The influence of the BCL11A polymorphism on the phenotype of patients with beta thalassemia could be affected by the beta globin locus control region and/or the Xmn1-HBG2 genotypic background

Maryam Neishabury a,⁎, Fahimeh Zamani a, Elahe Keyhani a, Azita Azarkeivan c, Seyedeh Sedigheh Abedini a, Masumeh Sadat Esfami c, Setareh Talebi Kakroodi a, Mahjoobeh Jafari Vesiehsaria a, Hossein Najmabadi b

a Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran
b Kariminejad-Najmabadi Pathology & Genetics Center, Tehran, Iran
c Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Department of Thalassaemia Clinic, Tehran, Iran

corresponding author at: Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Daneshjoo Blvd, Koodakyar St, Evin, Tehran, Iran
E-mail address: nneisha@gmail.com (M. Neishabury).

ARTICLE INFO

Article history:
Submitted 23 January 2013
Revised 20 February 2013
Available online 28 March 2013

(Communicated by Sir D. Weatherall, F.R.S., 23 February 2013)

Keywords:
Beta thalassemia
BCL11A
5’HS4
Beta globin LCR
Xmn1-HBG2

ABSTRACT

To study the influence of the β globin locus control region (LCR) genotypic background on the phenotype modifying role of BCL11A polymorphisms, 100 cases of thalassemia, 48 homozygous for the A allele and 52 homozygous for the G allele at the 5’HS4-LCR palindromic polymorphic site were genotyped for two BCL11A single nucleotide polymorphisms (rs11886868 and rs766432) in the intronic region of this gene. The effect of these polymorphisms on HbF variation was also examined in 122 normal individuals. The 5’HS4-LCR had the most significant role in determining the phenotype of these thalassemia patients. BCL11A polymorphisms showed a significant role in determining the phenotype of patients homozygous for the G allele at 5’HS4-LCR. However, the majority of patients homozygous for the A allele at 5’HS4-LCR, showed a severe phenotype, regardless of the BCL11A genotype. These results, without undermining the strength of BCL11A as a silencer of the γ globin gene, suggest that the LCR background, by governing the state of the LCR background on phenotype modifying effect of 2 BCL11A SNPs within intron 2 of this gene, (rs11886868 and rs766432) [8,11], was examined in 100 Iranian patients with different 5’HS4-LCR backgrounds in the homozygous state and in 122 normal individuals.

Design and methods

After ruling out mild and silent β thalassemia, alpha globin gene mutations and delta beta deletions [1,12], a cohort of patients, homozygous or compound heterozygous for severe β thalassemia mutations, 52 homozygous for the G allele and 48 homozygous for the A allele at 5’HS4-LCR, were selected for this study (Supplementary Table 1). As we have tried to select equal number of patients with different 5’HS4-LCR polymorphisms, both associated with the clinical phenotype in patients with thalassemia [1–3]. Based on the chromatin modifying potential of the latter region [4–6], we suggested that the phenotype modifying role assigned to Xmn1-HBG2 could be played by more functionally potent elements linked to it in LCR [1,2].

The palindromic stretch in 5’HS4 plays a role in regulating globin gene expression by influencing the binding of transcription factors [6]. On the other hand, ChIP-chip experiments have demonstrated binding of BCL11A, a silencer of the γ globin gene, to LCR-HS1, HS2 and HS3 [7,8]. We propose that 5’HS4-LCR polymorphic alleles, which are in linkage disequilibrium with other LCR polymorphic sites, including those in 5’HS2 [9,10], could affect interactions of BCL11A with LCR either directly or indirectly, thereby affect the phenotype modifying role of BCL11A polymorphism in patients with thalassemia. To further examine this hypothesis, the influence of the 5’HS4-LCR genotypic background on phenotype modifying effect of 2 BCL11A SNPs within intron 2 of this gene, (rs11886868 and rs766432) [8,11], was examined in 100 Iranian patients with different 5’HS4-LCR backgrounds in the homozygous state and in 122 normal individuals.

Introduction

We previously have observed a linkage disequilibrium between the positive/negative Xmn1-HBG2 profile and the G/A allele at the polymorphic palindromic sequence of 5’HS4 (TGGGG A/G CCCCA), both associated with the clinical phenotype in patients with thalassemia [1–3]. Based on the chromatin modifying potential of the latter region [4–6], we suggested that the phenotype modifying role assigned to Xmn1-HBG2 could be played by more functionally potent elements linked to it in LCR [1,2].

The palindromic stretch in 5’HS4 plays a role in regulating globin gene expression by influencing the binding of transcription factors [6]. On the other hand, ChIP-chip experiments have demonstrated binding of BCL11A, a silencer of the γ globin gene, to LCR-HS1, HS2 and HS3 [7,8]. We propose that 5’HS4-LCR polymorphic alleles, which are in linkage disequilibrium with other LCR polymorphic sites, including those in 5’HS2 [9,10], could affect interactions of BCL11A with LCR either directly or indirectly, thereby affect the phenotype modifying role of BCL11A polymorphism in patients with thalassemia. To further examine this hypothesis, the influence of the 5’HS4-LCR genotypic background on phenotype modifying effect of 2 BCL11A SNPs within intron 2 of this gene, (rs11886868 and rs766432) [8,11], was examined in 100 Iranian patients with different 5’HS4-LCR backgrounds in the homozygous state and in 122 normal individuals.

Design and methods

After ruling out mild and silent β thalassemia, alpha globin gene mutations and delta beta deletions [1,12], a cohort of patients, homozygous or compound heterozygous for severe β thalassemia mutations, 52 homozygous for the G allele and 48 homozygous for the A allele at 5’HS4-LCR, were selected for this study (Supplementary Table 1). As we have tried to select equal number of patients with different 5’HS4-LCR.
The clinical phenotype of the patients was assigned according to their stable blood transfusion profile, based on current definitions [14] and at least 10 years follow-up. Sixty-seven patients were blood transfusion-dependent, with a severe phenotype (S), having started receiving transfusion before 2 years of age and dependent on regular blood transfusion at less than 30 day intervals. Thirty-three patients were not blood transfusion-dependent. These included 24 patients with mild phenotype (M), who did not require either blood transfusion while on hydroxyurea treatment, and 9 patients, who showed an exceptionally mild phenotype (E), who did not require blood transfusions or hydroxyurea treatment. The other study group included 122 individuals with normal hematological indices (normal hemoglobin, mean cell volume (MCV), mean cell hemoglobin (MCH), and HbA2 levels) (Supplementary Table 2).

The alkali denaturation method was used to determine the HbF levels in normal individuals. A standard salting out procedure was used for DNA extraction. The Xmn1 polymorphism was detected using PCR-RFLP [15].

The effect of 5′HS4-LCR on phenotype of patients

There was a significant difference in the frequency in the patients population in Iran [13]. This is due to the linkage disequilibrium relationships that exist between 5′HS4, Xmn1-HBG2 and certain beta thalassaemia mutations [1].

### Results

#### Allelic frequencies and associations

A strong linkage disequilibrium was observed between the A allele at 5′HS4 and negative Xmn1-HBG2 profile, both in the normal and patient population (92.3% association in normal population and 95.8% in patient population). No linkage disequilibrium was detected between the G allele at 5′HS4 and the positive Xmn1 profile in the normal population (59.1% with A, 40.9% with G), while a strong linkage disequilibrium (96.1%) was observed between the G allele at 5′HS4 and + Xmn1-HBG2 in the patient population. The allelic frequencies for BCL11A SNPs, rs766432 and rs11886868, were analyzed by the polymerase chain reaction (PCR) and DNA sequencing was performed using a pair of primers: 5′ GCAATCTCATTTCTCTGAAATGT and 5′ ATCTACACAGTGTCCATTGTAGCACT. The ABI Prism 377 DNA Automatic Sequencer (Perkin Elmer, Foster City, CA) and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit were applied for DNA extraction. The Xmn1 polymorphism was detected using PCR-RFLP [15].

5′HS4-LCR was analyzed as previously reported [13]. Two BCL11A SNPs, rs766432 and rs11886868, were analyzed by the polymerase chain reaction (PCR) and DNA sequencing was performed using a pair of primers: 5′ GCAATCTCATTTCTCTGAAATGT and 5′ ATCTACACAGTGTCCATTGTAGCACT. The ABI Prism 377 DNA Automatic Sequencer (Perkin Elmer, Foster City, CA) and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit were applied for DNA sequencing.

### Table 1

<table>
<thead>
<tr>
<th>Allelic frequencies among 100 thalassemia patients and 122 normal individuals.</th>
<th>Total no. and allelic freq.</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low HbF</td>
<td>HS4-LCR: A/G</td>
</tr>
<tr>
<td>S (n = 67)</td>
<td>M (n = 24)</td>
<td>E(n = 9)</td>
</tr>
<tr>
<td>G</td>
<td>96 (48%)</td>
<td>92 (64.3)</td>
</tr>
<tr>
<td>C</td>
<td>104 (52%)</td>
<td>72 (35.7)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T (n = 26)</td>
<td>A (n = 24)</td>
<td>C(n = 2)</td>
</tr>
<tr>
<td>A</td>
<td>190 (77.9%)</td>
<td>186 (76.2)</td>
</tr>
<tr>
<td>C</td>
<td>45 (22.1%)</td>
<td>39 (44.7)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL11A (rs766432)</td>
<td>A (n = 24)</td>
<td>C(n = 18)</td>
</tr>
<tr>
<td>A</td>
<td>155 (77.5%)</td>
<td>104 (103.8)</td>
</tr>
<tr>
<td>C</td>
<td>45 (22.5%)</td>
<td>30 (30.1)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>134</td>
</tr>
</tbody>
</table>

The thalassemia patients included 67 with severe (S), 24 with mild (M), and 9 exceptionally mild (E) phenotypes. Normal individuals included 25 with low HbF (L) and 18 with high HbF values (H).
the patients with milder clinical phenotype (M and E) was significantly higher in patients with a G/G 5′HS4 genotype than among patients with an A/A 5′HS4 genotype ($P = 1 \times 10^{-7}$) (Table 2). The effect of BCL11A polymorphism on phenotype of patients or HbF values of normal individuals

The frequency of the ameliorating allele (C) for both BCL11A SNPs, rs11886868 (C/T) and rs766432 (C/A), was not significantly higher in patients with milder phenotype or in normal individuals with high HbF levels (Table 1). There was no difference in frequency of different clinical phenotypes in patients or in the mean HbF values in normal individuals between groups with different BCL11A alleles in the homozygous state (Table 2).

The effect of BCL11A SNPs in patients homozygous for the G allele at 5′HS4-LCR

From 48 patients homozygous for the A allele in 5′HS4-LCR, 46 showed a severe phenotype regardless of the BCL11A genotype. However, in 52 patients homozygous for the G allele, there was phenotypic heterogeneity, as expected [16]. In the latter case, the BCL11A polymorphism did have a significant effect, with P-values of 0.032 and 0.047 for rs11886868 and rs766432, respectively (Table 3). Of the15 patients that were homozygous for the C allele at BCL11A/rs11886868, and had a G/G 5′HS4 background, only 3 (20%) had a severe phenotype. Twelve (80%) of these had mild, including 5 with exceptionally mild (E), clinical phenotypes. On the other hand, of 37 patients with a T allele for this SNP in the homozygous or double heterozygous state, the severe and mild phenotypes showed almost equal frequencies. For the other BCL11A SNP rs766432, the effect of the ameliorating allele (C) was stronger, observed in the heterozygous state as well. Of 17 patients that had the BCL11A-rs766432 (C) allele in the homozygous or heterozygous state, only 4 (23.5%) showed a severe phenotype, while 13 (76.5%) of them showed milder phenotypes, including 6 with exceptionally mild clinical phenotypes. Of 35 patients homozygous for the A allele at rs766432, almost half had a severe and half had a mild phenotype (Table 3). Because of the low number of normal individuals with GG 5′HS4-LCR background (only 6 out of 122), the same comparison could not be performed (Supplementary Table 2).

Different haplotypes of 5′HS4 and BCL11A

Three different haplotypic groups comprising 5′HS4 and BCL11A SNPs in the homozygous state were identified in our patients in this study (Table 4). For individuals with the same BCL11A haplotype, the frequency of milder phenotypes was significantly higher in patients with a G/G 5′HS4-LCR background than those with an A/A 5′HS4-LCR background. However, no significant difference was observed in the pattern of phenotype distribution among the different groups.

Discussion

Our findings support the association between the G/A polymorphic alleles and +/− Xmn1-HBG2 profile, as well as their association with certain β thalassemia mutations (Supplementary Table 1). The significant role of 5′HS4-LCR polymorphism in determining patient’s phenotype is confirmed, as we have previously reported [1,2]. In addition, a higher frequency of the 5′HS4-LCR G allele, observed in normal individuals with higher HbF values, provide quantitative support for the role of 5′HS4 polymorphism in controlling γ globin gene expression [1,2]. We did not observe a significant difference in mean HbF values between normal individuals with 5′HS4-LCR A and G alleles in the homozygous state. Also, no significant influence was observed for BCL11A SNPs on HbF values of normal individuals

<table>
<thead>
<tr>
<th>Locus (5′HS4)</th>
<th>Genotype</th>
<th>Patient</th>
<th>Observed no. (expected no.)</th>
<th>Mid-P exact P-value</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCR (5′HS4)</td>
<td>A/A</td>
<td>48 (48%)</td>
<td>46 (32.16) 2 (11.52) 0 (4.32)</td>
<td>2 (18.84) 1 × 10 −7</td>
<td>74 (92.5%) 0.7 0.22 0.1968</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>52 (52%)</td>
<td>21 (34.84) 22 (12.48) 9 (4.58)</td>
<td>31 (17.6)</td>
<td>6 (7.5%) 0.82 0.17</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>100</td>
<td>67 24 9</td>
<td>33</td>
<td>80 (100%) 0.71 0.21</td>
</tr>
<tr>
<td>BCL11A (rs11886868)</td>
<td>T/T</td>
<td>19 (38.8%) 13 (20.3) 5 (4.7)</td>
<td>2 (2.2) 6 (7)</td>
<td>0.2862</td>
<td>36 (61%) 0.7 0.22 0.4762</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>30 (61.2%) 18 (36.0) 7 (7.3)</td>
<td>5 (7.1) 12 (11.0)</td>
<td>0.4415</td>
<td>23 (39%) 0.74 0.19</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49</td>
<td>31 6 2</td>
<td>21</td>
<td>59 (100%) 0.7169 0.2118</td>
</tr>
<tr>
<td>BCL11A (rs766432)</td>
<td>A/A</td>
<td>62 (89.9%) 42 (62.25) 17 (15.3) 3 (4.5)</td>
<td>20 (19.8)</td>
<td>0.4415</td>
<td>71 (91%) 0.74 0.22 0.1578</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>7 (10.1%) 5 (7.45)</td>
<td>0 (1.7)</td>
<td>2 (2.2)</td>
<td>7 (9%) 0.86 0.08</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>69</td>
<td>47 17 5</td>
<td>22</td>
<td>78 (100%) 0.7487 0.2161</td>
</tr>
</tbody>
</table>

The thalassemia patients included those with severe (S), mild (M), and exceptionally mild (E) phenotypes. Normal individuals included those with low HbF (L) or high HbF values (H).
(Table 2). These findings could be explained by the fact that these modifying factors are more active under conditions of erythropoietic stress in thalassemia patients. [17] To detect their effect on HbF values of normal individuals, a larger sample size is needed.

No significant influence was observed of BCL11A SNPs on the phenotypic variation of patients (Table 1–2). However, when we studied the influence of BCL11A polymorphism on the patient phenotype, in two different 5′HS4-LCR homozygous backgrounds separately, we observed a different result. A significant role was observed for BCL11A polymorphisms on phenotypic variation in patients with GG 5′HS4 LCR background (Table 3). On the other hand, 46 out of 48 patients with A/A 5′HS4-LCR background, show a severe phenotype, regardless of BCL11A genotype, implying that AA 5′HS4-LCR background might have a masking effect on influence of the BCL11A polymorphisms on patient phenotype. Furthermore, the pattern of phenotype distribution in the thalassemia patients with the same genotype for BCL11A and different LCR backgrounds showed a significant difference (Table 4) which confirms the influence of the 5′HS4-LCR background on the phenotype modifying role of BCL11A. In 7 patients, homozygous for ameliorating alleles of 2 BCL11A SNPs, 5 cases with AA 5′HS4-LCR background (AA/CC/CC), showed a severe phenotype. Both patients with the same BCL11A genotype but a GG 5′HS4-LCR background (GG/CC/CC) showed an extremely mild phenotype (Table 4). Our studies showed a more significant impact of the 5′HS4-LCR polymorphism on the thalassemia phenotype as compared to BCL11A. A more significant role for Xmn1 compared to BCL11A polymorphisms on patients’ phenotype has also been reported in other studies [18].

The more significant effect of 5′HS4 polymorphism on patients’ phenotype compared to BCL11A single nucleotide variations, can have several explanations. First, the chromatin modifying potential of 5′HS4-LCR could affect binding of transcription factors throughout the LCR [5]. Although no evidence has shown a direct interaction between BCL11A and 5′HS4, and the exact positions on the LCR where BCL11A interacts with LCR are still unknown, BCL11A binding to 5′HS1, HS2, and HS3 has been observed in ChIP–chip experiments in mouse basophilic erythroblasts [8]. The 5′HS4 is in linkage disequilibrium with a motif in 5′HS2, which is associated with high HbF levels [9,10]. Therefore, it is possible that the 5′HS4-LCR locus is linked to other causal variants in the β-globin LCR, which could directly affect BCL11A binding to LCR.

Also, it has been suggested that when BCL11A is absent, the conformation of the β-globin locus changes, such that the LCR is juxtaposed with the transcriptionally activated γ-globin genes [7]. The same situation may arise, when BCL11A is present, but cannot bind to the LCR. Therefore, if the LCR polymorphisms affect binding of transcription factors such as BCL11A, to this region, they probably are able to influence LCR-γ globin interactions as well. Furthermore, the +/− Xmn1-HBG2 profile associated with A/A 5′HS4-LCR and +/− Xmn1-HBG2 profile associated with G/G 5′HS4-LCR, play their own role inhibiting or favoring LCR interactions with the γ-globin gene. Yet, it is also possible that +/− Xmn1-HBG2 alleles are only markers of other causal haplotypes linked to them in this region.

Assuming that the ameliorating alleles of BCL11A SNPs, such as rs11886868 and rs766432, decrease the expression level of BCL11A protein, but do not cause its expression to stop, our results suggest that the A/A 5′HS4-LCR, as a causal genotype or marker of other causal genotypes linked to it in the LCR, might provide such a good interaction condition for BCL11A and LCR that in patients with this LCR background even decreased amounts of BCL11A, in homozygotes with ameliorating alleles of both the BCL11A SNPs, which are underlined (AA/CC/CC), are enough to engage LCR, preventing it to juxtapose with the γ-globin genes. As a result, all of these patients (5 out of 5) show a severe phenotype, despite having BCL11A ameliorating alleles in the homozygous state (Table 4). On the other hand, the G/G 5′HS4-LCR, might be creating a condition inhibiting BCL11A binding to LCR, such that in patients with the same BCL11A genotype as above, but with a GG 5′HS4-LCR background (GG/CC/CC), any chance of interaction of BCL11A with LCR is prevented. Therefore, LCR is free to juxtapose with the γ-globin gene locus, inducing F-hemoglobin, and we see an extremely mild phenotype in these cases (Table 4). Meanwhile, higher levels of BCL11A expression, in patients homozygous for the non-ameliorating alleles of BCL11A in both SNPs, which are underlined (GG/TT/AA), could increase the chance of an interaction of BCL11A with LCR, leading to a severe phenotype in about half of the patients, that probably carry other unidentified modifying factors in favor of this condition.

As the majority of our patients are either receiving hydroxyurea or regular blood transfusion, an inability to measure their steady-state HbF values is a major shortcoming of our study, as in prior reports [12], where genotypic variations could only be correlated to the variations in patients’ blood transfusion dependencies, assumed to reflect their intrinsic ability to produce F-hemoglobin under erythropoietic stress. Furthermore, although we have excluded patients with mild and silent mutations, our results show that the A or G 5′HS4-LCR alleles are associated with certain specific mutations (Supplementary Table 1), which are themselves specific to certain parts of Iran with different ethnic backgrounds. The most prominent mutations with these associations are IVS1-1 and IVS5-1. Eighty percent of our patients with G/G 5′HS4-LCR background have IVS1-1 mutation, which is prevalent in North and West Iran. On the other hand, 35.4% of our patients with an A/A genotype have IVS5-1 mutation, which is more prevalent in the East and South East of this country [19]. Therefore, the effect of association of these mutations with other unknown factors or haplotypes that influence the patients’ phenotype under erythropoietic stress conditions, together with the specific intrinsic properties of each mutation in producing different globin chains cannot be excluded from the total phenotypic outcomes that we see in these patients.

Therefore, considering its pharmaceutical relevance, functional studies to determine the effect of a β-globin 5′HS4-LCR background in binding of transcription factors important in γ-globin gene regulation, and its influence on LCR-γ globin interactions, would be necessary to support our hypothesis. Furthermore, factors in this study are only a fraction of the complex mechanism of globin gene regulation. Many other determinants including other SNPs in BCL11A, the β-globin locus and HBS1L-MYB should be determined to explain the phenotype of these patients. Also, other determinants remain unknown [7,20].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2013.02.007.

Conflict of interest

No conflict of interest exists in this study.

References


