGLs with known driver mutations, but also highlights the relevance of studying other hypoxia-related genes in apparently sporadic tumors. Finally, the detection of a specific copy number alteration affecting chromosome 2p in EPAS1-mutated tumors may guide the genetic diagnosis of patients with this disease.

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INTRODUCTION

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare neural crest tumors that arise from the adrenal medulla and from the extra-adrenal sympathetic and parasympathetic paraganglia, respectively. The genetics of PCCs/PGLs is extremely puzzling, with up to 40% of patients carrying a germline mutation that affects 1 of 10 major susceptibility genes (1–10). Depending to a great extent on the gene mutated, the clinical phenotype of mutation types can vary dramatically (e.g. patients carrying germline mutations in VHL versus RET) as can risk of malignancy or age-related penetrance (e.g. patients carrying germline mutations in SDHB versus SDHD). Furthermore, some of the genes (i.e. SDHD, SDHAF2 and MAX) are affected by maternal imprinting or paternal transmission. In addition to this high and heterogeneous genetic predisposition, there is also a substantive percentage of hereditary cases, either with familial antecedents of PCC/PGL or with other clinical characteristics suggestive of hereditary disease, that do not harbor mutations in any of the susceptibility genes mentioned above. The sporadic presentation of the disease appears to also have a complex genetic etiology; NF1 that, of the known susceptibility genes, has one of the lowest associated incidences of PCC/PGL has recently been found to play a very important role in the sporadic presentation of the disease (11). For all these reasons, the genetic diagnosis, genetic counseling and clinical follow-up of patients with PCC/PGL syndromes are major challenges.

The most recent addition to the growing list of PCC/PGL-associated genes is endothelial PAS domain-containing protein 1 (EPAS1) (12). EPAS1, also known as HIF2A, encodes one of the members of the hypoxia inducible factor (HIF) family of transcriptional regulators involved in hypoxic response (13). Zhuang et al. (12) described somatic mutations in EPAS1 in two unrelated patients presenting with multiple PGLs. The mutations seemed to occur during early embryogenesis because both patients also had congenital erythrocytosis and one of them developed multiple somatostatinomas. Despite the fact that germline gain-of-function mutations of this gene are associated with increased erythropoietin and familial erythrocytosis (14–16), and unlike what happens with mutations in other polycythemia-related genes such as VHL and EGL nine homolog 1 (17), none of EPAS1 mutation carriers developed cancer. The discovery of somatic mutations affecting EPAS1 in PCC/PGL not only once again links polycythemia and cancer, but it also adds further weight to the hypothesis that the stabilization of HIF-alpha is important in chromaffin tumor development. This process, known as pseudo-hypoxia, has been described in PCC/PGL harboring mutations in VHL or in the succinate dehydrogenase (SDH) genes (18). Like EPAS1 mutations, pseudo-hypoxia prolongs the half-life of HIF-alpha and triggers the activation of the cellular adaptation to hypoxia in VHL- and SDH-related tumors (19), which seems to be of pivotal importance in the development of other neural crest tumors (20).

In the present study, we tested for EPAS1 mutations in three patients with multiple PCC/PGLs and erythrocytosis, as well as an additional series of 38 PCCs/PGLs, and described the clinical, molecular and genetic features of seven patients carrying somatic EPAS1 mutations. Of note, only three of these seven patients with EPAS1-mutated tumors presented with congenital erythrocytosis and three had a single sporadic PCC/PGL.

RESULTS

Somatic EPAS1 mutations are frequently found in PCC/PGL

We first genotyped exon 12 of EPAS1 in three tumors from three unrelated individuals who had developed multiple PCC/PGLs and congenital erythrocytosis. Three missense mutations, p.Ala530Thr, p.Pro531Ser and p.Pro531Leu, were found in three tumors (Table 1). To assess the involvement of EPAS1 mutations in PCC/PGL development, genotyping of exon 12 was also performed in 38 apparently sporadic tumors. Four non-truncating mutations were identified in four independent tumors: two were missense variants (p.Ala530Val and p.Asp539Tyr) and two were in-frame deletions (p.Ile533_Pro534del and p.Pro534_Asp536del) (Table 1). The p.Pro531Ser and p.Pro531Leu mutations affect the primary prolyl hydroxylation site, Pro-531, in EPAS1, whereas the remaining variants affect amino acids in proximity to this site. The two mutations involving Ala-530 had been previously reported (12); the other five were novel variants. None of the mutations were found in the corresponding patients’ germline DNA. The subsequent analysis of additional tumors developed by cases 1, 3 and 4 (Table 1) confirmed the presence of the same mutations (p.Ala530Thr, p.Pro531Ser and p.Pro531Leu, respectively), suggesting that the mutations occurred in a chromaffin precursor cell in these three cases. Moreover, the mutations found in tumors were neither present in extra-adrenal normal tissue (cases 2 and 3) nor in a non-neural-crest tumor obtained from case 2, supporting that a genetic mosaicism occurred in these patients. The analysis of normal adrenal tissue adjacent to the tumor obtained from one of the patients who developed a single tumor (case 7, Table 1) revealed the absence of the mutated allele, demonstrating that, at least in this case, the somatic mutation occurred only in the tumor.

The screening for mutations in exon 12 of the EPAS1 gene carried out in germline DNA from 186 patients without mutations in the known susceptibility genes revealed a missense variant (c.1700T>C; p.Met567Thr) in two apparently sporadic cases (Table 1). Alamut software predicts that the variant is not deleterious because it affects a moderately conserved residue, and the change to threonine is not physicochemically relevant. Furthermore, this variant was found in 1 of 4300 European individuals in the NHLBI Exome Sequencing Project and in 3 of 254 Spanish controls included in the present study. Based on these findings and the mild phenotype of the corresponding patients, we concluded that this variant is not disease causing.

Gain of 2p is an alteration exclusive to tumors harboring EPAS1 mutations

High-density single nucleotide polymorphism (SNP) genotyping performed in four EPAS1-mutated tumors revealed that three carried a gain of the short arm of chromosome 2 that
includes EPAS1 (Fig. 1A; Table 1). To investigate whether this somatic mechanism could be exclusive to tumors carrying EPAS1 mutations, we interrogated SNP array data from a further 94 PCC/PGLs with or without mutations in other PCC/PGL susceptibility genes (Data not shown), 34 of which had no somatic mutations in EPAS1 and found that none presented a gain of chromosome 2p \((P = 4.7 \times 10^{-3})\). We found by allele-specific PCR that the duplication of 2p had no somatic mutations in PCC/PGL susceptibility genes (Data not shown), 34 of which had no somatic mutations in EPAS1 and found that none presented a gain of chromosome 2p \((P = 4.7 \times 10^{-3})\). We found by allele-specific PCR that the duplication of 2p included EPAS1 and found that none presented a gain of chromosome 2p \((P = 4.7 \times 10^{-3})\). We found by allele-specific PCR that the duplication of 2p included EPAS1 and found that none presented a gain of chromosome 2p \((P = 4.7 \times 10^{-3})\).

**EPAS1-mutated tumors showed overexpression of several hypoxia-induced genes**

It has been reported that gain-of-function mutations in EPAS1 lead to the up-regulation of hypoxia-related genes (12). To assess whether the tumors carrying EPAS1 mutations exhibited a characteristic pseudo-hypoxic transcriptional profile, we used previously reported gene expression data from four of the seven EPAS1-mutated tumors (21). A previous hierarchical cluster analysis had grouped one of the EPAS1-mutated tumors in ‘cluster 1’, composed primarily of pseudo-hypoxic PCCs, whereas the remaining three cases were spread across the Ras/PI3K/mammalian target of rapamycin cluster (‘cluster 2’) (21). To decipher the particular hypoxic profile of EPAS1-mutated tumors, we carried out a supervised cluster analysis of the 4 altered tumors when compared with 22 sporadic tumors negative for mutations in EPAS1 included in the original expression profile study. This analysis revealed 12 probes (corresponding to 11 genes) significantly up-regulated in EPAS1 tumors (Fig. 1B) and 3 of these \((TMEM45A, PCSK6 and LMO4)\) were known hypoxia-induced genes (22–24). After excluding 6 tumors suspected to carry NF1 somatic mutations (because they exhibited known chromosome 17 losses), the up-regulation of 4 of the 11 genes remained significant: PCSK6, GNA14, AMZI and THCA2316750 (denoted in red in Fig. 1B). Furthermore, we found significantly higher levels of EPAS1 mRNA expression in the EPAS1-mutated tumors when compared with ‘cluster 2’ cases \((P = 0.002, \text{Supplementary Material, Fig. S2})\); no differences in expression were found when we compared EPAS1-mutated tumors with pseudo-hypoxic cases (‘cluster 1’, SDH and VHL). Finally, the sporadic tumor group, which included cases belonging to both PCC transcriptional clusters, showed an intermediate level of expression, suggesting that it includes both pseudo-hypoxic and non-pseudo-hypoxic cases. When we subclassified these cases depending on the transcriptional cluster they belonged to, we found significant differences with the EPAS1-mutated tumors within each subgroup, confirming that ‘cluster 1’ sporadic cases are even more pseudo-

**Table 1. Clinical presentation and genetic data for patients carrying variants in exon 12 of EPAS1**

<table>
<thead>
<tr>
<th>ID</th>
<th>Age at onset</th>
<th>Sex</th>
<th>Tumor</th>
<th>Secretion</th>
<th>Other tumors (age at onset)</th>
<th>Tumor variant</th>
<th>Protein</th>
<th>Germline variant</th>
<th>2p gaina</th>
<th>EPAS1 gainb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18, 22, 26</td>
<td>F</td>
<td>PCC, TA PGLs (6)</td>
<td>Yes (1 year)</td>
<td>EPO, NE, DA</td>
<td>No</td>
<td>c.1588G &gt; A</td>
<td>p.Ala530Thr</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>F</td>
<td>TA PGLs (3)</td>
<td>No</td>
<td>E, NE</td>
<td>Basal cell carcinoma (75 years)</td>
<td>c.1589C &gt; T</td>
<td>p.Ala530Val</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>13, 22</td>
<td>M</td>
<td>PCC, TA PGLs (3)</td>
<td>Yes (7 years)</td>
<td>EPO</td>
<td>Parotid adenoma (21 years)</td>
<td>c.1591C &gt; T</td>
<td>p.Pro531Ser</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>18, 20</td>
<td>F</td>
<td>PCC, TA PGLs (5)</td>
<td>Yes (1 year)</td>
<td>EPO</td>
<td>No</td>
<td>c.1592C &gt; T</td>
<td>p.Pro531Leu</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>F</td>
<td>PCC</td>
<td>No</td>
<td>n.a.</td>
<td>No</td>
<td>c.1599_1604del</td>
<td>p.He333Pro534del</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>F</td>
<td>PCC</td>
<td>No</td>
<td>E, NE</td>
<td>Uterine myoma (41 years)</td>
<td>c.1600_1608del</td>
<td>p.Pro534_Asp536del</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>F</td>
<td>TA PGL</td>
<td>No</td>
<td>n.a.</td>
<td>No</td>
<td>c.1615G &gt; T</td>
<td>p.Asp539Tyr</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>F</td>
<td>PCC</td>
<td>No</td>
<td>E, DA</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td>c.1700T &gt; C</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>F</td>
<td>PCC</td>
<td>No</td>
<td>NE, DA</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td>c.1700T &gt; C</td>
<td>–</td>
</tr>
</tbody>
</table>

F, female; M, male; PCC, pheochromocytoma; PGL, paraganglioma; TA, bilateral PCC; T, thoracic-abdominal; NE, norepinephrine; E, epinephrine, EPO, erythropoietin; DA, dopamine; n.a., not available; ND, not done due to poor DNA quality.

aGain detected by SNP-array analysis.
bGain detected by multiplex-PCR analysis.
hypoxic and that ‘cluster 2’ sporadic tumors exhibit a non-hypoxic expression profile similar to the mutated tumors from this cluster ($P = 0.038$ and $P = 0.029$, respectively).

**DISCUSSION**

The presence of known germline mutations in almost 40% of patients means that PCC/PGL is one of the human tumor entities with the highest explained heritability worldwide. In addition, the demonstrated relevance of somatic mutations affecting some of the susceptibility genes (especially NF1) in the sporadic forms of the disease has rapidly increased during recent years to 24–35% of cases (11,25). The discovery of somatic post-zygotic $EPAS1$ mutations in patients developing multiple PCC/PGLs represents a new twist in the genetics of the disease and means that some patients who apparently exhibit a familial phenotype are really non-hereditary. In the present study, we found somatic $EPAS1$ mutations not only in three additional patients with multiple PCC/PGLs, but also in three cases presenting with single tumors, the latter result revealing $EPAS1$ as a new gene involved in the sporadic presentation of the disease. Overall, after excluding the three selected cases with multiple tumors and erythrocytosis, mutations affecting the $EPAS1$ gene accounted for 10% of the consecutive tumors analyzed in this study which suggests that this somatic oncogenic event is one of the most relevant in the development of sporadic PCC/PGL, together with $NF1$, $VHL$ and $RET$ alterations.

The Pro-531 mutations constitute the first example of variants causing the direct abrogation of prolyl hydroxylation in $EPAS1$. In addition, as occurs with mutations found in patients with familial erythrocytosis, the remaining $EPAS1$ variants identified in the present study affect amino acids in proximity to the primary prolyl hydroxylation site (Fig. 2) and could, therefore, affect the conformation of the hydroxylation domain interfering with prolyl hydroxylase recognition, as

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**Figure 1.** (A) SNP-array analysis of chromosome 2 performed with tumor DNA from the patient carrying the p.Ile533, Pro534del mutation reveals a gain of the short arm. The lower panel shows the genomic plots of the log R ratio $\log_2(\text{R}_{\text{patient}}/\text{R}_{\text{reference}})$, indicating the presence of three alleles along the short arm. The upper panel gives the allele frequency parameters along chromosome 2, showing that the heterozygous state splits into two clusters. The chromosomal location of $EPAS1$ is also indicated. (B) Heat map showing the 11 genes (represented in the rows) differentially up-regulated (FDR < 0.15) in the supervised cluster analysis of $EPAS1$-mutated and $EPAS1$-non-mutated tumors (represented in the columns). Significantly up-regulated genes after ruling out tumors potentially NF1 mutated are highlighted in red. Color bar: green and red colors represent $\geq 2$-fold relative under- and over-expression, respectively.
demonstrated for the Ala-530 substitutions (12). The identification of two new cases harboring mutations in Ala-530, and another one involving Asp-539, a residue mutated in a patient with erythrocytosis (26), suggests that these two amino acids could be hotspots for both erythrocytosis and PCC/PGL. On the other hand, the absence of PCCs and PGLs in the reported cases of familial erythrocytosis carrying EPAS1 germline mutations suggests that, as occurs with mutations in the VHL gene, different mutations in EPAS1 predispose to different diseases. Nevertheless, we cannot rule out that additional genetic factors modulate the clinical phenotype of EPAS1 mutation carriers. In the case of the patient with multiple tumors found to carry one of the Ala-530 mutations (previously reported in a patient with erythrocytosis and PGLs) (12), the absence of erythrocytosis could be related to the time at which the mutation occurs. Studying a larger number of patients with erythrocytosis and/or PCC/PGL who carry EPAS1 mutations could help to solve this new phenotypic puzzle.

As recently reported for PCCs carrying somatic mutations in NF1, the detection of a specific copy number alteration may guide the genetic diagnosis of the disease. Indeed, it is nowadays accepted that negative immunostaining for SDHB, partially due to the somatic loss of chromosome 1p, almost guarantees the detection of a mutation in one of the SDH genes (27). The exclusive gain of chromosome 2p identified in three of the four EPAS1-mutated tumors analyzed, and the specific gain of the gene detected in another mutated case, could, therefore, be used to select candidate tumors to be analyzed for mutations in this particular gene, avoiding unnecessary expenses. Unexpectedly, we found that the chromosomal gain of 2p affected both the wild-type and the mutated alleles alike (Supplementary Material, Fig. S1A and B). There are three well-known genetic mechanisms to activate oncogenes in human neoplasm: activating mutations, gene amplifications and chromosomal rearrangements. Although it is widely accepted that a single altered copy of an oncogene is sufficient to cause alterations in cell growth, it has been reported that PCCs in patients carrying germine activating mutations in RET may harbor either somatic duplications of the mutated allele or loss of the wild-type RET allele (28). Although duplication of the mutated allele would lead to higher amount of stabilized EPAS1 protein, somatic gain of an extra copy of the wild-type allele can also activate the hypoxic response in the tumor. This is because, in the case of a monomeric protein such as EPAS1, a dominant negative effect is not required. Finally, the detection of the specific gain of the gene in three EPAS1-mutation-negative cases strongly suggests that somatic gain of EPAS1 could be a driving oncogenic event in some PCCs/PGLs.

A hierarchical cluster analysis suggested that EPAS1-mutated tumors do not exhibit the classical pseudo-hypoxic profile of VHL and SDH cases. Nevertheless, we found two hypoxia-related genes that were up-regulated in EPAS1-mutated cases when compared with sporadic tumors without EPAS1 mutations: PCSK6 and GNA14. Although the latter has not been reported as a hypoxia-related gene, it seems that it has an oxygen concentration-dependent expression and may play an important role in placental and fetal vascular endothelial functions, especially under chronic hypoxia (http://www.erp.wisc.edu/symposium/2012_abstracts.pdf). In addition, it has been reported that activation of the GNA14 protein can lead to STAT3 stimulation that plays a critical role in the development and function of normal hematopoietic cells (29). On the other hand, it is known that PCSK6 overexpression resulted in enhanced susceptibility to carcinogenesis and tumor progression (30), and it has been proposed as an invasion-associated gene in malignant gliomas (31). The low number of hypoxia-related genes significantly up-regulated in EPAS1-mutated cases suggested that some of the sporadic tumors could harbor alterations in other hypoxia-related genes. That we found high EPAS1 mRNA levels in ‘cluster 1’ sporadic tumors (Supplementary Material, Fig. S2) is consistent with this hypothesis. Interestingly, for two of these samples, the high mRNA expression of EPAS1 was associated with the gain of the specific gene. Further studies are needed to assess the involvement of still unknown pseudo-hypoxic alterations in the development of sporadic tumors. Whereas HIF1A is ubiquitously expressed, EPAS1 is encountered only in a limited number of cell types, with particularly elevated mRNA levels found in catecholamine-producing cells of the sympathetic nervous system (32). Interestingly, it has been proposed that the pattern of EPAS1 abundance may be responsible for the observed organ specificity of the VHL syndrome (33). Thus, considering that other organs affected in this syndrome, such as the retina, the kidney and the pancreas, also have high EPAS1 expression levels, it is plausible that EPAS1 driver mutations would be found in non-VHL heman-gioblastomas, clear cell renal cell carcinomas or pancreatic neuroendocrine tumors. Indeed, the EPAS1 locus has been recently identified as a new genomic region associated with renal cell carcinoma risk (34).

In summary, the finding of somatic post-zygotic mutations affecting EPAS1 in PCC/PGL patients suggests an alternative genetic explanation for the etiology of the disease in patients with multiple tumors, but not germine mutations. In addition, the discovery of the involvement of this gene in the sporadic presentation of the disease (i.e. one single tumor) not only increases the percentage of tumors with known driver mutations, but also highlights the relevance of studying other
hypoxia-related genes in apparently sporadic PCC/PGLs. Finally, the detection of a specific copy number alteration affecting EPAS1 may guide the genetic diagnosis of the disease in these patients.

MATERIALS AND METHODS

Tumors and patients

Three tumors, one frozen and two formalin-fixed paraffin-embedded (FFPE), were obtained from patients with multiple PGLs/PCCs and congenital erythrocytosis. In addition, frozen tumors were obtained through the Spanish National Tumor Bank Network in Madrid (Spain) for a total of 38 unrelated consecutive patients with PCC/PGL who were tested negative for germline and somatic mutations in the major susceptibility genes VHL, RET, SDHB, SDHC, SDHD, TMEM127 and MAX. Additional FFPE PCCs/PGLs were obtained from three of the four cases with somatic EPAS1 mutations who developed multiple tumors (a second tumor from cases 1 and 3 and the five additional tumors from case 4; Table 1); other tissue specimens were obtained from case 2 (bone and basal cell carcinoma), case 4 (lymphatic ganglia and intestine) and case 7 (normal adrenal medulla). A further 186 consecutive non-related index patients without germ-line mutations in VHL, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127 and MAX were also included in the study. All patients were clinically diagnosed in public Spanish hospitals with functioning or non-functioning PCC/PGL. Genomic DNA was obtained from tumor tissue using the DNAeasy (Qiagen Inc., Valencia, CA, USA) kit and from blood samples using a standard procedure (35). Clinical data were collected for all patients by means of detailed questionnaires, and written informed consent was obtained from each patient. We used DNA from 254 unrelated unaffected individuals as a control sample for the study of the variant of unknown significance detected in the germline genetic screening.

Mutational and molecular analyses

Analysis of point mutations affecting exon 12 of EPAS1 (ENST00000263734, NM_001430.4), which contains the primary hydroxylation site of the protein, was performed both in tumor and germline DNA, as well as in the non-paraganglionic tissue samples, using a standard PCR amplification protocol. Additional analysis of the 2 remaining EPAS1 hydroxylation sites (i.e. Pro-405 and Asn-847 located in exons 9 and 16, respectively), and exon 12 of HIF1A (ENST00000337138, NM_001530.3) and HIF3A (ENST00000377670, NM_152795.3), was performed in the 34 frozen tumors without mutations at the primary site. PCR conditions and primers are available on request. The Alamut® mutation interpretation software (http://www.interactivbiosoftware.com/software.html) was used to assess the pathogenicity of the germline variants identified.

SNP-array and multiplex-PCR analyses

To investigate the presence of chromosomal rearrangements in the tumors, we performed high-density SNP-array analysis in four EPAS1-mutated cases (three of the tumors were not analyzed due to poor DNA quality). A genome-wide scan of 616795 markers was conducted on 250 ng of tumor DNA, using the Illumina Human610-Quad BeadChip according to the manufacturer’s specifications. Image data were analyzed using the Chromosome Viewer tool contained in GenomeStudio 2010.2 (Illumina). The metric used was the log-R ratio that is the binary logarithm of the ratio of the observed to expected normalized R values for a given SNP (36). The allele frequency was also estimated for all SNPs. EPAS1 genomic gains were assessed in the 40 tumors by multiplex-PCR, including 2 pairs of primers for exons 12 and 14, as previously described for other genes (37). A similar semi-quantitative multiplex-PCR method was used to investigate which allele was duplicated in the three tumors exhibiting chromosome 2p gain in the array-SNP analysis. Briefly, we first designed and labeled (5’-FAM) two pairs of primers for each mutation that discriminated the wild-type allele from the mutated allele. Following that, three cocktails containing each specific pair of primers mixed with three control-labeled pairs of primers for chromosomes 5 and 16 (as internal controls) were prepared and used to amplify the corresponding tumor DNA by multiplex-PCR. The subsequent normalization was performed for each PCR product by overlapping each tumor sample with a control sample using the Peak Scanner™ software (Applied Biosystems, Foster City, CA, USA), as previously described for other genes (37).

Gene expression analysis

To identify specific transcripts related to EPAS1 deregulation, we used gene expression data for four of the 7 mutated tumors and for 22 sporadic cases negative for mutations affecting EPAS1 exon 12, as deposited in the National Center for Biotechnology Information GEO database under the accession number GSE19422 (21). A t-test was applied using POMELOII (http://pomelo2.bioinfo.cnio.es/) to compare the expression of individual genes between the two groups (38). Benjamini’s false discovery rate (FDR) correction was used to account for multiple testing (39); genes up-regulated in the mutated tumors with an FDR < 0.15 were selected as differentially expressed. In addition, we assessed the mRNA expression of EPAS1 in the whole series of PCC/PGLs (21), by the gene mutated. Considering that some of the sporadic cases included in the present study could have NF1-mutated tumors, we performed a second analysis excluding the six that exhibited chromosome 17 losses, based on the report that 84–90% of tumors with these losses harbor mutations in NF1 (11,25).

Statistical analysis

Difference in frequency of 2p gain between EPAS1-mutation-positive and -negative cases was assessed by Fisher’s exact test, using the SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
Conflict of Interest statement. A.A.d.C. and V.M. are predoctoral fellows of ‘la Caixa’/CNIO International PhD Programme. L.I.-P. and I.C.-M. are predoctoral fellows of the CIBERER and the Fundacion Ferrer, respectively.

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