



Case Report

A Novel α -Globin Gene Variant: Hb Romagna [$\alpha 61(\text{E}10)\text{Lys}>\text{Gln}$; *HBA1*:c.184A>C] Co-Inherited with Hb A2-Lampang [$\delta 47(\text{CD}6)\text{Asp}>\text{Asn}$; *HBD*:c.142G>A] in an Italian Diabetic Woman

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Abstract

Background: Screening for hemoglobinopathies, particularly in Mediterranean countries, is primarily aimed at preventing beta-thalassemia. However, during such screening, numerous other Hb defects can be diagnosed, each with different clinical significance, including qualitative and quantitative variations in the alpha genes. **Aims:** In the present study, we describe a new variant of the *HBA1* gene co-inherited with a previously described variant of the *HBD* gene in a diabetic patient tested for HbA1c. **Methods:** HbA1c and Hb fraction separation were performed using capillary electrophoresis (CE), and the Hb components were confirmed by high-performance liquid chromatography (HPLC). Molecular analysis was performed using next-generation sequencing (NGS). **Results:** The results of capillary electrophoresis and HPLC tests showed the presence of two anomalous peaks. The molecular study revealed the presence of a new variant of the *HBA1* gene, which was named Hb Romagna, and a co-inherited variant of the *HBD* gene already known as Hb A2-Lampang. **Discussion:** The coexistence of the two variants poses certain diagnostic challenges with clinical implications that could be relevant to patient management.

Keywords: Hb A1c; capillary electrophoresis; Hb variant; Hb A2-Lampang; Hb Romagna; HPLC



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1. Introduction

Diagnostic testing for hemoglobin disorders is performed worldwide in various settings, primarily for preventive purposes. In the Mediterranean region, these tests are primarily aimed at identifying carriers of β -thalassemia (β -thal) and at preventing or diagnosing other clinically significant conditions. To date, at least 500 defects of the *HBB* gene [1,2] have been known to be expressed as β -thal associated with increased levels of HbA2 [3]. Sometimes, first-level tests can reveal hemoglobin mutations: point variants in the Hb tetramer, microdeletions, or insertions that contribute to more complex diagnostic

conclusions, requiring appropriate characterization and genetic counseling. Currently, approximately 700 variants of the *HBB* gene are known, two-thirds of which are clinically asymptomatic and normally do not affect the relative percentage of Hb A2, with the exception of some unstable variants [4]. Measurement of Hb A2 is more directly influenced by the more than 140 variants involving the *HBD* gene and the numerous mutations of the *HBA1* and *HBA2* genes. Approximately 500 variants of the α -globin chains have been described, whose amino acid substitutions are known, but over a third of these have not been classified as belonging to the *HBA1* or *HBA2* gene [1]. The *HBA1* and *HBA2* genes are paralogues, differing from each other only by a small number of intronic nucleotides and normally producing identical α -globin chains. They differ mainly in their synthetic capacity, as both genes can have identical variants yet produce them in different quantities, as observed in some cases [5]. It may be important to know which of the two alpha-globin genes is mutated. The differing synthesis capacities of the two α -globin genes could have a significant impact when dealing with unstable variants and/or variants with altered oxygen (O_2) affinity, particularly if these variations are inherited alongside thalassemic defects [6]. It should be noted that amino acid analysis, and therefore experimental identification of the mutated amino acid sequence, may not correspond to the sequence translated. It is known that spontaneous post-translational deamidation processes occur that may involve asparagine (Asn) and glutamine (Gln) residues when these are close to certain amino acids or in alkaline pH conditions or depend on particular polypeptide conformations of the globin chain [7,8]. In these cases, the nature of the amino acid involved in a possible substitution may be unclear. Finally, although the diagnosis and prevention of Hb disorders are a persistent problem, it is not uncommon to detect Hb variants incidentally during HbA1c measurement for the diagnosis or monitoring of diabetes mellitus (DM), even in populations with a low prevalence of Hb variants, especially in the case of Hb separation systems such as the CE Hb A1c method [9,10]. In this diabetic context, using separation methods such as CE or HPLC grants the advantage of revealing clinically silent and unsuspected Hb defects that potentially interfere with the accurate evaluation of Hb A1c values.

2. Materials and Methods

We present the case of a 58-year-old Italian woman living in Emilia-Romagna, a region in northern Italy. The patient signed an informed consent as required. The 1975 Declaration of Helsinki, as amended in 1996, was respected and enforced. The patient had a known history of diabetes mellitus, and the medical history revealed no other medical conditions or previous episodes of anemia. She visited the Clinical Pathology Unit at the Laboratory Hub in Cesena to undergo routine tests, including an assessment of glycated hemoglobin (HbA1c). The HbA1c measurement was performed using capillary electrophoresis (CE) (CAPILLARYS3 Tera with the Capi3 HbA1c kit; Sebia, Lisses, France). To confirm the presence of hemoglobin variants, analyses were subsequently performed using capillary electrophoresis (CAPILLARYS3 Tera with the Hemoglobin(e) kit, Sebia) and high-performance liquid chromatography (HPLC) with cation exchange (VARIANT IITM Dual kit (Bio-Rad Laboratories, Hercules, CA, USA), as well as the Premier Hb9210TM Resolution (Trinity Biotech, Bray, Co Wicklow, Ireland). All procedures were conducted according to the manufacturers' recommendations. The blood count parameters were evaluated with the Automated Hematology Analyzer Sysmex XN-series (Sysmex Co., Ltd., Kobe, Japan). Two specific tests were performed to determine the stability of the detected Hb variant: Carrell's isopropanol test at 37 °C [11] and a test to detect the presence of erythrocyte inclusions in red blood cells after incubation at 37 °C with brilliant cresyl blue (BCB) [12]. Further analyses were conducted at the Genetics Laboratory of the IRCCS

Giannina Gaslini Institute in Genoa, Italy, for the purpose of molecular characterization of the proband regarding the *HBA2*, *HBA1*, *HBB*, and *HBD* genes. These analyses were performed using NGS, the validity of which was verified and confirmed on an independent sample using conventional molecular tests (Sanger sequencing and MLPA), as described previously [13,14]. Unfortunately, it was not possible to examine other family members to determine how the variants had been transmitted or whether these were isolated cases; the parents are deceased, and the patient has no siblings.

3. Results

HbA1c was measured using CE, and the system flagged an “atypical profile” after detecting two anomalous peaks: one between HbA1c and HbA0 and a smaller peak following HbA2 (Figure 1A). Despite these anomalies, the system was able to calculate the HbA1c value: 39.0 mmol/mol (5.7%) (normal range: 3.9–5.5%). This value was deemed correct by the laboratory, as the two anomalous peaks do not interfere with the normal peaks of HbA1c and HbA0. The presence of abnormal hemoglobin fractions was explored through the use of the CE kit (Sebia) for hemoglobinopathies, which identified the presence of an abnormal hemoglobin in zone Z12 (18.6%) and a minor peak in zone Z1 (0.7%), while Hb A2 was 0.9% (normal range: 2.2–3.2%) (Figure 1B). Further confirmation of the presence of the two additional peaks was obtained using the HPLC VARIANT II™ Dual Kit (Bio-Rad), which revealed an anomalous fast peak (15.8%), not completely separated from Hb A; Hb A2 at 0.9%; and an additional minor peak at 0.7% (Figure 1C). A further analysis was performed using the HPLC Premier Hb9210 Resolution analyzer (Trinity Biotech), which again showed an abnormal fast peak (18.1%), HbA2 at 1.46%, and an additional minor peak at 0.8% (Figure 1D).

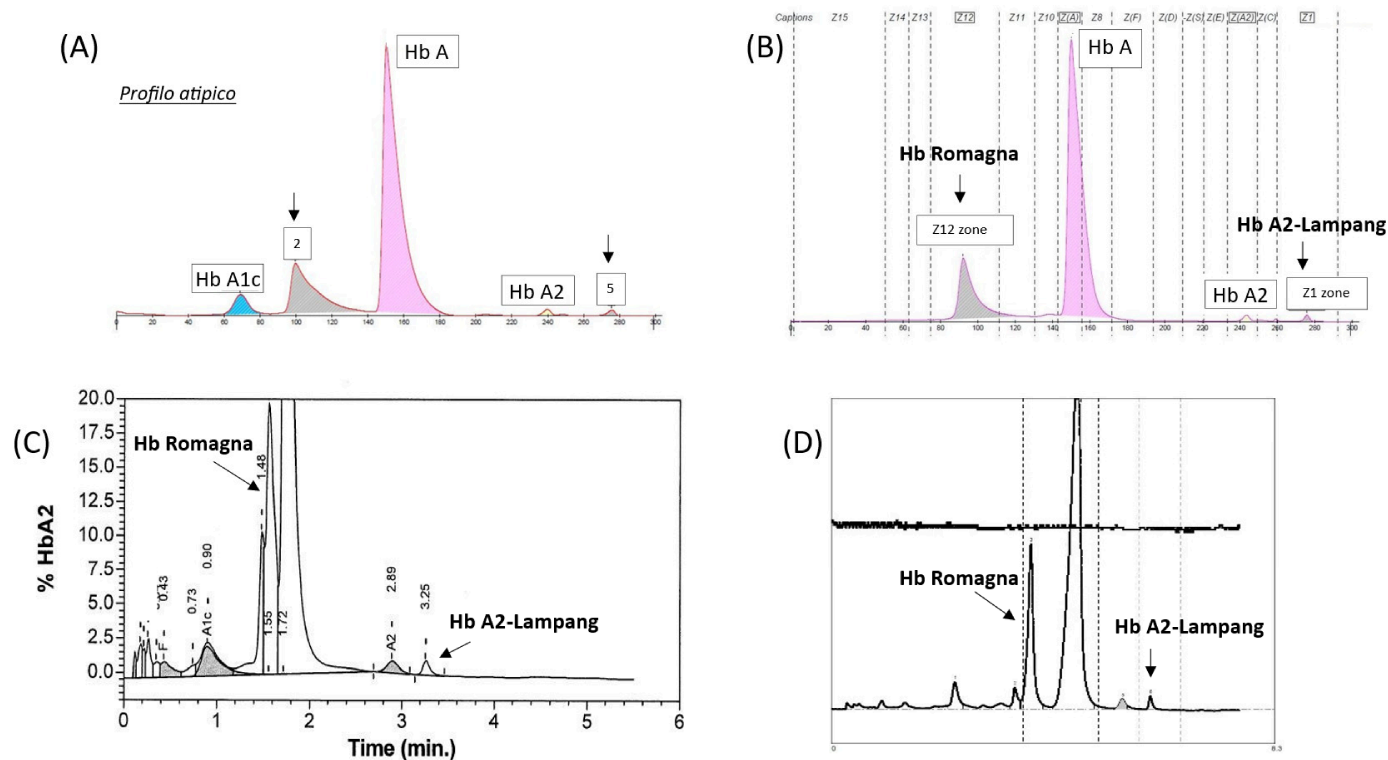


Figure 1. (A) Separation methods used for measuring glycated Hb in CE with the Hb A1c kit (Sebia). Confirmation of the presence of two different types of abnormal Hb: (B) Hemoglobin Kit by CE (Sebia), (C) HPLC VARIANT Dual Kit program (Bio-Rad), and (D) HPLC Premier Hb9210 Resolution (Trinity Biotech).

The data obtained from the three separation methods are consistent and indicate that the small fraction of abnormal hemoglobin, amounting to approximately 0.7–0.8% of the total, is likely a variant of Hb A2 resulting from a mutation in the δ chains rather than the same mutation producing the “fast” Hb variant. The results of the blood tests performed on the patient were within the normal range: red blood cell (RBC) 5.22 $10^{12}/L$ (4.00–5.20), hemoglobin (HGB) 144 g/L (120–155), hematocrit (HCT) 0.43 L/L (0.37–0.47), mean corpuscular volume (MCV) 81.8 fL (80–95), and mean corpuscular Hb (MCH) 27.6 pg (27.0–32.0). Both of the specific tests used to determine whether the hemoglobin variant was unstable came back negative.

The diagnosis was subsequently confirmed by direct sequencing, which revealed the presence in the patient of a novel heterozygous transversion in the *HBA1* gene: c.184A>C, resulting in an amino acid substitution from lysine (Lys) to Gln at residue 61 (Figure 2). These results led to the discovery of a previously unknown hemoglobin variant, which we have named Hb Romagna in honor of the region where the subject lives. This novel point mutation was included in HbVar [1] with ID number 3306 and in the ITHANET portal with assigned ithaID 4142 [15]. No mutations were found in the *HBA2* and *HBB* genes, and no α -thalassemia (α -thal) deletions were detected.

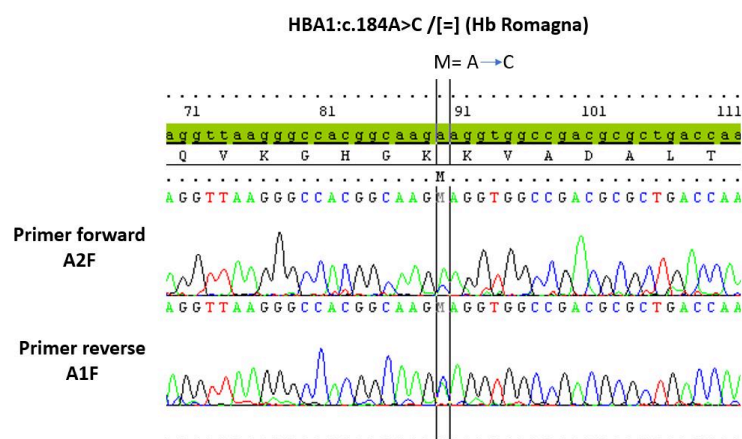


Figure 2. Detail of DNA sequence analysis (forward and reverse) of the proband for the region where the nucleotide variant was expected to be found, namely, the position shown in exon 2 of the *HBA1* gene, confirming the presence of the AAG>CAG mutation (M) at codon 61 (Hb Romagna) of the *HBA1* gene. The *HBA1* exon2 consensus sequence is highlighted in green. The four nitrogenous sequence bases are expressed with different letters and colors: A (adenine) in green, G (guanine) in black, T (thymine) in red, and C (cytosine) in blue. The M (gray) represents the overlap of an A and a C, one for each allele of *HBA1*.

The hypothesis that the proband carried a δ -globin variant was confirmed by sequencing of the *HBD* globin gene. A heterozygous G>A missense mutation was detected, resulting in an amino acid substitution at residue 47. This variant corresponded to Hb A2-Lampang [δ 47(CD6)Asp>Asn; *HBD*:c.142G>A], which is considered a clinically silent mutation and has previously been described in a Thai individual [16].

4. Discussion

The reasons for conducting a study on this patient stemmed from the need to accurately assess her HbA1c level in the presence of a variant detected by the separation methods used in our laboratory. Although this variant did not interfere, from a separation standpoint, with the measurement of HbA1c, we decided to characterize it in accordance with the recommendations of the National Glycohemoglobin Standardization Program (NGSP) (ngsp.org). After confirming the presence of the variant using three different

methods, it was essential to evaluate its stability to rule out any potential underestimation of the HbA1c value. The identification of a hemoglobin variant always requires molecular characterization, regardless of other considerations, as stipulated by the recommendations of the Italian Society of Thalassemia and Hemoglobinopathies (SITE) [17].

When a hemoglobin variant is characterized by analyzing the amino acid sequence of the globin chains and a substitution is observed that results in the insertion of a glutamic acid (Glu) or asparagine (Asp) residue, this could, but need not, be the result of post-translational deamidation of Gln or Asn residues [7]. These deamidation processes can be demonstrated by correlating the results of DNA analysis with those of amino acid analysis [18,19]. DNA sequencing alone cannot predict potential subsequent post-translational modifications, but it can accurately identify nucleotide mutations and, consequently, the specific Gln amino acid substitutions that could subsequently undergo deamidation. Conversely, amino acid analysis alone cannot definitively indicate whether the presence of a Glu residue is the result of genetic determination [18].

In the new variant of Hb Romagna, a nucleotide variation has been observed that results in a Lys>Gln substitution at codon 61. We believe that the Gln residue cannot be involved in secondary post-translational modifications. This conclusion is based on an analysis of the amino acid residues adjacent to the substitution and of the electrophoretic migration zone of Hb Romagna; the latter corresponds to that described for other hemoglobin variants with a similar substitution but differs significantly from variants with a Lys>Glu substitution (Table 1). Therefore, a comparison between Hb Romagna and the phenotypes of the hemoglobin variants listed in Table 1 allows us to conclude that the electrophoretic and/or chromatographic separation methods commonly used in laboratories are sufficiently sensitive to distinguish between amino acid substitutions with different isoelectric points, particularly between Gln and Glu. However, a comparative analysis of the hematological and clinical data for the two groups of Hb variants in Table 1, based on published data, reveals no significant phenotypic differences.

Table 1. Comparison between some Hb variants in which a Lys residue is replaced by Glu or Gln residues, respectively. The amino acids adjacent to the substitutions and the migration zone in the CE are shown.

Mutation Sequence Analysis				
Hb Variants	DNA Studied ^a	Protein Studied	CE Zone	Reference
<u>Hb variants with Lys>Glu substitution:</u>				
Hb Kurosaki: <i>HBA2/A1:c.22A>G</i> ; p.Lys7Glu	N.A.	-Asp- <i>Glu</i> -Thr-	15	[20]
Hb I: <i>HBA2:c.49A>G</i> ; p. Lys16Glu	-Gly- <i>Glu</i> -Val-	N.A.	15	[21]
Hb Shaare Zedek: <i>HBA2/A1:c.169A>G</i> ; p.Lys56Glu	N.A.	-Val- <i>Glu</i> -Gly-	15	[22]
Hb Miyagi: <i>HBA2/A1:c.184A>G</i> ; p.Lys61Glu	N.A.	-Asp- <i>Glu</i> -Val-	N.A.	[1]
Hb Sudbury: <i>HBA1:c.271A>G</i> ; p.Lys90Glu	-His- <i>Glu</i> -Leu-	-His- <i>Glu</i> -Leu-	14	[1]
<u>Hb variants with Lys>Gln substitution:</u>				
Hb J-Wenchang-W.: <i>HBA2:c.34A>C</i> ; p.Lys11Gln	-Val- <i>Gln</i> -Ala-	-Val- <i>Gln</i> -Ala-	12	[19]
Hb Romagna: <i>HBA1:c.184A>C</i> ; p.Lys61Gln	-Asp- <i>Gln</i> -Val-	N.A.	12	This report
Hb Bergerac: <i>HBA2:c.271A>C</i> ; p.Lys90Gln	-His- <i>Gln</i> -Leu-	N.A.	12	
Hb Luo Cheng: <i>HBA1:c.271A>C</i> ; p.Lys90Gln	-His- <i>Gln</i> -Leu-	N.A.	12	[1]
Hb Jilin: <i>HBA2:c.418A>C</i> ; p.Lys139Gln	-Ser- <i>Gln</i> -Tyr-	N.A.	12	[1]

N.A.: not available; *HBA2/A1*: *HBA2* or *HBA1* unknown; ^a: DNA analysis made it possible to determine the amino acid sequence.

Today, in the diagnosis of hemoglobinopathies caused by structural defects, following first-line tests, which constitute an essential step, and functional tests, DNA analysis is much more widely used than amino acid sequencing. In most cases, this may be sufficient for the structural characterization of a hemoglobinopathy. In addition, the potential pathogenicity of the identified variant, assessed using the predictive tools of the Alamut Plus software in accordance with the ACMG/AMP standards for variant classification, is increasingly being taken into account [23]. Although these tools have several limitations and approximations, they can still help define the clinical phenotype resulting from globin gene defects. In our case, however, this evaluation led to the classification of Hb Romagna as a “variant of uncertain significance (VUS)”. We therefore sought to consider, as an alternative, the fact that the Lys amino acid at residue 61 is not substantially involved in binding to the other chains of the tetramer and to heme, meaning that any substitutions do not affect the structural stability and/or function of the hemoglobin [24]. To date, five substitutions at codon 61 of the α -chains have been observed, including Hb Romagna, and the documented cases do not appear to manifest distinct phenotypes, although the characterization of some variants is incomplete and, in all cases, their association with thalassemic defects is unknown [1]. Among the variants listed, Hb Miyagi [$\alpha 61(\text{E10}) \text{Lys} > \text{Glu}$; HBA2:c.184A>G (or HBA1)] is a typical example of this incomplete characterization, as it has never been reported in the literature, and the only evidence comes from Harano T. in 1998 (HbVar ID 92), concerning a Japanese subject who, strangely, presented with methemoglobinemia (metHb) [1]. Given the lack of supporting documentation, it is unclear whether the reported methemoglobinemia is actually due to the Hb Miyagi mutation. In the case described here, the new variant of the α chains is co-inherited with a variant of the δ chains (Hb A2-Lampang). When this minor component is present in a heterozygous state and in the absence of other globin defects, it accounts for approximately 1.2–1.6%, as reported in the literature [16]. In our case, when assessing the relative amounts of Hb A2-Lampang and, above all, normal Hb A2 present, it is necessary to consider the extent to which normal and mutated α and δ genes contribute to the synthesis of minor tetramers: Hb A2 ($\alpha 2\delta 2$), Hb A2-Lampang ($\alpha 2\delta 2$ -Lampang), and the minor Hb ($\alpha 2$ -Romagna $\delta 2$) and ($\alpha 2$ -Romagna $\delta 2$ -Lampang). These last two were not detected by the various separation systems used because they were present in quantities below the instrumental detection limit. The coexistence of Hb Romagna, a variant of the globin alpha chains, and Hb A2-Lampang, a variant of the globin delta chains, poses diagnostic challenges with clinical implications that could be relevant to patient management.

In conclusion, we would like to summarize some key points that this case has helped to highlight. The use of separation techniques, particularly electrophoresis, has proven to be highly sensitive in distinguishing the presence of a Gln residue from that of a Glu residue. Furthermore, this capability, combined with the use of DNA analysis in characterizing a variant, is far more informative than protein analysis alone (amino acid sequencing or mass spectrometry), as demonstrated by the cases reported in Table 1. Even the most recent studies on new defects—particularly Hb variants—and the related information reported in databases are incomplete and/or misleading precisely because of the lack of correlation between protein studies and genetic analysis. The hypotheses we have formulated here may also be confirmed by further studies.

Finally, in this case, the measurement of Hb A1c, which is not subject to interference, and the accurate assessment of Hb A2, which is instead strongly influenced by both variants, are issues that the laboratory must be able to address by proposing and/or performing appropriate confirmatory tests. The laboratory is, in fact, responsible for the report that supports physicians in the proper therapeutic management of patients and in the prevention of genetic risk.

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Institutional Review Board Statement: Ethical review and approval were waived according to institutional policy and national regulations, as this study describes a single retrospective case report based on routine clinical practice, without experimental intervention and with fully anonymized patient data. Written informed consent was obtained from the patient's parents/legal guardians for publication of this case report.

Informed Consent Statement: Written informed consent has been obtained from the patient to publish this paper.

Data Availability Statement: The original contributions presented in this study are included in the article and in the HbVar database (ID 3306).

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Conflicts of Interest: Author Sauro Maoggi was employed by the company Sebia Italia srl, Bagno a Ripoli, Firenze (Italy). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CE	capillary electrophoresis
HPLC	high-performance liquid chromatography
NGS	next-generation sequencing
BCB	brilliant cresyl blue

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