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Somatic *HIF2α* gain-of-function mutations in paraganglioma and somatostatinoma associated with polycythemia

Zhengping Zhuang*, Chunzhang Yang*, Felipe Lorenzo, Maria Merino, Tito Fojo, Electron Kebebew, Vera Popovic-Brkic, Constantine A. Stratakis, Josef T. Prchal, and Karel Pacak
Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda (Z.Z., C.Y.); Laboratory of Pathology (M.M.), Medical Oncology Branch (T.F.), Endocrine Oncology Branch (E.K.), National Cancer Institute, National Institutes of Health, Bethesda; Division of Hematology, University of Utah School of Medicine, ARUP and VAH, UT (F.L., J.T.P.); Eccles Institute of Human Genetics, University of Utah School of Medicine, UT (J.T.P.); Neuroendocrine Unit, Institute of Endocrinology, University Clinical Center, Belgrade, Serbia (V. PB.), Program on Developmental Endocrinology and Genetics (C.A.S) and Program in Reproductive and Adult Endocrinology (K.P.), Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

SUMMARY

Hypoxia inducible factors (HIFs) are transcription factors controlling energy, iron metabolism, erythropoiesis, and development, and, when dysregulated, contribute to tumorigenesis, cancer progression, and invasion. However, HIFα mutations have not previously been identified in any cancer. Here we report two novel somatic gain-of-function *HIF2α* mutations in two patients, one presenting with a paraganglioma and a second with both paraganglioma and somatostatinoma. Both mutations were shown to confer increased *HIF2α* activity and protein half-life. While germline mutations of regulators of HIFα, including *VHL* and *EGLN1*, have been reported in pheochromocytomas/paragangliomas, this is the first report of a somatic gain-of-function mutation in *HIF*.

Keywords

HIF2α; paraganglioma; somatostatinoma; polycythemia

INTRODUCTION

Hypoxia-inducible factors (HIFs), originally described by Semenza et al., are transcription factors that respond to changes in tissue oxygen concentration.¹ HIFs are highly conserved proteins composed of α and β subunits. The HIFβ subunit is constitutively expressed, whereas the α subunit is inducible by hypoxia and associated with the aggressive, treatment-refractory behavior of tumors.^{2, 3} Under normoxic conditions, HIF1α, HIF2α, and HIF3α are hydroxylated on specific prolyl residues, allowing for recognition by the von Hippel-Lindau (VHL) tumor suppressor protein and rapid degradation via the proteasome.⁴ Under hypoxic conditions, prolyl hydroxylation of HIFα proteins is reduced, resulting in their

To whom correspondence should be addressed: Karel Pacak, MD, PhD, DSc, Chief, Section on Medical Neuroendocrinology, Professor of Medicine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Building 10, CRC, Room 1E-3140, 10 Center Drive MSC-1109, Bethesda, MD 20892-1109 USA, Tel.: (301) 402-4594, Fax: (301) 402-4712, karel@mail.nih.gov.

*Dr. Zhuang and Dr. Yang contributed equally to this article.

stabilization and in turn transcription of genes involved in the hypoxia response, including angiogenesis, glycolysis, apoptosis, proliferation, and growth.^{5, 6} Parangliomas/pheochromocytomas are catecholamine-producing tumors derived from the chromaffin cells of extra-adrenal paraganglia.⁷ The pioneering work of Neumann et al. showed that about one fourth of these tumors were hereditary, a fraction now closer to one third since the identification of mutations in several additional genes, including *SDHA*, *SDHAF2*, *TMEM127*, and *MAX*.^{8, 9} Importantly, the identification of these genetic drivers led to the demonstration of HIF α stabilization and dysregulation in pheochromocytoma and paraganglioma tumors harboring mutations in *VHL* and succinate dehydrogenase (*SDH*).^{10, 11}

Somatostatinomas are endocrine tumors producing somatostatin that are also believed to originate from neural crest cell origin. They are occasionally diagnosed in patients with *VHL* syndrome, multiple endocrine neoplasias type 1 or 2, or neurofibromatosis type 1.¹²

Polycythemia, a disease state in which the proportion of blood volume that is occupied by red blood cells increases, can be acquired or congenital; both forms can be primary (abnormality in erythroid progenitors causing increased erythroid proliferation), secondary (mediated by circulating erythropoietin), or related to abnormalities in hypoxia-sensing pathways (increased erythropoietin production and increased sensitivity of progenitors to erythropoietin).¹³ Germline mutations in *VHL*, *EGLN1*, or *HIF2 α* result in increased erythropoietin and congenital polycythemia.¹⁴

While mutations of *VHL* and *EGLN1* have been associated with tumorigenesis of neural crest tumors due at least in part to a dysregulation of HIF α , until now no HIF mutations have been identified in these tumors. In this study, we have identified two novel somatic gain-of-function mutations in the *HIF2 α* gene in multiple tumors from patients presenting with paraganglioma and somatostatinoma associated with polycythemia. These particular mutations disrupt prolyl hydroxylation of HIF2 α , and in turn recognition by the *VHL* protein, resulting in a failure of HIF2 α ubiquitination and degradation. Because these mutant HIF2 α proteins have a longer half-life, targets downstream of HIF2 α , including erythropoietin, are upregulated, leading to polycythemia. Our findings suggest the multiple phenotypes in these patients may represent an acquired disease syndrome initiated by gain-of-function mutations in *HIF2 α* .

CASE REPORT

Patient 1.

A thirty-year-old female was referred to our institution for evaluation of metastatic paraganglioma. From infancy the patient had polycythemia. At age 14 she was found to have a left periaortic paraganglioma, and at age 15 an aortocaval additional paraganglioma was detected. At age 23 an abdominal MRI showed multiple masses that were resected with diagnoses of paragangliomas (Table 1, Figure 1A). One year later she presented with a recurrent paraganglioma and 3 years later with at least 5 abdominal paragangliomas. At the time of her referral, CT and MRI demonstrated masses in the duodenum and pancreatic head, which were found to be a paraganglioma and somatostatinoma at the time of surgical resection (Figure 1A).

Patient 2.

An eighteen-year-old female was referred to our institution for resection of a right “adrenal pheochromocytoma” and an aortic bifurcation paraganglioma. From birth she had cyanosis and was then diagnosed with polycythemia with normal sensitivity of erythroid progenitors to erythropoietin (Table 1). At age 18, after having episodes of hypertensive crises, she was

found to have multiple paragangliomas at the time of surgical exploration. Two years later she again presented with another 3 cm periaortic paraganglioma that was successfully removed.

METHODS

This study was approved by the IRB of the NICHD/NIH, and patients gave written informed consent.

Mutation analysis of *HIF2α*

Genomic DNA was extracted from either tumor tissue or white blood cells with the NucleoSpin Tissue Kit (Macherey-Nagel). *HIF2α* exons were amplified from genomic DNA by polymerase chain reaction (PCR). The primer sets for exon amplification were as previously reported.¹⁵ The DNA sequence of each exon was identified by forward and reverse sequencing.

Peptides

Peptides corresponding to wild type HIF2α (ELDLETLPYIPMDGEDFQ), ΔHIF2α-A530T HIF2α (ELDLETLPYIPMDGEDFQ), and ΔHIF2α-A530V HIF2α (ELDLETLPYIPMDGEDFQ) were synthesized and N-terminal biotinylated (Peptide 2.0).

Plasmids and mutagenesis

The HA-HIF2α-pcDNA3 plasmid containing the human HIF2α gene coding sequence was obtained from Dr. William Kaelin (Addgene plasmid 18950¹⁶). The ΔHIF2α-A530T and ΔHIF2α-A530V mutations in the HIF2α gene were introduced into this plasmid using the Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent). DNA sequences of the plasmids were verified by sequencing the entire coding region of the HIF2α gene. The coding sequence of the human VHL gene was inserted into a pCMV6-Entry vector (Origene). The HA-EGLN1 plasmid containing the human PHD2 gene was a generous gift from Dr. Richard Bruick.¹⁷

HIF2α hydroxylation assay

A HIF2α hydroxylation assay was performed as previously described.¹⁸ Peptide hydroxylation was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as previously described.¹⁹ For detection of VHL binding after HIF2α hydroxylation, a radioactively labeled VHL protein was synthesized using the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of [³⁵S]-methionine (Perkin Elmer). EGLN1-treated beads were washed and incubated with [³⁵S]-VHL for 2 hr at 4°C. Bound VHL was eluted by heating in an SDS-containing sample buffer and resolved on NuPAGE Bis-Tris Gels (Invitrogen).

Real-time PCR

Total RNA was extracted from microdissected tumor samples using the RNeasy Extraction Kit (Qiagen). The mRNA expression of hypoxia-related genes was examined through real-time PCR on an ABI 7500 real-time PCR system. The primer sets used were EDN1 (Origene HP205717), EPO (Origene HP200740), GLUT1 (Origene HP209446), VEGFA (Qiagen QT01682072), and β-Actin (Promega G5740).

RESULTS

Up-regulation of hypoxia-related gene expression in tumors

We analyzed canonical hypoxia-related pathways by measuring gene expression in tumors from the index patients. A normal adrenal medulla specimen was used as a control. We found a marked up-regulation of HIF2 downstream-regulated genes, including EDN1, EPO, GLUT1, and VEGFA, in tumor specimens from both patients (Figure 1B), suggesting the clinical presentation of polycythemia was a consequence of hypoxia-related signaling.

Mutation analysis of *HIF2α*

To determine the causative gene responsible for polycythemia, we sequenced both germline DNA from leukocytes and DNA from the tumors. We identified two novel heterozygous mutations in the tumor specimens in both patients (Figure 1C). In patient 1, both the paraganglioma and somatostatinoma were found to have an identical G to A substitution at base 1588 in exon 12 of the *HIF2α* gene. In patient 2, the paraganglioma was found to have a C to T substitution at base 1589 in exon 12 of *HIF2α*. Neither of the mutations, nor other *HIF2α* mutations, was found in the germline DNA. Both the G1588A and C1589T mutations in *HIF2α* resulted in a substitution of alanine 530 in the HIF2α protein, with threonine in patient 1 and valine in patient 2. Analysis of tumor cDNA from both patients found equal proportions of the mutant and wild-type HIF2α alleles and no evidence of aberrant mRNA splicing.

Disruption of prolyl hydroxylation of HIF2α

Alignments of multiple HIF2α peptide sequences indicated that Ala530 is located in the vicinity of the primary hydroxylation site of the HIF2α protein and is conserved across different species and other HIF family members (Figure 1D). Amino acid substitution of Ala530 is likely to affect the conformation of the HIF2α hydroxylation domain and possibly interfere with prolyl hydroxylase recognition and VHL binding. To test this hypothesis, we performed *in vitro* hydroxylation assays, as previously described.¹⁸ Synthetic HIF2α peptides were incubated in the presence of PHD2. Prolyl hydroxylation of the HIF2α peptides was investigated by MALDI-TOF mass spectrometry (Figure 2A). Consistent with previous reports, 60.6% of wild type HIF2α peptides were hydroxylated. In contrast, only 9.1% and 16.7% of the ΔHIF2α-A530T and ΔHIF2α-A530V peptides were hydroxylated in the same assay. To further investigate the impact of the mutations on HIF2α signaling, we analyzed the binding of VHL to the HIF2α peptide. Radioactively labeled VHL protein showed strong binding to wild type HIF2α peptide (Figure 2B), consistent with efficient E3 ligase binding and the possibility of proteasomal degradation (Figure 2C). By comparison, the VHL protein had much less affinity for the ΔHIF2α-A530T and ΔHIF2α-A530V peptides, with only 18.4% and 24.0% of the VHL protein bound to the mutant peptides, respectively.

Stability of mutant HIF2α

Finally, we determined the stability of mutant HIF2α using cycloheximide. The wild type HIF2α protein had a 14.4 min lifespan, in agreement with the rapid turnover of HIF2α in normoxic conditions. In contrast, ΔHIF2α-A530T and ΔHIF2α-A530V were more stable, with half-lives of 57.6 and 79.8 min, respectively (Figures 2D and 2E).

DISCUSSION

Previously, HIFα germline mutations have been reported in patients with familial polycythemia, with mutations at hot spots in exon 12 of the HIF2α gene.^{20, 21} Members of the HIFα family have also been demonstrated to play an important role in tumorigenesis.²²

The interplay between HIF1 α and HIF2 α in tumorigenesis is unclear, but recent evidence suggests these two transcription factors may promote, initiate, or maintain different cell functions, resulting in varied tumor development and behavior, including prognosis.^{3, 23} Dysfunction of the VHL and SDH proteins as a result of gene mutations leads to HIF α dysregulation and overexpression and induces pseudo-hypoxia, a mechanism described in the pathogenesis of hereditary pheochromocytomas and paragangliomas.¹⁴ Furthermore, altered hydroxylation of proline residues on HIF α due to mutations of the prolyl hydroxylase gene can also cause downregulation of HIF α , which may also play a role in tumorigenesis.²⁴ In aggregate, these studies have shown changes in the regulation of, but not mutations in, HIF α subunits in various tumors.

We now report somatic gain-of-function *HIF2 α* mutations in paragangliomas and somatostatinoma associated clinically with polycythemia. These mutations were identified in the vicinity of the primary hydroxylation site of the HIF2 α protein. They affected prolyl hydroxylation and VHL protein binding, resulting in reduced HIF2 α degradation. We found increased erythropoietin mRNA and protein levels in both tumors that may result in a high serum erythropoietin level and polycythemia. However, we cannot rule out the possibility that HIF2 α somatic mutations were present in precursor cells that secret excessive erythropoietin resulting in the early onset of polycythemia before gross tumor formation.

The identical somatic mutation detected in two separate and functionally distinct tumors, paraganglioma and somatostatinoma, in two young patients indicates the mutation may have occurred in a cell at early embryogenesis. The HIF2 α mutant cells may have then been distributed throughout multiple organs during development. A similar genetic mechanism can be found in McCune-Albright syndrome, in which a post-zygotic mutation in *GNAS1*, a protein involved in G protein signaling, presents as a mosaic in only a fraction of cells.

The patients described herein presented with a rare combination of disease phenotypes, yet shared mutations of the same gene at a similar domain. Our findings raise several important questions, including (a) whether the *HIF2 α* mutation is unique to paraganglioma and somatostatinoma associated with polycythemia, and (b) whether patients presenting with paragangliomas or somatostatinsomas need to be screened for *HIF2 α* mutations.

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Figure 1.

Panel A: Histopathological findings of pheochromocytoma (middle) and paraganglioma (left). Multiple pheochromocytoma masses were well encapsulated, solid, and pink-tan in color. Morphologically, they were composed of large spindle and round cells with prominent nuclei and granular cytoplasm. Some cells had a large irregular and hyperchromatic nucleus with large nucleoli. The tumor cells were arranged, forming nests or growing in a diffuse pattern, and appeared to be embedded in a rich vascularized stroma. Occasionally a brown pigment was identified in the tumor. Immunohistochemistry showed positive staining for chromogranin A and synaptophysin. MiB1 staining was weak, consistent with a low proliferative index. Neuroendocrine somatostatinoma involved the duodenal wall and was composed of round uniform cells with small compact nucleoli and scant cytoplasm. The cells were arranged in nests and cords that infiltrated the lamina propria and superficial muscularis. These cells stained positive for chromogranin A and synaptophysin and somatostatin receptor (right). Panels B, C, and D present evidence of pseudohypoxic signaling and HIF2 α mutations in tumor specimens from the patients. **Panel B:** Quantitative mRNA expression measurement of four hypoxia related genes - EDN1, EPO, GLUT1, and VEGFA - performed by qPCR assay. **Panel C:** Genomic DNA sequencing of nucleotides 1580 to 1597 in exon 12 of HIF2 α . Heterozygous G1588A (patient 1) and C1589T (patient 2) mutations were identified. A similar mutation was not found in germline DNA obtained from the peripheral blood. **Panel D:** Alignment of amino acid sequences of HIF2 α residues 524 to 542 (human HIF2 α nomenclature) in human, mouse, rat, and *Xenopus laevis*, as well as the same conserved domain in human HIF1 α and HIF3 α .

Figure 2.

Functional characterization of the Ala530 mutation in the HIF2α protein. **Panel A:** Reduced hydroxylation of peptides encoding HIF2α mutant sequences demonstrated through matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Biotinylated HIF2α peptides were incubated with recombinant EGLN1 and purified through streptavidin magnetic beads. Native peptides (downward pointing arrows) incubated with EGLN1 (labeled +) were hydroxylated (downward pointing arrowhead), and this was recorded as an increase in the molecular mass of the peptide. **Panel B:** Binding assay demonstrating the reduced association of the VHL protein with mutant HIF2α peptides when compared with a peptide encoding the wild type HIF2α sequence. Input represents 10% of the total amount of [³⁵S]-labeled recombinant VHL protein. VHL binding to the HIF2α peptides was analyzed by autoradiography. **Panel C:** Ubiquitination of the HIF2α mutant protein. The binding of VHL and HIF2α ubiquitination were analyzed by immunoprecipitation assay. After incubating the various His-tagged HIF2α proteins with VHL, comparable immunoprecipitations were performed with an anti HA antibody, as shown in the middle blot. The upper blot shows greater ubiquitination of the wild type HIF2α protein, coinciding with the greater association of the VHL protein shown in the lower blot. **Panel D:** Enhanced stability of mutant HIF2α proteins evidenced by the higher levels seen on Western blot analysis over time following the addition of cycloheximide (CHX). **Panel E:** Quantification and half-life of the HIF2α mutants based on data in panel D.

Table 1**Age at presentation**

Upper reference limits are as follows: norepinephrine: 498 pg/ml; epinephrine: 83 pg/ml; dopamine: 46 pg/ml; normetanephrine: 112 pg/ml; metanephrine: 61 pg/ml; chromogranin A: 225 ng/ml. 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. Upper reference limits established at NIH for plasma catecholamines, metanephrines, complete blood count, and erythropoietin or at Mayo Medical Laboratories in Rochester, MN for chromogranin A. Both patients were tested for VHL, SDHB/C/D, PHD, and JAK2 gene mutations and were found to be negative.

Value	Patient 1	Patient 2
Age at presentation (yr)	14	18
Complete blood count		
Erythrocytes (per mm ³)	7,780,000	7,850,000
Hematocrit (%)	50.50%	59.30%
Hemoglobin (g/dl)	14.7	18.8
Leukocytes (per mm ³)	7,190	8,130
Platelets (per mm ³)	194,000	249,000
Erythropoietin (mIU/ml)	150	180
Plasma biochemical test		
Normetanephrine (pg/ml)	4,834	858
Metanephrine (pg/ml)	121	9
Norepinephrine (pg/ml)	10,951	1,760
Epinephrine (pg/ml)	100	7
Dopamine (pg/ml)	28	20
Chromogranin A (ng/ml)	1,640	320
Age of symptom diagnosed (yr)		
Polycythemia	At birth	At birth
Multiple paragangliomas	15	18
Somatostatinoma	30	None