A novel heterozygous HIF2A^{M535I} mutation reinforces the role of oxygen sensing pathway disturbances in the pathogenesis of familial erythrocytosis

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ABSTRACT

HIF2A transcription factor plays a central role in the regulation of the hypoxia responding pathway in mammalian cells, by modulating erythropoiesis and angiogenesis. Molecular alterations of oxygen sensing pathway constituents are implicated in hereditary erythrocytosis. Here we show that 2 members of a family with idiopathic erythrocytosis exhibited a new heterozygous G to A mutation at base 1605 of the exon 12 of hypoxia-inducible factor-2A (*HIF2A*) gene. This mutation determines the replacement of methionine by isoleucine at the position 535, very close to the position 531, where the hydroxyl acceptor prolyne is located. In addition, we found that mRNA expression of erythropoietin receptor, vascular endothelial growth factor, transferrin receptor, adrenomedullin and N-myc downstream regulated gene 1, up-regulated by *HIF2A* or hypoxia, were significantly higher in patients carrying the mutation than in normal controls. These results suggest that the *HIF2A*^{M535I} gene mutation could induce hereditary erythrocytosis at a young age.

Key words: familial erythrocytosis, *HIF2A*.

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Introduction

The association between polycythemia and people living at high altitudes was reported more than 100 years ago.[1] Nonetheless, the molecular mechanism underlying the regulation of erythropoietin (Epo) production by oxygen tension was only clarified a few years ago.[2] The molecular network responsible for cell response to hypoxia can be simplified into three major constituents: the prolyl hydroxylase domain-containing proteins (PHD1, PHD2, and PHD3), the hypoxia-inducible factor (HIF, consisting of the labile α-subunit and of the constitutively expressed β-subunit) and the protein product of the Von Hippel Lindau (*VHL*) oncosuppressor gene (pVHL).[3-5] Under normal oxygen tension, PHDs hydroxylate the α-subunit of HIF and target HIF-α for degradation by the pVHL. In conditions of hypoxia, HIF-α subunit hydroxylation by PHDs is hampered: as a consequence, the pVHL binding is reduced and HIF-α subunits accumulate, dimerize with HIF-β subunits, migrate into the nucleus and induce the expression of several target genes such as *Epo, Transferrin Receptor (TfR) and Vascular Endothelial Growth Factor (VEGF)*.[3-5] The important role played by the HIF pathway in maintaining the oxygen homeostasis is highlighted by the fact that molecular defects occurring in one of its members induce exaggerated erythropoiesis. Thus, the familial and congenital polycythemia observed among Chuvash people, leading to premature mortality related to arterial or venous thrombosis, is due to the homozygous VHL^{R200W} mutation.[6,7] Moreover, other functionally similar *VHL* gene mutations have been described in individuals belonging to different ethnicities, who are affected by congenital polycythemia.[7,8] More recently, two different mutations of the *PHD2* gene which resulted in

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defective HIF hydroxylation and inhibition, with consequent familial erythrocytosis have been reported.[9-11] Finally, Percy *et al*. highlighted that also gain-of-function mutations of *HIF2A* gene cause hereditary erythrocytosis.[12,13] In this study we report a novel mutation of the *HIF2A* gene in 2 members of a familiy affected by idiopathic erythrocytosis.

Design and Methods

Patients. The patients investigated were observed at the Department of Hematology of the University "La Sapienza" of Rome for erythrocytosis (Table 1). Patient samples were obtained after receiving informed consent in accordance with the Declaration of Helsinki and with the approval from the local Institutional Review Boards. At the time of the first observation, the mother was aged 38 years and the child was 14 years old. This latter had been included in a previously reported series of children with familial polycythemia.[14-16] The woman reported that her father had erythrocytosis and had died of pulmonary embolism at the age of 56 years. She also has one brother, having normal hematologic findings (Figure 1A). The serum erythropoietin level was above the normal range in the mother. On the contrary, the child showed normal serum erythropoietin level, despite increased Hb and Hct values (Table 1). Patients had normal arterial oxygen saturation and no liver or spleen enlargement. Possible causes of secondary erythrocytosis, such as pathological hemoglobins, were ruled out. Indeed, both patients started therapy with low dose aspirin and periodical phlebotomies. During the follow-up, they were analyzed for *Epo Receptor (EpoR)* mutations, *JAK2*^{V617F} mutation and *JAK2* exon 12 mutations, all of which proved negative.[14-15] Furthermore, when evaluated by the human androgen receptor assay, the mother showed polyclonal hematopoiesis, and endogenous erythroid growth was absent in both patients.[14]

Mutational analysis of HIF2A gene. DNA was extracted from peripheral blood granulocytes as previously described.[14] *HIF2A* exon 12 was amplified from DNA of peripheral granulocytes by PCR. Primers and annealing temperature are indicated in the *Online Supplementary Table S1*. PCR-direct sequencing was performed using standard protocols and the same amplification primers.[14] The G1605A mutation screening was performed in granulocytes colleted from 100 healthy blood donors by the amplification-refractory mutation system (ARMS)-PCR for G1605A mutation, using primers designed by the primer-design program devised by Ye *et al*.[17] (*Online Supplementary Table S1*).

Real-time assay. RNA was extracted from mononuclear cells as previously described.[14] The mRNA levels of EpoR, VEGF, TfR, adrenomedullin (ADM), and N-myc downstream regulated gene 1 (NDRG1) were evaluated by real-time PCR in the mononuclear cell fraction. After cDNA synthesis, 4 μl of cDNA were diluted 1:20 and were added to a PCR mixture containing 8.4 μl sterile water, 12.5 μl 2x SYBR mix (Bio-Rad, Hemel Hempstead, United Kingdom) and 0.05 μl of each forward and reverse primers (100 μM), to a final volume of 25 μl. Cells obtained from patients 2 and 3 and from 15 normal controls were investigated in duplicate. Three separate experiments were performed and each assay was carried out by using an iCycler-iQ multicolor Real-Time PCR detection system (Bio-Rad). Results were analyzed by the iCycler-iQ optical system software (Bio-Rad). For each sample, the average of the duplicate assays was obtained and normalized to the average amount of β-actin, to determine the relative changes in mRNA expression. The primers used for each gene and the annealing temperatures are listed in *Online Supplementary Table S1*.

Statistical analysis. Statistical analysis was performed by the Kruskal-Wallis or the Mann-Whitney U-test, as appropriate. p values <0.05 were considered statistically significant.

Results and Discussion

We investigated 2 members of a family with hereditary erythrocytosis for the presence of exon 12 *HIF2A* gene mutations. By PCR-direct sequencing we found that both patients (mother and son) harbored a heterozygous G→A change at base 1605 (Figure 1B and 1C). The mutation was not detected in the father of the child, who showed normal hematologic parameters (Figure 1B and 1C) and in a large series of normal controls (*data not shown*). These results indicate that the mutation segregates with the erythrocytosis phenotype in the members of the index family, and that it is not a polymorphism. We did not find the mutation in the other 4 children with familial erythrocytosis. Notably, this G→A transition determines the replacement of methionine by isoleucine at amino-acid 535, very close to the 531 position, where the hydroxyl acceptor prolyne is located, in a highly conserved region of HIF2A.[18,19] Percy *et al*. have recently described an hereditary heterozygous gain-of-function mutation of HIF2A gene, resulting from the G→T change at 1609 position with consequent substitution of glycine 537 by tryptophane.[12] The authors demonstrated that this HIF2A^{G537W} mutation affects both the hydroxylation by PHD2 and the subsequent recognition by VHL, leading to a reduced degradation of HIF2A mutant as compared to

Table 1. Clinical and laboratory findings at diagnosis in patients with *HIF2A*^{M535I} gene mutations. *Normal range 3.0–30 mU/mL.

Pt	Age (yrs)	Hb (g/dL)	Htc (%)	RBC (10^{12}/L)	WBC (10^{9}/L)	PLT (10^{9}/L)	Serum Epo mU/mL *
2	38	20.5	59.6	6.69	7.37	271	51
3	14	18.6	53.2	6.29	6.76	207	7.4

the wild type counterpart.[12] Indeed, they concluded that the HIF2A^{G537W} mutation induces familial erythrocytosis.[12] Subsequently, the same group reported two additional heterozygous HIF2A mutations (M535V and G537R) in 4 patients with idiopathic erythrocytosis.[13] Interestingly, we observed a 1605 G to A transition located at the same codon as the M535V mutation,[13] suggesting that the M535I mutation affects the HIF2A degradation and might be causative of erythrocytosis. To address this hypothesis, since the hypoxic responding system operates in all kinds of mammalian cells, we evaluated the expression level of several genes up-regulated by HIF2A[3-5] or hypoxia,[20,21] (*EpoR, VEGF, TfR, ADM*, and *NDRG1*) in mononuclear cells isolated from the 2 patients with HIF2A^{M535I} mutation and from 15 normal controls (Figure 1D). Results obtained clearly show that mutated patients have an increased expression of HIF2A induced genes as compared to normal controls, indirectly suggesting their exaggerated up-regulation by HIF2A^{M535I}.

The family reported in this study had been affected by idiopathic erythrocytosis for three generations and the grandfather had died because of pulmonary embolism. Moreover, thrombotic manifestations were also observed in families studied by Percy *et al.*[12,13] Indeed, these findings suggest that, similarly to patients carrying *VHL* or *PHD2* gene mutations,[6-11] also patients with a *HIF2A* mutation might have an increased thrombotic risk and require appropriate therapy. In addition, one of the affected members was 14 years old at the time of the first examination, indicating that this mutation induces hematologic alterations from the first years of life, whereas a significant increase of serum Epo levels could be evidenced only subsequently. Recent studies published by our group highlighted that a significant proportion of children diagnosed as having

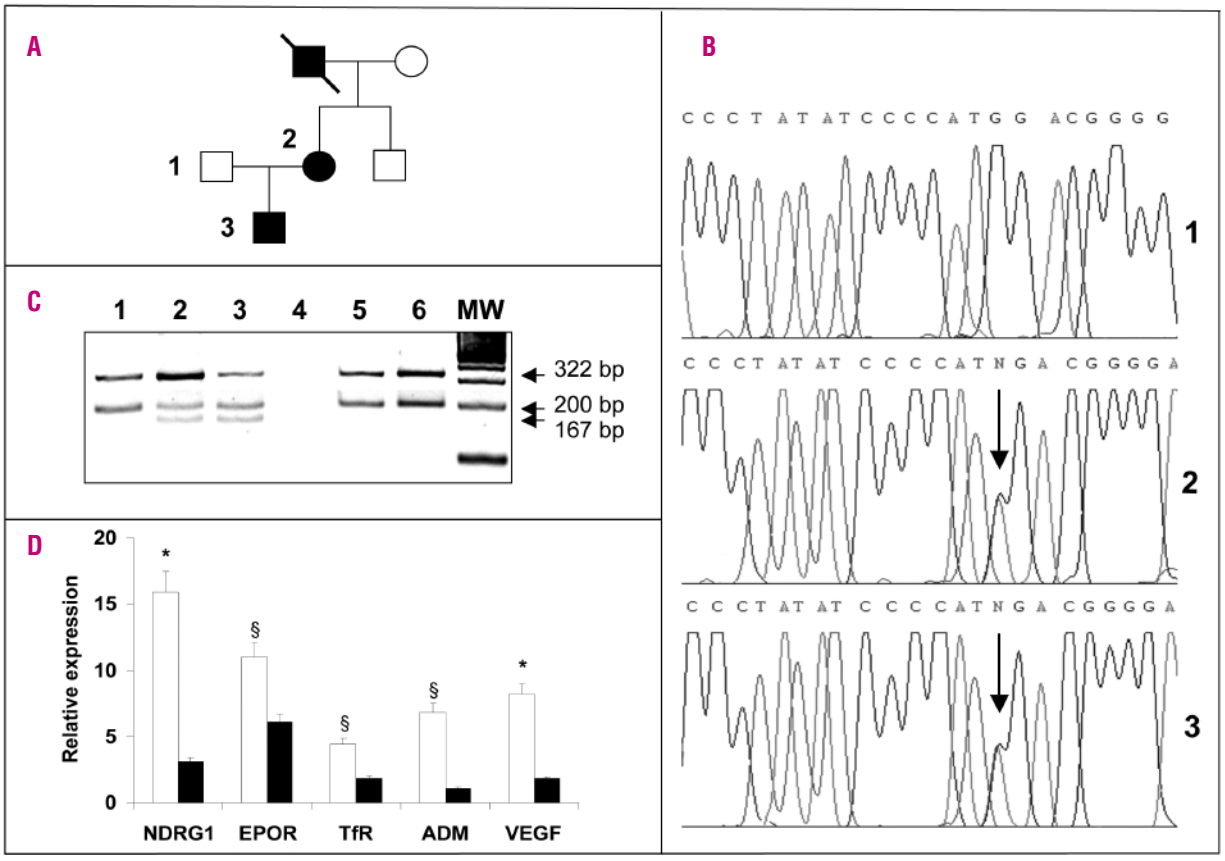

Figure 1. Figure 1. (A) The pedigree of family with hereditary erythrocytosis. Squares represent males, circles females, solid symbols members with erythrocytosis and slashes indicate deceased members. DNA samples from patients 1, 2 and 3 were analyzed. Patient 2 confirmed that also her grandfather had been affected by erythrocytosis. (B) Nucleotide sequence (1591 to 1612) of *HIF2A* exon 12. The wild type sequence was detected in the father (sequence 1), while a heterozygous G to A change at base 1605 (indicated by arrow) was detected in the son (patient 3) and in the mother (patient 2). (C) Amplification-refractory mutation system (ARMS)-polymerase chain reaction (PCR) for G1605A mutation. The wild type allele produced a 200 bp band and the mutant allele a 167 bp band; an internal PCR control, yielding a band of 322 bp, is inserted in each lane. Samples in lanes 2 and 3 (mother and son respectively) show wild type and mutant alleles, while samples in lanes 1, 5 and 6 (father and two normal controls respectively) show the presence of the wild type allele only. Lane 4 corresponds to negative control. MW indicates molecular weight markers. Primers used are shown in *Online Supplementary Table S1*. (D) RNA levels of genes induced by HIF2A or hypoxia (abbreviations are detailed in the text) evaluated by real-time PCR in mononuclear cells isolated from patients with HIF2A^{M535I} mutation (white columns) and from 15 normal controls (black columns). Patients 2 and 3 and 15 normal controls were investigated in duplicate. Results pooled from three separate experiments are shown. Primers used are indicated in the *Online Supplementary Table S1*. T bars indicate the standard deviations. *= p values < 0.01; §= p values < 0.05.

essential thrombocythemia really have an hereditary mutation of the thrombopoietin receptor, causing familial thrombocytosis.[14-16] The present study emphasizes that also children with erythrocytosis must be carefully investigated for its familial occurrence and that all idiopathic cases should be tested during follow-up for any inheritable molecular alterations subsequently identified.

Authorship and Disclosures

MM, LTe contributed to the study design, analyzed data and wrote the manuscript. FG and MR enrolled patients and recorded clinical data. TC and Lo performed cell separation and molecular analysis. GL and RF critically reviewed the manuscript. LML designed the study and wrote the manuscript. The authors reported no potential conflicts of interest.

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