A novel deletion of $\beta$ globin promoter causing high HbA2 in Indian population

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A novel deletion of β-globin promoter causing high HbA2 in Indian population

Running Title: β-thalassaemia causing high HbA2

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β-thalassaemia is the most common inherited disorder characterized by reduction or absence of β-globin chain synthesis. Over 200 mutations have been so far identified which result in β-thalassaemia. Most of the mutations are single nucleotide substitutions or deletions or insertions in b-globin gene or its flanking sequences. Heterozygous β-thalassaemia usually presents with mild microcytic and hypochromic anaemia with slight increase in haemoglobin A2 (HbA2) levels (3.5-5.5%).\textsuperscript{1,2} The rare large deletions in the b-globin cluster cause abnormal haemoglobin patterns in heterozygous states; deletions involving d- and b-globin genes increase foetal haemoglobin (HbF) levels and those involving promoter regions of b-globin gene, without the deletion of d-globin gene, cause increase in HbA2 levels.\textsuperscript{2} Identification and characterization of these deletions are important for understanding the molecular mechanisms involved in regulation of globin genes in adults. In this study we characterized a novel 4056bp deletion of β-globin gene and its promoter causing increased HbA2 in an Indian family. We characterized the deletion using a combination of gene dosage analysis, multiplex ligation-dependent probe amplification (MLPA) and PCR amplification across the breakpoints.

The patient was a four year old boy born of a consanguineous marriage from Gujarat, India presented with severe anemia requiring frequent blood transfusions from the age of 2 years. His pre-transfusion haemoglobin level was 5.5 g/dL. He had hepatomegaly but the spleen was not palpable. Complete blood count showed that both the parents had hypochromic microcytic anaemia (64.8 and 61.6fL) and haemoglobin analysis using cation exchange chromatography (VARIANT, CA, Bio-Rad) revealed that they had increased HbA2 (7.7 and 7.1%) with normal HbF (1.1 and 1.4%) (Table S1). Reverse dot blot analysis showed that the common point mutations were absent in this family. To detect possible deletions in the β-globin cluster a multiplex PCR using fluorescently labeled primers was performed for g-, d- and β-globin genes with albumin gene as the control. For accurate determination of the copy numbers of the genes in heterozygotes, 200ng of DNA was used with 0.4mM primers and the amplification was carried out for 20 cycles. Amplified products were separated by a capillary electrophoresis in an ABI-3130 Genetic Analyzer (Applied Biosystems) and results were analyzed by GeneMapper v4.0 (Applied Biosystems). The peak heights obtained for amplified
products of globin genes were divided by those obtained from albumin gene from each sample and subsequently, the ratios obtained for patient and parents were divided by those obtained from normal individuals. This gene dosage analysis showed absence of amplification from β-globin gene in the patient in the homozygous state and the parents were heterozygous for a deletion involving β-globin gene (Figure 1A). For further identification of the extent of deletion in the β-globin cluster we performed Multiplex Ligation-dependent Probe Amplification (MLPA) for β-globin cluster in this family using the SALSA MLPA Kit P102 HBB, version 09 (MRC Holland, Amsterdam, the Netherlands) which contain 29 probes for 73 kb region of the β-globin gene cluster targeting the locus control region, coding genes in the cluster and the intergenic sequences. The MLPA products were separated by capillary electrophoresis in Genetic Analyzer and analyzed using GeneMapper v4.0. MLPA analysis showed loss of amplification of the probes targeted to d-b globin intergenic region and promoter, exons 1 and 2 and introns 1 and 2 confirming the presence of a large deletion of a region from HBD-HBB intergenic region to IVS-2 of HBB (Figure 1B). To characterize the breakpoints we performed PCR with three forward primers that bind to different regions downstream of d globin gene with a constant reverse primer 5’ AGCAGAATGGTAGCTGGATTG 3’ that binds to sequences in β IVS-2. Using a forward primer 5’ CAGGCCTACTTGAGGGTTGA 3’ that binds at ~2kb downstream of HBD we obtained a ~1.1 kb amplification product from the patient and the parents while the expected fragment size from a normal individual was 5.1kb (Figure 2A). DNA sequencing of the amplified product showed that the deletion encompasses 4056 bp region that extends from 2.7 kb downstream of d-globin gene to IVS-2 of β-globin gene (Figure 2B).

MLPA analysis identified an additional deletion present in heterozygous state in the g-globin region in the father (Figure 1B) and this is probably due to Gg-Ag fusion gene, which is present frequently in population. However, this coinheritance of g-globin gene deletion along with β-globin gene in single allele does not alter the phenotype in heterozygous β-thalassaemia. For further investigation of more severe anaemia in the mother (Hb=9.1 g/dL and MCV=61.5 fL), we performed multiplex PCR analysis for α-
globin genes and found that she was heterozygous for ααα3.7 (ααα3.7/aa) (Figure S1) and it is well known that this genotype is consistent with severe heterozygous β-thalassaemia.

So far 11 deletions, varying from 260bp-67kb in length, of β-globin gene promoter and its flanking sequence have been reported. In Indian population a deletion of 10.3 kb that extends from 3011 bp 5' to the mRNA cap site to an L1 repeat element present downstream of the β-globin gene has been found to cause elevated levels of HbA2 (7.1-7.8%) in heterozygote. The novel mutation that we identified is similar to 4237 bp deletion reported in a Czechoslovakian family which caused elevated levels of HbA2 (8.1%-9.0%) in heterozygous state and DNA sequence analysis showed that the 5' and 3' break points are a few bases apart (Figure 2C). We used robust, reliable and easier assays for identifying the deletion in b-globin cluster which are useful for proper genetic counseling and diagnosis of β-thalassaemia and helps in genotype-phenotype prediction. The exact mechanism for unusual levels of HbA2 in these deletions is not known and it may be hypothesized that it is due to increased availability of transcription factors at the δ globin promoter when b-globin gene promoter is deleted and this promoter competition is not evident between b and g globin genes.
References


**Figure Legends**

**Figure 1:** (A) Genomic quantitative-PCR for gene dosage analysis to calculate the copy numbers of γ-, δ- and β-globin genes. The peak heights of amplified products of the globin and genes and the control albumin gene are shown. (B) MLPA analysis of the β-globin cluster in the family. Arrows indicate the genomic regions that are deleted. Father is heterozygous for an additional deletion of Gg-globin.

**Figure 2:** (A) Location of the primers used for amplification across the break points and agarose electrophoresis of 1127bp amplified products from the patient and the parents. From normal control the expected amplified product 5.1kb was not obtained by the conditions used for amplification. (B) DNA sequencing of the PCR product showing the breakpoints. (C) Comparison of Czechoslovakian and Indian deletions.
Online Supplementary Figure S1. Genomic quantitative-PCR for gene dosage analysis to calculate the copy numbers of α2- and α1- globin genes. (A) Electropherogram showing the peaks obtained from the amplified genes in normal control, patient and parents. Mother DNA shows increase in copy number of HBA2 globin gene, patient and father DNA showing normal copy number of HBA2 gene. (B) Normalized peak heights showing the exact copy numbers of the globin genes in the family.
Table 1: Hematological parameters in the patient and the parents
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<th>Proband</th>
<th>Father</th>
<th>Mother</th>
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<td><strong>Age/Sex</strong></td>
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<td>36 / M</td>
<td>28 / F</td>
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<td><strong>Hb (g/dL)</strong></td>
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