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Original Article

Identification of compound heterozygosity for a rare beta-globin gene mutation, codon 15 (-T) with a common mutation IVS1-5 (G>C) by direct sequencing in a Bangladeshi patient.

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ABSTRACT: Most of the beta-thalassemia cases in Bangladesh are caused by 5-7 common mutations in the beta-globin gene. The present study reports a rare mutation in compound heterozygosity with a common mutation in beta-globin gene of a thalassemic patient first time in Bangladesh. A Bangladeshi individual, born to a family of carrier mother and normal father was diagnosed with abnormal hemoglobin electrophoresis result. Sequencing analysis revealed the presence of a rare mutation caused by the deletion of a thymine at c.46 of β-globing gene, HBB: c.46delT [codon 15 (-T)]. A second common mutation HBB: c.92+5 G>C [IVS1-5 (G>C)] was also identified along with the rare mutation. This compound heterozygous condition produced significant reduction in the synthesis of beta-globin chain. This study recommends the necessity of integrating direct sequencing evaluation during prenatal screening program and antenatal diagnosis of thalassemia cases.

KEYWORDS: Thalassemia, hemoglobin, mutation, PCR, sequencing analysis

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INTRODUCTION

Beta thalassemia (β-thalassemia) is one of the most prevalent monogenic disorders affecting more than 150 million people from about 60 countries worldwide.[1] It is caused by mutations in β-globin gene of hemoglobin (Hb) located on chromosome 11. It contains three exons, two intervening sequences (IVS1 and IVS2) and the 5′ and 3′ untranslated region (Figure 1). β-globin gene defect leads to a reduction (β^+) or absence (β^o) in β-globin chain production, causing ineffective erythropoiesis. The spectrum of β-thalassemia ranges from silent carrier to mild or sever transfusion dependent anemia with variable degree of clinical manifestations and disease prognosis. [2,3]

At molecular level β-thalssemia is extremely heterogeneous. There are more than 200 documented mutations involving β-globin gene.[4] In most cases these mutations involve single base change such as, substitutions, deletions or insertions. However, insertion or deletion of small oligonucleotides and rarely large deletions are also reported.[5] These mutations may occur either within the β-globin gene or in the regulatory regions resulting in reduced or diminished synthesis of β-globin chain in many ways. Some reduce mRNA splicing efficiency resulting in aberrant β-globin chain formation, some result in nonfunctional mRNA, while others lead to abnormal post-transcriptional modification of mRNA.[6]

The mutations in β-globin gene though are diverse in number within each affected population, a few common mutations together with a variable number of rare mutations are reported.[7–10] In Bangladesh, about 4% of the populations are carriers of β-thalassemia and consistently 5 to 7 mutations of beta-thalassemia have been reported so far.[11] Mutations that cover more than 70 – 90% of identified mutations among beta thalassemia patients included IVS1-5 (G>C), IVS1-1 (G>T), HbE [Cd 26 (G>A)], Cd 41/42 (-G>C), Cd 8/9 (+G) and 619 bp deletion at 3′ end of beta-globin gene. Off these, three common [Cd 26 (G>A), IVS1-5(G>C) and Cd 41/42 (-TCTT)] and three rare mutations (HBB: c.-80T>C, HBB: c. 92G>C, HBB: c.-92C>G) have been reported in many studies.[12,13] Prenatal screening program and diagnosis are supposed to be recommended nationwide to identify mutations responsible for the disease and carrier detection as the strategic approach for the management of the disease and prevention of emergence of new cases in the Bangladeshi population. However, through this program, accurate and reliable identification of mutations might not be accomplished if advanced molecular methods are not applied. Moreover, some rare mutations might remain undetected that lead to thalassemia. This study describes the direct sequencing based finding of compound heterozygosity for a rare mutation [codon 15 (-T)] with a common mutation IVS1-5 (G>C) in a patient for the first time in Bangladesh.

MATERIALS AND METHODS

Subject: A 14-month old-female Bangladeshi individual was referred to DNA Solution Limited (Molecular Diagnostic Institute), by the specialist physician for the molecular characterization of HBB gene. Informed consent was obtained from the individual and the institutional ethical review committee approved the study protocol. Hemoglobin electrophoresis reports of the family carried out using Helena SAS Electrophoresis System (Helena Biosciences, UK) demonstrated compatible beta thalassemia pattern. The father was diagnosed to be normal and the mother was diagnosed as suggestive of beta thalassemia trait. Hemoglobin electrophoresis reports of the case showed Hb F level 5.5%, reduced HbA level 82.1%, Hb E zone 9.4 % and HbA2 level 3%. The baby was suffering from thalassemia major like complications. The specialist physician suggested identifying mutations in the HBB gene by direct sequencing in order to determine whether the baby is trait or affected.

DNA Extraction: Approximately 3.0 mL peripheral blood samples in duplicate were collected in a VACUETTE® EDTA K_3 (Greiner Bio-One GmbH) tube, from the patient. Genomic DNA was extracted using QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Genmany). Extracted DNA was quantified by Quantus® fluorometer (Promega Corporation, USA). The extracted DNA was used as a template for PCR reaction and stored at – 20°C until further use.

Amplification and Sequencing Analysis: For the detection of mutation two segments of the β-globin gene were amplified separately that cover almost full of HBB (1425 bp out of 1605 bp) by polymerase chain reaction using specific primers designed in-house. Segment 'A' covered 680 bp of the gene containing the 5′UTR, exon 1, IVS-I, exon 2 and and part of IVS-II. On the other hand, segment 'B' covered 745 bp of the gene and included part of IVS-II, exon 3 and part of 3′UTR (Figure 1). Both the amplified segments cover about 99% of the mutations reported for β-globin gene. The thermocycling conditions consisted of one denaturing cycle at 95°C for 10 minutes followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 58.5°C for 1 minute, and extension at 72°C for 2 minutes. Final extension was at 72°C for 10 minutes. The amplification products were then electrophoresed in a 2% agarose gel to assess PCR efficacy and to detect the presence of the 619-bp deletion.

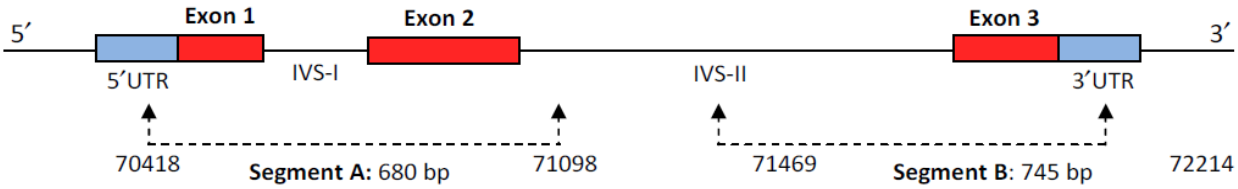

Figure 1: Schematic diagram of beta-globin gene showing relative positions of the two segments amplified by PCR. PCR = polymerase chain reaction

Excess primers and unincorporated dNTPs in each PCR products were cleaned by EXOSAP IT (USB Corporation, USA). After purification, 5 µL of the amplicons and 1 µmol/L of either forward or reverse primers were used for sequencing. The cycle sequencing reaction was carried out using BigDye Terminator cycle sequencing kit v3.1 (Life Technologies, USA) following the protocol provided by the manufacturer. The sequencing amplicons were purified with 3M sodium acetate followed by ethanol precipitation and separated by capillary electrophoresis on 3500 Dx Genetic Analyzer (Life Technologies, USA).

The resultant sequence was compared with NCBI Ref Seq entry of HBB (NG_000007.3) using SeqScape software (Applied Biosystems, USA). HbVar

(http://globin.bx.psu.edu/cgi-bin/hbvar/query_vars3) was used to identify mutations reported in other population.

RESULTS AND DISCUSSION

Direct sequencing of the beta-globin gene showed that the patient had a rare mutation at codon 15 of the β-globin gene (HBB: c.46delT) [codon 15 (-T)]. This mutation was caused by the deletion of a thymine at codon 15 of beta-globing gene changing the reading frame which usually results in premature termination of translation (Figure 2).

The patient also had a second common mutation of the β-globin gene (HBB: c.92+5 G>C) [IVS1-5 (G>C)]. Mutation at this position reduces splicing efficiency which results in aberrant beta-globin chain formation (Figure 3).

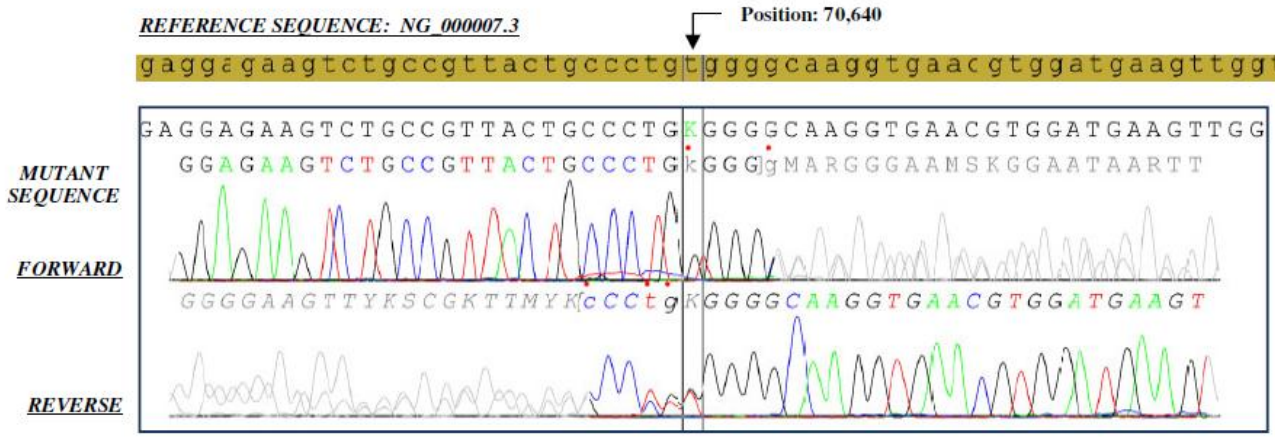

Figure 2: Sequencing results of the affected individual showing deletion of T at codon 15 [codon 15 (-T)] in exon 1 of the HBB gene. The deletion was confirmed by sequencing with both forward primer and reverse primer.

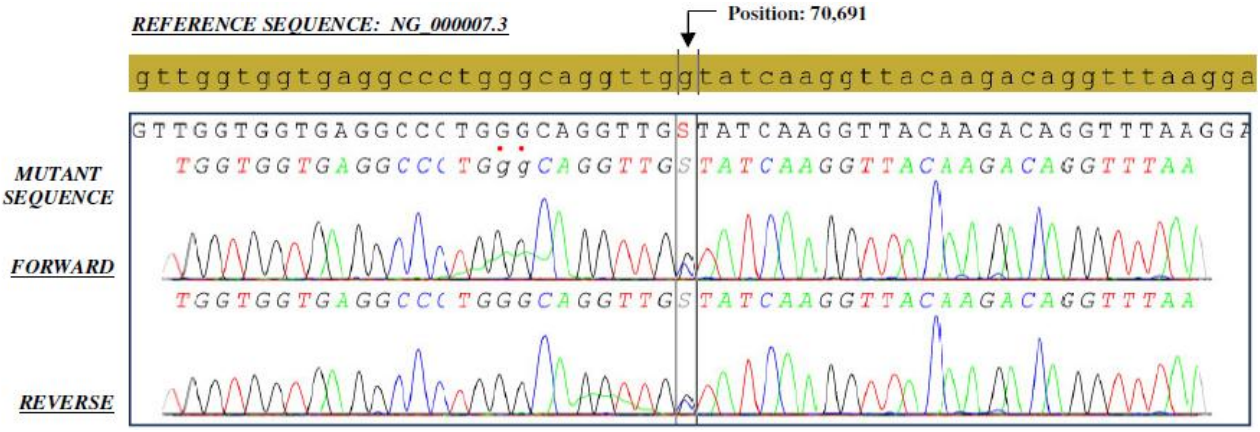

Figure 3: Sequencing results of the same affected individual showing IVS 1-5 (G >C) in intron 1 of the HBB gene. The deletion was confirmed by sequencing with both forward primer and reverse primer.

This compound heterozygote condition explained the generation of significant reduction in the synthesis of beta-globin chain as exhibited the patient and complications compatible with β thalassemia major.

The enormous heterogeneity in beta-globin gene mutation and finding of increasingly new types of mutation sometime pose a challenge to determine an improved protocol for carrier screening and prevention of affected child birth. On a global scale approximately 200 mutations account for 90% of the mutation of beta-globin gene. In Southeast Asia about 5 to 8 mutations are commonly found and about 20 mutations account for 95% of the mutations.[7,9,10,14,15] Clinical laboratories use a battery of mutations for routing diagnosis or screening purpose based on the most common mutations specific for a particular population. However, this approach sometime misses rare mutations if present. Therefore, an undetected rare mutation if co-inherited with a common mutation may be wrongly diagnosed as beta-thalassemia trait. As a consequence, the result may mislead the physician and the family members in their clinical decision making. In this study we report such a rare mutation HBB:c.46delT [codon 15 (-T)] which was co-inherited with a common mutation, HBB: c.92+5 G>C [IVS1-5 (G>C)] found in a Bangladeshi patient.

HBB c.46delT is located in codon 15 of exon 1 of Hb gene. This deletion caused a frame-shift in premature termination of translation at codon 18(TGA) resulting in beta- thalassemia major. A review of literature suggests

that this mutation is very rare and previously reported to be present only in a Malay family.[16] IVS 1-5 (G->C) on the other hand is one of the most common mutations found in Bangladeshi population. Mutation in this position greatly reduces the efficiency of the splicing of the normal 5′ plicing site resulting in aberrant beta-globin chain formation with a β^{0}/β^{+} phenotype.[17] The mutation is also prevalent in Indian Subcontinent, Mediterranean countries, the Middle East and Southeast Asia with highest frequencies in India, Pakistan, Indonesia, Malaysia, Iran and United Arab Emirates. Though adequate references are available about the prevalence of compound heterozygous conditions where IVS 1-5 (G->C) is co-inherited with other mutations, no information so far is available about the co-inheritance of codon 46delT with any common mutation in Bangladesh.

In conclusion, the study clearly demonstrates that individuals harboring rare mutations can be missed during the screening program of beta-thalassemia mutation if analysis is carried out using most common mutation specific to a particular population. This study therefore emphasizes the need for identifying mutation in the beta-globin gene by direct sequencing of beta-globin gene during prenatal screening program and antenatal check up for beta-thalassemia in Bangladesh.

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DECLARATION OF INTEREST
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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