Molecular genetic analyses in familial and sporadic congenital primary erythrocytosis

Susana Rives, Heike L. Pahl, Lourdes Florensa, Beatriz Bellosillo, Andrea Neususess, Jesus Estella, Klaus-Michael Debatin, Elisabeth Kohne, Klaus Schwarz, Holger Cario

From the Department of Pediatric Hematology and Oncology, Hospital Sant Joan de Déu, Barcelona, Spain (SR, JE); Department of Anaesthesiology, University Hospital Freiburg, Freiburg, Germany (HLP); Laboratori de Citologia Hematologica, Servei de Patologia, Hospital del Mar-IMAS, Barcelona, Spain (LF); Laboratori de Citogenètica i Biologia molecular, S. Patologia, Hospital del Mar, Barcelona, Spain (BB); Department of Pediatrics, University Hospital Schleswig-Holstein, Campus Kiel, Germany (AN); Department of Pediatrics, University Hospital Ulm, Ulm, Germany (K-MD, EK, HC); Institute for Transfusion Medicine, University Hospital Ulm, Ulm, Germany and Institute for Clinical Transfusion Medicine and Immunogenetics, Ulm (KS).

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Correspondence:
Holger Cario, M.D.. Department of Pediatrics, University Hospital Ulm, Eythstrasse 24, D-89075 Ulm, Germany.
E-mail: holger.cario@uniklinik-ulm.de

ABSTRACT

Dominant mutations in the erythropoietin receptor (EPOR) gene account for only about 15% of cases of primary congenital erythrocytosis. To search for molecular alterations in patients with this disorder. Sixteen patients with Epo <10 mU/mL were studied, 3 were related. Analyses included *EPOR* and *JAK2* gene sequencing, quantitative PRV-1 RT-PCR, and erythroid colony assays. A novel sporadic *EPOR 1453G→A* (Trp439Stop) mutation was detected. All familial cases, varied in phenotype, presented the *EPOR 1414C→G* (Tyr426Stop) mutation. *JAK2* mutations are not involved in the pathogenesis of primary congenital erythrocytosis.

Key words: congenital erythrocytosis, polycythemia, erythropoietin receptor, primary familial erythrocytosis, PCFP, JAK2.

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Congenital erythrocytoses are rare, heterogeneous disorders. Primary congenital erythrocytosis is caused by an intrinsic defect of erythroid precursors. In contrast, secondary erythrocytoses are driven by extrinsic factors, predominantly erythropoietin (Epo). Elevated Epo may either constitute a physiological response to tissue hypoxia of various etiologies, or reflect a disorder of oxygen-sensing, as for example in Chuvash polycythemia.[1] Primary familial congenital polycythemia (PFCP) is a rare autosomal dominant disorder.[2] Sporadic cases with identical clinical, hematological, and molecular findings have also been described.[3,4] PFCP is caused by mutations in the Epo receptor (*EPOR*) gene. Eleven different mutations resulting in truncation of the C-terminal cytoplasmic receptor domain have been reported to date.[4-15] The truncated Epo receptor lacks negative feedback regulation causing hypersensitivity to circulating serum Epo.[2,9] Due to the impaired regulatory feedback, the majority of patients present with serum Epo levels below the normal range. However, *EPOR* gene mutations account for less than 15% of patients with primary congenital erythrocytosis.[3] Thus, in the majority of patients, the underlying defect remains unknown. While the $JAK2^{V617F}$ mutation which is characteristic for polycythemia vera (PV) does not seem to be involved in primary congenital erythrocytosis,[16] other *JAK2* mutations might lead to this disorder.

Design and Methods

Sixteen patients were selected from a group of 70 patients with congenital erythrocytosis of unknown origin. Selection criteria was low serum Epo (< 10 mU/mL). Given that Epo levels in PFCP patients may be in the lower normal range and since different Epo assays were applied, we used this rather high Epo level as the cut-off to define a group of patients with presumed primary congenital erythrocytosis. The patients did not present additional clinical or hematological characteristics of PV (Table 1). All patients/parents gave their written informed consent to the genetic analysis, data analysis and publication. The study was approved by the local ethics committee and performed in accordance with the World Medical Association Declaration of Helsinki of 1975, as revised in 2000.

Table 1. Hematological parameters in patients with presumed primary congenital erythrocytosis.

	All Patients (n=16)		*Patients with EPOR mutation*			
	Median	*Range*	*Pt. 10*	*Pt. 12*	*Pt. 13*	*Pt. 14*
Age [years]	12.5	5-66	5	40	14	22
Gender [m/f]	8/8		f	f	m	m
Hemoglobin [g/dL]	17.5	12.9-22.8	16.1	12.9	22.8	19.7
Hematocrit [%]	52	41-66	50	41	66	57
MCV [fL]	85.5	73-109	84	73	82	85
Leukocytes [$\times 10^3/\mu L$]	6.5	4.9-11.1	7.9	7	5.8	5.4
Platelets [$\times 10^3/\mu L$]	208	111-332	315	200	156	130
Erythropoietin [mU/mL]	7	2.3-9.4	< 1	2.3	< 7.8	n.d.
(Normal range)			(4-20)		(7.8-24.6)	

Erythropoietin receptor (EPOR) gene analysis

PCR reactions were performed using the (Hot-Star) Taq DNA Polymerase Kit (QIAGEN, Hilden, Germany) in a GeneAmp® PCR System 9700 (Applied Biosystems, Darmstadt, Germany). Details and primer sequences are available upon request. After PCR product purification, sequencing reactions were performed with the DNA Sequencing Kit (Big Dye™, Applied Biosystems). Products were analyzed on an ABI 3100 DNA Genetic Analyzer (Applied Biosystems).

Janus kinase 2 (JAK2) gene analysis

JAK2 gene analysis was performed as described for *EPOR* with different PCR conditions (details primer sequences available upon request).

Allele-specific PCR of JAK2 exon 12

PCR reactions from genomic DNA were performed using Hot-Star Taq DNA polymerase, with 0.5 μM of each of the two forward primers and 1 μM of a common reverse primer (primer sequences as reported by Baxter *et al.*[17] with some modifications to the allele-specific primer). PCR products were separated by a 1% agarose gel and visualized by ethidium bromide staining.

Endogenous erythroid colony (EEC) assay

The EEC assay was conducted and scored precisely as previously described.[18]

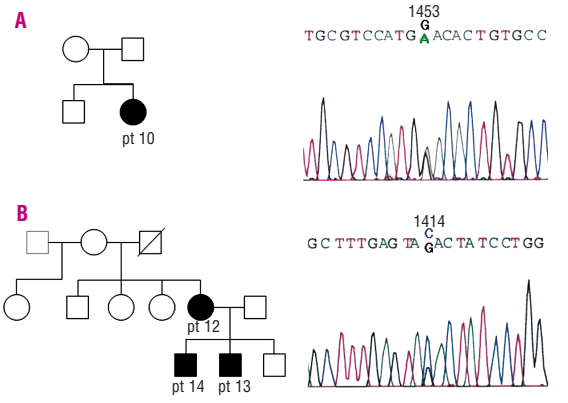

Figure 1. Pedigrees (left side) and *EPOR* gene sequence analysis of patients with *EPOR* gene mutation *EPOR 1453G→A* (A) and *EPOR 1414C→G* (B).

Erythropoietin sensitivity analysis

Peripheral blood mononuclear cells from patients and controls were plated in methylcellulose medium (2×10^5/mL; Methocult H-4533, Stem Cell Technology). Epo was added at the following concentrations: 0.0, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 3 and 4 IU/mL. Cultures were maintained in a humidified atmosphere with 5% carbon dioxide in the air at 37°C. Large erythroid colonies (BFU-E) were scored at day 14.

PRV-1 mRNA quantification

Granulocyte *PRV-1* mRNA levels were determined as previously described.[19]

Results and Discussion

Sequencing analysis of exon 7 and 8 of the EPOR gene encoding the C-terminal part of the receptor was performed for all patients. A mutation in *EPOR* exon 8 was detected in four patients. *EPOR* exons 1-6 were sequenced in 10/12 patients with normal exon 7 and 8 sequences. Only wild type sequences were found.

The same patients were examined for the presence of a genomic *JAK2* gene mutation. None of the patients presented a *JAK2* mutation. Since the acquired $JAK2^{V617F}$ mutation may be missed by direct sequencing, an additional allele-specific PCR was performed which revealed only wild type alleles in all patients.

PRV-1 mRNA expression in granulocytes and EEC formation were studied in 7/12 patients without *EPOR* mutation. None of them presented an increased *PRV-1* mRNA expression or EEC's growth.

A heterozygous mutation EPOR1453G→A (mRNA RefSeq. NM_000121) as yet unreported was detected in a 5-year old girl (pt. 10, Figure 1A). The parents and the brother had wild type *EPOR* and normal hemoglobin.

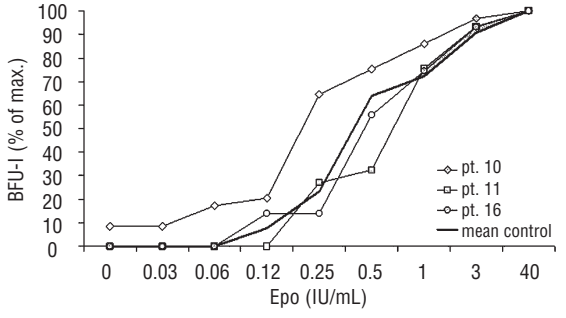

Figure 2. ***In vitro*** **studies of erythroid progenitor cells. Erythroid progenitor of patient 10 exhibits hypersensitive response to Epo compared to the control (mean of three samples) and the other two patient samples.**

Using short tandem repeat analysis (Identifiler Kit, ABI Biosystems) we confirmed paternity (*data not shown*). Since the parents themselves did not have an *EPOR* mutation, the change of the *EPOR* gene of the patient has to be regarded as *de novo*. The girl was phlebotomized shortly after birth because of *neonatal polyglobuly* with a hematocrit of 0.70. Erythrocytosis was later diagnosed by chance during a diagnostic work-up for failure to thrive at the age of 16 months. The family history was negative for erythrocytosis, occurrence of thrombosis, infarction, or phlebotomies. The patient presented high hemoglobin and hematocrit but normal leukocyte and platelet counts, and low Epo. The mutation *EPOR1453G→A* creates a premature stop codon (TGG to TGA) replacing tryptophan (Trp439Stop). The first reported *EPOR* gene mutation in a large Finnish group of related subjects affected the same codon (*EPOR1452G→A*).[7] This mutation was later identified in an English patient with sporadic erythrocytosis.[4] It leads to a 70 amino acid truncation of the intracellular domain of the Epo receptor, deleting six tyrosine residues (Tyr454, 456, 468, 485, 489, and 504), causing Epo hypersensitivity.

Epo sensitivity of erythroid progenitors could be studied in three patients (patients #10, 11, and 16). Only cells of patient #10, who carries the novel *EPOR* mutation, showed the typical hypersensitive response to Epo. These cells formed low numbers of BFU-E colonies even in the absence of Epo (Figure 2). Epo-independent erythroid colony growth, similar to that found in PV, had previously been reported in another PFCP patient.[9]

The second EPOR gene mutation was detected in a mother and two of her three sons (patients #12, 13, 14, Figure 1B). They presented a heterozygous mutation *EPOR 1414C→G* (mRNA RefSeq. NM_000121) creating a stop codon at position 426 (Tyr426Stop) leading to the deletion of 83 cytoplasmic amino acids including seven tyrosine residues (Tyr426, 454, 456, 468, 485, 489, and 504). This mutation was previously described in the study of the family that had led to the original description of the *PFCP* entity.[10] The 14-year old propositus (#13) of our family was diagnosed with erythrocytosis when examined by a pediatric endocrinologist because of apparent gynecomasty. The family's medical history revealed previous erythrocytosis of the 40-year old mother (#12) and current erythrocytosis of the 23-year old brother (#14). In both cases, the origin of the erythrocytosis was unknown. At the time of the propositus' presentation, his mother had normal hemoglobin with low MCV and MCH. It is interesting to note that a young girl from the original PFCP family who inherited the EPOR mutation also appeared to be phenotypically normal.[10] In contrast to this girl, it is known that the mother of our propositus had erythrocytosis during childhood and adolescence. At the age of 27 her hemoglobin was 17.5 g/dL (MCV 86 fl). At present, she has a very low serum ferritin concentration of 5 ng/mL (nL 15-150) and transferrin saturation of 4 % indicates iron deficiency, offering a possible explanation for her apparently normal hematological presentation. In contrast, the girl from the original PFCP family was reported with erythrocyte indices within the age-specific normal range.[10]

The mother presented with a serum Epo concentration below the normal range despite her low normal hemoglobin. The origin of the low Epo synthesis and release is unknown since she did not display features of impaired renal function. It is possible that an additional feedback loop other than the hemoglobin level that suppresses Epo synthesis and release when the Epo receptor is continuously activated could be involved.

The propositus' brother (#14) had suffered a deep vein thrombosis at the age of 18. Later additional hyperviscosity symptoms occurred at intervals and were relieved by phlebotomies. A 12-year old brother displayed no hematological abnormalities and does not carry the EPOR mutation. The four siblings of the mother and the propositus' maternal grandmother have normal hemoglobin and erythrocyte indices. The propositus' maternal grandfather died of unknown cause at the age of 73.

Since only a minority of primary congenital erythrocytoses is caused by *EPOR* mutations, it has been suggested that subsequent parts of the Epo signalling pathway might be involved in other cases. In PV – the acquired form of primary erythrocytosis – the *JAK2*^{V617F} mutation plays a central pathogenetic role.[17,20] In a group of 42 patients with idiopathic erythrocytosis with Epo < 15 mU/mL, the mutation was identified in a single female patient presenting primary erythrocytosis for 9 years without any additional features of PV.[16] We excluded similar cases among our patients by allele-specific *JAK2* PCR and *PRV-1* mRNA expression analysis. We also excluded genomic mutations in cod-

ing *JAK2* regions as the potential cause of primary congenital erythrocytosis. Further studies to identify the underlying cause of the disease in the majority of patients with primary congenital erythrocytosis are necessary. Further investigation of the phenotypic variability observed in some cases will also increase our knowledge on the regulation of hematopoiesis and may contribute to the development of treatment strategies.

Authors' Contributions

SR and HC wrote the manuscript. SR, AN, JE, and HC were responsible for the clinical management of patients and data acquisition; HLP, LF, BB, and KS were involved in all aspects of the realization of the study, in particular concerning laboratory analyses and the interpretation of experimental data. In addition, HLP and KS contributed essentially to the drafting and editing of the article; EK contributed essentially to the recruitment of patients and the evaluation of specific hematological aspects, and reviewed the manuscript critically. KMD promoted this trial, was involved in the study conception, and reviewed the manuscript critically; HC was the principal investigator, conceived and designed the study. All co-authors approved of final version of the manuscript to be submitted.

Conflict of Interest

A part of the results was presented as poster presentation at the EHA conference in Amsterdam in June 2006.

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