Molecular study of Congenital Erythrocytosis in 70 unrelated patients revealed a potential causal mutation in less than *half* of the cases. (Where is/are the missing gene(s)?)

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Abstract

Introduction

Congenital Erythrocytosis can be classified as primary, when the defect is intrinsic to the RBC progenitors and independent of the serum erythropoietin (Epo) concentration, or secondary, when the erythrocytosis is the result of an up-regulation of Epo production. Primary erythrocytosis is associated with mutations in the *EPOR* gene, secondary congenital erythrocytosis can de due to mutations that stabilize the hemoglobin in the oxygenated form or to mutations in the genes that control the transcriptional activation of the *EPO* gene – *VHL*, *EGLN1*, *EPAS1*.

Material and Methods

With the main objective of describing the etiology and molecular basis of congenital erythrocytosis we have studied 70 consecutive unrelated patients presenting with idiopathic erythrocytosis from our hematology clinic or referred from other centers.

According to a study algorithm we have sequenced all the genes described as associated with congenital erythrocytosis.

Results and Discussion

Erythrocytosis molecular etiology was identify in 25 (36%) of the 70 subjects. High-affinity Hb variants were the most common cause, present in 20% of the cases. New mutations were identified in the *JAK2*, *EPOR*, *VHL* and *EGLN1* genes.

Conclusions

High affinity hemoglobin variants are a very rare cause of secondary congenital erythrocytosis, but it seems likely that their incidence may be underestimated. Our experience shows that in erythrocytosis with a dominant inheritance and normal or inappropriate high Epo levels, the *HBB* and *HBA* genes should be the first to be studied. In spite of the seven genes known to be involved in congenital erythrocytosis, the majority of the cases have unknown etiology.

Introduction

Erythrocytosis is caused by an increased red blood cell production, associated with a rise in hemoglobin and hematocrit. Erythropoietin (Epo), along with its receptor (EpoR), regulates the proliferation, survival, and differentiation of erythroid progenitor cells, leading to a tight control of red blood cell production (1, 2).

Erythrocytosis can be congenital or acquired. The most frequent forms are acquired, due to an intrinsic defect of the erythroid compartment, as in Polycythemia Vera (PV), or due to secondary factors, such as chronic lung or cardiac disease, sleep apnea, which due to hypoxia induce an elevation in the circulating Epo (3,4), or caused by some types of tumor in which there is an intrinsic EPO production.

Congenital erythrocytosis (CE) can also be primary secondary. Primary congenital erythrocytosis are caused by an intrinsic defect in the red blood cell progenitors and there for is independent of the serum Epo levels. The only known form is caused by mutations in the EpoR gene (*EPOR*) and is designated Primary Familial and Congenital Polycythemia (PFCP) (5).

Secondary CE are the result of an up-regulation of Epo production caused by an increased stability of the hypoxia inducible transcription factors (HIF). The activation of the Epo gene (*EPO*) is regulated by HIF, alterations that induce tissue hypoxia, like the presence of high oxygen affinity hemoglobins (Hbs) and 2,3-bisphosphoglycerate mutase (BPGM) deficiency, or alterations that deregulate the hypoxia sensing pathway, cause an increased stability of HIF with consequent increased *EPO* mRNA transcription and Epo synthesis (6,7).

The knowledge of the genetic causes of CE has substantially improved in the last few years with the identification of mutations in the *EPOR* gene and in the genes coding for the proteins implicated in the regulation of the HIF transcription complex: von Hippel-Lindau (*VHL*), prolyl hydroxylase domain 2 (PHD2, *gene EGLN1*) and hypoxia-inducible factor-2 *α* (HIF2α*, gene EPAS1)(8)* .

CE due to *HBB, HBA (HBA2 and HBA1)*, *EPOR, EGLN1* and *EPAS1* mutations have a dominant pattern of inheritance with sporadic cases of *de novo* mutations; CE due to *BPGM* and *VHL mutations* have a recessive pattern of inheritance, although some cases of unexplained *VHL* heterozygous presenting the disease have been described.

With the main objective of describing the etiology and molecular basis of congenital erythrocytosis we studied 70 consecutive unrelated patients presenting with idiopathic erythrocytosis, followed in our hematology clinic or referred from other centers. All the acquired secondary causes of increased hemoglobin/hematocrit levels were excluded as well as the presence of the *JAK2* V617F mutation (9).

Samples were sequenced for the genes associated with CE according to an algorithm based on serum Epo levels, familial history, and Hb studies (determination of P50 and high-performance liquid chromatography (HPLC))(Fg.1). Acquired mutations in the exon 12 of the *JAK2* gene, associated with PV, were excluded in selected samples (as mentioned in the algorithm), as previously published data indicates that mutations in this exon have been found in patients

initially diagnosed with idiopathic erythrocytosis, since their phenotype is mainly with erythrocytosis (10,11).

The elevated number of remaining cases of erythrocytosis with unknown etiology shows that other yet unidentified genes have to be implicated in this pathology.

Design and Methods

Patients

We studied 70 consecutive samples (designated Pt1 – Pt70) from patients referred for investigation of congenital erythrocytosis. All patients had normal leukocyte and platelet counts, and none had splenomegaly. Secondary acquired causes of increased hemoglobin/hematocrit levels, as cardiac, pulmonary, renal, or hepatic disorders had been excluded, as well as the presence of the *JAK2* V617F mutation. All the adult patients have a long history of erythrocytosis, most of them requiring regular phlebotomies.

Patients were mainly from Portugal (41 samples) and Spain (23 samples) , due to collaboration with the Red blood cell diseases group of the Portuguese Society of Hematology and the Spanish Group of Erythropathology. Six samples were from French patients. The study was approved by the local Ethical Committees and all subjects or representatives gave their informed consent.

Thirty five patients (50%) had familial history of erythrocytosis, twenty eight with at least one first-degree relative affected (parents, children or siblings). The remaining thirty five were either sporadic cases or with no information concerning familial history.

Nineteen (27%) of the samples were from female patients. Five of the patients (4 male and one female) had ages between seven and 18 years-old.

The clinical and hematologic data of the patients are summarized in Table 1.

Methodology

Molecular studies were guided according to the data on serum Epo level, P50, study of hemoglobins by HPLC and the familial history, based on the algorithm published by McMullin (12), with adaptations according to the data available in our patients (Fig. 1).

Samples with markedly reduced serum Epo levels (<3.0 mUI/mL; normal range 3 - 34 mUI/mL) were first screened for *JAK2* exon 12 mutations. Negative samples for *JAK2 exon12* mutations were then sequenced for *EPOR* mutations in the specific exons that code for the cytoplasmic domain of the Epo-R (exons 7 and 8). We have also sequenced exons 7 and 8 of *EPOR* in the samples with a normal/high serum Epo level without mutations in all the other genes studied.

Samples with normal/high Epo levels and a P50<22 mgHb or no P50 determination and/or a variant in the HPLC, were sequenced for *HBB* and *HBA* genes. In the cases were a variant was found in HPLC, according to the percentage of the variant, samples were first sequenced for *HBB* (when HbX>30%) or *HBA* (when HbX<30%).

From the remaining samples, those with a positive familial history were first sequenced for *EGLN1* (all the exons) and exon 12 of *EPAS1* (coding for the N-terminal activation domain were CE mutations have been described) and those with a negative familial history were sequenced for *VHL* (all the exons).

All the samples studied where a causative mutation was not found were screened for the other genes described as associated with CE (Fig.1).

As a complementary study we sequenced the exon 9 of the *EPAS1* gene in the samples without a mutation in all the genes studied.

Mutations nomenclature is according to the Human Genome Variation Society (http://www.hgvs.org/mutnomen/), the name of new mutations was checked with Mutalyzer (https://mutalyzer.nl/).

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using the DNA extraction kit QIAamp® DNA Blood Mini (QIAGEN, Hilden, Germany).

HBB, HBA1, HBA2, BPGM, EPOR (exons 7- 8), VHL, EGLN1, EPAS1 (exon 9 and12) and JAK2 (exon 12) genes were selectively amplified by PCR in a Biometra® TPersonal Thermal Cycler (AlphaGen, Iowa, USA). Details and primer sequences are available upon request. The resulting amplicons were then sequenced on a Hitachi 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.
The germline or somatic origin of the new mutations was confirmed by their identification on DNA extracted from hair follicle cells of the probands.

Founding mutations that were not described before have been tested by single strand conformational polymorphism (SSCP) in 100 DNA samples from normal controls.

***In Silico* Analysis**

Software programs SIFT (sorting intolerant from tolerant, http://blocks.fhcrc.org/sift/SIFT.html) PolyPhen-2 (polymorphism phenotyping, http://genetics.bwh.harvard.edu/pph2/) and Project HOPE (13-15) were used to predict the impact of the new variants on the structure and function of the affected proteins. The level of conservation of affected residues among orthologous *EGLN1* genes was evaluated by using the NCBI HomoloGene tool (http://www.ncbi.nlm.nih.gov/homologene).

Results and Discussion

Results are divided in three groups of samples, group 1 (n=8) patients with markedly reduced serum Epo levels (<3.0 mUI/mL); group 2 (n=50) patients with normal/high Epo level; group 3 (n=12) patients without Epo and P50 determination at diagnosis.

The results obtained are summarized in tables 2 and 3.

None of the mutations described here for the first time were found in a control group of 100 DNA samples from healthy subjects.

Primary Familial Congenital Polycythemia (PFCP)

Sequencing of the *JAK2* exon 12 in the eight patients of group 1 revealed, in two patients (Pt35 and Pt60), the presence of two different mutations already reported in the literature as associated with PV, *JAK2* N542-E543del and *JAK2* R541-E543delinsK (10,17). These are somatic mutations as none of them was present in the DNA extracted from the hair follicles. These patients have been reclassified as PV, according to the WHO guidelines (18). In Pt 32 we found a mutation in the exon-intron boundary *JAK2* c.1641+6T>C. This mutation will probably interfere with the consensus splicing but this was not investigated as the patient and other family members were not available for complementary studies.

Sequencing of the *EPOR* in the remaining 5 patients of group 1 revealed, in one of the patients (Pt30) a heterozygosity for a *frameshift* mutation due to the deletion of two nucleotides (*EPOR* c.1311_1312delTC) resulting in a premature termination at codon 443 which predicts the production of an EPOR molecule truncated for 66 amino acids at the C-terminal end of the protein (p.Pro438Metfs*6). Familial studies revealed two family members, belonging to three different subsequent generations, heterozygous for the same mutation, that was absent in the son of the propositus with normal hematological parameters (16).

In another patient of group 1 (Pt49) we found a heterozygous state for a nonsense mutation (*EPOR* c.1235C>A). The mutation creates a premature stop codon (p.Ser412*) predicting the production of a truncated EPOR for the last 97 amino acids at the C-terminal end of the molecule. Unfortunately, neither children nor other family members were available to be studied (16).

Sequencing of *EPOR* in the patients of group 2 and 3 revealed heterozygosity for a missense mutation, c.1460A>G (p.Asn487Ser) in two patients (Pt21 and Pt65), both with a moderate erythrocytosis, not requiring phlebotomies. This mutation was previously reported in other cases of CE (19, 20) but it doesn't seem to interfere with the negative regulatory domains of the protein. Functional studies are in course in order to explain, or refute, the pathogenic effect of this mutation.

Still in group 2, we have identified, in the heterozygous state, a not yet reported missense mutation (*EPOR* c.1310G>A; p.Arg437His) in one patient (Pt40). Pt40 is a 35-year-old man requiring regular venosection (his father was also c.1310G>A heterozygous with erythrocytosis). This mutation is predicted to be benign according to *in silico* analysis, in divergence with the familial data indicating a co-segregation of the mutation with the disease. Functional studies are in course in order to confirm or refute the pathogenic effect of this mutation.

High affinity Hbs

Sequencing of the *HBB* and *HBA* genes revealed the presence of a high affinity hemoglobin variant in 14 patients (Table 2).

We did not find pathological mutations in the *BPGM* gene.

Hypoxia sensing pathway

VHL

Sequencing of the *VHL* gene revealed a homozygous missense mutation (*VHL* c.586A>G) leading to a change of a lysine into a glutamic acid at CD196 (p.Lys196Glu; K196E) (Fig. 2A) in a 65 years old woman form Seville, Spain (Pt1). Her parents are second-degree relatives. Her two children, heterozygous, have normal Hb values.
This female patient, diagnosed at 33 years old, with Hb 20 g/dL, $7.0x10^6$ red blood cells (RBC), Hct 60%, also has a Type 1 diabetes mellitus under insulin treatment. She had no history or evidence of thrombotic complications or cancer. The patient had undergone regular phlebotomy treatment until the age of 60 years when her Hb stabilized at around 16.4 g/dl.

The missense mutation *VHL* K196E has never been described although a nonsense mutation in the same codon, *VHL* K196X, was found in patients with von Hippel-Lindau disease (21). This is the third description of a homozygous *VHL* mutation causing erythrocytosis (22, 23). This mutation is predicted to be probably damaging by *in silico* analysis (Fig. 2B). Molecular dynamics simulations and binding energies of wild type and mutated VHL showed that this mutation perturbs the Gln195 closure to Pro95 located at β-sheet forming the PQP site (Pro95-Gln96-Pro97). This change resulted in inducing effect on Tyr98 residue, a critical residue for VHL and HIF2α interactions (24)(Fig. 2C,D).

Another *VHL* mutation (c.524 A>G; p.Tyr175Cys) was found in the heterozygous state in a Portuguese girl who, beyond the erythrocytosis, also had ataxia – telangiectasia (Pt 39). The *VHL* mutation was inherited from the father, with normal hematological parameters (25). Similar cases of CE heterozygous for *VHL* mutations, with a normal carrier parent, have been described in the literature; the subjacent mechanism was not found yet.

PHD2 (*EGLN1)*

We found a *EGLN1* c.1000 T>C (p.Trp334Arg) mutation, in heterozygous state, in a French woman with a family history of polycythemia (Pt 26) (Fig. 3A). The same mutation was found in three members of the family, affecting 3 generations, with an autosomal dominant mode of inheritance (Fig. 3B). This mutation was not previously described in the literature. The Trp334Arg substitution, located in a highly conserved amino acid of the catalytic domain (residues 181-426), was predicted to be probably damaging by *in silico* analysis. Amino acid alignment of PHD2 orthologs showed conservation of this residue suggesting that they may exert a fundamental role in protein structure and/or function (Fig. 3C).

Three patients (Pt10, Pt58 and Pt69) are heterozygous for a previously reported polymorphism frequent in caucasians and asiatics *EGLN1* c.380G>C, p.Cys127Ser (rs12097901) (26) so we didn´t consider it as a casual mutation.

HIF2α (*EPAS1*)

Sequencing of the exons 9 and 12 of the *HIF2*α gene was done in the 45 samples without a causative mutation in the other genes. No mutations were found.

Characterization of the patients without mutations

Although a potential causal mutation was identified in 25 (36%) of the erythrocytosis' patients, 45 remain without a definitive diagnostic.

Of these 45 patients with idiopathic erythrocytosis, three have Epo levels <3.0 mUI/mL, 17 have a positive familial history, and three are under 16 years-old.

Conclusions

We were able to identify the molecular etiology of erythrocytosis in 25 (36%) of the 70 subjects studied. The presence of a high-affinity Hb variant was the commonest cause (14 unrelated carriers). New mutations in the *JAK2*, *HBB*, *EPOR, VHL* and *EGLN1* were found for the first time in these patients, some of them already published by the authors. Mutations *JAK2* c.1641+6T>C*, EPOR (*p.Arg437His), *VHL* (p.Lys196Glu) and *EGLN1* (p.Trp334Arg) were described here for the first time. None of the subjects showed disease causing mutations in *BPGM* or *EPAS1 (*exon 12*)*. BPGM mutations are very rare with only three mutations already described.

As a mutation in the *EPAS1* exon 9, coding for the secondary oxygen-degradation domain, was described in association with erythrocytosis associated with a recurrent paraganglioma (27) we sequenced also this exon but we didn´t found any mutation in the 45 samples studied.

Among the seven patients with low Epo levels three carried a *JAK2* exon12 mutation and two an *EPOR* truncation mutation. Three subjects presented missense *EPOR* mutations but the consequences of these mutations were inconclusive and need further studies.

We did not find a causal mutation in the remaining 45 subjects (3 with Epo levels <3.0 mUI/mL) but this is less than what was expected from the results of other groups (28-32), even because, to our knowledge, this is the only study where all the genes have been tested.

High affinity hemoglobins are a very rare cause of secondary erythrocytosis but it seems likely that their incidence may be underestimated (33). We propose that in individuals with a dominant form of erythrocytosis and normal or high Epo levels, the determination of P50 should be done as a first line diagnostic. If it was below the normal range or if it was not available than the *HBB* and *HBA* genes should be sequenced.

The phenotypic variability of patients' presentation that remains without an identified mutation suggests that more than one gene has to be implicated. Recently published data using NGS technology identified new genes associated with red blood cell diseases (34,35); probably some of them are "the missing genes" in Congenital Erythrocytosis.

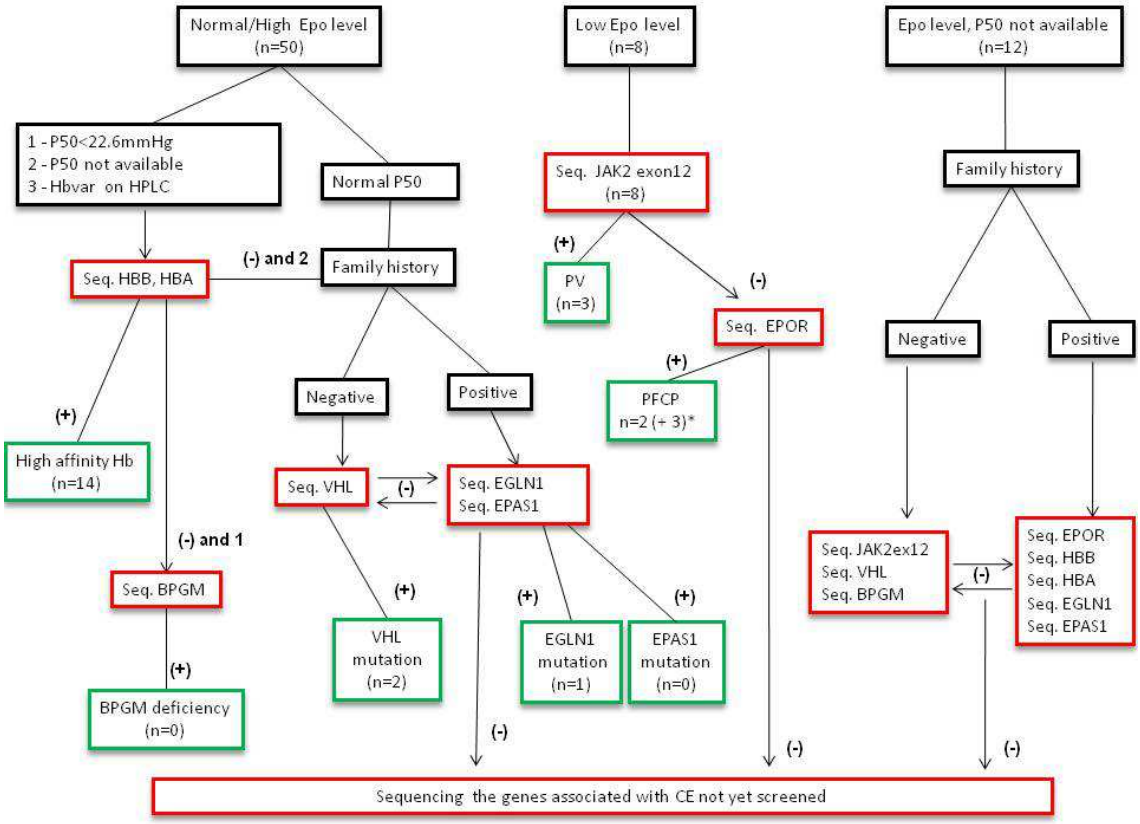

Figure 1. Algorithm followed in the orientation of the biochemical and molecular studies of the samples with erythrocytosis. (-) indicate that no mutations were found; (+) indicates the presence of a causative mutation; *EPOR mutations found in patients from group 2 and group 3; Seq. means sequencing; all the other abbreviations used are explained in the text.

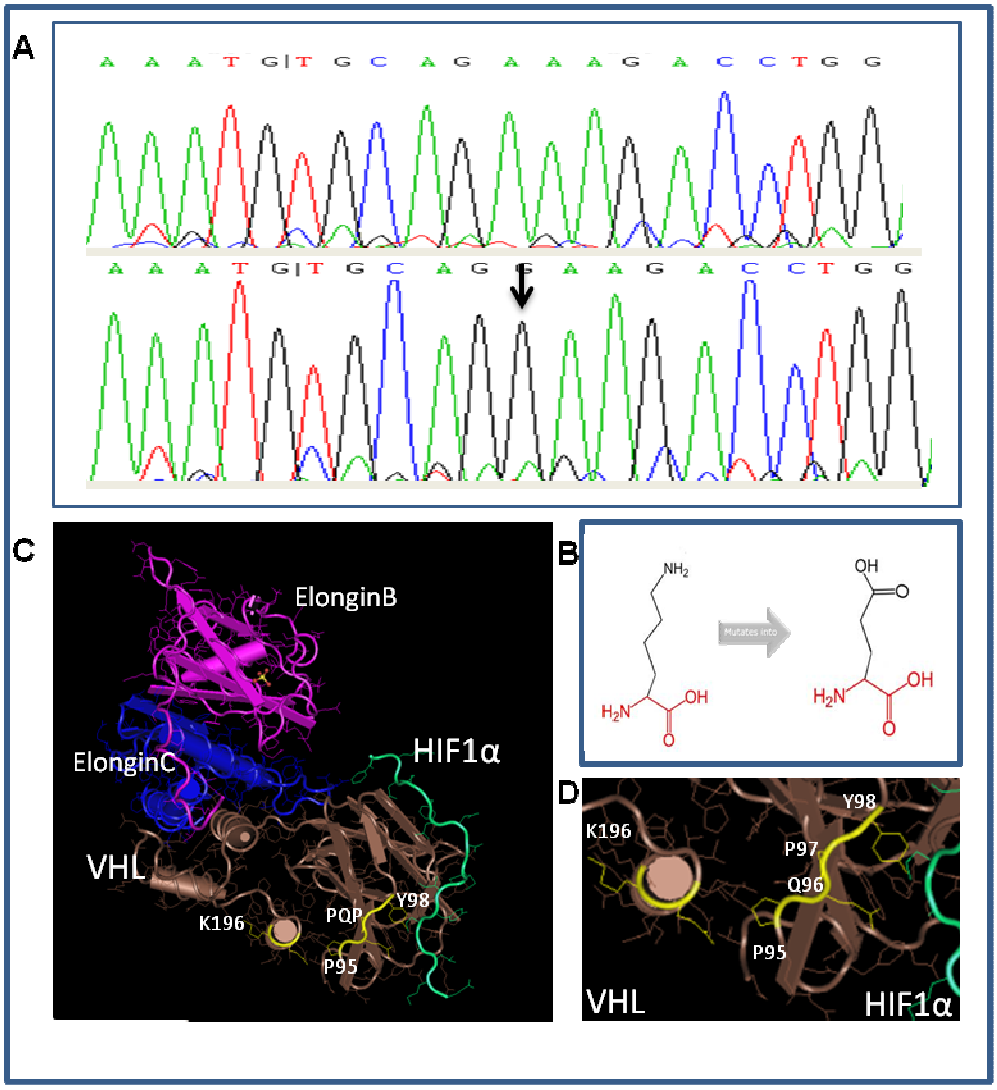

Figure 2. **A**. DNA sequence chromatogram of *VHL* exon 3 from a normal control (upper) and from our patient (Pt1) (down) showing the mutation c.586A>G (arrow) in the homozygous state. **B**. Schematic structures of the wild-type (left) and the mutant (right) amino acid. According to Project HOPE the wild-type residue and newly introduced mutant differ in size, charge, and hydrophobicity. These possibly disrupt contacts with other molecules and cause a loss of external interactions. **C**. Molecular dynamics (MD) simulations of the VHL -ElonginC - ElonginB complex and interaction with HIF-1a. VHL, ElonginC and Elongin B are shown in brown, blue and pink, respectively. K196 residue is part of the beta/alpha domain. K196E mutation perturbs the Gln195 close to P95 located at β-sheet forming the PQP site. This change resulted in inducing effect on Y98 residue, a critical residue for VHL and HIF1α interactions (implicated amino acids are shown in yellow). **D**. Close-up view of the amino acid locations.

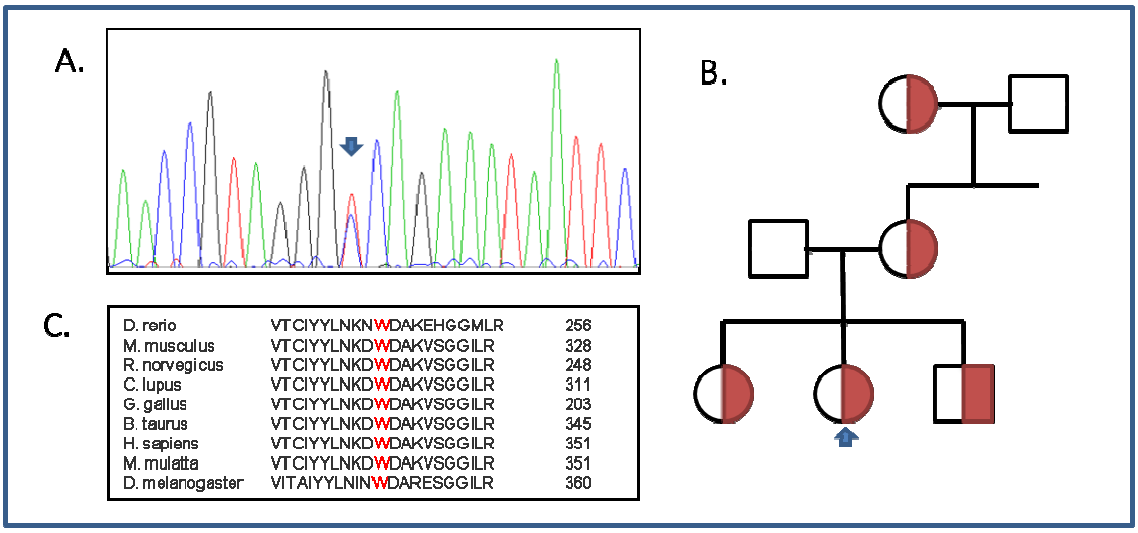

Figure 3. **A**. Sequencing of sample Pt26 showing the *EGLN1* c.1000 T>C mutation; **B.** Pedigree of the family. The propositus is indicated with an arrow. Mutation *EGLN1* c.1000 T>C was found in five members of the family, affecting 3 generations, with an autosomal dominant mode of inheritance. **C.** Amino acid alignment of PHD2 orthologs showing conservation of the Trp residue.

Table 1. Clinical and laboratory data of the patients

Sex	Number of patients	Age at diagnosis (years) Median (Range)	Hb (g/dL) (Median + STD)	Hct (%) (Median + STD)	Patients with Epo <3.0mUI/mL	Positive familial history
Female	19	42 (18 ; 63)	16,65 ± 1,02	51,5 ± 3,59	0	15
Male	51	38 (7; 76)	18,87 ± 0,79	56,58 ± 4,1	8	20
Total	70	39 (7; 76)	18,45 ± 1,10	55,84 ± 4,12	8	35

Table 2. High affinity Hb variants found in this study

Hb Variant	HGVS name	HPLC	Nº Patients (total =14)
Hb Olympia	HBB:c.61G>A; p.V21M	No separation	1
Hb Vila Real	HBB:c.110C>A; p.P37H	Hb X=36%	3
Hb Barcelona	HBB:c.283G>C; p.D95H	Hb X=37%	1
Hb Malmo	HBB:c.294C>A; p.H98Q	Hb X=48%	1
Hb Coimbra	HBB:c.300T>A; p.D100E	No separation	1
Hb Yakima	HBB:c.298G>C; p.D100H	Hb X=38%	2
Hb Jonhstown	HBB:c.328G>T; p.V110L	No separation	1
Hb San Diego	HBB:c.328G>A; p.V110M	Hb X=36%	3
Hb Saratoga Springs	HBA1:c.123G>T; p.K41N	Hb X=14%	1

Table 3. Data of patients were a causal mutation was identified

Gene	Patient ID	Sex	Age at diagnosis (y)	Mutation	Inheritance	Familial History	Epo levels (mUI/mL)	Hb (g/dL)
JAK2 exon12	Pt32	M	41	c.1641+6T>C	unknown	No	<2.0	19.0
	Pt35	M	54	N542-E543del	Clonal	No	<2.0	19.2
	Pt60	M	27	R541-E543delinsK	Clonal	No	<2.0	19.8
EPOR	Pt21	M	60	CD 487 (AAC-AGC)	Heterozygous	yes	5.6	18.2
	Pt30	M	37	CD 437 (-TC)	Heterozygous	yes	2.0	18.9
	Pt40	M	32	CD 437 (CGT-CAT)	Heterozygous	yes	6	19.3
	Pt49	M	70	CD 412 (TCG-TAG)	Heterozygous	unknown	2.9	18.9
	Pt65	M	45	CD 487 (AAC-AGC)	Heterozygous	yes	not determinated	18.4
HBB	Pt5	M	18	Hb Vila Real	Heterozygous	yes	32	18.3
	Pt9	F	37	Hb Yakima	Heterozygous	yes	17	16.6
	Pt13	F	28	Hb Malmo	Heterozygous	yes	27	16.4
	Pt16	M	34	Hb Coimbra	Heterozygous	yes	10	19.1
	Pt22	M	45	Hb Olympia	Heterozygous	yes	18	18.8
	Pt27	M	27	Hb San Diego	Heterozygous	yes	34	18.1
	Pt42	F	41	Hb Johnstown	Heterozygous	yes	16	16.1
	Pt45	F	49	Hb Yakima	Heterozygous	yes	21	16.3
	Pt47	M	43	Hb Vila Real	Heterozygous	yes	18	19.3
	Pt53	F	48	Hb Vila Real	Heterozygous	yes	14	18.3
	Pt54	F	26	Hb Barcelona	Heterozygous	yes	20	16.5
	Pt63	F	43	Hb San Diego	Heterozygous	yes	32	16.5
	Pt64	M	19	Hb San Diego	Heterozygous	yes	21	18.6
HBA	Pt56	F	44	Hb Saratoga-Springs	Heterozygous	yes	33	16.2
VHL	Pt1	F	33	CD196 (AAA-GAA)	Homozygous	no	13	20.0
	Pt39	F	17	CD175(TAC-TGC)	Heterozygous	no	14	20.2
EGLN1	Pt26	F	31	CD334 (TGG-CGG)	Heterozygous	yes	6	17.4

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