A novel deletion of β-globin promoter causing high HbA_2 in an Indian population

β-thalassemia is the most common inherited disorder characterized by a reduction or absence of β-globin chain synthesis. So far, over 200 mutations have been identified that result in β-thalassemia. Most of the mutations are single nucleotide substitutions or deletions, or insertions in the β-globin gene or its flanking sequences. Heterozygous β-thalassemia usually presents with mild microcytic and hypochromic anemia with a slight increase in hemoglobin A_2 (HbA_2) levels (3.5-5.5%).[1,2] The rare large deletions in the β-globin cluster cause abnormal hemoglobin patterns in heterozygous states; deletions involving δ- and β-globin genes raise fetal hemoglobin (HbF) levels and those involving promoter regions of β-globin gene, without the deletion of δ-globin gene, raise HbA_2 levels.[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 HbA_2 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 4-year old boy born of a consan-

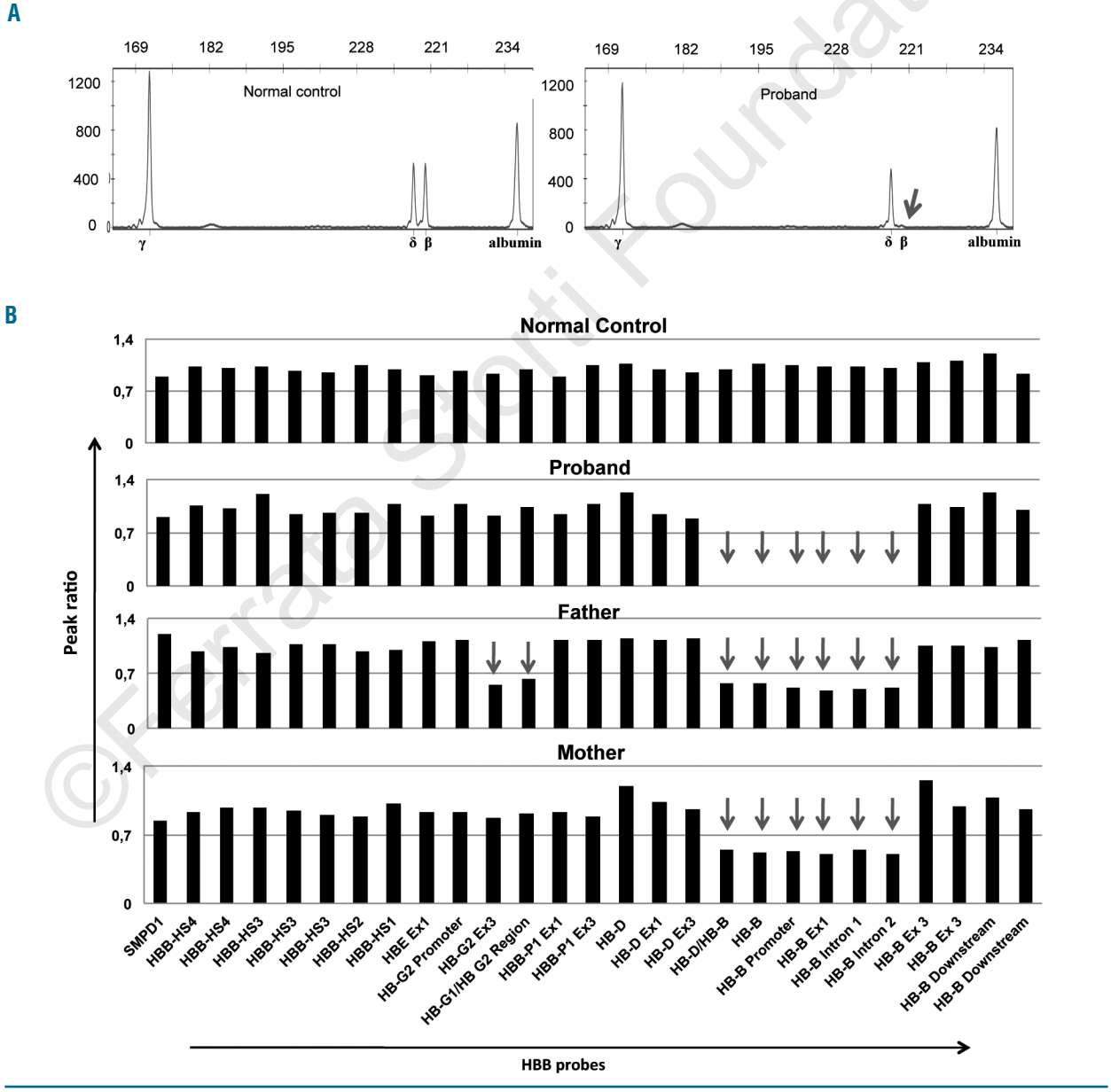

Figure 1. (A) Genomic quantitative-PCR for gene dosage analysis to calculate the copy numbers of γ-, δ- and β-globin genes. The peak heights of amplified products of the globin genes and the control albumin gene are shown. (B) MLPA analysis of the β-globin cluster in the family. Arrows indicate the genomic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

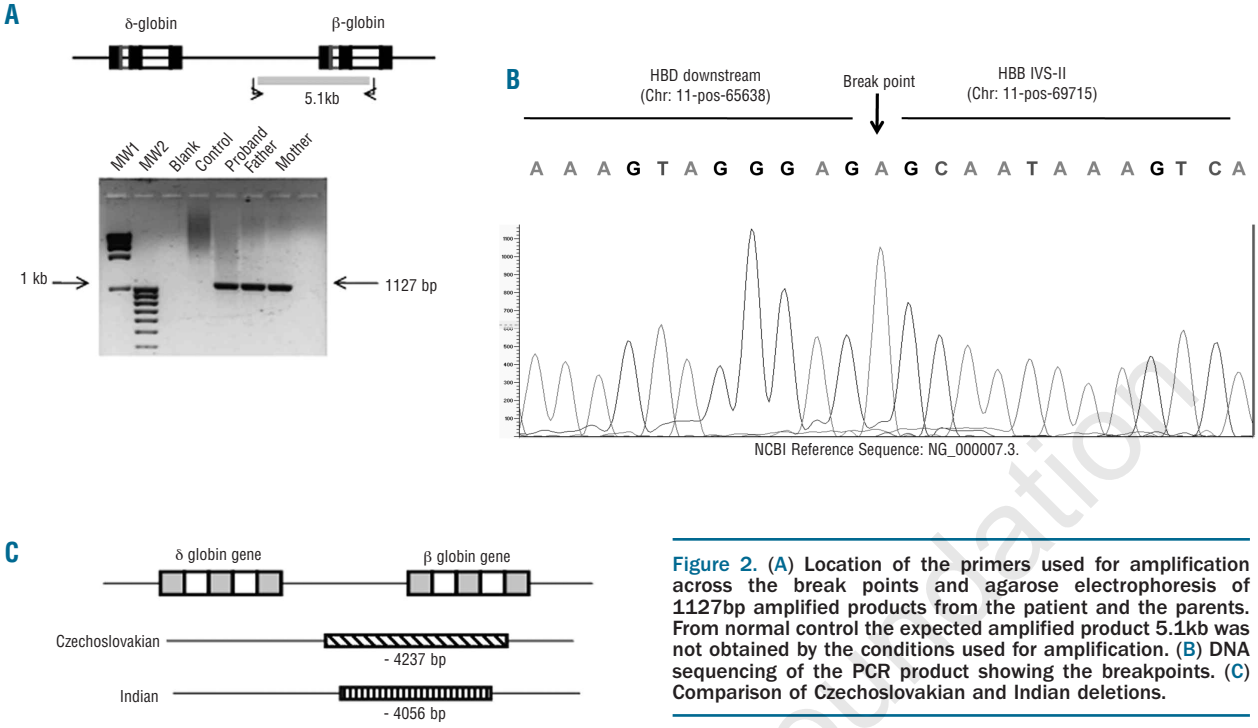

Figure 2. (A) Location of the primers used for amplification across the break points and agarose electrophoresis of 1127bp amplified products from the patient and the parents. From normal control the expected amplified product 5.1kb was not obtained by the conditions used for amplification. (B) DNA sequencing of the PCR product showing the breakpoints. (C) Comparison of Czechoslovakian and Indian deletions.

guineous marriage from Gujarat, India, who presented with severe anemia requiring frequent blood transfusions from the age of two years. His pre-transfusion hemoglobin 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 anemia (64.8 and 61.6 fL) and hemoglobin analysis using cation exchange chromatography (Bio-Rad, VARIANT, CA, USA) revealed that they had increased HbA_2 (7.7 and 7.1%) with normal HbF (1.1 and 1.4%). Reverse dot blot analysis showed that in this family the common point mutations were absent. To detect possible deletions in the β-globin cluster a multiplex PCR using fluorescently labeled primers was performed for γ, δ- and β-globin genes with the albumin gene as the control. For accurate determination of the copy numbers of the genes in heterozygotes, 200 ng of DNA was used with 0.4 μM 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 software version 4.0 (Applied Biosystems). The peak heights obtained for amplified products of the globin genes were divided by those obtained from the albumin gene from each sample, and the ratios obtained for patient and parents were then divided by those obtained from normal individuals. This gene dosage analysis showed absence of amplification from the β-globin gene in the patient in the homozygous state and the parents were heterozygous for a deletion involving the β-globin gene (Figure 1A and *Online Supplementary Figure S1*). For further identification of the extent of deletion in the β-globin cluster, we performed Multiplex Ligation-dependent Probe Amplification (MLPA) for the β-globin cluster in this family using the SALSA MLPA Kit P102 HBB software version 09 (MRC Holland, Amsterdam, The Netherlands) which contain 29 probes for the 73 kb region of the β-globin gene cluster targeting the locus control region, coding genes in the cluster and the intergenic sequences.[3] The MLPA products were separated by capillary electrophoresis in a Genetic Analyzer and analyzed using GeneMapper software version 4.0. MLPA analysis showed loss of amplification of the probes targeted to the δ-β 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 δ-β intergenic region to IVS-2 of β globin gene (Figure 1B). To characterize the breakpoints, we performed PCR with three forward primers that bind to different regions downstream of the δ-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 an ~1.1 kb amplification product from the patient and the parents while the expected fragment size from a normal individual was 5.1 kb (Figure 2A). DNA sequencing of the amplified product showed that the deletion encompasses 4056 bp region that extends from 2.7 kb downstream of the β-globin gene to IVS-2 of the β-globin gene (Figure 2B).

MLPA analysis identified an additional deletion present in a heterozygous state in the γ-globin region in the father (Figure 1B) and this is probably due to the Gγ-Aγ fusion gene which is frequently present in this population.[4] However, this co-inheritance of a β-globin gene deletion along with the β-globin gene in a single allele does not alter the phenotype in heterozygous β-thalassemia. For a further investigation of the more severe anemia in the mother (Hb 9.1 g/dL, MCV 61.5 fL), we performed multiplex PCR analysis for α-globin genes and found that she was heterozygous for $\alpha\alpha\alpha^{3.7}$ ($\alpha\alpha\alpha^{3.7}/\alpha\alpha$) (*Online Supplementary Figure S1*); it is well known that this genotype is consistent with severe heterozygous β-thalassemia.

So far, 11 deletions, varying from 260bp-67kb in length, of the β-globin gene promoter and its flanking sequence have been reported.[5] In an 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 a heterozygote state.[6] The novel mutation that we identified is similar to the 4237 bp deletion reported in a Czechoslovakian family which caused elevated levels of HbA_2 (8.1%-9.0%)[7] in a heterozygous state and DNA sequence analysis showed that the 5' and 3' breakpoints are a few bases apart (Figure 2C). We used robust, reliable and easier assays to identify the deletion in the β-globin cluster that are useful for appropriate genetic counseling and diagnosis of β-thalassemia, and help predict genotype-phenotype prediction. The exact mechanism for unusual levels of HbA_2 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 the β-globin gene promoter is deleted, and this promoter competition is not evident between the β- and γ-globin genes.

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Molecular characterization of identical, novel *MLL-EPS15* translocation and individual genomic copy number alterations in monozygotic infant twins with acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) occurring in the first year of life is rare, accounting for 2-5% of pediatric ALL cases. Infant ALL is distinguished by unique clinical and biological characteristics with an aggressive course following a short latency period. The mixed lineage leukemia (*MLL*) gene, located on chromosome 11q23, is involved in 80% of cases. Over 70 different *MLL*-fusion partner genes have been molecularly characterized,[1] with t(4;11), t(9;11) and t(11;19) occurring most frequently in infant ALL.

The outcome of *MLL*-rearranged infant ALL remains poor with up to 50% 5-year survival. The uncommon nature of ALL in infancy limits the rate of accrual for clinical trials and evidence for the best therapeutic approach has been conflicting, particularly regarding the benefit of hematopoietic stem cell transplantation. The clinical heterogeneity of ALL in infancy, such as poorer outcome under 90 days of age,[2] underlies the need for stratification on these trials. The age-related difference in outcome may be a reflection of underlying molecular characteristics with differences in gene expression profiles according to age.[3]

Risk stratification according to *MLL* translocation partners has not been undertaken due to small patient numbers and as the same biological process was thought to occur irrespective of the partner gene, for *MLL*-mediated leukemogenesis. However, recent identification of distinct molecular differences among the *MLL* subtypes, including variations in epigenetic[4] and gene expression profiles,[3] has given rise to the notion that the many fusion partners of *MLL* should be considered as distinct entities.[1] The complex interplay between *MLL* translocation and these additional molecular differences is not fully understood but further characterization of cases will contribute to the current understanding of the clinical heterogeneity of the disease.

The t(1;11) (*MLL-EPS15*) translocation is rare with only a small number of clinical cases published and three reports of molecular characterization at the transcriptional level.[5-7] The epidermal growth factor receptor pathway substrate 15 (*EPS15*) gene encodes a protein that is involved in receptor-mediated endocytosis of epidermal growth factor. We present an *MLL-EPS15* rearrangement with novel breakpoints at the transcriptional and DNA level in monozygotic infant twins with ALL and characterization of the molecular changes in their leukemic cells. The methods are described in the *Online Supplementary Appendix*. Both twins were diagnosed at seven weeks of age, with a peripheral blood blast population of 99.96×10^9/L in Twin One and 154.87×10^9/L in Twin Two. $CD19^+$, $CD24^+$, $CD10^-$, B-precursor ALL was confirmed on immunophenotyping. Banded chromosomal analysis revealed a 46,XX,t(1;11)(p32;q23)[13]/46,XX[7] karyotype in Twin One and a 46,XX,t(1;11)(p32;q23)[8]/ibid+X[4]/46,XX[8] karyotype in Twin Two, with fluorescence *in situ* hybridization confirming MLL involvement.[8]

The *MLL*-fusion transcripts were sequenced and novel, identical breakpoints were identified in both twins (Figure 1A). For the *MLL-EPS15* transcript, the breakpoint was located at the end of exon 8 of *MLL* which was fused to exon 10 of *EPS15*. For the *EPS15-MLL* transcript,