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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

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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 BCL11A binding to this region, plays a more significant role in determining the thalassemia phenotype than the level of BCL11A protein expression, that might be influenced by single nucleotide polymorphisms in intronic regions of the BCL11A gene. Functional studies to confirm the interactions between BCL11A and LCR could be useful in designing pharmacogenetic strategies for the treatment of beta thalassemia major.

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

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backgrounds, the frequency of 5'HS4 polymorpic alleles and the frequency of the mutations that we observe in this study, would not reflect the true frequency in the patient population in Iran [13]. This is due to the linkage disequilibreum relationships that exist between 5'HS4, Xmn1-HBG2 and certain beta thalassaemia mutations [1]. 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]. 5′HS4-LCR was analyzed as previously reported [1,3]. 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′ GCATTCTCATTTCCCTGAAATGT 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.

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 (96.15%) was observed between the G allele at 5′ HS4 and + Xmn1-HBG2 in the patient population. The allelic frequencies for BCL11A SNPs, rs11886868 (T/C) and rs766432 (A/C) are shown (Table 1). The C allele in rs766432 was only associated with the C allele in rs11886868 both in the normal (27 out of 27) and patient (26 out of 26) populations. The association of A allele in rs766432 with the T allele in rs11886868 in the normal population (67.1%) was more frequent than in the patient population (51.5%). (Supplementary Tables 1–2).

The effect of 5′HS4-LCR on mean HbF values in the normal population

There was a significant difference in the frequency of A/G alleles in 5′HS4-LCR between the low HbF and high HbF groups of normal individuals ($P = 0.0338$) (Table 1). Based on our HbF distribution curve in our population, HbF values between 0.3 and 0.5% were indicated as low, whereas HbF values between 1 and 1.2% were indicated as a high HbF range in our normal population (Supplementary Table 2). No difference in mean HbF was detected between normal individuals with G/G and A/A 5′HS4-LCR backgrounds (Table 2).

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

There was a significant difference in the frequency of G/A at 5′ HS4-LCR between severe (S) and milder (including M and E) phenotypic groups of patients ($P = 1 \times 10^{-7}$) (Table 1). The frequency of

Table 1
The allelic frequencies among 100 thalassemia patients and 122 normal individuals.

Polymorphic region	SNP	Patients (n = 100)					Normal (n = 122)			
		Total no. and allelic freq.	S (n = 67)	M (n = 24)	E(n = 9)	(M + E) (n = 33)	Statistical analysis	Total no. and allelic freq.	Low HbF 0.3%–0.5%	High HbF 1%–1.2%	Statistical analysis
			Observed and (expected) no. of alleles in different phenotypic groups				Mid-P exact S/(M + E)	Observed and (expected) no. of alleles in different HbF groups			Mid-P exact L/H
LCR (5′HS4)	A	96 (48%)	92 (64.3)	4 (23.0)	0 (8.64)	4 (31.7)	$1 \times 10 - 7$	190 (77.9%)	45(38.9)	27 (28.0)	0.034
	G	104 (52%)	42 (69.7)	44 (25.0)	18 (9.36)	62 (34.3)		54 (22.1%)	5 (11.0)	9 (7.96)	
	Total	200	134	48	18	66		244	50	36	
BCL11A (rs11886868)	T	89 (44.5%)	62 (59.6)	22 (21.3)	5 (8.01)	27 (29.4)	0.24	135 (55.3%)	27 (27.6)	22 (19.9)	0.26
	C	111 (55.5%)	72 (74.4)	26 (26.6)	13 (9.99)	39 (36.6)		109 (44.7%)	23 (22.3)	14 (16.1)	
	Total	200	134	48	18	66		244	50	36	
BCL11A (rs766432)	A	155 (77.5%)	104 (103.8)	41 (37.2)	10 (13.9)	51 (51.1)	0.95	186 (76.2%)	39 (38.1)	29 (27.4)	0.39
	C	45 (22.5%)	30 (30.1)	7 (10.8)	8 (4.05)	15 (14.8)		58 (23.8%)	11 (11.9)	7 (8.6)	
	Total	200	134	48	18	66		244	50	36	

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).

Table 2
For each SNP (1) the frequency of different phenotypes has been compared between two groups of patients homozygous for one of the alleles and (2) the mean HbF value has been compared between two groups of normal individuals homozygous for one of the alleles. (Abbreviations are as in Table 1).

Locus	Genotype	Patient						Normal			
		Genotype freq.	Observed no./(expected no.)				Mid-P exact P-value S/(M + E)	Genotype freq.	MEAN	STDEV	ANOVA P-value
			S	M	E	M + E					
LCR (5′HS4)	A/A	48 (48%)	46 (32.16)	2 (11.52)	0 (4.32)	2 (18.84)	$1 \times 10 - 7$	74 (92.5%)	0.7	0.22	0.1968
	G/G	52 (52%)	21(34.84)	22 (12.48)	9 (4.68)	31 (17.16)		6 (7.5%)	0.82	0.17	
	Total	100	67	24	9	33		80 (100%)	0.71	0.21	
BCL11A (rs11886868)	T/T	19 (38.8%)	13 (12.03)	5 (4.7)	1 (2.3)	6 (7)	0.2862	36 (61%)	0.7	0.22	0.4762
	C/C	30 (61.2%)	18 (18.97)	7 (7.3)	5 (3.7)	12 (11.0)		23 (39%)	0.74	0.19	
	Total	49	31	12	6	18		59 (100%)	0.7169	0.2118	
BCL11A (rs766432)	A/A	62 (89.9%)	42 (42.25)	17 (15.3)	3 (4.5)	20 (19.8)	0.4415	71 (91%)	0.74	0.22	0.1578
	C/C	7 (10.1%)	5 (4.75))	0 (1.7)	2 (0.5)	2 (2.22)		7 (9%)	0.86	0.08	
	Total	69	47	17	5	22		78 (100%)	0.7487	0.2161	

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).

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 (76.47%) 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).

Table 3
In patients who are homozygous for G allele at 5′HS4-LCR, the frequency of different phenotypes, observed and (expected) has been compared between groups of patients with different genotypes of BCL11A SNPs in a homozygous or heterozygous state.

Homozygous for G in 5′HS4-LCR				
	Phenotype			Mid-P exact
	S	M	E	
rs11886868				
CC (15)	3 (6.01)	7 (6.35)	5 (2.60)	CC versus TC and TT
TC (28)	14 (11.31)	11 (11.85)	3 (4.85)	P = 0.03192
TT (9)	4 (3.63)	4 (3.81)	1 (1.56)	
rs766432				
CC (2)	0 (0.81)	0 (0.85)	2 (0.35)	CC and AC versus AA
AC (15)	4 (6.06)	7 (6.35)	4 (2.60)	P = 0.04737
AA (35)	17 (14.13)	15 (14.81)	3 (6.06)	

The thalassemia patients included those with severe (S), mild (M), and exceptionally mild (E) phenotypes.

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 4
The phenotype distribution pattern in 3 groups of individuals with the same genotype for BCL11A SNPs (rs11886868 and rs766432) and different 5′HS4-LCR backgrounds (homozygous for the A or G alleles shown in bold) were compared. (Abbreviations are as in Table 2).

Group	LCR/rs11886868/ rs766432	No of individuals among 100 patients	S	M	E	M + E	Mid-P exact S/(M + E)
1	**AA**/CC/AA	4	4	0	0	0	0.023
	GG/CC/AA	7	2	4	1	5	
2	**AA**/TT/AA	10	9	1	0	1	0.026
	GG/TT/AA	9	4	4	1	5	
3	**AA**/CC/CC	5	5	0	0	0	0.024
	GG/CC/CC	2	0	0	2	2	

(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 [1,2], 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 IVSII-1 and IVSI-5. Eighty percent of our patients with G/G 5′HS4-LCR background have IVSII-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 IVSI-5 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].

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Conflict of interest

No conflict of interest exists in this study.

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