CASE REPORT

CHARACTERIZATION OF A HEMOGLOBIN VARIANT : HbQ-INDIA / IVS 1-1 [G >T] - β -THALASSEMIA

V S Bhat, K K Dewan*, P R Krishnaswamy*, A K Mandal** and P Balaram***

Department of Biochemistry, Manipal Hospital Diagnostic Services, Manipal Hospital, Old Airport road, Bangalore-560017.

*Cauvery Medical Center, 43/2 Sahakarnagar, Bellary Road, NH-7, Bangalore 560 092,

**Molecular Medicine & Clinical Proteomics dept, St. John's Research Institute, Bangalore

***Department of Molecular Biophysics, Indian Institute of Science, Bangalore

ABSTRACT

Hemoglobin Q- India (α) 64 Asp $\rightarrow$ His is an α chain variant which is generally found in heterozygous state and presents normal hematological blood picture. Here we report a rare case of HbQ-India with a thalassemic phenotype that has been analyzed using a combination of mass spectrometry, gene sequencing and PCR analysis. This combined analyses revealed the HbQ variant to be associated with a beta chain mutation, IVS 1-1 [G>T]. Though HbQ has earlier been reported with thalassemic trait using different techniques, this is the first report of a compound α and β chain Hb heterozygous mutant involving HbQ and IVS1-1 being validated using Mass Spectrometry and Reverse dot blot hybridization.

KEY WORDS

Hemoglobin Q, Mass Spectrometry, Intervening sequence, Reverse dot blot hybridization, Amplification Refractory Mutation System.

INTRODUCTION

The molecular characterization of Hemoglobin variants is usually conducted at two levels. The first level involves gel electrophoresis or cation exchange high performance liquid chromatography (HPLC) while the second level of analysis engages Mass Spectrometry (MS) (1,2) and/or DNA sequencing (1). The combination of liquid chromatography and Electrospray ionization Mass Spectrometry (LC-ESI-MS) (3) and DNA sequencing analysis are complementary techniques, with the latter usually being used to confirm deductions based upon mass spectrometric analysis.

Hemoglobin Q is a rare α chain variant first described by Vella et al (4) in association with α -Thalassemia in a Chinese family. The first case of HbQ-India was reported by Sukumaran

(5) in 1972 in a Sindhi family with associated β -Thalassemia and later by Desai (6). The present study describes the HPLC and molecular findings of HbQ hemoglobinopathy with a concomitant β -Thalassemia case from a 22year old lady who hailed from the state of Uttar Pradesh in North India was referred for comprehensive hematologic investigation, as part of prenatal check up to Manipal Hospital, Bangalore. The work reported here describes the molecular characterization of the variations on the Hb α and β chains.

MATERIALS AND METHODS

Blood sample collected in EDTA tube was analyzed for a routine blood count using Sysmex XT 1800i Hematology analyzer. For Hemoglobin variant analysis a Biorad D10 HPLC instrument was used as per standard procedures. From the patient blood sample, plasma and buffy coat was aspirated away following centrifugation and the packed cells then washed with 0.9% NaCl. The washed cells were lysed with ice cold distilled water and the hemolysate centrifuged at 12880 xg for 10 minutes to remove the erythrocyte membranes. The clear supernatant was diluted 100 times with distilled water

Address for Correspondence :

Mr. Vijay S. Bhat

Chief Biochemist

Manipal Hospital, Old Airport road, Bangalore-560017

E-mail : vijay.bhat@manipalhealth.com

prior to Mass Spectrometric analysis.

Enzymatic digestion of hemoglobin was performed using Trypsin (TPCK treated). The digested sample was fractionated through a C18 reverse phase column. Fractions were collected and concentrated in a Speed Vac system and spotted onto a MALDI plate for MS analysis. MALDI Mass spectra were obtained on trypsin digested sample using an Ultraflex MALDI-TOF/TOF Mass spectrometer (Bruker Daltonics), using 200-ns time delay and a 25 KV accelerating voltage in positive ion mode. The system utilizes a 50-Hz pulsed nitrogen LASER, emitting at 337 nm. α -cyano-4-hydroxy cinnamic acid was used as a matrix.

Genomic DNA was extracted from the blood sample using the DNEasy kit from Qiagen. Based on MS data, HbQ-India mutation specific primers were used to amplify DNA using Amplification Refractory Mutation System (ARMS) PCR.

The ARMS primer sequence used was 5' – CACGTGCGCCACGGCGTTTGGTCAGCGCGAG- 3' along with a common primer 5' – CTGGTCCCCACAGACTCAGA – 3'. The common reverse primer used was 5' AGGCCCAAGGGGCAAGAAGCAT 3' which produced a mutation specific PCR product of 370bp with the ARMS primer and a control band of 766bp with the common primer. PCR reactions were conducted on a PTC-100 Peltier Thermal cycler (M.J.Research) using 50 μ l reactions including 5 μ l of DNA template. The reaction conditions were: Initial precycling hold at 94°C for 2min, Thermocycling : 94°C denaturation for 45 secs, annealing at 54°C for 45secs, 72°C for 45secs (30cycles), and final extension at 72°C for 2 mins. Sequencing of PCR product were conducted using ABI Prism Big Dye Terminator technology from Applied Biosystems, USA to confirm the mutation.

A screen for beta chain mutation(s) was conducted using the β -globin StripAssay SEA (ViennaLab 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 colour substrates. The conditions for the PCR reaction and protocol for the RBDH assays were conducted following instructions from the kit.

RESULTS

The examination of blood in a pre-natal workup from a patient from North India presented an anomalous blood picture. Based

on the hematological data, the patient had Hb levels of 12.7gm% and MCV of 61.8 fl. Peripheral smears showed a mildly hypochromic and microcytic blood picture. Biorad D10 HPLC profiles (reverse phase HPLC) (Fig1) showed an intense peak at 4.46 min assigned as unknown and HbA₂ levels of 5.1% at 3.21 min.

To investigate the nature of the large unknown peak detected by HPLC, mass spectral analysis was conducted. Primary mass assignments of deconvoluted LC/ESI-MS spectral data (Fig 2, upper right) revealed the presence of a peptide with a

Peak table - ID: 4942

Peak	R.time	Height	Area	Area %
Unknown	0.14	5663	11215	0.5
Alb	0.27	7133	51469	2.1
F	0.44	9261	47316	1.6
LA1c/CHb-1	0.62	3107	21840	0.9
Alc	0.76	7855	72795	4.3
P3	1.45	9255	89694	3.6
A0	1.73	378272	1876193	75.5
A2	3.21	6185	110618	5.1
Unknown	4.46	71648	204221	8.2
Total Area:				2485361

Concentration:	
% F	1.6
% Alc	4.3
% A2	5.1

Fig 1: shows the Biorad D10 HPLC profile of a patient possessing a variant chain. The intense peak at 4.46 min is assigned as an unknown and shows an HbA₂ level of 5.1% at 3.21 min

molecular weight of 15147Da, which is +22 Da greater than the normal alpha chain (15125 Da). One of the putative alterations, among others, that could generate such a mass difference of + 22 Da could be an Aspartate (115 Da) to Histidine (137 Da) alteration. Such an alteration has been earlier encountered as an HbQ-India alpha chain variant from this laboratory (7).

Fig 2: *Upper left* :Total Ion Chromatogram [TIC]of alpha and beta chains of hemoglobin depicting a putative Hb- α chain mutant peak at 15147 Da along with the normal Hb- α peptide (15125 Da). *Upper right* : deconvoluted mass spectra of the normal and mutant alpha chains along with the beta chain. Mass differences between the normal and mutant alpha chains are 22 Da implicating a single Asp to His mutation. *Lower right* :3 putative sites on the alpha chain where Asp to His alterations can occur

Identification of Hemoglobin Q: To further characterize the suspected HbQ variant, hemoglobin preparations from the patient along with a normal control was subject to tryptic digestion followed by MALDI-MS. The HPLC profile of tryptic digest is shown in (Fig 3a), along with the MS spectra of the tryptic peptide finger print of HbQ is shown in (Fig 3b). Comparisons of the MALDI-MS spectra of tryptic digests for the normal Hb sample and putative HbQ, reveal a tryptic fragment with an m/z value of 2997.3 (Fig 4). In the case of the variant sample, an additional peak is detected at m/z 3019.3 which corresponds to the 22Da increase in mass noted above.

The tryptic fragment at positions 62 to 90 would have a normal mass of of 2997.3 while a single change from D to H in this fragment would result in a mass of 3019.3 Da (Fig 5). However, since the concerned fragment includes four Aspartate residues the question of which of these would have undergone the mutation required further investigation. Earlier reports have

Fig 3a: (Upper panel) HPLC profile of tryptic digest of variant Hb and the (lower panel) MALDI MS spectra related to peptides having mass of 3015.3 Da and 2997.3 Da, displaying a difference of 22 Da

Fig 3b: MALDI-MS spectra of tryptic peptide finger print of HbQ. The peptide fragment at 2997.3 Da and 3019.3 Da differing by 22Da indicates the specific fragment harbouring the putative D to H mutant. Three possible sites for this change have been shown in the primary sequence (inset)

Fig 4: MALDI-MS of Tryptic Peptide Finger Print of Human Hemoglobin

Fig 5: Primary structure for the Hb alpha chain depicting the concerned fragment in red (position 62 – 90). The four possible sites where a single Asp(D) to His(H) change could have occurred is depicted by arrows (lower section), all of which could potentially generate the mass of 3019.3 Da. Three of these (shown in arrows) have earlier been reported as Hb Q -India, Hb Q -Thailand and Hb Q -Iran

detected D>H mutations at position 64(HbQ-India)(7), 74 (HbQ-Thailand)(8) and 75 (HbQ-Iran)(9).

DNA sequence level characterization of HbQ: Given that the mass spectrometric analysis of tryptic fragments conducted above would not resolve the specific position of the Asp>His alteration from the fragment, a DNA based analysis of this

region was conducted using two approaches. First, DNA preparations of peripheral blood isolated from the patient was subjected to molecular analysis by an amplification refractory mutation system PCR (ARMS-PCR)(10). In this case, an HbQ-India specific primer was used to see if the site of the alteration was at this location. The predicted 370 bp fragment resulting from the PCR reaction confirmed that the mutation indeed

Fig 6: a) Ethidium bromide stained 2% Agarose gels showing the results for HbQ specific ARMS-PCR. Lane 1: Normal control sample Lane2: Patient sample, Lane 3: Molecular size markers. Presence of the characteristic 370 base pair fragment (bold arrow) for the patient sample shows the DNA sample to possess an HbQ mutation. This band is missing in the normal control sample DNA The upper 766 base pair band is a reaction control and is expected to be present in both samples. b) Sequence electrophoretogram of HbQ location. Arrows indicate the site of HbQ mutation. Overlapping peaks (G and C) of nearly equal intensity at site of mutation indicates the heterozygosity at the site

Lane 1   2   3

Fig 7: Scanned results of Vienna lab's assay strip used to screen for β -thal mutations. Lane 1: Assay strip of a normal sample with upper panel showing no bands, for the 22 mutations and lower panel has the bands of all the wild types. **Lane 2:** Assay strip showing a single band positive for the mutation IVS 1-1 [G > T] in the upper panel screening for 22 mutations. The lower wild type panel on the strip shows the presence of a copy of all the wild type alleles related to the 22 screened mutations indicating that the detected IVS 1-1 [G > T] mutation also retains a wild type allele and is therefore heterozygous. **Lane 3:** Key to the strip assay

was HbQ-India (Fig 6a). The ARMS-PCR was then further cross verified by sequencing the concerned region. Sequencing electropherograms clearly demonstrated the specific location of the mutation as HbQ-India and further showed that the codon GAC encoding Aspartic acid (D) was mutated to codon CAC encoding for Histidine (H) (Fig 6b). The overlapping peaks of about equal intensity for both G and C at the point of the mutation reflect the heterozygosity of the mutation with both the normal and mutant allele being present in the genomic DNA of the patient.

Since the HbA₂ level and blood cell indices of the patient indicated the concomitant presence of beta thalassemia trait, a screening kit from Vienna Labs was used for identifying any beta chain mutation. The kit uses β -globin gene specific

primers in a multiplex PCR reaction and the amplified product is subsequently analysed to investigate if any of the 22 known common mutations that have been reported in the South East Asian region has been amplified in a reverse dot blot hybridization strip assay.

As seen in (Fig7) the amplification products selectively hybridized to the IVS 1-1 band (arrow) among the other 22 mutations in its panel confirming that the patient harbours a β -thalassemia mutation, more specifically IVS 1-1 [G > T]. 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.

DISCUSSION

HbQ-India variant is an alpha chain variant due to structural mutation. The other molecular variants identified and documented have been HbQ-Thailand and HbQ-Iran. Since HbQ-India is a silent variant, in this case its association with beta thalassemia trait IVS 1-1[G>T] also has not produced any clinical abnormality. Based on earlier references HbQ-India with presence of beta thalassemia trait though have been reported using electrophoresis, HPLC (11) and ARMS –PCR techniques, our emphasis has been on using Mass Spectrometry with RDBH and DNA sequencing at arriving at a definitive diagnosis.

Electro spray ionization Mass Spectrometry provides a rapid method of identifying structural hemoglobin variants, and its characterization. Specific sequence level mutations are also done using MS-MS fragmentation approaches, though in this case we followed a sequencing and molecular biology approach. In the process of identifying the site of mutation, it is evident that MS and DNA sequencing are complementary techniques. The Vienna Lab β -globin strip can be used for in vitro amplification of β -globin gene sequences, and the subsequent detection of nearly twenty two mutations based on reverse hybridization technique. In case of this patient due to presence of an alpha chain variant along with β thalassemia trait an imbalance in the α^A , α^Q and β^A chain ratio occurred and so favored the $\alpha^Z\alpha^Q\beta^Z$ formation.

ACKNOWLEDGEMENTS

We sincerely acknowledge the staff of Dept. of Molecular Biophysics, Indian Institute of Science, Bangalore in assisting us in carrying out Mass Spectral analysis at their laboratory and Dept. of Molecular Pathology, Manipal Hospital for doing the PCR work.

REFERENCES

1. Shackleton CHL, FalickAM, Green BN, Witkowska HE. Electrospray mass spectrometry in the clinical diagnosis of variant hemoglobins. *J Chromatography B* 1991; 562: 175-90.
2. Wada Y. Advanced analytical methods for hemoglobin variants. *J Chromatography B* 2002; 781: 291-301.
3. Wild BJ, Green BN, Cooper EK, Lalloz RA. Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry. *Blood Cells Mol Dis* 2001; 27: 691-704.
4. Vella F, Wells RHC, Ager JAM, Lehmann H. A hemoglobinopathy involving hemoglobin H and a new(Q) hemoglobin. *Br Med J* 1958; 1: 752-5.
5. Sukumaran PK, Merchant SM, Desai MP. Hemoglobin Q India (alpha 64 (E13) aspartic acid to histidine) associated with beta-thalassemia observed in three Sindhi families . *J Med Genet* 1972; 9: 436-42.
6. Desai DV, Dhanani H, Kapoor AK, Yeluri SV. HbQ-India in a Sindhi family : an unknown hemoglobin variant. *Lab Hematol* 2004; 10: 212-4.
7. Mandal AK, Bisht S, Balaram P, Bhat VS, Krishnaswamy PR. Electrospray mass spectrometric characterization of hemoglobin Q (HbQ-India) and a double mutant hemoglobin S/D in clinical samples. *Clin Biochem* 2008; 41: 75-81.
8. Ching - Nan Ou, Rognerud CL. Rapid Analysis of Hemoglobin Variants by Cation -Exchange HPLC. *Clin Chem* 1993; 39/5 : 820-24.
9. Lorkin PA, Charlesworth D, Lehmann H, Rahbar S, Tuchinda S, Lie-Injo LE. Two hemoglobin Q, alpha-74(EF3) and alpha-75 (EF4) aspartic acid to histidine. *Br J Hematol* 1970; 19: 117- 25.
10. Newton CR, GrahamA, Heplinstall LE. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acid Res* 1989; 17: 2305 -16.
11. Desai D, Parmar C, Dhanani H, Patel R, Master D. A Rare case of Co-Existent HbQ India-Beta Thalassemia Trait. *Int J Hematol* 2007; 3: 1.