

REVIEW ARTICLE

Role of *XmnI* Polymorphism in HbF Induction in HbE/ β and β -Thalassaemia Patients

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Abstract

Background: Thalassaemia is one of the most common genetic blood disorders worldwide. Patients with β -thalassaemia major and HbE/ β -thalassaemia are blood transfusion dependent. Foetal haemoglobin or HbF can play a role in disease manifestations in these patients and there is evidence that a homozygous state for *XmnI* polymorphic site, associated with increased expression of *G γ -gene*, may play an important role among other factors in ameliorating the clinical severity of homozygous β -thalassaemia and thalassaemia intermedia. The aim of this review was to provide a comprehensive review of the role of *XmnI* polymorphic site for increased HbF production in HbE/ β and β -thalassaemia patients.

Methods: Published literatures were reviewed on the allelic frequency of *XmnI* polymorphism and its effect on HbF induction among thalassaemia patients of different countries.

Results: In all β -thalassaemias, Hb F levels are relatively increased due to the selective survival of the erythroid precursors that synthesize relatively more γ -chains. The expression of HbF level is dominated by three different loci: *HBG2*: γ -158C>T, *BCL11A*, and *HBS1L-MYB* intergenic region. Genetic determinants influencing Hb F response can be within the β -globin complex or *trans*-acting. The published literature showed that the C>T substitution (rs7482144) at position -158 of the *G γ -globin* gene, referred to as the *XmnI-G γ* polymorphism, is a common sequence variant in all population groups, present at a frequency of 0.32 to 0.35. It was found in some studies, response to Hydroxyurea (HU) has been shown to be largely associated with the presence of the C>T polymorphism at -158 *XmnI* site (*HBG2*:c.- 53-158C>T) upstream of the *G γ -globin* gene and HU therapy exerts a 2- to 9- fold increase in γ -mRNA expression in β -thalassaemia patients.

Conclusion: A number of various study groups around the world suggests that *XmnI* polymorphism is an important key regulator of disease severity of HbE/ β and β -thalassaemia patients.

Key words: β -thalassaemia, HbE/ β -thalassaemia, *XmnI-G γ* polymorphism, Hydroxyurea.

Introduction

The word “thalassaemia” derived from the Greek words “Thalassa” (sea) and “Haema” (blood) refers to the disorders associated with defective synthesis of α - or β -globin subunits of haemoglobin (Hb) A ($\alpha_2\beta_2$). The diseases are inherited as pathologic alleles of one or more of the globin genes located on chromosomes 11 (β) and 16 (α).¹ The thalassaemia syndrome is characterized based on the affected globin chains. Alpha (α) thalassaemia is caused by reduced (α^+) or absent (α^0) synthesis of alpha globin chains, and beta (β) thalassaemia is caused by reduced (β^+) or absent (β^0) synthesis of beta globin chains.^{1,2} More

than 200 deletions or point mutations that impair transcription, processing, or translation of α - or β -globin mRNA have been identified.^{1,3,4,9} However, only 20 mutations account for 90% of the abnormal β -genes.⁵ Haemoglobin E (HbE) is another abnormal structural variant of haemoglobin, resulting from a substitution mutation G>A in codon 26 (Glu>Lys) of the β -globin gene, mostly prevalent in South-East Asian populations.^{6,7,8,10} HbE/ β -thalassaemia results from co-inheritance of a β -thalassaemia allele from one parent and the structural variant Haemoglobin E from the other.^{7,8,11} Worldwide, HbE/ β -thalassaemia may be one of the most important haemoglobinopathies because of the high gene frequencies for both HbE and β -thalassaemia.¹²⁻¹⁴

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Thalassaemia is one of the most common genetic blood disorders worldwide.¹⁵⁻¹⁷ It is estimated that more than 300,000 infants are born with major haemoglobinopathies worldwide each year of whom 60,000 to 70,000 are β -thalassaemia major cases especially in the Mediterranean area, Middle East, Far East, and East Asia.¹⁸⁻²⁰ Globally every year, severe form of β -thalassaemia accounts for 50,000 to 100,000 deaths in all age group and about 0.5%- 0.9% deaths of under-5 children of low or middle income countries.²¹ According to Thalassaemia International Federation (TIF), about 23,000 children are born with transfusion-dependent β -thalassaemia major each year, while a smaller ill-defined number have the non-transfusion dependent thalassaemia (NTDT), a form of β -thalassaemia intermedia.²² Bangladesh, the most densely populated countries in the world, with a population of over 160 million people. According to World Health Organization (WHO), approximately 3% of the carriers of β -thalassaemia and 4% are the carriers of haemoglobin E (HbE) in Bangladeshi population.²³ It is highly concerning that with the birth rate of 21.6/1000, it could be estimated that nearly 2500 thalassaemia major cases are added every year in Bangladesh.²⁴ As, thalassaemia is a hereditary disease, it is only manageable when it is prevented. So, proper and effective public awareness should be built up among the population of Bangladesh.

However, the thalassaemias are heterogeneous at the molecular level, with more than 200 disease causing mutations having been identified.¹² In erythroid development, the γ -globin expression was regulated by interactions between *cis*-acting sequences within the β -globin cluster and *trans*-acting factors such as BCL-11A, cMYB, and TOX.^{12,25} The expression of HbF level is dominated by three different loci: *HBG2*: γ - 158C>T on 11p15.4, BCL11A on 2p16.1 and HBS1L-MYB intergenic region on 6q23.3. The most significant genetic factor in *cis* associated with high HbF is *XmnI* polymorphism located at -158 upstream to the *G γ -globin* genes.²⁶ Although the production of Hb F is almost switched off at birth, all adults continue to produce residual amounts of Hb F. In all β -thalassaemias, HbF levels are relatively increased due to the selective survival of the erythroid precursors that synthesize relatively more γ -chains.⁴ *XmnI* polymorphism is responsible for the induction of γ -chains in adult patients with β -thalassaemias.

Materials and Methods

A literature review was performed with the aim to probe the role of *XmnI-G γ* polymorphism on HbF induction in thalassaemia patients. The articles were searched at PubMed, google scholar and other Journal databases. The key words such as thalassaemia, β -thalassaemia, HbE/ β -thalassaemia, *XmnI-G γ* polymorphism, Haemoglobin F and Hydroxyurea were used while searching these sources. Primarily, the articles which addressed the modifying effect of *XmnI-G γ* polymorphism on disease severity of the HbE/ β and β -thalassaemia patients were screened. Finally, a total of 81 articles, both original and review, were selected for this review purpose. Articles not written in English were excluded.

Pathophysiology and Clinical Variability of β -thalassaemia and HbE/ β -thalassaemia: Although clinical spectra vary depending on coinheritance of other genetic modifiers, the underlying pathology among the types of thalassaemia is similar.²⁷ This pathology is characterised by decreased Hb production and red blood cell (RBC) survival, resulting from the excess of unaffected globin chain, which form unstable homotetramers that precipitate as inclusion bodies. α -homotetramers in β -thalassaemia are more unstable than β -homotetramers in α -thalassaemia and therefore precipitate earlier in the RBC life span, causing marked RBC damage and severe haemolysis associated with ineffective erythropoiesis (IE) and extramedullary hemolysis.²⁸ (figure:1) Without transfusion support, 85% of patients with severe homozygous or compound heterozygous β -thalassaemia will die by 5 years of age because of severe anaemia.²⁹

β -thalassaemia includes three main forms: Thalassaemia Major, variably referred to as "Cooley's Anaemia" and "Mediterranean Anaemia", Thalassaemia Intermedia and Thalassaemia Minor also called " β -thalassaemia carrier", " β -thalassaemia trait" or "heterozygous β -thalassaemia". Individuals with β -thalassaemia major (β^0/β^0 , β^0/β^+ , and sometimes β^+/β^+) usually come to medical attention within the first two years of life and require regular RBC transfusions to survive.⁹ (figure 2a) Patients with β -thalassaemia intermedia (β^+/β^0 or β^+/β^+) have milder anemia and do not require or only occasionally require transfusion.⁹ (figure 2c).

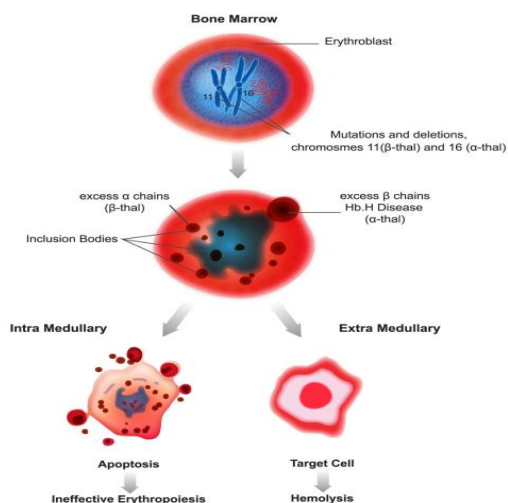


Figure 1: Mechanism of Ineffective Erythropoiesis (IE) and hemolysis in thalassaemia. (Rachmilewitz EA and Giardina PJ, 2011)²⁸

And individuals with β -thalassaemia minor (β/β^+ , β/β^0 or mild β/β^+) are carriers and they are usually clinically asymptomatic but sometimes have a mild anemia. Table-1 illustrates common genotypes leading to a β -thalassaemia intermedia phenotype.³⁰ (figure 2b)

The *HBB* variants are represented in grey exons while the wild type alleles are represented in blue exons. Production of β -globin from a single/double wild type alleles are represented by one/two colored schematic of the β -globin protein respectively. Grey colored β -globin diagrams refer to below-normal synthesis levels of the protein, created by mutant *HBB* variants. Bright red-colored RBCs represent normal cell phenotype, while pink colored ones represent microcytic, hypochromic cells characteristic of beta-thalassaemia phenotype. Relative number of RBC reflects relative levels of anaemia amongst the three classes of β -thalassaemia and in comparison to the wild type RBC pool.

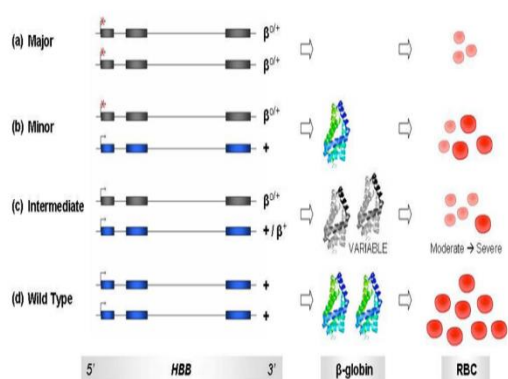


Figure 2: Schematic representation of inherited β -globin variants and related beta-chain and red blood cell (RBC) phenotype.

Table I: Genotype-phenotype associations in β -thalassaemia.³⁰

Phenotype	Genotype	Clinical severity
Silent carrier	• Silent β/β	• Asymptomatic • No hematological abnormalities
Trait/minor	• β^0/β , β^+/β or mild β^+/β	• Borderline asymptomatic anemia • Microcytosis and hypochromia
Intermedia	• $\beta^0/\text{mild } \beta^+$, $\beta^+/\text{mild } \beta^+$ or mild $\beta^+/\text{mild } \beta^+$ • $\beta^0/\text{silent } \beta$, $\beta^+/\text{silent } \beta$, mild $\beta^+/\text{silent } \beta$, or silent $\beta/\text{silent } \beta$ • β^0/β^0 , β^+/β^+ , or β^+/β^+ and deletion or non-deletion α -thalassaemia • β^0/β^0 , β^+/β^+ , or β^+/β^+ and increased capacity for γ -chain synthesis • Deletion forms of $\delta\beta$ -thalassaemia and HPFH • β^0/β or β^+/β and $\alpha\alpha\alpha$ or $\alpha\alpha\alpha\alpha$ duplications • Dominant β -thalassaemia (inclusion body)	• Late presentation • Mild-moderate anemia • Transfusion dependent • Clinical severity is variable and ranges between minor to major
Major	• β^0/β^0 , β^+/β^+ , or β^0/β^+	• Early presentation • Severe anemia • Transfusion-dependent

Hb E/ β -thalassaemia results from co-inheritance of a β -thalassaemia allele from one parent and the Haemoglobin E, structural variant of haemoglobin, from the other. Haemoglobin E results from a G>A substitution in codon 26 of the β globin gene, which produces a structurally abnormal Haemoglobin (HbE).³¹ The pathophysiology of Hb E/ β -thalassaemia is related to many factors including reduced β -chain synthesis resulting in globin chain imbalance, ineffective erythropoiesis, apoptosis, oxidative damage and shortened red cell survival.^{32,33} Depending on the severity of symptoms, HbE/ β -thalassaemia can be divided into three categories.⁹ Individuals with mild HbE/ β -thalassaemia maintain Hb levels between 9 and 12 g/dl and usually does not develop clinically significant problems. Individuals with moderately severe HbE/ β -thalassaemia maintain Hb levels between 6 and 7 g/dl and the clinical symptoms are

similar to thalassaemia intermedia. The Hb level can be as low as 4-5 g/dl in the patients with severe HbE/ β -thalassaemia usually manifest symptoms similar to thalassaemia major and are treated as thalassaemia major patients.

The Globin Genes: There are eight functional globin genes as well as several pseudo genes. The globin genes are found in two loci, each of which has an associated upstream regulatory element. The α -globin locus on chromosome 16^{34,35} contains three of globin genes. Listed in 5' to 3' order these are Haemoglobin subunit zeta (*HBZ*), Haemoglobin subunit alpha 2 (*HBA2*) and Haemoglobin subunit alpha 1 (*HBA1*)³⁶ (figure:3)

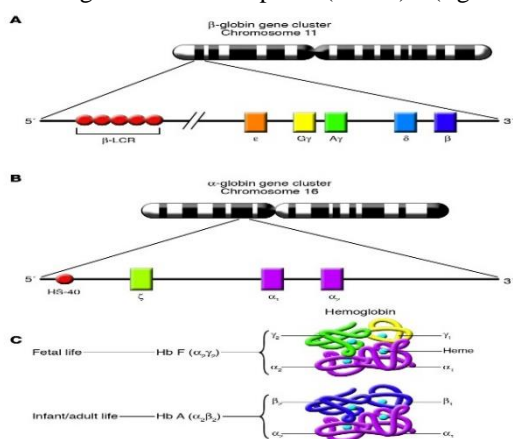


Figure 3: Chromosomal organization of the α - and β -globin gene clusters. A)

The genes of the β -globin gene cluster (ϵ , γ , δ , and β) are present on chromosome 11 in the same order in which they are expressed during development. The β -locus control region (β -LCR) is a major regulatory element located far upstream of the genes of the cluster that is necessary for the high level of expression of those genes. (B) The genes of the α -globin gene cluster (ζ , α_1 , and α_2) are present on chromosome 16, also in the same order in which they are expressed during development. HS-40 is a major regulatory element located far upstream of the genes of the cluster that is necessary for their high level of expression. (C) During fetal life, Hb F ($\alpha_2\gamma_2$) is the predominant type of Haemoglobin. Haemoglobin switching refers to the developmental process that leads to the silencing of γ -globin gene expression and the reciprocal activation of adult β -globin gene expression. This results in the replacement of Hb F by Hb A ($\alpha_2\beta_2$) as the predominant type of Haemoglobin in adult life. (Frenette PS and Atweh GF. 2007)

The remaining five functional globin genes are found in the β -globin locus on chromosome 11.^{37,38} Listed in 5' to 3' order these are Haemoglobin subunit epsilon 1 (*HBE1*), Haemoglobin subunit

gamma 2 (*HBG2*), Haemoglobin subunit gamma 1 (*HBG1*), Haemoglobin subunit delta (*HBD*) and Haemoglobin subunit beta (*HBB*). An upstream regulatory element known as the β -Locus Control Region (β -LCR) is required for expression of these genes.³⁹ Fetal Hb (HbF, $\alpha_2\gamma_2$) is the predominant form during fetal development but is largely replaced by adult Hb (HbA, $\alpha_2\beta_2$) following a shift from gamma (γ)- to β -globin gene expression that begins around birth.⁴⁰ Two main mechanisms control globin gene switching, competition for access to the upstream regulatory element and autonomous gene silencing.⁴¹ Autonomous gene silencing plays an important role in the switching from foetal to adult Haemoglobin.

Haemoglobin F: Haemoglobin F (HbF, $\alpha_2\gamma_2$) accounts for up to 90% of the circulating Haemoglobin at birth. It's synthesis starts to decline during the third trimester, and over the first year of life it is gradually replaced by adult Haemoglobin, HbA ($\alpha_2\beta_2$). Normal adults have less than 1% of HbF, apparently confined to a subset of red blood cells called F cells,⁴² which constitute about 3% of the erythrocytes.⁴³ Several inherited and acquired conditions are associated with the persistence or the reactivation of HbF production.⁴⁴

Most of the genetic disorders associated with persistent HbF production involve alterations of the structure of the β -globin cluster. The highest adult levels of HbF are seen in β - and $\delta\beta$ -thalassaemia, or hereditary persistence of foetal haemoglobin (HPFH), in which HbF can constitute up to 100% of the Haemoglobin. It is now clear that HPFH is an extremely heterogeneous group of conditions, some of which result from deletions of the β -globin gene or point mutations in the γ -globin gene promoter regions, whereas others arise from genetic determinants that segregate independently of the β -globin gene cluster.⁴⁵

Several acquired conditions are associated with modest elevations of HbF. They include pregnancy, recovery from marrow hypoplasia, aplastic anaemia, leukemia, thyrotoxicosis, haepatoma, and juvenile chronic myeloid leukaemia.⁴⁶ The latter condition is exceptional in that it seems to reflect a genuine reversion to fetal erythropoiesis.⁴⁷ The remainder seem to be examples of the transient reactivation of HbF under conditions of acute erythropoietic stress, that is, rapid expansion of the erythron.⁴⁸

Mechanism for increased HbF production: According to Rees DC *et al*, a proposed possible mechanism by which HbF may be increased.⁴⁴ In this model, the absolute numbers of F-cell progenitors would expand, proportionate to the increase in all red blood cell precursors. In both transfused and non-

transfused patients, the F-cell precursors would have a selective advantage because of their lesser degree of globin chain imbalance, leading to the observed increases in HbF levels. The observed change in α/δ ratio is not compatible with this mechanism alone, and suggests the possibility that there is a genuine increase in HbF and/or F-cell production. This preferential production of F reticulocytes has typically been thought to be important in acute increases in Epo, rather than the chronic elevations seen in thalassaemia figure 4.^{48,49}

Association of XmnI polymorphism with Hb F induction: Genetic determinants influencing Hb F response can be within the β -globin complex or *trans*-acting.

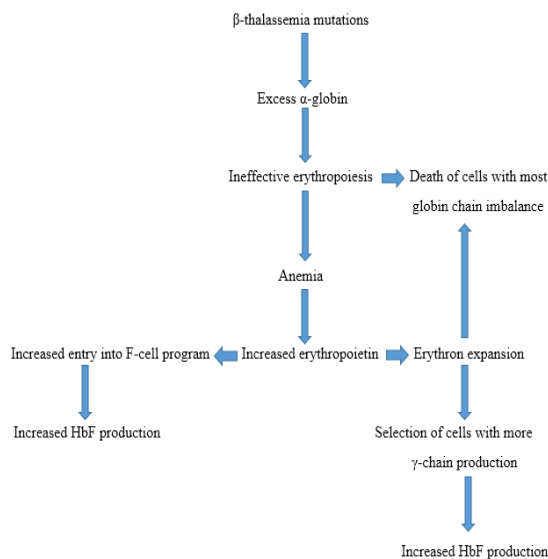


Figure 4: Proposed mechanism for increased HbF production in β -thalassaemia syndromes. (Rees DC et al., 1999)⁴⁴

The C>T substitution (rs7482144) at position -158 of the *G γ -globin* gene, referred to as the XmnI-*G γ* polymorphism, is a common sequence variant in all population groups, present at a frequency of 0.32 to 0.35.^{50,51} Although the increases in Hb F and F cells are minimal in normal people, clinical studies have shown that, under conditions of hematopoietic stress, for example in homozygous β -thalassaemia and sickle cell disease, the presence of the XmnI-*G γ* site favors a higher Hb F response.^{52,53} However, the -158 C > T polymorphism is located near a nuclease hypersensitive site at 50 to 150 bp upstream region of the *G γ -globin* gene. Perhaps the -158 substitution reduces the binding of transcription factor(s) that silence(s) the *γ -globin* gene expression in adult cells. Therefore, the *γ -globin* gene is reactivated in adult life.^{54,55}

The Hb F is a mixture of two molecular species $\alpha_2\text{G}\gamma_2$ and $\alpha_2\text{A}\gamma_2$ in which the constituent γ -chains contain a glycine or an alanine at position 136. During the switch from fetal to adult, there is a quantitative change in the γ -chain composition. Normally the *G γ :A γ* ratio is 70:30 at the time of birth and 40:60 in the trace amounts of Hb F found in the adult. This ratio is modified in many Haemoglobin disorders, but in the presence of XmnI polymorphic site almost this ratio look like the time of birth.⁵⁶

The frequency of XmnI polymorphic site: The prevalence of XmnI polymorphic site 5' to the *G γ -gene* is different among various population (table II). There is evidence that a homozygous state for XmnI polymorphic site, which is associated with increased expression of *G γ -gene*, may play an important role among other factors in ameliorating the clinical features of homozygous β -thalassaemia and its clinical presentation as thalassaemia intermedia.⁵⁷ Furthermore, the presence of XmnI polymorphic site 5' to the *G γ -globin* promoter region was positively correlated with elevated synthesis of fetal Hb and its *G γ -globin* component in term newborn infants and is associated with delayed switch over from fetal to adult Haemoglobin. It is unknown that how XmnI polymorphic site influences the expression of the *G γ -globin* gene. It seems that interaction of a multi-protein transcription complex to be involved. In a genome-wide linkage study of large Asian Indian kindred, a genetic interaction between the XmnI polymorphic site and a locus on chromosome 8q was reported to influence on adult F cell (FC) levels.^{58,59} Unlike the rare mutations in the *γ -globin* promoter that are consistently associated with large discrete effects of increased HbF levels of 10–35% in heterozygotes, the so-called pancellular hereditary persistence of fetal Haemoglobin (HPFH), the change at *G γ -158* does not always raise the Hb F levels in otherwise normal individuals. The XmnI polymorphic site is not a recognized binding motif for any of the known transcription factors.⁶⁰

However, the Hb F response associated with the XmnI polymorphic site is usually moderate and may not be sufficient to explain the wide difference in phenotype observed in some cases.^{61,62} According to Ballas SK et al.⁶³ there was a significant correlation between the presence of XmnI polymorphic site and increased *G γ :A γ* ratio. However, the Hb F level was not significantly increased in the presence of XmnI polymorphic site in their study. Although XmnI polymorphic site maintains a *G γ :A γ* ratio typical of fetal life but does not necessarily cause elevation of Hb F. The latter seems to depend on factors other than the XmnI polymorphic site.⁶³ Hooshang N et al

revealed that in the presence of *XmnI* polymorphic site $G\gamma$ percent and $G\gamma$: $A\gamma$ ratio were significantly increased ($p=0.01$) and the clinical features such as splenomegaly and bone marrow expansion were significantly improved ($p=0.01$). It was found that in the presence of *XmnI* polymorphic site on both chromosomes (+/+) the level of Hb F tended to be increased compared to the absence of *XmnI* (-/-) (table III).⁶⁰

Table II: Allelic Frequency of *XmnI* polymorphic site in different population groups

Country/ Population groups	Sample Size (n)	Types of population	Frequency	References no.
Caucasian French Canadian	100	Normal infants	0.32	56
European	300	Healthy	0.32–0.35	50
India	64	β -thalassaemia	0.25	77
Eastern India	64	β -thalassaemia and HbE/ β -thalassaemia	0.48	78
Northern India	101	β -thalassaemia major and intermedia	0.28	79
Southern Iran	48	β -thalassaemia intermedia	0.41	80
Western Iran	197	β -thalassaemia major	0.39	60
Malaysia	107	β -thalassaemia major	0.66	81

Table III: Parameters associated with *XmnI* polymorphism in β -thalassaemia patients studied by Hooshang N et al. (2010)⁶⁰

Parameter	<i>XmnI</i> C/C (+/+)	<i>XmnI</i> C/T (+/-)	<i>XmnI</i> T/T (-/-)	<i>p</i> value
Hb F level %	97.1	94.4	91.8	0.08
$G\gamma$ %	74.8	71.4	66.7	0.01
$A\gamma$ %	25.1	28.6	33.3	0.01
$G\gamma$ / $A\gamma$ ratio	3 \pm 0.5	2.5 \pm 0.4	2 \pm 0.3	0.01
Age of first blood transfusion (months)	12 \pm 8	11 \pm 5	10 \pm 5	0.16
Facial bone deformity %	17.5	25.4	57.1	0.02
Splenectomy %	15.9	19.1	65	0.01

G\gamma-XmnI polymorphism genotyping: *XmnI* polymorphism is heterogeneously distributed in different parts of the world.⁶⁴ The archived genomic DNAs are genotyped employing Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) technique. The genotypes were categorised into homozygous wild type (CC, +/+), heterozygous (CT, +/-) and homozygous variant (TT, -/-)^{60,65-68} Another study was performed to detect the *G\gamma-XmnI* polymorphism genotype in patients using the Tetra-Primer ARMS-PCR technique.⁶⁹

Association of G\gamma-XmnI polymorphism with *Hydroxyurea (HU)* treatment: HU therapy has been successfully used in thalassaemia intermedia patients and was associated with a significant improvement in hematological parameters and quality of life.^{70,71} Hydroxyurea efficacy in β -thalassaemia major has been variable in different studies.⁷²⁻⁷⁴ Response to HU has been shown to be largely associated with the presence of the C>T polymorphism at -158 *XmnI* site (*HBG2*:c.-53-158C>T) upstream of the $G\gamma$ -globin gene and it is thus far the most studied nucleotide change to have a significant association to drug response. This particular polymorphism acts as an enhancer of HbF expression during erythropoietic stress, resulting in a beneficial effect in SCD patients. HU is a myelo suppressive agent that may enhance fetal Haemoglobin production. Several pharmacologic agents, such as 5-azacytidine, erythropoietin, butyrates including Hydroxyurea have been shown to stimulate γ -globin gene expression *in vivo* and therefore might reduce the severity of clinical symptoms in patients with intermediate thalassaemia. Moreover, one study on β -thalassaemia patients treated with Hydroxyurea has revealed a significant correlation between the presence of T allele in *XmnI* polymorphic site and the better treatment response. Hydroxyurea therapy exerts a 2- to 9- fold increase in γ -mRNA expression in β -thalassaemia patients,⁷⁴ leading to improvement in the α /non- α -chain imbalance and more-effective erythropoiesis.⁷⁵ However, Kosaryan et al. suggested that β -thalassaemia major or intermedia with *XmnI* polymorphism of (C/T or +/-) show better response to hydroxyurea therapy than (T/T or -/-) genotype, this finding was not proved by other studies.⁷⁶ No previous study on association of *G\gamma-XmnI* polymorphism with Hydroxyurea (HU) treatment in Bangladeshi population is found.

Conclusion

The high level of HbF can ameliorate the severity of the disease by reducing the excess alpha chain imbalance in patients with Sickle cell disease (SCD), β -thalassaemia and HbE/ β -thalassaemia. Therefore, for investigating the success of Hydroxyurea medication in diseased population, a follow-up study is required to determine the HbF induction effect of Hydroxyurea in presence of T allele in thalassaemia patients. However, till date there is no study regarding the frequency of *XmnI-G γ* polymorphism and its effect on HbF production among thalassaemia patients in Bangladesh. So, study on the effect of *XmnI-G γ* polymorphism on disease severity of Bangladeshi thalassaemia patients is recommended in order to validate the use of Hydroxyurea as therapeutic intervention for these patients.

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