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ORIGINAL ARTICLE

Germ-line *PHD1* and *PHD2* mutations detected in patients with pheochromocytoma/paraganglioma-polycythemia

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Abstract
We have investigated genetic/pathogenetic factors associated with a new clinical entity in patients presenting with pheochromocytoma/paraganglioma (PHEO/PGL) and polycythemia. Two patients without hypoxia-inducible factor 2α (*HIF2A*) mutations, who presented with similar clinical manifestations, were analyzed for other gene mutations, including prolyl hydroxylase (*PHD*) mutations. We have found for the first time a germ-line mutation in *PHD1* in one patient and a novel germ-line *PHD2* mutation in a second patient. Both mutants exhibited reduced protein stability with substantial quantitative protein loss and thus compromised catalytic activities. Due to the unique association of patients' polycythemia with borderline or mildly elevated erythropoietin (EPO) levels, we also performed an in vitro sensitivity assay of erythroid progenitors to EPO and for EPO receptor (EPOR) expression. The results show inappropriate hypersensitivity of erythroid progenitors to EPO in these patients, indicating increased EPOR expression/activity. In addition, the present study indicates that HIF dysregulation due to *PHD* mutations plays an important role in the pathogenesis of these tumors and associated polycythemia. The *PHD1* mutation appears to be a new member contributing to the genetic landscape of this novel clinical entity. Our results support the existence of a specific *PHD1*- and *PHD2*-associated PHEO/PGL-polycythemia disorder.

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Key message

- A novel germ-line *PHD1* mutation causing pheochromocytoma/paraganglioma and polycythemia.
- Increased EPOR activity and inappropriate hypersensitivity of erythroid progenitors to EPO.

Keywords Paraganglioma · Pheochromocytoma · Polycythemia · Erythropoietin

Introduction

Hypoxia-inducible factors (HIFs) function as central players in the cell response to hypoxia [1]. HIF-α protein degradation is initiated through its hydroxylation by prolyl hydroxylase domain proteins (PHDs), of which there are three main isoforms: PHD1, PHD2, and PHD3 (encoded by *EGLN2*, *EGLN1*, and *EGLN3*, respectively) [2, 3]. In normoxia, PHDs use O_2 and α-ketoglutarate to hydroxylate HIF prolyl residues that are subsequently recognized by the von Hippel-Lindau (VHL) protein, a component of the E3 ubiquitin ligase complex, which modifies HIFs for their degradation in proteasomes [4]. Abnormal regulation of the HIF pathway (e.g., during hypoxia) is tightly linked to the induction of many downstream genes and promotes cell transformation and tumorigenesis [1, 4].

Genes regulated specifically by HIF-1α or HIF-2α have been reported; one well-studied example is erythropoietin (EPO), which was recently discovered to be mainly regulated by HIF-2α [5]. Elevated EPO levels cause secondary polycythemia, a disease where the absolute red cell mass is increased [6]. Secondary polycythemia is caused by genetic defects in the oxygen-sensing pathway, impaired oxygen delivery, local tissue hypoxia, or it can be tumor- and drug-induced [7]. Mutations in *PHD2*, *VHL*, and *HIF2A* have been implicated in the pathogenesis of polycythemia in humans [8–10]. This is in contrast to primary polycythemia where intrinsic abnormalities of hematopoeietic progenitors or their EPO receptors (EPORs) result in the constitutive overproduction of red cell mass without EPO elevations [11, 12]. Among genetically induced polycythemias, *PHD*-related polycythemias typically present with normal or slightly increased EPO levels [13].

Paragangliomas (PGLs) are neural crest tumors derived either from the adrenal medulla (called pheochromocytomas (PHEOs)) or extra-adrenal chromaffin cells [14]. These tumors produce catecholamines, often with serious consequences, including hypertension, stroke, or arrhythmia. Ladroue et al. first described a germ-line *PHD2* mutation in a patient presenting with recurrent abdominal PGLs and polycythemia [15]. *VHL* and somatic *HIF2A* mutations were later reported in patients with an early onset of polycythemia induced by high EPO followed by the development of PHEOs/PGLs, often recurrent and multiple [16, 17]. The presence of a germ-line *HIF2A* mutation in a patient with PGL and polycythemia was described by Lorenzo et al. [18].

However, some patients with similar clinical phenotypes have not been found to be associated with the mutations described above, even though up-regulation of HIF-1α or HIF-2α is present in their PHEOs/PGLs. Given their clinical presentation of early-onset polycythemia with slightly elevated EPO levels followed by the development of PHEO/PGL, we decided to screen for mutations in other HIF regulators, including *PHDs*, in these patients.

In this study, we have found for the first time a *PHD1* (*EGLN2*) germ-line mutation in one patient and a *PHD2* (*EGLN1*) germ-line mutation in another patient, who would be the second patient reported to date with a *PHD2* mutation. Both patients shared similar clinical phenotypes. Patients with *PHD1* and *PHD2* mutations associated with polycythemia appear to have borderline or mildly elevated EPO levels, in contrast to *HIF2A* mutation patients who have markedly elevated EPO levels. *PHD1* and *PHD2* mutations caused loss of protein function, triggering activation of HIF-1α and HIF-2α and their downstream-regulated genes (*EDN1*, *EPO*, *GLUT1*, *GNA14*, *LDHA*, *POU5F1*, *SOX2*, and *VEGFA*) in the tumors. Increased EPOR activity and inappropriate hypersensitivity of erythroid progenitors to erythropoietin resulted in polycythemia with borderline or mildly elevated EPO in these patients. Our study provides important evidence indicating the existence of a specific association of *PHD1* and *PHD2* mutations with PHEO/PGL and polycythemia and may help explain the molecular pathogenetic mechanism of this disease.

Case series

Patient 1

A 48-year-old female referred herself to NIH for evaluation of recurrent PHEOs/PGLs and polycythemia with borderline or mildly elevated EPO levels (Table 1). The patient was diagnosed with polycythemia at age 6, followed by phlebotomies at age 10 (Table 1). At age 14, she presented with a left adrenal PHEO and later with recurrent PHEOs along with a thoracic periaortic lymph node positive for metastatic PHEO/PGL. After each operation, her polycythemia improved but did not normalize; usually after 2–4 months, it worsened and the patient again required phlebotomies. At age 48, she was found to have 2 lesions in the right adrenal gland, 1 lesion in the aortocaval region, and 1 in the right pelvic area suggestive of multiple and metastatic PHEOs/PGLs. Furthermore, a urinary bladder tumor was detected, surgically removed, and confirmed as PGL.

Table 1 Clinical and genetic features, laboratory and imaging findings in *PHD* index patients

	Patient 1	Patient 2	URL
Age of diagnosis of polycythemia and tumors (year)			
Polycythemia	6	16	
Pheochromocytoma	14, 48	39, 60	
Paraganglioma	48	39 (multiple)	
Recurrent pheochromocytoma	33, 37	No	
Recurrent paraganglioma	No	60	
Metastatic tumors (based on histopathology)	Yes	No	
Clinical characteristics			
Red cheeks or lips	5	5	
Red eyes	11	7	
Flushing	No	5	
Red/blue feet	5	15	
Nose bleed	5	No	
Headache	5	38	
Episodic chest pain	Yes	Yes	
Palpitations	Yes	Yes	
Anxiety, nervousness	Yes	Yes	
BP and HR before the first operation	Very high[a]	190/100	
BP/HR (at NIH)	126/71; 80[b]	132/66; 88[b]	
Pertinent family history	None	None	
Therapy	Phlebotomy[c]	Phlebotomy	
Other clinical diagnoses	Enlarged heart? (age 6) Colonic diverticulosis Prominent ascending aorta Uterine fibroid Arthromyalgia Five miscarriages	Cystic kidney disease Primary hyperparathyroidism Diabetes mellitus Hypothyroidism Gout Hyperlipidemia Three miscarriages, one stillborn	
Complete blood count and erythropoietin at NIH or outside NIH[d]			
Erythrocytes (per mm^3)	4,540,000 5,730,000[d]	5,540,000 5,980,000[d]	5,220,000 5,200,000[d] 5,000,000[d]
Hematocrit (%)	38.90 % 48.6 %[d]	44.50 % 49 %[d]	44.90 % 46 %[d] 46 %[d]
Hemoglobin (g/dl)	12.8 16.2[d]	13.9 15.8[d]	15.7 16[d] 15.2[d]
Leukocytes (per mm^3)	5470 6500[d]	6280 5900[d]	10,040 11,300[d] 10,300[d]
Platelets (per mm^3)	226,000 274,000[d]	175,000 198,000[d]	369,000 450,000[d] 415,000[d]
Erythropoietin (mIU/ml)	32.5 29.0[d]	40.5 55[d]	31.5 19.5[d] 31.5[d]
Biochemical tests at NIH			
Pl normetanephrine (pg/ml)	418	227	112
Pl metanephrine (pg/ml)	23	<10	61

Table 1 (continued)

	Patient 1	Patient 2	URL
Pl norepinephrine (pg/ml)	1639	982	750
Pl epinephrine (pg/ml)	<27	5	51
Pl dopamine (pg/ml)	24	17	30
Pl chromogranin A (ng/ml)	350	189	225
Pl somatostatin (pg/ml)	8	4	21
U normetanephrine (μg/24 h)	1770	686	521
U metanephrine (μg/24 h)	90	45	180
U norepinephrine	244	54	80
U epinephrine	2	<1.2	21
U dopamine	259	99	400
Imaging tests at NIH			
CT/MRI	+	+	
18F-FDG	+	+	
18F-FDOPA	+	+	
Genes tested: *VHL*, *SDHB*/*C*/*D*, *JAK2*, *HIF1*/*2A*, *RET*, *PHD3*	No germ-line or somatic mutations found	No germ-line or somatic mutations found	

The listed upper reference limits for laboratory values were established at the National Institutes of Health (NIH) for plasma catecholamines, metanephrines, complete blood count, and EPO and at Mayo Medical Laboratories in Rochester, Minnesota, chromogranin A. To convert values for plasma measurements to picomoles per liter, multiply by 5.46 for normetanephrine, 5.08 for metanephrine, 5.91 for norepinephrine, 5.46 for epinephrine, and 6.53 for dopamine. The last biochemical tests (8/2013) for patient 1 also showed elevated plasma epinephrine (66 pg/ml) and dopamine (74 pg/ml). Note that blood count and erythropoietin values at NIH were measured at the same time. In patient 1, one daughter's, two brothers', and the mother's blood sample and the father's and a second daughter's hair, nail, and saliva tested negative for the *PHD1* mutation. In patient 2, three sisters', a daughter's, and a brother's blood samples tested negative for the *PHD2* mutation

HIF1/*2A* hypoxia-inducible factor 1 α and 2α, *JAK2* Janus kinase 2, *PL* plasma, *PHP* primary hyperparathyroidism with 3 abnormal glands, *RET* rearranged during transfection, *SDHB*/*C*/*D* succinate dehydrogenase subunits B, C, D, *U* urine, *URL* upper reference limit, *VHL* von Hippel-Lindau

[a] The patient did not remember the BP values but was told they were very high. Before her subsequent operations, her BP was about 150–190/100–110 mg/Hg. The blood test at NIH for patient 1 was done 2 months after an outside phlebotomy

[b] At the time, patient 1 was on losartan 25 mg QD, terazosin 5 mg QD, and metoprolol 25 mg QD; patient 2 was on Toprol XL 25 mg QD

[c] Last phlebotomy performed in February 2012

[d] Outside NIH values: In patient 1, EPO was measured in 2010 and the complete blood count in 2012 before her last operation outside NIH in 2012; in patient 2, they were measured in 2012 before her last operation in 2013 at NIH

Patient 2

A 60-year-old female was referred to NIH for evaluation of multiple PGLs and a right adrenal PHEO (Table 1). The diagnosis of polycythemia was made at age 16, and the patient was treated with phlebotomies from then on. Her EPO levels were found to be only borderline or mildly elevated. At age 39, she was found to have a left adrenal PHEO and 2 PGLs near the left renal artery. After resection of the tumors, her polycythemia significantly improved, but worsened after 6 months. At NIH, the patient was found to have multiple tumors; surgical resection was performed in 2013 and histopathology confirmed the presence of a right adrenal PHEO and 3 periaortic PGLs.

No relative of either patient was found to have any history of polycythemia or PHEO/PGL (Table 1). The parents of patient 2 are deceased and were therefore not evaluated.

Materials and methods

This study was approved by the IRB of the *Eunice Kennedy Shriver* NICHD/NIH, and all patients gave written informed consent.

DNA sequencing

Genomic DNA was extracted from tumors and white blood cells. Exon sequencing was performed to screen for mutations in *VHL*, *HIF1A*, *HIF2A*, *JAK2*, *PHD1*, *PHD2*, and *PHD3* using polymerase chain reaction (PCR). DNA sequences of each exon were determined by Sanger DNA sequencing.

Loss of heterozygosity analysis

Loss of heterozygosity (LOH) was determined through microsatellite analysis of the *PHD1* and *PHD2* gene loci; the

primer pairs for the microsatellite markers are summarized in Supplementary Table S1.

Real-time PCR

Total RNA was extracted from tumor specimens. Messenger RNA (mRNA) expression of hypoxia-related genes was measured using quantitative real-time PCR (qPCR). The primers used in present study include *EDN1* (QT00088235), *EPO* (QT00001484), *GLUT1* (QT00068957), *GNA14* (QT00099379), *LDHA* (QT00001687), *POU5F1* (QT00210840), *SOX2* (QT00237601), *VEGFA* (QT01010184), and *ACTB* (QT00095431).

PHD1 and PHD2 plasmid and mutagenesis

PHD1 and *PHD2* mutations were introduced into the pCMV6-PHD1/2 vector (Origene) using the Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent). DNA sequences of the plasmids were verified by DNA sequencing.

Luciferase assay

A luciferase assay was performed as previously described [19]. A hypoxia-responsive element (HRE)-driven luciferase gene was introduced into Hep3B cells through lentivirus infection and puromycin selection. Zero to 25 ng of *PHD1* or *PHD2* expression vectors were co-transfected with a *HIF2A* expression vector (50 ng) into the Hep3B HRE-Luc cell line using Lipofectamine 2000 (Life Technologies, Invitrogen). A pcDNA3 vector was used as a control plasmid. The HRE-associated transcriptional activity was determined after 36 h using the ONE-Glo luciferase assay system (Promega).

Cycloheximide pulse-chase assay

HeLa cells were transfected with recombinant vectors encoding *PHD1* or *PHD2* mutants using Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h, the cells were treated with cycloheximide (50 μM). Cells were collected after 0, 3, 6, 12, or 24 h. The quantity of protein residue was determined by Western blot.

Immunohistochemistry

Five-micrometer tissue sections were probed with anti-HIF-1α (Sigma-Aldrich), anti-HIF-2α (Novus Biologicals), anti-EPOr (Santa Cruz), anti-phosphorylated EPOR, anti-PHD1, and anti-PHD2 (Abcam) antibodies and counterstained with hematoxylin. The intensity of the staining was scored as follows: 0=negative, 1+=weak, 2+=intermediate, 3+=strong; original magnification, ×200. Immunohistochemistry (IHC) data were reviewed in a double-blinded manner.

Microarray processing and analysis

PHEO/PGL tissue and normal adrenal medulla were collected for the study. One sample from the *PHD2* patient and six from two *HIF2A* patients were used for microarray analysis, as previously described [20, 21].

In vitro assay of erythroid progenitor's sensitivity to EPO

An in vitro sensitivity assay of erythroid progenitors to EPO was performed, as previously described [22]. Mononuclear cells isolated from the peripheral blood were plated in methylcellulose media (MethoCult, StemCell Technologies) supplemented with various concentrations of EPO (0.015 to 3.0 U/ml; StemCells Technologies). Cell cultures were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C for 14 days. Erythroid burst-forming unit colonies (BFU-Es) were scored by standard morphological criteria.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Results were expressed as mean±SEM with p values <0.05 considered significant.

Results

Mutation and allelic deletion analyses of *PHD1* and *PHD2*

In both blood and tumor specimens, we identified a novel heterozygous *PHD1* mutation (c.188T>A, p.Ser61Arg (S61R)) in one patient and *PHD2* mutation (c.682G>T, p.Ala228Ser (A228S)) in another patient [Fig. 1a, Supplementary Figure S1]. LOH analysis was performed with two sets of microsatellite markers flanking the *PHD1* and *PHD2* genes, and both tumors showed allelic loss for the markers at the *PHD1* (Q^{LOH}=0.33–0.37) and *PHD2* (Q^{LOH}=0.04–0.10) gene loci, respectively (Fig. 1b). The allelic deletions were also noticed in a sequencing study, with mutant nucleotide signals more dominant compared to wild-type nucleotides (Supplementary Figure S2).

Protein stability analysis of PHD1 and PHD2 mutants

Alignment of the amino acid sequences of PHD1 residues 41 to 65 and PHD2 residues 216 to 240 in different species

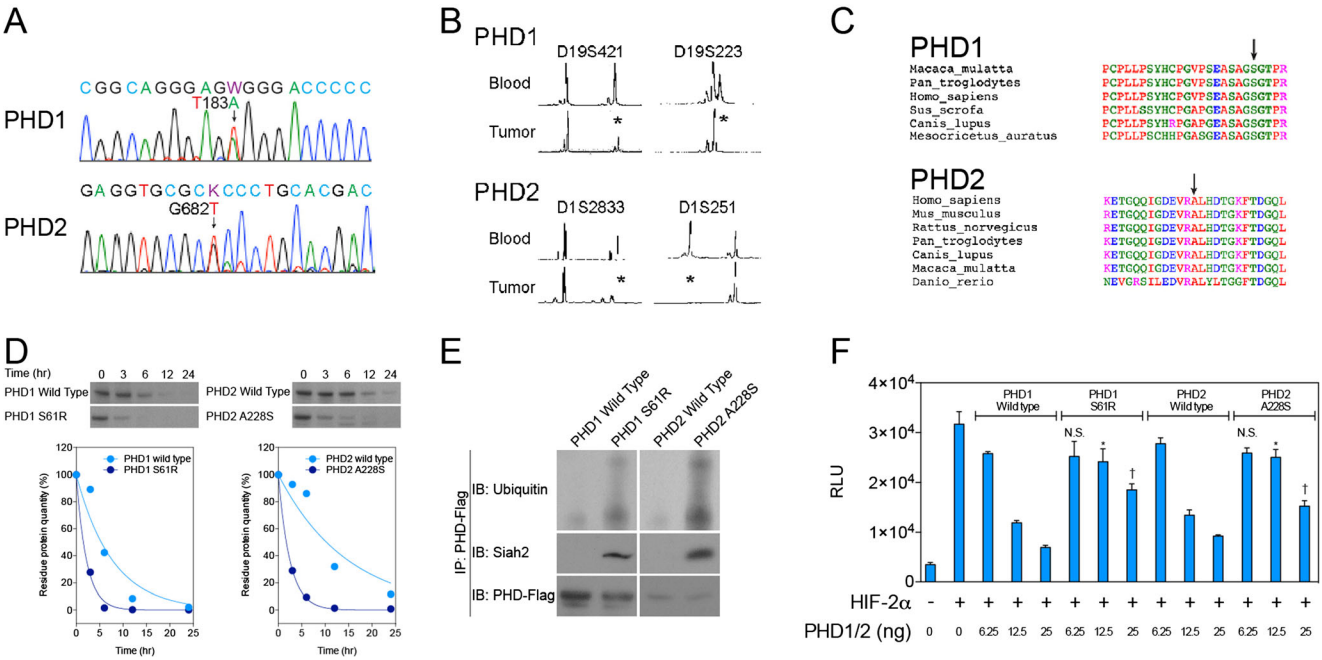

Fig. 1 *PHD1* and *PHD2* mutations, LOH, peptide sequencing and stability, and ubiquitination in PHEO/PGL and other samples. **a** Sanger sequencing showing heterozygous *PHD1* and *PHD2* mutations in blood samples. **b** DNA fragment analysis showing LOH in *PHD1* or *PHD2* loci, respectively, in tumors. Loss of allele is indicated by *asterisk*. **c** Amino acid sequence alignment of mutation sites in different species of *PHD1* and *PHD2*. **d** CHX assay measuring PHD1 and PHD2 stability and showing the shortened protein stability in mutant proteins. **e** Immunoprecipitation assay showing enhanced Siah2 recognition and protein ubiquitination of PHD1 and PHD2 mutants. **f** Luciferase assay showing that reduction of HIF-2α induced target gene expression was significantly less diminished after transduction with the mutant than the WT PHDs (*$p<0.01$, PHD wild type vs. PHD mutant, 12.5 ug transfection; †$p<0.01$, PHD wild type vs. PHD mutant, 25 ug transfection)

showed that the mutations were located in the evolutionarily conserved area (Fig. 1c). The amino acid substitutions were likely to affect the folding and stability of the PHD1 and PHD2 proteins, as previously reported [15]. To test this hypothesis, we performed a cycloheximide pulse-chase assay. Mutations in *PHD1* and *PHD2* resulted in remarkably reduced protein half-lives, indicating quantitative losses of the PHD1 and PHD2 proteins due to their reduced stability (Fig. 1d). Reduced stability of mutant PHD1 and PHD2 was evidenced by the rapid degradation over time after the addition of cycloheximide (CHX) to block protein synthesis as seen on Western blot. The wild-type PHD1 and PHD2 proteins showed half-lives of 5.33 and 10.27 h, respectively. In contrast, PHD1-S61R and PHD2-A228S were less stable, with half-lives of 1.52 and 1.7 h, respectively. Enhanced protein degradation of the mutant PHD1 and PHD2 was supported by increased association of the mutant PHDs with E3 ubiquitin ligase 3 (Siah2) and protein ubiquitination compared to the wild-type PHDs in an immunoprecipitation assay (Fig. 1e).

Measurement of HIF-2α transcriptional activity

We next studied HIF-2α induction of transcriptional activity in the presence of wild-type and mutant PHDs using a luciferase assay (Fig. 1f). HIF-2α transcriptional activity was effectively reduced by an overexpression of wild-type PHD1 or PHD2 with an increasing amount of PHDs. Overexpression of PHD1-S61R or PHD2-A228S mutants led to a much less pronounced effect on HIF-2α-induced transcriptional activity, indicating reduced HIF degradation due to decreased protein half-lives of the mutant proteins (Supplementary Figure S3).

Expression of PHD1 and PHD2 proteins in tumors with *PHD1* and *PHD2* mutations in comparison to human adrenal medulla and a sporadic non-*PHD1*- or non-*PHD2*-mutated PHEO

The tumor from the *PHD1* patient shows negative staining for PHD1, while the expression of PHD2 is heterogeneous, with areas of negative staining (0) and areas with weak (1+) staining. PHD1 expression in the tumor from the *PHD2* patient was scored 1–2+; PHD2 staining in this sample was weak (1+). In the sporadic PHEO, PHD1 staining exhibited intermediate/strong (2–3+) intensity and PHD2 staining was scored 1–2+. Human adrenal medulla shows strong (3+) staining for PHD1 and intermediate (2+) staining for PHD2 (Fig. 2). A PHEO sample was incubated without primary antibody as a negative control (Supplementary Figure S4).

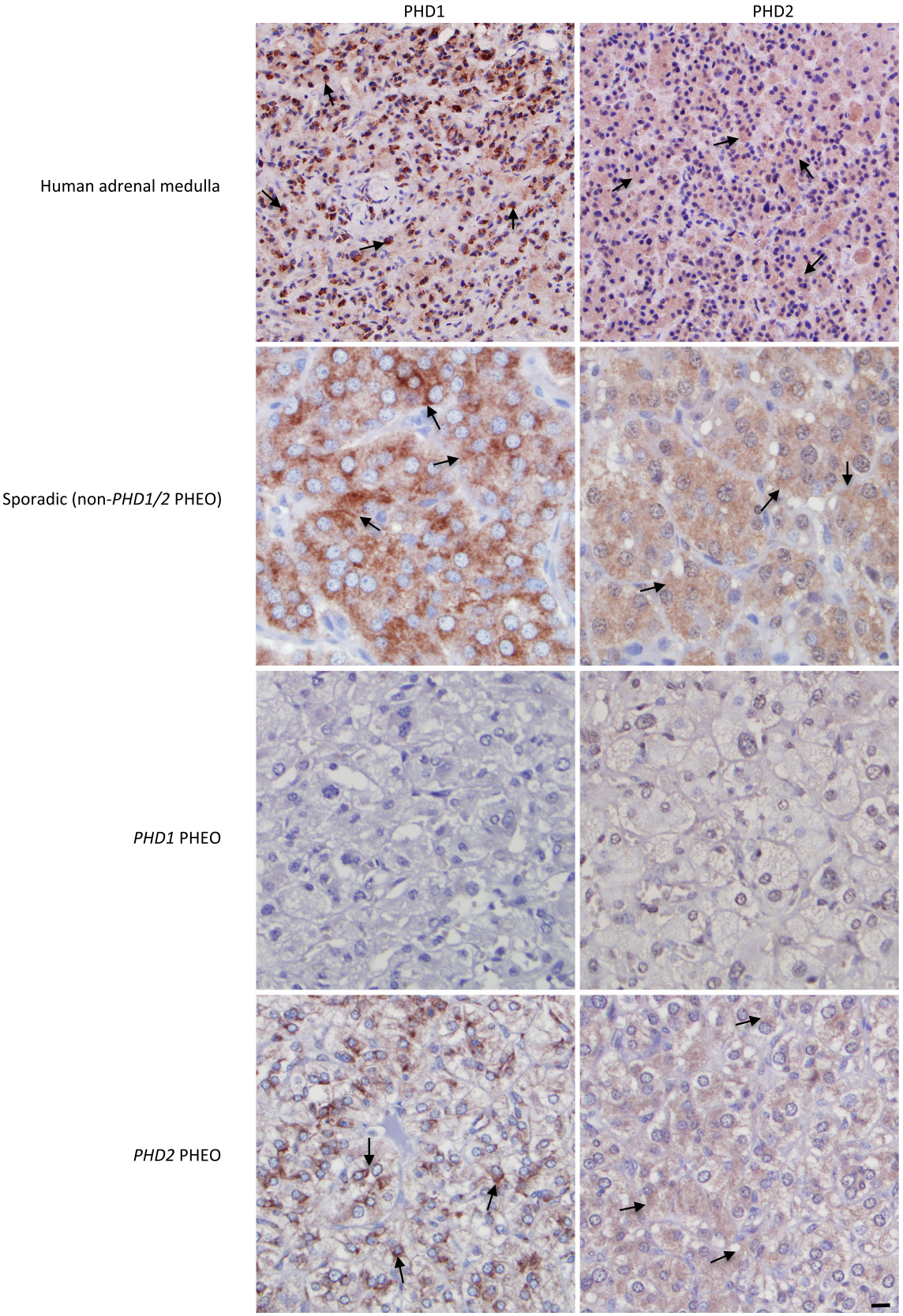

Fig. 2 Immunohistochemical staining for PHD1 and PHD2 in tumors from patients with *PHD1* and *PHD2* mutations in comparison to human adrenal medulla and a sporadic (non-*PHD1*- or *PHD2*-mutated) PHEO. Original magnification, human adrenal medulla ×150; sporadic, *PHD1* and *PHD2* PHEOs ×200. *Scale bar*, 100 μm

Up-regulation of HIF-1α and HIF-2α and downstream hypoxia-related gene expression

HIF-1α and HIF-2α levels were increased in tumor tissue, as detected by IHC analysis (Fig. 3a). The figure shows positive cytoplasmic immunostaining for HIF-1α and nuclear staining for HIF-2α. The tumor mass from the *PHD1* mutation patient showed weak (1+) staining for HIF-1α and intermediate (2+) heterogeneous staining for HIF-2α. HIF-1α and HIF-2α expression in the sample from the *PHD2* mutation patient was scored 1–2+. In comparison, in the tissue of a patient with a *HIF2A* mutation, expression of HIF-1α was weak (1+) and HIF-2α intensity was scored 2+. In normal adrenal medulla, HIF-1α was absent and only rarely did nuclei stain for HIF-2α. Increased activity of hypoxia pathways was confirmed

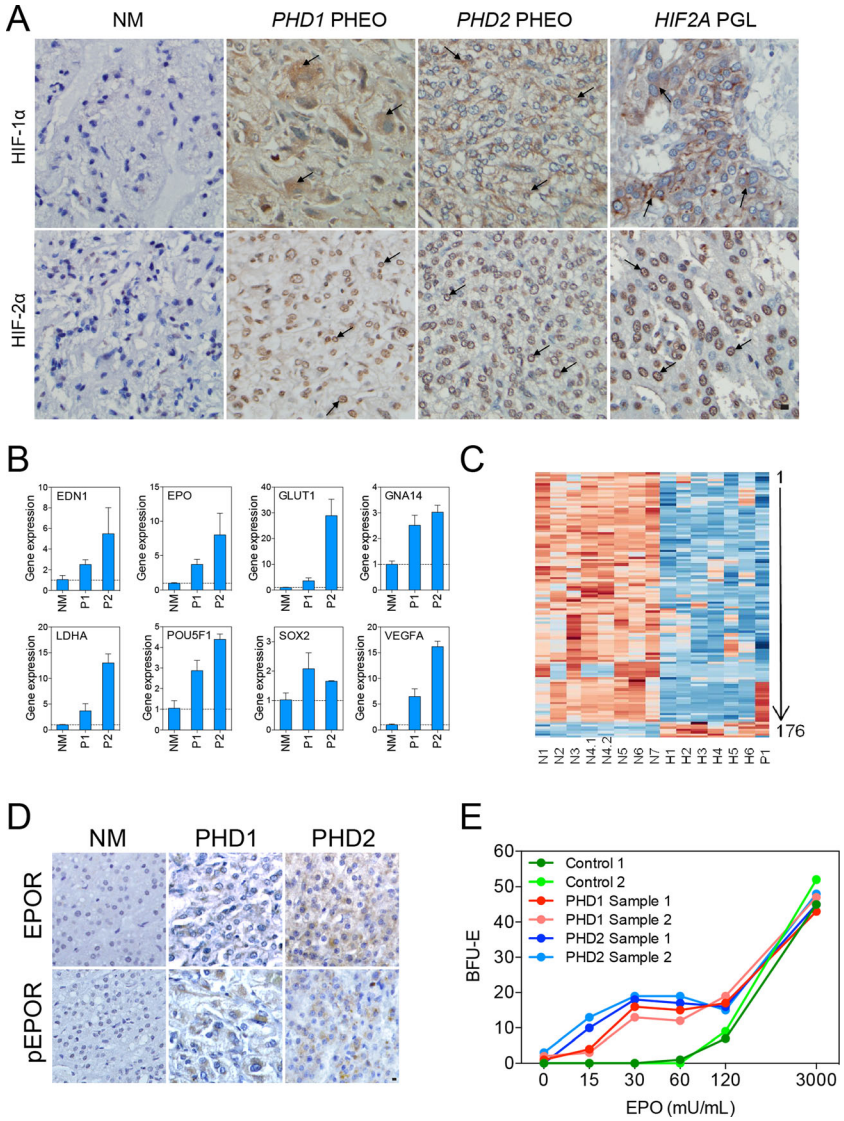

Fig. 3 Effects of *PHD1* and *PHD2* mutations on HIFs, HIF-regulated gene expression, microarray gene profiling, EPO receptor activity, and in vitro analysis of native erythroid progenitors in tumor and blood samples. **a** IHC of HIF-1α and HIF-2α in PHEOs with *PHD1* and *PHD2* mutations in comparison to a PGL with a *HIF2A* mutation. Original magnification, ×200. *Scale bar*, 10 μm. **b** qPCR assay showing the expression of eight hypoxia-related genes—*EDN1*, *EPO*, *GLUT1*, *GNA14*, *LDHA*, *POU5F1*, *SOX2*, and *VEGFA*—in tumor specimens from *PHD1* (P1) and *PHD2* (P2) patients. A normal adrenal medulla (NM) was used as a control. **c** Heatmap of 176 differentially expressed genes in normal adrenal medulla (N1-7) vs. *HIF2A* (H1-6) and *PHD2* (P1) PHEOs/PGLs identified by significance analysis of microarray with an FDR of 0.01. P1 showed an expression pattern more similar to *HIF2A* PHEOs/PGLs than normal adrenal medulla. However, expression differences also existed between *HIF2A* and *PHD2* tumors, as can be seen based on the different color distributions (*red*, up-regulation; *blue*, down-regulation). Rank product analysis identified 20 genes to be significantly differentially expressed between *HIF2A* and *PHD2* ($p<0.05$; genes are indicated in Supplemental Table 3). **d** IHC showing EPOR expression in tumor specimens. *Scale bar*, 10 μm. **e** BFU-E assay showing EPO sensitivity of hematopoietic precursor cells from patients. The *y*-axis represents the absolute number of clones per 1,000,000 blood cells from patient blood.

by induction of HIF-1α and HIF-2α target gene expression relative to normal adrenal medulla, namely *EPO*, *EDN1*, *GLUT1*, *GNA14*, *LDHA*, *POU5F1*, *SOX2*, and *VEGFA*, in tumors from both patients by qPCR (Fig. 3b).

Comparison of gene expression profiles in tumors associated with *PHD2* and *HIF2A* mutations

We further compared the gene expression profiles of *PHD2* and *HIF2A* tumors with normal adrenal medulla by microarray (Fig. 3c). Genes that showed similar expression levels in the *PHD2* tumor and normal adrenal medulla were excluded (ratio of 0.5 to 1.5), resulting in 1940 genes that passed the filter. Then, differentially expressed genes between a combination of *HIF2A* and *PHD2* PHEOs/PGLs vs. normal adrenal medulla were estimated by significance analysis of microarray (SAM) with a two-class comparison option. False discovery rates were estimated with 1000-fold permutations, and significant expression changes were selected at a 1 % false discovery rate, revealing 176 genes (*y*-axis). Figure 3c shows a heat map of these 176 genes, which indicates differences in the expression pattern of *HIF2A* (H1-6)- and *PHD2* (P1)-related PGLs compared to normal medulla (N1-7). Twenty significantly changed genes between *HIF2A* and *PHD2* PHEOs/PGLs were identified by rank product analysis ($p<0.05$). Sample information is presented in Supplementary Table 2; gene identifiers and expression values are given in Supplementary Table 3. All data are available at the GEO database (GSE 39716). Of note, we only had the chance to analyze one tumor with the *PHD2* mutation, since adequate RNA from *PHD1*-mutated tumors was not available, because these tumors were removed outside the NIH and frozen tissue was not collected.

EPO receptor IHC and in vitro analysis of native erythroid progenitors

We determined the expression of EPOR in PHEO specimens by IHC (Fig. 3d). We identified up-regulation of both the receptor and its phosphorylation in tumor specimens from *PHD1* and *PHD2* patients compared to a normal adrenal medulla. A BFU-E assay was carried out to assess erythroid progenitor sensitivity to EPO. Progenitor cells from both *PHD1* and *PHD2* patients' peripheral blood exhibited hypersensitivity to EPO compared to progenitor cells from control persons (Fig. 3e).

Discussion

The present study shows for the first time a *PHD1* germ-line mutation associated with the pathogenesis of PHEO/PGL-polycythemia and further extends the findings of *PHD2* mutations in this disease. Both mutations caused amino acid substitutions, abnormal recognition of intrinsic E3 ligase Siah2, and resulted in substantial loss of protein stability of both PHD1 and PHD2. The dysregulation of the HIF-α signaling pathway caused by a mutation in either *PHD1* or *PHD2* affected HIF-α protein degradation and HIF-1α and HIF-2α target gene expression. We also found increased EPO and EPOR activity in *PHD1* and *PHD2* tumors compared to normal adrenal medullary tissue. Furthermore, we discovered hypersensitivity of erythroid progenitors to EPO in both patients. These findings are consistent with the clinical presentation that in addition to activation of the HIF-α signaling pathway, both patients also developed polycythemia, at least partially, due to the high activity and hypersensitivity of the EPOR signaling pathway to EPO.

PHDs and VHL are known to play essential roles in regulating HIFs. The pioneering studies of Ratcliffe and Kaelin showed that HIF ubiquitination and proteasomal removal are preceded by its proper hydroxylation by PHDs [2, 3]. Mutations in *PHDs*, *VHL*, and *HIF2A* can result in HIF-α stabilization, which may play a central role in the pathogenesis of some hereditary PHEOs/PGLs [23].

Almost all tumors are hypoxic, and tumor hypoxia, specifically via up-regulation of HIFs, contributes to tumor growth, progression, migration, metastasis, and resistance to various cancer treatments. Although the role of PHDs in cancer development remains inconclusive, in neural crest tumors such as PHEOs/PGLs, PHDs may act as tumor suppressor genes [24]. PHDs use 2-oxoglutarate as an electron donor, which is oxidized into succinate. High succinate levels inhibit PHD activity [25]. The accumulation of succinate is a typical metabolic feature of succinate dehydrogenase-deficient PHEOs/PGLs, and it is hypothesized that their tumorigenesis is also linked to the inhibition of PHDs, resulting in the up-regulation of HIF-α target genes. PHDs may also affect tumorigenesis in HIF-independent signaling, as reviewed by Jokilehto and Jaakkola [24]. This can involve the regulation of the NF-κB, NOTCH, and perhaps mTOR signaling pathways, resulting in abnormal cell differentiation and proliferation, angiogenesis, apoptosis, and immune responses [24, 26]. Finally, both mutations cause abnormal recognition of intrinsic E3 ligase Siah2 and result in the quantitative loss of both PHD1 and PHD2, further contributing to dysregulation of the HIF signaling pathway [27]. In summary, the precise role of PHDs as tumor suppressor genes or oncogenes depends on their relative abundance in a specific tissue, interplay among the three isoforms, as well as other genetic and epigenetic mechanisms.

Furthermore, a very recent study provided new evidence that *PHD* mutations are indeed involved in the pathogenesis of polycythemia via HIF-2α up-regulation and hypersensitivity of erythroid progenitors to EPO [28]. Consistent with these findings, one case of a germ-line *PHD2* mutation has been

described in the pathogenesis of PGL associated with polycythemia, presenting with normal EPO levels [15]. Nevertheless, the precise pathogenic mechanism of polycythemia, particularly extramedullary and extratumoral sources of erythropoiesis, is still unclear in our patients. As evident from the medical history of our patients, removal of their PHEOs/PGLs only partially improved their polycythemia or normalized their condition for a short period of time, despite no evidence of residual or additional tumors by biochemical and imaging assessment. This suggests that other sources of extratumoral and extramedullary erythropoiesis could exist. A recent study by Arsenault et al.[28] showed that heterozygous *Phd2* loss in the neural crest-derived renal interstitial EPO-producing cells alone accounts for a significant portion of the erythrocytosis in both $Phd2^{P294R/+}$ and $Phd2^{+/-}$ mice. Furthermore, other neural crest cells, beside chromaffin cells, may be additional sources of EPO. This is well documented in previous studies that suggest astrocytes, retinal cells, and, perhaps, enterochromaffin cells in the gastrointestinal tract, among others [29]. Thus, *PHD* germ-line mutations in these cells could lead to abnormal EPO production, further supporting the recent data of Arsenault et al. in a *Phd2* knock-in mouse model [28].

Our findings regarding up-regulation of EPOR and its hypersensitivity to EPO support the notion that these two mechanisms may contribute to tumorigenesis in both patients. Some studies demonstrated that EPO could stimulate proliferation, growth, and metastatic spread as well as inhibit apoptosis in cancer cells via JAK2-STAT, ERK1/2, and the PI3K/AKT pathways [30, 31]. These effects can be further potentiated during hypoxia. Interestingly, and related to the present study, Um et al. found that EPO via EPOR exerts an antiapoptotic effect on rat PHEO (PC-12) cells [32]. Nevertheless, detailed molecular mechanisms related to EPO/EPOR signaling and tumor growth remain to be established in this population of patients, as suggested by Yoon et al. [33].

Mutations in different HIF regulators and HIF itself appear to have similar disease manifestations but also present with some different clinical phenotypes, especially the recently described *HIF2A*- and now *PHD1*- and *PHD2*-associated PHEO/PGL and polycythemia [16]. The differences could result from the subtly distinct impacts of these gene mutations in the activation of HIF-1α and HIF-2α target genes and their effects on tumorigenesis [34]. For this reason, we decided to look at the differential expression of several HIF target genes in *PHD2*-associated tumors. We found that the *PHD2* mutation affected both HIF-1α and HIF-2α target genes in PHEO/PGL. In contrast to *HIF2A* mutations where HIF-2α target genes are preferentially affected [16], there seems to be an interplay in the induction of both HIF-1α and HIF-2α target genes in *PHD1* and *PHD2* loss-of-function mutations. To gain a more global perspective, microarray-based gene expression profiling was performed. A total of 176 genes in *PHD2*- and *HIF2A*-related PHEOs/PGLs were identified as differentially expressed compared to normal adrenal medulla. In addition to commonly expressed genes, however, there was clear evidence for differential expression between *PHD2* and *HIF2A* tumors. None of these genes was identified as an exclusive HIF-1α or HIF-2α target gene; thus, transcriptional co-factors or other mechanisms in the complex pathway of HIF transcriptional regulation may contribute to the differential expression pattern. This is in agreement with previous studies showing that PHDs affect both HIF-1α and HIF-2α signaling pathways [24]. When well-established human PHEO/PGL-derived cell lines become available, several experiments performed in the present study should be validated and performed under both normoxic and hypoxic conditions. Moreover, further experiments with additional *PHD1* and *PHD2* samples are needed to derive more conclusive information.

Patients with *PHD1*, *PHD2,* or *HIF2A* mutations share some clinical similarities, including multiple or recurrent PHEOs/PGLs, noradrenergic phenotypes, and an early onset of polycythemia, but they also differ in several aspects of their clinical presentations. First, patients with *PHD1* and *PHD2* mutations present with borderline or mildly elevated serum EPO levels compared to those with *HIF2A* mutations, where EPO levels are usually several-fold increased. Polycythemia does not seem to occur at birth. Second, *HIF2A* mutation patients present with another neuroendocrine tumor—somatostatinoma—that has not yet been detected in patients with *PHD1* and *PHD2* mutations. Third, in patients with *HIF2A* mutations, metastatic PHEO/PGL has not yet been described. Finally, both these diseases diverge from primary polycythemias where the EPOR/JAK-STAT pathway is constitutively activated, resulting in polycythemia with inappropriately low EPO levels.

In conclusion, the *PHD1* mutation is the latest inherited abnormality that contributes to the genetic landscape of PHEOs/PGLs. The present study further provides evidence that the PHD-HIF-EPOr pathway plays a role in the pathogenesis of these tumors and their association with polycythemia. Its close link to the HIF-1α and HIF-2α signaling pathways further supports the hypothesis that these and other hereditary PHEOs/PGLs could be therapeutically approached by modulating HIF signaling in these tumors [23]. The discovery reported here suggests that patients with polycythemia associated with borderline or mildly elevated EPO levels should be screened for the presence of *PHD1* and *PHD2* mutations. Finally, patients carrying a germ-line mutation in genes of the HIF signaling pathway should have stringent follow-up to detect and treat PHEO/PGL early.

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