

Frequent *EPAS1/HIF2 α* exons 9 and 12 mutations in non-familial pheochromocytoma

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Abstract

Pheochromocytomas are neuroendocrine tumors arising from the adrenal medulla. While heritable mutations are frequently described, less is known about the genetics of sporadic pheochromocytoma. Mutations in genes involved in the cellular hypoxia response have been identified in tumors, and recently *EPAS1*, encoding HIF2 α , has been revealed to be a new gene involved in the pathogenesis of pheochromocytoma and abdominal paraganglioma. The aim of this study was to further characterize *EPAS1* alterations in non-familial pheochromocytomas. Tumor DNA from 42 adrenal pheochromocytoma cases with apparently sporadic presentation, without known hereditary mutations in predisposing genes, were analyzed for mutations in *EPAS1* by sequencing of exons 9 and 12, which contain the two hydroxylation sites involved in HIF2 α degradation, and also exon 2. In addition, the copy number at the *EPAS1* locus as well as transcriptome-wide gene expression were studied by DNA and RNA microarray analyses, respectively. We identified six missense *EPAS1* mutations, three in exon 9 and three in exon 12, in five of 42 pheochromocytomas (12%). The mutations were both somatic and constitutional, and had no overlap in 11 cases (26%) with somatic mutations in *NF1* or *RET*. One sample had two different *EPAS1* mutations, shown by cloning to occur in *cis*, possibly indicating a novel mechanism of HIF2 α stabilization through inactivation of both hydroxylation sites. One of the tumors with an *EPAS1* mutation also had a gain in DNA copy number at the *EPAS1* locus. All *EPAS1*-mutated tumors displayed a pseudo-hypoxic gene expression pattern, indicating an oncogenic role of the identified mutations.

Key Words

- ▶ *EPAS1*
- ▶ HIF2A
- ▶ pheochromocytoma
- ▶ mutation
- ▶ copy number
- ▶ gene expression

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Introduction

Pheochromocytomas and abdominal paragangliomas are catecholamine-producing tumors derived from neuroendocrine chromaffin cells in the adrenal medulla or the extra-adrenal paraganglia. The tumors can be a manifestation of different hereditary tumor syndromes such as multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), neurofibromatosis type 1 (NF1) or familial pheochromocytoma–paraganglioma syndrome, which are caused by mutations in the genes *RET*, *VHL*, *NF1*, and *SDHx* respectively (Welander *et al.* 2011, Dahia 2014). In recent years, additional susceptibility genes for pheochromocytoma and paraganglioma have been discovered, including *TMEM127* (Qin *et al.* 2010), *MAX* (Comino-Mendez *et al.* 2011), and *FH* (Castro-Vega *et al.* 2014), and more rarely *EGLN1* (Ladroue *et al.* 2008) and *KIF1Bβ* (Schlisio *et al.* 2008). The majority of pheochromocytomas and paragangliomas are apparently sporadic, i.e. isolated and non-familial. Until recently, somatic mutations in many of the genes known to be associated with familial disease have been found only in a small proportion of the cases (Burnichon *et al.* 2011). This changed when somatic *NF1* mutations were revealed in about 20–25% of sporadic pheochromocytomas (Burnichon *et al.* 2012, Welander *et al.* 2012), but still the majority of sporadic tumors remain unexplained. Studies of gene expression have revealed that pheochromocytomas and paragangliomas can be divided into two groups: *VHL*- and *SDHx*-mutated tumors form one cluster (Cluster 1) with a hypoxia-related transcription signature, whereas *RET/NF1/TMEM127/MAX*-mutated tumors form another cluster (Cluster 2) and display transcription profiles characterized by the activation of kinase signaling pathways (Eisenhofer *et al.* 2004, Dahia *et al.* 2005, Dahia 2014). Sporadic tumors cluster into either of the two distinct groups, instead of forming clusters of their own, indicating that similar molecular mechanisms are involved.

Hypoxia-inducible factors (HIFs) are transcription factors involved in the cellular hypoxia response. Active HIF is a dimer composed of an α and a β subunits. Whereas the β subunit is stably expressed, the levels of the α subunits HIF1 α , HIF2 α , and HIF3 α are regulated by oxygen-dependent prolyl hydroxylases (Kaelin & Ratcliffe 2008, Keith *et al.* 2012). Prolyl hydroxylation occurs at two specific proline residues in the oxygen-dependent degradation domain of HIF α . This allows recognition and ubiquitination of the protein by the VHL tumor suppressor E3 ubiquitin ligase complex, thereby targeting

it for proteasomal degradation. At low oxygen levels, the prolyl hydroxylation is reduced, resulting in stabilization of HIF α and subsequent transcription of target genes involved in the hypoxia response, including metabolic changes and angiogenesis.

Mutations in *VHL*, *EGLN1* (encoding the prolyl hydroxylase PHD2), and *SDHx* (encoding the subunits of succinate dehydrogenase) result in an accumulation of HIF α and thus a pseudo-hypoxic response, allowing activation of target genes proposed to contribute to chromaffin cell tumorigenesis (Maher 2013). Rarely, patients with polycythemia, a disease state characterized by an elevated concentration of red blood cells, have mutations in *VHL*, *EGLN1* or in *EPAS1*, the gene encoding HIF2 α (Bento *et al.* 2014). Recently, somatic gain-of-function mutations in *EPAS1* have been reported for the first time, to our knowledge, in paragangliomas associated with polycythemia (Zhuang *et al.* 2012), providing an additional link between polycythemia and neuroendocrine tumors and also supporting the hypothesis of pseudo-hypoxia as an important process in their development. A subsequent study revealed a germline *EPAS1* mutation in a patient with polycythemia and paraganglioma (Lorenzo *et al.* 2013) which, just like the first mutations identified, was demonstrated to decrease ubiquitination and degradation, thereby increasing the stability of HIF2 α . Subsequent studies identified somatic *EPAS1* mutations in pheochromocytomas and paragangliomas also in the absence of polycythemia (Favier *et al.* 2012, Comino-Mendez *et al.* 2013, Toledo *et al.* 2013), and demonstrated that *EPAS1* mutations promote tumor growth in mice (Toledo *et al.* 2013). So far, only a few cases of *EPAS1* mutations have been reported in single sporadic pheochromocytomas/paragangliomas without polycythemia. Herein, we further investigated this new class of mutations by analyzing *EPAS1* in a cohort of adrenal pheochromocytomas from patients with isolated, non-familial disease without polycythemia. In contrast to several previous studies focusing only on exon 12, we sequenced both exons that cover the HIF2 α hydroxylation sites, as an instance of a gain-of-function mutation in exon 9 has been described (Lorenzo *et al.* 2013). As one mutation of unknown pathogenicity has been described in exon 2, adjacent to the DNA-binding domain (Toledo *et al.* 2013), this exon was also included. The findings were evaluated in view of copy number and gene expression results, clinical presentation and previously detected somatic mutations in these tumors.

Subjects and methods

Patients and biological samples

The study included 42 primary adrenal pheochromocytomas from 42 patients presenting as sporadic without family history or syndromic features of the disease. None of the patients had polycythemia. Clinical data for the cohort have previously been published (Welander *et al.* 2012) and are presented in detail in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. Two patients had developed metastases during follow-up and were therefore classified as malignant according to the WHO criteria (Thompson *et al.* 2004), while 40 cases were without metastases. In addition, all cases were classified according to Armed Forces Institute of Pathology (AFIP) criteria (Lack 2007) ([Supplementary Table S1](#)). All samples were collected and studied with informed consent and approval from the local ethics committees. DNA was extracted from tissue and blood samples using the Maxwell 16 Tissue DNA Purification Kit (Promega) and the Maxwell 16 Blood DNA Purification Kit (Promega) respectively. RNA was extracted using the RNeasy Minikit (Qiagen), and cDNA synthesis was carried out with the Maxima First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

The tumors had previously been investigated for mutations in *NF1*, *RET*, *VHL*, *SDHB*, *SDHD*, and *MAX* genes, which revealed somatic *NF1* mutations in ten samples and a somatic *RET* mutation in one sample (Welander *et al.* 2012). Two patients had non-synonymous single-nucleotide polymorphisms in *SDHB* (rs11203289) and *SDHD* (rs11214077) respectively. Subsequent mutation analysis of *TMEM127* in all cases revealed a novel, constitutional missense sequence variant in one sample (c.10C>T, [Supplementary Figure S1](#), see section on [supplementary data](#) given at the end of this article), which was predicted to be benign by the PolyPhen-2 algorithm (Adzhubei *et al.* 2010).

Mutation screening and DNA copy number analysis of *EPAS1*

Exons 2, 9, and 12 of *EPAS1* were analyzed by direct Sanger sequencing of tumor DNA using previously described methodology (Welander *et al.* 2012) and primers as specified in [Supplementary Table S2](#), see section on [supplementary data](#) given at the end of this article. Mutations were confirmed by sequencing of a second, independent PCR product from the same sample. When available, mutation

analysis was also carried out on corresponding normal DNA from patients with mutations in the tumor DNA. In case 38, which had two different mutations, PCR amplification and cloning of *EPAS1* cDNA into *Escherichia coli* were carried out to determine if the mutations occurred in *cis* or *trans*. A previously described cloning technique (Welander *et al.* 2012) was used, with the primers 5'-CGTCCTGAGTGAGATTGAGAAG-3' (in the exon 8–9 boundary) and 5'-CCAGTGGCTGGAAGATGTTT-3' (in exon 12). *EPAS1* cDNA was then PCR amplified from each clone and sequenced.

The effects of missense mutations were predicted using PolyPhen-2 (Adzhubei *et al.* 2010). To study conservation of the affected residues, a multiple sequence alignment of the HIF2 α amino acid sequence between different species and a comparison with the human HIF1 α protein was carried out using the Clustal Omega algorithm (Sievers *et al.* 2011). The sequences used were HIF2 α (*EPAS1*) from *Homo sapiens* (UniProt accession number Q99814), *Mus musculus* (P97481), *Bos taurus* (A5PJT1), *Gallus gallus* (Q9W7C6), and *Danio rerio* (B3DJ1), together with HIF1 α from *H. sapiens* (Q16665) for comparison.

Previously retrieved data from SNP microarray analysis (Affymetrix GeneChip Human Mapping 250K) of tumor DNA (Welander *et al.* 2012) was analyzed for copy number alterations in *EPAS1* using Genotyping Console v. 4.0 (Affymetrix, Santa Clara, CA, USA).

Gene expression analysis

Total RNA was quantified with a NanoDrop-1000 spectrophotometer (Thermo Scientific) and 250 ng were used for whole-transcriptome analysis with GeneChip Human Gene 1.0 ST arrays (Affymetrix), covering 28 869 annotated genes with a median of 26 probes per gene. Two of the 42 sporadic tumor samples (number seven and 20) for which no RNA was available were excluded. Five hereditary tumors were used as controls ([Supplementary Table S3](#), see section on [supplementary data](#) given at the end of this article): two with *RET* mutations, one with a *NF1* mutation (Welander *et al.* 2012), one with a *VHL* mutation, and one with a *SDHA* mutation (Welander *et al.* 2013). Included samples had RNA integrity numbers (RIN) between 6.6 and 9.1 (mean 8.3) when analyzed using a 2100 Bioanalyzer system using the Eukaryote Total RNA Nano assay (Agilent Technologies, Santa Clara, CA, USA). Sense-strand cDNA was generated using the Ambion WT Expression Kit (Life Technologies) and then fragmented, labeled and hybridized to arrays in a GeneChip

Hybridization Oven 640 using the GeneChip WT Terminal Labeling Kit (Affymetrix), all according to the protocols supplied by the manufacturers. The arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 3000 7G (Affymetrix).

Microarray data files (CEL) were opened in Expression Console v. 1.3 (Affymetrix) and normalized using the robust multi-array average (RMA) algorithm. Normalized signal intensities for *EPAS1*, its target genes *VEGFA*, *CCND1*, *EDN1*, *EPO*, and *SLC2A1* (Jochmanova et al. 2013), and the gene for phenylethanolamine N-methyltransferase (*PNMT*) were exported for separate statistical analysis as described below. The CEL files were also imported into GeneSpring GX v. 12.6 (Agilent Technologies) for transcriptome-wide statistical analysis and clustering. After RMA normalization, a quality filter was applied to remove probesets for which none of the 45 samples had signal intensity values greater than the 20th percentile of all signal intensity values of the sample. To test how the transcription profiles of *EPAS1*-mutated samples would cluster compared with genotypes with previously known expression patterns, a set of genes previously shown to separate the *SDHx*, *VHL*, and *RET/NF1* groups was downloaded (Burnichon et al. 2011). These genes overlapped with 454 probesets that had passed the quality filter in our analysis, which was used to perform a hierarchical clustering with a Euclidean distance metric and Ward's linkage rule.

Statistical analysis

Gene expression levels of the seven candidate genes described above were compared between *EPAS1*-mutated and *EPAS1*-WT sporadic tumors using two-tailed Student's *t*-tests on the exported log₂ signal values. After back-transformation ($2^{(\log_2 \text{ signal})}$), expression levels were normalized to the mean of the three hereditary Cluster 2 controls (samples eight, 12, and 14) for visualization in GraphPad Prism v.6.03 (GraphPad Software, La Jolla, CA, USA). Transcriptome-wide statistical analysis was carried out on all genes passing the quality filter in GeneSpring, in which genes with differential expression between *EPAS1*-mutated and *EPAS1*-WT sporadic tumors were identified by *t*-tests. The Benjamini–Hochberg method (Benjamini & Hochberg 1995) was used to control for multiple testing. Genes were regarded as differentially expressed when they had a false discovery rate (FDR) <0.05 and an absolute fold change >2.

The clinical variables such as age, tumor size, and hormone levels were compared between cases with and

without *EPAS1* mutations using two-tailed Student's *t*-tests. Fisher's exact test was used to test for association with malignancy and gender. *P* values <0.05 were regarded as significant.

Results

Mutations in *EPAS1*

Among 42 non-familial pheochromocytomas from 42 unrelated patients, five tumors harbored six missense mutations in *EPAS1* (Table 1 and Fig. 1A), of which three were found in exon 9 and three in exon 12. No mutations were detected in exon 2. Two of the mutations were present in blood or normal tissue, whereas two were confirmed to be somatic, and two are unknown because neither blood nor non-tumor tissue was available (Fig. 1B). Two of the *EPAS1* mutations occurred in the same patient, of which one was present in DNA from blood whereas the other was somatic. Molecular cloning showed that the two mutations occurred in *cis* (Supplementary Figure S2, see section on supplementary data given at the end of this article). All but one of the identified mutations occurred at highly conserved residues, and the three mutations in exon 12 occur in or in the vicinity of the primary hydroxylation site (Fig. 1C). PolyPhen analysis predicted four of the six amino acid substitutions to be damaging (Table 1).

Based on SNP microarray data, case 11, which also carried a mutation, had a gain (three copies) of the 2p21 chromosomal region where *EPAS1* is located (Supplementary Figure S3, see section on supplementary data given at the end of this article). The other 41 samples showed no copy number gain, but two samples without mutations (cases 2 and 34) had heterozygous loss of 2p, including *EPAS1*. No copy number alterations in the *EPAS1* locus were found in 60 blood DNA samples from healthy controls analyzed by SNP microarrays. Previously known intronic or synonymous polymorphisms in *EPAS1* (rs7557402, rs116510029, rs35606117, rs184760160, and rs41281469) were detected at the expected frequencies according to information in the Ensembl database.

Gene expression patterns in *EPAS1*-mutated pheochromocytomas

Tumors with *EPAS1* mutations had a significantly higher gene expression of both *EPAS1* (*P*=0.0031) and its target gene *VEGFA* (*P*=0.0039) than tumors without mutations (Fig. 2A). In addition, the *EPAS1*-mutated tumors had a very low expression of *PNMT* (*P*<0.0001). Notably,

Table 1 Details for apparently sporadic pheochromocytomas with mutation(s) in *EPAS1/HIF2A*

Case number	Gender	Age	Malignancy, size	Exon	Mutation ^a	Present in normal DNA ^b	PolyPhen prediction (score)	<i>EPAS1</i> gain	Biochemical phenotype NE/EPI/DA
11	F	54	Benign, 25 mm	12	c.1592C>T ^c , p.Pro531Leu	Unknown	Damaging (1.000)	Yes	I/N/N
19	F	56	Benign, 50 mm	9	c.1121T>A ^d , p.Phe374Tyr	Yes	Benign (0.074)	No	I/I/N
22	F	61	Benign ^e , 120 mm	9	c.1104G>A, p.Met368Ile	Unknown	Benign (0.022)	No	I/N/N
38	M	47	Benign, 30 mm	9	c.1234T>A, p.Ile412Asn	Yes	Damaging (0.999)	No	I/I/–
				12	c.1595A>G ^f , p.Tyr532Cys	No	Damaging (1.000)	No	
52	F	43	Benign, 37 mm	12	c.1589C>A, p.Ala530Glu	No	Damaging (1.000)	No	I/N/–

F, female; M, male; EPI, epinephrine levels in plasma or urine before surgery; NE, norepinephrine levels in plasma or urine before surgery; DA, dopamine levels in urine before surgery; N, normal; I, increased; –, no value (further details can be found in Supplementary Table S1).

^aMutation nomenclature is based on the human *EPAS1* sequence ENST00000263734 in the Ensembl database.

^bNon-tumor DNA was extracted from blood (samples 19 and 38) or adrenal cortex (sample 52).

^cPreviously reported in pheochromocytoma/paraganglioma (Comino-Mendez et al. 2013, Toledo et al. 2013).

^dPreviously reported in pheochromocytoma/paraganglioma (Lorenzo et al. 2013).

^eMalignant according to AFIP criteria (Lack 2007), local invasion.

^fPreviously reported in pheochromocytoma/paraganglioma (Yang et al. 2013).

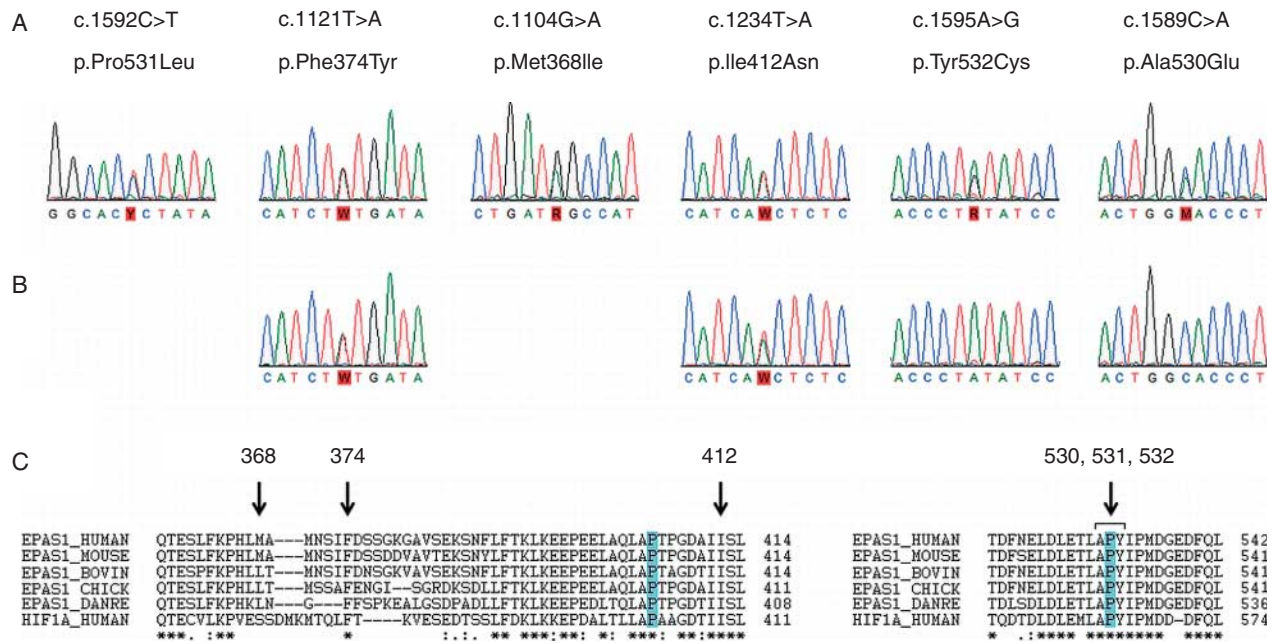
the target genes *CCND1*, *EDN1*, *EPO*, and *SLC2A1* (Jochmanova et al. 2013), which have been frequently investigated by others (Zhuang et al. 2012, Lorenzo et al. 2013, Toledo et al. 2013), did not show any difference in expression in this study (Supplementary Figure S4, see section on supplementary data given at the end of this article). Possible explanations may include differences in detection methods or sampling, and/or that these genes, though clearly regulated by HIF2 α in some tissues, may have a more variable expression in the adrenal medulla. Six tumors without identified mutations but with high *EPAS1* expression were sequenced for all the remaining exons of *EPAS1* (Supplementary Table S2). No mutations were detected, but one case (case 6) had a missense polymorphism in exon 15 predicted to be benign (rs59901247, PolyPhen score 0.001, allele frequency 0.15 in Ensembl).

One of the recent large gene expression studies of pheochromocytomas and paragangliomas identified a set of genes that could separate tumors with *SDHx*, *VHL*, and *RET/NF1* mutations by hierarchical gene expression clustering (Burnichon et al. 2011). To investigate how the gene expression profiles of *EPAS1*-mutated tumors would cluster compared with the previously known genotypes, we utilized the same set of genes (454 probesets) for hierarchical clustering of the present cohort. As expected, tumors with known somatic *NF1* and *RET* mutations clustered together with hereditary *NF1* and *MEN2A* cases (Fig. 2B). In contrast, all five tumors with *EPAS1* mutations clustered together with the hereditary

VHL and *SDH* cases, in agreement with the hypothesis of a pseudo-hypoxic behavior. The sporadic tumors without identified mutations were distributed between the two main clusters. After transcriptome-wide comparison of sporadic tumors with and without *EPAS1* mutations, 22 genes were significantly and more than twofold differentially expressed between the groups (Supplementary Table S4, see section on supplementary data given at the end of this article) after correction for multiple testing. Among those upregulated in *EPAS1* cases were several genes involved in cell metabolism (*COX4I2*, *NOX4*, *FOLH1*, *FOLH1B*, *COX17*) and angiogenesis (*KDR* (*VEGFR*), *ANGPT2*). The most significantly upregulated gene, *COX4I2*, has previously been reported to be strongly upregulated in *VHL*/*SDH*-related pheochromocytomas (Favier et al. 2009) and another one, *GNA14*, has been recently observed by others to be significantly upregulated in *EPAS1*-mutated tumors (Comino-Mendez et al. 2013). Among the most downregulated genes are *NCAM2* (a neural cell adhesion molecule, previously found to be upregulated in Cluster 2 tumors (Burnichon et al. 2011)), *HCN1* (a cation channel contributing to neuron currents), and *PNMT*.

Clinical presentation of *EPAS1*-mutated pheochromocytomas

All five patients with *EPAS1* mutation(s) had a single pheochromocytoma without recurrence or metastasis,

**Figure 1**

Mutations in *EPAS1* identified in adrenal pheochromocytomas with sporadic presentation. (A) Mutations identified in tumor DNA. (B) Sequences from corresponding normal DNA (when available), showing that two of the mutations are constitutional. (C) Multiple sequence alignment of HIF2 α

(*EPAS1*) from different species together with the human HIF1 α amino acid sequence. Prolyl hydroxylation occurs at Pro405 and Pro531 in human HIF2 α , indicated by the blue background. (*), identical residues; (:), conserved substitutions; (;), semi-conserved substitutions.

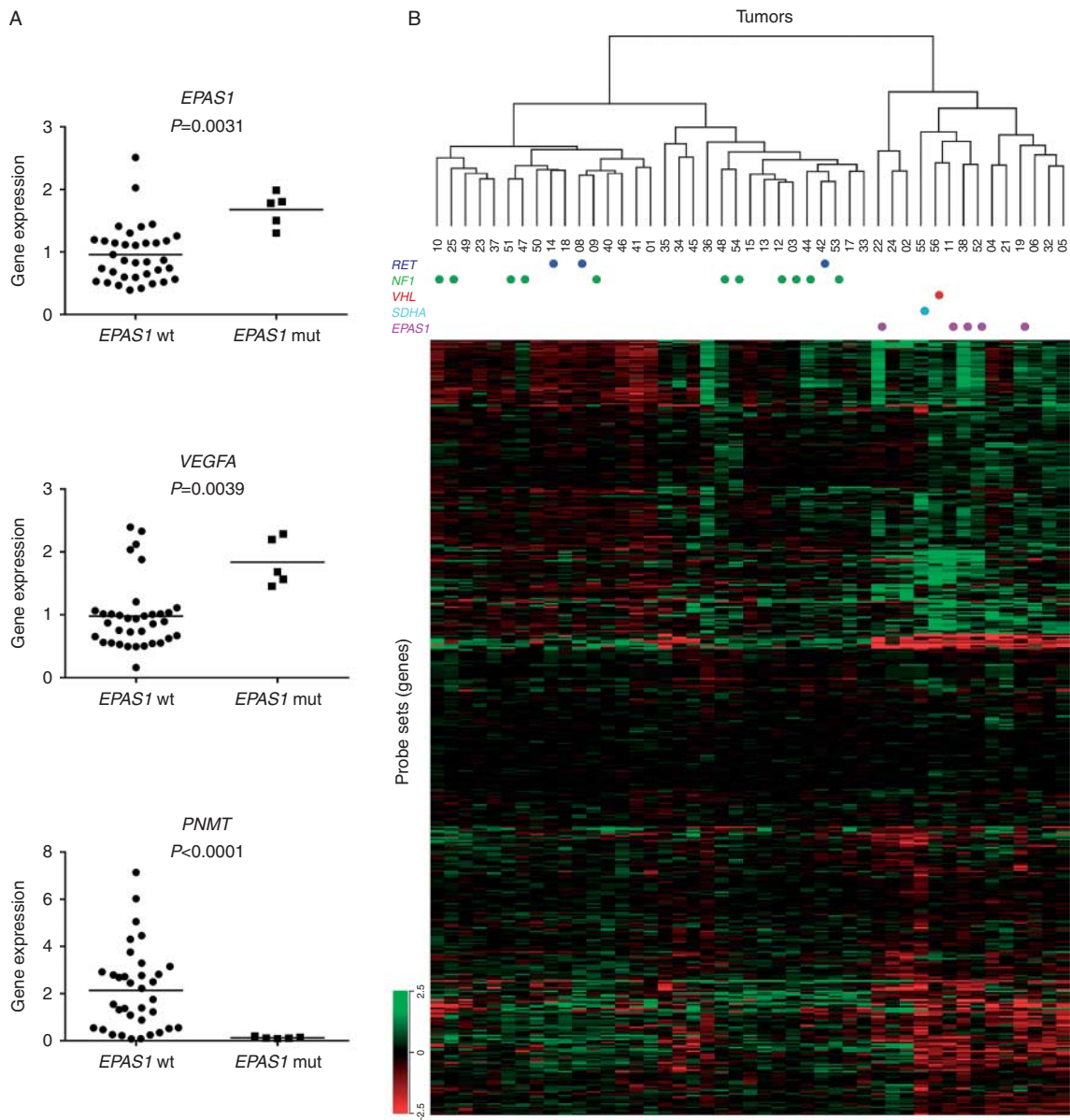
and none suffered from polycythemia. Re-evaluation of medical records showed that none of the patients had signs of hereditary tumor disease, but one presented with a ductal breast cancer *in situ* 4 years after the pheochromocytoma diagnosis (patient 22) and one suffered from sickle cell anemia (patient 52). Histopathologically, it can be noted that the tumor from case 19 showed slightly increased proliferation with 5% Ki-67-positive cells, and case 22 presented local invasion in agreement with histopathological malignancy according to AFIP criteria (Lack 2007). Patients with *EPAS1* mutations tended to be younger than those without *EPAS1* mutations (mean 52.2 ± 7.2 vs 61.6 ± 12.9 years), but the difference was not statistically significant ($P=0.12$). No associations were found between *EPAS1* mutation status and tumor size, malignancy, or gender, but it can be noted that 80% of the patients with *EPAS1* mutations were females, in agreement with the female dominance in the cases reported so far (Comino-Mendez *et al.* 2013, Pacak *et al.* 2013, Toledo *et al.* 2013). However, in another cohort where all susceptibility genes were investigated, *EPAS1* mutations were detected in four males and one female (Welander *et al.* 2014). Data on hormone profiles were incomplete and did not allow any statistically significant conclusions. It can be noted that all five patients with

EPAS1 mutations were observed to display increased norepinephrine levels (Table 1 and Supplementary Table S1). In addition, these patients had normal or only slightly increased epinephrine levels (Table 1 and Supplementary Table S1), which is consistent with their low expression of *PNMT* (Fig. 2A).

None of the cases with *EPAS1* mutation(s) had mutations in any of the tested susceptibility genes *NF1*, *RET*, *VHL*, *SDHB*, *SDHD*, or *MAX*, i.e. they were mutually exclusive with the somatic mutations identified in *NF1* (24%) and *RET* (2.4%) in the cohort (Supplementary Table S1 and Figure S5, see section on supplementary data given at the end of this article). One case (number 19) had a constitutional, probably benign sequence variant in *TMEM127* (Supplementary Figure S1, see section on supplementary data given at the end of this article).

Discussion

Mutation analysis of *EPAS1* in 42 non-familial pheochromocytomas resulted in the identification of six different mutations, of which three have not been reported previously in pheochromocytoma or paraganglioma. The three mutations identified in exon 12 occur in or in close proximity to the primary hydroxylation site. Thus, they

**Figure 2**

Gene expression in pheochromocytomas in relation to *EPAS1* mutation status. (A) RNA levels of the genes *EPAS1*, *VEGFA*, and *PNMT* compared between sporadic pheochromocytomas with (*EPAS1* mut, $n=5$) and without (*EPAS1* wt, $n=35$) *EPAS1* mutations. The gene expression has been normalized to the mean value of cases with hereditary *NF1* and *MEN2A*

defects. Horizontal bars represent mean values. (B) Hierarchical clustering of tumors based on RNA expression levels of 454 genes (implicated in pheochromocytoma subgroups (Burnichon et al. 2011)). High and low levels of expression are indicated by green and red colors respectively. Cases with mutations are highlighted below the dendrogram.

are likely to affect the conformation of the hydroxylation domain and disrupt prolyl hydroxylation and degradation of *HIF2 α* , as has previously been functionally demonstrated for this type of mutation (Zhuang et al. 2012, Toledo et al. 2013, Yang et al. 2013). The Phe374Tyr mutation in exon 9 was predicted to be non-pathogenic by PolyPhen and has been reported as a very rare polymorphism (rs150797491 with allele frequency

0.00092 in Ensembl), but, nevertheless, it has been demonstrated to be a gain-of-function mutation that decreases VHL protein binding and ubiquitination, increasing the stability of *HIF2 α* (Lorenzo et al. 2013). In both this and the previous study, the mutation was present in the constitutional DNA of a pheochromocytoma/paraganglioma patient. Met368Ile has also been reported as a rare polymorphism (rs61757375 with allele

frequency 0.0014). This could be in agreement with the proposition that *EPAS1* mutations are pheochromocytoma/paraganglioma-promoting, but not sufficient for tumor development (Lorenzo *et al.* 2013), but further studies will be required to determine its pathogenicity. The novel Ile412Asn mutation occurs in a highly conserved residue close to the hydroxylation site and is bioinformatically predicted to be damaging.

Interestingly, two heterozygous *EPAS1* mutations were identified in *cis* in the same tumor. It seems probable that the constitutional mutation, affecting an amino acid seven residues from the Pro405 hydroxylation site, is less severe than the somatic mutation close to the primary hydroxylation site Pro531 (Fig. 1C). Speculatively, the combination of the two may lead to an even higher stability of the HIF2 α protein than one mutation would cause alone, as previous results have indicated for the HIF1 α protein (Chan *et al.* 2005). One patient with an *EPAS1* mutation also had copy number gain of the *EPAS1* locus. The combination of activating mutations and copy number gain has previously been reported for *EPAS1* (Comino-Mendez *et al.* 2013), as well as for other oncogenes (Modrek *et al.* 2009).

Our findings are in agreement with previous reports (Comino-Mendez *et al.* 2013, Toledo *et al.* 2013), and merging the present study with the two previous ones implies a total frequency of *EPAS1* mutations of around 5.3% in sporadic tumors. In this cohort, the frequency of *EPAS1* mutations was 11.9% or, more conservatively, 7.1% when not considering the two variants not immediately in the hydroxylation sites for which no functional studies have been performed. *EPAS1* mutations were mutually exclusive with somatic mutations in other susceptibility genes in our cohort of pheochromocytomas, supporting an oncogenic role.

All tumors with *EPAS1* mutations showed increased expression of the HIF2 α target gene *VEGFA*, which has also previously been shown to be upregulated in pheochromocytomas and paragangliomas with *EPAS1* mutations (Zhuang *et al.* 2012, Lorenzo *et al.* 2013, Toledo *et al.* 2013). Notably, the tumors with mutations also had a higher expression of *EPAS1* itself. The reason for this is not clear, as the mutations are thought to act at the protein level, but the same result has been observed previously (Comino-Mendez *et al.* 2013). We also show that the tumors with *EPAS1* mutations have a very low expression of *PNMT*, encoding the enzyme PNMT which catalyzes the methylation of norepinephrine to form epinephrine. This is in agreement with the loss of *PNMT* expression in other Cluster 1 tumors as compared with Cluster 2 tumors, which

have a high expression, resulting in high epinephrine levels (Eisenhofer *et al.* 2004). Furthermore, the five tumors with *EPAS1* mutations clustered together with hereditary VHL and SDH cases, supporting the hypothesis that the mutations have a role in the disease by inducing a similar pseudo-hypoxic response. Interestingly, some sporadic tumors without any known mutations also clustered with the VHL/SDH/*EPAS1* cases, some of which also had an increased expression of *EPAS1*. Those with high *EPAS1* expression had the sequences of all *EPAS1* exons determined, but no additional mutations were identified. This indicates that other, so far unknown, alterations of hypoxia response mechanisms may be involved in a subset of the sporadic tumors.

EPAS1 mutations have now been identified in association with a variety of phenotypes, including polycythemia without tumors (Percy *et al.* 2008), polycythemia in combination with single or multiple pheochromocytomas/paragangliomas, and sometimes multiple somatostatinomas (Zhuang *et al.* 2012, Pacak *et al.* 2013, Taieb *et al.* 2013, Yang *et al.* 2013), as well as single or multiple pheochromocytomas/paragangliomas without polycythemia (Comino-Mendez *et al.* 2013, Toledo *et al.* 2013). Previous findings of *EPAS1* mutations in different tumors from the same patient, but not in germline DNA, indicate that somatic *EPAS1* mutations may occur in a cell during embryogenesis and predispose the affected tissues to tumor formation (Zhuang *et al.* 2012). However, it has been demonstrated that at least one reported case harbored a somatic mutation only in the tumor and not in adjacent normal tissue (Comino-Mendez *et al.* 2013). Possibly the variable phenotypes, constituted by different combinations of polycythemia and/or single or multiple tumors, may be directed by the specific time point of a somatic *EPAS1* mutation, e.g. during early embryogenesis or later in life. Plausibly, the type of mutation as well as additional genetic, epigenetic, or environmental factors is also part of the explanation. In addition to somatic mutations, one case with an inherited mutation, identical to one of the constitutional mutations reported in this study, has been described previously (Lorenzo *et al.* 2013), but in contrast to the study by Lorenzo and colleagues, the patient reported here did not suffer from polycythemia. The penetrance of pheochromocytoma/paraganglioma for constitutional mutations is still unknown and will require further family studies, but the lack of family history of disease in the cases reported here supports an incomplete penetrance. It is also not possible to exclude the possibility that some of the variants do not play a pathogenic role in the clinical phenotype.

In conclusion, we show that somatic as well as germline *EPAS1* mutations are of importance in pheochromocytoma development. Our results indicate that mutations in both exon 9 and exon 12 of *EPAS1* are frequent in isolated, non-familial pheochromocytomas, and that they are associated with a pseudo-hypoxia-related gene expression pattern.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0384>.

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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