ORIGINAL ARTICLE

Hb Oslo [β42(CD1)Phe→Ile; *HBB*: c.127T>A]: A Novel Unstable Hemoglobin Variant Found in a Norwegian Patient

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Hb Oslo [β42(CD1)Phe→Ile; *HBB*: c.127T>A]: A Novel Unstable Hemoglobin Variant Found in a Norwegian Patient

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Abstract

Unstable hemoglobin (Hb) variants are the result of sequence variants in the globin genes causing precipitation of Hb molecules in red blood cells (RBCs). Intracellular inclusions derived from the unstable Hb reduce the life-span of the red cells and may cause hemolytic anemia. Here we describe a patient with a history of hemolytic anemia and low oxygen saturation. She was found to be carrier of a novel unstable Hb variant, Hb Oslo [β42(CD1)Phe→Ile (*T*TT>*A*TT), *HBB*: c.127T>A] located in the heme pocket of the β-globin chain. Three-dimensional modeling suggested that isoleucine in position 42 creates weaker interactions with distal histidine and with the heme itself, which may lead to altered stability and decreased oxygen affinity. At steady state, the patient was in good clinical condition with a Hb concentration of 8.0-9.0 g/dL. During virus infections, the Hb concentration fell and on six occasions during 4 years, the patient needed a blood transfusion.

KEYWORDS β-Globin gene; hemoglobinopathy; hemolysis; hemolytic anemia; unstable hemoglobin

Introduction

Hereditary hemolytic anemia is a blood disorder where the survival of the erythrocytes is shortened as a consequence of a defective hemoglobin (Hb) molecule (*e.g.* unstable Hb), a defect in the structure of the erythrocyte membrane or by a defect in the enzymatic machinery [1]. If the cause of the hemolytic anemia is not clearly defined, the presence of an unstable Hb variant should always be considered [2]. At present, more than 1400 Hb variants have been identified according to the HbVar database and IthaGenes [3,4], of which nearly 150 are unstable Hb variants of different clinical severity. Depending on the nature of the substitution and its location, the sequence variants associated with unstable Hb can weaken or modify the heme-globin interactions, interfere with the secondary or tertiary structure, or disrupt the packing contacts at the α1β1 interface [2,5]. Inheritance follows an autosomal dominant pattern, but a significant number of the unstable Hb variants are *de novo* variants with no evidence of Hb instability in other family members [5]. Due to gene dosage effect, the clinical severity of β-globin gene sequence variants are generally greater than those of α-globin variants, with hemolytic anemia

presenting in early childhood as Hb F becomes replaced by Hb A as the major Hb [5]. Here, we describe a novel unstable Hb variant found in a 5-year-old Norwegian girl with hemolytic anemia caused by a missense substitution in exon 2 of the β-globin gene. The sequence variant was located close to the heme binding site, a domain important for the stability of the Hb molecule. To evaluate and classify the novel sequence variant, the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants were used [6].

Materials and methods

Blood samples from the proband, her newborn sister and her parents were collected in EDTA and serum separation tubes. All samples went through standard sample evaluation, including a complete blood count (CBC) performed on an XE-2100 (Sysmex Corporation, Kobe, Japan), hemoglobin high performance liquid chromatography (HPLC) analysis using the β-Thalassemia Short Program (VARIANT™; Bio-Rad Laboratories, Hercules, CA, USA), capillary electrophoresis (CE) (Capillarys 2; Sebia, Lisses, France) and measurement of serum or plasma ferritin (Cobas 8000, e602; Roche Diagnostics, Mannheim, Germany).

Genomic DNA was prepared from 200 μL EDTA whole blood using the MagNA Pure LC DNA Isolation Kit I protocol according to the manufacturer's instructions (Roche Diagnostics). The samples were examined for seven common α-thalassemia (α-thal) deletions using multiplex gap-polymerase chain reaction (gap-PCR) described elsewhere [7]. The α-globin genes were amplified using the following primers: αF 5'-CGC GCA TTC CTC TCC GCC C-3' (common forward primer for *HBA1* and *HBA2*) α1R 5'-ATG CCT GGC ACG TTT GCT GAG GG-3' and α2R 5'-CAC CTC CAT TGT TGG CAC AT-3. The β-globin gene was amplified using the following primers: βF 5'-AAC TCC TAA GCC AGT GCC AGA AGA GC-3' and βR 5'-ATG CAC TGA CCT CCC ACA TTC CCT-3'. Polymerase chain reaction was performed on a Thermal Cycler (VWR, Radnor, PA, USA) using HotStar Taq DNA Polymerase Kit (Qiagen Benelux BV, Venlo, The Netherlands) and dNTP mix PCR Grade (Qiagen). The cycling conditions comprised 15 min. of enzyme activation at 95 °C followed by 35 cycles 1 min. at 94 °C, 1 min. at 60 °C and 90 seconds at 72 °C, and a final extension step at 72 °C for 10 min. The PCR products were purified and sequenced in both directions using an ABI PRISM™ 3730 high-throughput CE instrument (Life Technologies, Foster City, CA, USA) at the University of Oslo, Oslo, Norway. Sequence data were analyzed using FinchTV v.1.4.0 (Geospiza Inc., Seattle, WA, USA) and HbVar tracks in the University of California Santa Cruz (UCSC) Genome Browser [4,8]. Copy number analysis of the α-globin genes and the regulatory region hypersensitive-40 (HS-40), was performed using real-time quantitative PCR [9].

Inclusion body analysis was performed by incubating two drops of fresh EDTA whole blood and one drop of brilliant cresyl blue (1.0% w/v) for 15 min. or for 2 hours at 37 °C and then making smears. May-Grünwald-Giemsa (MGG) stained smears (Merck Millipore, Burlington, MA, USA) were made according to the instructions of the vendor. Isopropanol stability test was performed as described elsewhere [10]. In short, hemolysate was made from the proband and two control samples; one fresh sample from a healthy donor as a negative control and a 1-week-old sample as a positive control. A 17.0% isopropanol solution was equilibrated at 37 °C in a water bath. A test or control sample of 2 mL was added and placed in the water bath. The degree of

flocculent precipitate was visually evaluated after 5, 20 and 30 min.

To predict the impact of amino acid substitution on the structure and function of the protein, we used the Alamut Visual version 2.4 (Interactive Biosoftware, Rouen, France) that includes four different missense prediction tools (Align GVGD, SIFT, Mutation Taster and PolyPhen-2 [11-14]). The atomic coordinates of human Hb at high resolution (1.25Å, deoxy form) was retrieved from the Protein Data Bank (PDB ID 2dn2) [15] and analyzed using the molecular graphics program PyMol from Schrödinger. Three different population databases; Exome Aggregation Consortium (ExAc) [16]. The Exome Sequencing Project data set (ESP) [17] and dbSNP [18] were used to assess the frequency of the sequence variant in a general population. The PhyloP conservation tracks [19] in the UCSC Genome Browser [8] and Alamut Visual were used to evaluate the conservation of the affected amino acid.

This study was approved by the Norwegian Regional Ethics Committee (#2015/2352). Informed consent was obtained from all the included subjects.

Results

The proband was 2 years old when she was first diagnosed with hemolytic anemia during an upper airway tract infection at a local hospital. The direct antiglobulin test was negative, as were tests for hereditary spherocytosis (performed at the local hospital, data not shown). The girl was in good clinical condition with no visible jaundice or cyanosis, and there was no palpable hepatosplenomegaly, but pulse oximetry showed low values of 80.0-90.0% saturation at rest. She was then referred to Oslo University Hospital, Oslo, Norway, for hemoglobinopathy evaluation. Hematology and biochemistry data showed anemia with marked reticulocytosis and anisocytosis with no sign of iron deficiency (Table 1). Peripheral blood staining with the supravital dye brilliant cresyl blue showed numerous cells with inclusion bodies and pronounced reticulocytosis (Figure 1A, compare with Figure 1B). May-Grünwald Giemsa staining showed marked anisocytosis, moderate poikilocytosis and basophilic stippling (Figure 1C). Hemoglobin analysis by both HPLC and CE showed a Hb pattern with normal Hb A and Hb A_2, elevated Hb F and a small additional peak (Figure 2). The isopropanol stability test showed significant flocculent precipitation in the hemolysate after 5 min. (data not shown). To confirm the presence of an unstable Hb variant, DNA sequencing was performed and revealed a heterozygous missense variant in exon 2 of the β-globin gene causing a substitution of phenylalanine by isoleucine (Figure 3A). According to the conventional numbering systems for the globin genes [20], this sequence variant is reported as *HBB*: c.127T>A, β42(CD1)Phe→Ile. Any molecular defects on the α-globin genes that might contribute to the phenotype of the proband were excluded by DNA sequencing and copy number variation analysis of the α-globin genes (data not shown). The novel Hb variant was designated Hb Oslo after the location of its discovery and it was entered into the HbVar database [4] (HbVar ID 2885). Different *in silico* analyses were used for interpretation of the novel sequence variant. All the in silico tools, except from Align GVGD, predicted that the amino acid substitution had a deleterious effect and was disease causing (Table 2). It was not listed in the population databases, and it was strongly conserved during evolution. PyMol analysis indicated that isoleucine at position β42 creates weaker interactions with both Phe45, His63 and the heme itself (Figure 3B). To investigate whether it was a *de novo* variant, the β-globin genes of both parents were analyzed by DNA sequencing. They were found not to

carry the sequence variant and their hematological parameters were normal. The proband's younger sister was also not a carrier of the variant. The hematological parameters and molecular analysis are summarized in Table 1.

The proband was followed up at her local hospital. At 'steady state' the proband was in good clinical condition, with a Hb concentration of 8.0-9.0 g/dL (reference interval 11.0-15.5 g/dL), reticulocytes of about 300.0×10^9/L (reference interval $30.0\text{-}100.0 \times 10^9$/L), lactate dehydrogenase (LDH) 700.0-800.0 U/L (reference interval 110.0-295.0 U/L) and with no need of regular blood transfusion. Furthermore, transferrin receptor was increased (4.05-11.52 mg/L, reference interval 1.9-4.4 mg/L) and haptoglobin concentration was below 0.1 g/L (reference interval 0.4-1.9 g/L). C-reactive protein (CRP) and ferritin were both within reference intervals, except at times when the proband had infections. On six occasions, the proband needed blood transfusions.

Discussion

This report describes a Norwegian girl referred to our laboratory for evaluation of an undefined hemolytic anemia. DNA sequencing showed a novel unstable Hb variant, named Hb Oslo, caused by a heterozygous sequence variant located in the heme pocket of the β-globin chain. Demonstration of inclusion bodies in the proband's red blood cells is supportive of Hb Oslo being an unstable Hb variant that may precipitate intracellularly and cause hemolysis [21]. The genetically dominant effects of the novel variant and its absence in all other family members, suggesting that it was a *de novo* variant, are also in accordance with what is often reported for unstable Hb variants [5].

The sequence variant in Hb Oslo is located in the heme pocket of the β-globin chain, in which hydrophobic interactions between heme and the amino acids inside the pocket constitute important stabilization forces, both for the heme and for the globin itself [5] (Figure 3B). Substitution of the amino acid phenylalanine by the much smaller isoleucine in position β42 creates weaker interactions with both Phe45, His63 (distal histidine) and heme. It would be expected that an alteration from a larger to a smaller residue in the heme binding interface of the β-globin unit would give a more flexible protein, with altered stability and possibly also functional consequences for oxygen binding. Unfortunately, we were not able to measure the oxygen affinity (p50). Several sequence variants located at the same position of the heme pocket give rise to unstable Hb variants with decreased oxygen affinity [5]. Hb Oslo represents the fifth β-globin variant at the CD1 position, the previously described are Hb Sendagi [β42(CD1)Phe→Val; *HBB*: c.127T>G] [22], Hb Louisville [β42(CD1)Phe→Leu; *HBB*: c.127T>C] [23], Hb Hammersmith [β42(CD1)Phe→Ser; *HBB*: c.128T>C] [24] and Hb Little Venice [β42(CD1)Phe→Cys; *HBB*: c.128T>G] [25]. A number of case reports of patients with these Hb variants describe clinical presentations similar to our proband in several respects [23,26-29]. All produce chronic hemolytic anemia of varied severity with inclusion bodies, anisocytosis and reticulocytosis, and many of the reported patients have experienced hemolytic crises requiring blood transfusions during viral infections [25,28]. Hb Hammersmith, Hb Sendagi and Hb Louisville have all shown decreased oxygen affinity, and patients were described as having cyanotic appearance [26,29] or to have low oxygen saturation by pulse oximetry on room air [30-32]. Our proband showed partly compensated hemolytic anemia with numerous inclusion

bodies, marked reticulocytosis and increased transferrin receptor. Her pulse oximetry showed 80.0-90.0% saturation on room air, suggesting that the sequence variant has functional consequences for oxygen binding. Together, our findings are compatible with Hb Oslo being an unstable Hb variant with reduced oxygen affinity.

Parvovirus B19 may cause transient aplastic crises in patients with chronic hemolytic anemia [33]. When our proband was 3 and a half years old, she had a Parvovirus B19-infection and the Hb concentration fell to 4.5 g/dL, and treatment with blood transfusion was required. In addition, on five other occasions, the Hb concentration fell and she received blood transfusions (data not shown). This reflects that the erythrocytes have considerably increased turnover due to the unstable Hb variant.

PhyloP conservation tracks in the UCSC Genome Browser and Alamut Visual showed that the β42Phe is a highly conserved amino acid across species, indicating an important functional role. *In silico* tools tend to have low specificity and the use in sequence variant prediction should be carefully implemented [6]. We used multiple programs for sequence variant interpretation; with only one exception (Align GVGD), all predicted a deleterious effect and were used as supporting evidence of pathogenicity of the sequence variant. According to the ACMG standards and guidelines [6], the combination of these observations, together with positive isopropanol stability test and detectable inclusion bodies in peripheral blood, Hb Oslo was classified as a pathogenic variant explaining the proband's phenotype (Table 2).

Due to natural selection through relative resistance of heterozygous carriers against severe malaria, some inherited Hb disorders, such as thalassemia and the Hb variants Hb S (*HBB*: c.20A>T), Hb E (*HBB*: c.79G>A) and Hb C (*HBB*: c.19G>A), are common in many tropical and subtropical countries [34], and are extremely rare among the Northern European population. Unstable Hb variants, on the other hand, are often *de novo* variants and generally limited to a single pedigree [5], and described in patients in all parts of the world. Hence, unstable Hb variants should always be a consideration in cases with undefined hemolytic anemia [2].

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Figure legends

Figure 1. Blood smears. A) Fresh peripheral blood from the proband stained with brilliant cresyl blue revealed intracellular inclusions (red arrows) and pronounced reticulocytosis (green arrows). B) Healthy control sample. The RBCs appear as regular round cells with minimal variation of the size and no proof of intracellular inclusions. C) Fresh peripheral blood from the proband stained with May-Grünwald-Giemsa showed marked anisocytosis, moderate poikilocytosis and basophilic stippling (red arrows).

Figure 2. Hemoglobin fractionation. Hemoglobin pattern from HPLC (A) and CE (B) analyzed in two different samples. Both methods showed increased Hb F and an additional peak (marked with black arrow), suggesting a Hb variant.

Figure 3. DNA sequencing and location of the sequence variant. A) Sequencing of the β-globin gene revealed a heterozygous sequence variant on exon 2, causing a substitution of phenylalanine by isoleucine. B) Three dimensional modelling (PyMol) shows that the sequence variant, β42(CD1)Phe→Ile, is located in the heme pocket, close to the heme binding site, an important domain for the stability of the Hb molecule.

Table 1. Hematological, biochemical and molecular data at the time of diagnosis.

Parameters	Proband	Sister	Mother	Father
Sex-age	F-5	F-1 day	F-36	M-37
Age at diagnosis	2 years	1 day	36	37
Hb (g/dL)	8.1	21.7	12.3	14.6
MCV (fL)	95.3	104.0	91.2	93.4
MCH (pg)	27.4	35.8	30.9	30.3
RBC (10^{12}/L)	2.96	6.10	3.98	4.82
Reticulocytes (10^9/L)	390.7	NA	45.8	74.7
RDW (%)	27.7	NA	13.2	13.4
Reticulocytes-Hb (pg)	32.3	33.1	33.0	37.1
Ferritin (μg/L)	73.0	283.0	13.0	187.0
Hb A_2 (%)	3.1	0.8	2.6	2.4
Hb X (%)	17.0	58.4	0.6	–
HBB sequencing[a]	*HBB*: c.127T>A[b]	normal	normal	normal
α-Thal gap-PCR	negative	negative	negative	negative

Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; RBC: red blood cell count; NA: not available; RDW: RBC distribution width; Retic-Hb: reticulocyte Hb.

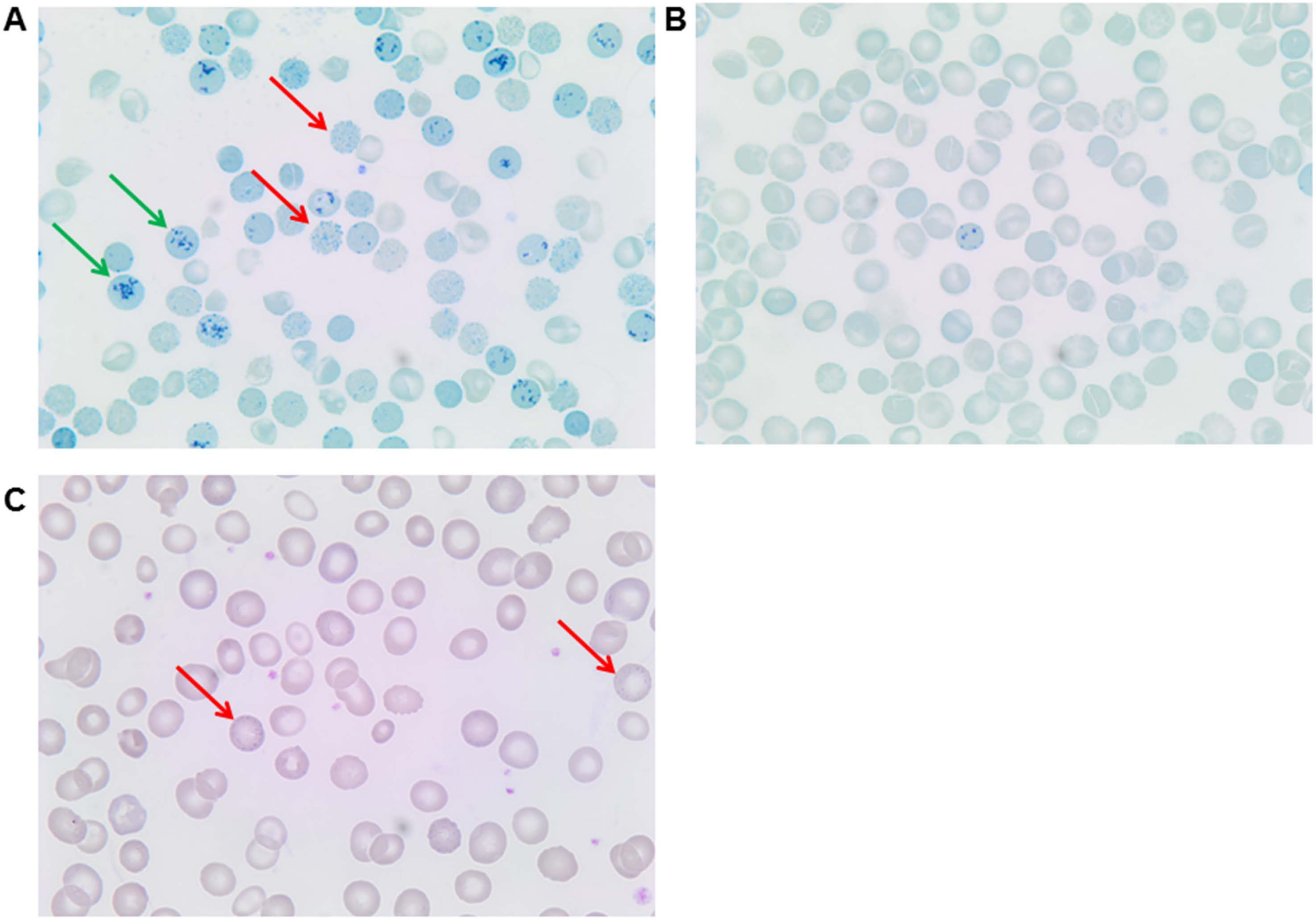
[a] Sanger sequencing of transcript NM_000518.4.

[b] Heterozygous state.

Table 2. Classification of the sequence variant, *HBB*: c.127T>A, based on the American College of Medical Genetics standards and guidelines.

Observation	Evidence of Pathogenicity[a]	Comments
Functional studies supportive of a damaging effect on the gene product	Strong	Blood smears show inclusion bodies and stability test is positive
De novo variant	Moderate	Without paternity and maternity confirmed by genetic testing
Located in a mutational hotspot and/or in a critical well-established domain	Moderate	Located in the heme pocket. CD1 is in direct contact with the heme pocket.
Novel missense change at an amino acid residue where a different missense change, determined to be pathogenic, has been seen before	Moderate	Hb Sendagi (*HBB*: c.127T>G) and Hb Louisville (*HBB*: c.127T>C) are unstable variants
The amino acid is highly conserved	Supportive	PhyloP: highly conserved amino acid
The sequence variant is absent in population databases (ExAc, ESP and dbSNP)		The population databases are not race-matched
Deleterious effect is predicted by *in silico* tools		Align GVGD: less likely; MutationTaster: disease causing; SIFT: deleterious; PolyPhen2: probably damaging
The patient's phenotype is specific for the disease	Supportive	Hemolytic anemia and low oxygen saturation
Total	Class 5	Pathogenic variant

[a] According to the ACMG standards and guidelines [6], the evidence of pathogenicity is weighted as very strong, strong, moderate, or supporting. A *de novo* variant is only considered as strong evidence if both maternity and paternity are confirmed by genetic testing.


A
B
C

A

ANALYTE ID	%	TIME	AREA
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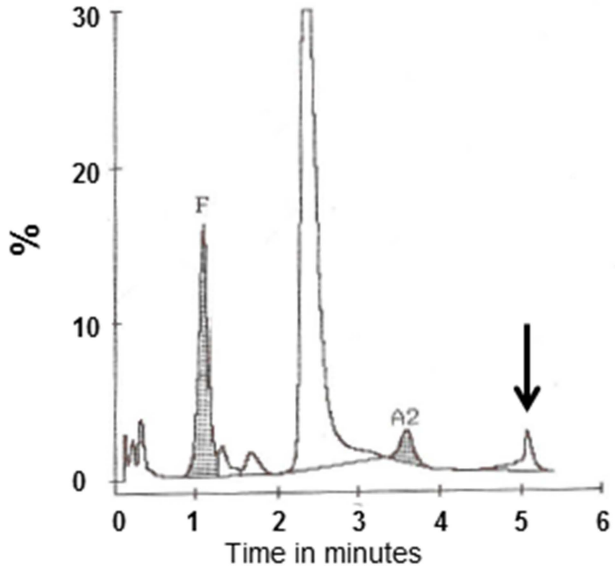
P3	2.3	1.66	44757
A0	72.0	2.34	1373726
A2	3.1	3.58	63627
C-WINDOW	3.9	5.07	73984



B

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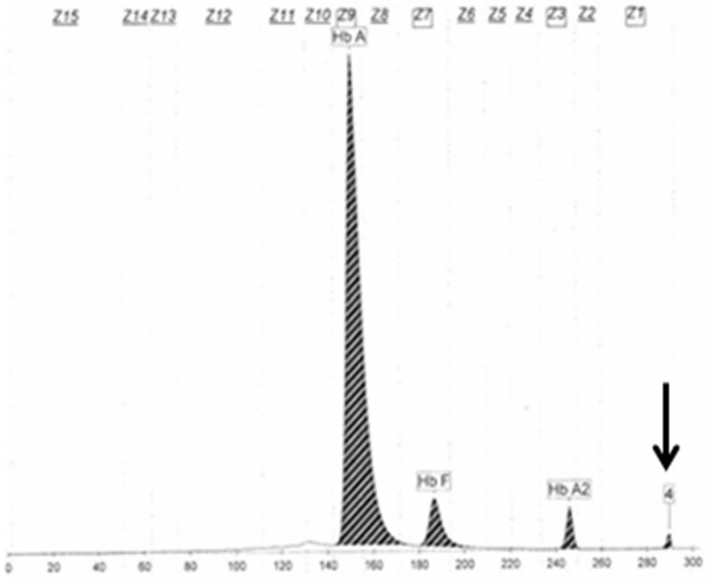
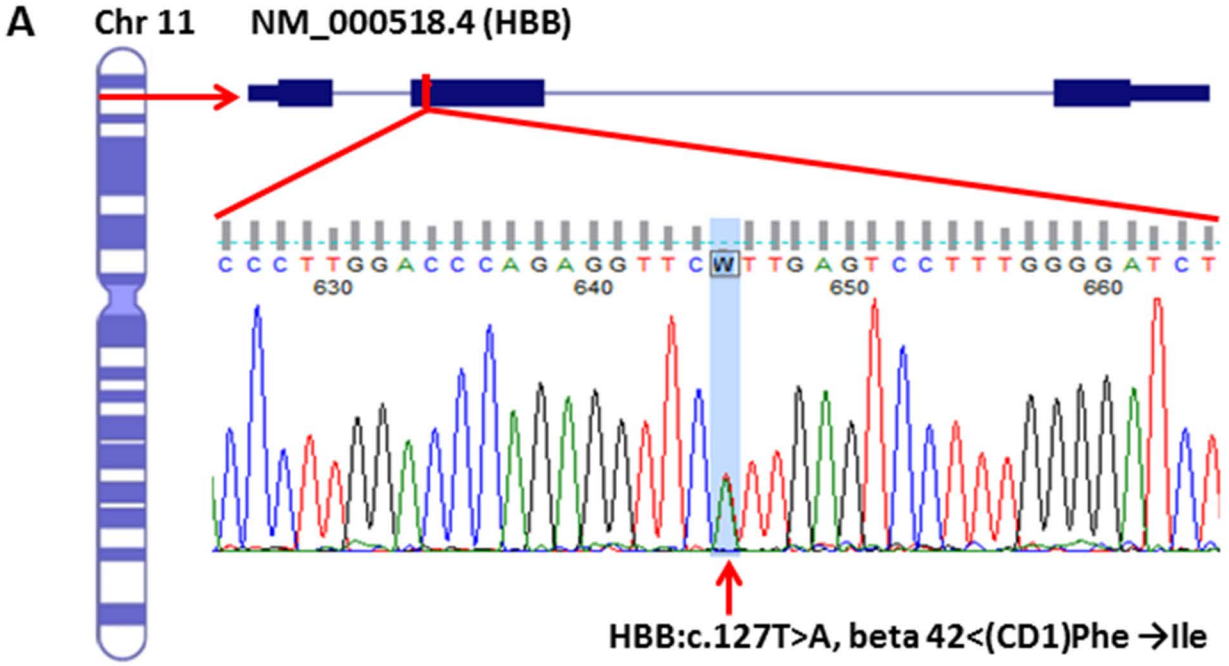
Hb F	7,6		
Hb A2	3,4		
4	0,7		




A
Chr 11
NM_000518.4 (HBB)
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640
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660
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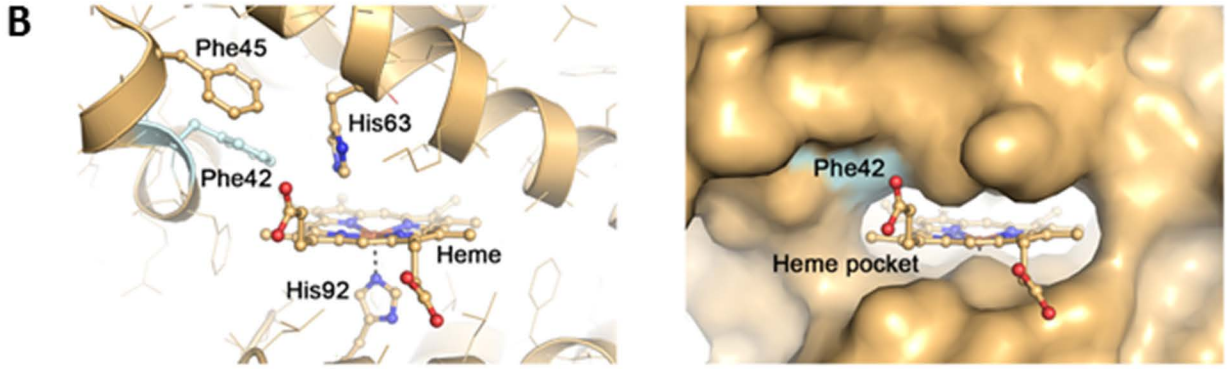
B
Phe45
His63
Phe42
Heme
His92
Phe42
Heme pocket