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Case Report

Capillary electrophoresis and mutational images of hemoglobin sendagi [B42 (CD1) PHE → VAL; HBB: C.127T→G]

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ABSTRACT

We report two cases of hemoglobin Sendagi in a Romanian family residing in Spain: a four-year-old boy and his mother, who had been previously diagnosed with another type of congenital hemolytic anemia and had undergone splenectomy in her country during childhood. The unstable hemoglobin variant, hemoglobin Sendagi, is characterized by decreased oxygen affinity caused by replacement of one of the critical amino acid residues, phenylalanine beta 42 (CD1) of the beta-chain, with valine in the heme pocket, resulting in methemoglobin formation. As a result of migratory movements in Europe, new disease-causing hemoglobin variants are emerging in our country. Here, capillary electrophoresis enabled the identification of the variant and a molecular study was used to establish an accurate diagnosis.

1. Introduction

Hemoglobin (Hb) Sendagi, an unstable hemoglobin variant, was first described in 1986 in a Japanese male and his daughter who presented with moderate hemolytic anemia and decreased stability upon heat and isopropanol precipitation tests [1]. Also known as Hb Warsaw, the disorder has additionally been reported in four members from two generations of a Polish-American family presenting with congenital Heinz-body hemolytic anemia and cyanosis [2,3].

In Hb Sendagi, the amino acid valine (Val) replaces phenylalanine (Phe), which is normally present at the heme contact position beta 42(CD1), [β42 (CD1) Phe → Val]. Patients with Hb Sendagi present with moderate hemolytic anemia, reticulocytosis, Heinz bodies, and cyanosis due to lower oxygen affinity and decreased heme-heme interaction. Findings of a hemolytic state in this variant may lead to misdiagnosis if confounded with other congenital hemolytic anemias.

2. Case description

A 4-year-old Romanian male was referred to our institution due to hemolytic anemia. At age one year, when he was still living in his country of origin, he was diagnosed with hemolytic anemia presumed to be due to glucose-6-phosphate dehydrogenase (G6PDH) deficiency, requiring hospitalization on two occasions for hemolytic crises (July 2014 and October 2015). At the time he was taking folic acid and vitamin C for his condition. While in Romania, his mother had also been diagnosed with hereditary anemia due to G6PDH deficiency and had undergone a splenectomy at the age of 8.

The patient arrived at the emergency department for decay, pallor, fever (38 °C), and earache. Upon arrival, a blood sample was taken (see Table 1 for details). Unexpectedly, these results did not reveal G6PDH deficiency. A physical examination was unremarkable except for 87% oxygen saturation determined by pulse oximetry and significantly pale skin. A peripheral blood smear showed anisopoikilocytosis, basophilic stippling, and presence of Heinz bodies. Except for low concentrations of haptoglobin, the patient had no other test results that were indicative of hemolysis and no other abnormal biochemical parameters.

Beyond these findings, a red blood cell transfusion was ordered and treatment with amoxicillin-clavulanic acid was initiated, causing prompt disappearance of his fever. Despite this favorable response, his oxygen saturation remained at 92%, and oxygen therapy was continued. Findings from a chest X-ray were normal, and cardiological assessment by electrocardiogram and echocardiogram revealed no evidence indicating the causes of cyanosis.

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Table 1
Laboratory values for the patient and his mother.

Variable	Patient (4 years)	Mother (41 years)	Reference value
RBC (10^{12}/L)	3.78	2.9	3.88–4.99
Hb (g/dl)	10.4	8.2	12.2–16.5
PCV (L/L)	0.32	0.28	0.36–0.48
MCV (fl)	85.8	96.7	82–98
MCH (pg)	27.4	28.2	27.3–32.6
MCHC (g/dl)	31.9	29.2	31.6–34.9
RDW (%)	22	14.6	9.9–15.5
Reticulocytes (%)	6.34	0.59	0.8–4.1
Hemoglobin quantification (%)			
• Hb A	91.6	94.8	
• Hb F	4.7	0	
• Hb A_2	3.7	5.2	
Ferritin (ng/ml)	338	815	12–300
Transferrin saturation (%)	28	13	20–40
Erythropoietin (mUI/ml)	33	14.4	3.7–31.5
Hemoglobin-oxygen affinity (P_{50}) (mmHg)[a]	33	Non–quantifiable	23–27
Haptoglobin (mg/dl)	< 10	104	30–200
Total bilirubin (mg/dl)	1.33	1.14	0.3–1.2
Direct bilirubin (mg/dl)	0.31	Not measured	0–0.2
Lactate dehydrogenase (U/l)	442	214	0–247
Antiglobulin			
• Direct	Negative	Negative	Negative
• Indirect	Negative	Negative	Negative
Folic acid (ng/ml)	12.9	14.6	3.1–19.9
Vitamin B_{12} (pg/ml)	471	159	180–914
G6PDH (U/10^{12} RBC)	568	832	146–376
G6PDH (U/g Hb)	20.6	29.4	4.6–13.5

[a] The P_{50} value represents the concentration of oxygen for 50% saturation for the high-affinity state of the hemoglobins.

Given these results, hemoglobinopathy was suspected. Capillary hemoglobin electrophoresis and cation-exchange high-performance liquid chromatography (HPLC) were requested, the results of which showed no signs of abnormal hemoglobin (Fig. 1). The Hb separation pattern was normal, with a slight increase in Hb A_2 and Hb F levels for both techniques (Fig. 1A: Hb A_2 5.9%, Hb F 5.5%; Fig. 1B: Hb A_2 3.7%, Hb F 4.7%). However, the oxygen P_{50} value (hemoglobin dissociation curve) was increased, at 33 mmHg, as demonstrated by the rightward shift of the oxygen-hemoglobin dissociation curve, because of the low affinity for oxygen. Neither methemoglobin nor carboxyhemoglobin was increased.

Sequencing of the β gene revealed the mutation TTT > GTT in CD42 in the second exon, in an allele that determines the substitution of the amino acid phenylalanine (Phe) with valine (Val) at position 1 of the CD segment of the β chain, encoded by the mutated allele (Fig. 1C). This finding was confirmed by sequencing the other strand (data not shown). Using direct sequencing, no point mutations were observed in the promoter region, exons, introns, or 5′- and 3′- ends of the alpha genes.

Diagnosis of heterozygous hemoglobin Sendagi [β42(CD1) Phe > Val; HBB:c.127T > G] was therefore established. Associated alpha thalassemia was ruled out.

3. Material and methods

Hematological data were determined by electrical impedance in an automated cell counter, the UniCel® DxH 800 Coulter® cellular analysis system (DxH 800; Miami, FL, USA). Hemoglobin fractions were measured using HPLC (VARIANT™; Bio-Rad Laboratories, Hercules, CA, USA). HPLC analysis was performed in accordance with the manufacturer's instructions for the BioRad VARIANT II β-Thalassemia Short Program (Bio-Rad, Hercules, CA, USA).

Hemoglobin was studied by capillary electrophoresis techniques, Minicap® CDT (Sebia, Lisses, France), and also in Sebia Capillarys 2 Flex piercing, following the manufacturer's guidelines for the Sebia Capillarys Flex system and using reagents provided in the Capillarys Hemoglobin(E) kit (Sebia, Norcross, GA, USA). Hemoglobin–oxygen affinity (P_{50}) was determined by a compact blood-gas CO-Oximeter and electrolyte analyzer, GEM® Premier 4000 (Instrumentation Laboratory, Bedford, USA).

A spectrophotometric assay was used to quantify glucose-6-phosphate dehydrogenase (G6PD) enzyme activity (Ben S.r.l. Biochemical Enterprise, Milan, Italy) by measuring the formation of NADPH molecules (based on absorbance at 340 nm). Fluorescence was detected by means of Specord 200 Plus Analytik Jena spectrophotometer (Jena, Germany).

Following automatic isolation (Biorobot® EZ1; Qiagen GmbH, Hilden, Germany), genomic DNA was quantified using a NanoDrop 1000 instrument (Thermo Scientific, Wilmington, DE, USA). The most frequent α-globin mutations were ruled out by multiplex PCR followed by reverse-hybridization with a commercial Alpha-Globin StripAssay kit (ViennaLab Diagnostic GmbH, Vienna, Austria).

The β globin gene, including the promoter regions and the 3′ UTR, was amplified by polymerase chain reaction (PCR) using the following pairs of primers: β1F 5′-TCC TAA GCC AGT TGC CAG AAG-3′ [specific for the 5′ region of the β-globin gene (from nucleotide (nt) -160 to −142)] and β1R 5′-TGC TTC GTC TGT TTC CCA TTC TAA AC-3′ (from nt +610 to +585); CD1 5′-TGC CTC TTT GCA CCA TTC TA-3′ (from nt +1098 to +1118); and CD2 5′-GAC CTC CCA CAT TCC CTT TT-3′ (from nt +1659 to +1643) (all positions are given relative to the Cap site, nt 1 from NCBI GenBank®). The PCR products (770 and 575 bp in length, respectively) were purified and directly sequenced with β1F and CD1 primers. α genes were amplified with primers P1A (5′-AGCGCCG CCCGGCCGGGCGT-3′) and C3R (5′-CCATTGTTGGCACATTCCGG-3′) for the α2 gene and C2R (5′- CAGAGAGGTTCTAGCCATGTGTG -3′) for the α1 gene; the amplicons (947 bp) were automatically sequenced using primers P1A, PB (5′-CCC GCC CGG ACC CAC A-3′), and P1C (5′-AGATGGCGCCTTCCTCTCAG-3′) using the BigDye Terminator V3.1 Cycle Sequencing kit and an ABI prism 3130 Genetic Analyzer (Applied-Biosystems, Foster City, CA, USA).

All hematological data and clinical findings were collected with the prior informed consent of the patient.

4. Discussion

Phenylalanine (Phe) is a critical amino acid in position 42 (the first position of the region between the C and the D helices, CD1) of the β chain. Phe participates in contacts with the heme pocket, maintaining solubility and stability. In Hb Sendagi, the amino acid valine (Val) replaces the amino acid phenylalanine (Phe), which is normally present at the heme contact position beta 42(CD1), [β42 (CD1) Phe → Val].

Other hemoglobinopathies affect the same amino acid, phenylalanine beta 42 (CD1), such as Hb Louisville [5] [β42 (CD1) Phe → Leu] and Hb Oslo [4] [β42 (CD1) Phe → Ile] in the HBB:c.127 position, and both Hb Hammersmith [6] [β42 (CD1) Phe → Ser] and Hb Little Venice [4] [β42 (CD1) Phe → Cys] in the HBB:c.128 position.

Although no stability test was performed, it can be assumed that this is an unstable hemoglobin, especially since it has already been described. It is impossible to separate these hemoglobins by electrophoretic techniques and/or by ion-exchange HPLC.

All of these hemoglobin variants present similar clinical and analytical data, although at different intensities. Hb Louisville is also known as Hb Bucuresti and manifests clinically as mild hemolytic anemia, reticulocytosis, and laboratory findings showing Heinz bodies, decreased affinity for oxygen. Hemoglobin Oslo was first described in a Norwegian girl born in 2010. It presents with marked hemolytic anemia and is presumed to be characterized as hyper-unstable Hb because of its association with hemolytic anemia. It is less evident on the phenotypic level and the mutated amino acid is located in the heme pocket. This Hb

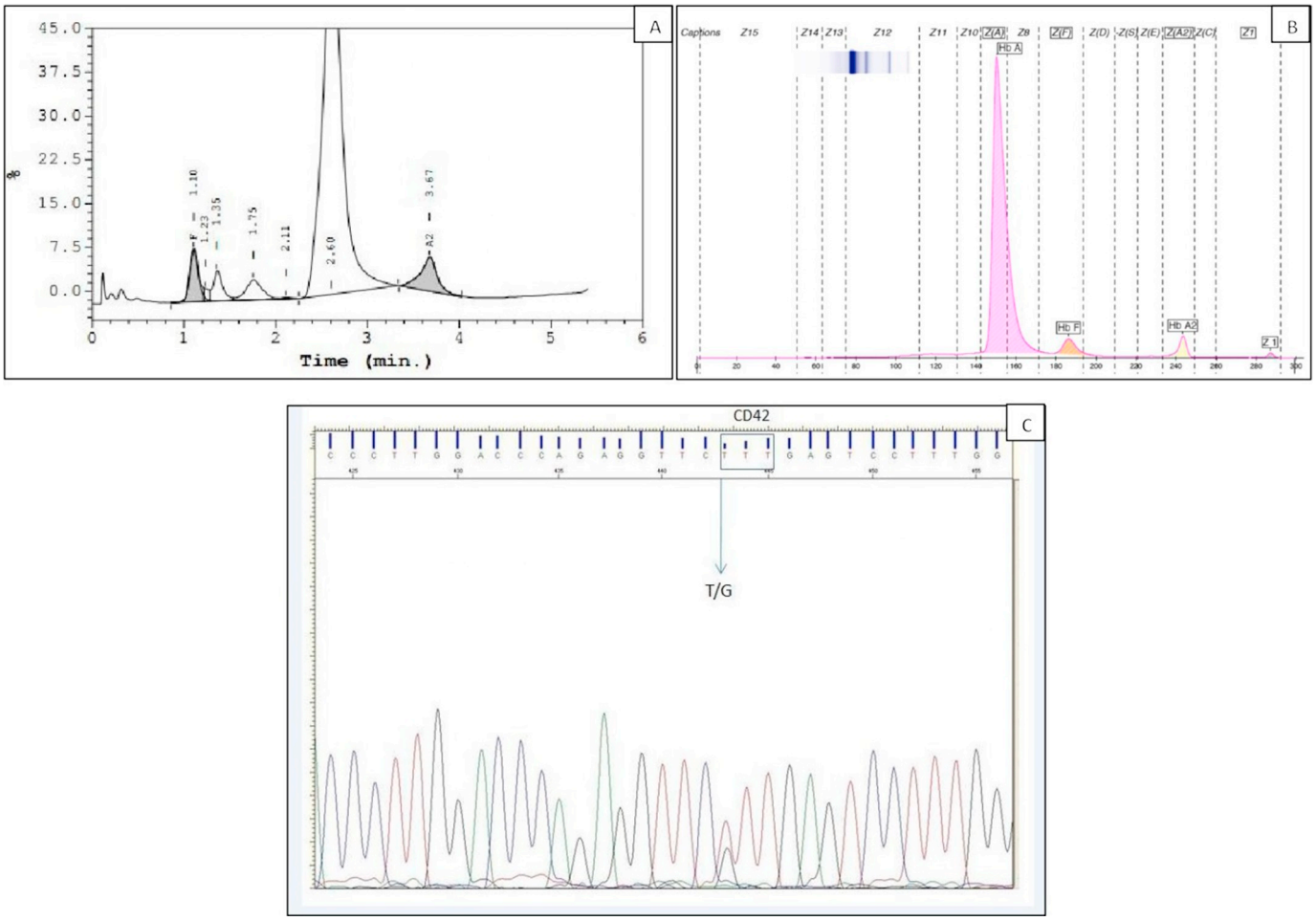

Fig. 1. A) Capillary electrophoresis by Sebia Showing an increase in Hb A2 and in Hb F. B) Hemoglobin fractions measured by high-performance liquid chromatography (HPLC) (VARIANTTM). C) DNA sequencing of the beta gene showing the segment containing the transversion at codon 42 (blue arrow) corresponding to the TTG > GTT mutation and the (Phe→Val) substitution of hemoglobin Sendagi.

variant cannot be determined by any technique. Hemoglobin Hammersmith [6] also presents as hemolytic anemia, cannot be determined by any technique, and exhibits unstable hemoglobin with a decreased affinity for oxygen. Hemoglobin Little Venice [4] presents clinically as marked hemolytic anemia, severe chronic hemolytic anemia, and laboratory findings include anisocytosis and poikilocytosis.

Sendagi hemoglobinopathy is an unstable Hb with a low affinity for oxygen, causing a rightward shift in the oxygen-dissociation, with high P_{50} values and decreased oxygen saturation. In response to an increase in oxygen supply to the tissues, the oxygen sensor decreases the production of erythropoietin, resulting in anemia and even cyanosis in cases of marked affinity reduction.

More than 1000 different hemoglobin variants have been described, most due to a point mutation causing the substitution of a single amino acid, although in a majority of cases this change in structure does not cause significant alteration in solubility, stability, or function. Other variants resembling the one described here have been described, also manifesting clinically.

Molecular study is key for establishing an accurate diagnosis and for subsequent treatment. As evidenced in this study, such methodologies may limit the influence of potentially confounding observations that could lead to an ill-advised splenectomy to treat hemolytic anemia, as in the mother's case reported here.

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