Influence of *Xmn* 1^G γ (HBG2 c.-211 C $\rightarrow$ T) Globin Gene Polymorphism on Phenotype of Thalassemia Patients of North India

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Received: 17 April 2012 / Accepted: 8 August 2013 / Published online: 25 August 2013
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Abstract Beta (β) thalassemia is the most common single gene disorder in India. It has been reported that in patients with β -thalassemia in the presence of *Xmn* 1^G γ polymorphic site the level of fetal hemoglobin (HbF) is increased thereby reducing the severity of disease. To determine the prevalence of *Xmn* 1^G γ polymorphic site and its effect on the clinical phenotype and HbF level in 39 β -thalassemia major and 62 thalassemia intermedia patients, along with response to hydroxyurea therapy in thalassemia intermedia cases. Status of *Xmn* 1^G γ polymorphism was determined by polymerase chain reaction-restricted fragment length polymorphism procedure. The HbF level was determined using high performance liquid chromatography. Genotypes and allele frequencies of the *Xmn* 1^G γ polymorphism did not vary significantly between the various thalassemia groups. HbF levels were observed to be significantly increased and age at presentation was significantly greater in presence of *Xmn* 1^G γ polymorphic site on both alleles as compared to its absence in thalassemia major but not in thalassemia intermedia cases. The response of hydroxyurea in thalassemia intermedia was found only in a few patients irrespective of their *Xmn* 1^G γ status. *Xmn* 1^G γ polymorphisms appear to significantly influence HbF levels and age at presentation in thalassemia major but not in thalassemia intermedia patients. Small numbers precluded a definitive correlation of the polymorphism with response to hydroxyurea therapy.

Keywords Beta thalassemia · HbF · Hydroxyurea · *Xmn* 1^G γ polymorphism

Introduction

Beta (β) thalassemia is characterized by absence or decreased production of the β -globin chains, resulting in an imbalance between the proportion of the alpha and beta chains (α/β ratio) of HbA (adult hemoglobin, $\alpha_2\beta_2$) [1]. The severity of β -thalassemia is directly related to the severity of the globin chain imbalance. This imbalance can be reduced by stimulating the production of the γ chain, which combines with the excess of α chains to form fetal hemoglobin (HbF, $\alpha_2\gamma_2$). This happens inherently in all patients suffering from β -thalassemia (thalassemia major or intermedia). The HbF levels of the patients are relatively increased due to the selective survival of the erythroid precursors that synthesize relatively more γ chains. However, patients with β -thalassemia differ considerably in their ability to synthesize γ chains and this result in differences in their HbF levels. Genetic studies are being done to determine the determinants influencing HbF response that may lie within the β globin gene complex or can be trans-acting.

The *Xmn* 1^G γ polymorphism (HBG2 c.-211 C $\rightarrow$ T) is a sequence variant described in several population groups. It is present with an allele frequency ranging from 0.32 to 0.35 [2]. This genetic variant accounts for about one-third of the variation in the HbF levels in normal adults. Although this seems to have little effect in normal people, there is evidence that individuals homozygous (genotype described as +/+ here for the mutant type) have an increased propensity to produce HbF under haemopoietic stress, and that this can have the effect of elevating HbF levels in patients with β -thalassemia [3, 4]. This polymorphism is well described across various population groups and is considered an important genetic determinant to look at in making genotype–phenotype correlations among patients suffering from β -thalassemia [5].

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Hydroxyurea is a pharmacological inductor of HbF that significantly increases Hb levels and reduces transfusion needs in patients with sickle cell disease [6, 7]. Hydroxyurea is expected to benefit patients with thalassemia, as the increase in γ -chain synthesis may decrease the imbalance between α and non- α chains. In the presence of $Xmn\ 1^G\gamma$ polymorphic site the effect of hydroxyurea is noted to be better [8].

The aim of present study was to determine the prevalence of $Xmn\ 1^G\gamma$ polymorphic site in the north Indian population of thalassemia patients and its association with the clinical phenotype of thalassemia (age of onset of disease, blood requirements, RBC indices, HbF levels) and response to hydroxyurea among thalassemia intermedia patients.

Materials and Methods

Ethical approval and a written informed consent were taken from all patients or their guardians at the time of enrollment in the study. The study was done on 101 patients who were being treated as either β -thalassemia major or intermedia, in the Department of Medical Genetics, SGPGIMS, Lucknow from 2007 to 2011. The study had a retrospective design and was intended to compare the molecular and the clinical phenotype of β -thalassemia.

A very distinct labeling of an individual case is sometimes difficult however largely the policy was to label a case as thalassemia intermedia if the age of first blood transfusion was ≥ 2 year or the child had failure to thrive with other systemic manifestations such as skeletal changes of thalassemia. The laboratory diagnosis was done by Cation Exchange-High Performance Liquid Chromatography (Bio-Rad, Hercules, CA–USA) on venous blood samples collected in EDTA-vacutainers before any treatment was started in the form of blood transfusion or hydroxyurea drug trial. DNA was extracted by the standard phenol–chloroform method. Detection of β globin gene (HBB) mutations was carried out by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) as described by Agarwal et al. [9].

The status of $Xmn\ 1^G\gamma$ polymorphism was determined by restriction fragment length polymorphism (PCR-RFLP) procedure as described by Sutton et al. [10] using forward primer 5'-AACTGTTGCTTTATAGGATTTT-3' and reverse primer 5'-AGGAGCTTATTGATAACCTCAGAC-3'.

Statistical Analysis

The relationship between mean values of the HbF levels and age of onset of disease in patients with different $Xmn\ 1^G\gamma$ genotypes was analyzed by analysis of variance (ANOVA) followed by Bonferoni's test for posthoc analysis if the ANOVA was significant. Effect of $Xmn\ 1^G\gamma$ polymorphism on age of onset of disease, red cell indices and HbF levels was analyzed using one way ANOVA. The statistical significance of the differences in the frequency of the $Xmn\ 1^G\gamma$ polymorphism among the thalassemia groups was analyzed by Chi square test. All the statistical analysis was performed on SPSS 15.0.

Results

Out of the 101 patients, clinical phenotype corresponded to thalassemia major in 39 and intermedia in 62. The genotype and the allele frequencies of the $Xmn\ 1^G\gamma$ polymorphism are as shown in Tables 1 and 2 respectively. Between the various thalassemia groups no significant difference in genotype and allele frequencies of $Xmn\ 1^G\gamma$ polymorphism (P value = 0.147 and 0.0981 respectively) was noted.

In patients with the clinical diagnosis of thalassemia major and the molecular testing showing the presence of $Xmn\ 1^G\gamma$ polymorphism in homozygous mutant (+/+) state, the mean pre-transfusion HbF level was $94.4 \pm 0.8\%$, whereas it was $73.5 \pm 32.7\%$ in patients with heterozygous ($\pm$) state and $56.7 \pm 25.7\%$ in patients with absence of $Xmn\ 1^G\gamma$ polymorphic site (–/–) on any allele. The ANOVA test revealed a statistically significant difference in HbF levels of thalassemia major patients with different $Xmn\ 1^G\gamma$ genotypes ($P = 0.042$). In this group, the HbF levels were increased in patients with presence of

Table 1 $Xmn\ 1^G\gamma$ polymorphism genotype frequency

Diagnosis	$Xmn\ 1^G\gamma$ polymorphism genotype			P value
	–/– ^a	$\pm$ ^a	+/+ ^a	
Thalassemia major ($n = 39$)	26 (0.667)	10 (0.256)	3 (0.77)	0.147
Thalassemia intermedia ($n = 62$)	29 (0.468)	26 (0.419)	7 (0.113)	
Total	55 (0.545)	36 (0.356)	10 (0.99)	

Numbers in parenthesis represents genotype frequency. χ^2 test was used to obtained P value

^a $Xmn\ 1^G\gamma$ polymorphism in homozygous mutant (+/+), heterozygous ($\pm$) state, (–/–) absence of the $Xmn\ 1^G\gamma$ polymorphic site on any allele

Table 2 *Xmn* 1^Gγ polymorphism allele frequency

Diagnosis	<i>Xmn</i> 1 ^G γ allele		<i>P</i> value
	–	+	
Thalassemia major (<i>n</i> = 39)	62 (0.795)	16 (0.205)	0.0981
Thalassemia intermedia (<i>n</i> = 62)	84 (0.677)	40 (0.323)	
Total	146 (0.723)	56 (0.277)	

Numbers in parenthesis represents allele frequency. *P* value was obtained by χ^2 test

Xmn 1^Gγ polymorphic site on both alleles (+/+) as compared to patients with absence of *Xmn* 1^Gγ polymorphic site (–/–) (*P* = 0.027). However the ANOVA test failed to show any significant difference between *Xmn* 1^Gγ polymorphism and HbF level in thalassemia intermedia group (Table 3). Instead Bonferroni test showed a significantly increased HbF level in presence of homozygous mutant (+/+) state of the *Xmn* 1^Gγ polymorphism than in the absence of the polymorphic site (i.e. –/–) on any allele

(*P* value = 0.034). Effect of *Xmn* 1^Gγ polymorphism on mean HbF levels were also evaluated in thalassemia intermedia sub groups and we found no significant association of *Xmn* 1^Gγ and HbF (Table 3).

Table 4 shows the red cell indices of patients among various genotypes of *Xmn* 1^Gγ polymorphism in the two thalassemia groups. We did not find any association of red cell indices and *Xmn* 1^Gγ polymorphism in both group of patients (major or intermedia).

In the presence of *Xmn* 1^Gγ polymorphic site in homo/heterozygous state, the average age of onset of the disease symptoms was increased compared to the absence of site in thalassemia major group of patients (Table 5). However no similar significant relationship was found in the thalassemia intermedia group.

On calculating the blood requirement of our patients we found that the average packed red blood cells requirement to maintain a pre transfusion Hb of 8–9 g/dl among the three groups of patients was 180 ± 32.5 , 207 ± 29.1 and 225 ± 82.5 ml/kg body weight/year, in *Xmn* 1^Gγ +/+, ± and –/– groups respectively. Although this data is not

Table 3 Table showing genotypes of *Xmn* 1^Gγ and the mean HbF of the patients with thalassemia

Diagnosis	<i>Xmn</i> 1 ^G γ genotype			<i>P</i> value*
	–/–	±	+/+	
Thalassemia major (39)	56.7 ± 25.7	73.5 ± 32.7	94.4 ± 0.8	0.042
Thalassemia intermedia (62)	$27.8 \pm 27.1^\dagger$	29.8 ± 22.6	$52.4 \pm 39.3^\dagger$	0.097
Thalassemia intermedia sub type				
Heterozygous (9)	5.3 ± 8.1	8.3 ± 1.2	1.9	0.695
Homozygous (12)	62.5 ± 20.2	72.8 ± 19.8	92.5 ± 0.1	0.203
SB (15)	13.8 ± 12.5	22.8 ± 10.6	22.9 ± 1.6	0.481
EB (23)	21.5 ± 12.5	22.9 ± 10.0	41.2	0.270
DB (3)	4.4 ± 2.2	–	–	

Values are given as mean HbF(%) $\pm$ standard deviation

SB Sickle-beta thalassemia, EB HbE-beta thalassemia, DB HbD Punjab-beta thalassemia

* *P* value was calculated by using “one way ANOVA” test

† *P* value for relationship between HbF levels and the genotype in the thalassemia intermedia group = 0.034 (Bonferonni test) (as mentioned in text)

Table 4 *Xmn* 1^Gγ polymorphism and red cell indices

Thalassemia type	<i>Xmn</i> 1 ^G γ genotypes	Hb (g/dl)	MCV (fl)	MCH (pg)
Thalassemia major (<i>n</i> = 39)	–/–	5.3 ± 2.2	72.4 ± 6.2	22.4 ± 6.3
	±	5.8 ± 2.9	74.2 ± 7.3	22.5 ± 2.9
	+/+	4.0 ± 0.6	74.1 ± 2.7	20.9 ± 2.1
Thalassemia intermedia (<i>n</i> = 62)	–/–	6.9 ± 2.4	70.7 ± 11.6	20.7 ± 4.6
	±	6.8 ± 2.5	70.4 ± 9.5	20.6 ± 3.3
	+/+	6.8 ± 2.1	73.6 ± 5.5	21.5 ± 1.6

Values are given as mean $\pm$ standard deviation

Table 5 Age of onset and *Xmn* 1^Gγ polymorphism

Thalassemia type	<i>Xmn</i> 1 ^G γ genotypes	Age of onset (years)	<i>P</i> value*
Thalassemia major (<i>n</i> = 39)	(−/−)	1.1 ± 0.7	0.011
	(±) and (+/+)	2.1 ± 1.5	
Thalassemia intermedia (<i>n</i> = 62)	(−/−)	17.6 ± 16.1	0.150
	(±) and (+/+)	12.5 ± 10.8	

Values are given as mean ± standard deviation

* *P* value was calculated by using “Student *t*” test

significant (*P* value = 0.765) but there is decreasing trend of blood requirement with increase in the number of alleles from 0 to 2 for the mutant *Xmn* 1^Gγ polymorphic site.

In case of thalassemia intermedia 36 patients did not require any blood transfusion or hydroxyurea to maintain their Hb 6–9 g/dl. Splenomegaly was found in 35/62 (56.4 %) patients out of which splenectomy had been performed in 12 patients. Hydroxyurea therapy was started in 25 patients including these 12 patients. Out of these 25 patients, 3 were homozygous (+/+), 9 were heterozygous (±) and 13 were wild type (−/−) for the *Xmn* 1^Gγ polymorphism. The response to hydroxyurea in thalassemia intermedia patients, was considered satisfactory in terms of maintenance of Hb >7 g/dl and no requirement of blood transfusions. A satisfactory response was found in two patients who were homozygous (+/+) for the mutant of *Xmn* 1^Gγ polymorphism and in six patients with heterozygosity for *Xmn* 1^Gγ polymorphism. On the other hand, 7 out of 13 patients with the genotype being homozygous wild type (−/−) showed a satisfactory response to hydroxyurea.

Regarding the molecular phenotype of thalassemia, in the present study 12 patients with thalassemia intermedia were homozygous for the beta globin gene mutations detected. Four out of these 12 homozygous thalassemia intermedia harboured one mild β-thalassemia mutation (β+), therefore their intermedia status may be due to the presence of mild β-thalassemia mutation. Another five patients had two alpha (α−/α−) gene deletions explaining their intermediate phenotype. However the remaining three patients did not have any of these (β+ type of mutation or α deletion), instead they were homozygous for the *Xmn* 1^Gγ mutant polymorphic site (+/+).

Discussion

Thalassemia is a genetic disorder with variable severity. Several genetic factors have been proposed to be important in contributing to this variability [11]. Among the well

explained genetic factors are those that can reduce the imbalance between α and the non-α chains, changing the clinical phenotype of such patients from severe to relatively less severe. Such genetic factors include alpha gene deletions and *Xmn* 1^Gγ polymorphism among others. The *Xmn* 1^Gγ site is 5′ to the ^Gγ gene and has been associated with increased expression of ^Gγ gene which is expected to reduce the severity of thalassemia.

The mutant allele frequency for the *Xmn* 1^Gγ polymorphism in our study group of patients from northern India was 0.277 which is similar to the frequency (0.25) reported by Nadkarni et al. [12] from western part of India. The allele frequency reported from other population groups such as Caucasian French Canadian term infants, European individuals and from Southern Iran are as 0.32 [13], 0.32–0.35 [2], 0.41 [14] respectively. Wong et al. [15] demonstrated a higher allele frequency of 0.66 for this polymorphism in the Malaysian population.

Regarding the genotypes ± and +/+ status was more common in thalassemia intermedia group than thalassemia major. The *Xmn* 1^Gγ mutant allele frequency in the thalassemia intermedia group was found higher (i.e. 0.323) in comparison to the thalassemia major (0.205), but the difference was not found significant (*P* value = 0.0981). Also in thalassemia major patients the average age at onset of disease was increased in presence of *Xmn* 1^Gγ polymorphic site on two or one alleles. This resembles the observation made by Panigrahi et al. [16] who reported that the Eβ thalassemia patients heterozygous for *Xmn* 1^Gγ polymorphism had later onset of the disease compared to the Eβ thalassemia patients where *Xmn* 1^Gγ site was absent on both the alleles. In the presence of the homozygous state the percentage levels of HbF were found highly elevated than the polymorphisms of ± and −/− groups. Recently Nemati et al. [17] also showed that in the presence of *Xmn* 1^Gγ polymorphic site on both alleles (+/+) the level of HbF was increased compared to the absence of *Xmn* 1^Gγ (−/−).

Bandyopadhyay et al. [18] reported that homozygosity of the *Xmn* 1^Gγ site (+/+) was strongly correlated with a mild β-thalassemia phenotype and its absence (−/−) with a severe phenotype. In our study also a tendency of decrease in the blood requirement of patients, was seen with an increase in the number of mutant alleles for the *Xmn* 1^Gγ polymorphism from 0 to 2.

A satisfactory response of hydroxyurea in thalassaemia intermedia group of patients was found only in few patients and it seems irrespective of the *Xmn* 1^Gγ polymorphism. Also the number of thalassemia intermedia patients in our study is small which was unimprovable due to the retrospective design of the study. However there appears to be a trend for better response to hydroxyurea in the presence of *Xmn* 1^Gγ polymorphism. Instead the *Xmn* 1^Gγ

polymorphism is neither a sole nor a strong genetic determinant and hence at present cannot be used as a marker for predicting response of hydroxyurea among thalassemia intermedia patients. Our report is similar to Italia et al. [19] who studied the effect of hydroxyurea on 13 E β thalassemia patients with respect to *Xmn* 1 γ and found response of hydroxyurea in selected patients only.

Presence of homozygous mutant *Xmn* 1 γ (+/+) genotype in a patient is expected to reduce the globin chain imbalance thereby changing the severity of the disease from severe to less severe but we also found 2 thalassemia major cases in which *Xmn* 1 γ polymorphism was present in homozygous mutant state. Therefore it is expected that there are some other genetic factors also affecting the clinical phenotype of thalassemia, the role of which needs to be studied further.

Conclusion

The present study from Northern India supports the earlier reports from various parts of the world that the presence of the *Xmn* 1 γ site may be a contributor in enhancement of HbF levels in thalassemia major patients. The high level of HbF might be responsible for reducing the severity of the disease in some patients. Further studies on other potential genetic markers which may also help in predicting the therapeutic intervention are required such as the occurrence of gamma-globin gene triplications [20] and a number of cis-elements, like motifs in the –530 bp position 5' to beta-globin gene cap site, other motifs in the LCR (locus control region) of the beta-globin locus, or the pre-G-gamma haplotypes and the intergenic A-gamma-delta globin genes haplotypes [21].

Acknowledgments We would like to thank Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, India for providing us infrastructural facilities to run thalassemia screening program. Ravindra Kumar is thankful to CSIR for his fellowship.

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