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Hb Riesa or β93 (F9) Cys → Ser, a new electrophoretically silent haemoglobin variant interfering with haemoglobin A1c measurement

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ABSTRACT

A new β variant was found in a German diabetic patient whose blood samples appeared to contain 45% Hb A_{1c} using Bio-Rad Variant V-II A1c-analyzer but 7.6% on boronate affinity chromatography. Structural studies using, HPLC, mass spectrometry, and the genomic DNA analysis revealed a new substitution in which the cysteine residue at position β93 was replaced by serine. The variant was named Hb Riesa or β93 (F9) Cys → Ser and accounted for 54.3% of the total haemoglobin. This suggests that the protein-synthesis processes for the mutant could be slightly more promoted than those of the wild-type. Hb Riesa is clinically and electrophoretically silent.

1. Introduction

Hb A_{1c} is used to evaluate long-term control in patients with diabetes mellitus and most accurately reflects the previous 2–3 months of glycemic control. The Diabetes Control and Complications Trial (DCCT) [1] and the United Kingdom Prospective Diabetes Study [2] have demonstrated that Hb A_{1c} is directly related to the risk of diabetes complications. Therefore, the evaluation of glycohaemoglobin levels (Hb A_{1c}) is generally accepted as the most suitable marker for the management of diabetes, and clinical diabetes guidelines recommend specific treatment goals related to Hb A_{1c} worldwide. An international effort has been made to develop a reference method with the aim of assigning Hb A_{1c} target values for human-matrix-based secondary reference materials. Medical laboratories are therefore recommended to use assay methods that are certified as traceable to the "DCCT Hb A_{1c} reference method". In spite of these improvements the interferences due to the haemoglobin variants remain obstacles in the methods for glycohaemoglobin determination. Many analytical procedures have been developed for this purpose and more than 20 different assays are commercially available. These assays are based on the physical or chemical properties of glycohaemoglobin species and on antibodies specifically for glycated N-terminal amino acid of the beta chains. The degree of interferences due to the haemoglobin variants differs considerably with different assays. Here, we report a case of German diabetic male patient whose Hb A_{1c} peak was falsely increased using Bio-Rad Variant V-II A1c-analyzer. Structural analysis showed that he was heterozygous for a new haemoglobin variant, designated haemoglobin Riesa after the name of the city where the patient lived.

2. Materials and methods

2.1. Blood samples and haemoglobin analyses

Fresh blood samples were collected in tubes with K3EDTA as anticoagulant.

Haematologic characteristics were determined by routine examinations. Routine haemoglobin analyses are briefly reported: the determination of Hb A_{1c} level was done on Bio-Rad Variant V-II A1c-analyzer. Hb analysis was done on standard agarose gel electrophoresis, on cation exchange high-performance liquid chromatography (CE-HPLC) using PolyCATA column [3] and on reverse HPLC (rp-HPLC). The total glycohaemoglobin (GHb) concentration was determined by boronate affinity chromatography [4]. Hb instability was measured using heat stability and isopropanol stability

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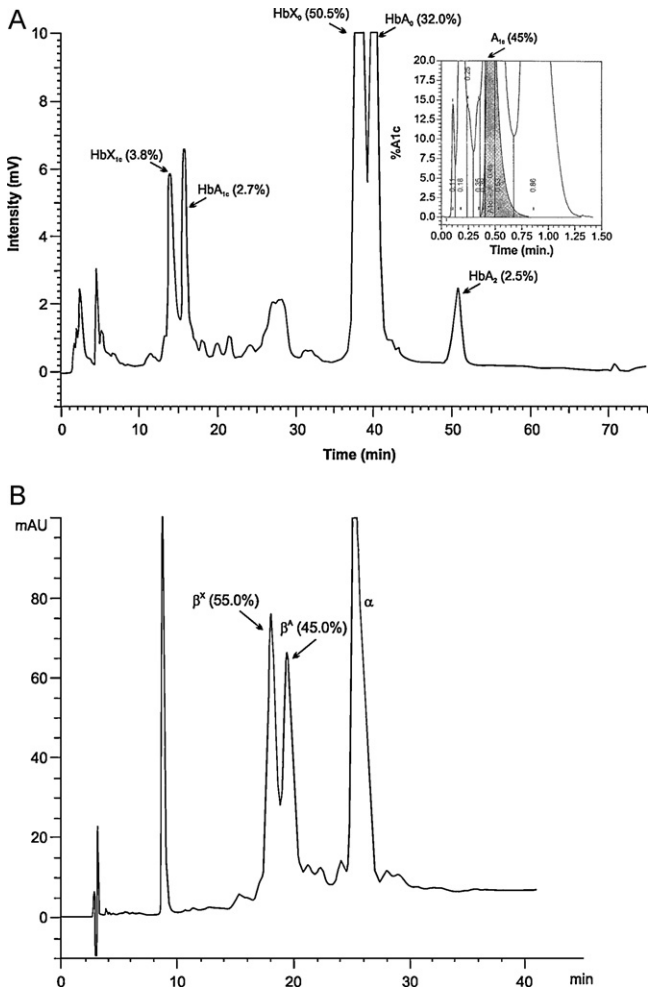

Fig. 1. Elution patterns of haemoglobin components in a blood sample from a carrier of Hb Riesa depicted by HPLC-PolyCAT A (A), by Bio-Rad Variant V-II A1c-analyzer (A, inset), and by reverse-phase HPLC (B).

tests [5]. The oxygen-binding properties of whole blood were studied using oximetric parameters as reported previously [6].

2.2. Mass spectrometry

2.2.1. Material and reagents

All chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany). Tris–HCl was from Euromedex (Souffelweyersheim, France). Porcine trypsin was from Promega (Madison, WI, USA). Distilled water produced from a Milli-Q Water System^{TM} (Millipore, Guyancourt, France) was used for the preparation of the buffer and solvents.

2.2.2. Mass spectrometry analysis

Isolation of globin chains was performed using a RP-HPLC Vydac TP C18 column (2.1 mm × 250 mm, 5 μm, 300 Å). The HPLC solvents were water with 0.1% trifluoroacetic acid (A) and acetonitrile with 0.08% trifluoroacetic acid (B). The gradient elution was as follows: 40% B at 0 min, 45% B at 2 min, 47% B at 15 min, 70% B at 16 min. The flow rate was 0.3 ml/min. The separation was performed at 40 °C.

An electrospray spectrum of each collected fraction was obtained on a TOF mass spectrometer equipped with an ESI source (LCT Micromass, Manchester, UK). The main MS conditions were the following: temperature source 90 °C, electrospray needle voltage at 3.5 kV and cone voltage at 35 V. Mass spectra were acquired in the positive mode, in the range of *m*/*z* 500–2500. Acquisitions were performed in the positive ion mode after external calibration performed with the multiply charged ions produced by a 2 μM horse heart myoglobin solution diluted in a 1:1 water/acetonitrile mixture (v/v) acidified with 1% formic acid (v/v). Data analysis was performed with MassLynx 4.0 (Waters, Manchester, UK).

The HPLC fraction containing the modified beta chain was further digested using trypsin. A 5 μl aliquot of purified Hb mutant fraction was digested overnight at 39 °C after addition of 5 μl of 2,2,2-trifluoroethanol, 30 μl of 50 mM Tris–HCl (pH 8.5) buffer and 20 μg of porcine trypsin The digestion was stopped by adding 1 μl of 10% formic acid, and the solution was analyzed by micro liquid chromatography coupled to an ion trap mass spectrometer (microLC–MS/MS). Analysis was performed using an Agilent 1100 series micro-HPLC system (Agilent Technologies, Palo Alto, USA) coupled to an HCT Ultra ETD ion trap (Bruker Daltonics, Bremen, Germany). Tryptic digest was loaded on a RP-capillary enrichment column (ZORBAX 300SB-C18, 5 mm × 0.3 mm, 5 μm) with 0.1% trifluoroacetic acid (TFA) in water at a flow rate of 50 μl/min. After 3 min of trapping the desalted peptides were then eluted from the enrichment column to the analytical RP-capillary column (ZORBAX 300-SB-C18, 0.3 mm × 150 mm, 3.5 μm). The solvent system consisted of 0.05% TFA in water (solvent A) and 0.045% TFA in acetonitrile (solvent B). Elution was performed at a flow rate of 4 μl/min with a 10–50% gradient (solvent B) over the first 55 min followed by a 70% stage (solvent B) over 5 min before the reconditioning of the column at 90% of solvent A.

The voltage applied to the capillary cap was optimized to −4000 V. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides, preferring doubly charged ions, were selected on each MS spectrum for further isolation and fragmentation. The MS/MS scanning was performed in the ultrascan resolution mode at a scan rate of 26.000 *m*/*z* per second. A total of 6 scans were averaged to obtain a MS/MS spectrum. The complete system was fully controlled by ChemStation B.01.03 (Agilent Technologies) and EsquireControl 6.1 (Bruker Daltonics) softwares.

Mass data collected during microLC–MS/MS analysis were processed, converted into *.mgf files, and interpreted using a local Mascot 2.2.0. (Matrix Science, London, UK) server. Searches were performed against the SwissProt database without any taxonomy, molecular weight, or isoelectric point restrictions. Searches were performed with a tolerance on mass measurements of 0.25 Da in both MS and MS/MS modes. For peptides not corresponding to expected masses for normal beta haemoglobin, the corresponding MS/MS spectrum was submitted to manual *de novo* sequencing and Peaks Studio version 4.2 (BSi, Waterloo, Canada).

2.3. DNA analysis

Genomic DNA was extracted from peripheral blood using the standard salting out method [7]. The three exons of the β-globin gene were amplified in two formats using the oligonucleotides CD7–CD6 and CD1–CD2 as published [8,9]. The amplified products were sequenced directly with the Dye Primer Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's recommendations (oligonucleotides as for amplification). Sequencing reactions were analyzed on an ABI 3730 automated sequencer.

3. Results

3.1. Patient

The propositus was a 56-year-old man of German origin with insulin-dependent diabetes mellitus. Haematological studies of his

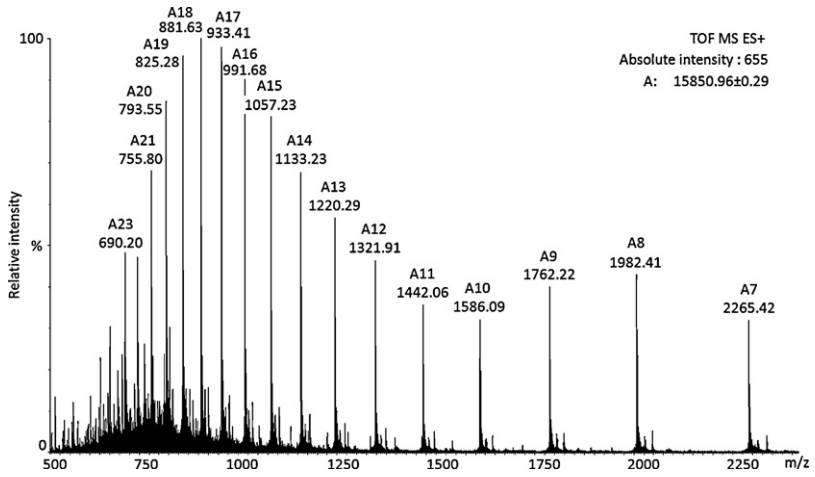

Fig. 2. MS spectrum of the Hb mutant fraction. Transformed data revealed molecular mass of 15,851.2 ± 0.5 Da (A) which matches with mutant beta chain 16 Da lighter than the mass of the normal beta chain (15,867.2 Da).

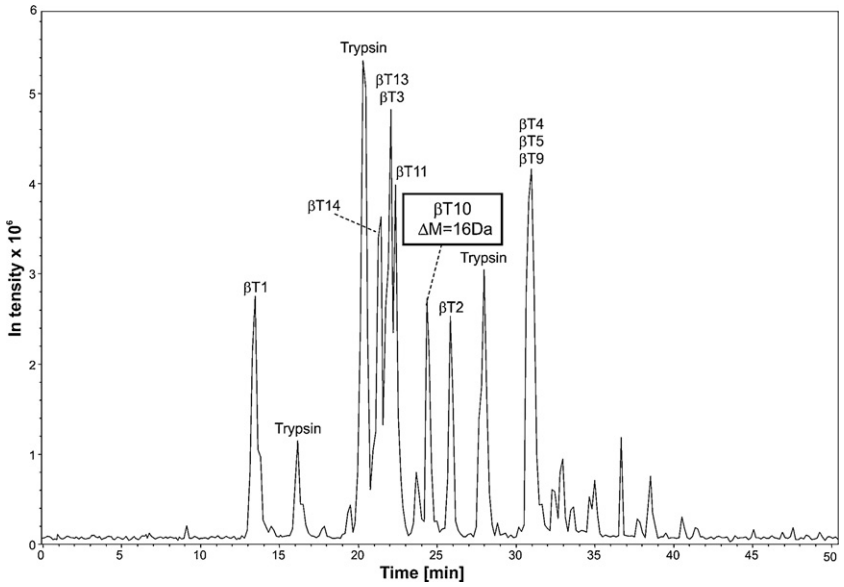

Fig. 3. Base Peak Chromatogram (BPC) obtained from micro-LC–MS/MS analysis of tryptic beta chain mutant digestion. The peak at 24 min exhibited a mass of 1404.7 Da which does not correspond to any peptide expected for normal beta chain.

peripheral blood were within the normal range: Hb 14.2 g/dl, mean corpuscular volume (MCV) 83.3 fl, mean corpuscular Hb (MCH) 27.9 pg, mean corpuscular Hb concentration (MCHC) 32.6 g/dl, the packed cell volume (PCV) 0.433 l/l. The analysis of his blood sample on Bio-Rad Variant V-II A1c-analyzer showed a large peak overlapping Hb A_{1C} and constituting 45% of the patient's total haemoglobin, and was identified as Hb A_{1C} by the instrument software (Fig. 1A inset). The concentration of the total GHb determined by affinity chromatography was 7.6%. The immunoturbidimetric method (Tina-Quant; Roche Diagnostics, Mannheim, Germany) and Tosoh-G5-A1c-Analyzer gave a result of 7.2%. and 7.0% respectively. The Hb variant was identified neither on Tosoh-G8-A1c-Analyzer using the β-thalassaemia program nor on Tosoh-G5-A1c-Analyzer. Both analyzers do not provide any warning flags.

3.2. Haemoglobin analyses

By electrophoresis on agarose the abnormal haemoglobin was not detected. Chromatography of the hemolysate carried out with CE-HPLC using PolyCAT A column showed two abnormal Hb fractions, Hb X_{1C} and Hb X_0 eluting before Hb A_{1C} and HbA_0. The glycated Hb variant (Hb X_{1C}) was separated from Hb A_{1C}, whereas the major abnormal Hb fraction (Hb X_0) eluted close to normal Hb A_0. The percentages of these Hb variants were 3.8% and 50.5% of the patient's total haemoglobin respectively (Fig. 1A). The separation of globin chains by rp-HPLC (Fig. 1B) showed a mutant globin (β^X) eluting before the wild-type β^A The relative quantity of β^X in the heterozygote was 55.0% of the total β chains ($\beta^X + \beta^A$).

Oxygen-binding properties of the whole blood showed a normal halfsaturation tension (P_{50}) of 26.3 mmHg versus 25.2 mmHg for the wild-type control (reference interval: 23.8–28.8 mmHg). A haemoglobin heat stability test and the isopropyl alcohol test were inconspicuous.

3.3. Mass spectrometry analysis

The total hemolysate was fractionated by HPLC and the collected fractions were then analyzed by ESI-MS to determine

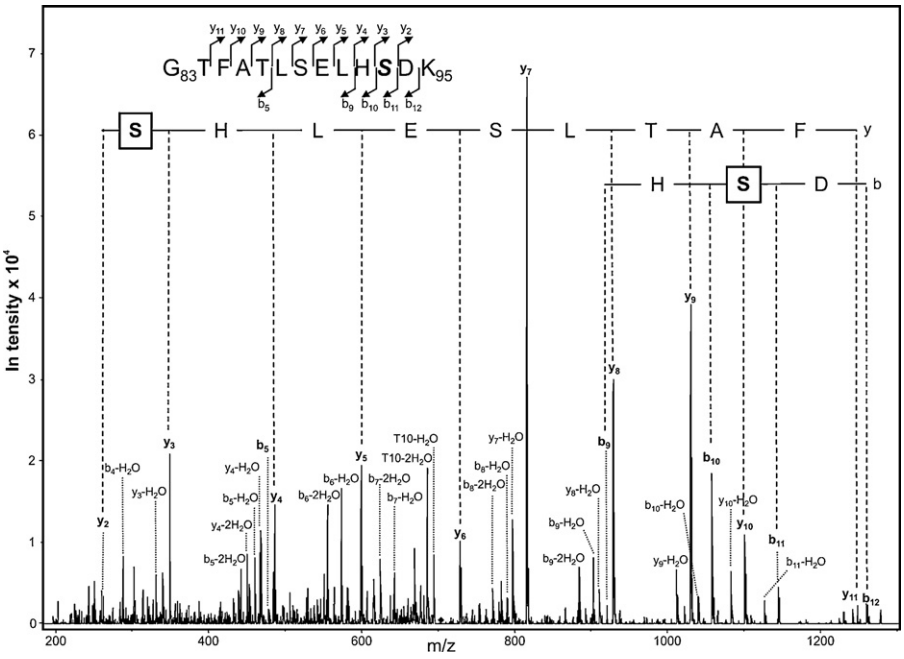

Fig. 4. MS/MS fragment ion spectrum obtained from the 2^+ molecular ion at m/z 703.3 from the 13 amino acids mutated tryptic peptide βT10 (^{83}GTFATLSELHSDK^{95}).

the molecular masses of the different globin chains present. Three major peaks were observed with masses measured at 15,851.2 ± 0.5 Da, 15,867.2 ± 0.3 Da and 15,126.4 ± 0.1 Da. The two last masses fitted respectively with the molecular masses of normal beta and alpha chains which was also in agreement with the retention times observed. The first peak, with a retention time slightly shorter than the beta chain, corresponds to a variant with a molecular mass 15.9 Da lower than the normal beta chain (Fig. 2). According to the single nucleotide-mutation in coding triplet, this delta mass of −16 Da could be due to five different amino acid substitutions. In order to characterize the mutated amino acid, the abnormal peak was collected, digested with trypsin, and was further analyzed by microLC–MS/MS.

MS/MS data obtained were interpreted using MASCOT algorithm and allowed us to identify with good sequence coverage (74%) the beta chain. The identification of the major ions detected is reported in Fig. 3. Among ions with masses not corresponding to expected tryptic peptides from beta chain, doubly charged ion at m/z 703.3 was the most intense.

The MS/MS spectrum of ion at m/z 703.3 was submitted to *de novo* sequencing using Peaks software and validated manually (Fig. 4). The sequence identified corresponds to the tryptic peptide T10 from the beta chain with a mutation in position 93 (Cys → Ser). This mutation induces a mass difference of −15.98 Da, which is consistent with the data obtained by ESI-MS on the abnormal globin chain fraction.

3.4. DNA analysis

The DNA analysis of the patient's β-globin gene revealed a single base mutation G to C in codon 93 in the second exon of the β-globin gene, producing a Ser → Cys substitution.

4. Discussion

In the past two decade the number of haemoglobin variants all over the world has been increased. Accordingly over 1000 structural haemoglobin variants have been recorded [10] and many of them are more or less rare. So far as we know no haemoglobin variant of substitution at β93 (F9) Cys → Ser has been recorded. Two amino acid substitutions have been reported at the β93 (F9) Cys position: Hb Okazaki β93 (F9) Cys → Arg [11] and Hb Fort Dodge β93 (F9) Cys → Tyr [12]. Therefore Hb Riesa β93 (F9) Cys → Ser is the third substitution observed at this position. The residue β93 has no special function, whereas Hb Okazaki has been reported as having high oxygen affinity and molecular instability, but without associated haematological abnormalities. It is conceivable for Hb Okazaki that arginine having a bulky extended guanidino group could induce moderate changes in the stability and function of Hb molecule. In contrast serine and threonine are small and moderately polar, so their introduction in the interchain region of the alpha and beta chains ($\alpha_2\beta_1$) is supposed to have no effect on the stability and the functional behaviour of the Hb molecule. Indeed like Hb Fort Dodge, Hb Riesa has no functional abnormalities and does not appear to be associated with clinical or haematological anomalies. The quantity of Hb Riesa in the hemolysate of the patient was 54.3% of the total haemoglobin and the mutant β-chain polypeptide also accounts for 55.0% of the total β-chains. This high percentage of the variant suggests that the protein-synthesis processes for Hb Riesa could be promoted slightly more than those of Hb A.

In this case we provide an explanation for the extreme high Hb A_{1C} value obtained on Bio-Rad Variant V-II A1c-analyzer. Therefore Hb Riesa is yet another example to be added to the expanding list [13,14] of Hb variants affecting Hb A_{1C} analysis. The immunoturbidimetric method (Tina-Quant) and Tosoh-G5-A1c-Analyzer demonstrated minimum interference from the presence of Hb Riesa. However boronate affinity should be recommended in the management of diabetic carriers of Hb Riesa.

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