DR. KAREN G. SCHEPS (Orcid ID : 0000-0002-8812-714X)

Article type : Original Article

Two novel unstable hemoglobin variants due to in-frame deletions of key amino acids in the β-globin chain

Karen Gabriela Scheps[1,2,§], Marcia Anahí Hasenahuer[3,§], Gustavo Parisi[3], Héctor M. Targovnik[1,2], Eliana García[4], Ernesto Samuel Veber[5], Renée Crisp[4], Graciela Elena[5], Viviana Varela[1,2], María Silvina Fornasari[3].

[1]Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Microbiología, Inmunología y Biotecnología/Cátedra de Genética, Buenos Aires, Argentina

[2] CONICET-Universidad de Buenos Aires, Instituto de Inmunología, Genética y Metabolismo (INIGEM), Buenos Aires, Argentina

[3]Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología, Bernal, Buenos Aires, Argentina.

[4] Sección Hematología y Oncología, Servicio de Pediatría, Hospital Nacional A. Posadas, Pcia de Bs As. Argentina

[5]Servicio de Hemato-Oncología Pediátrica. Hospital General de Niños "Dr. Pedro de Elizalde", Ciudad Autónoma de Buenos Aires, Argentina.

[§] Both authors contributed equally to this work

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ejh.13029

Correspondence to: Dra. Karen Scheps

Cátedra de Genética, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires

Junin 956, C1113AAC, Ciudad de Buenos Aires, Argentina

Tel.: 54-11-49648296; Fax: 54-11-49648296

E-mail: karenscheps@gmail.com

Short running title: Two novel unstable hemoglobin variants

ABSTRACT

Hemoglobinopathies are the most common autosomal recessive disorders and are mostly inherited in a recessive manner. However, certain mutations can affect the globin chain stability, leading to dominant forms of thalassemia.

The aim of this work was the molecular and structural characterization of two heterozygous in-frame deletions, leading to β-globin variants in pediatric patients in Argentina. The *HBB* gene of the probands and their parents was sequenced, and other markers of globin chain imbalance were analyzed. Several structural analyses were performed and the effect of the mutations on the globin chain stability was analyzed.

In **Hb JC-Paz**, *HBB:*c.29_37delCTGCCGTTA (p.Ala10_Thr12del), detected in an Argentinean boy, one α-helix turn is expected to be lost. In **Hb Tavapy**, *HBB*:c.182_187delTGAAGG (p.Val60_Lys61del), the deleted residues are close to distal histidine (His63) in the heme pocket. Both mutations are predicted to have a destabilizing effect.

The development of computational structural models and bioinformatics algorithms is expected to become a useful tool to understand the impact of the mutations leading to dominant thalassemia.

KEYWORDS

Unstable hemoglobin variants; *HBB* gene; Dominant beta-thalassemia; Bioinformatics; Hemolytic anemia

INTRODUCTION

Hemoglobinopathies are the most common autosomal recessive disorders worldwide, with an incidence of over 350,000 affected newborns per year [1]. At least 5.2% of the world's population carries a significant variant and hemoglobin (Hb) disorders contribute the equivalent of 3.4% of mortality in children aged under 5 years [2]. To date there are more than 1,500 sequence variants reported in the databases "HbVar database" [3] and "ITHANET" [4], associated with this group of syndromes.

In terms of the quality and quantity of the Hb synthesized as a result of these changes, Hb disorders can be broadly classified into two general categories [5]:

- **Structural hemoglobin variants**, due to qualitative defects in one of the globin subunits that result in the production of structurally abnormal globin chains. Although this category includes over 1,200 variants, only a fraction have phenotypic impact. Within this group can be included variants that produce unstable Hbs, that can lead to hemolytic anemia. The most common mutations that decrease the stability of the Hb are small deletions or substitutions that usually affect the heme pocket. The resulting instability is often caused by premature dissociation of the heme from the globin chain. Such heme-depleted globin is precipitated as intracellular material known as Heinz bodies [6]. These mutations affect most frequently the *HBB* gene. Despite their inherent instability, the chains are able to combine with α-globin subunits to produce a hemoglobin tetramer that precipitates in the mature red cell in the peripheral blood [7].

- **Thalassemias,** characterized by decreased production of one of the subunits that comprises adult hemoglobin and consequently, a relative excess of the remaining subunit, normally synthesized. They are classified into α or β-thalassemias (thal), depending respectively on the affected gene.

Although these syndromes are typically inherited in a Mendelian recessive manner, dominant forms of β-thal (OMIM: 603902) have been described, due to mutations that affect both the structure as well as the quantity of the synthesized β-globin chains. These frequently arise as *de novo* point mutations [8]. The highly unstable nascent globin chains are unable to ensemble the $\alpha_1\beta_1$ dimers. This leads to ineffective erythropoiesis, also exacerbated by the concomitant relative excess of the α-chains [9]. Our group has already identified and characterized 2 elongated variants associated with dominant β-thal in 2 pediatric Argentinean patients [10].

In this report we present 2 novel small deletions of 2 and 3 amino acids, respectively, leading to unstable variants in 2 pediatric patients, one with thalassemia intermedia-like features and the other with a β-thal major phenotype, both with hematological normal parents. We propose a putative pathogenic mechanism for the observed phenotype based on β-globin sequence and structural analysis.

PATIENTS AND METHODS

Proband 1 was an Argentinean 5-year-old boy, full term born from a non-consanguineous couple. He was admitted to the National Hospital A. Posadas with respiratory symptoms and non-immune hemolytic anemia, requiring a transfusion with packed red blood cells. Upon physical examination the patient presented moderate splenomegaly and an ultrasound revealed the presence of gallstones. The subsequent evolution of the patient was characterized by mild basal hemolysis with elevated LDH levels, exacerbated during hemolytic crises that were accompanied by fever, jaundice, vomiting, headaches and hemoglobinuria. The proband presented delayed growth and weight gain

and developed severe splenomegaly and nephropathy. The patient required, after the initial crisis, 6 additional packed red blood cells transfusions; 3 of these hemolytic crises were triggered by an infection. When the patient turned 6 years-old, his phenotype resembled a thalassemia intermedia, with occasional acute hemolytic crises.

Proband 2 was an 11-year-old patient born in Colonia Tavapy, Paraguay. She was the first daughter of a non-consanguineous couple, who also had a second healthy child. At the time of the admission to the Hematology Service of the "Dr. Pedro de Elizalde" Hospital, the patient presented a regular transfusion requirement and had already been splenectomized in her home country when she was 3 years old. The proband did not present hepatomegaly, and the biliary tract was normal, although jaundice of skin and mucous membranes was observed.

The parents of both probands were normal upon physical examination, with normal hematological features.

Written informed consents following the current version of the Helsinki Declaration were obtained from the individuals involved in this study and the research project was approved by the institutional bioethical committee.

Peripheral blood cell counts and erythrocyte indices were determined using an electronic cell counter (Sysmex XT2000i; Sysmex Corporation, Kobe, Japan). Hemoglobin electrophoreses were carried out with a semiautomatic agarose gel system at both alkaline and acid pH (Sebia,Lisses, Évry, France). Hb A_2 was assessed with microcolumn chromatography and Hb F using an alkali denaturation method. Heinz bodies were evaluated by means of incubation with brilliant cresyl blue [11]. Tests for stability were carried out by the isopropanol precipitation test [12].

Genomic DNA from the probands and their asymptomatic parents was isolated from peripheral blood leucocytes using standard methods [13]. The complete *HBB* gene was analyzed as previously described [14]. In order to study the alleles separately and identify precisely the variants found, the

patients' *HBB* PCR products, from c.-209 to c.315+144 were cloned in the pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA) as described in [15] and sequenced.

The most common α-thal^{+} deletions, -$\alpha^{3.7}$, and the $\alpha\alpha\alpha^{anti3.7}$ insertion were analyzed by GAP-PCR [16, 17]. Three SNPs in the *loci* that showed the strongest association with Hb F levels were studied [18]: *rs7482144*, in the promoter region of *HBG2* (minor allele T), *rs1188686* in the second intron of *BCL11A* (minor allele G) and *rs4895441* in the intergenic region of *HBS1L-MYB* (minor allele G).

To improve our understanding on the impact of the β-globin sequence changes in functional or structural perturbations, the altered amino acid sequences were studied analyzing their physicochemical properties, sequence conservation, secondary and tertiary structure predictions and structural stability calculations. Structural and/or functional importance of the positions altered in these patients were analyzed using Evolutionary Trace method [19], sequence variation impact on protein function was studied using PROVEAN [20]. Sequence based secondary structure prediction was performed on the novel variants using PSIPRED [21]. Moreover, in order to obtain structural models of both variants, homology modelling of β-globin chains was performed. Modeller program [22] was used for this purpose including as templates previously selected structures deposited in PDB (www.rcsb.org) [23]. These structures, PDB IDs 2DN1 and 2DN2 [24], correspond, respectively, to an oxy dimer and to a deoxy tetramer human consensus wild type Hb (UniProtKB codes P69905 for HBA_HUMAN and P68871 for HBB_HUMAN). These structures were selected due to their high crystal resolution taking into account that as higher is the resolution more structural details could be confidently inferred [25]. In order to describe the impact of these sequence variants in the hemoglobin structure stability, and to obtain significant results, it was necessary to assess several structural models. Following this objective, and for each variant, 100 structural models were obtained for β-globin oxy and deoxy forms. These models were structurally corrected using *repairpdb* routine and then stability energy estimations were performed using, for both steps, the program FoldX [26]. This routine allows the free energy of folding (ΔG) estimation of a target protein

structure. Structural model quality assessments were performed using the programs Procheck and ProSAWeb [27, 28]. In order to compare structure stability changes with respect to wild type hemoglobins, all available RX crystal structures of wild type human Hb β-chains were downloaded from CoDNaS database [29], avoiding those with carbon monoxide, nitric oxide, Niquel, Xenon, abnormal salt concentration or relative humidity, as well as structures not published in papers. Stability estimations were done for these selected 45 structures. Looking for electrostatic changes, structure electrostatic calculations were also performed with Bluues server for wild type and variants structures [30].

RESULTS

The hematological parameters of the probands are summarized in **Table 1.** The values for **Proband 1** were obtained during the initial hemolytic crisis. None of the patients presented an elevated Hb A_2 fraction: it was within normal range for **Proband 1** and slightly below the cutoff for **Proband 2**. A low percentage of an abnormal Hb X fraction was observed in the electrophoresis from **Proband 2**, which hinted the presence of an unstable Hb variant. No abnormal hemoglobin fraction was present in the electrophoresis of **Proband 1**. However, the proband presented a positive isopropanol test and Heinz bodies were detected, suggesting the synthesis of an unstable variant in this patient, as well. In both probands, erythroblasts in peripheral blood were observed and the levels of Hb F were incremented, which would further indicate that they had been subjected to hematopoietic stress. To study a possible genetic influence in the Hb F levels exhibited, the 3 major quantitative *loci* associated with the modulation of this fraction after birth were analyzed in the probands. Both exhibited the same genotypes: C/T for the SNP *rs7482144* in the promoter region of *HBG2*; A/A, for *rs4895441* in the intergenic region of *HBS1L-MYB* and homozygosity for the protection allele G for *rs1188686* in the second intron of *BCL11A*. The G/G genotype for *rs1188686* could influence, at least slightly, the expression of Hb F. Other *loci* and environmental factors, may be contributing to the differential expression of the *HBG1* and *HBG2* genes.

The presence of mutated Hb variants was established by DNA sequencing and confirmed by cloning the mutated alleles. The DNA sequences of the patients and the translations of the abnormal β-globin chains are shown in **Figure 1**. Both probands presented novel in-frame deletions in the *HBB* gene: **Proband 1** presented the change *HBB:*c.29_37delCTGCCGTTA (p.Ala10_Thr12del) in heterozygote state. As a result of this mutation, an Alanine, a Valine and a Threonine of the "A" α-helix of the β-globin chain are deleted. The name **Hb JC-Paz** was given to this new variant, since the patient was born in this region of the Buenos Aires Province.

According to physicochemical properties, the isoelectric points of both variants have values close to wild type β-chains, unlike the two previously Argentinean variants reported by our group [31]. Sequence conservation derived results showed an important degree of conservation in the affected positions. PROVEAN sequence based prediction of p.Ala10_Thr12del indicates a deleterious effect with a strong score (-14.38, cutoff −2.5 for high balanced accuracy). These deleted residues are close to N-term and at least one α-helix turn is expected to be lost. **Figure 2a** includes the structural characterization of the altered region. Moreover, this alteration could affect the polar interactions of Val1 and His2 with the allosteric modulator 2,3-diphosphoglycerate (2,3-DPG). However, alterations affecting this interaction have been previously reported in several variants and its impact has been proposed as not destabilizing enough to explain the observed instability: reports of Hb Rahere (*HBB*:c.248A>C, p.Lys82Thr), Hb Providence (*HBB*:c.249G>C/*HBB*:c.249G>T, p.Lys82Asn) and Hb Helsinki (*HBB*:c.248A>T, p.Lys82Met), which are variants caused by different amino acid substitutions at the 2,3-DPG binding site on Lys82, describe an altered oxygen affinity with no consequences regarding the stability of the resulting variants [32]. The most important finding in terms of structural characterization for both variants is the associated instability of both compared with their wild type counterparts (these results are commented below taking together the two variants).

Proband 2 presented the mutation *HBB*:c.182_187delTGAAGG (p.Val60_Lys61del) in heterozygous state. As a consequence of this deletion in exon 2, the Valine and Lysine of codons 60 and 61 are lost (positions 4 and 5 of the "E" α-helix), altering the globular structure of the modified chain.

Considering the birthplace of the patient, **Hb Tavapy** was the name given to this new Hb variant. As well as in **Hb JC-Paz**, sequence conservation derived scores showed that the positions involved in this deletion have an important degree of conservation. Moreover, PROVEAN sequence based prediction of p.Val60_Lys61del indicates a deleterious effect with a strong score (-16.74). Additionally, deleted residues are close to distal histidine (His63) in the heme pocket. **Figure 2b** includes the structural characterization of the altered region.

Aiming at compare structural stability of the different variants and of the wild-type Hbs, **Figure 3** shows the distributions of the estimation of ΔG free energy of folding for the 100 models of each template and wild type structures (in oxy and deoxy forms). As it can be observed in the boxplots, the models for both variants (last four boxplots, right side) show higher ΔG energies, compared to the ones for the wild type conformers (first two boxplots, left side), meaning that they are more unstable structures. Also, oxy forms are slightly more unstable that deoxy ones.

The patients did not present any other alterations in the *HBA* genes that would contribute to the observed phenotype. In both cases, the mutations analyzed were absent in their parents' DNA.

DISCUSSION

As sequencing is becoming a more accessible strategy worldwide to identify β-thal mutations and the taboo associated with the genetic studies to identify carriers is disappearing, the number of reports of novel mutations affecting the globin genes is increasing. In fact, our group has previously reported 2 novel α-thal mutations and 2 variants that lead to elongated β-globin chains in Argentinean pediatric patients [10]. In this study, we identified 2 novel *de novo* occurring in-frame

deletions that decrease the stability of the β-globin chain, resulting in thal intermedia features with hemolytic anemia in an Argentinean boy and dominant β-thal in a Paraguayan girl. There are 24 entries of strictly in frame-deletions affecting the *HBB* gene reported in the HbVar and ITHANET Databases. Interestingly, many of these variants lead to a decreased stability of the β-globin chain. The mutations that map in exon 1 are mostly associated with recessive structural hemoglobinopathies with altered O_2 affinity. The deletions affecting the second exon also lead to mild hemolytic anemia, although 2 variants that affect the β-chain, Hb Korea (*HBB*:c.100_102delGTG) [31] and Hb Dresden (*HBB*:c.101_106delTGGTCT) [33] trigger dominant β-thal phenotypes. All except 2 mutations affecting Exon 3 (Hb Coventry, *HBB*:c.424_426delCTG- and Hb Birmingham, *HBB*:c.425_433delTGGCCCACA-) produce both structural and thalassemic variants that lead to dominant β-thal.

The deletion that gives rise to **Hb JC-Paz** involves 9 pb: CTGCCGTTA. Interestingly, the pentanucleotide CTGCC is repeated immediately downstream the deleted sequence, so it may have contributed to the genesis of the deletion. As a consequence, the Alanine, Valine and Threonine from codons 10 to 12 are lost. This mutation is the in-frame deletion that affects the β-globin gene located most 5'. Conservation analysis reveals that Valine is the most conserved residue of these three residues. There is only 1 variant with clinical repercussions for the substitution of this residue: Hb Windsor (*HBB*:c.35T>A, *p.Val11Asp*) [34], that leads to hemolytic anemia. There are 2 other variants described: Hb Washtenaw (*HBB*: c.34G>T, *p.Val11Phe*), slightly unstable with decreased affinity for the O_2 and Hb Hamilton (*HBB*:c.34G> A, *p.Val11Ileu*) without any particular phenotype associated. None of the structural variants that result from the substitution of the Alanine (Hb Belleville: *HBB*:c.31G>A, *p.Ala10Thr*; Hb Ankara: *HBB*:c.32C>A, *p.Ala10Asp*; Hb Iraq-Halabja: *HBB*:c.32C>T, *p.Ala10Val*) or the Threonine (Hb William-Harvey: *HBB*:c.38C>T, *p.Thr12Ileu*) have any clinical repercussions. The sequence, structural and electrostatic analyses performed in the present work have revealed that the deletion would have an important deleterious effect. While an alteration of contacts with 2,3-DPG can be suspected, there are not many reports that would

indicate that the alteration of 2,3-DPG interaction could be responsible for the hematological alterations observed, especially since the clinical picture of the patient is different from those described by other in-frame deletions affecting exon 1: in basal state it resembles thalassemia intermedia, with most significant clinical features (splenomegaly and growth retardation) starting to develop around 5-6 years old. It is notorious that the patient also experienced hemolytic crises, making it more difficult to specify his phenotype.

The deletion presumably, and analyzing several wild type structures, does not involve any non-covalent interaction including interface residues, not directly nor through a network of residues involved in non-covalent interactions. The main finding was the obtained by homology modeling obtained structures followed by stability calculations, showing a notorious destabilizing effect caused by this deletion.

The deletion originating the **Hb Tavapy** involves 6 pb and deletes the Valine and Lysine of codons 60 and 61. Unlike the 2 other variants reported that affect the "E" helix (Hb Saint-Antoine: *HBB*:c.223_228delGGCCTG and Hb Vicksburg: *HBB*:c.226_228delCTG) that lead to unstable structural variants, the loss of these residues leads to a thalassemic structural variant.

The affected Valine is highly conserved among species, so the loss of this amino acid would already have an impact. In fact, there are 3 reports of structural variants due to substitution of this residue: Hb Yatsushiro (*HBB*:c.181G>C, *p.Val60Leu*), Hb Collingwood (*HBB*:c.182T>C, *p.Val60Ala*) and Hb Cagliari (*HBB*:c.182T>A, *p.Val60Glu*) [35]. The last one is a thalassemic variant that leads to dominant β-thal. There are 2 variants that involve the substitution of the Lysine 61: Hb Pocos de Caldas (*HBB*:c.184A>C, *p.Lys61Gln*) and Hb N-Seattle (*HBB*:c.184A>G, *p.*Lys61Glu). Neither leads to clinical consequences. Sequence, structural and electrostatic analyses have revealed that this two amino acid deletion have deleterious effect and besides, the heme binding pocket is affected.

The analysis of secondary modifiers could not contribute to explain the differences in the phenotype exhibited by both patients. Although the levels of Hb F could play a role modulating the clinical outcome, the primary modifier -the mutations in *HBB*- seems to be mainly to blame for the clinical behavior of the probands.

It is difficult to establish a correlation between sequence, structural and physicochemical alterations predicted and the clinical outcome of these two patients. Allosteric regulation alteration should seriously impact in the adequate O_2 delivery. But, heme pocket integrity is essential. The results of structural modelling performed in this work show a notorious change in the structural stability of both forms compared with wild type structures. In **Hb JC-Paz** as well as in **Hb Tavapy**, both oxy and deoxy structural models show an increment in the instability of beta chains, being more important the reduction in stability for oxy-forms. These changes correlate with the hematological and clinical features of both patients.

The synthesis of novel abnormal hemoglobins confers a challenge for the treating physician, when it is not possible to frame the clinical behavior of the patient in those described in literature. It is expected that the development of structural models and bioinformatic algorithms could improve the characterization of these variants and their relationship with the patients' clinical behavior.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

Nothing to report.

SOURCES OF FUNDING

This study was supported by Research Grants: 20020100200073 and 20020120200092BA from the Universidad de Buenos Aires (UBA), 112-200801-02849, Multi-year Research Projects from the

Consejo Nacional de Investigaciones Científicas y Técnicas (PIP CONICET), and 1004/11 from the Universidad Nacional de Quilmes (UNQ).

REFERENCES

1. Giordano PC, Harteveld CL, Bakker E. Genetic epidemiology and preventive healthcare in multiethnic societies: The hemoglobinopathies.Int J Environ Res Public Health 2014; 11(6): 6136-6146.

2. Modell B, Darlison, M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ 2008; 86(6): 480-487.

3.Patrinos GP, Giardine B, Riemer C, Miller W, Chui DH, Anagnou NP, Wajcman H, Hardison RC. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies. Nucleic Acids Res 2004; 32(suppl 1): D537-541.

4. Kountouris P, Lederer CW, Fanis P, Feleki X, Old J, Kleanthous M. IthaGenes: an interactive database for haemoglobin variations and epidemiology. PloS One 2014; 9(7): e103020.

5. Forget BG, Bunn HF. Classification of the Disorders of Hemoglobin. Cold Spring HarbPerspect Med 2013; 3(2): a011684.

6. Patrinos GP, Antonarakis SE. Human hemoglobin. In: Speicher M, Antonarakis SE, Motulsky AG, eds. Vogel and Motulsky's Human Genetics. Springer-Verlag Berlin Heidelberg; 2010: 365-401.

7. Weatherall DJ. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet 2001; 2(4): 245-255.

8. Luo HY, Tang W, Eung SH, Coad JE, Canfield P, Keller F, Crowell EH Jr, Steinberg MH, Chui DH. Dominantly inherited beta thalassemia intermedia caused by a new single nucleotide deletion in

exon 2 of the beta globin gene: Hb Morgantown (beta91 CTG>CG). J ClinPathol 2005; 58: 1110-1112.

9. Cao A, Galanello R. Beta-thalassemia. Genet Med 2010; 12:61–76.

10. Scheps KG, Hasenahuer MA, Parisi G, Fornasari MS, Pennesi SP, Erramouspe B, Basack FN, Veber ES, Aversa L, Elena G, Varela V. Hb Wilde and Hb Patagonia: two novel elongated beta-globin variants causing dominant beta-thalassemia. Eur J Haematol. 2015; 94: 498-503.

11. Eisinger J, Flores J, Tyson JA, Shohet SB. Fluorescent cytoplasm and Heinz bodies of hemoglobin Köln erythrocytes: evidence for intracellular heme catabolism. Blood 1985; 65: 886-893.

12. Carrell RW, Kay R. A simple method for the detection of unstable heamoglobins. Br J Haematol 1972; 23: 615-619.

13. Murray MG, Thompson WF. Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res 1980; 8: 4321-4325.

14. Rossetti LC, Targovnik HM, Varela V. The molecular basis of beta-thalassemia in Argentina. Influence of the pattern of immigration from the Mediterranean Basin. Haematologica 2004; 89: 746-747.

15. Scheps, KG; Binaghi A; Varela V. Identification of a new HBA1 gene mutation (HBA1:c.301-2A>T) in cis with the Hb Riccarton (*HBA1*:c.154G>A) [alpha1 51(CE9) Gly>Ser]. Hemoglobin 2012; 36: 504-507

16. Tan AS, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for α-thalassemia. Blood 2001 ;98:250-251.

17. Wang W, Ma ES, Chan AY, Prior J, Erber WN, Chan LC, Chui DH, Chong SS.Single-tube multiplex-PCR screen for anti -3.7 and anti -4.2 α-globin gene triplications. ClinChem 2003; 49:1679-1682.

18. Badens C, Joly P, Agouti I, Thuret I, Gonnet, K, Fattoum, S, Francina A, Simeoni MC, Loundou A, Pissard S. Variants in genetic modifiers of β-thalassemia can help to predict the major or intermedia type of the disease. Haematologica 20110; 96:1712-1714.

19. Lichtarge O, Bourne HR, Cohen FE. An evolutionary trace method defines binding surfaces common to protein families. J MolBiol 1996; 257: 342-358.

20. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics 2015: btv195.

21. Jones DT. Protein secondary structure prediction based on position-specific scoring matrices. J MolBiol 1999; 292: 195-202.

22. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol. 1993; 234: 779-815.

23. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The Protein Data Bank. Nucleic Acids Res. 2000; 28: 235-242.

24. Park SY, Yokoyama T, Shibayama N, Shiro Y, Tame JR. 1.25 A resolution crystal structures of human haemoglobin in the oxy, deoxy and carbonmonoxy forms. J Mol Biol. 2006; 360: 690-701.

25. Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. Annu Rev BiophysBiomol Struct 2000; 29: 291-325.

26.Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Serrano L. The FoldX web server: an online force field. Nucleic Acids Res. 2005; 33 (Web Server issue):W382-8.

27. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. J ApplCrystallogr 1993; 26: 283-291.

28.Wiederstein M, Sippl MJ. ProSA-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 2007; 35(suppl 2): W407-410.

29. Monzon AM, Rohr CO, Fornasari MS, Parisi G. CoDNaS 2.0: a comprehensive database of protein conformational diversity in the native state. Database (Oxford). 2016 Mar 28; 2016.

30. Walsh I, Minervini G, Corazza A, Esposito G, Tosatto SC, Fogolari F. Bluues server: electrostatic properties of wild-type and mutated protein structures. Bioinformatics. 2012; 28: 2189-2190.

31. Park SS, Barnetson R, Kim SW, Weatherall DJ, Thein SL. A spontaneous deletion of beta 33/34 Val in exon 2 of the beta globin gene (Hb Korea) produces the phenotype of dominant beta thalassaemia. Br J Haematol. 1991; 78: 581-582

32. Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin variants: biochemical properties and clinical correlates. Cold Spring HarbPerspect Med. 2013; 3:a011858.

33. Vetter B, Neu-Yilik G, Kohne E, Arnold R, Sinha P, Gaedicke G, Ivancevic V, Kulozik AE. Dominant beta-thalassaemia: a highly unstable haemoglobin is caused by a novel 6 bp deletion of the beta-globin gene. Br J Haematol. 2000; 108:176-181.

34. Gilbert AT, Fleming PJ, Sumner DR, Hughes WG, Holland RA, Tibben EA. Hemoglobin Windsor or beta 11 (A8)Val----Asp: a new unstable beta-chain hemoglobin variant producing a hemolyticanemia. Hemoglobin. 1989; 1: 437-453.

35. Podda A, Galanello R, Maccioni L, Melis MA, Rosatelli C, Perseu L, Cao A. Hemoglobin Cagliari (beta 60 [E4] Val----Glu): a novel unstablethalassemic hemoglobinopathy. Blood. 199; 77: 371-375.

TABLES

	Proband 1	Father 1	Mother 1	Proband 2	Father 2	Mother 2
Hb (g/dL)	6.5	15.4	13.4	7.2	16.4	12.6
RBC (10^{12}/L)	2.29	4.77	4.50	7.2	5.16	4.52
PCV (L/L)	21	41	39	26	46	38
MCV (fL)	90	86	87	112	90	84
MCH (pg)	28	32	30	30	32	28
MCHC (g/dL)	28	37	34	27	35	33
Reticulocyte (%)	9.9	-	-	50	2	0.5
Heinz Bodies	+	-	-	+	-	-
Hb A_2 (%)	2.5	2.8	2.6	1.9	1.9	2.6
Hb F (%)	5.9	1.4	1.3	13.5	0.26	0.27
Hb X (%)	-	-	-	6.9	-	-
Isopropanol test	+	-	-	+	-	-

Table 1: Hematological parameters and results of the laboratory tests of the affected pediatric patients.

Proband 1 was 5 years old. The data was obtained during the initial hemolytic crisis. **Proband 2** was 11 years old and splenectomized when the studies were performed

FIGURES

Figure 1: Identification of mutations by DNA sequencing of the PCR product of *HBB* gene (left) and direct sequencing of the clones (mutated and wild-type) obtained by with the pGEM®-T Easy Vector system (right). **(a)** The electropherogram showed the presence of the mutation *HBB:*c.29_37delCTGCCGTTAin a heterozygous state. **(b)** The electropherogram showed the presence of the mutation *HBB*:c.182_187delTGAAGG in a heterozygous state.

Figure 2: Affected residues in **Hb JC-Paz** and **Hb Tavapy**, highlighted in red and in sticks representation over deoxy Hemoglobin tetramer in cartoon representations (PDB ID 1B86). α chains are in black and β chains in white. 2,3 DPG and β chain residues in contact (Val1, His2, Lys82, His143), His92, His63 and Heme groups are in sticks representation. **(a)** Three residues deletion (p.Ala10_Thr12del, in red) of **Hb JC-Paz** in α helix "A", and their neighbors in sequence (Ser9 and Ala13, in yellow). As it can be observed almost a complete α-helix turn would be lost, affecting the amino terminal residues of β chain. **(b)** Two residues deletion (p.Val60_Lys61del, in red) of **Hb Tavapy** in α helix "E" and neighboring residues (Lys59 and Ala62, in yellow). As it can be better observed in the zoom panel, this region, at the beginning of α helix "E", is immediately previous to His63 (distal Histidine).

Figure 3: Distributions of ΔG energies of unfolding for (from left to right) 14 wild type conformers in oxy form and 31 wild type conformers in deoxy form obtained from CoDNAS, 100 models for **Hb Tavapy** using an oxy template (PDB 2DN1) and 100 using a deoxy template (PDB 2DN2), and in the same way for models of **Hb JC-Paz**.