One gene, two opposite phenotypes: a case report of hereditary anemia due to a loss-of-function variant in the *EPAS1* gene

Erythropoiesis is a tightly regulated and complex process originating in the bone marrow from a multipotent stem cell and terminating in a mature, enucleated erythrocyte.[1] Erythropoiesis is controlled by erythropoietin (EPO) and signaling through its receptor, EPOR. Defects in erythropoiesis can lead to erythrocytosis.[2] Erythrocytosis embraces disorders characterized by an increase in the red cell mass, hemoglobin, hematocrit, or red blood cell count.[3] Erythrocytosis can be distinguished as familial or acquired and can be divided into primary (due to an intrinsic defect in the erythroid cell) and secondary (extrinsic to the erythroid cell).[4,5] Primary familial erythrocytosis (ECYT1) is usually an autosomal dominant condition, associated with low EPO levels due to germline *EPOR* variants. Secondary familial erythrocytosis (ECYT2-5) is caused by germline pathogenic variants in different genes involved in the hypoxia-sensing pathway, such as *VHL* (ECYT2), *EGLN1* or *PHD2* (ECYT3), *EPAS1* or *HIF-2α* (ECYT4), and *EPO* (ECYT5). Secondary erythrocytosis can result from defects causing tissue hypoxia, such as hemoglobin variants with high oxygen affinity, due to mutations in the globin genes (*HBB* or *HBA*) or defective bisphosphoglycerate mutase leading to 2,3-bisphoshoglycerate deficiency.[6] These genes are involved in key mechanisms of erythropoiesis including the HIF-EPO pathway in kidneys, the EPO-EPOR signaling pathway in the bone marrow, and the regulation of hemoglobin-oxygen affinity in red blood cells.[6] Indeed, mutations in these genes are associated with hemoglobin with high oxygen affinity and with augmented EPO production.[7] In normoxia, the VHL protein binds to HIF-1α or HIF-2α (product of the *EPAS1* gene) when hydroxylated by PHD2 (product of the *EGLN1* gene). This complex is then degraded by ubiquitination.[8] Any variation in any one of these genes can induce either loss or gain of function and results in the specific phenotype of erythrocytosis.

EPAS1 is the causative gene of ECYT4, a dominantly inherited disorder. It encodes a transcription factor involved in the induction of oxygen-regulated genes in hypoxic conditions. Most of the *EPAS1* causative variants are located in exon 12, encoding the oxygen-dependent degradation domain.[9] These variants prevent the binding of PHD2 hydroxylase to the EPAS1 protein and subsequent hydroxylation and binding of the VHL protein. This leads to an increase in EPO production, resulting in a gain-of-function phenotype. Interestingly, most of the ECYT4 causative variants are missense, possibly because stop gain variants would lead to deletion of the C-terminus.[9] On the other hand, common loss-of-function variants in *EPAS1* have been associated with physiological adaptation to low oxygen in healthy individuals living at high altitudes.[10] These polymorphic variants are mainly located in the non-coding region, probably affecting the regulation of *EPAS1* expression.

We herein describe an 8-year-old male proband referred to our Medical Genetics Laboratory Unit from the Hematology Unit of Ancona (Hospital Riuniti, Ancona, Italy) in 2021. At 4 years, the patient was firstly evaluated for microcytic anemia (hemoglobin 9.6 g/dL, mean corpuscolar volume 76 fL) unresponsive to iron treatment. First-line investigations revealed that he was negative for fecal occult blood, IgA anti-transglutaminase, and *Helicobacter pylori* antigens. The levels of vitamin B12 and folic acid were in the normal range. Hemoglobin electrophoresis and *HBB* and *HBA* genetic testing did not identify alterations. As all performed tests were almost normal, a primary bone marrow defect was suspected. Diepoxybutane and erythrocyte adenosine deaminase tests were performed. Negative results excluded Fanconi anemia and Diamond-Blackfan anemia. Subsequently, bone marrow biopsy showed a slight hypocellularity and abnormalities of the erythroid lineage, which was mostly immature, suggesting a congenital bone marrow deficiency. Thus, the patient was originally suspected to have a myelodysplastic syndrome.

When he was referred to our Medical Genetics Unit, he had chronic normocytic anemia with a reduced red blood cell count (Table 1). The family history revealed that the proband's father (I.1, 45 years old) and sister (II.1, 11 years old) also suffered from anemia, suggesting a dominant condition. The mother (I.2, 48 years old) and older brother (II.2, 15 years old) were healthy (Figure 1A, Table 1). All the affected subjects had variable degrees of anemia (hemoglobin levels ranging from 10.2 g/dL to 12.5 g/dL) and low hemocrit (ranging from 31.2% to 38.4%). The reticulocyte count was almost normal in all family members, as were the leukocyte and granulocyte counts, while the red blood cell count was slightly reduced in all three affected subjects (proband $3.8x10^{12}$/L, father $4.2x10^{12}$/L, and sister $4.1x10^{12}$/L). Similarly, slightly reduced EPO levels were observed in both the proband (4.7 U/L) and father (3.8 U/L), while EPO levels close to the lower range value (7.6 U/L)

Table 1. Clinical and genetic features of the proband and his affected/unaffected family members.

	Proband II.3			Father I.1	Mother I.2	Brother II.1	Sister II.2	Reference ranges*
Blood count and biochemical data								
Age, years	4	5	8	45	48	15	11	-
RBC, 10^{12}/L	-	-	3.8	4.2	4.4	5.3	4.1	F: 4.13-5.15 M: 4.71-5.82 6-12 yrs: 4–5.2
Hemoglobin, g/dL	9.8	9.6	10.2	12.5	13.9	15.8	11.6	F: 12.5-15.5 M: 14.2-17.2 6-12 yrs: 11.5-15.5 2-6 yrs:11.5-13.5
Hematocrit, %	-	-	31.2	38.4	40.9	47.7	35.6	F: 37.9-46.1 M: 43.1-51.5 6-12 yrs: 35-45
MCV, fL	76	80	83.0	91.0	92.0	90.0	87.0	F/M: 81.8-95.3 6-12 yrs: 77-95 2-6 yrs:75-87
Retic. count, 10^{9}/L	-	-	35.8	40.1	45.6	50.5	30.6	22.0-139.0
Reticulocytes, %	-	-	0.9	1.0	1.0	0.9	0.8	0.5-2.0
Erythropoietin, U/L	-	-	4.7	3.8	12.8	6.5	7.6	5.0-30.0
EPAS1 genotype	c.(61del)/wt			c.(61del)/wt	wt/wt	wt/wt	c.(61del)/wt	-

*Reference ranges from AOU Ospedali Riuniti, Ancona, Italy. *EPAS1* reference sequence, NM_001430.4. RBC: red blood cell count; MCV: mean corpuscular volume; F: female; M: male; yrs: years; retic.: reticulocyte.

were observed in the sister (Table 1).

The proband (II.3) was investigated through a custom gene panel for hereditary red blood cell defects.[11,12] Genomic DNA preparation, genetic testing by targeted next-generation sequencing for hereditary red blood cell defects, and validation of the variant by Sanger sequencing were performed as previously described.[13] Next-generation sequencing revealed the presence of a novel heterozygous deletion NM_001430.4:c.(61del) in the *EPAS1* gene that is predicted to affect the protein sequence by the introduction of a premature stop codon p.(Asp21Metfs*27) (Figure 1B, C). According to the American College of Medical Genetics and Genomics guidelines, this variant was predicted as pathogenic. To confirm the pathogenicity of the variant, sequencing was also carried out on the other family members. In agreement with the dominant inheritance, both the father and the sister presented the *EPAS1* variant in the heterozygous state, while the mother and the brother were negative for it (Figure 1B, C).

To characterize the effect of the EPAS1 variant, functional studies on blood samples obtained from family members were carried out. Samples were obtained after signed informed consent according to the Helsinki Declaration, allowing, by the way, anonymous use of clinical, genetic, and functional data for research purposes. The local University Ethical Committee approved the collection of the data (DAIMedLab, 'Federico II' University of Naples; N. 252/18, October 2018). First, total RNA and proteins were extracted from peripheral blood leukocytes as previously described.[12] We searched for the c.(61del) variant at the cDNA level by amplification of the specific exon region encompassing the mutation. We observed the presence of the deletion as heterozygous, suggesting that it does not account for nonsense-mediated mRNA decay. Accordingly, mRNA expression of *EPAS1* showed no variations in patients compared to healthy controls (n=15) (Figure 1D).

Additionally, we tested the effect of the variant on protein structure and synthesis. Computational predictions of the variant (*phyloP100way, http://hgdownload.soe.ucsc.edu/goldenPath/hg38/phyloP100way/*) revealed that the mutated residue is highly conserved among vertebrates and is comprised among a helix-loop-helix domain, necessary for heterodimerization. As shown by the protein sequence prediction and the general protein model in Figure 2A, B, the deletion introduces a premature stop producing a mutated protein of 46 amino acids. To evaluate the effect of the mutation on the expression of EPAS1 protein, we measured the amount of wildtype protein in patients and compared it to the amount in healthy controls using a multiple reaction monitoring approach.[14] Briefly, 50 μg of protein extracts were obtained from three patients and three controls. For each protein, at least three double-

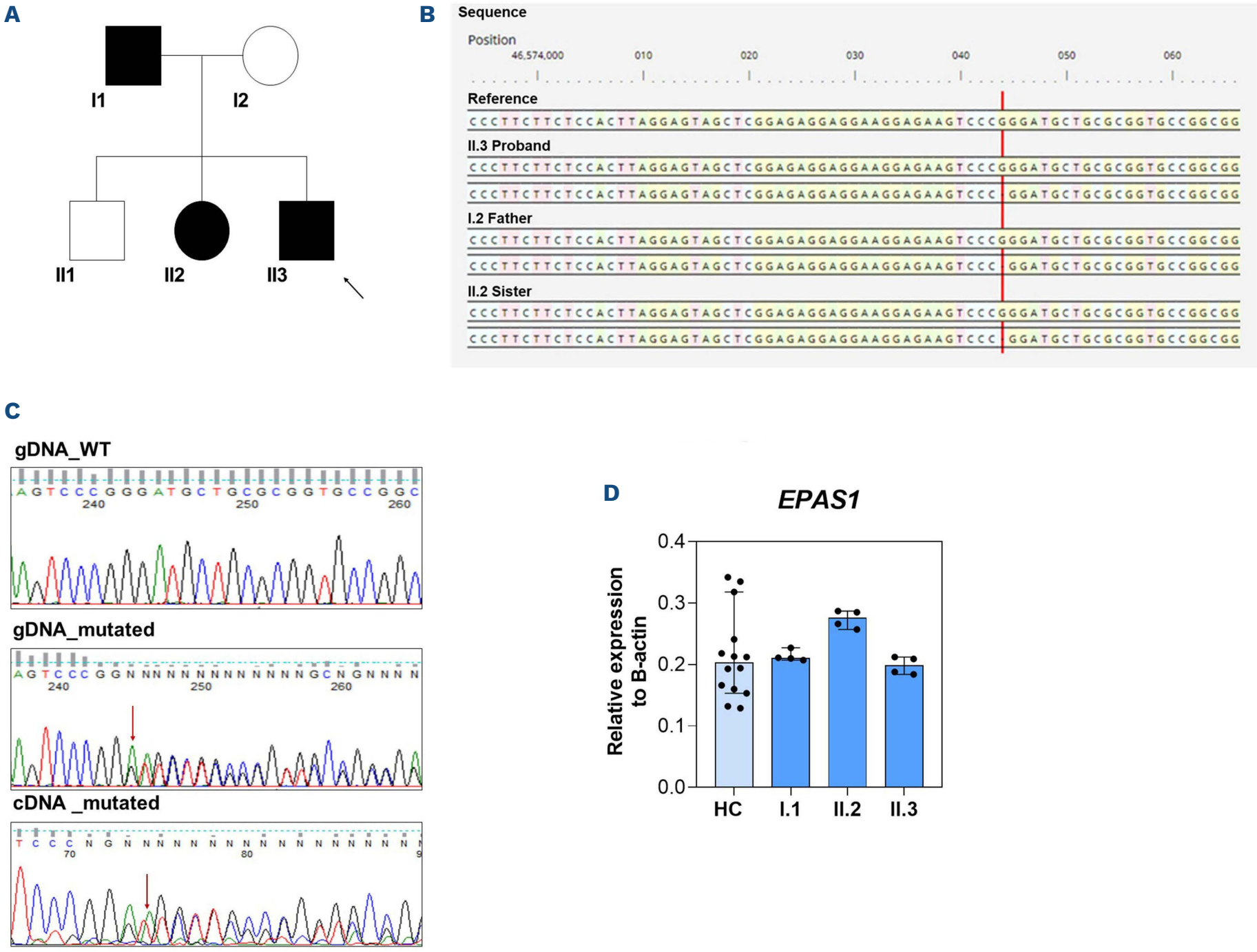

Figure 1. Genetic analysis and functional assessment of the identified *EPAS1* loss-of-function variant. (A) Family pedigree of the proband. Squares, males; circles, females; solid symbols, affected subjects; a black arrow indicates the proband. I.1, II.2, II.2 show the subjects carrying the heterozygous variant c.(61del), p.(Asp21Metfs*27) in the *EPAS1* gene. (B) Genome browser visualization of the *EPAS1* reference sequence and heterozygous mutated sequence of the affected proband and relatives (Alissa Interpret software v5.2.10; Agilent Technologies). (C) Representative Sanger sequence of the wildtype *EPAS1* gDNA (upper), mutated gDNA (middle), and mutated cDNA sequence (lower). The red arrow indicates the deleted nucleotide. (D) *EPAS1* gene expression in peripheral blood leukocytes isolated from the proband and healthy controls (HC, N=15). No significant changes in *EPAS1* gene expression were observed in patients compared to HC (P=0.127 Kruskal-Wallis test; HC *vs.* I.1 P>0.99; HC *vs.* II.2 P=0.21; HC *vs.* II.3 P>0.99, *post-hoc* correction by the Dunn multiple comparisons test). Data are presented as the median with 95% confidence interval. WT: wildtype; HC: healthy controls.

charged prototypic peptides were selected with at least three transitions (Figure 2A), *in silico* predicted by using Skyline. Fold changes were determined by normalization to GAPDH-derived peptides. Interestingly, no EPAS1-mutated protein was detected in either the healthy controls or affected subjects. Accordingly, we observed a strong reduction of EPAS1 wildtype protein in patients compared to healthy controls (Figure 2C).

Although gain-of-function variants in *EPAS1* are classically associated with ECYT4, hematochemical and biochemical data from this family did not recapitulate the clinical picture of erythrocytosis but an opposite phenotype due to a loss-of-function variant. Indeed, our data suggest that the deletion herein described accounts for EPAS1 haploinsufficiency. Interestingly, EPAS1/HIF-2α null mice presented a similar phenotype, with normocytic anemia in the presence of reduced hematocrit, normal reticulocyte count, and reduced erythrocyte count, accompanied by pancytopenia.[15]

To the best of our knowledge, this is the first report of a null variant in the *EPAS1* gene that accounts for an anemic phenotype. This case underlines once again the relevance of next-generation sequencing in the diagnostic workflow of the complex landscape of hereditary erythrocyte de-

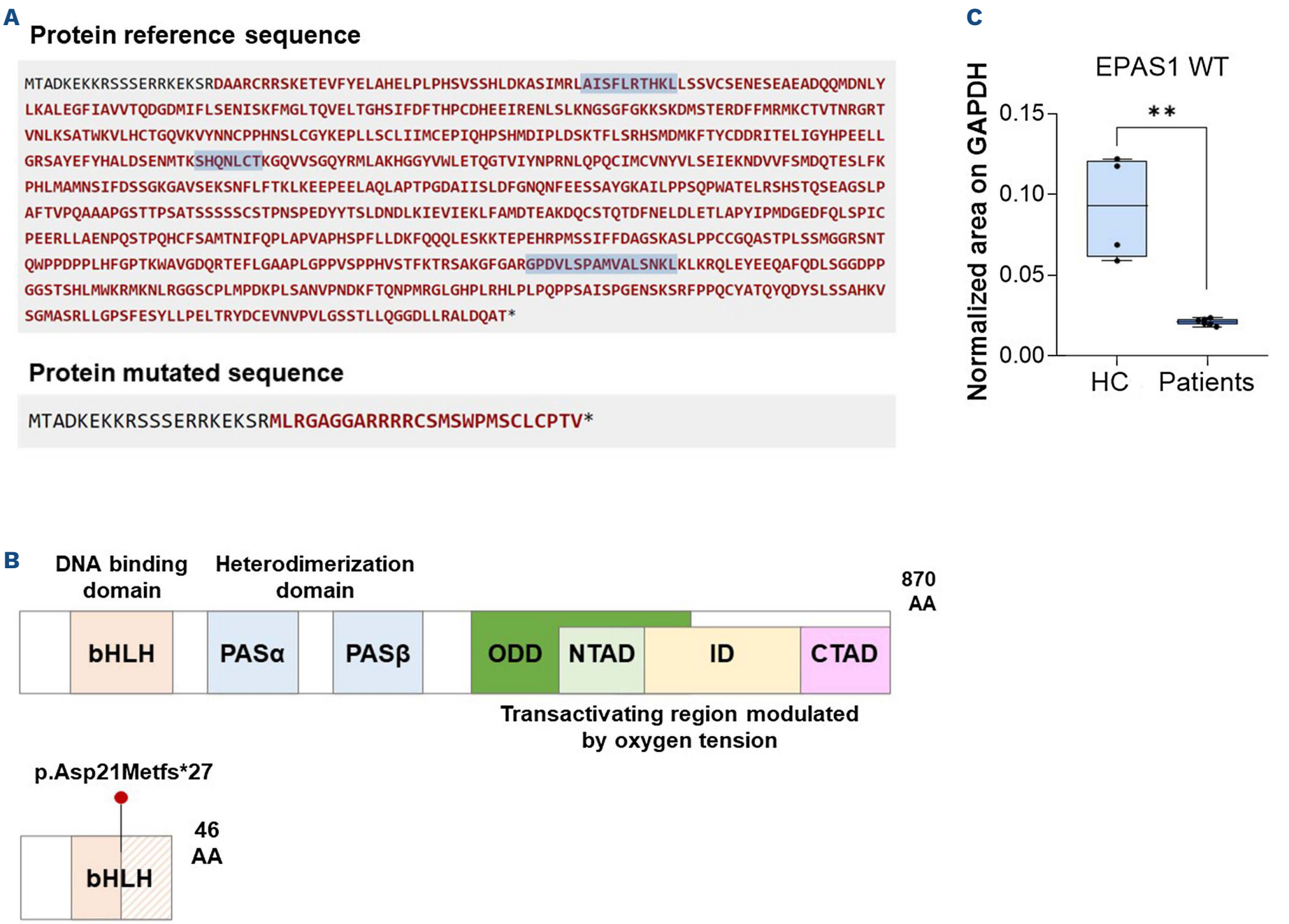

Figure 2. The EPAS1 deletion variant accounts for EPAS1 haploinsufficiency. (A) Predicted protein reference (upper) and mutated (lower) sequence by Mutalyzer (v 3.0.4, *https://mutalyzer.nl/*). The three EPAS1 wildtype-derived peptides for multiple reaction monitoring analysis are highlighted in light blue. (B) General protein model of EPAS1 representing EPAS1 wildtype (upper) and hypothesized mutated (lower) protein structure. (C) Boxplot showing normalized mean areas for the amount of EPAS1 wildtype protein in patients compared to that in healthy controls. The mean of the total transition area relative to each peptide of the EPAS1 protein was normalized to the mean of the total transition area of the GAPDH protein. All samples were run in technical duplicates. **P<0.01. P value by the Mann-Whitney test.

fects. Moreover, this paradigmatic clinical case highlights how the effect of genetic variants strictly depends on the variant itself, suggesting that the gene-to-disease paradigm should be replaced by the variant-to-disease one in the current era of precision medicine.

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Disclosures

No conflicts of interest to disclose.

Contributions

AI, IA, and RR designed and conducted the study. BER prepared the initial draft of the manuscript. RM, FDG, and AN prepared the library enrichment, and performed the Sanger sequencing analysis and gene expression analysis. SG, BB, and PC cared for the patient. VM and MM performed the multiple reaction monitoring analysis. AI critically revised the manuscript.

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Data-sharing statement

The original data and protocols are available to other investigators upon request.

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