Effect of a group of genetic markers around the 5′ regulatory regions of the β globin gene cluster linked to high HbF on the clinical severity of β thalassemia

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A R T I C L E   I N F O
Article history:
Communicated by Sir D. Weatherall, F.R.S.,
3 November 2012
(Submitted 26 September 2012
Available online 2 December 2012)

Keywords:
β Thalassemia intermedia
Pre G γ globin haplotype
Ay–δ intergenic region haplotype,
β-LCR

A B S T R A C T

The clinical and hematological course of β thalassemia intermedia is influenced by a number of genetic factors which play a role in increasing fetal haemoglobin levels. Several polymorphisms located in the promoters of β and γ globin gene are involved in influencing the disease severity. Our objective was to study the effect of cis-DNA haplotypes, motifs, or polymorphisms (Pre G γ globin gene haplotypes, Ay–δ intergenic region haplotypes XmnI and (AT)x(T)y polymorphisms, β-LCR HS2 and HS3 site motifs) that may contribute to higher HbF levels and a milder clinical course. We found that a combination of T haplotype of the Ay–δ intergenic region, TAG Pre-Gγ haplotype, presence of the XmnI polymorphism along with the (AT)x(T)y motif constitutes a topography that co-relates with raised HbF levels which may contribute in ameliorating the disease severity.

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I N T R O D U C T I O N

There is considerable heterogeneity in the clinical presentation of β thalassemia homozygotes which spans from the transfusion-dependent cases of thalassemia major to the various forms of thalassemia intermedia having a relatively milder course. The molecular basis of the variable clinical manifestations comprises the type of defective genes and a large number of known or unknown DNA alterations. The contribution of cis-acting elements to HbF production is critical to understand the variable clinical phenotype of β-thalassemia and sickle cell anemia. There are reports showing that sequence variations within regulatory sequences of the human β-globin and Aγ globin gene are involved in increasing fetal haemoglobin levels. Several polymorphisms located in the promoters of β and γ globin gene are involved in influencing the disease severity. Our objective was to study the effect of cis-DNA haplotypes, motifs, or polymorphisms (Pre G γ globin gene haplotypes, Ay–δ intergenic region haplotypes XmnI and (AT)x(T)y polymorphisms, β-LCR HS2 and HS3 site motifs) that may contribute to higher HbF levels and a milder clinical course. We found that a combination of T haplotype of the Ay–δ intergenic region, TAG Pre-Gγ haplotype, presence of the XmnI polymorphism along with the (AT)x(T)y motif constitutes a topography that co-relates with raised HbF levels which may contribute in ameliorating the disease severity.

MATERIALS AND METHODS

Peripheral blood samples were collected from 79 β thalassemia homozygous, 11 sickle cell anemia and 14 sickle-β thalassemia individuals. Fifty age and sex matched healthy individuals were enrolled as the normal control group. The study was approved by the Institutional Ethics Committee. An informed consent was taken from the families before sample collection.

All the hematological parameters in transfused cases were measured 2 months after the last transfusion. Red cell indices were measured on an automated blood cell counter (Sysmex K 1000). HbA2 and HbF levels were measured using cation exchange HPLC on the Variant Hemoglobin Testing System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Genomic DNA was isolated from peripheral blood leucocytes using the QiAamp Blood Mini Kit. The β globin gene mutations were characterized using covalent reverse dot blot hybridization (CRDB) and amplification refractory mutation system (ARMS) techniques. The uncharacterized samples were subjected to DNA sequencing. XmnI and the Ay–δ intergenic region polymorphisms were studied using PCR-RFLP [6]. Gene scan analysis was used to study the (AT)x(T)y motif at the −530 region upstream of the 5′ β-globin gene. The −530 region of the β globin gene was amplified using specific primers. The PCR product was then used for fragment analysis and the sizing was done using ROX 500 as the size standard. Size calling of the peaks was done using the fragment analysis software. The polymorphic sites in the Pre Gγ globin gene region and the β globin LCR region motifs were studied by direct DNA sequencing. Fig. 1 shows the schematic representation of all the modifiers studied across the β globin gene cluster on chromosome 11.
Results

Clinical evaluation

Table 1 summarizes the clinical and hematological data of all the patients. Of our 79 β-thalassemia homozygotes, 40 patients had a severe clinical presentation requiring regular blood transfusions (β-thalassemia major). They presented within 2 years of age with pallor and icterus. The remaining 39 individuals showed a milder clinical presentation (β-thalassemia intermedia). They presented later in life (2–31 years). In the thalassemia intermedia group, 6 patients had received intermittent transfusions while 4 cases were on regular transfusion after 5 years of age and 29 cases were untransfused. Hepatosplenomegaly was pronounced in the thalassemia intermedia group. Fifty-four percent of the cases showed a spleen size more than 6 cm (1–14 cm). One patient from this group was splenectomized.

Hematological analysis

There were no significant differences seen in the mean values for all the parameters in the two groups [p > 0.05] except the HbF levels (Table 1). Most of our patients from the thalassemia major group were on regular blood transfusion therapy. The HbF values among them ranged from 0.03 to 7.49 g/dL. The ages of the patients when HbF was measured varied from 7 months to 10 years. In the thalassemia intermedia group the HbF values ranged from 1.0 to 7.55 g/dL with a mean of 2.72 g/dL. The ages of the patients in this group ranged from 2 years to 31 years.

Genotypic analysis

β Globin genotype

Thirteen different β globin gene mutations were encountered among the thalassemia homozygotes. IVS1 nt 5 (G→C) was the most prevalent mutation (57%) followed by IVS1 nt 1 (G→T) (10.75%). Among the sickle-β thalassemia group, 8 patients had a β^0 mutation (codon 15 (G→A) and codon 30 (G→C)) while the remaining 6 cases showed presence of the β^+ IVS1 nt 5 (G→C) mutation.

Polymorphisms and motifs. The detailed genotypic analysis was carried out in short repeat sequences—motifs, polymorphism along the β globin gene cluster that are involved in the regulation of transcription of the β globin gene.

XmnI polymorphism. Chromosomes (62.5%) in the milder group showed the presence of the XmnI polymorphism as against only 27.5% in the thalassemia major group (p < 0.0001). All the sickle cell anemia cases were homozygous for the XmnI polymorphism. Among the normal controls, 35.5% of the chromosomes showed the presence of the −158G→C→T polymorphism, among them the Hbf levels ranged from 0.01 to 0.22 g/dL.

The (AT)_3(T)_9 motifs −530 bp β globin gene. The analysis of the (AT)_3(T)_9 region showed three different motifs—(AT)^7(T)$_9$, (AT)$_9$(T)$_9$ and (AT)$_{10}$(T)$_9$ which gave rise to 6 genotypes. Among the β thalassemia homozygotes, 25.7% of the chromosomes in the thalassemia intermedia group showed the presence of the (AT)$_{10}$(T)$_9$ motif. Chromosomes (25%) from the milder group showed the presence of both the XmnI polymorphism and the (AT)$_{10}$(T)$_9$ motif. The Hb F levels among these patients varied from 2.27 to 7.55 g/dL. All the 4 chromosomes carrying the codon 15 (G→A) mutation in the thalassemia intermedia group were linked to the (AT)$_{10}$(T)$_9$ motif. Sickle chromosomes (77.27%) showed the presence of this motif. Among the control group the (AT)$_{10}$(T)$_9$ reference motif was predominantly present (97%).

Motifs of β globin LCR. The β-LCR region is involved in developmental stage and tissue specific expression of the β globin gene. We studied the HS2 and the HS3 motifs which are thought to be enhancers of the linked β globin gene. DNA sequencing of the HS2 region of the LCR showed six polymorphic repeat patterns of (TA)$_n$(TA)$_m$. The configuration (TA)$_n$(TA)$_m$ associated with thalassemia intermedia phenotype was well spread among both the severe and milder group. However among the sickle cell cases the (AT)$_{10}$(TA)$_{12}$ motif was seen in 54.54% of the patients. Among the control group the motif was equally distributed. The HS3 sequence did not show any change with respect to the reference sequence in both the samples as well as the control individuals.

Pre G γ globin gene haplotypes. The combination of point mutations T, A, G at position −1450, −1280 and −1225 relative to the cap site of G γ globin gene form the Pre G γ globin gene haplotypes. A combination of these gave rise to four Pre G γ globin gene haplotypes—TAG, TAA, TGA and TGG. The TAG haplotype is shown to be associated with the Senegal Sickle cell β haplotype and with elevated levels of Hbf. The TAG haplotype was encountered in 56.4% of the chromosomes from the thalassemia intermedia group as compared to 15.8% of the chromosomes of the thalassemia major group (p < 0.001). Interestingly here also the 4 chromosomes carrying the Codon 15 (G→A) mutation from the thalassemia intermedia group showed linkage to the TAG haplotype like the (AT)$_{10}$(T)$_9$ motif. All the sickle chromosomes showed the presence of this haplotype showing its linkage to the Arab Indian haplotype like XmnI polymorphism. Among the control group, the haplotype TGG was predominantly seen (64%).

Ay–δ intergenic region haplotypes. The 2 haplotypes are known for Ay–δ intergenic region, one referred as R (reference) and another as T (mutant). These 2 haplotypes differ in at least 8 polymorphic sites [6]. The haplotype T linked to high Hbf level was predominantly present among the thalassemia intermedia cases with 72% of the patients showing homozygosity. The patients showing presence of the T haplotype showed higher Hbf levels as compared to those showing its absence (Table 2). All the sickle cell chromosomes were homozygous for the T haplotype. Among our control population the reference haplotype R was predominantly present (54.54%).

Discussion

The clinical manifestation of β thalassemia homozygous cases is influenced by many factors. The disease severity varies considerably, even among those with identical beta-thalassemia mutations and when known epistatic genetic factors, such as alpha-thalassemia, are considered. Most of this heterogeneity can be linked to the capacity to produce variable amounts of Hbf [7]. It is believed that there is genetic...
### Table 1
Clinical and haematological details of β thalassemia patients.

<table>
<thead>
<tr>
<th>Hemoglobinopathy</th>
<th>Age at presentation</th>
<th>RBC ×10^6/μL (mean±SD)</th>
<th>Hb (gm/dL) (mean±SD)</th>
<th>MCV (fl) (mean±SD)</th>
<th>MCH (pg) (mean±SD)</th>
<th>HbA2 % (mean±SD)</th>
<th>HbF (g/dL) (mean±SD)</th>
<th>HBS (mean±SD)</th>
<th>Liver (cm) (mean±SD)</th>
<th>Spleen (cm) (mean±SD)</th>
<th>Transfusion requirement (units/year) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Thalassemia major (n = 40)</td>
<td>4 months–2 years</td>
<td>3.22±0.90</td>
<td>8.8±2.68</td>
<td>78.97±5.78</td>
<td>27.19±2.79</td>
<td>3.05±0.55</td>
<td>2.04±3.04</td>
<td>1.67±2.25</td>
<td>2.05±2.81</td>
<td>11.92±3.83</td>
<td>9.54±3.83</td>
</tr>
<tr>
<td>β Thalassemia intermedia (n = 39)</td>
<td>2–31 years</td>
<td>3.47±0.67</td>
<td>7.65±1.56</td>
<td>68.86±8.60</td>
<td>22.14±3.12</td>
<td>3.39±1.46</td>
<td>5.38±2.72</td>
<td>2.24±2.67</td>
<td>4.55±3.96</td>
<td>2.48±4.58</td>
<td>3.06±4.58</td>
</tr>
<tr>
<td>Sickle cell anemia (n = 11)</td>
<td>1–24 years</td>
<td>3.23±0.57</td>
<td>8.64±1.57</td>
<td>79.7±9.37</td>
<td>26.76±3.33</td>
<td>2.98±0.89</td>
<td>1.91±0.78</td>
<td>72.44±6.16</td>
<td>2.72±4.10</td>
<td>4.5±5.56</td>
<td>0.36±0.50</td>
</tr>
<tr>
<td>Sickle-β thalassemia (n = 14)</td>
<td>1–21 years</td>
<td>6.61±10.21</td>
<td>9.14±2.13</td>
<td>72.8±7.43</td>
<td>23.94±2.44</td>
<td>4.55±0.77</td>
<td>2.24±1.37</td>
<td>59.7±14.27</td>
<td>1.28±1.43</td>
<td>2.35±3.20</td>
<td>0.07±0.26</td>
</tr>
</tbody>
</table>

* Some of our thalassemia major patients were on regular transfusion.

### Table 2
The effect of the presence of different DNA motifs and polymorphisms linked to raised Hb F on the clinical severity among β thalassemia homozygotes.

<table>
<thead>
<tr>
<th>Pre G γ globin haplotype (no.)</th>
<th>HbF (g/dL) (mean±SD)</th>
<th>p value</th>
<th>Aγ–δ intergenic region haplotype (no.)</th>
<th>HbF (g/dL) (mean±SD)</th>
<th>p value</th>
<th>Xml polymorphism (no.)</th>
<th>HbF (g/dL) (mean±SD)</th>
<th>p value</th>
<th>(AT)T5 polymorphism (no.)</th>
<th>HbF (g/dL) (mean±SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of TAG</td>
<td>Absence of TAG</td>
<td>Presence of T</td>
<td>Absence of T</td>
<td>Presence of T</td>
<td>Absence of T</td>
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<td>Presence of T</td>
<td>Absence of T</td>
</tr>
<tr>
<td>β Thalassemia intermedia (n = 39)</td>
<td>(22) 5.66±2.50</td>
<td>(17) 4.96±3.16</td>
<td>p = 0.47</td>
<td>(29) 5.83±2.55</td>
<td>(10) 3.23±2.45</td>
<td>p = 0.07</td>
<td>(25) 5.83±2.61</td>
<td>(14) 3.96±2.90</td>
<td>p = 0.12</td>
<td>(10) 5.64±2.87</td>
<td>(29) 5.02±2.59</td>
</tr>
<tr>
<td>β Thalassemia major (n = 40)</td>
<td>(6) 3.73±2.71</td>
<td>(34) 1.79±3.04</td>
<td>p = 0.19</td>
<td>(12) 0.97±1.87</td>
<td>(28) 2.94±3.35</td>
<td>p = 0.03</td>
<td>(11) 1.68±2.13</td>
<td>(29) 2.31±3.59</td>
<td>p = 0.50</td>
<td>(6) 1.93±3.78</td>
<td>(34) 2.08±2.81</td>
</tr>
<tr>
<td>Sickle cell chromosomes (n = 36)</td>
<td>(33) 2.19±1.09</td>
<td>(3) 1.41±1.35</td>
<td>p = 0.42</td>
<td>(36) 2.09±4.12</td>
<td>(36) 2.09±4.12</td>
<td>p = 0.03</td>
<td>(23) 2.19±1.06</td>
<td>(13) 1.83±1.33</td>
<td>p = 0.54</td>
<td>(23) 2.19±1.06</td>
<td>(13) 1.83±1.33</td>
</tr>
</tbody>
</table>
variation in cis-acting elements and trans-acting factors implicated in gamma-globin gene expression, in modulation of HbF concentration within erythrocytes, and in regulation of erythroid cell differentiation and proliferation. Several genetic loci can modify γ chain production. It is now clear that many different genes are involved in this. Some are located within the β globin cluster and others on different chromosomes. In this study we tried to identify some of these genetic variations on the β globin cluster and their effect on clinical severity. Many studies on analysis of Hbf regulating variants using genetic association done recently have shown two main quantitative trait loci (QTLs) on chromosome 6 (HBS1L/MYB) and chromosome 2 (BC11/1A) which are associated with raised Hbf levels [8,9]. A study by He et al. reported similar findings in β thalassemia patients [10]. Few studies have shown the association of a region on chromosome 8 (TOX) and the K chromosome with raised Hbf levels [11,12]. We have not looked into these determinants in our study.

Several genetic loci are candidates for cis acting regulators of β globin gene transcription [13]. In addition to the well documented markers like Xmml and (AT)x(T)y polymorphisms, we tried to study the configuration of Pre G γ, Aγ-δ intergenic region haplotypes and the β globin LCR (HS2,HS3) which are proposed to contribute to raised Hbf levels [6].

The Pre G γ globin haplotypes have been shown to be linked to the β globin haplotype and Hbf levels in studies on sickle patients [14]. The Pre G γ globin haplotype TAG was shown to be associated with the Senegal haplotype with elevated levels of Hbf. In our study all sickle cell chromosomes except 3 chromosomes showed presence of the TAG Pre G γ globin haplotype showing its linkage to the Arab-Indian haplotype. Similar observation was reported by Pissard and Beuzard [15]. The association of both these β globin gene haplotypes with the TAG Pre G γ globin haplotype and elevated Hbf levels may be due to sharing of the same RFLP pattern around the 2 γ globin genes. The study of Ofori-Acquah et al. [16] has reported a dominant influence of the γ globin gene promoter on Hbf expression in sickle cell anemia patients. Another study on analysis of hybrid haplotype chromosomes of sickle cell anemia patients showed that variability in Hbf F level is associated with the Pre G γ frame work [14]. Our thalassemia intermedia patients (56.4%) showed the presence of the TAG haplotype. The patients showing presence of this haplotype showed higher levels of Hbf as against patients showing absence of the TAG haplotype (Table 2). Similar results were documented by Papachatzopoulos et al. [6], only none of their thalassemia major cases showed the TAG haplotype. However we did see the presence of this haplotype in 15% of our thalassemia major patients also (Table 3).

The intergenic region between the Aγ and δ globin genes has been reported to contribute in intergenic transcription [17]. It is thought to contribute when the γ globin gene is switched-off and δ and β globin genes are switched on in adults [18]. Webster et al. [19] analyzed the intergenic region in 840 chromosomes from a global sampling of human populations. Two divergent haplotypes R (reference) and T (mutant) were found to predominate in all populations studied. Papachatzopoulos et al. [6] reported in their study that the intergenic haplotype T even in heterozygosity was sufficient in shifting the disease severity to an intermedia phenotype. The haplotype T was absent among their thalassemia major patients, while in our study 30% of our thalassemia major patients showed presence of this haplotype against 75% of thalassemia intermedia patients. All our sickle cell chromosomes showed the presence of the T haplotype confirming its linkage with the Arab-Indian haplotype and raised Hbf.

The (TA)xN12(TA)z motifs generate several configuration isomers which are involved in chromatin remodeling and play a role in gene regulation. The (TA)10N12(TA)12 motif is found to be associated with raised Hbf in thalassemia intermedia patients [20, 21]. Kukreti et al. [21] in their study on β thalassemia reported that there was linkage disequilibrium between the G allele at HS4 and the (TA)9N12(TA)10 motif. In our study, we did not find significant differences in the prevalence of the (TA)9N12(TA)10 motif among the severe and milder groups. The (TA)10N12(TA)12 motif has been shown to be associated with the Arab-Indian haplotype by Perichon et al. [5]. Among our patients 54.4% of sickle cell chromosomes showed presence of the (TA)10N12(TA)12 motif.

Association of haplotypes with a milder phenotype

This is a 1st large scale study carried out to see the regulatory effect of 6 different cis-DNA haplotypes, motifs, or polymorphisms that may correlate with the presence of high Hbf and clinical outcome of the disease. It is also a 1st report showing the linkage of TAG Pre G γ globin haplotype and T Aγ-δ intergenic haplotype to Arab Indian haplotype in Sickle cell anemia patients. When we tried to look at the association between presence of modifying factors with the disease severity, we observed that the presence of all the 4 modifying factors (TAG haplotype, intergenic T haplotype, − 158G γ T allele and (AT)x(T)y motif) was seen only among the thalassemia intermedia cases (Fig. 2). Thalassemia intermedia patients (89.74%) showed presence of 2 or more modifiers as compared to 41.05% of the thalassemia major cases. Any 3 of the factors studied were required to ameliorate the disease severity (Fig. 2).

Thus our study of the sickle cell chromosomes confirm the linkage of the γ globin gene determinants with the Arab Indian haplotype in our sickle cell anemia patients. Though we did not find a statistically significant association between high Hbf genetic determinants and Hbf levels except in the T Aγ-δ intergenic haplotype (p = 0.07) and the TAG Pre G γ haplotype (p = 0.47), there was a trend of high Hbf levels in most of our patients showing presence of the Hbf genetic determinants against the patients showing their absence (Table 2). It seems that a combination of the TAG Pre G γ globin haplotype, T Aγ-δ

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Table 3

<table>
<thead>
<tr>
<th>Pre G γ globin haplotype (TAG)</th>
<th>p value</th>
<th>Aγ-δ intergenic region haplotype (no. of chr [%])</th>
<th>p value</th>
<th>Xmml polymorphism (no. of chr [%])</th>
<th>p value</th>
<th>(AT)x(T)y polymorphism (no. of chr [%])</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Thalassemia intermedia chromosomes (n = 78)</td>
<td>44 (56.4%)</td>
<td>61 (78.2%)</td>
<td>49 (62.5%)</td>
<td>20 (25.6%)</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β Thalassemia major chromosomes (n = 80)</td>
<td>12 (15.2%)</td>
<td>24 (30%)</td>
<td>11 (13.7%)</td>
<td>0.09</td>
<td></td>
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</tbody>
</table>
intergenic region haplotype and the (AT)_30(T)5 configuration of the β-globin silencer region constitutes a topography that co-relates with raised HbF levels. This combination might have helped our patients in decreasing the disease severity.

Author's contribution

All authors have contributed sufficiently to the project to be included as authors.

• PD performed the research and wrote the paper
• AN designed the research study and helped in writing the paper
• RC contributed in designing of research study
• KG contributed in data analysis

Conflict of interest

All authors have read and approved the manuscript. None of the authors had a conflict of interest related to this manuscript.

Acknowledgments

This work was funded by Indian Council of Medical Research (ICMR), New Delhi, India (grant to AN and PD).

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