



## Correspondence

### Rapid molecular identification of a rare $\beta$ -globin gene deletion & its clinical implication

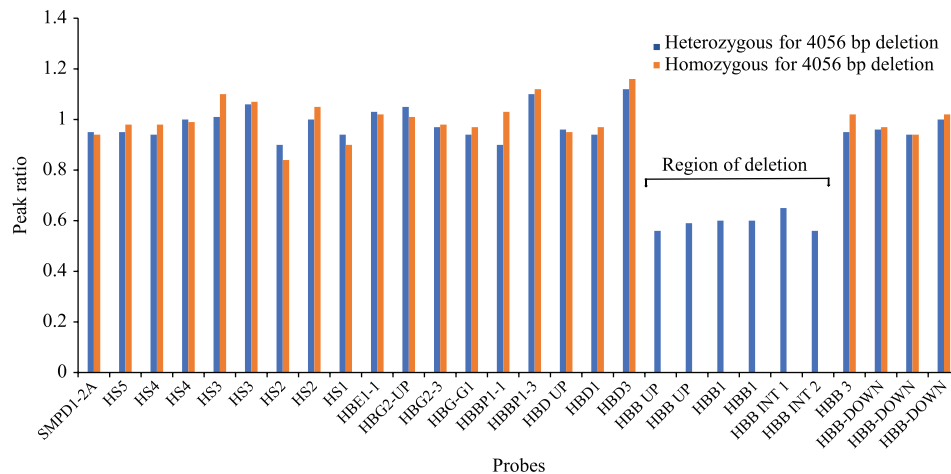
Sir,

$\beta$ -thalassaemia is one of the most common inherited monogenic disorders prevalent in India, with an overall frequency of 3–4 per cent and an estimate of 10,000–12,000 children born every year as  $\beta$ -thalassaemia major<sup>1</sup>. This emphasizes on the precise identification of  $\beta$ -thalassaemia carriers and characterization of the  $\beta$ -globin gene mutation as the same will be required for prenatal diagnosis. The standard test for detecting a typical  $\beta$ -thalassaemia carrier is the elevated HbA<sub>2</sub> level which ranges from 4.0 to 6.5 per cent<sup>2</sup>. However, some atypical cases show an elevated HbA<sub>2</sub> level >7.0 per cent, wherein the large deletion is suspected. Identification of large deletions in the  $\beta$ -globin gene cluster has recently gained importance because of its significance in unsolved thalassaemia cases and disease prevention. However, conventional diagnostic tests [covalent reverse dot blot (CRDB) hybridization, amplification-refractory mutation system (ARMS) polymerase chain reaction (PCR) and  $\beta$ -globin gene sequencing], targeting point mutations and small insertions–deletions in the  $\beta$ -globin gene fail to detect large deletions<sup>3</sup>. Thus, a differential approach combining techniques such as southern blotting, quantitative fluorescent (QF) PCR, fluorescent *in situ* hybridization, multiplex ligation-dependent probe amplification (MLPA) and gap-PCR could be utilized<sup>4</sup>. In the present study, a combined strategy of MLPA and simplified gap-PCR technique was used to detect a rare deletion of 4056 bp which was mainly detected in couples from the Gujarat region of India.

The study was approved by the Institutional Ethics Committee, ICMR-National Institute of Immunohaematology (NIIH), and the samples were obtained after obtaining written informed consent from the patients. Five families with a history of

$\beta$ -thalassaemia were referred for haemoglobinopathy workup and eventually for prenatal diagnosis during January 2016 to March 2020. Preliminary screening by analyzing the complete blood count and HbA<sub>2</sub> and HbF levels by high performance liquid chromatography (HPLC) was carried out. Genomic DNA was extracted and  $\beta$ -globin gene analysis was carried out. MLPA was performed using SALSA MLPA Kit P102 HBB (MRC-Holland, Amsterdam, The Netherlands)<sup>5</sup>, which contained 29 probes for the 73 kb region of the  $\beta$ -globin gene cluster targeting the locus control region, coding genes in the cluster, the intergenic sequences and 10 reference probes<sup>5</sup>. The manufacturer's protocol was followed. The MLPA products were separated on an automated DNA sequencing machine (Applied Biosystems, Genetic Analyzer 3130xl, ThermoFisher Scientific, USA), and the results were analyzed using GeneMapper™ software version 4.0 (ThermoFisher Scientific, USA) and Coffalyser.Net software (Coffalyser.Net - MRC, Holland). Further, a simplified single tube gap-PCR was performed with the help of previously reported primers sequences<sup>6</sup>. The gap-PCR programme employed to detect the deletion was as follows: initial denaturation at 94°C for five minutes, followed by 30 cycles of denaturation: 94°C for one minute, annealing at 58°C for one minute, extension at 72°C for one minute and a final extension at 72°C for five minutes.

In five families, both or either of the parents (total 7) had an elevated A<sub>2</sub> in the range of 7.5–8.8 per cent (mean±SD; HbA<sub>2</sub>: 7.95±0.45%). In these patients, initial molecular screening by CRDB hybridization, ARMS and  $\beta$ -globin gene sequencing was carried out at ICMR-NIIH, Mumbai and was found to be normal. Out of the five families, four were referred to our centre for prenatal diagnosis as well. In families 1 and 3, one parent had typical beta-thalassaemia indices

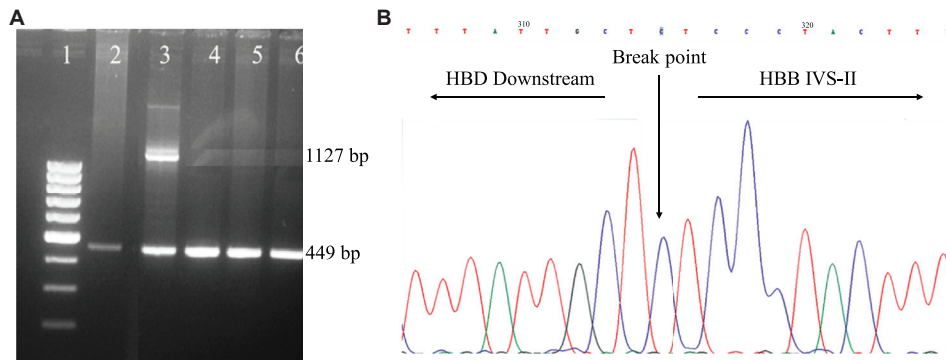


**Fig. 1.** MLPA analysis of two different patient samples.  $\beta$ -globin cluster showing genomic regions showing 4056 bp deletion in heterozygous (blue) and homozygous (red) patients. The Y axis represents the probe dosage ratio and the X axis represents the MLPA probes. MLPA, Multiplex ligation-dependent probe amplification.

and DNA analysis identified IVS1-5 (G→C) mutation (HBB: c.92+5G>C) [NM\_000518.5 (HBB): c.92+5G>C]. The other parent had high A<sub>2</sub>, 7.5-8.8 per cent. Each of the above families had a child presenting with severe anaemia requiring frequent blood transfusions. Reverse dot blot analysis of both the affected children showed the presence of HBB: c.92+5G>C homozygous pattern, however, the prenatal report was given (family 1 and 3) as the chorionic villus sample showed the absence of HBB: c.92+5G>C mutation. In the second family, both the parents had high HbA<sub>2</sub> and they had a clinically presenting child, who required regular blood transfusion (they had come for prenatal diagnosis). CRDB analysis and whole  $\beta$ -globin gene sequencing showed a normal pattern. As the  $\beta$ -globin gene mutation was undetermined, the patient was called for cordocentesis (family 2). On HPLC analysis of this cordocentesis sample, the HbA<sub>2</sub> level of foetal blood was 2.6 per cent, by ruling out maternal contamination this was suggestive of  $\beta$ -thalassaemia minor and the report was issued. Similarly, in family 4, both the parents had raised HbA<sub>2</sub> levels, suggesting that they were  $\beta$ -thalassaemia carriers and they were referred for prenatal diagnosis. Family 5 was referred for molecular diagnosis of  $\beta$ -globin gene mutation, as the mother had a raised HbA<sub>2</sub> level. In the individuals, where the HbA<sub>2</sub> was found to be elevated, deletion in the  $\beta$ -globin gene was suspected, that was being missed by the conventional diagnostic methods. Hence, with the standardization of MLPA technique, these uncharacterized samples were analyzed again. In MLPA, probe amplification spanning from the  $\delta\beta$  intergenic region,  $\beta$ -globin promoter, exon 1, 2, up

to the intron 2 of the  $\beta$ -globin gene was found to be reduced in the individuals with elevated HbA<sub>2</sub> and their affected children (Fig. 1). The results of MLPA analysis helped in designing a gap-PCR which generated two bands in heterozygous condition: an internal control band of 449 bp and a mutant band of 1127 bp; and a single band each of 449 bp and 1127 bp under normal and homozygous condition for the deletion, respectively (Fig. 2A). Further, DNA sequencing of the mutant band showed a deletion of 4056 bp that extends from 2.7 kb downstream of the  $\delta$ -globin gene to IVS-2 of the  $\beta$ -globin gene (Fig. 2B). Table shows the haematological and molecular details of the families studied. We observed that the individuals heterozygous for 4056 bp deletion had microcytic anaemia and the  $\beta$ -thalassaemia homozygous patients for 4056 bp deletion presented with a severe clinical condition requiring regular blood transfusion.

$\beta$ -thalassaemia occurs due to mutation in the  $\beta$ -globin gene which leads to reduced or absent  $\beta$ -globin chain synthesis. In rare cases, the deletional form of  $\beta$ -thalassaemia, involving variable-sized deletion of the  $\beta$ -globin gene cluster region with an exceptionally high HbA<sub>2</sub> level with mild elevation in HbF level, is observed<sup>7</sup>. In this study, a 4056 bp deletion was identified in heterozygous form in seven individuals showing high HbA<sub>2</sub> levels (7.5-8.8%) with slight elevation in the HbF levels (1.1-3.8%). Furthermore, two affected cases of compound heterozygous for 4056 bp deletion and HBB: c.92+5G>C  $\beta$ -globin gene mutation, and one affected case homozygous for 4056 bp deletion were identified in this study.



**Fig. 2.** (A) 1.5 per cent agarose gel showing amplified products of gap-PCR. Lane 1: 100 bp ladder. Lane 2, 4, 5, 6: Normal control, showing the presence of control band of 449 bp. Lane 3: Sample heterozygous for 4056 bp deletion showing an internal control band of 449 bp and a mutant band of 1127 bp. (B) Automated DNA sequencing of the patient harbouring 4056 bp deletion in heterozygous condition. The electropherogram shows the breakpoint position of the 4056 bp deletion.

The molecular characterization of 4056 bp deletion showed that the deletion extended from the 3157 bp 5' upstream from the cap site to the 3'-IVS-2 region of the  $\beta$ -globin gene. Previous studies have reported around 15 deletions in different populations that remove a variable genomic segment from the  $\beta$ -globin gene and result in  $\beta$ -thalassaemia phenotype<sup>8</sup>. Popovich *et al*<sup>9</sup>, 1986, characterized a  $\beta$ -globin gene deletion of 4237 base pairs, which showed a similar extent of deletion from 3.3 kb upstream from the cap site to the middle of IVS-2 of the  $\beta$ -globin gene in a family of Czechoslovakian descent with higher HbA<sub>2</sub> levels ranging from 8.1 to 9.0 per cent and HbF levels ranging from 3.3 to 5.5 per cent. Dimovski *et al*<sup>10</sup>, 1993, detected a 1605 bp deletion of the 5' beta-globin promoter region leading to  $\beta^0$ -thalassaemia in a Croatian family with unusually high HbA<sub>2</sub> (7.6-8.2%) with HbF levels: 5.8-8.5 per cent. Similarly, Gallienne *et al*<sup>11</sup>, 2010, identified a novel deletional mutation of 909 base pairs in the  $\beta$ -globin gene, which involved the removal of the  $\beta$ -globin gene promoter, exon 1, IVS-I entirely and most of exon 2 of the  $\beta$ -globin gene (-478 to +432) with exceptionally high HbA<sub>2</sub> phenotype (HbA<sub>2</sub>: 8.6-9.0%; HbF: 1.7-3.9%). In the Indian population, the most common  $\beta$ -globin deletional mutation observed is the 619 bp deletion; however, these patients also show an average HbA<sub>2</sub> level of 6.5 per cent, slightly higher than the typical  $\beta$ -thalassaemia<sup>12</sup>. Thein<sup>13</sup>, 2013, reported that the deletional mutations removing the 5' end of  $\beta$ -globin gene and promoter sequences involving TATA, CCAAT and CACCC boxes, which are involved in regulating the transcription, may result in unusually higher HbA<sub>2</sub> level and mild elevation in the HbF level. A similar observation is found in the present study as

well. The plausible mechanism of raised HbA<sub>2</sub> and HbF levels may be due to the increased interaction of the locus control region with the cis- $\delta$ -globin and  $\gamma$ -globin promoter region in the absence of competing  $\beta$ -globin gene, thus enhancing their expression<sup>13</sup>. Wayne *et al*<sup>14</sup>, 2017, also characterized two novel deletions of 538 bp and 1517 bp which removed the  $\beta$ -globin gene promoter, 5' UTR and exons 1 and 2. Majority of the deletional mutation of  $\beta$ -thalassaemia span from the 5' promoter region of the gene to the  $\beta$ -IVS-2 region at the 3' end which could be attributed to the presence of a recombinational hot spot.

Large deletions in the  $\beta$ -globin cluster escape traditional diagnostic techniques<sup>6</sup>. In our study as well, the CRDB, ARMS PCR and direct DNA sequencing failed to identify the presence of 4056 bp deletion. During routine diagnosis, as the HbA<sub>2</sub> level in the presence of the deletion is comparatively higher than the average HbA<sub>2</sub> value for  $\beta$ -thalassaemia trait, a deletion in the  $\beta$ -globin gene can be easily suspected. A combined strategy of MLPA, QF-PCR and gap-PCR was thus used to detect this deletion. Previously, the 4056 bp deletion was first identified by Mayuranathan *et al*<sup>6</sup>, 2012, in a patient of Gujarat origin. They used combined MLPA and PCR techniques to identify this deletion wherein the PCR generated only one deletion-specific band of 1.1 kbp. Hence, heterozygosity or homozygosity for the deletion could not be distinguished. In this study, we have described a fast, reliable, relatively cost-effective gap-PCR approach to identify 4056 bp deletion that generates an amplification product of 449 bp in the presence of a normal chromosome, and a 1.1 kb deletion specific band, which can easily distinguish between a normal, heterozygous and homozygous condition of the deletion.

**Table.** Haematological and molecular data of families

	Age	Caste	Origin	RBCs (10 <sup>6</sup> /μl)	Hb (g/dl)	MCV (fl)	MCH (pg)	HbA <sub>2</sub> (%)	HbF (%)	β-globin genotype by conventional technique (CRDB)	β-globin genotype after MLPA and gap-PCR
<b>Family 1</b>											
Father	23 yr	Rajput	Saurashtra	5.23	10.5	61.4	20.1	5.0	0.1	HBB: c. 92+5G>C Heterozygous	HBB: c. 92+5G>C Heterozygous
Mother	22 yr	Rajput	Saurashtra	4.50	9.8	63.6	21.8	8.1	3.8	Normal	4056 bp deletion Heterozygous
Affected child*	3 yr	Rajput	Saurashtra	4.70	12.0	74.6	20.6	2.1	3.2	HBB: c. 92+5G>C Homozygous pattern	HBB: c. 92+5G>C +4056 bp deletion
Foetus (CVS)	12 wk gestation	Rajput	Saurashtra	-	-	-	-	-	-	HBB: c. 92+5G>C Absent	Follow up
<b>Family 2</b>											
Father	30 yr	Rajput	Rajkot	6.19	12.2	61.6	19.7	7.5	1.1	Normal	4056 bp deletion Heterozygous
Mother	28 yr	Rajput	Rajkot	4.13	8.3	62.2	20.1	8.8	2.8	Normal	4056 bp deletion Heterozygous
Affected child*	4 yr	Rajput	Rajkot	4.89	12.9	75.5	26.4	3.2*	4.6	Not amplified	4056 bp deletion homozygous
Foetus (AF)	17 wk gestation	Rajput	Rajkot	-	-	-	-	-	-	Normal	4056 bp deletion heterozygous
<b>Family 3</b>											
Father	28 yr	Patel	Surat	5.58	11.8	69.4	21.1	8.2	3.2	Normal	4056 bp deletion Heterozygous
Mother	25 yr	Patel	Surat	5.10	10.2	64.5	20.0	5.0	0.7	HBB: c. 92+5G>C Heterozygous	HBB: c. 92+5G>C Heterozygous
Affected child*	5 yr	Patel	Surat	3.81	11.3	88.7	29.7	3.2	4.0	HBB: c. 92+5G>C Homozygous pattern	HBB: c. 92+5G>C +4056 bp deletion

*Contd...*

	Age	Caste	Origin	RBCs (10 <sup>6</sup> /μl)	Hb (g/dl)	MCV (fl)	MCH (pg)	HbA <sub>2</sub> (%)	HbF (%)	β-globin genotype by conventional technique (CRDB)	β-globin genotype after MLPA and gap-PCR
<b>Family 3</b>											
Foetus (CVS)	12 wk gestation	Patel	Surat	-	-	-	-	-	-	HBB: c. 92+5G>C Absent	Follow up
<b>Family 4</b>											
Father	32 yr	Ahir	No blood samples provided, only DNA samples were referred along with HbA2 and HbF levels					7.5	2.0	Normal	4056 bp deletion Heterozygous
Mother	28 yr	Ahir						8.05	3.2	Normal	4056 bp deletion Heterozygous
Foetus (AF)	17 wk gestation	Ahir						-	-	Normal	4056 bp deletion Heterozygous
<b>Family 5</b>											
Father	34 yr	Singh	No blood samples provided, only DNA samples were referred along with HbA2 and HbF levels					2.9	0.6	Normal	Normal
Mother	30 yr	Singh						7.5	2.1	Normal	4056 bp deletion Heterozygous
*Post-transfused sample. CVS, chorionic villus sample; AF, amniotic fluid; RBCs, red blood cells; Hb, haemoglobin; HbA <sub>2</sub> , haemoglobin A <sub>2</sub> ; HbF, foetal Hb; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MLPA, multiplex ligation-dependent probe amplification; CRDB, covalent reverse dot blot; HBB, Hemoglobin subunit beta											

The molecular characterization of large deletional mutations in the *β-globin* gene cluster is extremely critical for at-risk couples seeking prevention as these deletions escape identification and compromise the prenatal diagnosis in laboratories that follow the conventional methodology to detect *β-globin* gene mutations. The 4056 bp deletion seems to be a region-specific mutation, hence gap-PCR could be easily performed, instead of going for an expensive technique; however, if the finding of gap-PCR are negative then MLPA, QF-PCR can be performed in suspected cases.

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