Characterization of a Double Heterozygote HbE/ β^+ Thalassemia IVS 1-1 [G>T] in a Juvenile Diabetic

Vijay S. Bhat · S. Lokeshwarappa ·
Amit Kumar Mandal

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Abstract The present case report describes the molecular and proteomic based study of Hb variant HbE associated with β^+ thalassemia IVS 1-1 G>T, in a juvenile diabetic patient. Given the ethnic origin and mobility of the variant hemoglobin at alkaline pH, HbE would be suspected. But hematologically and clinically abnormality being detected, HPLC and Electrophoresis not being able to characterize due to retention time and band being in region of HbA₂, respectively, further characterization of hemoglobinopathy was made using MALDI and IVS 1-1 G>T being validated by reverse dot blot hybridization. Capillary electrophoresis was also employed in order to separate HbE and HbA₂ bands. This case report being first of its kind, wherein a HbE/ β^+ thalassemia has been characterized using multiple techniques.

Keywords Hemoglobin E · Matrix assisted laser desorption ionization mass spectrometry · High performance liquid chromatography · Reverse dot blot hybridization

Introduction

Hemoglobin E Genotype: $\alpha 2\beta 2$ 26 Glu → Lys was first described by Chernoff et al. [1]. It is a structural variant of

the hemoglobin beta chain and is most common one among Southeast Asians. The prevalence of HbE carriers in India is 0–3.5 % of which 50–80 % are in Assam [2]. HbE trait (heterozygous HbE, Hb AE) is an asymptomatic benign condition associated with microcytosis, but no anemia.

HbE/ β -thalassemia results from co-inheritance of a β -thalassemia allele from one parent and the structural variant hemoglobin E from the other. Patients with combined HbE/ β -thalassemia have a variable clinical presentation with symptoms varying from a mild form of thalassemia to thalassemia major. Peripheral blood smear demonstrates the target cells characteristic of HbE and the basophilic stippling characteristic of β -thalassemia. The first case of Hb E- β -thalassemia was reported in a Kulita caste family from Western Orissa by Balgir [3] wherein Hemoglobin electrophoresis showed a predominance of HbE(40–75 %), with increased HbF (5–40 %) and variable HbA (1–30 %).

The present case study describes the HPLC and molecular findings of HbE with a concomitant β -thalassemia from a 8 year old boy who hailed from the state of Assam in North East India, was referred to Manipal Hospital, Bangalore, for a diabetic work up.

The issue to be addressed is that HbE migrates in same position and retention time with HbA₂, using alkaline electrophoresis and HPLC methods, respectively. In laboratories using HPLC, compound heterozygotes for HbE/ β -thalassemia are not being able to estimate HbA_{1c} levels in diabetics, mainly due to interference of HbF and HbE.

Materials and Methods

Blood sample collected in Beckton Dickenson vacutainer EDTA tube was analysed for a routine blood count using Sysmex XT 1800i fully automated Hematology analyser.

V. S. Bhat (✉) · S. Lokeshwarappa
Department of Biochemistry, Manipal Hospital Diagnostic Services, Manipal Hospital, HAL Airport Road, Bangalore 560017, Karnataka, India
e-mail: vijay.bhat@manipalhospitals.com

A. K. Mandal
Molecular Medicine & Clinical Proteomics Department, St. John's Research Institute, Bangalore, Karnataka, India

As the patient was diabetic, the sample was analysed for glycated hemoglobin (HbA1c) on BioRad D10 HPLC instrument as per standard procedures. During HbA1c testing a variant window was detected in HPLC, and no result was obtained for A1c, due to the interference of the variant. This prompted us to proceed with its identification on this same platform, but by using the Beta Thal short program mode. The same sample was also run on the SEBIA capillarys 2 analyser, in order to segregate the HbA2 from the HbE, and also to determine the percentage of HbF which was detected by HPLC.

The sample was also centrifuged at 3,000 rpm for 10 min and the obtained packed cells were lysed with hemoglobin lysing reagent. Electrophoresis was carried out on HELENA SAS-II analyser 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 mmol 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 Chymotrypsin (TLCK treated, SIGMA, USA) in 50 mmol 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), followed by sonication for 10 min. This solution was centrifuged at 6,000 rpm for 1 min at 4 °C and supernatant consisting of peptides were collected.

MS analysis were 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 analysed using MassLynx V4.1 Software. Denovo sequencing was done by manual assignment of daughter ions [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.

A screen for beta 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 Southeast Asia. Biotinylated primer products were detected using streptavidin alkaline phosphatase and colour substrates. The conditions for the PCR reaction and protocol for the RDBH assay were conducted following instructions from the kit. Basically the method is based on multiplex DNA amplification (including gap-PCR) and involves three steps: (1) DNA isolation (2) PCR

amplification using biotinylated primers (3) hybridization of amplification products to test strips containing allele specific oligonucleotide probes immobilized as an array of parallel lines. Some of the mutations covered by the β -globin strip assay are located within a few nucleotides on the β -globin gene. On the test strips these are represented by a common wild type probe, so that the 22 mutations are covered by 12 wild type probes only [5].

Results and Discussion

The examination of blood from a 8 year old boy from Assam in North East India presented an anomalous blood picture. Based on the hematological data, the patient had Hb levels of 9.8 gm %, MCV of 70 fl and MCH of 20 pg, reticulocyte count was 12 %. Peripheral smear showed microcytic hypochromic blood picture with many nRBCs, and basophilic stippling. Clinically the boy was anemic and had mild hepatosplenomegaly.

BioRad D10 HPLC profile showed an intense peak (variant window) of 50 % and HbF of 34 %. HbA1c could not be measured, and so the same was checked in Beta thal short program mode, when HbF of 31.8 % and HbA2 of 66.9 % was obtained (Fig. 1a). This gave an indication that the patient could be harbouring a HbE/Beta thalassemia mutation. In order to separate out? HbE from HbA2 fraction, the blood sample was subjected to capillary electrophoresis using the SEBIA capillarys 2 system. Here we could obtain a HbA0 of 4.3 %, HbF of 39.9 %, HbE of 50.8 % and HbA2 of 5.0 % (Fig. 1b) which suggested that the boy was a double heterozygote for HbE and Beta thalassemia.

As the patient was diabetic and HbA1c could not be measured by HPLC, an alternate method was used. HbA1c was estimated on Roche COBAS 6000 using the immunoturbidometry method, and A1c value of 9.6 % was obtained.

In order to clarify the β -thalassemia mutation, a screening kit from Vienna Labs was used. This kit which uses β -globin gene specific primers in a multiplex PCR reaction, had the amplified product identified as IVS1-1[G>T], as seen in (Fig. 2). 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.

Meanwhile as part of the routine procedure, the sample was subjected to alkaline gel electrophoresis using the HELENA SAS-II system.

Figure 3 depicts the electrophoretogram of the sample in comparison to other samples of HbE which are traits and homozygous.

Fig. 1 a HPLC pattern b Electrophoretogram by capillary electrophoresis

Fig. 2 Reverse dot blot hybridization

But post HPLC and alkaline hemoglobin electrophoresis, the identification of HbE variant remains ambiguous, due to presence of other variants like HbC, HbO (Arab), and also the HbA2. So this variant band was isolated from gel and digested with chymotrypsin and analysed in MALDI-MS. The peptide mass fingerprint was compared with that of normal hemoglobin. The beta globin gene sequence is represented by amino acids with one letter code which reads as:

1 11 21 31 41 51
 1 MVHLTPEEKS AVTALW**GKVN** **VDEVGG**EALG RLLVVYPWTQ RFFESFGDLS TPDVAMGNPK 60
 61 VKAHGKKVLG AFSDGLAHLN NLKGT**FATLS** ELHCDKLHVD PENFRLLGNV LVCVLAHHFG 120
 121 KEFTTPPVQAA YQKVVGAVAN ALAHKYH

Fig. 3 Alkaline gel electrophoresis depicting various patterns of HbE. Lane 2 is pattern of the propositus

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 chymotryptic peptide finger print.

The wild type T3 sequence of **GKVN**VDEVGG**EAL** is altered to **GKVN**VDEVGG**KAL**. No other single amino acid substitution in this peptide will cause a mass alteration of minus 1, therefore the mass alteration of the T3 wild type fragment from $[M+H]^+$ 1,286.7 Da to $[M+H]^+$ 1,285.7 is highly specific for haemoglobin E.

Thus comparison of chymotryptic peptide profile for HbE band showed appearance of a new fragment at 1,285.7 m/z in place of 1,286.7 m/z in HbA (Fig. 4a). Assignment of MS/MS spectra of parent molecular ion with m/z 1,285.7 confirm that it is a β globin fragment corresponds to residue 16–28 with the site of mutation

E(26) to K (Fig. 4b). HbE arises from the substitution of lysine for glutamic acid at position 26 of the beta-globin chain.

Little et al. [6] showed that most HPLCs gave artificially low HbA1c results when compared to affinity chromatography. No clinically significant difference was found for HbA1c results measured with immunoassay. This could be due to the fact that the amino acid substitution at position 26 was far from the N-terminus of the beta globin chain where HbA1c glycation and antibody binding took place. It was noticed that HbA1c values were lower on HPLC when compared to immunoassay, as was also stated by Tsai et al. [7]. In the heterozygous state, HbE leads to a very mild thalassemic phenotype. HbE and HbA2 elute together in all the CE-HPLC system, therefore HbA2 cannot be measured in presence of HbE. A study by Pravatmuang et al. [8] found significantly higher HbA1c results, when measured by HPLC, as compared to immunoassay.

Panigrahi et al. studied the factors affecting the phenotype of HbE/ β -Thalassemia in 30 cases from North India and as per their findings they found Hb E/ β -thalassemia, 42 (6.36 %) HbE carriers, 16 (2.42 %) HbE homozygous and 10 (1.52 %) carriers of other mutations. Genotypic study of beta mutations revealed the prevalence of IVS1-5 mutation among the studied beta carriers to be 46.6 %, and codon 26 (G>A) mutation to be 31.54 %. Other prevailing mutations among the screened individuals included codon 30

Fig. 4 a MALDI-MS spectra of chymotryptic peptides obtained from in-gel digestion of HbE band in alkaline gel electrophoresis. **b** MALDI-MS spectra of the peptide ion with m/z 1285.7. Series 'b', 'y' ions and neutral losses are labeled

(7.53 %), codon 15 (5.01 %), codon 41/42 (3.58 %), and codon 8/9 (1.07 %) [1–3, 9]. These studies have given enough evidence of the prevalence of HbE and HbE/ β Thalassemia in India.

In 2003, Rai et al. [10] showed that variants differing by 1 Da from normal can be detected if present at >10 % abundance. In that report, the normal β -chain mass was determined with a precision of 0.05 Da SD, which resulted in a 0.10 Da mass change being detectable with 95 % confidence. Of course, although ESI-MS of the globin chains can detect ± 1 Da mutations, precise identification requires enzymatic digestion. Hemoglobins C, D-Punjab, E, and O-Arab can all be positively identified directly from the mass spectra of 30 min digests with trypsin, which was reconfirmed by Wild BJ et al. [11]. Rai et al. have mentioned about the usage of ESI-MS in the study of hemoglobin variants, whereas the method adapted here is unique, as it involves characterizing hemoglobin variant, which is obtained through the steps of performing alkaline gel electrophoresis, excising and proteolytically digesting the hemoglobin gel bands to obtain peptides; and finally analyzing the peptides by MALDI-MS. There are now references to multiplexed genotyping of beta globin mutations with MALDI-TOF mass spectrometry as MS method is turning out to be more sensitive, having high throughput, highly automated, flexible, and cost effective alternative to conventional beta thalassemia genotyping methods [12].

Though there are adequate references to the prevalence of HbE with and without concomitant β -Thalassemia, the emphasis in this manuscript is more in favour of how in a diabetic individual harbouring a HbE/ β^+ Thalassemia mutation, selection of an appropriate technique to characterize the mutation and also estimate HbA1c by using an alternate method.

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