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ORIGINAL ARTICLE

Identification of rare and novel deletions that cause $(\delta\beta)^0$-thalassaemia and hereditary persistence of foetal haemoglobin in Indian population

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Abstract

Objectives: Hereditary persistence of foetal haemoglobin (HPFH) and $(\delta\beta)^0$-thalassaemia are conditions caused by large deletions that involve δ- and β-globin genes in the β-globin cluster, and they are characterized by increased haemoglobin (HbF) levels in adults. Significant phenotypic diversity is observed between the different mutations that cause these conditions. Molecular characterization of these deletions is important for accurate molecular diagnosis, and they will also provide the information on the *cis*-acting genetic regulatory elements present in the β-globin cluster. *Methods:* We performed gap-PCR, multiplex ligation-dependent probe amplification (MLPA), quantitative fluorescent multiplex PCR (QF-MPCR) and DNA sequencing to detect and characterize the deletions in the β-globin cluster. *Results:* We characterized six different deletions resulting in $(\delta\beta)^0$-thalassaemia or HPFH in 51 unrelated families. *Conclusion:* With the help of multiple genetic tools, we performed comprehensive genetic analysis of HPFH and $(\delta\beta)^0$-thalassaemia in Indian population and could define the molecular basis of these conditions in this population. We also identified two novel HPFH mutations, 49.98 kb (HPFH-9) and 86.7 kb (HPFH-10) deletions, in this population.

Key words $(\delta\beta)^0$-thalassaemia; hereditary persistence of foetal haemoglobin; haemoglobin; β-thalassaemia; mutation

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Hereditary persistence of foetal haemoglobin (HPFH) and $(\delta\beta)^0$-thalassaemia are conditions caused by large deletions in the β-globin cluster involving δ- and β-globin genes, with or without $^A\gamma$-globin gene. These mutations are characterized by high foetal haemoglobin (HbF) levels in heterozygous states, and the distinction between HPFH and $(\delta\beta)^0$-thalassaemia is made on clinical and haematological grounds (1). Heterozygotes of HPFH have normal blood indices and pancellular distribution of HbF, with the levels ranging from 15% to 30%, and, in contrast, $(\delta\beta)^0$-thalassaemia heterozygotes have hypochromic and microcytic red cells with lower levels of HbF, 5%–15%, with heterocellular distribution (2). Homozygotes of $(\delta\beta)^0$-thalassaemia and compound heterozygotes of $(\delta\beta)^0$-thalassaemia and β-thalassaemia show severe clinical picture similar to β-thalassaemia intermedia or major. However, HPFH homozygotes are clinically asymptomatic and compound heterozygotes of HPFH and β-thalassaemia are also clinically asymptomatic or, in some rare cases, they show thalassaemia intermedia phenotype (1).

More than 40 different types of deletions with varying 5′ and 3′ breakpoints which result in $(\delta\beta)^0$-thalassaemias and HPFH have been reported (3). Although the breakpoints of most of these deletions have been identified, it is not clear why some deletions cause milder phenotype and some others cause more severe anaemia. Complete characterization of these deletions including their breakpoints is important for

accurate molecular diagnosis of these conditions and genotype–phenotype correlation. Additionally, study of different deletions that cause activation of HbF is useful for identification of the regulatory elements in the β-globin cluster that determine the developmental-stage expression of globin genes.

The conventional approaches to the characterization of large deletions, Southern blot analysis and comparative genome hybridization (CGH) (4), are labour-intensive, time-consuming and expensive and they cannot 'pinpoint' the break points. PCRs that amplify across the break points can be applied only for frequent mutations for which the deletion breakpoints have been characterized. Multiplex ligation-dependent probe amplification (MLPA), a recently described quantitative method for detecting unknown deletions, can detect the presence of deletions in the genomic regions in both heterozygous and homozygous states. This method has been applied successfully in a number of genomic regions in which deletions and duplications are common (5). However, it is more expensive than PCR-based methods and technically challenging as multiple sensitive steps are required, which makes this method not easier in a routine diagnosis set up. Using this method, the breakpoints of the deletions cannot be located as the probes that bind to the genomic locus are widely spaced and do not span the whole genomic region where these deletions occur. We developed quantitative fluorescent multiplex PCR (QF-MPCR), which are cheaper and easier to perform, to detect the large deletions in the β-globin cluster (6). This method could also facilitate locating the breakpoints successfully. Using this method, we identified six different deletions that cause $(\delta\beta)^0$-thalassaemia or HPFH in Indian population.

Materials and methods

Patients

The subjects of this study were the patients with the phenotype of β-thalassaemia major or intermedia, who had with at least one of their parents with increased HbF levels (>10%) and normal HbA_2 levels (<3.5%).

Detection of common deletions using Gap-PCR

Two previously reported mutations in the Indian population, $^{G}\gamma(^{A}\gamma\delta\beta)^0$-deletion/inversion (del-inv) and HPFH-3, were detected by Gap-PCRs using the primers described by Craig *et al.* (7).

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed using the SALSA MLPA Kit P102 HBB (MRC Holland, Amsterdam, Netherlands) according to the manufacturer's protocol. This kit had 29 probes spanning a 73-kb region of the β-globin gene cluster targeting hypersensitive sites and ε, $^{G}\gamma$, $^{A}\gamma$, δ and β globin genes and intergenic regions. Approximately, 100 ng concentration of DNA was used for the assay. Ligation and amplification were carried out on a thermal cycler, the amplified products were separated by capillary electrophoresis in an ABI-3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the results were analysed by GeneMapper 4.0 (Applied Biosystems). The peak heights obtained from the patients and/or their parents were normalized with a normal control which was processed with every batch of samples.

Quantitative fluorescent multiplex PCRs (QF-MPCRs)

Copy numbers of γ-, δ- and β-globin genes were measured by a gene dosage quantitative multiplex fluorescent PCR-PCR (GD-QF-MPCR) as we described recently (6). We used Hot Star Master Mix (Qiagen, GmbH, Hilden, Germany) with 0.5 μM primers for 20–22 cycles with 200 ng of DNA, and the fluorescently labelled amplified products were analysed by capillary electrophoresis using ABI genetic analyser and the peak heights were determined by GeneMapper 4.0 (Applied Biosystems). The data were intranormalized by dividing the peak heights of γ-, δ- and β-globin genes by that of albumin gene for each sample. Subsequently, internormalization was carried out by dividing the intranormalized peak heights of globin genes in the test sample with those from the normal control sample. For the QF-MPCRs to detect the various 3′ break points (3′β QF-MPCRs), we amplified genomic regions at 14, 18, 21, 25, 30, 36, 40, 55 and 70 kb downstream of β-globin gene. The primers were designed on non-repeat sequences and the sizes of the amplified products were limited to <250 bp. The multiplex PCRs contained 4–5 primer pairs including the one for amplification of albumin gene which was used as the control for normalization. The fluorescently labelled PCR products were analysed by capillary electrophoresis, and the peak heights were subjected to intra- and internormalization as performed for GD-QF-MPCR.

Characterization of the break points of the deletions

Primers were designed at the genomic regions close to the deletions, which were determined by MLPA and QF-MPCRs. The amplified products were sequenced to characterize the exact breakpoints of each deletion.

The sequences of the primers used in this study are shown in the Table S1.

Results

A total of 90 individuals from 51 families were screened for $(\delta\beta)^0$-thalassaemia or HPFH mutations. They included 49 heterozygotes and 12 homozygotes for $(\delta\beta)^0$-thalassaemia or

HPFH and 29 compound heterozygotes for these deletions and β-thalassaemia. Initial screening by Gap-PCR for the two common mutations in Indian population showed that the Indian $^{G}\gamma(^{A}\gamma\delta\beta)^0$ deletion–inversion (del/inv) was present in 25 families and HPFH-3 mutation in 14 families. Of the 12 families who did not have the two common mutations, GD-QF-MPCR showed γ:δ:β ratio of 1 : 0.5 : 0.5 in four families suggestive of $(\delta\beta)^0$-deletions and 0.75 : 0.5 : 0.5 in eight families suggestive of $(^{A}\gamma\delta\beta)^0$-deletion (Figure S1).

To detect the approximate locations of genomic regions where the break points are present in the 12 families with unknown mutations, we initially performed MLPA in the heterozygotes in these families. In all the samples, the probes from HS-5 region till HBG2/HBG1 ($^{G}\gamma/^{A}\gamma$) intergenic region showed the peak height ratio of ~1 and the remaining probes from ψβ-exon1 region till the last 3′ MLPA probe, which binds at the region 9 kb downstream of β-globin gene, the ratio was 0.5 (Fig. 1). Due to the lack of probes in 7.7 kb region between $^{G}\gamma$-$^{A}\gamma$ intergenic region and ψβ-globin gene, we could not determine a short span of region where the 5′ break points of the deletions are present. MLPA also could not distinguish between $(\delta\beta)^0$- and $(^{A}\gamma\delta\beta)^0$-deletions due to insufficient probes in and around $^{A}\gamma$ globin gene. However, GD-QF-PCRs in these families could help us distinguish between $(\delta\beta)^0$- and $(^{A}\gamma\delta\beta)^0$-deletions, and we could further narrow down the genomic regions where the 5′ break points are present in these families. In four families, GD-QF-MPCR showed that $^{A}\gamma$ globin gene is intact and MLPA showed that ψβ globin gene is deleted. We concluded that the 5′ break points in these families were present in the 7.7 kb region between $^{A}\gamma$- and ψβ-globin genes. In eight families, GD-QF-MPCR showed that $^{A}\gamma$-globin gene is deleted. As MLPA showed that $^{G}\gamma/^{A}\gamma$ intergenic region is intact, we concluded that the 5′ breakpoints in these families were in 1.3 kb region between $^{G}\gamma/^{A}\gamma$ intergenic probe and $^{A}\gamma$ globin gene. Thus, MLPA and GD-QF-MPCR together could locate the genomic regions where the 5′ break points are present in the 12 families with uncharacterized deletions.

The 3′β-QF-MPCR analysis showed that in three families with unknown mutations, the PCR product from 14 kb downstream of β-globin gene showed normal peak height ratio. As the MLPA probe at 9 kb downstream showed deletion of this region, we concluded that the 3′ breakpoint in these families is between 9 and 14 kb downstream of β globin gene. In seven families, the peak height ratio from 18 and 21 kb downstream of β globin was 0.5 and that from 25 kb was 1, which suggested the 3′ breakpoint in these cases is between 21 and 25 kb region downstream of β globin gene. Similarly, we found two more deletions: one with the 3′ break point between 30 and 36 kb and the other between 55 and 70 kb (Fig. 2A,B). Taken together, 3-β-QF-MPCR helped us to detect short span of genomic regions where the break points are present in all the 12 families with uncharacterized mutations. The results are summarized in the Fig. 3B.

For amplification across the break points, we used suitable forward and reverse primers based on the results provided by MLPA, GD-QF-MPCRs and 3′BP QF-MPCRs. We

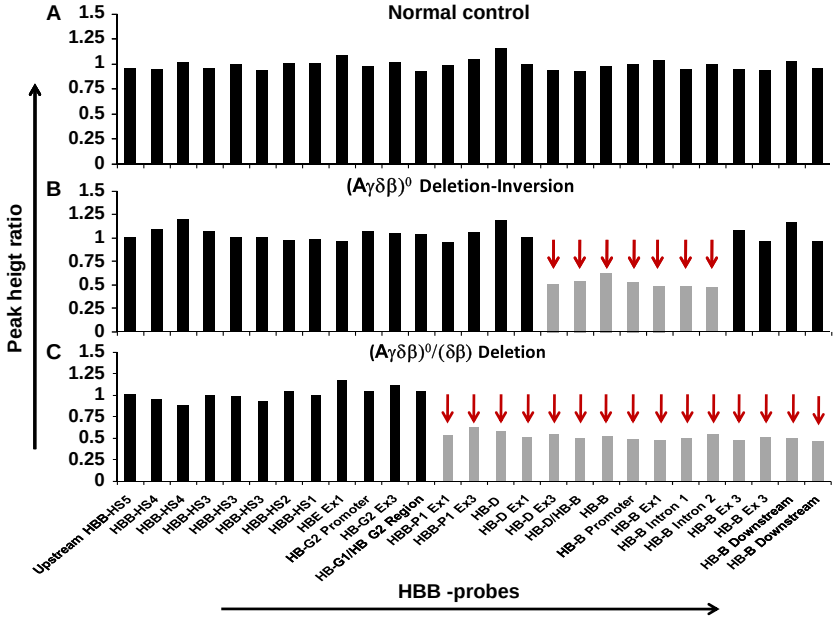


Figure 1 MLPA analysis of β-globin cluster in the patients with different types of deletions. (A) Pattern in a normal individual showing a peak height ratio of ~1.0 for all the probes. (B) The heterozygotes for $(^{A}\gamma\delta\beta)^0$-del/inv mutation showing the peak height ratio of ~0.5 for β-globin probes. As $^{A}\gamma$ and δ globin genes are inverted, they do not show reduced peak heights. (C) The pattern obtained in all other cases of $(\delta\beta)^0$- or $(^{A}\gamma\delta\beta)^0$-deletions. (Arrows indicate the reduction in copy number of the genes).

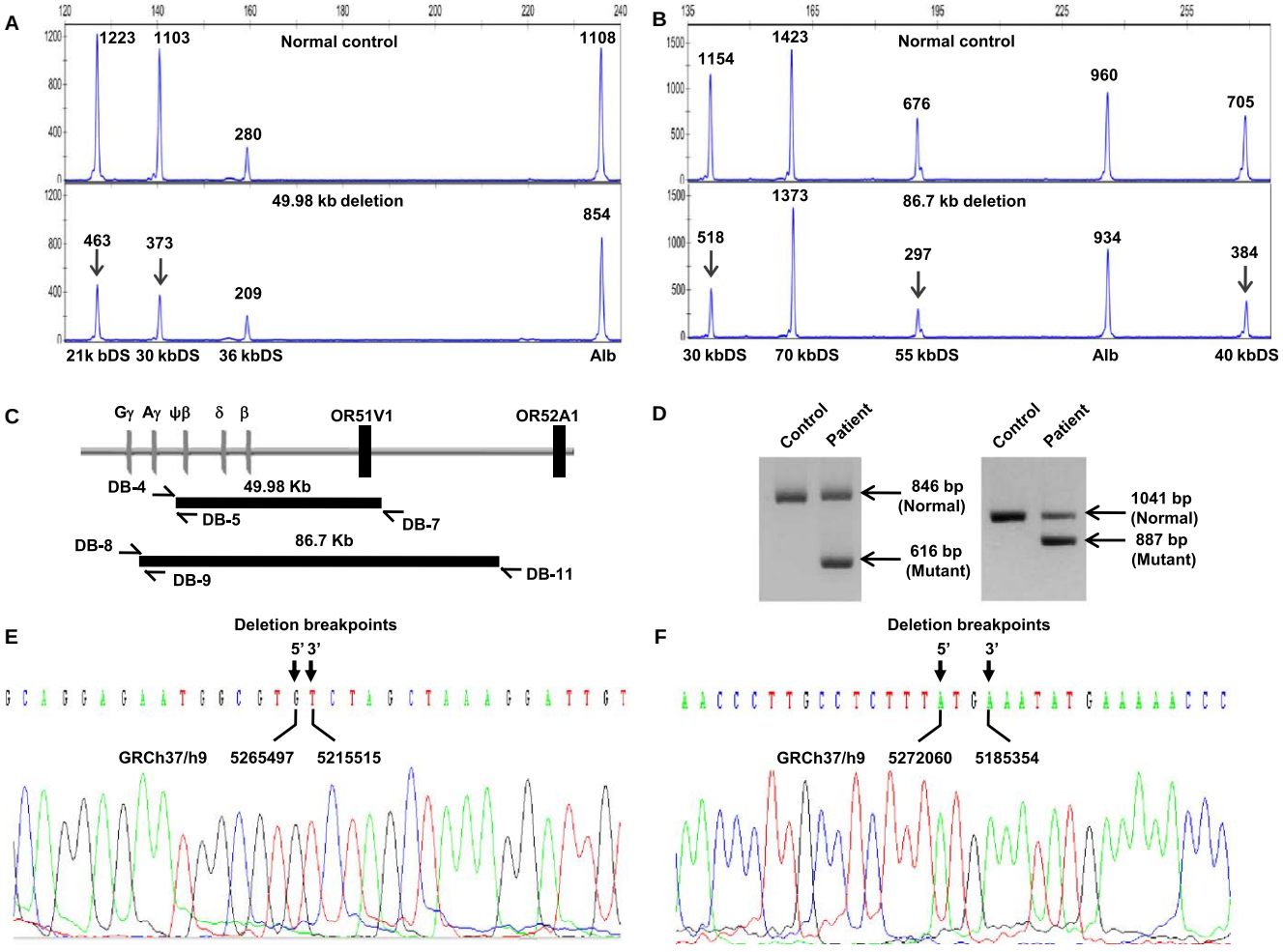
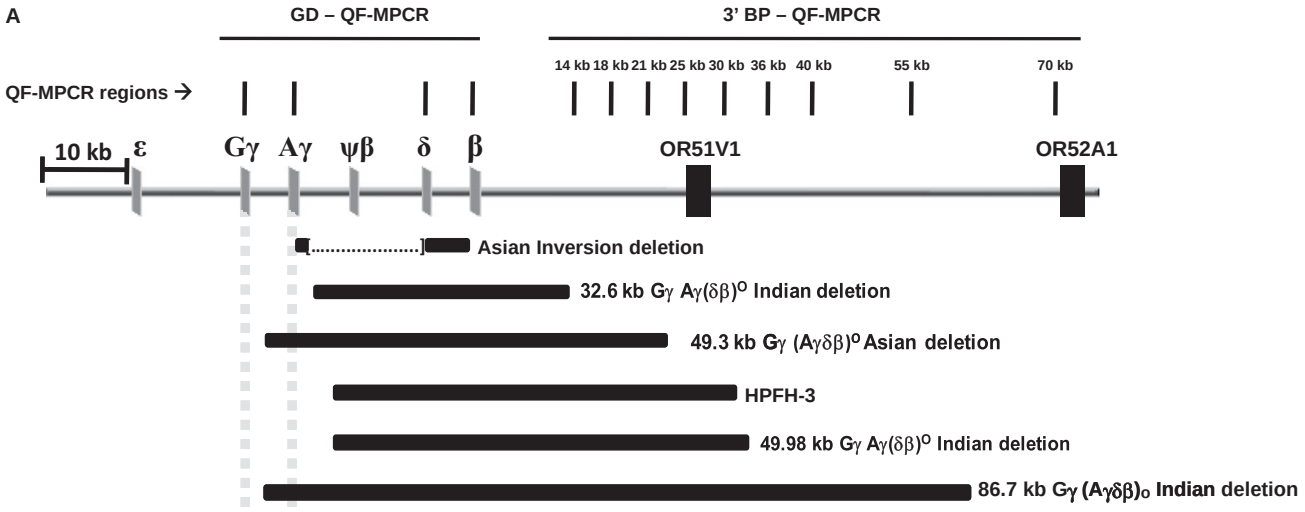


Figure 2 Identification of novel 49.98 and 86.7 kb deletions. (A and B) Electropherograms of labelled amplified products obtained from 21, 30, 36, 40, 55 and 70 kb downstream (DS) of β-globin gene. Arrows indicate the reduction in copy number of genes. (C and D) Position of the primers used for Gap PCRs to amplify across the break points and the agarose gel electrophoresis of the amplified products. The amplified products of 616 and 887 bp are obtained from 49.98 and 86.7 kb deletions, respectively. (E and F) Characterization of the breakpoints by DNA sequencing of the amplified products obtained by Gap PCRs. In 86.7 kb deletion, an orphan 2 bp (+TG) insertion is inserted between the breakpoints. (OR51V1 – olfactory receptor, family 51, subfamily V, member 1; OR52A2 – olfactory receptor, family 52, subfamily A, member 2).

designed three forward primers: one binds at the $^{G}\gamma$-$^{A}\gamma$ and two at $^{A}\gamma$-ψβ intergenic regions (Fig. 2 and Table S1). The reverse primers were designed between the two adjacent regions which showed the peak height ratios of 0.5 and 1 in the 3′ β-QF-MPCRs. The amplified products were sequenced to characterize the breakpoints of the deletions. In three families, we found a 32.6 kb deletion which had been reported earlier in one Indian subject with (δβ)^0 thalassaemia (8, 9), and in seven families, we identified a 49.3 kb deletion which had been reported earlier in Asian Indian families in Canada (10) (Figure S2). The five heterozygotes for the 32.6 kb deletion had haematological parameters, suggesting that this mutation is associated with HPFH (Table 1) and the three patients who were compound heterozygous for this deletion and β-thalassaemia had thalassaemia intermedia phenotype. The seven heterozygotes for the 49.3 kb deletion showed the haematological parameters suggestive of (δβ)^0-thalassaemia as reported earlier (Table 1). Patients who were compound heterozygous for this deletion with β-thalassaemia mutations had thalassaemia major phenotype, requiring regular blood transfusions.

In one family, we found a novel 49.98 kb (δβ)^0-deletion (HPFH-9) with the breakpoints very close to those of HPFH-3 deletion (Fig. 3). The haematological parameters in the heterozygotes suggested that this deletion is also associated with HPFH (Table 1). The peripheral blood analysis of one of the heterozygotes showed pancellular distribution of HbF. A patient with this deletion and β-thalassaemia had mild thalassaemia intermedia phenotype with Hb = 8.8 g/dL at the age of 8 yr without significant organomegaly. The 5′ break points of both 49.98 kb and HPFH-3 deletions lie in Alu repeats at ~4 kb downstream of $^{G}\gamma$-globin gene and

A



B

	MLPA		3' BP QF-MPCRs								
	0.5 kb	9 kb	14 kb	18 kb	21 kb	25 kb	30 kb	36 kb	40 kb	55 kb	70 kb
HPFH-3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	1
32.6 kb Indian deletion	0.5	0.5	1	1	1	1	1	1	1	1	1
49.3 kb Asian deletion	0.5	0.5	0.5	0.5	0.5	1	1	1	1	1	1
49.98 kb Indian deletion	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	1
86.7 kb Indian deletion	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1

Figure 3 (A) Representation of different types of deletions found in Indian population and the locations of genomic regions analysed by GD-QF-MPCRs to detect the deletions. (B) Peak heights obtained in MLPA and 3′ BP QF-MPCRs for different deletions (the peak heights are approximated to 0.5 and 1).

Table 1 The haematological parameters of six different deletions in heterozygous states

Deletion type	Hb (g/dL)	MCV (f/L)	HbF (%)	HbA2 (%)
Indian $^{G}\gamma$ $(^{A}\gamma\delta\beta)^0$ del/Inv (n = 15)	12.0 ± 1.8	72.9 ± 5.1	19.1 ± 4.8	2.5 ± 0.3
HPFH-3 (n = 9)	13.4 ± 0.8	81.7 ± 4.7	30.9 ± 5.6	2.1 ± 0.2
32.6 kb Indian $^{G}\gamma^{A}\gamma$ $(\delta\beta)^0$ del (n = 5)	13.9 ± 1.0	85.6 ± 4.4	16.4 ± 2.5	2.8 ± 0.6
49.3 kb Asian $^{G}\gamma$ $(^{A}\gamma\delta\beta)^0$ -Thal (n = 7)	11.3 ± 0.8	68.3 ± 2.7	16.5 ± 7.0	2.2 ± 0.2
49.98 kb Indian HPFH (HPFH-9) (n = 4)	12.5 ± 1.0	79.7 ± 5.2	26.9 ± 4.6	2.2 ± 0.2
86.7 kb Indian HPFH (HPFH-10) (n = 1)	12.9	80.8	32.2	2.3

their 3′ breakpoint lie in L1 repeat and a perfect 160 bp palindrome sequence (11), suggesting a common mechanism for the origin of these two mutations. The second novel 86.7 kb $(^{A}\gamma\delta\beta)^0$-deletion (HPFH-10) found in one of the families (Fig. 3) had the 5′ break point at 4.3 kb downstream of $^{G}\gamma$-globin gene and the deletion extended up to 61 kb downstream of β-globin gene where the 3′ break point lies in L1 repeat sequence. Haematological parameters in the only one heterozygote for the 86.7 kb deletion showed that this mutation is also associated with HPFH (Table 1), and we did not find this mutation in compound heterozygous state with β-thalassaemia in any patients.

Discussion

The analysis of naturally occurring mutations that increase HbF in adults and their correlation with the clinical and haematological phenotypes provides information on *cis*-acting genetic elements required for developmental-stage-specific regulation of globin genes, which will help in developing therapeutic approaches for increasing HbF for the treatment of β-thalassaemia and sickle cell disease (12). Several hypotheses have been proposed for increased γ-chain production in conditions with $(\delta\beta)^0$-thalassaemia and HPFH. These include the deletion of regulatory sequences where

transcriptional repressors of γ-globin gene bind, lack of promoter competitions when δ- and β-globin genes are deleted and juxtaposition of putative enhancer sequences and γ-globin gene when the deletion occurs (4, 13–20). However, the exact mechanisms that cause increased HbF in these cases have not been understood well. Characterization of the break points of the different deletions that cause $(\delta\beta)^0$-thalassaemia and HPFH will help us understand the molecular mechanisms which cause increased γ-globin production observed in the individuals with these deletions. Several transcriptional and epigenetic regulators, miRNAs, non-coding RNAs and epigenetic factors, have been found to regulate repression and activation of genes in mammalian cells. Identification of the breakpoints of these deletions will help us isolate genomic regions involved in γ globin gene reactivation by carrying out experiments using *ex vivo* erythropoiesis systems. Identification of the breakpoints is also important for the accurate molecular diagnosis as the haematological parameters, HbF levels and the phenotypes of the patients who are compound heterozygous for one of these mutations with a β-thalassaemia mutation will be very similar to those with homozygous β-thalassaemia. Identification of the mutations in the patients is useful for genotype–phenotype correlation and antenatal diagnosis in the families with these mutations.

We performed the most comprehensive genetic screening in the largest number of patients with $(\delta\beta)^0$-thalassaemia and HPFH in Indian population. A very few studies have been carried out in understanding the molecular basis of $(\delta\beta)^0$ -thalassaemia and HPFH in Indian population. A recent study (21) which attempted to identify the frequency of the deletions showed that in addition to the common mutations, del-inv and HPFH-3, which constituted 75% of cases, the Vietnamese/Chinese deletion (22) was present in 9% of cases. They could not identify the deletions in 16% of cases.

Only one patient with 32.6 kb deletion has been reported earlier (8, 9), and our study identified this mutation in five individuals from three more Indian families. The 49.3 kb deletion has not been earlier reported in any of the studies carried out in India, and it was identified earlier in seven unrelated Canadian families who had some degree of Asian Indian ancestry (10); we identified this mutation in seven more families, suggesting the importance of screening this mutation for molecular diagnosis of $(\delta\beta)^0$-thalassaemia. We also identified two novel mutations, 49.98 kb (HPFH-9) and 86.7 kb (HPFH-10) deletions, which have haematological parameters suggestive of HPFH.

As α thalassaemia can affect the haematological parameters and HbF levels, we screened for α globin gene deletions using a QF-MPCR (data not shown) in five individuals who were heterozygous for 49.3 kb deletion and three who were compound heterozygous for 49.3 kb deletion and β-thalassaemia. We found $-\alpha^{4.2}/\alpha\alpha$ in one individual who was compound heterozygous for β-thalassaemia and the deletion. These data suggest that 49.3 kb deletion causes $(\delta\beta)^0$-thalassaemia and the lower MCV and HbF levels in the individuals with this mutation are not due to co-inheritance of α-thalassaemia.

We found that QF-MPCRs are efficient to detect and characterize break points of large deletions in the genome. Compared with other approaches that include extensive restriction endonuclease mapping and Southern blot analysis or comparative genomic hybridization, QF-MPCRs approach is easier to perform and less expensive and feasible for high-throughput analysis. Although MLPA is successful in detecting deletions, it has poor resolution to 'pinpoint' the breakpoints as only a limited number of probes can be used in one MLPA assay. Use of multiple MLPA assays containing tiled probes will make the process cumbersome and expensive. Although QF-MPCRs have been described earlier, this method has not been used extensively which may be due to the lack of reproducibility of the previously reported protocols (23–25). We found the design of primers, amplification reaction conditions using specific enzymes and careful determination of the number of cycles in the exponential phase at which the quantitation is performed are important parameters for successful and reproducible copy number analysis. We have successfully applied this strategy to detect six different deletions including two were novel deletions (Fig. 3) and characterized the molecular basis for $(\delta\beta)^0$-thalassaemia and HPFH in all of the 51 unrelated families in Indian population.

Acknowledgement

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. GD-QF-MPCR for the identification of copy numbers of γ-, δ- and β-globin genes. (A) Electropherograms of labeled PCR amplified products of γ-, δ- and β-globin and albumin genes. The peak heights of amplified products of the globin genes and the control albumin gene are shown. (B) Intra- and internormalized peak heights for globin genes in samples with different mutations. Arrows indicate the reduction in the copy number of the genes.

Figure S2. Detection of 32.6 and 49.3 kb deletions. (A and D) Position of the primers used for Gap-PCR to amplify across the break points and the results of agarose gel electrophoresis. (B and E) Characterization of the breakpoints by DNA sequencing of the amplified products obtained by Gap PCRs (C) Electropherograms of labelled amplified products obtained from 18, 21 and 28 kb downstream (DS) of β-globin gene in the 49.3 kb deletion. (Arrows indicate the reduction in copy number of genes).

Table S1. Details of the oligonucleotide primers used in this study.