A study of 36 unrelated cases with pure erythrocytosis revealed three new mutations in the erythropoietin receptor gene

Maha Al-Sheikh,[1,2] Elodie Mazurier,[1] Betty Gardie,[5] Nicole Casadevall,[4] Frédéric Galactéros,[1,3] Michel Goossens,[1,2] Henri Wajcman,[1,2] Claude Préhu,[1,2] and Valérie Ugo[6]

[1]INSERM, U841, Créteil; [2]Service de Biochimie et Génétique, AP-HP, Groupe Henri Mondor, Albert Chenevier, Créteil; [3]Unité de Génétique du Globules Rouges, AP-HP, Groupe Henri Mondor, Albert Chenevier; [4]Service Hématologie Biologique, AP-HP, Hôpital Hôtel Dieu, Paris; [5]Institut Gustave Roussy, Villejuif; [6]Laboratoire d'hématologie, Centre Hospitalier Universitaire de Brest, Université de Bretagne Occidentale, Brest, France

ABSTRACT

Thirty-six unrelated cases with erythrocytosis of unknown origin were investigated. Exons 5-8 of the erythropoietin receptor gene (*EPOR*), the von Hippel-Lindau gene, and the prolyl hydroxylase domain protein 2 gene (*PHD2*) were screened by direct DNA sequencing. The Janus kinase 2 mutation, JAK2 (Val617Phe), was screened by allele specific PCR. In this study, three new mutations of *EPOR* causing deletions in exon 8 were found: the first led directly to a stop codon [g.5957_5958delTT (p.Phe424X)], the second to a stop codon after one residue [g.5828_5829delCC (p.Pro381GlnfsX1)] and the third to a stop codon following a frameshift sequence of 23 residues [g.5971delC (p.Leu429TrpfsX23)]. One patient had a previously reported *EPOR* mutation [g.6146A>G (p.Asn487Ser)] and another, a silent one (g.5799G>A). All were heterozygotes. In addition, 2 patients were positive for JAK2 (Val617Phe), and 2 reported elsewhere, were mutated in the *PHD2* gene [c.606delG (p.Met202IlefsX71).

Key words: polycythemia, erythrocytosis, erythropoietin receptor, von Hippel-Lindau, JAK2, prolyl hydroxylase domain protein 2.

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Introduction

Polycythemia refers to a group of disorders with increased hematocrit and hemoglobin levels, and increased red cell mass. Most polycythemia cases are acquired, secondary to a chronic tissue hypoxia, or to a primary bone marrow disease, such as polycythemia vera (PV). In this latter case activating mutations in the Janus kinase 2 (*JAK2*) have been found: *JAK2* (Val617Phe)[1] is the most frequent, however, 10 others have been recently reported in exon 12.[2-3] Congenital polycythemia can result from various etiologies such as hemoglobins with high oxygen affinity, deficiencies in 2,3-diphosphoglycerate mutase,[4] mutations of the *EPOR*, and of genes coding for factors involved in the oxygen sensing pathway[5] [Von Hippel-Lindau (*VHL*), prolyl hydroxylase domain 2 (*PHD2*)].[6-8]

Percy recently reviewed the mutations in the *EPOR* associated with erythrocytosis[9] (an absolute increase in red cell mass and hematocrit without elevation of the megakaryocytic or granulocytic lineages). This subgroup of abnormalities is usually characterized by low to normal plasma erythropoietin levels, and hypersensitivity of erythroid progenitors to exogenous erythropoietin *in vitro*.[10] The patients are usually clinically asymptomatic or presenting with mild symptoms, however, this condition could still contribute to cardiovascular problems.[11] We investigated 36 unrelated cases with erythrocytosis of unidentified origin. In all samples we screened for *EPOR* abnormalities, focusing on the exons encoding for the cytoplasmic region (exons 7-8) which interacts with kinases, and the transmembrane region (exon 6) which is important for proper receptor activation.[12] All these patients have already been investigated for *PHD2* mutations:[8] two sibs and four patients with missense mutations were found. In addition, we searched for *JAK2* (Val617Phe) and *VHL* mutations. This report focuses on the mutations found in *EPOR*.

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Correspondence: Valérie Ugo, Laboratoire d'Hématologie, Hôpital Morvan, Centre Hospitalier Universitaire de Brest, 5 Avenue Foch, 29200 Brest, France.
E-mail: valerie.ugo@chu-brest.fr

Design and Methods

Patients

The patients were referred to our laboratory for molecular diagnosis of erythrocytosis. Polycythemia vera or causes of secondary erythrocytosis were eliminated. All patients had initially elevated hemoglobin and hematocrit levels. None of them carried hemoglobin with increased oxygen affinity. In 10 cases a familial history of erythrocytosis was found, and a total of 44 samples were analyzed corresponding to 36 unrelated cases. The hematologic data of the patients are summarized in Table 1.

Erythroid colony formation assays

Bone marrow or peripheral blood mononuclear cells were used, and the assays performed according to standard procedures.[13]

Molecular biology studies

All patients gave their signed informed consents. The study was approved by the local ethics committee and performed in accordance with the World Medical Association Declaration of Helsinki. Genomic DNA was prepared from peripheral blood by phenol chloroform procedure. Screening for *JAK2* (Val617Phe) mutation was performed by allele-specific polymerase chain reaction (PCR), as previously described.[14] Exons 5-8 of *EPOR* gene were amplified by PCR using Ampli Taq® DNA polymerase (Roche, New Jersey, USA), in a Gene Amp® PCR system 2700 (Applied Biosystems, Foster city, CA, USA). Details and primer sequences are available upon request. The PCR products were purified. Sequencing reactions were carried out using Big Dye® sequencing kit, (Applied Biosystems) and analyzed on the ABI Prism® 3100 Genetic analyzer (Applied Biosystems) according to the manufacturer's protocol. The three exons of *VHL* gene were analyzed according to a similar procedure (details for primer sequences and PCR conditions are available upon request).

Table 1. Hematologic and clinical presentation of the 36 cases including 44 patients with presumed pure erythrocytosis.

			Median	*Range*
Age at diagnosis (years)	Males: 32	Females: 12	45.5	6-76
Gender				
Hb (g/dL)		18.5	16.2-21.9	
Hct (%)		55	47-66	
WBC ($\times 10^9$/L)		7.1	3.5-13.1	
Platelets($\times 10^9$/L)		206.5	134-324	
epo (mUI/mL)	[1]:4	N: 13	[2]: 9	NA: 18
RCM	[1]: 22	NA: 22		
EEC assays (neg, NA)	Neg: 18	NA: 26		
Familial history of erythrocytosis	A: 11	P: 10	NA: 23	

epo: erythropoietin, normal range (5-24mUI/mL); 1: number of patients with elevated values; N: number of patients with normal values; 2: number of patients with low values; NA: data not available RCM; red cell mass; 1: number of patients with elevated values. NA: data not available; EEC: endogenous erythroid colony. neg: negative result; NA: data not available. Familial history of erythrocytosis; A: absent; P: present; NA: not available.

Results and Discussion

In this study, three different mutations of *EPOR*, and one frameshift mutation of *PHD2* appeared to be responsible for the observed erythrocytosis. In addition, 2 patients had the JAK2 (Val617Phe) mutation. The first *EPOR* mutation resulted in a stop codon at position 424 (p.Phe424X), predicting a protein shortened by 85 residues. It was found, over 3 generations, in 4 members of a family (Figure 1A). Erythropoietin level in the third generation was low. Erythroid colony formation assays showed hypersensitivity of erythroid progenitors to exogenous erythropoietin (Figure 1B). Electrophoresis of the PCR product showed a heteroduplex. Sequencing of the PCR revealed a heterozygous deletion of two thymines at positions 5957 and 5958 in exon 8 (g.5957_5958delTT) (Figure 1C).

The second *EPOR* mutation was a two nucleotides deletion encoding at position 381 for a glutamine followed by a stop. This was observed in a 47-year-old woman (Hb: 18.5g/dl, Hct: 56%). One of her cousins was erythrocytosic as well, with a low EPO level. Both patients were treated by venesection. Electrophoresis of the PCR product revealed a heteroduplex. Sequencing showed a heterozygous deletion of cytosines 5828 and 5829 in exon 8 (g.5828_5829delCC) (Figure 1D), replacing codon 382 by a stop codon. This led to a 127 amino acid truncation (p.Pro381GlnfsX1), which is the largest reported to date in the *EPOR* molecule.

The third *EPOR* mutation led to a 57 amino acid truncation. This case of familial erythrocytosis, observed in a 31-year-old mother, and in her two daughters (9 and 11 years old), was discovered when the youngest was hospitalized at 9 years old for a convulsive episode associated with fever. Because erythrocytosis was observed (Hb 20.2 g/dL, Hct 58%) a complete blood count was carried out for the sister and the mother, revealing Hb levels of 18.8 and 20.6 g/dl, and Hct 54.8 and 60.2% respectively. The proband had an EPO level of less than 5 mUI/mL, (normal range: 5-24 mUI/mL). Electrophoresis of the PCR products showed no special feature, but DNA sequencing revealed a heterozygous deletion of a cytosine at position 5971 in exon 8 (g.5971delC) (Figure 1E). This caused a frameshift at position 429, predicting an introduction of 23 amino acids followed by a premature stop codon (p.Leu429TrpfsX23). The three mutations associated with familial erythrocytosis described above result in truncation in the distal region of the protein, involving the loss of 6 or 7 functionally important tyrosines. In addition, a previously described mutation[15] was found in a 62-year-old man with erythrocytosis (Hb 17.4 g/dl, Hct 54%, normal serum EPO level), treated by venesection. Sequencing of the PCR product of exon 8 showed a heterozygous A>G mutation of nt 6146 resulting in an aspargine to serine substitution at position 487

[g.6146A>G (p.Asn487Ser)]. However, the patient had a chronic respiratory failure with an arterial SaO2 of 86%. This raised the question of whether this base change might be a polymorphism or whether it has some relation with the erythrocytosis. The *in vitro* studies in murin Ba/F3 cell line could not demonstrate if this mutation had biological consequences.[15] We found the last *EPOR* mutation in a 6-year-old erythrocytosic child (Hb 19 g/dL), with an elevated serum EPO level (30.8 mUI/mL) and no familial history of erythrocytosis. Sequencing of the PCR product of exon 8 showed a heterozygous guanine to adenine substitution at nucleotide 5799 (g.5799G>A), which does not modify the encoded amino acid, and thus is unlikely to be the cause of erythrocytosis.

This work emphasizes the negative growth-regulatory role of the distal region of the *EPOR* molecule in erythropoiesis. After EPO binding and conformational change of *EPOR*, JAK2 triggers the signaling cascade by autophosphorylation and phosphorylation of *EPOR* on tyrosine residues, which become docking sites for positive and negative regulators. The former group includes: signal transducers and activators of transcription (STAT5a/b), p85α regulatory subunit of PI-3Kinase, and Lyn tyrosine kinase.[16] Among the negative regulatory signals are protein tyrosine phosphatase (SHP-1), CIS (cytokine inducible Src homology-2 containing proteins) or SOCS (suppressors of cytokin signaling), and Lnk.[17] According to residue positions in the human *EPOR*, it has been suggested that SHP1 interacts with P-Tyr454,[18] CIS3, also referred to as SOCS3, interacts with P-Tyr426, P-Tyr454 and P-Tyr456,[19-20] down regulating cytokine signaling in each case. To date, including this work, 16 mutations affecting the intracellular domain of *EPOR* have been described.[9] The percentage of familial and congenital polycythemias found, in this study, to be associated with *EPOR* mutations in exon 8 is similar to that described in the literature (<15%). This suggests that mutations in other regions of the *EPOR*, or in other genes, could be responsible for the unresolved cases.[21] Mutations affecting the negative or the positive regulators of *EPOR* signaling cascade are candidates for further exploration. In this study, screening for JAK2 (Val617Phe) mutation was found positive in 2 patients, which were reclassified as PV (Table 2). This observation is in agreement with two reports that JAK2 (Val617Phe) is found at a low incidence in patients with idiopathic erythrocytosis,[22,23] and has to be screened for. The 2 patients did not fulfill the criteria of PV since both had normal leukocyte and platelet counts, no splenomegaly, and negative endogenous erythroid colony (EEC) assay. This is also in agreement with the reported patients' characteristics.[22,23] For the second patient, the relative percentage of the JAK2 (Val617Phe) allele could be quantified on granulocytes (stored before cytotoxic treatment) and was found to be quite low, about 5%. This may argue for a *false negative* result of the EEC assay in this patient, because if only few

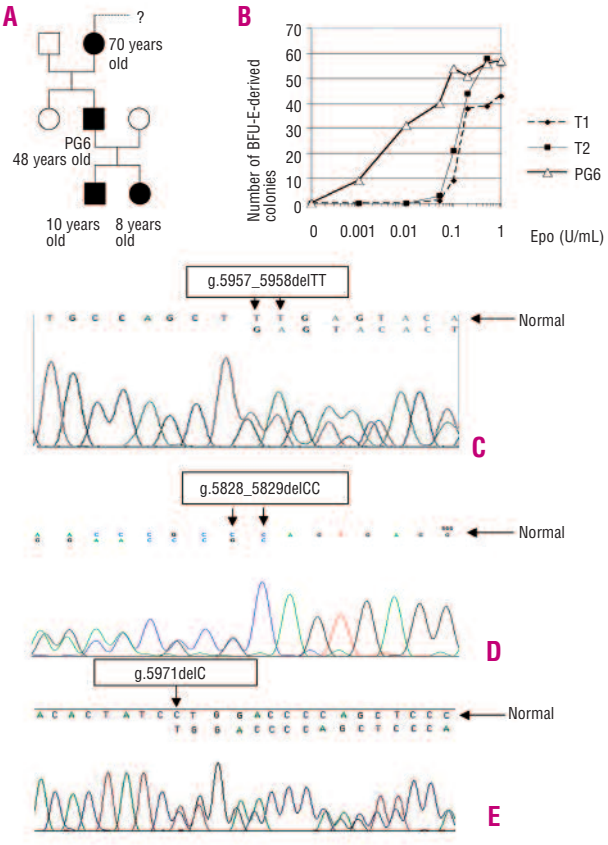

Figure 1. Experimental data concerning the first (A, B, C), the second (D), and the third (E) *EPOR* mutations. (A) Pedigree of the 3-generation family. (B) Erythroid colony formation assays in one member of the family (PG6) and in 2 normal controls (T1 and T2). The vertical axis indicates the number of BFU-E-derived colonies per $2.5x10^5$ peripheral blood mononuclear cells. The final EPO concentration added to the cultures is indicated in the horizontal axis. It shows *in vitro* hypersensitivity of BFU-E to erythropoietin at low concentrations. (C) DNA sequencing showing g.5957_5958delTT. (D) DNA sequencing showing g.5828_5829delCC. (E) DNA sequencing showing g.5971delC.

Table 2. Main hematologic characteristics of the two patients found positive for the JAK2(Val617Phe).

Age at diagnosis	*Hb (g/dL)*	*Hct (%)*	*WBC (x10⁹/L)*	*Platelets (x10⁹/L)*	*EPO mUI/mL*	*RCM (%)*	*EEC assays*	*Treatment*	*Comments*
64	19.5	58.3	7.1	191	NA	NA	neg	Venesection	Died at the age of 85 years (cancer of prostate)
53	16.9	49.5	6.7	246	Normal	>30.2	neg	Venesection	

hematopoietic progenitors harbored the JAK2 (Val617Phe) allele conferring EPO hypersensitivity, the number of endogenous colonies might be low and difficult to detect. In contrast to other studies reporting that mutations in the VHL gene constitute around 10%[24] of all cases with idiopathic erythrocytosis, the screening for VHL mutations was negative in all our patients who presented with variable serum EPO levels and a wide age range. The *PHD2* mutation found in the two sibs [c.606delG (p.Met202IlefsX71)] led to a protein truncated by its 154 C-terminal amino acids. Further studies are in progress to verify biological consequences of the point mutations.

The screening for exon 12 mutations in JAK2 must still be performed on new samples of the DNA extracted from granulocytes, or from that of the endogenous erythroid colonies.[3] In conclusion, our findings add to the spectrum of the molecular defects identified so far reflecting the heterogeneity of the erythrocytosis. However, in the majority of the patients, the genetic defect(s) remain elusive and require further research.

Authorship and Disclosures

MA-S performed PCR and sequencing experiments, collected the clinical data and wrote the manuscript

EM contributed to the experimental work; BCG performed VHL sequencing; HW wrote the manuscript and discussed the results; CP designed the study, performed sequencing experiments and wrote the manuscript; VU designed the study, performed EPOR sequencing, JAK2 V617F screening, and in vitro cultures, and contributed to writing the manuscript. CP and VU contributed equally to this work. The authors reported no potential conflicts of interest.

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