Published in final edited form as:

Am J Hematol. 2012 April ; 87(4): 439–442. doi:10.1002/ajh.23123.

Two new mutations in the *HIF2A* gene associated with erythrocytosis†

Melanie J. Percy¹, Yu Jin Chung², Claire Harrison³, Jane Mercieca⁴, A. Victor Hoffbrand⁵, Carla L. Dinardo⁶, Paulo C.J.L. Santos⁶, Guilherme H.H. Fonseca⁷, Sandra F.M. Gualandro⁷, Alexandre C. Pereira⁶, Terence R.J. Lappin⁸, Mary Frances McMullin^{1,8}, and Frank S. Lee^{2,*}

¹Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland, BT9 7AB, United Kingdom

²Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

³Department of Haematology, St. Thomas' Hospital, London, SE1 7EH, United Kingdom

⁴St. Helier Hospital, Carshalton, SM5 1AA, United Kingdom

⁵Department of Haematology, Royal Free Hospital, London, NW3 2QG, United Kingdom

⁶Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), University of Sao Paulo Medical School, Sao Paulo, Brazil

⁷Department of Hematology, University of Sao Paulo Medical School, Sao Paulo, Brazil

⁸Centre for Cancer Research and Cell Biology, Queen's University, Belfast, Northern Ireland, BT9 7AB, United Kingdom

Abstract

Congenital or familial erythrocytosis/polycythemia can have many causes, and an emerging cause is genetic disruption of the oxygen-sensing pathway that regulates the *ERYTHROPOIETIN* (*EPO*) gene. More specifically, recent studies have identified erythrocytosis-associated mutations in the *HIF2A* gene, which encodes for Hypoxia Inducible Factor-2 α (HIF-2 α), as well as in two genes that encode for proteins that regulate it, Prolyl Hydroxylase Domain protein 2 (PHD2) and the von Hippel Lindau tumor suppressor protein (VHL). We report here the identification of two new heterozygous *HIF2A* missense mutations, M535T and F540L, both associated with erythrocytosis. Met-535 has previously been identified as a residue mutated in other patients with erythrocytosis, although the mutation of this particular residue to Thr has not been reported. In contrast, Phe-540 has not been reported as a residue mutated in erythrocytosis, and we present evidence here that this mutation impairs interaction of HIF-2 α with both VHL and PHD2.

Keywords

erythropoietin; erythrocytosis; Hypoxia Inducible Factor-2 α ; oxygen-sensing; prolyl hydroxylation

†Conflict of interest: All authors declare there are no conflicts of interests.

*Correspondence: Frank S. Lee, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, 605 Stellar Chance Labs, 422 Curie Blvd., Philadelphia, PA, 19104. Tel: (215) 898-4701. Fax: (215) 573-2272. franklee@mail.med.upenn.edu.

The three principal proteins of the oxygen-sensing pathway regulating the *EPO* gene are PHD2, HIF-2 α , and VHL [1]. PHD2 is a prolyl hydroxylase that site-specifically modifies HIF-2 α in an oxygen-dependent manner[2]. The primary site of hydroxylation is Pro-531 of HIF-2 α , and this posttranslational modification allows recognition by VHL, a component of an E3 ubiquitin ligase complex [3–5]. VHL recognizes hydroxylated, but not unmodified, HIF. Under normoxic conditions, VHL targets HIF-2 α for constitutive degradation. Under hypoxic conditions, this modification is attenuated, allowing stabilization of HIF-2 α . HIF-2 α then transactivates genes that promote adaptation to hypoxic conditions. A key gene is that encoding for EPO, the central regulator of red cell mass, and the transcriptional upregulation of the *EPO* results in increased circulating levels of EPO, increased red cell mass, and hence increased oxygen delivery to tissues[6, 7].

Recent studies have identified erythrocytosis-associated mutations in the genes that encode for these three proteins of the oxygen-sensing pathway[1, 8–11]. These include heterozygous mutations in the *PHD2* gene, heterozygous mutations in the *HIF2A* gene, and either homozygous or compound heterozygous mutations in the *VHL* gene[12–14]. Current evidence indicates that the *PHD2* and *VHL* mutations lead to loss of function of the respective proteins, while the *HIF2A* mutations lead to a gain of function of HIF-2 α [1]. Intriguingly, haplotypes in the *HIF2A* and *PHD2* genes have also been associated with adaptation to high altitudes in Tibetans, highlighting a central role for these genes in hypoxic adaptation[15–17]. All of these issues make the documentation of human mutations in this pathway of considerable interest. In the present report, we identify two new *HIF2A* mutations associated with erythrocytosis.

Patient A, a 27 year old female, presented with dizzy episodes, and her routine blood picture showed a hemoglobin (Hb) of 17.4 g/dl, a hematocrit (Hct) of 0.51 with a white cell count of $5.8 \times 10^9/l$ and normal platelet counts. The oxygen dissociation curve and abdominal ultrasound were both normal. She was a smoker. There is no history of thrombosis or pulmonary hypertension and no family history of erythrocytosis. No family members were available for screening. No splenomegaly was detected. No mutations of *JAK2* V617F, *JAK2* exon 12, *VHL*, or *PHD2* were detected. Repeat Hb level was 17.6 g/dl and at this time her serum EPO was 6.3 mU/ml (reference range 5.0–25.0 mU/ml). She remains asymptomatic with a Hb at this level.

Patient B, an asymptomatic 49-year-old Brazilian male, presented with an increased Hb of 21.0 g/dl, Hct of 0.65, white cell count of $7.3 \times 10^9/l$, and platelet count of $236 \times 10^9/l$ during routine blood tests. There was no history of either thromboembolic events or pulmonary hypertension, nor any family history of erythrocytosis. His father and grandfather both died of acute ischemic cerebral vascular events. He did not smoke, nor did he use any medications. Arterial blood gas analysis showed normal oxygen saturation and p50 values. EPO level was 38.2 mU/mL (reference range 5.0–25.0 mU/ml). Abdominal ultrasound was normal, as were ferritin and C-reactive protein levels. No mutations of *JAK2* V617F, *JAK2* exon 12, *EPOR* exon 8, *VHL*, or *PHD2* were detected. The patient has been treated with phlebotomies and acetylsalicylic acid.

Patient C presented at age 35 with a raised Hb of 18.2 g/dl and Hct of 0.52. His white cell count was $3.5 \times 10^9/l$ and platelet count $200 \times 10^9/l$. He is a smoker. There was no splenomegaly and no evidence of renal disease or pulmonary hypertension. His serum EPO level was 7.8 mU/ml (reference range 5.0–25.0 mU/ml). Sequencing both *VHL* and *PHD2* did not detect any mutations. He remains asymptomatic.

Sequencing of exon 12 of *HIF2A* in these three individuals revealed two novel mutations (Figure 1A). Patient A was heterozygous for a c.1604 T>C mutation (middle panel), which

exchanges Met for Thr at amino acid 535 (p.Met535Thr; M535T). Patient B had an identical heterozygous mutation, and this mutation was not present in his only son, who had a normal Hb level (data not shown). Patient C possessed a C to G change at c.1620 (c.1620C>G), resulting in a p.Phe540Leu (F540L) mutation (lower panel). In the case of patient C, a family history of erythrocytosis was confirmed, but no family members were available for screening. The serum EPO levels for patients A and C were within the reference range, while the EPO level for patient B was elevated. It may be noted that in many of the described cases with *HIF2A* mutations, it is well above the reference range [18–20].

Both mutations share two key features with previously described *HIF2A* mutations[1]. First, they are heterozygous. Second, they affect residues that are C-terminal and in close proximity to the primary site of prolyl hydroxylation in HIF-2 α , Pro-531(Figure 1B). In fact, the M535T mutation is now the third mutation that has been reported to affect Met-535, the first two being M535I and M535V[18, 19]. The M535V mutation has previously been shown to impair interaction of HIF-2 α with PHD2 [21]. Furthermore, HIF-1 α Met-568, which corresponds to HIF-2 α Met-535, is a contact residue for PHD2 in the cocrystal structure of HIF-1 α (556–574):PHD2[22], therefore making it likely that Met-535 of HIF-2 α is one as well.

The F540L affects a residue that has, thus far, not been a mutational target in the context of erythrocytosis. To examine the functional consequences of the F540L mutation, we proceeded as follows. To assess its effect on the interaction between HIF-2 α and VHL, we performed a competition assay in which we incubated immobilized (biotinylated) and hydroxylated (Hyp-564) HIF-1 α (556–574) peptide with VHL in the absence or presence of wild type or F540L Hyp-531 HIF-2 α (527–542) peptide. In the absence of HIF-2 α peptide, VHL binds to immobilized Hyp HIF-1 α , as expected (Figure 2A, lane 3). In the presence of Hyp HIF-2 α peptide, this binding is diminished, and we find that the mutant HIF-2 α is a substantially weaker inhibitor than wild type (compare lanes 5 and 4), implying that this mutation weakens the interaction with VHL.

To assess the effect of the F540L mutation on the interaction of HIF-2 α and PHD2, we performed a direct binding assay in which we examined the binding of PHD2 to immobilized glutathione S-transferase fused to either wild type or F540L HIF-2 α (516–549). We find substantially less binding to the latter than the former (compare lanes 4 and 3), indicating that this mutation also weakens the interaction with PHD2. We therefore conclude that the F540L mutation impairs the interaction of HIF-2 α with both PHD2 and VHL.

Taken together, these observations support the assignment of the M535T and F540L *HIF2A* mutations as new causes of erythrocytosis. The F540L mutation, in addition, is noteworthy for several reasons. First, the F540L mutation affects a residue that is the most distant from Pro-531 of any of the erythrocytosis-associated mutations that have been reported thus far. Second, like many, though not all, HIF-2 α mutations, it impairs interaction with both its upstream modifying enzyme, PHD2, and its downstream ubiquitin ligase, VHL. Third, it is a seemingly conservative substitution of a bulky hydrophobic amino acid with another bulky hydrophobic residue, yet it produces significant functional defects. It may be noted in this regard that Phe-540 is conserved in HIF-2 α proteins from mammalian species, human HIF-1 α and HIF-3 α , as well as HIF-2 α proteins from chicken, frog, and zebrafish (Figure 1B). The importance of this residue is further highlighted by x-ray crystallographic studies, which show that the corresponding residue in HIF-1 α , Phe-572, is a contact residue for both PHD2 and for VHL [22–24].

Methods

Mutation Screening

PCR-direct sequencing was performed for exon 12 of *HIF2A* as described previously [13].

Binding Assays

Proteins—Polyhistidine and Flag-tagged VHL and PHD2 were purified from baculovirus-infected insect cells using Ni-NTA-agarose (Qiagen) as described [25]. Bacterial expression vectors for glutathione S-transferase (GST)-HIF-2 α (516–549) and GST-HIF-2 α (516–549) F540L were prepared using pGEX-5X-1 and oligonucleotides encoding the indicated sequences by standard recombinant DNA methods. Authenticity of the constructs was verified by DNA sequencing. GST, GST-HIF-2 α (516–549), and GST-HIF-2 α (516–549), were purified from *E. coli* transformed with the appropriate vectors using glutathione (GSH)-agarose (GE Healthcare).

VHL binding—Ten ng of recombinant VHL was incubated without or with 1 μ g of biotinylated hydroxyproline (Hyp)-564 HIF-1 α (556–574) prebound to 10 μ l of streptavidin-agarose (Sigma) in the absence or presence of 5 nM wild type (WT) or F540L Hyp-531 HIF-2 α (527–542) peptide. Peptides were obtained from Genscript. The resins were washed, eluted, and the eluates subjected to SDS-PAGE and western blotting using anti-Flag antibodies (Sigma), essentially as described [13, 21]. Quantification was performed using a Chemi Doc-It system (UVP Inc).

PHD2 binding—Ten ng of recombinant PHD2 was incubated with 1 μ g of *E. coli*-expressed GST, GST-HIF-2 α (516–549), or GST-HIF-2 α (516–549) F540L prebound to 10 μ l GSH-agarose. The resins were washed, eluted, and the eluates subjected to SDS-PAGE and western blotting using anti-Flag antibodies, as described [12, 21]. Quantification was performed using a Chemi Doc-It system.

Statistical analysis—Student's *t* test was employed to analyze binding data.

Acknowledgments

We thank the patients who participated in the study. This work was supported in part by NIH grant R01-CA153347 (FSL), the University of Pennsylvania Scholars program (YJC), and a fellowship from FAPESP, Proc. 2010-17465-8, Brazil (PCJLS). The technical assistance of the Laboratory of Genetics and Molecular Cardiology group, Heart Institute group (University of Sao Paulo Medical School) is gratefully acknowledged.

References

1. Lee FS, Percy MJ. The HIF pathway and erythrocytosis. Annual review of pathology. 2011; 6:165–192.
2. Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol. 2004; 5:343–354. [PubMed: 15122348]
3. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell. 2010; 40:294–309. [PubMed: 20965423]
4. Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 2008; 30:393–402. [PubMed: 18498744]
5. Semenza GL. Oxygen homeostasis. Wiley Interdiscip Rev Syst Biol Med. 2010; 2:336–361. [PubMed: 20836033]
6. Fandrey J. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. Am J Physiol Regul Integr Comp Physiol. 2004; 286:R977–988. [PubMed: 15142852]

7. Jelkmann W. Erythropoietin after a century of research: younger than ever. *Eur J Haematol.* 2007; 78:183–205. [PubMed: 17253966]
8. Percy MJ, Lee FS. Familial erythrocytosis: molecular links to red blood cell control. *Haematologica.* 2008; 93:963–967. [PubMed: 18591620]
9. Yoon D, Ponka P, Prchal JT. Hypoxia. 5. Hypoxia and hematopoiesis *American journal of physiology. Cell physiology.* 2011; 300:C1215–1222. [PubMed: 21368293]
10. Wenger RH, Hoogewijs D. Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. *Am J Physiol Renal Physiol.* 2010; 298:F1287–1296. [PubMed: 20219824]
11. McMullin MF. HIF pathway mutations and erythrocytosis. *Expert Rev Hematol.* 2010; 3:93–101. [PubMed: 21082936]
12. Percy MJ, Zhao Q, Flores A, et al. A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci U S A.* 2006; 103:654–659. [PubMed: 16407130]
13. Percy MJ, Furlow PW, Lucas GS, et al. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med.* 2008; 358:162–168. [PubMed: 18184961]
14. Ang SO, Chen H, Hirota K, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet.* 2002; 32:614–621. [PubMed: 12415268]
15. Beall CM, Cavalleri GL, Deng L, et al. Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan highlanders. *Proceedings of the National Academy of Sciences of the United States of America.* 2010; 107:11459–11464. [PubMed: 20534544]
16. Simonson TS, Yang Y, Huff CD, et al. Genetic evidence for high-altitude adaptation in Tibet. *Science.* 2010; 329:72–75. [PubMed: 20466884]
17. Yi X, Liang Y, Huerta-Sanchez E, et al. Sequencing of 50 human exomes reveals adaptation to high altitude. *Science.* 2010; 329:75–78. [PubMed: 20595611]
18. Percy MJ, Beer PA, Campbell G, et al. Novel exon 12 mutations in the HIF2A gene associated with erythrocytosis. *Blood.* 2008; 111:5400–5402. [PubMed: 18378852]
19. Martini M, Teofili L, Cenci T, et al. A novel heterozygous HIF2AM535I mutation reinforces the role of oxygen sensing pathway disturbances in the pathogenesis of familial erythrocytosis. *Haematologica.* 2008; 93:1068–1071. [PubMed: 18508787]
20. Gale DP, Harten SK, Reid CD, et al. Autosomal dominant erythrocytosis and pulmonary arterial hypertension associated with an activating HIF2 alpha mutation. *Blood.* 2008; 112:919–921. [PubMed: 18650473]
21. Furlow PW, Percy MJ, Sutherland S, et al. Erythrocytosis-associated HIF-2alpha mutations demonstrate a critical role for residues C-terminal to the hydroxylacceptor proline. *J Biol Chem.* 2009; 284:9050–9058. [PubMed: 19208626]
22. Chowdhury R, McDonough MA, Mecinovic J, et al. Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. *Structure.* 2009; 17:981–989. [PubMed: 19604478]
23. Hon WC, Wilson MI, Harlos K, et al. Structural basis for the recognition of hydroxyproline in HIF-1alpha by pVHL. *Nature.* 2002; 417:975–978. [PubMed: 12050673]
24. Min JH, Yang H, Ivan M, et al. Structure of an HIF-1alpha -pVHL complex: hydroxyproline recognition in signaling. *Science.* 2002; 296:1886–1889. [PubMed: 12004076]
25. Huang J, Zhao Q, Mooney SM, et al. Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. *J Biol Chem.* 2002; 277:39792–39800. [PubMed: 12181324]

Figure 1.

Genetic testing for *HIF2A* mutations. (A) Detection of the c.1604 T>C and c.1620C>G mutations by PCR-direct sequencing. PCR-direct sequencing was performed on total peripheral blood DNA using specific primers to amplify exon 12. Sequencing detected a heterozygous T to C change at base 1604 in patient A (asterisk, middle panel) and C to G at base 1620 in patient C (asterisk, lower panel), as indicated by arrows as compared to wild type sequence (upper panel). Shown are nucleotides 1594–1634. Bases are as follows: G = black; A = green; T = red; C = blue. (B) Comparison of HIF-2 α sequences from human (h), mouse (m), chicken (c), *X. laevis* (x), and zebrafish (z), as well as those of human HIF-1 α , human HIF-3 α . Shading indicates identical residues, asterisk indicates the hydroxylacceptor Pro-531, and underlining indicates residues that have been sites of HIF-2 α mutations

previously associated with erythrocytosis[1]. The two new missense mutations described in the present report are indicated by the inverted triangles.

Figure 2.

Binding assays. (A) Recombinant FlagVHL was incubated without (lane 2) or with (lanes 3 to 5) biotinylated Hyp-564 HIF-1 α (556–574) peptide prebound to streptavidin-agarose, in the absence (lane 3) or presence of 5 nM wild type (WT) or F540L Hyp-531 HIF-2 α (527–542) peptide (lanes 4 and 5, respectively). The resins (lanes 2 to 5) were washed, eluted with $2 \times$ SDS loading buffer, and the eluates subjected to SDS-PAGE and western blotting using anti-Flag antibodies. Quantification was performed using a Chemi Doc-It system. Input represents 1% of the total VHL added to each reaction. The degree of inhibition observed with WT HIF-2 α (527–542) peptide was $84 \pm 1.2\%$ ($n = 3$), while that observed with the mutant peptide was $34 \pm 2.8\%$ ($n = 3$) ($P < 0.01$). (B) Recombinant FlagPHD2 was incubated with GST, GST-HIF-2 α (516–549), or GST-HIF-2 α (516–549) F540L prebound to GSH-agarose, as indicated. The resins were washed, eluted with $2 \times$ SDS loading buffer, and the eluates subjected to SDS-PAGE and western blotting using anti-Flag antibodies. Quantification was performed using a Chemi Doc-It system. Input represents 1% of the total PHD2 added to each reaction. The degree of binding observed to GST-HIF-2 α (516–549) was 100 ± 2.4 (arbitrary units, $n = 3$), while that observed with the mutant GST-HIF-2 α (516–549) was 11 ± 1.5 ($n = 3$) ($P < 0.01$).