Congenital erythrocytosis associated with gain-of-function *HIF2A* gene mutations and erythropoietin levels in the normal range

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ABSTRACT

Hypoxia-inducible factor 2α (HIF-2α) plays a pivotal role in the balancing of oxygen requirements throughout the body. The protein is a transcription factor that modulates the expression of a wide array of genes and, in turn, controls several key processes including energy metabolism, erythropoiesis and angiogenesis. We describe here the identification of two cases of familial erythrocytosis associated with heterozygous *HIF2A* missense mutations, namely Ile533Val and Gly537Arg. Ile533Val is a novel mutation and represents the genetic *HIF2A* change nearest to Pro-531, the primary hydroxyl acceptor residue, so far identified. The Gly537Arg missense mutation has already been described in familial erythrocytosis. However, our patient is the only described case of a *de novo HIF2A* mutation associated with the development of congenital polycythemia. Functional *in vivo* studies, based on exogenous expression of hybrid HIF-2α transcription factors, indicated that these genetic alterations lead to the stabilization of HIF-2α protein. All the identified polycythemic subjects with *HIF2A* mutations show serum erythropoietin in the normal range, independently of the hematocrit values and phlebotomy frequency. The erythroid precursors obtained from the peripheral blood of patients showed an altered phenotype, including an increased rate of growth and a modified expression of some HIF-2α target genes. These results suggest the novel proposal that polycythemia observed in subjects with *HIF2A* mutations might also be due to primary changes in hematopoietic cells and not only secondary to increased erythropoietin levels.

Introduction

Regulation of oxygen homeostasis is critical to survival. Accordingly, eukaryotes have developed molecular mechanism(s) for the maintenance of proper oxygen tissue levels. A major physiological consequence of hypoxia is the enhanced renal synthesis of erythropoietin (EPO) and the increase of serum EPO. In turn, the cytokine stimulates red cell production restoring the oxygen supply to tissues.[1,2]

EPO is produced by kidney interstitial fibroblasts in close association with peritubular capillary and tubular epithelial cells.[3,4] The cytokine is also synthesized in the perisinusoidal cells in the liver.[5] However, while liver synthesis predominates in the fetal and perinatal periods, renal production is clearly predominant during adulthood. The hypoxia-dependent up-regulation of renal EPO production is almost exclusively due to the activation of *EPO* gene transcription.[3,4] The protein is then rapidly modified and released in the serum. Erythroid precursors at specific stages of differentiation express high levels of EPO receptor (EPOR) whose engagement results in increased red cell production.[6-8]

Induction of *EPO* gene transcription by hypoxia has led to the discovery of the transcription factor hypoxia-inducible factor (HIF) and the molecular pathways that allow the modulation of HIF levels by oxygen levels.[9] HIF consists of two subunits, HIF-α and HIF-β, whose nuclear heterodimerization results in the active form. Only the levels of HIF-α, which exists in two isoforms HIF-1α and HIF-2α, are regulated by oxygen content.[10-13] In particular, normoxia induces the ubiquitin-mediated degradation of HIF-α. Conversely, cellular HIF-α isoforms levels are increased by low O_2 partial pressure.

The ubiquitination of HIF-α requires a previous prolyl-4-hydroxylation that commits the transcription factor to the interaction with an E3 ubiquitin-protein ligase.[14-17] The E3 complex is formed by various proteins including the von Hippel-Lindau protein (VHL), elongins B and C, cullin 2, and RBX1.[14-17] Three prolyl hydroxylases (PHD 1-3), identified in mammalian cells, use O_2 as a substrate to generate 4-hydroxyproline at specific prolyl residues of HIF-α.[18,19]

The primary site of hydroxylation of HIF-2α is Pro-531, which corresponds to proline 564 in HIF-1α.[20,21] The hydroxy-

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proline represents a specific degron that allows HIF-2α recognition by VHL.[22] Under normoxic conditions, the activities of PHD (mainly PHD2) and VHL maintain the HIF-α (both HIF-1α and HIF-2α) content at almost undetectable levels.[23] Conversely, hypoxic conditions rapidly decrease HIF-α prolyl hydroxylation thus allowing the accumulation of the transcription factor.[23] In the nucleus, HIF-α associates with HIF-β forming an active complex endowed with transcriptional activity.[24] The HIF-α/HIF-β heterodimer binds to target consensus sequences in the regulatory regions of several hundreds of genes and activates their expression.[25-27]

HIF target genes are involved in the control of a plethora of molecular processes including the regulation of cell growth and differentiation, the activation of apoptosis and the modulation of cell movement and migration.[25-27] Accordingly, several complex physiological and pathological events are regulated by HIF levels, such as hormone regulation, energy metabolism, angiogenic signaling, vasomotor regulation, matrix and barrier functions, transport and virus-related genes.[25-27] Although still debated, various data suggest that HIF-1α/HIF-β and HIF-2α/HIF-β regulate different genes and modulate, in part, distinct processes.[28]

Recent studies have identified congenital erythrocytosis-associated mutations in the genes that encode for VHL, PHD2 and HIF-2α.[29-41] The genetic changes embrace homozygous or compound heterozygous mutations of the *VHL* gene, heterozygous *PHD2* gene changes, and heterozygous mutations of the *HIF2A* gene.[29-41] Specific *HIF2A* and *PHD2* haplotypes have also been associated with adaptation to high altitudes in Tibetans, confirming the central role of these genes in the hypoxic response.[42-44] Interestingly, no mutation of *HIF1A* has been reported so far.[35,45] Mechanistically, *PHD2* and *VHL* mutations lead to loss of function of the respective proteins, while *HIF2A* mutations probably lead to a gain of function of HIF-2α.[29-41] A small number of subjects affected by inherited *HIF2A* mutations (Pro534Leu, Met535Val, Met535Ile, Met535Thr, Gly537Trp, Gly537Arg, Asp539Glu, Phe540Leu) in heterozygosity have been described. These patients show a dominant erythrocytosis and increased or inappropriately normal EPO serum levels.[34-41] In some cases, the patients present a history of thrombosis or pulmonary hypertension.[35] Increased serum EPO is supposed to be the cause of erythrocytosis in these patients. In addition to these patients, three cases of somatic gain-of-function *HIF2A* mutations (Ala530Thr, Ala530Val, Phe374Tyr) associated with paraganglioma and polycythemia as well as with increased serum EPO have been reported.[46-48]

In the present report, we identify two *HIF2A* mutations associated with erythrocytosis and analyze the effect of the genetic change on transcription factor stability and activity. One change is a *de novo* mutation, a condition that represents the first direct genetic proof that *HIF2A* mutations might be the cause of hereditary erythrocytosis. We also describe, for the first time, the features of red cell progenitors prepared from the patients bearing *HIF2A* genetic changes.

Methods

Patients

We analyzed a series of 75 patients with sporadic, apparently congenital or familial erythrocytosis. All patients gave informed written consent on entering the study, which had been approved by the Ethics Committee of the Second University of Naples, in accordance with the Declaration of Helsinki.

Case reports

Patient 1 is an asymptomatic 15-year old Italian male who presented with an increased hemoglobin (Hb) of 17.3 g/dL, hematocrit (Hct) of 0.52, white cell count of 9.3x10^9/L, and a platelet count of 245x10^9/L during routine blood tests at 9 years of age. The oxygen dissociation curve and abdominal ultrasound were both normal. His serum EPO was 6.3 mU/mL (reference range, 3.7-31 mU/mL). He has no history of thrombosis or pulmonary hypertension and no family history of erythrocytosis. His last EPO measurement (July 2012) was 8.7 mU/mL (Hb, 17.8 g/dL; Hct, 0.53). Both parents have normal Hb and Hct levels.

Patient 2 is a 30-year old Italian male who presented with headache, dizziness and fatigue. He had a Hb of 19.1 g/dL and a Hct of 0.55 with normal white cell and platelet counts. His spleen was normal on ultrasound. He was a light smoker with no history of either thromboembolic events or pulmonary hypertension. He did not use any medications. Arterial blood gas analysis showed normal oxygen saturation and p50 values. His EPO level was 18 mU/mL (reference range, 3.7-31 mU/mL). His carboxyhemoglobin value was 1.6% (normal range, <1.5%). He has been managed for the past 15 years by regular venesection and remains well at the age of 45 years. His last EPO level was 25 mU/mL (August 2012; Hb, 17.1 g/dL; Hct, 0.51). His asymptomatic 16-year old daughter also had an erythrocytosis (Hb of 18.0 g/dL, Hct of 0.53) with EPO level near to the low limit of the normal range (6.1 mU/mL).

Mutation screening

Genomic DNA were collected at diagnosis from all patients and relatives.

Erythroid precursor cultures

Liquid cultures of peripheral erythroid precursors were prepared by employing peripheral blood CD34$^+$ cells as a source of progenitors.[8]

Quantitative polymerase chain reaction analysis

cDNA was prepared from patients' mRNA from erythroid precursors with an I-Script kit (Bio-Rad). Real-time polymerase chain reaction (PCR) was performed for adrenomedullin (*ADM*), N-myc downstream regulated gene 1 (*NDRG1*), vascular endothelial growth factor (*VEGF*) and transferrin receptor (*TFR*) genes, in accordance with the manufacturers' instructions. The reactions were run on an ABI 7300 real-time PCR system (Applied Biosystems). Assays were performed in triplicate. We used the $2^{-\Delta\Delta Ct}$ method to analyze the data obtained.

Plasmids

A PCR product spanning the human HIF-2α oxygen-dependent degradation (ODD) region (amino acids 404-569) was cloned into the EcoRI site of pM3-VP16 (Clontech) to obtain one-hybrid constructs harboring a N-terminal GAL4 DNA-binding domain and a C-terminal VP16 transactivation domain. The plasmid was mutated at specific residues as reported in the text. Human PHD expression vectors have been described earlier.[49,50]

In vitro *prolyl-4-hydroxylation assays*

Enzymatic activities of recombinant PHD1, PHD2 and PHD3 on HIF-2α peptide mutants were determined by an His6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) binding assay, essentially as described elsewhere.[49,50]

Luciferase assays

The experiments were performed essentially as reported by Wirthner *et al.*[49] and Stiehl *et al.*[50]

More details of the design and methods of this study are available in the *Online Supplementary Appendix*.

Results

Clinical and genetic characterization of patients

A cohort of 75 patients affected by congenital erythrocytosis were investigated for the presence of mutations in the *JAK2*, *EPOR*, *VHL*, *PHD1*, *PHD2*, *PHD3*, *HIF1A* and *HIF2A* genes. In detail, the following regions were sequenced: *JAK2* (exons 12 and 14), *EPOR* (exons 7 and 8), *VHL* (entire coding sequence), *PHD1* (exons 1-5), *PHD2* (exons 1-4), *PHD3* (exons 1-3), *HIF1A* (exons 9, 12 and 15), and *HIF2A* (exon 12). Two patients (1 and 2) were found to have two different *HIF2A* mutations.

Patient 1 is heterozygous for a c.1609 G>A mutation, which predicts replacement of glycine with arginine at residue 537 (Gly537Arg) (Figure 1). This mutation has been previously reported by us and others.[35-37] The sequence of exon 12 of the *HIF2A* gene of both the parents of patient 1 did not evidence the Gly537Arg change. Genetic analysis confirmed paternity with an index of 99% (*data not reported*). Of note, this patient is the first case described in literature in which the *HIF2A* alteration appeared as a *de novo* mutation. The patient's serum EPO level was repeatedly found to be near the lower value of the reference range.

Patient 2 is heterozygous for a novel A to G change at c.1597 (c.1597A>G), corresponding to an Ile533Val mutation (Figure 2). The change creates a *BmsF1* restriction site (Figure 3). The alteration was also found in the patient's son confirming the family history of erythrocytosis. To test whether this mutation was a single-nucleotide polymorphism, a control panel of 200 DNA samples was screened by means of *BsmF1* digestion. No case of mutation was identified. Serum EPO levels were within the reference range in patient 2 and his affected son.

Effect of HIF-2α mutations on protein metabolism

The detected mutations share two key features with previously described pathogenetic *HIF2A* changes. First, both the mutations are present in heterozygosity. Second, they affect residues that closely follow the main and primary site of prolyl hydroxylation in HIF-2α (i.e. Pro-531). However, the occurrence of HIF-2α-dependent familial erythrocytosis with normal EPO serum levels might cast some doubts on the effect of mutations on HIF-2α stability and, in turn, on the molecular mechanism(s) of the disease.

Previously reported *in vitro* experiments suggested that

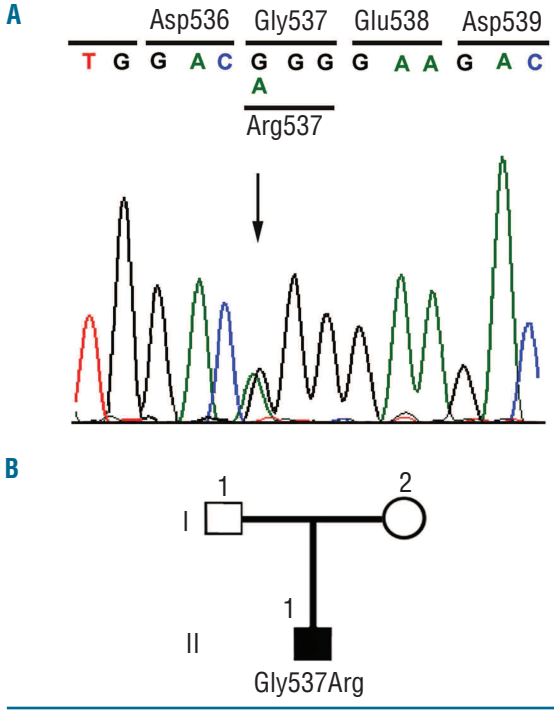

Figure 1. Identification of the Arg537Gly mutation in the *HIF2A* gene. (A) Detection of the c.1609 G>A (Gly537Arg) mutation by PCR-direct sequencing. PCR-direct sequencing was performed on total peripheral blood DNA using specific primers to amplify exon 12 of the *HIF2A* gene. Sequencing detected a heterozygous G to A change at base 1609 in patient 1, as indicated by the arrow, as compared to the wild-type sequence (upper panel). Nucleotides 1604–1617 are shown. Bases are as follows: G black; A green; T red; C, blue. (B) The pedigree of the family of patient 1 is shown. Squares represent male family members, circles represent female family members, and solid symbols represent family members with erythrocytosis; the genotype is shown under the symbol.

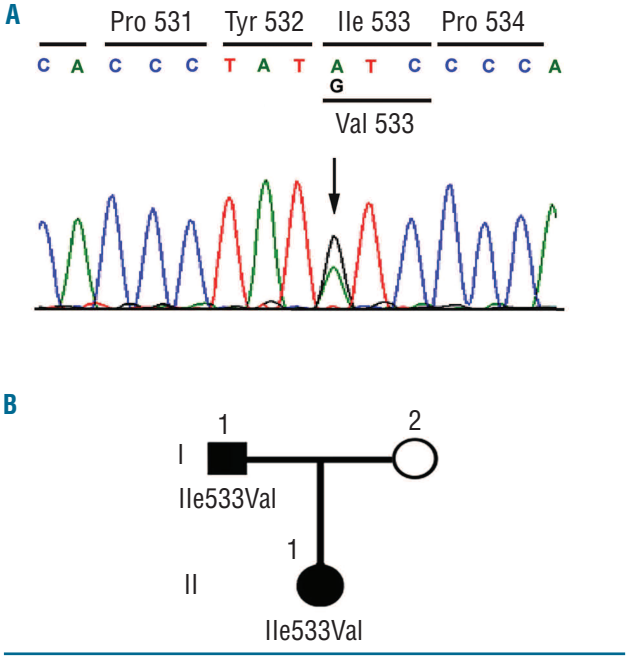

Figure 2. Identification of the Val533Ile mutation in the *HIF2A* gene. (A) Detection of the c.1597 A>G (Ile 533Val) mutation by PCR-direct sequencing. PCR-direct sequencing was performed on total peripheral blood DNA using specific primers to amplify exon 12. Sequencing detected a heterozygous A to G change at base 1597 in patient 2, as indicated by the arrow, as compared to the wild-type sequence (upper panel). Nucleotides 1589–1603 are shown. Bases are as follows: G, black; A, green; T, red; C, blue. (B) The pedigree of the family of patient 2 is shown. Squares represent male family members, circles represent female family members, and solid symbols represent family members with erythrocytosis; genotypes are shown under each symbol.

the Gly537Arg mutation affects the interaction of HIF-2α peptides with PHD2 and VHL as well as their ability to act as hydroxyl acceptors.[39] Thus, we performed experiments to evaluate the ability of different PHD (PHD1-3) to hydroxylate Pro-531 on peptides including Gly537Arg or Ile533Val changes. Therefore, wild-type (Figure 4A) and mutated biotin-HIF-2α-derived peptides (amino acids 523-542) were bound to NeutrAvidin-coated 96-well plates. The peptides were hydroxylated by the addition of recombinant GST-PHD (PHD1, PHD2 and PHD3). The degree of hydroxylation was evaluated by the binding of a recombinant thioredoxin-tagged protein complex formed by VHL/elongin B/elongin C (VBC) to the hydroxylated peptide. The amount of bound VBC was then estimated by an enzyme-linked immunosorbent assay (ELISA) including rabbit antithioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies. Thus, the method allows the assessment of PHD activity on a putative substrate peptide by quantifying the binding to the hydroxylated peptide of the VBC complex (and not simply to VHL protein).

As shown in Figure 4B, biotinylated peptides (from HIF-1α and HIF-2α) containing increasing proportions of synthetically generated hydroxyproline residue demonstrate a direct proportionality between the absorbance at 405 nm and the percentage of peptide hydroxylation. Addition of recombinant active PHD1, PHD2 and PHD3 to wild-type peptides also increased VBC binding (Figure 4C). Finally, since we preferred to use oxidation-insensitive peptides in which methionine 535 is substituted by alanine, we confirmed that this change did not affect the efficiency of hydroxylation by the three PHD (Figure 4D).

Next, we evaluated the hydroxylation of five different peptides, encompassing the region from 523 to 542, which carry the following changes: Gly537Arg, Ile533Val, Gly537Ala, Gly537Trp and Ala535Val (Figure 5A). We also tested a wild-type peptide (as a positive normal control) and the Pro531Ala (P531A) mutant peptide (as the negative control). In the absence of a recombinant PHD, no Pro-531 hydroxylation occurs (Figure 5B). In contrast, in the presence of all PHD isoforms, VBC binding to the wild-type peptide is approximately 8- to 10-fold higher than to the P531A mutant peptide (Figure 5C). None of the five HIF-2α mutations analyzed affected the efficiency of PHD-dependent hydroxylation, except the repeatedly observed diminished PHD3-dependent hydroxylation of the Gly537Arg mutant (Figure 5C).

To investigate whether *HIF2A* mutations might affect the *in vivo* stability of HIF-2α protein, Hek293 cells were transfected with an expression vector encoding a mutant fusion protein formed by the GAL4 DNA-binding domain, the HIF-2α ODD (amino acids 404-569) domain and the VP16 transactivation domain. The cells were co-transfected with a GAL4-responsive reporter plasmid containing the firefly *luciferase* gene under control of the E1b promoter and five GAL4 response elements. Whereas the wild-type ODD domain decreased fusion protein-dependent luciferase activity under normoxic conditions, double-mutation of the ODD region (by replacing the two hydroxyl-acceptors Pro-531 and Pro-564 with alanine) strongly induced fusion protein activity (Figure 5D). Interestingly, the fold increase was similar to that induced by the proline double mutation when one of the three indicated mutations was introduced into the ODD domain, suggesting that these mutations led to comparable protein stabilization (Figure 5D).

Following overexpression of PHD 1 to 3, a strong reduction of luciferase activity was observed in the wild-type control sample (Figure 5D). Conversely, no decrease of luciferase activity was observed in the samples transfected with ODD lacking the two pivotal prolines. In contrast to the normoxic basal conditions, overexpression of any of the three PHD led to a partial inhibition of luciferase activity mediated by the three mutant fusion proteins, most likely due to partial inhibition of their VHL-mediated degradation (Figure 5D). Of note, diminished proline hydroxylation of Gly537Arg by PHD3 could not be confirmed by the transfection experiments.

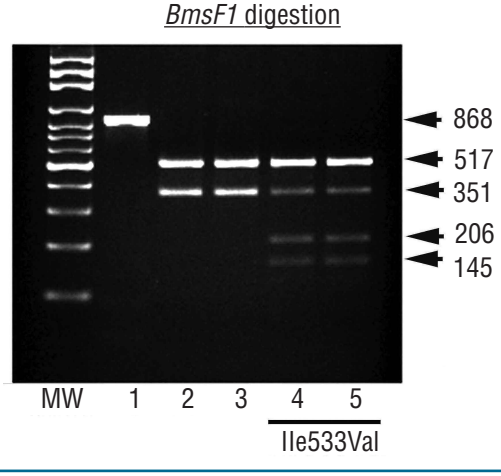

Figure 3. Identification of the Ile533Val mutation by *BsmF1* digestion. Lymphocytic cDNA from a control (lane 2), patient 2 (lanes 1 and 4), his daughter (lane 3) and his affected son (lane 5) were amplified using primers localized in *HIF2A* exon 12. The amplified materials were not digested (lane 1) or digested with *BmsF1* (lanes 2-5). The unrestricted PCR product is 868 bp long. *BmsF1* digestion results in two fragments of 517 and 351 bp in the absence of the Val533Ile mutation (lanes 2 and 3). The presence of the c1597 A>G mutation creates a second *BmsF1* restriction site in the 351 bp fragment and the formation of two further fragments of 206 and 145 bp each (lanes 4 and 5). MW, molecular weight.

Effect of HIF-2α mutations on erythroid precursor phenotype

As reported above, the serum EPO levels did not increase in the identified polycythemic patients with *HIF2A* mutations. Conversely, EPO concentrations were frequently near the lower end of the range and would not, therefore, explain the erythrocytosis in these patients.

Since HIF-2α might affect the expression of a significant number of genes modifying cell phenotypes, we wondered whether erythroid precursors of patients showed phenotypic alterations. The expression of a number of HIF-2α target genes, i.e. *ADM*, *NDRG1*, *VEGF* and *TFR* was examined by quantitative PCR analysis. As shown in Figure 6A, the Gly537Arg and Ile533Val mutations induced a significant increase in transcript levels of *ADM*, *NDRG1*, *VEGF* and *TFR* genes in circulating erythroid progenitors when compared to the levels in corresponding cells from control subjects.

Previous experiments demonstrated that peripheral erythroid progenitors of congenital polycythemic subjects are hypersensitive to EPO through an as of yet unknown

underlying mechanism. This feature is particularly evident at EPO concentrations lower than 1 U/mL. Thus, to investigate the phenotypic features of erythroid precursors of our patients, we characterized the *in vitro* proliferation of these precursors. In these experiments, EPO was employed at a low concentration (0.1 U/mL) to identify a putative hyper-responsivity to the cytokine. As shown in Figure 6B, erythroid precursors from our patients showed an increased growth rate when compared to a control, as determined by direct cell counting.

To confirm the findings obtained by reverse transcriptase PCR, vascular endothelial growth factor and soluble transferrin receptor concentrations were determined in the growth medium of the erythroid precursors after 7 days (i.e. from day 0 to day 7) and 14 days (i.e. from day 8 to day 14) of culture. As shown in Figure 6C, protein data correlated with the mRNA data shown in Figure 6A. Soluble transferrin receptor could be detected only after 14 days of culture, in accordance with the known observation that only mature erythroid precursors (CFU-E cells) synthesize transferrin receptor.

Discussion

In the present study a novel *HIF2A* mutation (Ile533Val) occurring in patient with congenital erythrocytosis has been characterized. Moreover, we identified a polycythemic subject in whom the presence of a *de novo HIF2A* genetic change (Gly537Arg) is associated with the development of the disease. The *HIF2A*-dependent erythrocytosic patients did not have increased serum levels of EPO. The levels of

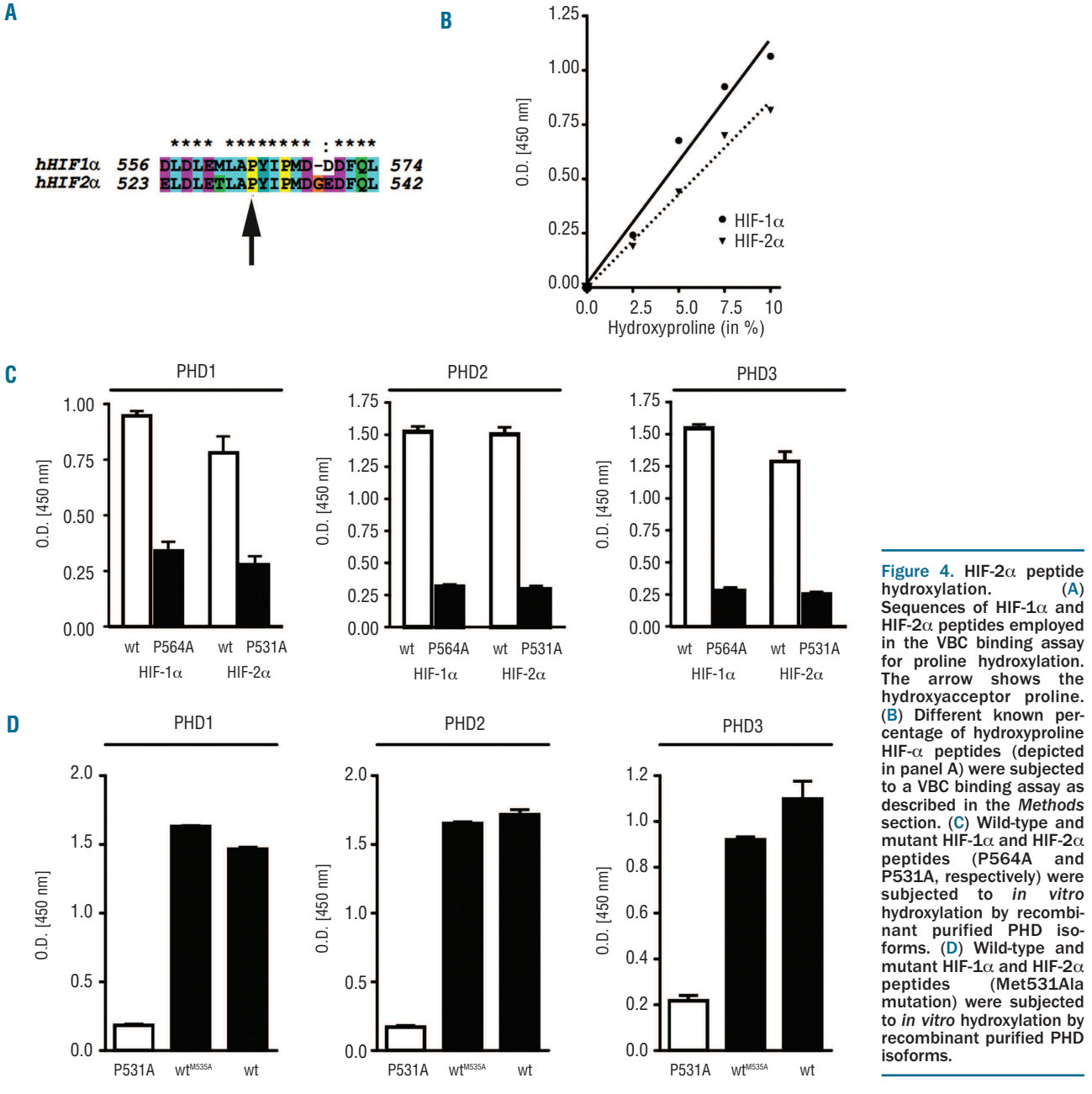

Figure 4. HIF-2α peptide hydroxylation. (A) Sequences of HIF-1α and HIF-2α peptides employed in the VBC binding assay for proline hydroxylation. The arrow shows the hydroxyacceptor proline. (B) Different known percentage of hydroxyproline HIF-α peptides (depicted in panel A) were subjected to a VBC binding assay as described in the *Methods* section. (C) Wild-type and mutant HIF-1α and HIF-2α peptides (P564A and P531A, respectively) were subjected to *in vitro* hydroxylation by recombinant purified PHD isoforms. (D) Wild-type and mutant HIF-1α and HIF-2α peptides (Met531Ala mutation) were subjected to *in vitro* hydroxylation by recombinant purified PHD isoforms.

the cytokine were, conversely, repeatedly found near the lower end of the physiological range, independently of phlebotomy. These findings suggest that *HIF2A* mutations might induce erythrocytosis, at least in the described cases, by affecting processes independent of increased EPO production. Accordingly, we found that *HIF2A* genetic changes affect the phenotype of erythroid precursors.

The novel genetic *HIF2A* change identified, namely Ile533Val mutation, represents the hereditary alteration closest to Pro-531 reported so far.[33] Although the missense is clearly a conservative substitution, i.e. a bulky hydrophobic amino acid is substituted by another large hydrophobic residue, the change produces significant functional defects. Intriguingly, Ile-533 is conserved in

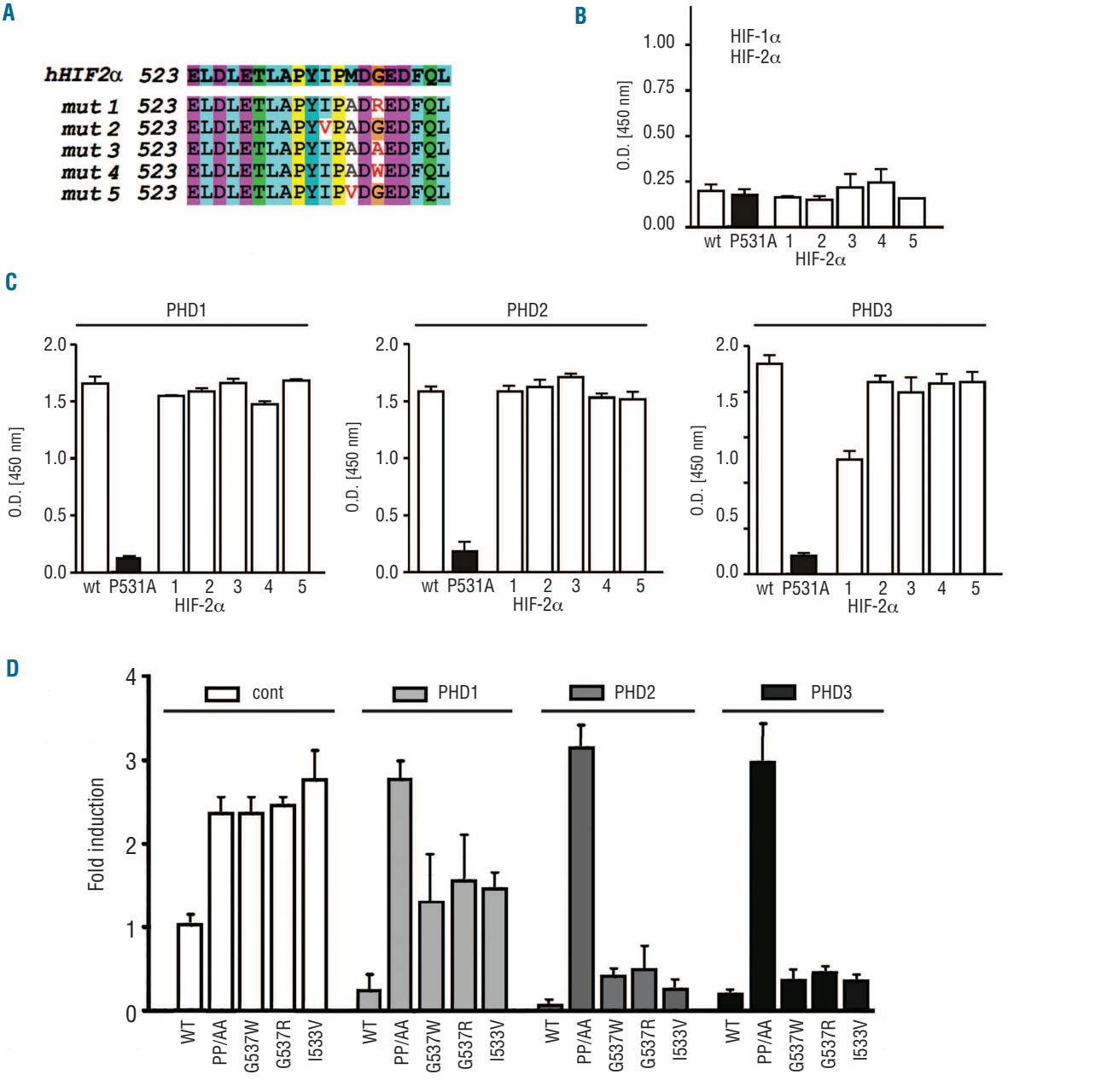

Figure 5. Functional effects of HIF-2α mutations. (A) Sequences of wild-type and mutant HIF-2α peptides employed. (B) HIF-2α peptides (depicted in panel A) and containing the indicated point mutations were used for a VBC binding assay in the absence of recombinant PHD isoforms. (C) The same HIF-2α peptides were used for the VBC binding assay following hydroxylation by the recombinant PHD as indicated. While wild-type peptides were efficiently hydroxylated by all PHD, the HIF-2α G456R mutation showed reduced hydroxylation specifically by PHD3 by approximately 40%. (D) Subconfluent Hek293 cultures were co-transfected with pGRE5xE1bluc, 250 ng of pM3-HIF-2α (amino acids 404-569) and 200 ng of the respective PHD expression construct or empty expression vector. Different mutants of pM3-HIF-2α were used, as reported in the figure, including: the wild-type (WT) form; a form mutated in both the hydroxylable proline residues (PP/AA) and three forms encoding G537W, G537R and I533V mutants. pRL-SV40 was used to normalize for transfection efficiency. Twenty-four hours post-transfection, cultures were equally distributed into 12-well plates and grown for an additional 24 h in 20% oxygen. Cells were subjected to a dual luciferase assay.

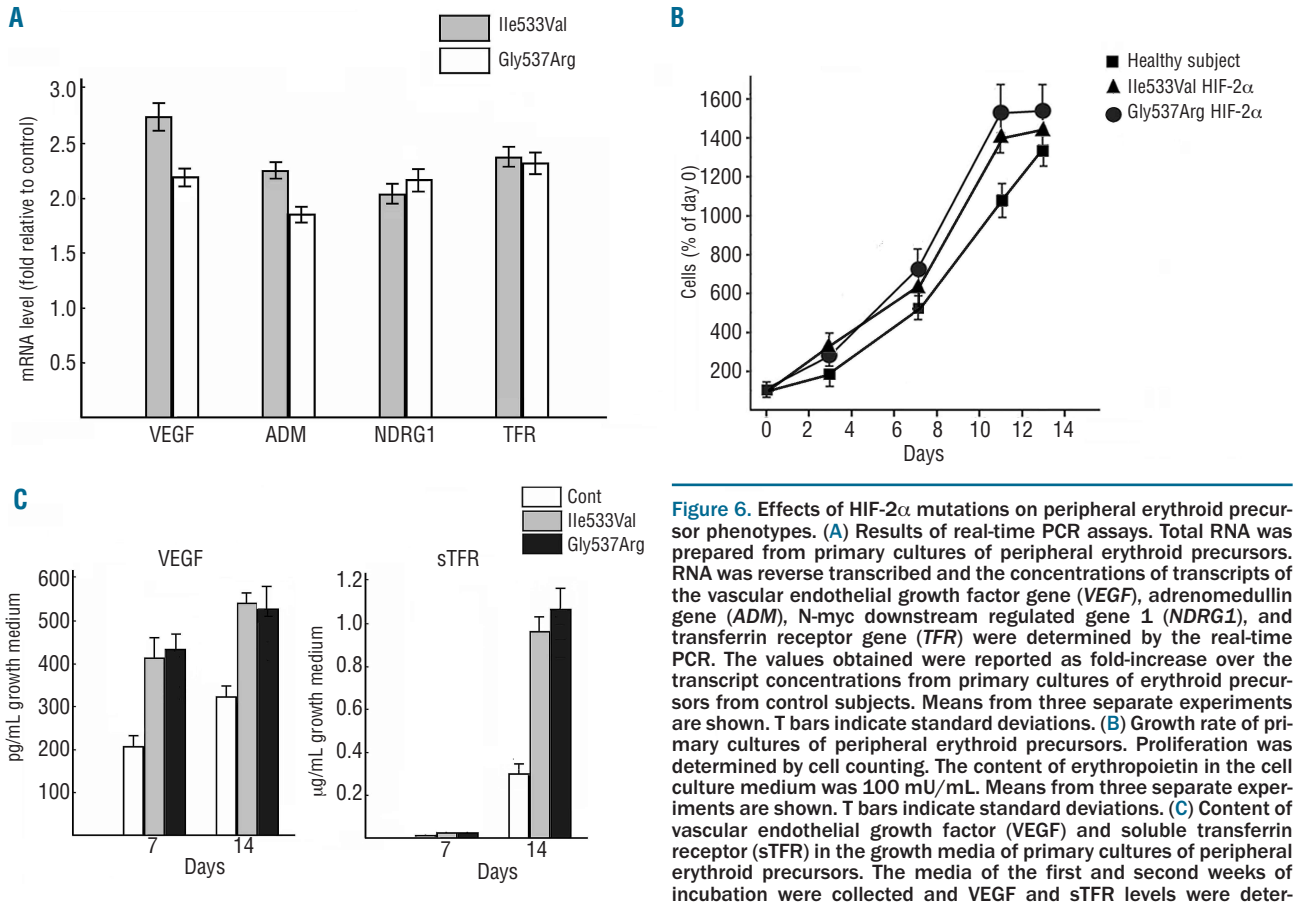

Figure 6. Effects of HIF-2α mutations on peripheral erythroid precursor phenotypes. (A) Results of real-time PCR assays. Total RNA was prepared from primary cultures of peripheral erythroid precursors. RNA was reverse transcribed and the concentrations of transcripts of the vascular endothelial growth factor gene (*VEGF*), adrenomedullin gene (*ADM*), N-myc downstream regulated gene 1 (*NDRG1*), and transferrin receptor gene (*TFR*) were determined by the real-time PCR. The values obtained were reported as fold-increase over the transcript concentrations from primary cultures of erythroid precursors from control subjects. Means from three separate experiments are shown. T bars indicate standard deviations. (B) Growth rate of primary cultures of peripheral erythroid precursors. Proliferation was determined by cell counting. The content of erythropoietin in the cell culture medium was 100 mU/mL. Means from three separate experiments are shown. T bars indicate standard deviations. (C) Content of vascular endothelial growth factor (VEGF) and soluble transferrin receptor (sTFR) in the growth media of primary cultures of peripheral erythroid precursors. The media of the first and second weeks of incubation were collected and VEGF and sTFR levels were determined by ELISA. Means from three separate experiments are shown. T bars indicate standard deviations.

HIF-2α proteins from mammalian species, human HIF-1α and HIF-3α, as well as HIF-2α proteins from chicken, frog, and zebrafish. The importance of this residue is further highlighted by X-ray crystallographic studies on the configuration of an HIF-1α peptide bound to VHL and the co-crystal structure of HIF-1α (556-574):PHD2.[51-53] These structures show that Ile 566 in HIF-1α (which corresponds to Ile-533 of HIF-2α) is localized in one of two regions that are predicted to make essential contacts with VHL and PHD2 (residues 528-533 and residues 539-542).

Our study also describes the first identified case of a *de novo HIF2A* mutation associated with the development of congenital polycythemia. The genetic change reported, glycine 537 to arginine, occurs in seven out of the 19 cases of *HIF2A* mutations (5 out of 12 families) associated with congenital polycythemia so far described (including ours).[35-37] As mentioned in the *Introduction*, a further change at Gly-537 (Gly537Trp) has been identified in three patients (1 family), allowing us to consider Gly-537 as a hot spot in the *HIF2A* gene.[34]

Following the identification of the erythrocytosis-associated Gly537Arg and Ile533Val *HIF2A* gene mutations, we functionally characterized the two mutant proteins. Functional studies on the Gly537Arg HIF-2α mutant have been published previously, although employing distinct experimental approaches.[37,39] Initially, we used peptides encompassing the mutations identified (Gly537Arg and Ile533Val), and compared the results with peptides showing a different change in Gly-537 (namely, Gly537Ala) or *HIF2A* mutations reported by others (i.e., Gly537Trp and Met535Val) and associated with familial erythrocytosis. By means of an ELISA-based test, originally developed and employed in HIF-1α studies,[49,50] we investigated both the PHD-dependent hydroxylation of the peptides and their subsequent binding to a VHL-containing complex. No differences were found between the wild-type and the mutated peptides. Previous studies employed peptides similar to those used in the present study (including Gly537Trp and Gly537Arg mutations) to evaluate either binding to PHD2 or the interaction with VHL.[34,39] These analyses showed that the mutations remarkably reduce the peptide/PHD2 association and the peptide/VHL interaction. Although we cannot definitely explain the bases of the different results, we may suggest that they could derive from the use of distinct approaches. For instance, binding between the peptides and PHD2 was investigated by co-immunoprecipitation but peptide hydroxylation by PHD2 was not analyzed.[39] Moreover, to investigate the binding between VHL and the hydroxylated peptides, we used the complex formed by VHL, elongin B and elongin C and not recombinant VHL alone (as previously done by others). Although the different interaction studies could explain the difference between our results and those reported previously, they cannot explain the difference in the hydroxylation observed in our studies. A critical reappraisal of the findings obtained from *in vitro* approaches

and based on the use of peptides seems necessary to determine the usefulness of these methodologies for investigating the functional consequences of HIF-2α mutations.

The *in vivo* experiments, on the other hand, clearly show that the identified HIF-2α mutants are more stable than the wild-type counterpart. This was demonstrated either in cells overexpressing all PHD or in unmodified cells with endogenous HIFα proline hydroxylases only. These results demonstrated that the identified genetic alterations result in increased transcriptional activity of the mutated protein.

A further observation of this study is that no increase of serum EPO was observed in the polycythemic patients identified here.[34-41] Similar data were reported in other studies on *HIF2A*-dependent polycythemias although scarce attention has been paid to this finding.[37-44] So far seven patients, including the case reported here, have been found to have the Gly537Arg *HIF2A* mutation. Three patients belong to the same family. Two of them had very high EPO levels (about 2000 mU/mL) while the third had normal EPO levels (26 mU/mL).[37] The EPO concentrations in three other subjects (from 3 families) ranged from 60 mU/mL to >200 mU/mL.[36] Our patient has an EPO level of about 6-18 mU/mL. There is, therefore, no apparent interplay between a specific *HIF2A* change and EPO level. Two hypotheses might explain the absence of correlation between genetic status and cytokine level. One is the different genetic background of subjects and the other is the age of patients (and their treatments, including phlebotomies).

Since variation of serum EPO content might not explain the polycythemic phenotype of our patients, we hypothesized that erythrocytosis might be due (at least in part) to intrinsic alterations of erythroid precursors and/or other components of the hematopoietic niche. We found that peripheral erythroid precursors from the *HIF2A*-dependent polycythemic patients showed an increased rate of growth *in vitro* when compared to the normal counterpart.

An increased hypersensitivity to EPO has also been observed in the erythroid progenitors from patients with congenital polycythemia due to the Chuvashian VHL (R200W) mutation.[29,54] Recently, it has been suggested that this could be due to impaired degradation of phosphorylated JAK2 related to altered binding of VHL R200W with SOCS.[55]

Moreover, a noticeable up-regulation of the expression of some HIF-2α target genes was also evidenced in these cells, confirming observations reported in other cell systems showing HIF-2α mutated protein.[34,38,39]

Two different approaches confirmed the last finding, namely quantitative PCR and the direct evaluation of HIF-2α-regulated protein released by the erythroid precursors in the culture medium. Collectively, these data demonstrate, for the first time, that erythroid precursors from patients with congenital erythrocytosis due to *HIF2A* gain-of-function mutations have a different phenotype with respect to the normal counterpart. This finding suggests, as also indicated by recent data,[56] that HIF-2α is essential for excessive erythropoiesis in defects of the hypoxia sensing pathway.

In conclusion, we identified a novel *HIF2A* mutation responsible for hereditary erythrocytosis and the occurrence of a *de novo HIF2A* change associated with development of the disease. Moreover, we suggest that the functional effects of the mutations should be evaluated mainly by *in vivo* experiments. Finally, we believe that the erythrocytosis due to *HIF2A* genetic changes is not simply a consequence of increased EPO but is probably due to more general effects on different cell phenotypes including erythroid precursor cells.

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