Isolated erythrocytosis: study of 67 patients and identification of three novel germ-line mutations in the prolyl hydroxylase domain protein 2 (*PHD2*) gene

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ABSTRACT

The oxygen sensing pathway modulates erythropoietin expression. In normal cells, intracellular oxygen tensions are directly sensed by prolyl hydroxylase domain (PHD)-containing proteins. PHD2 isozyme has a key role in tagging hypoxia-inducible factor (HIF)-α subunits for polyubiquitination and proteasomal degradation. Erythrocytosis-associated *PHD2* mutations reduce hydroxylation of HIF-α. The investigation of 67 patients with isolated erythrocytosis, either sporadic or familial, allowed the identification of three novel mutations in the catalytic domain of the PHD2 protein. All new mutations are germ-line, heterozygous and missense, and code for a predicted full length mutant PHD2 protein. Identification of the disease-causing genes will be of critical importance for a better classification of familial and acquired erythrocytosis, offering additional insight into the erythropoietin regulating oxygen sensing pathway.

Key words: isolated erythrocytosis, prolyl hydroxylase domain protein 2, oxygen sensing pathway, JAK2, Epo.

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Introduction

Isolated erythrocytosis (IE) defines a clinical phenotype characterized by a persistent increase of red cell mass and hematocrit without apparent cause in the absence of any Janus kinase 2 (*JAK2*) mutations. These patients typically present without splenomegaly and may show a wide range of serum erythropoietin (Epo) levels, reflecting the heterogeneous genetic origins of this disorder.[1-3] In the presence of *JAK2* mutations, these patients are usually classified as polycythemia vera (PV) according to the WHO 2008 classification.[4]

In IE without *JAK2* mutations, the defect may be intrinsic to the erythroid progenitor cells. These cases are often familial, show a subnormal serum Epo level and are associated with mutations in the erythropoietin receptor (*EPOR*) gene.[3] In other cases of sporadic or familial IE, the defects may be extrinsic to the erythroid progenitor cells and due to a defect in the oxygen sensing pathway.[5,6] This pathway includes the prolyl hydroxylase domain protein 2 (PHD2),[7] von Hippel-Lindau tumor suppressor (VHL)[6] protein and α-subunit of the hypoxia-inducible factor (HIF-α)[8] protein. The α subunit can be either HIF-1α or HIF-2α, while the aryl hydrocarbon nuclear translocator forms the β subunit. Epo levels in these patients may range from low to increased,[9] reflecting the complexity and, maybe, the redundancy of the oxygen sensing system.

Under normal oxygen tension, PHD2, belonging to the Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase family, hydroxylates HIF-α allowing its ubiquitination by VHL, with consequent degradation of HIF-α in the proteasome and downregulation of the Epo production at physiological values.[10] Under hypoxic conditions, the HIF-α subunit hydroxylation by PHD2 is hampered, thus allowing HIF-α to escape VHL-mediated degradation. HIF-α subunits, therefore, accumulate and dimerize with the HIF-β subunits, migrate into the nucleus and regulate the transcription of several target genes including those coding for Epo,[10] causing an increased mRNA and protein production of this cytokine.

This study aimed to identify *PHD2* mutations in a cohort of 53 subjects with sporadic or familial IE. Fourteen additional cases presenting with an IE phenotype but with *JAK*[2V617F] mutation were also included. In all patients with *PHD2* mutation, *VHL* and *HIF2A*-exon 12 were also investigated. DNA samples from all cases were screened for *PHD2* mutations in the entire coding region and three new mutations were found. *JAK2* V617F and exon 12 mutations were studied as previously described.[11,12]

Design and Methods

Patients

In 67 patients referred to our laboratory for investigation of increased hemoglobin/hematocrit level from 1980 to 2007, a clinical phenotype of IE was established by confirming an increased hematocrit over several years in the absence of any apparent cause. In addi-

tion, normal platelet and white blood cell counts, and absence of splenomegaly were required. Bone marrow biopsy showed erythroid hyperplasia without proliferation of megakaryocytic and myeloid lineages in all cases. Serum Epo level, when available, was consistently normal or low. In 12 cases, a familial history of erythrocytosis (almost one first-degree relative with the same diagnosis to the proband) was found, while 55 patients presented sporadic type. Sporadic forms included 14 cases positive for the *JAK2*[V617F] and 5 cases with *JAK2*-exon 12 mutation, thus requiring reclassification as PV according to the new WHO 2008 criteria.[4]

DNA extraction and amplification

Peripheral blood samples were collected at diagnosis from all patients and epithelial cells from the index cases. The study was approved by the local Ethical Committee and all subjects gave their informed consent. Genomic DNA was extracted with Blood Core Kit B (Qiagen, Hilton, Germany) according to the manufacturer's instructions. Amplifications of the entire *PHD2* coding region (exon 1 to 5) and of the intron/exon boundaries were performed under standard conditions by polymerase chain reaction (PCR) using FastStart Taq DNA Polymerase (Roche, New Jersey, USA), in a GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, CA, USA). The three *VHL* exons and *HIF2A*-exon 12 were analyzed in the 3 *PHD2* mutated patients, according to a similar procedure; details for PCR conditions are available upon request. Primer sequences (obtained from Primer3 software v4.0, *http://frodo.wi.mit.edu/primer3/input.htm*) and PCR fragment lengths are shown in *Online Supplementary Table S1*.

Sequencing analysis

The PCR products were purified. Sequencing reactions were carried out using Big Dye® sequencing kit (Applied Biosystems). Direct sequencing was performed in both directions for all samples on an automated sequencer ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Electropherograms were compared with the *PHD2* (NCBI GenBank accession n. NM_022051), *VHL* (NCBI GenBank accession n. NM_000551) and *HIF2A* (NCBI GenBank accession n. NC_000002) wild-type sequences. Mutations were named in accordance with the standard international nomenclature guidelines recommended by the Human Genome Variation Society (HGVS, *http://www.hgvs.org/mutnomen/*).

Characterization of the new PHD2 mutations

To confirm the identity of the newly identified mutations, allele-specific PCR (AS-PCR) was performed for each allelic variant (Figure 1B). Reaction mixtures were run on a GeneAmp® PCR System 2700 at standard conditions and PCR fragments were analyzed by agarose gel. Primer sequences and PCR fragment lengths are listed in *Online Supplementary Table S1*.

The germ-line origin of these new genetic lesions was confirmed by their identification on DNA obtained from epithelial cells (buccal cotton-swab sampling) of the probands.

DNA samples from peripheral blood mononuclear cells from 100 normal controls with the same ethnic background were also screened for the novel mutations, by means of AS-PCR. Control DNA was obtained from the DNA samples stored in a biobank of healthy subjects at San Bortolo Hospital. Informed consent from the donors to the biobank had already been obtained for research purposes.

Results and Discussion

In this study, three different new germ-line heterozy-

Table 1. Clinical and laboratory features of the 3 patients carrying *PHD2* mutation.

	Patient 1	Patient 2	Patient 3
Age at diagnosis (years)/gender	61/M	29/M	60/M
Diagnosis	IE°	IE	IE
Hb (g/dL)	23.0	17.6	16.4
Epo (IU/mL)	2	5	23
Ht (%)	68.0	52.0	51.8
Platelets (×10⁹/L)	179	358	192
WBC (×10⁹/L)	7.5	11.1	7.5
Splenomegaly	No	No	No
Thrombosis	No	No	No
Hemorrhage	Yes	No	No
Therapy*	P, A	No	P, A
Evolution	No	No	No
Follow up (years)	19	9	20
Familial history of erythrocytosis	No	Yes	No
PHD2 mutation	N203K	K291I	K423E
HIF2A mutation (exon 12)	No	No	No
VHL mutation	No	No	No
JAK2[V617F] mutation	No	No	No
JAK2-exon 12 mutation	Yes	No	No

*IE: isolated erythrocytosis; IE°: subsequently reclassified as polycythemia vera according to WHO 2008; Hb: hemoglobin; Epo: erythropoietin; Ht: hematocrit; WBC: white blood cells count; * P: phlebotomy; A: aspirin.*

gous mutations of *PHD2* were found in a cohort of 67 patients with IE, including 14 and 5 cases subsequently reclassified as PV on the basis of *JAK2* V617F or exon 12 mutations. None of the *PHD2* mutated subjects was *JAK2*[V617F] positive while one co-harbored *JAK2*-exon 12 mutation. Main clinical and laboratory features of mutated patients are listed in Table 1.

In Patient 1, molecular studies revealed a C>G missense mutation at coding nucleotide position c.609 resulting in a N203K replacement in the putative amino acid sequence (Figure 1A). This genetic variation occurred in a male aged 80 years referred 19 years before for erythrocytosis. A large increase was seen in hemoglobin and hematocrit values. The patient resulted positive for the *JAK2*[547insL+I540-F547dup8] mutation[13] in exon 12, and his serum Epo level was low (less than 5 mUI/mL, normal range 5-25 mUI/mL). The same N203K *PHD2* mutation was detected on DNA extracted from four single Epo-independent endogenous erythroid colonies obtained from the patient.[13] Low serum Epo level presented by Patient 1 typically characterizes *JAK2*-exon 12 mutated subjects suggesting that, in this patient, clinical manifestations are mainly driven by his acquired *JAK2* lesion.

Patient 2 harbored *PHD2* mutation consisting of an A>T substitution at position c.872 leading to K291I replacement (Figure 1A). He was referred at age 29 for a familial history of erythrocytosis observed in his father, uncle and first cousin (Figure 1C).

The last *PHD2* mutation was found in Patient 3, a male aged 60 years at diagnosis with sporadic IE consisting in an A>G substitution at coding position c.1267, resulting in a K423E replacement (Figure 1A).

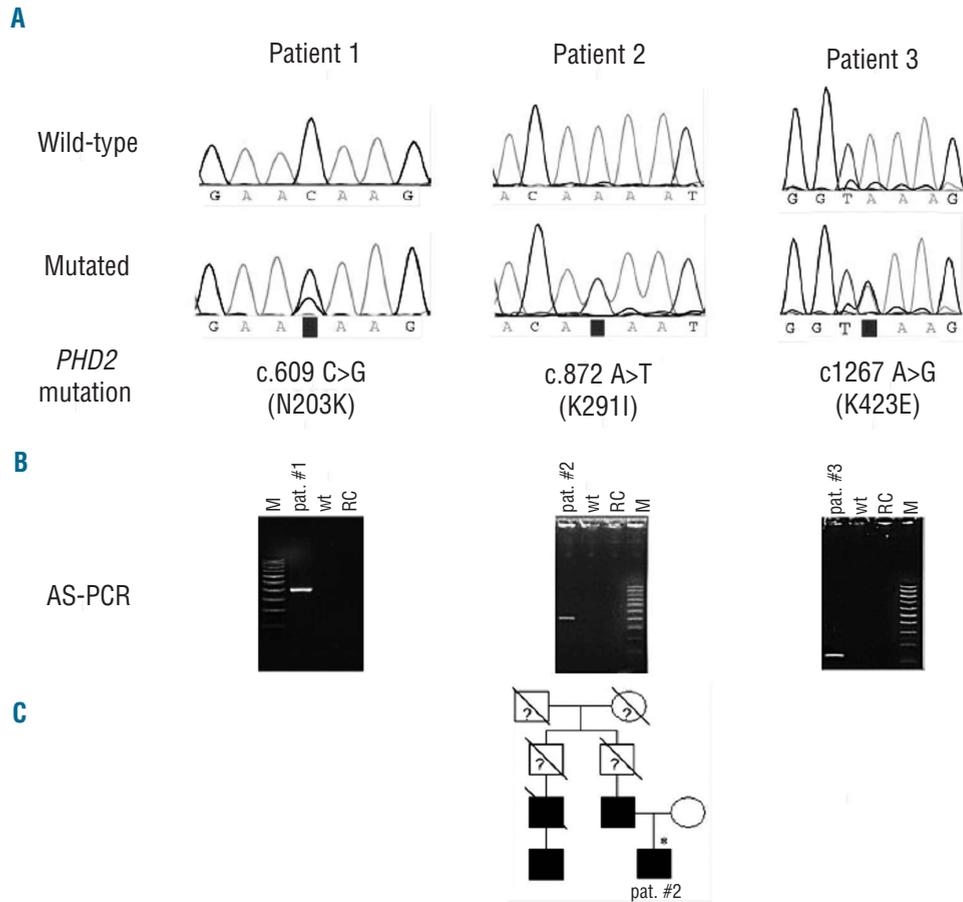To exclude the possibility that single-nucleotide poly-

A

B

C

Figure 1. Mutational analysis results and localization of the *PHD2* mutations. (A) Sequencing results of wild-type and mutated alleles in 3 patients. Nucleotide positions (GenBank accession, NM 022051), nucleotide changes and corresponding amino acid changes are indicated below. (B) Confirmation of the new mutations by allele-specific PCR (AS-PCR) and agarose gel electrophoresis. AS-PCR fragment lengths agree with the respective primer positions (*Online Supplementary Table S1*). Lane M: molecular weight marker, lane pat. #: patient's DNA amplified with mutation specific primer, lane wt: control DNA amplified with mutation specific primer, lane RC: reaction control (no template). (C) Pedigree of the family with erythrocytosis. Squares represent males, circles females, affected individuals are indicated in black and slashes indicate deceased members. Genetically tested individual is indicated by an asterisk.

morphisms (SNPs), increasingly reported during the last two years, could be interpreted as mutations, a control panel of 100 DNA samples from healthy subjects was screened by AS-PCR and found negative. All the three new erythrocytosis-associated *PHD2* mutations here reported involve the catalytic domain of the protein and add to those already known (Figure 2A-B).[16-19] We previously described as mutation a germ-line missense substitution,[20] Q157H, found in association with *JAK2*[V617F] in a propositus with polycythemia vera and found in an isolated form also in his son, presenting only with mild erythrocytosis. Q157H has been reported also as SNP (NCBI entry, rs61750991) with a frequency of around 2% in normal subjects but with a much higher frequency in cancer patients.[21] Whether this substitution contributes to the phenotypic expression of myeloproliferative neoplasms or erythrocytosis must be determined with more extensive studies. The most extensively studied and biochemically characterized *PHD2* mutations are P317R[16] and R371H.[17] P317 and R371 are close to the active site amino acids that directly interact with the Fe(II) (H313 and H374, respectively) (Figure 2C) and are likely to contribute to an HIF-α substrate binding groove. Three additional heterozygous *PHD2* mutations have been identified, producing truncated proteins lacking the catalytic domain (M202IfsX71, R281TfsX3)[18] or removing the last C-terminal amino acids tail (Q377X).[18] Finally, the H374R variation has been recently identified in a patient with erythrocytosis and recurrent paraganglioma,[19] suggesting that *PHD2* can also act as a tumor-suppressor gene. Our series of newly iden-

tified mutations are all germ-line and missense type, coding predicted mutant full length PHD2 proteins of 426 amino acids.

N203K, found in Patient 1, flanks the M202 residue in the helice α1 (Figure 2B), previously found to be mutated in IE,[18] and introduces a positive charged amino acid with a long side chain in place of a polar asparagine. The patient with this new genetic variation is also affected by a complex mutation in the exon 12 of *JAK2*.[13] The mutation presented by Patient 2, K291I, lies at the beginning of the strand β4 of the hydroxylase domain (Figure 2B) and, in contrast to the other heterozygous *PHD2* mutations, this novel mutation involves a codon that was neither conserved in other species, nor in the human isoforms PHD1 and PHD3. N203 and K291 map in the region responsible for the differential target preference of PHD2,[22] corresponding to residues 201-296 (N1 region, Figure 2A). It is worthy of note that this distinct domain is relatively far from the catalytic site; it contains the highest density of dissimilar residues between *PHD2* and *PHD3* and it was previously suggested to have a role on substrate discrimination.[22] The K291I genetic lesion points to haploinsufficiency, leading to a decreased prolyl hydroxylase activity disturbing the oxygen sensing pathway, as the cause of erythrocytosis in Patient 2. K423E substitution, harbored by Patient 3, causes the loss of a positive charged side chain with the acquisition of a complementary charged glutamate, three coding residues from the end of the enzyme. Lys423 is an evolutionary conserved residue in ortholog forms of PHD2 (*Mus musculus* and *Rattus norvegicus*,
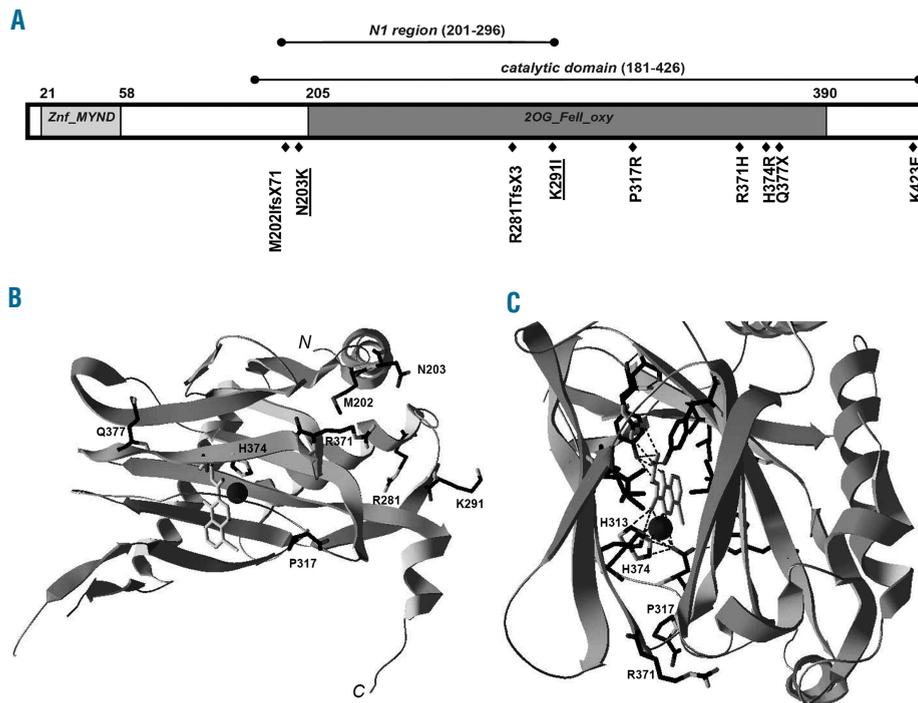
A

N1 region (201-296)

catalytic domain (181-426)

21 58   205   390

Znf_MYND | 2OG_FeII_oxy

M202IfsX71 N203K   R281TfsX3 K291 P317R   R371H H374R Q377X   K423E

B

C

Figure 2. (A) Schematic diagram representing the human PHD2 protein. *Znf_MYND*, Zinc finger MYND-like domain, *2OG_FeII_oxy*, 2-oxoglutarate and Fe(II)-dependent oxygenase-type domain. Diamonds indicate the location of the mutations, the novel genetic variations described in this study are underscored. Numbers indicate amino acid residue positions. **(B)** Three dimensional ribbon representation of the catalytic domain of the human PHD2, highlighting the locations of the mutated residues. The structure was generated using Swiss-PdbViewer v3.7 software[14] from Protein Data Bank coordinates of the X-ray structure, 2G1M.[15] Note that the C-terminal K423 is not visible as it falls outside the available PHD2 X-ray maps (residues from 188 to 403). **(C)** Active site contacts. P317 and R371 are close to the iron-chelating residues H313 and H374, respectively, both in the active site. Hydrogen bonds are shown as dashed lines.

http://toolkit.tuebingen.mpg.de/prot_blast) but it was absent in the two closely related human isozymes, PHD1 and PHD3, consisting of 407 and 239 amino acids, respectively. Mutation analyses of the catalytic domains of the PHDs as shown by X-ray crystallography for PHD2,[15] and results for other 2OG oxygenases,[23,24] suggested that removal of the C-terminus of the enzyme actually promotes uncoupled turnover of 2OG. However, due to the lack of the experimental solution of the C-terminal PHD2 structure,[15] it is not possible to anticipate the K423E mutation effects on protein stability and function.

A better identification of the disease-causing genes will improve classification of familial and acquired IE, in many cases still not characterized at a molecular level. Furthermore, the study of IE can give additional insight into the human oxygen sensing pathway that regulates Epo.

In conclusion, our findings emphasize the wide spectrum of genetic defects in IE. Furthermore, the co-presence of *PHD2* and *JAK2* mutations in one of the studied patients highlights the difficulties in clearly differentiating intrinsic (primary) from extrinsic (secondary) forms and suggests that a positivity for *JAK2* mutation should not prevent a more extensive investigation of other genes involved in the homeostatic system regulating erythrocyte production in sporadic or familial cases with erythrocytosis. Further studies will be required to assess the functional consequences on the substrate binding affinity and the differential effect of these new mutations on HIF-α hydroxylation.

Authorship and Disclosures

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