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Different forms of Hb Le Lamentin. An unexpected finding in Hba1c quantification

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ABSTRACT

Objectives: Glycated hemoglobin (HbA1c) is accepted as the most trusted marker for monitoring patients with *diabetes mellitus*. Ion-exchange high-performance liquid chromatography (HPLC) is one of the most widely used methods for HbA1c analysis. The presence of a hemoglobin variant can interfere with HbA1c quantification, requiring other analyses to clarify the results.
Herein, we present two cases of Hb Le Lamentin, which, although they were the same variant, were thought to correspond to different hemoglobinopathies because of their percentages.

Design and methods: Two male patients presented with an anomalous peak between HbA1c and HbA_0 during a routine analysis of HbA1c using ion-exchange HPLC (Variant™ II Turbo).
The hemoglobin variants were studied using capillary zone electrophoresis with the Sebia system, and the globin chains were analyzed by reverse-phase HPLC. A genetic analysis was performed using automated sequencing of the α2 and β genes.

Results: In this work, we describe the first case of homozygous Hb Le Lamentin and the first double-heterozygous case of Hb Le Lamentin/Hb City of Hope.

Conclusions: Although the presence of these variants does not lead to clinical anomalies, it also does not affect hematologic parameters. The variants have an impact on the determination of glycated hemoglobin levels using ion-exchange HPLC because the retention time interferes with the elution time of HbA1c, resulting in a falsely reduced value. Therefore, it is necessary to either recalculate the result or use another measurement method.

1. Introduction

Glycated hemoglobin (HbA1c) is widely accepted as the most trusted marker for the long-term monitoring of glucose control in patients with *diabetes mellitus* and for evaluating the risk of developing chronic complications associated with diabetes [1]. In laboratories in Spain, ion-exchange high-performance liquid chromatography (HPLC) is one of the most widely used routine methods for the determination of HbA1c levels. Careful inspection of the chromatogram during HbA1c analysis by HPLC allows for the detection of hemoglobin (Hb) variants due to changes in the normal chromatograph pattern. The presence of an Hb variant or hemoglobinopathy can interfere with HbA1c quantification, causing a discrepancy with other analytical and clinical data and requiring other analyses to clarify the specificity of the results.

Quantification of the anomalous Hb observed during HbA1c measurement can indicate which globin chain is altered or mutated. One diploid cell has four α genes and two β genes, such that the percentage of anomalous Hb is approximately 25% when the variant is in an α chain. If the β chain is affected, the proportions of both Hb molecules (anomalous Hb and HbA) would be similar (~50%). However, these percentages can lead to uncertainty depending on the mutation status (i.e., heterozygosity or homozygosity) or when the variants are associated with other erythrocyte abnormalities, as in the case of G-Hb Philadelphia where up to 50% of HbG Philadelphia may be observed due to the presence of the alpha thalassemia. Such uncertainty can delay diagnostics [2].

We present two Hb Le Lamentin cases corresponding to a variant in the α chain whose percentages in the heterozygous state should be approximately 25%. In both cases, even with the same variant, it was thought that they corresponded to different Hb molecules because of the percentages. The first Hb variant corresponds to the first described case of homozygous Hb Le Lamentin; the second Hb

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variant corresponds to the first association of heterozygous Hb Le Lamentin with Hb City of Hope, with the latter being the first report in Spain.

2. Materials and methods

The first case (I1)was a male, 51 years old, born in Albacete Capital, whose parents were cousin-siblings. The second case (I2)was a 46-year-old male born in the municipality of Madrigueras (Albacete); both are Caucasian, and they are not related. In both cases, an anomalous peak between the HbA1c and HbA_0 (=HbA) retention times (RTs) of 0.614 min and 0.703 min, respectively, was detected during a routine analysis of HbA1c by ion-exchange HPLC (Variant™ II Turbo; Bio-Rad Laboratories, Hercules, CA, USA)using a specific program for the quantification of HbA1c (HbA1c Kit-2.0).

Hematologic data were obtained using an automatic cell counter (Advia® 120 System, Siemens S.A., Germany). The HbA_2 and HbF levels were measured by ion-exchange HPLC (VARIANT™ II; Bio-Rad Laboratories, Hercules, CA, USA). The Hb molecules were studied using capillary zone electrophoresis with the Sebia system (Sebia Capillarys Flex) using the kit reagents provided by the manufacturer [Capillarys Hemoglobin (E) kit (Sebia, Norcross, GA)]. The HbA_2 and HbF levels were also analyzed by ion-exchange HPLC using the short program for the Bio-Rad Variant II [BioRad Variant II β-thalassemia Short Program (Bio-Rad, Hercules, CA)]. The globin chains were separated using reverse-phase HPLC as previously described [3].

Genetic material was extracted using an automated system (Biorobot® EZ1, Qiagen GmbH, Hilden, Germany) and subsequently quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The most frequent α-thalassemia mutations were analyzed using several PCRs with the *Alpha-Globin StripAssay* commercial kit (ViennaLab Diagnostic GmbH, Vienna, Austria).

The α2 gene was amplified with primers P1A (5′-AGCGCCGCCCGG CCGGGCGT-3′) and C3R (5′-CCATTGTTGGCACATTCCGG-3′), and the amplicon (947 bp) was automatically sequenced using primers P1A, PB (5′-CCC GCC CGG ACC CAC A-3′) and P1C (5′-AGATGGCGCCTTCCTC TCAG-3′). Sequencing of the β globin gene was performed according to a previously reported protocol [4].

All of our hematologic indices and clinical findings were collected with the informed, written consent of all subjects, and the study was approved by the Ethics Committee of the Hospital Clínico San Carlos, Madrid, Spain.

3. Results

The hematologic data are shown in Table 1. The peripheral blood smears were normal.

In both cases, peaks corresponding to HbA and HbA_2 were observed using capillary electrophoresis, with the latter peak showing a slight protuberance. The anomalous Hb variants were separated by RTs of 1.59 min and 1 min using ion-exchange HPLC (Fig. 1). In I1, the study of the globin chains by reverse-phase HPLC revealed the presence of a β chain and a double peak corresponding to α and α^x chains.Chains β^A, β^x, α and α^x were eluted from the second patient (I2) (Fig. 1).

Sequencing of the α2 gene revealed the mutation CAC > CAA in CD20, causing a His > Gln amino acid change known as Hb Le Lamentin [$\alpha_2$20(B1)His > Gln;BA2:c.63C > A]. This mutation was homozygous in I1 and heterozygous in I2. In case I2, sequencing of the β gene revealed the substitution of a guanine for an adenine (GGT > AGT) in codon 69 in a heterozygous state; this sequence corresponds to Hb City of Hope [β69(E13)Gly > Ser;HBB:c.208G > A] (Fig. 2).

In both cases, α-thalassemia deletion and non-deletion were ruled out.

4. Discussion

The Hb Le Lamentin variant is distributed worldwide. Cases have been identified in France, England, the USA, Germany and Japan [5–9]. Most variants have been detected in neonatal and population studies or during HbA1c quantification for diabetes control. In Spain, Hb Le Lamentin has been described only in the Canary Islands [10]. This study is the first report of Hb Le Lamentin on the peninsula of Spain. It is important to highlight that these cases are not related, although the subjects were born in two very close municipalities of the province of Albacete.

In Hb Le Lamentin, the histidine substituted in the 20th residue of the α globin chain is located on the outside of the molecule, and the functionality and stability are normal. However, the Hb variant cannot be separated from HbA using electrophoresis; thus, the variant can only be detected using more sophisticated techniques, such as isoelectric focusing (IEF), mass spectrophotometry and ion-exchange HPLC. In this study, the presence of the variant was a coincidental finding when processing glycated Hb using ion-exchange HPLC as was indicated by the anomalous peaks observed at different RTs. Currently, the use of capillary electrophoresis allows for the separation of HbA from structural variants, which cannot be achieved using conventional electrophoresis. Thus, Hb Le Lamentin, in both the homozygous and heterozygous

Table 1
Hematological data.

	Reference range	Hb Le Lamentin (heterozygous)	(I1) Hb Le Lamentin (homozygous)	(I2) Hb Le Lamentin/Hb City of Hope (double heterozygous)
Sex/Years	M/18–50	M/85	M/51	M/46
RBC ($x10^{12}$)	4.5–5.7	4.11§	5.62	5.13
Hb (g/dL)	13.5–18	12.4§	17.5	16.0
PCV (%)	42–55	39.7	52	49.2
MCV (fL)	78–100	96.4	92.5	95.9
MCH (pg)	26–33	30.3	31.1	31.2
RDW (%)	11–16	15.0	13.7	15.6
Hb A_2 (%)	2.5–3.5	1.8/2.6*	1.1/2.5*	1.8/2.6*
Hb F (%)	< 1	0.6	1.1	0.9
Hb Le Lamentin (%)	–	28.5**/26.4***	54.5**/47.9***	28.9**/25.8***
Hb A (%)	85–95	61.6	38.2	59.5
Hb A1c (%)	< 5.7	5.0	5.2	6.4

* Hb A_2 levels by Capillary Electrophoresis.
** Hb Le Lamentin levels by HPLC with Variant II with β-thalassemia Short Program.
*** Hb Le Lamentin levels by HPLC Variant II with HbA1c Kit-2.0.
§ Anemia due to age.