Role of *XmnI* polymorphism in HbF induction in HbE/β and β-thalassemia patients

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Abstract

Thalassemia is one of the most common genetic blood disorders worldwide. Patients with β-thalassemia major and HbE/β-thalassemia are blood transfusion-dependent. If no blood transfusion is given, 85% of patients with severe homozygous or compound heterozygous β-thalassemia will die by 5 years of age. Hemoglobin F or fetal hemoglobin has been known to play a role in ameliorating clinical manifestations in these patients. In all β-thalassemia, 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 is up-regulated by three different loci, namely, -158(C → T) (XmnI-Gγ), BCL11A, and HBS1L-MYB intergenic region. Genetic determinants influencing HbF response may lie within the *β-globin* complex or they may be *trans*-acting components. The C>T substitution (rs7482144) at position –158 of the *Gγ-globin* gene, referred to as the Xmn*I-Gγ* polymorphism, is a common sequence variant in all population and is present at a frequency of 0.32 to 0.35. Although HbF concentrations and F cells are minimal in normal adults, clinical studies have shown that, under conditions of hematopoietic stress, for example, in homozygous β-thalassemia and sickle cell disease, the presence of the *Xmn*1-Gγ site favors a higher HbF response. There is evidence that a homozygous state of Xmn1 polymorphic site, which is associated with increased expression of *Gγ-gene*, may play an important role, among other factors, in improving the clinical features of homozygous *β*-thalassemia and its clinical presentation as thalassemia intermedia. Favorable Response to Hydroxyurea (HU) treatment has been shown to be largely associated with the presence of the C>T polymorphism at -158 Xmn1 site (*HBG2*: c.- 53-158C>T), which is upstream of the Gγ-globin gene and HU therapy exerts a 2- to 9- fold increase in γ-mRNA expression in β-thalassemia patients. In addition to discussing some effects of Xmn1 polymorphism on beta-thalassemia, this review will give special focus on discussing the importance of Xmn1 polymorphism in determining the clinical heterogeneity of hemoglobin E-beta thalassemia.

Key words: Thalassemia, β-thalassemia, HbE/β-thalassaemia, Xmn*I-Gγ* polymorphism, Hydroxyurea.

Introduction

Thalassemia is known as the most common monogenic blood disorder. The disorder is associated with defective or even absent synthesis of α- or β-globin subunits of hemoglobin (Hb) A ($\alpha_2\beta_2$). The diseases are inherited as recessive manner. That is, both alleles of one or more of the globin genes located on chromosomes 11 (β) and 16 (α) must be inherited to cause the disorder.[1] The thalassemia syndrome is characterized based on the affected globin chains. Alpha (α) thalassemia is caused by reduced (α⁺) or absent (α°)

synthesis of alpha globin chains, whereas beta (β) thalassemia is caused by reduced (β⁺) or absent (β°) 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-gene.[5] Hemoglobin E (HbE) is another abnormal structural variant of hemoglobin, resulting from a substitution mutation G>A in codon 26 (Glu>Lys) of the β-globin gene, mostly prevalent in South-East Asian population.[6,7,8,10] HbE/β-thalassemia results from co-inheritance of a β-thalassemia allele from one parent and the structural variant hemoglobin E from the other.[7,8,11] Worldwide,

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HbE/β-thalassemia may be one of the most important hemoglobinopathies because of the presence high gene frequencies for both HbE and β-thalassemia.[12-14]

It is estimated that as many as 300,000 infants are born with major hemoglobinopathies worldwide each year and 60,000 to 70,000 of these patients with hemoglobinopathies are β-thalassemia major and the disorder is mainly seen in the countries of thalassemia belt including Mediterranean area, Middle East, Far East, and East Asia.[15-20] Globally, severe form of β-thalassemia accounts for 50,000 to 100,000 deaths per year in all age groups and about 0.5%- 0.9% deaths of under-5 children of low or middle income countries.[21] According to thalassemia International Federation (TIF), about 23,000 children are born with transfusion-dependent β-thalassemia major each year, while a smaller ill-defined number have the non-transfusion dependent thalassemia (NTDT), a form of β-thalassemia intermedia. According to World Health Organization (WHO), there are approximately 3% β-thalassemia carriers and 4% hemoglobin E (HbE) carriers in Bangladeshi population.[23] It is highly concerning that with the birth rate of 21.6/1000 in Bangladesh, it could be estimated that nearly 2500 thalassemia major cases are added every year in the country.[24] However, thalassemia is a preventable disorder and the effective preventive measures can be taken through social awareness and carrier screening program to discourage marriage between two carriers. Prenatal diagnosis followed by abortion can be another preventive measure provided that social and religious legislations support the matter.

However, the thalassemia is heterogeneous at the molecular level, with more than 200 disease causing mutations.[12] In erythroid development, the *γ*-globin expression is regulated by interactions between *cis*-acting sequences within the *β*-globin gene cluster and *trans*-acting factors such as BCL-11A, cMYB, and TOX.[12,25] The expression of HbF level is known to be up-regulated 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* sequence associated with high HbF is *XmnI* polymorphism located at -158 upstream to the *Gγ-globin* gene.[26] Although the production of Hb F is almost switched off at birth, all adults continue to produce residual amounts of Hb F, which is usually <1%. In all β-thalassemia, HbF levels are relatively increased due to the selective survival of the erythroid precursors that synthesize relatively more γ-chains.[4] *Xmn1* polymorphism is responsible for the induction of γ-chains in adult patients with β-thalassemia.

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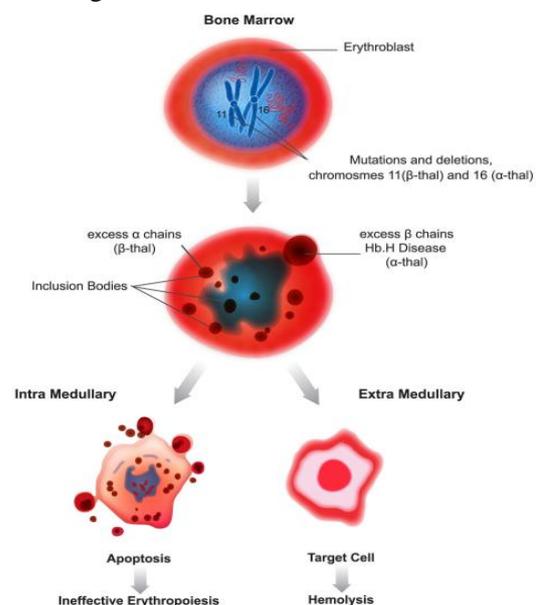

Figure 1: Mechanism of Ineffective Erythropoiesis (IE) and hemolysis in thalassemia. (Rachmilewitz EA and Giardina PJ, 2011) [28]

β-thalassemia includes three main forms: Thalassemia Major, variably referred to as "Cooley's Anemia" and "Mediterranean Anemia", Thalassemia Intermedia, and Thalassemia Minor, which is also called "β-thalassemia carrier" or "β-thalassemia trait" or "heterozygous β-thalassemia". Individuals with β-thalassemia major (β^0/β^0) 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-thalassemia intermedia (β^+/β^0 or, β^+/β^+) have milder anemia and do not require or only occasionally require transfusion.[9] (Figure:2c). And individuals with β-thalassemia 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)
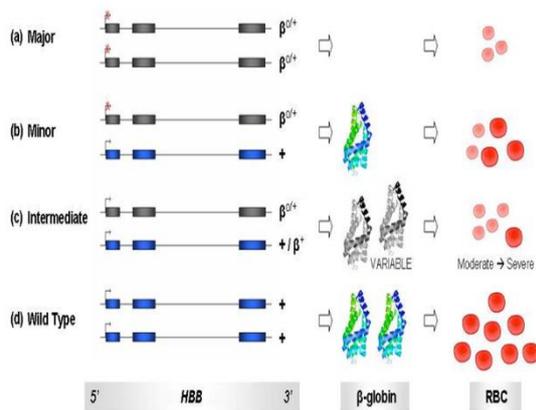

Figure 2: Schematic representation of inherited *β-globin* variants and related beta-chain and red blood cell (RBC) phenotype**.**

The *HBB* variants are represented by grey exons, whereas the wild type alleles are represented by blue exons. Production of *β-globin chain* from a single/double wild type alleles is represented by one-/two-colored schemata 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-thalassemia phenotype. Relative number of RBC reflects relative levels of anemia among the three classes of β-thalassemia compared to the wild type RBC pool.

Table I: Genotype-phenotype associations in β-thalassemia.

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

Hb E/β-thalassemia results from co-inheritance of a β-thalassemia allele from one parent and the Hemoglobin E, structural variant of hemoglobin, from the other. Hemoglobin E results from a G>A substitution in codon 26 of the β globin gene, which produces a structurally abnormal hemoglobin (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 of nucleated RBCs, oxidative damage and shortened red cell survival.[32,33] Depending on the severity of symptoms, HbE/β-thalassemia can be divided into three categories.[9] Individuals with mild HbE/β-thalassemia maintain Hb levels between 9 and 12

g/dl and usually do not develop clinically significant problems. Individuals with moderately severe HbE/β-thalassemia maintain Hb levels between 6 and 7 g/dl and the clinical symptoms are similar to thalassemia intermedia. The Hb level can be as low as 4-5 g/dl in the patients with severe HbE/β-thalassemia and usually manifest symptoms similar to thalassemia major and are treated as thalassemia 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 contains three of the globin genes.[34,35] Listed in 5′ to 3′ order these are Hemoglobin subunit zeta (*HBZ*), Hemoglobin subunit alpha 2 (*HBA2*) and Hemoglobin subunit alpha 1 (*HBA1*)[36] (figure:3)
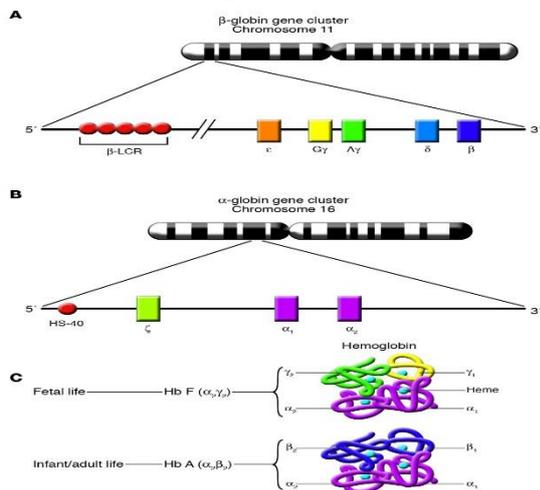

Figure 3: Chromosomal organization of the α- and β-globin gene clusters. A)

The genes of the β-globin gene cluster (ε, Gγ, Aγ, 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, α₁, and α₂) 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 hemoglobin. Hemoglobin 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 hemoglobin in adult life. (Frenette PS and Atweh GF. 2007)

The remainin five functional globin genes are found in the β-globin locus on chromosome 11.[37,38] Listed in 5′ to 3′ order, these are Hemoglobin subunit epsilon 1 (*HBE1*), Hemoglobin subunit gamma 2 (*HBG2*), Hemoglobin subunit gamma 1 ((*HBG1*), Hemoglobin subunit delta (*HBD*) and Hemoglobin 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, α2γ2) is the predominant form during fetal development but is largely replaced by adult Hb (HbA, α2β2) 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 fetal to adult hemoglobin.

Hemoglobin F: Hemoglobin F (HbF, α2γ2) accounts for up to 90% of the circulating hemoglobin at birth. Its synthesis starts to decline during the third trimester, and it is gradually replaced by adult hemoglobin, HbA (α2β2) over the first year of life. 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 δβ-thalassemia, or hereditary persistence of fetal hemoglobin (HPFH), in which HbF can constitute up to 100% of the hemoglobin. 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]

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Several acquired conditions are associated with modest elevations of HbF. They include pregnancy, recovery from marrow hypoplasia, aplastic anemia, leukemia, thyrotoxicosis, hepatoma, and juvenile chronic myeloid leukemia.[46] The latter condition is exceptional in that it seems to reflect a genuine reversion to fetal erythropoiesis.[47] The remainder seems 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,* (1999) proposed a possible mechanism by which HbF may be increased.[44] In this model, the absolute numbers of F-cell progenitors would expand in proportion 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 increase in HbF levels. The observed change in α/ γ 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 an acute increase in Epo, rather than the chronic elevations seen in thalassemia.[48,49] This proposed mechanism is summarized in (figure 4).
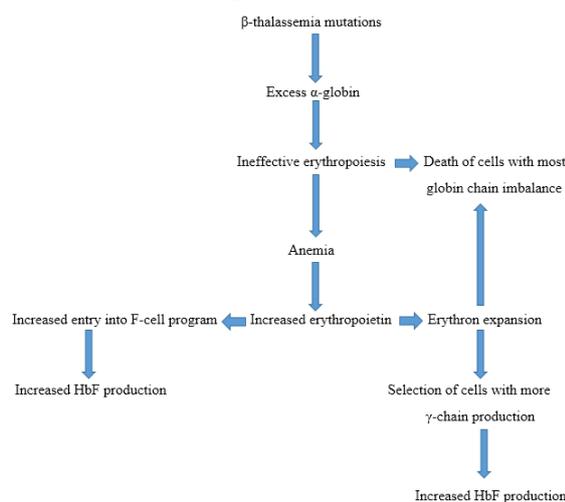

Figure 4: Proposed mechanism for increased HbF production in β-thalassemia syndromes. (Rees DC et. al.,1999)[44]

Association of XmnI polymorphism with Hb F induction: Genetic determinants influencing Hb F response reside within the β-*globin* complex or *trans*-acting elements. The C>T substitution (rs7482144) at position –158 of the *Gγ-globin* gene, referred to as the Xmn*I-Gγ* polymorphism, is a common sequence variant in all population groups and is present at a frequency of 0.32 to 0.35.[50,51] Although the increase in Hb F and F cells are minimal in normal people, clinical studies have demonstrated that under conditions of hematopoietic stress, for example, in homozygous β-thalassemia and sickle cell disease, the presence of the *Xmn*1-Gγ site favors a higher HbF 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 Gγ-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 HbF is a mixture of two molecular species $α_2Gγ_2$ and $α_2Aγ_2$ 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 Gγ:Aγ 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 hemoglobin disorders, but in the presence of *XmnI* polymorphic site, this ratio looks like as it is at the time of birth.[56]

The frequency of XmnI polymorphic site: The prevalence of *XmnI* polymorphic site, which is located at 5' to the Gγ-gene, is different among various population (Table:2). There is evidence that a homozygous state for Xmn1 polymorphic site, which is associated with increased expression of *Gγ-gene*, may play an important role among other factors in ameliorating the clinical features of homozygous *β*-thalassemia and its clinical presentation as thalassemia intermedia.[57] Furthermore, the presence of *XmnI* polymorphic site 5' to the Gγ-*globin* promoter region was positively correlated with elevated synthesis of fetal Hb and its Gγ-globin component in term newborn infants and is associated with delayed switch from fetal to adult hemoglobin. It is unknown how *Xmn1* polymorphic site influences the expression of the Gγ-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 adult F cell (FC) levels.[58,59] Unlike the rare mutations in the *γ-globin* promoter that are consistently associated with large discrete effects on the increase of HbF levels in the range of 10–35% in heterozygotes, the so-called pan-cellular hereditary persistence of fetal hemoglobin (HPFH), the change at Gγ -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]

Table II: Allelic Frequency of *Xmn1* 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-thalassemia	0.25	77
Eastern India	64	β-thalassemia and HbE/β-thalassemia	0.48	78
Northern India	101	β-thalassemia major and intermedia	0.28	79
Southern Iran	48	β-thalassemia intermedia	0.41	80
Western Iran	197	β-thalassemia major	0.39	60
Malaysia	107	β-thalassaemia major	0.66	81

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, there was a significant correlation between the presence of Xmn1 polymorphic site and increased Gγ: Aγ ratio.[63] However, the HbF level was not significantly increased in the presence

of *Xmn1* polymorphic site in their study. Although *XmnI* polymorphic site maintains a Gγ: Aγ ratio typical of fetal life, it does not necessarily cause elevation of HbF. 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 Gγ percent and Gγ: Aγ ratio were significantly increased (*p*= 0.01) and the clinical features such as splenomegaly and bone marrow expansion were significantly improved (p = 0.01).[60] It was found that the level of Hb F tended increase in the presence of *Xmn1* polymorphic site on both chromosomes (+/+) compared to the absence of *Xmn1* (-/-) (table 3).[60]

Table III: Parameters associated with Xmn1 polymorphism in β-thalassemia patients studied by Hooshang N et al (2010)

Parameter	*Xmn1* C/C (+/+)	*Xmn1* C/T (+/-)	*Xmn1* T/T (-/-)	*P value*
Hb F level %	97.1	94.4	91.8	0.08
Gγ %	74.8	71.4	66.7	0.01
Aγ %	25.1	28.6	33.3	0.01
Gγ/ Aγ ratio	3 ± 0.5	2.5 ± 0.4	2 ± 0.3	0.01
Age of first blood transfusion (months)	12 ± 8	11 ± 5	10 ± 5	0.16
Facial bone deformity %	17.5	25.4	57.1	0.02
Splenectomy %	15.9	19.1	65	0.01

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

Association of Gγ-XmnI polymorphism with Hydroxyurea (HU) treatment: HU therapy has been successfully used in thalassemia intermedia patients and is associated with a significant improvement in hematological parameters and quality of life.[70,71] Hydroxyurea efficacy in treatment of β-thalassemia 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 Xmn1 site (*HBG2*:c.- 53-158C>T) upstream of the Gγ-globin gene and it is thus far the most studied nucleotide change to have a significant association with drug response. This particular polymorphism acts as an enhancer of HbF expression during erythropoietic stress, resulting in a beneficial effect in Sickle cell disease (SCD) patients. HU is a myelo-suppressive agent that may enhance fetal hemoglobin 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 thalassemia. Moreover, one study on β-thalassemia patients treated with Hydroxyurea has revealed a significant correlation between the presence of T allele in *Xmn1* polymorphic site and the better treatment response. Hydroxyurea therapy exerts a 2- to 9- fold increase in γ-mRNA expression in β-thalassemia patients,[74] leading to improvement in the alpha/non–alpha chain imbalance and more-effective erythropoiesis.[75] However, Kosaryan et al. suggested that β-thalassemia major or intermedia with *Xmnl* polymorphism of (C/T or +/–) showed better response to hydroxyurea therapy than (T/T or –/–) genotype. However, this finding was not supported by other studies.[76] We did not find any report on association of *Gγ-XmnI* polymorphism with Hydroxyurea (HU) treatment in Bangladeshi population.

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-thalassemia major and HbE/β-thalassemia. Therefore, for investigating the success of Hydroxyurea medication in diseased population, a follow-up study is required to determine the effect of HU treatment on HbF induction in the presence of T allele in thalassemia patients. In addition, studies on other potential genetic markers which may help in predicting the therapeutic intervention are required. Till now, there is no study demonstrating the association between *XmnI-Gγ* polymorphism and HbE/β

thalassemia disease severity in Bangladesh. So, wet lab studies are necessary to observe the effect of hydroxyurea (HU) for treating Bangladeshi thalassemia patients, especially Hb E/β thalassemia patients.

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