Co-inheritance of HbD^{Iran}/Beta Thalassemia IVS1–5 (G > C) Trait in a Punjabi Lady with Diabetes

Vijay S. Bhat · Amit Kumar Mandal · Bobby Mathew

Received: 10 February 2012 / Accepted: 3 March 2012 / Published online: 24 March 2012
© Association of Clinical Biochemists of India 2012

Abstract The present report describes the molecular study of HbD^{Iran} (beta) 22 Glu → Gln associated with β -Thalassemia IVS1–5 (G > C) found in India, and the first case in which mutation has been identified using mass spectrometry. Given the apparent ethnic origin and the mobility of the variant hemoglobin at alkaline pH, hemoglobin D-Punjab would be suspected, but HPLC excluded this possibility. Further characterization of hemoglobinopathy was made by using nondenaturing gel electrophoresis and matrix assisted laser desorption ionization mass spectrometry and IVS1–5 being validated by reverse dot blot hybridization followed by sequencing of the β -globin gene.

Keywords Hemoglobin D-Iran · Mass spectrometry · Matrix assisted laser desorption ionization mass spectrometry · High performance liquid chromatography · Reverse dot blot hybridization

Introduction

The HbD^{Iran} genotype: $\alpha 2\beta 2$ 22 Glu–Gln (GAA → CAA) was first described by Rahbar in 1973 [1]. Found mainly in Iranian and Pakistani families, and generally in the heterozygous state, with no abnormality [2]. There is no

anemia or reticulocytosis, MCV and MCH are usually normal unless associated with an underlying iron deficiency.

But HbD^{Iran} in combination with β -thalassemia produces a moderate microcytic and hypochromic anemia that is not transfusion dependent. It is a mutation caused by replacement of glutamic acid by glutamine at $\beta 22$, and this is the fourth substitution to be described at this site of β -globin gene. The others being G Coushatta (Ala), E Saskatoon (Lys) and G Taipei (Gly).

The issue to be addressed is that HbD^{Iran} appears as HbSS on alkaline electrophoresis and migrates with HbA2 and HbE using HPLC methods. So in laboratories utilizing HPLC instruments HbD^{Iran}/ β -thalassemia compound heterozygotes are suspected of having either homozygous HbEE, if HbD^{Iran} is misinterpreted as elevated HbE, or β -thalassemia major if the HbD^{Iran} is thought to be elevated HbA2 [3].

The present case study describes the HPLC and molecular findings of HbD^{Iran} with a concomitant β -Thalassemia from a 32-year-old lady who hailed from the state of Punjab in North India, was referred to Manipal Hospital, Bangalore, for a comprehensive check up as part of diabetic work up.

Material and Methods

Blood sample collected in Beckton Dickenson vacutainer EDTA tube was analyzed for a routine blood count using Sysmex XT 1800i Hematology analyzer.

As the patient was diabetic, the sample was analyzed for glycated hemoglobin (HbA1c) on BioRad D10 HPLC instrument as per standard procedures. During A1c testing a variant window was detected in HPLC, which prompted

V. S. Bhat (✉)
Department of Biochemistry, Manipal Hospital Diagnostic Services, Manipal Hospital, HAL Airport Road, Bangalore 560 017, India
e-mail: vijay.bhat@manipalhospitals.com

A. K. Mandal · B. Mathew
Department of Molecular Medicine & Clinical Proteomics, St. John's Research Institute, Bangalore, India

us to proceed with its identification on this same platform, but by using the Beta Thal short program mode.

In this mode due to the presence of an elevated HbA2 percentage, the sample was later centrifuged at 3,000 rpm for 10 min and obtained packed cells were lysed with hemoglobin lysing reagent. Electrophoresis was carried out on HELENA SAS-II analyzer at alkaline pH.

Gel was stained using Acid Blue stain and subsequently destained at room temperature. The hemoglobin bands were later excised from the gel, destained and desalted by vigorous shaking in a solution containing acetonitrile and 50 mM NH_4HCO_3 buffer in 1:1 (v/v) ratio. Gel pieces were dehydrated with acetonitrile for 5 min. In-gel digestion was performed using trypsin (TPCK treated, SIGMA, USA) in 50 mM NH_4HCO_3 , pH 8.0, at 37 °C for 12 h. Proteolytic peptides were eluted from gel with elution buffer (acetonitrile:water = 60:40 (v/v), 0.1 % TFA).

Mass spectrometry analysis was performed on a MALDI mass spectrometer (Waters Synapt HDMS). PEG mix from Waters, UK was used as an external calibrant. The digested peptides were mixed with the matrix solution, α -cyano-4-hydroxycinnamic acid in 1:1 (v/v) ratio and spotted on a MALDI plate. Mass spectra for proteolytic peptides were recorded in the positive ion mode using a 200 Hz laser.

Mass spectral findings were analyzed using MassLynx software [4].

With prior consent and clearance from the Institutional Ethics committee, genomic DNA was extracted from the blood sample using the DNEasy kit from Qiagen. The β -thalassemia mutations were characterized by a PCR method based on ARMS by Newton et al. [5].

For molecular characterization, a region containing exon 3 of β -globin was amplified with the following primers:

RE (5'-CAATGTATCATGCCTCTTTGCACC-3') and RD (5'-GAGTCAAGGCTGAGAGAGATGCAGGA-3') the 861 bp PCR product of this amplicon was digested by *EcoRI* restriction enzyme (Roche, Germany). Direct β -globin gene sequencing was performed in both directions on DNA sequencer.

ARMS PCR consisted of 27 cycles, preheating at 94 °C for 4 min, denaturing at 94 °C for 1 min, annealing at 67 °C for 30 s and extension at 72 °C for 1.5 min. The PCR product was electrophoresed on a 3 % agarose gel stained in ethidium bromide with ϕ X 174 *HaeIII* digest as marker. The primers mentioned above for this run were selected from a published report.

The amplification reaction was performed using a DNA thermal cycler (PTC-100 from M.J. Research). Sequencing

Fig. 1 **a** BioRad D10 HPLC pattern depicting a variant window at 1.6 min RT. **b** BioRad D10 HPLC pattern in Beta Thal mode showing elevated HbA2 at RT of 2.9 min. **c** Alkaline hemoglobin electrophoresis depicting intense band in HbS/D/G region

of PCR product was conducted using ABI Prism Big Dye Terminator technology from Applied Biosystems, USA to confirm the mutation.

A screen for β -chain mutation(s) was conducted using the β -globin strip assay SEA (Vienna Lab Diagnostics, GmbH). The kit follows a PCR based reverse dot blot hybridization (RDBH) protocol that simultaneously screens for 22 mutations covering >90 % of the β -globin defects found and reported in South-East Asia. Biotinylated primer products were detected using streptavidin alkaline phosphatase and color substrates. The conditions for the PCR and protocol for the RDBH assay for this sample were conducted following instructions from the kit.

Results

The examination of blood in a comprehensive health diabetic workup from a patient from Punjab in North India presented an anomalous blood picture. Based on the hematological data, the patient had Hb levels of 12.5 gm%, RBC count of $4.7(10^{12}/l)$, MCV of 70 fl and MCH 23 pg. Peripheral smear showed a mildly hypochromic and microcytic blood picture with occasional target cells. BioRad D10 HPLC profiles (reverse phase HPLC) (Fig. 1a) showed an intense peak (variant window) at 1.6 min with a percentage of 44.7 % and glycated hemoglobin (HbA1c) was 10.4 %.

Fig. 2 Arrow depicting position of IVS1–5 (G > C) in chromatogram obtained by Big Dye Terminator DNA sequencer technology

Fig. 3 Arrow depicting position of band obtained by Reverse dot blot hybridization (RDBH) method using Vienna Lab Beta Globin strip assay, identifying heterozygosity of IVS1–5 (G > C)

To investigate the nature of the large unknown peak detected by HPLC, the same sample was run in beta thal short program mode, wherein a HbA2 level of 29.6 % was obtained (Fig. 1b). In a HPLC run a HbA2 value between 3 and 10 % is indicative of thalassemia carrier status, between 10 and 25 % Hb-Lepore, 25–60 % HbE heterozygosity and more than that as homozygous. As there was an ambiguity in the result, based on the ethnicity of the patient, the sample was subjected to alkaline hemoglobin electrophoresis using SAS-Mx electrophoresis from Helena

laboratories. An intense band in HbS/D/G position, as depicted in (Fig. 1c), was obtained.

As the HPLC findings and that of electrophoresis were contradictory, we proceeded with molecular analysis of sample using an amplification refractory mutation system PCR (ARMS-PCR). This analysis was done due to the fact that a concomitant presence of thalassemia trait was suspected by the finding in the index patient of microcytosis, hypochromia and occasional target cells in the peripheral smear.

Table 1

	1	11	21	31	41	51	
1	MVHLTPEEKS	AVTALWGKVN	VDEVGGEALG	RLLVVYPWTQ	RFFESFGDLS	TPDAVMGNPK	60
61	VKAHGKKVLG	AFSDGLAHL	NLKGTFATLS	ELHCDKLHVD	PENFRLLGNV	LVCVLAHHFG	120
121	KEFTTPVQAA	YQKVVAGVAN	ALAHKYH				

Fig. 4 a Depicts the MALDI-MS spectra of the tryptic peptide finger print for normal hemoglobin (mass of 1,314.7 Da) and (b) for the hemoglobin with the mutated peptide with mass 1,313.7 Da

Specific primers were used to see the site of alteration in the β -globin gene. The predicted 285 bp fragment resulting from the PCR confirmed the mutation to be IVS1–5 (G > C). The ARMS-PCR was then further cross verified by sequencing the concerned region using the Big Dye Terminator DNA sequencer. Sequencing electrophoretogram clearly demonstrated the specific location of the mutation (Fig. 2). A screening kit from Vienna Labs was also used for identifying the β -chain mutation. This kit uses β -globin gene specific primers in a multiplex PCR and the amplified product is subsequently analyzed to investigate if any of the 22 known common mutations that have been reported, has been amplified in a RDBH strip assay.

Presence of IVS1-5 (G > C) band (arrow), as seen in (Fig. 3), among the other 22 mutations in its panel confirming that the patient harbors a β -thalassemia. The presence of positive bands for the entire lower wild type panel in the assay strip further indicates that this mutation is present in the heterozygous state.

Post HPLC and alkaline hemoglobin electrophoresis, there was an ambiguity regarding the variant type. So the variant band was isolated from gel and digested with trypsin and analyzed in MALDI-MS. The peptide mass fingerprint was compared with that of normal hemoglobin. The β -globin gene sequence is represented by amino acids with one letter code which reads as (Table 1):

Using tandem mass spectrometry the characteristic peptide was fragmented and sequenced to identify and locate the mutation. Figure 4 depicts the MALDI-MS spectra of the tryptic peptide finger print.

The peptide fragment at 1,314.7 Da is for normal hemoglobin and 1,313.7 Da is the mutated peptide, differing by 1 Da, indicating the specific fragment harboring the putative E to Q mutant.

Discussion

Hemoglobin D-Iran is formed as a result of a substitution of the amino acid glutamine for the wild type glutamic acid at position 22 of the β -chain. It is a silent variant, and in this case its association with β -thalassemia trait IVS1–5

(G > C) also has not produced any clinical abnormality. HbD^{Iran} with presence of β -thalassemia trait though have been reported using electrophoresis, HPLC, and ARMS-PCR techniques, our emphasis has been on using non-denaturing gel electrophoresis and matrix assisted laser desorption ionization mass spectrometry with RDBH and DNA sequencing at arriving at a definitive diagnosis.

There is a masquerading effect seen between HbD^{Iran} and HbE, and this has been resolved aptly using the mass spectrometry as can be seen by the fact that: When subjected to digestion with trypsin it is found in the T3 peptide which contains amino acids 21–30. The wild type T3 sequence of **VNVDEVGGEALGR** is altered to **VNVDQVGGEALGR**. No other single amino acid substitution in this peptide will cause a mass alteration of –1, therefore the mass alteration of the T3 wild type fragment from (M + H) 1,314.7 Da to (M + H) 1,313.7 is highly specific for HbD^{Iran}.

In the process of identifying the site of mutation, it is also evident that MS and DNA sequencing are complementary techniques, and can be a very useful tool in a molecular approach in identification of hemoglobin variants.

References

1. Rahbar S. Haemoglobin D Iran: 2 22 glutamic acid leads to glutamine (B4). *Br J Haematol*. 1973;24(1):31–5.
2. Taghavi M, Karimipoor M, Amirian A, Jafarinejad M, Katouzian L, Valaei A, et al. Co-inheritance of hemoglobin D and β -thalassemia traits in three Iranian families: clinical relevance. *Arch Iran Med*. 2011;14(1):61–3.
3. Rohe RA, Sharma V, Ranney HM. Hemoglobin D Iran alpha A2 beta 22 2-Glu leads to Gln in association with thalassemia. *Blood*. 1973;42(3):455–62.
4. Mathew B, Mandal Amit Kumar, Bhat Vijay. Analysis of hemoglobin variants using nondenaturing gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry. *Anal Biochem*. 2011;416:135–7.
5. Newton CR, Graham A, Heptinstall LE. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucl Acids Res*. 1989;17:2503–16.