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Isolated Erythrocytosis Associated With 3 Novel Missense Mutations in the *EGLN1* Gene

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Abstract

Hypoxia-inducible factor-1 (HIF-1) is a key regulator of erythropoiesis. In this article, we report 3 novel mutations, P378S, A385T, and G206C, on the *EGLN1* gene encoding the negative HIF-1α regulator prolyl hydroxylase domain-2 (PHD2) in 3 patients with isolated erythrocytosis. These mutations impair PHD2 protein stability and partially reduce PHD2 activity, leading to increased HIF-1α protein levels in cultured cells.

Keywords

erythrocytosis, EGLN1, PHD2, HIF-1, polycythemia

Introduction

Hypoxia-inducible factor-1 (HIF-1) is a central transcriptional regulator of the physiological response to hypoxia.[1] HIF-1α protein levels are coupled to oxygen levels by an exquisite molecular mechanism.[2,3] Oxygen-dependent hydroxylation by the prolyl hydroxylase domain-2 (PHD2) protein marks HIF-1α for ubiquitination by the von Hippel Lindau (VHL) tumor suppressor protein, leading to proteasomal degradation (Figure 1).[4-6] Under hypoxic conditions, PHD2 activity is limited, and therefore HIF-1α protein is stabilized, leading to an increase in the transcription of erythropoietin as well as hundreds of target genes that coordinate diverse processes including erythrocytosis, angiogenesis, metabolism, cell proliferation, and autophagy.[7-9]

In this article, we describe 3 patients with unexplained long-standing erythrocytosis who were found to have novel heterozygous mutations on the *EGLN1* gene, which encodes PHD2. These PHD2 mutations reduce PHD2 protein stability and its prolyl hydroxylase activity, leading to an increase in HIF-1α protein levels in cultured cells.

Case Presentations

Patient 1

A 60-year-old female was referred to our clinic for polycythemia, with elevated hemoglobin in the range of 16 to 17 g/dL for at least 13 years. She is a nonsmoker with no known cardiac or pulmonary issue. She has no known family history of polycythemia. Physical examination was normal. The oxygen saturation was 99% on ambient air, carboxyhemoglobin was <1%. The P_{50} was 29 mm Hg (24-30 mm Hg), hemoglobin electrophoresis was negative for high-affinity hemoglobin variants, and erythropoietin level was 11 mU/mL (2.6-18.5 mU/mL). Workup for secondary causes of polycythemia, including transthoracic echocardiogram with bubble study, sleep study, pulmonary function tests, and abdominal ultrasound were normal. Peripheral blood polymerase chain reaction (PCR) for the JAK2 mutations in exons 12 to 14 was negative.

Given the long-standing erythrocytosis with no apparent cause, genetic testing for congenital erythrocytosis was pursued. EGLN1 gene sequencing revealed a heterozygous missense mutation on exon 3 at c.1132C>T, resulting in amino acid substitution p.Pro378Ser (p.P378S). Her hemoglobin has remained stable without the need for phlebotomy, and she has had no thrombotic events.

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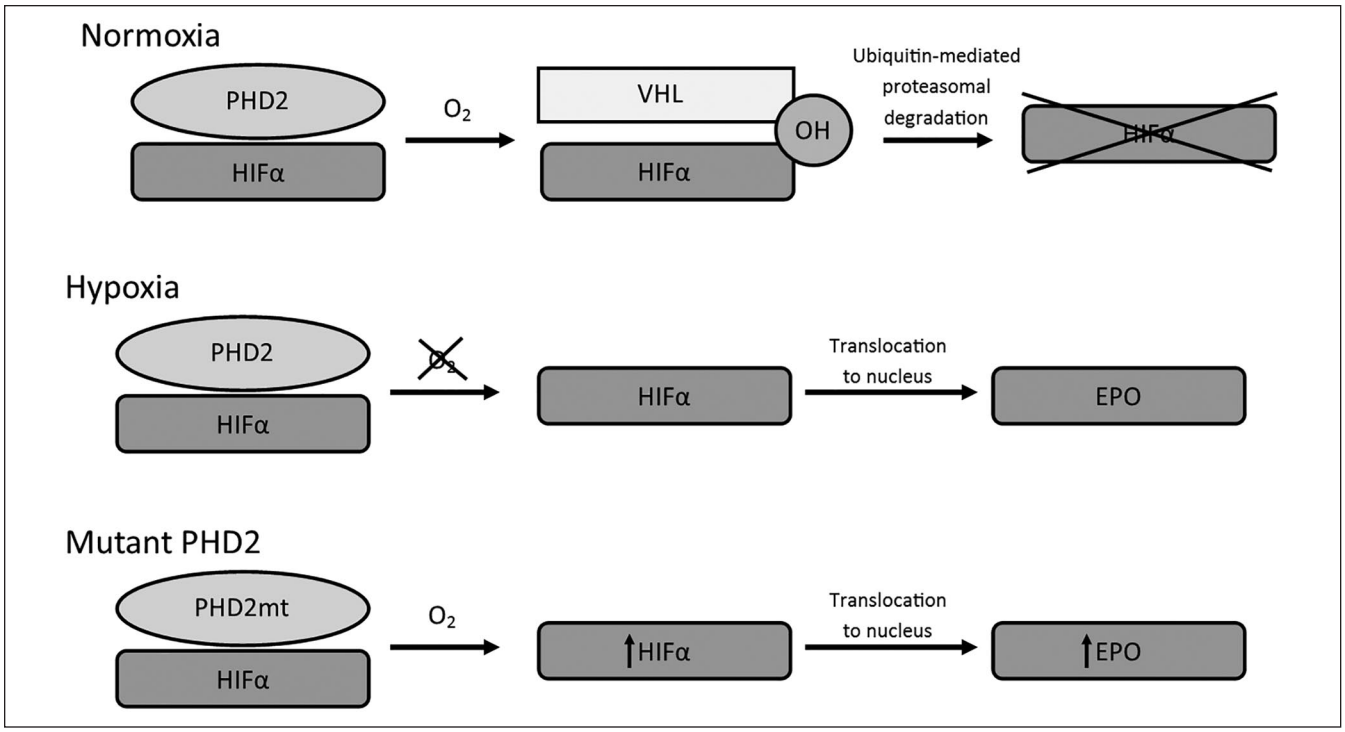

Figure 1. Schematic diagram of the oxygen sensing pathway under conditions of normoxia, hypoxia, and mutant prolyl hydroxylase domain-2 (PHD2) protein.

Patient 2

A 52-year-old male with a history of well-controlled HIV on HAART (highly active antiretroviral therapy) was referred to our clinic for polycythemia, with hemoglobin in the range of 18 to 19 g/dL as far back as records could be obtained. History did not reveal any obvious causes for secondary erythrocytosis: he was a life-long nonsmoker, had no cardiac or pulmonary issues, no travel to high altitude areas, and did not take testosterone supplementation or erythropoiesis-stimulating agents. Physical examination was normal. The oxygen saturation was 100% on ambient air and erythropoietin was 9 mU/mL (4-27 mU/mL). Peripheral blood PCR for JAK2 exon 12 to 14 mutations was negative.

Genetic testing for congenital erythrocytosis revealed a heterozygous missense mutation in the *EGLN1* gene at c.1153G>A, resulting in amino acid substitution p.Ala385Thr (p.A385T). He was seen by neurology for complaints of intermittent headaches and paresthesias of the left arm. Magnetic resonance imaging of the brain showed innumerable hypodensities concerning for chronic microvascular ischemic changes. The patient was initiated on antiplatelet therapy and phlebotomy to maintain hematocrit <50%, with improvement of his headaches.

Patient 3

A 28-year-old female was referred to our clinic for incidentally discovered erythrocytosis, with hemoglobin 17.2 g/dL. Physical examination was notable for oxygen saturation of 100%, obesity, and a large lower extremity hemangioma. The erythropoietin level was normal (14 mU/mL) and PCR for JAK2 mutation was negative. Workup for secondary etiologies of erythrocytosis, including a sleep study, was unrevealing. She was found to have a heterozygous missense mutation in the *EGLN1* gene at c.616G>T, resulting in amino acid substitution pGly206Cys (p.G206C). She has remained asymptomatic without the need for phlebotomies. It remains unclear if the large hemangioma is related to abnormal angiogenesis drive by the PHD2 mutation.

Methods

The cDNA of wild-type PHD2 was amplified by PCR and cloned to p3XFLAG vector. P378S and A385T PHD2 mutants were generated by the site-directed mutagenesis. Lentiviral PHD2 short hairpin RNA (shRNA) was purchased from Sigma (TRCN0000318549). HeLa cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 and 95% air incubator. PHD2 knockdown HeLa cells were generated by transducing lentivirus carrying PHD2 shRNA and subsequent treatment with puromycin. Cells were transfected with plasmid DNA using PolyJet (SignaGen). Twenty-four hours posttransfection cells were lysed in lysis buffer (150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM Tris-HCl [pH 8.0], 0.5% IGEPAL CA-630, protease inhibitor cocktail). Proteins were

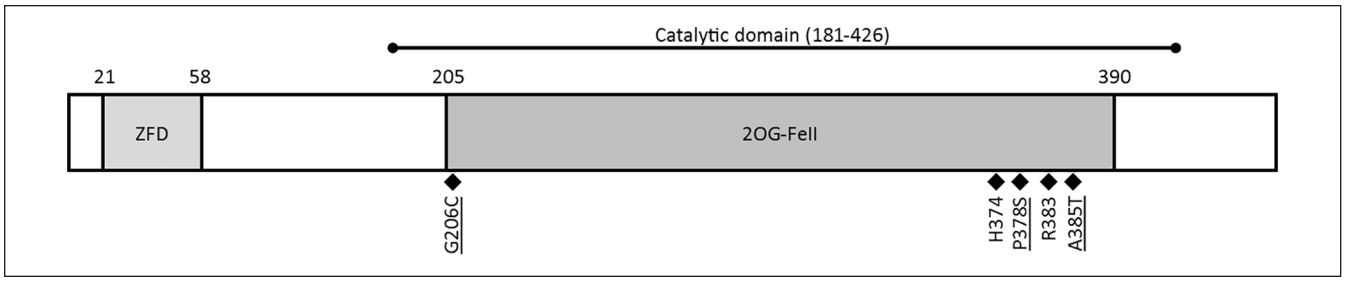

Figure 2. Schematic diagram representing the human prolyl hydroxylase domain-2 (PHD2) protein. ZFD, zinc finger domain; 2OG-FeII, 2-oxoglutarate and Fe(II)-dependent oxygenase-type domain. Diamonds indicate the location of the mutations described in this study (underlined), as well as normal functional elements of the PHD2 protein. Numbers indicate amino acid residue positions.

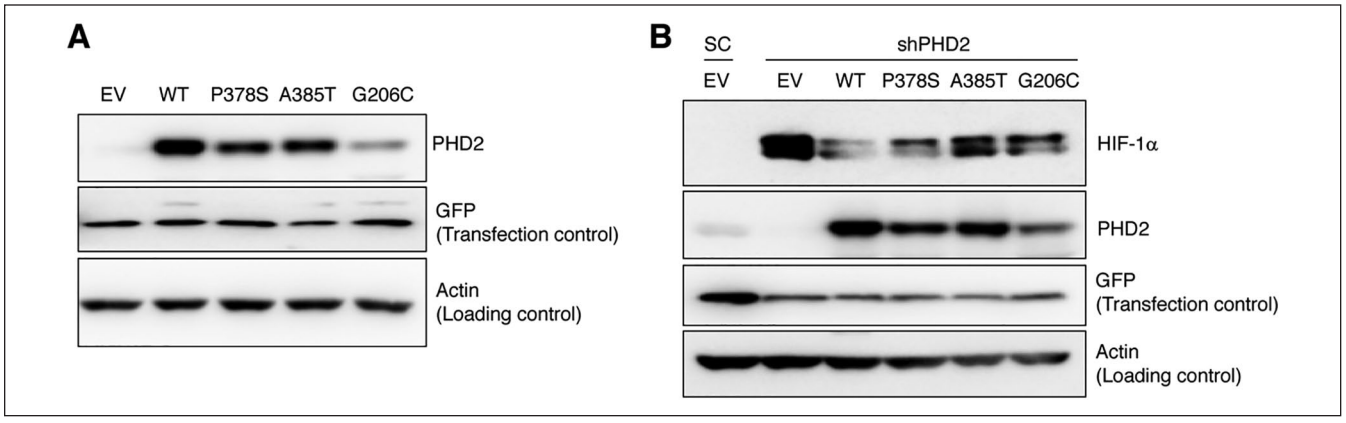

Figure 3. Effect of mutant prolyl hydroxylase domain-2 (PHD2) on hypoxia-inducible factor-1α (HIF-1α) protein stability. (A) HeLa cells were co-transfected with expression vectors encoding GFP and wild-type (WT) FLAG-PHD2, FLAG-PHD2 (P378S), FLAG-PHD2 (A385T), FLAG-PHD2 (G206C), or empty vector (EV). Lysates were probed with indicated antibodies. (B) Scrambled control (SC) and PHD2 knockdown HeLa cells were co-transfected with expression vectors encoding GFP and WT FLAG-PHD2, FLAG-PHD2 (P378S), FLAG-PHD2 (A385T), FLAG-PHD2 (G206C), or EV. Lysates were probed with indicated antibodies.

quantified and then fractionated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The following antibodies were used in immunoblot: HIF-1α (BD Biosciences, Cat. # 610959), β-actin (Proteintech, Cat. # 66009-1-lg), FLAG (Sigma, Cat. # F3165).

Results and Discussion

Previous work has identified genetic causes of polycythemia due to mutations in genes of the oxygen-sensing pathway, with rare cases associated with mutations on the *EGLN1* gene.[10-13] We report 3 cases of isolated erythrocytosis associated with novel missense mutations, P378S, A385T, and G206C, on the *EGLN1* gene. These mutations occur at amino acid residues that are highly conserved throughout evolution and have not previously been reported as a pathogenic mutation in the literature. The p.Pro378Ser mutation lies in close proximity to the active-site iron-coordinating residue at codon 374, whereas the p.Ala385Thr mutation is located in the catalytic domain in close proximity to the 2OG-binding residue R383, important for protein function (Figure 2).[12,14]

These mutations robustly decreased levels of the PHD2 protein in HeLa cervical carcinoma cells (Figure 3A). Modified HeLa cells that were stably transfected with shRNA targeting endogenous PHD2 were used to assess the effect of these PHD2 mutations on endogenous HIF-1α levels. Overexpression of wild-type PHD2 led to a decrease in HIF-1α protein levels, but this effect was partially lost by the P378S, A385T, and G206C mutations (Figure 3B). These cases add to the body of evidence identifying a central role of the PHD2–HIF axis in regulating erythropoiesis.

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Declaration of Conflicting Interests

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Ethics Approval

Our institution does not require ethical approval for reporting individual cases or case series.

Informed Consent

Verbal informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

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