Prevalence and Mutation Spectrum of Thalassemia Carriers and Study of Genetic Modifiers Affecting the Disease Severity of HbE/β-Thalassemia in Bangladeshi Population

Thesis for Doctoral Degree (Ph.D.)

A Dissertation Submitted to the University of Dhaka in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry and Molecular Biology

Submitted by

Farjana Akther Noor

Registration No: **12/ 2017-2018**

Faculty of Biological Sciences
Department of Biochemistry and Molecular Biology
University of Dhaka, Dhaka-1000, Bangladesh
PhD Fellow
Bangabandhu Science and Technology Fellowship Trust
Ministry of Science and Technology, Govt. of Bangladesh

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Declaration

I hereby declare that, this thesis entitled, **'Prevalence and Mutation Spectrum of Thalassemia Carriers and Study of Genetic Modifiers Affecting the Disease Severity of HbE/β-Thalassemia in Bangladeshi Population'** is an original work and any published or unpublished writings discussed have been clearly referenced in the text. To the best of our knowledge no part of the work presented here has been submitted for any other degree or qualification or to pass an examination.

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Farjana Akther Noor

PhD Student
Registration No: 12/ 2017-2018
Faculty of Biological Sciences
Department of Biochemistry and Molecular Biology
University of Dhaka, Dhaka-1000, Bangladesh
PhD Fellow
Bangabandhu Science and Technology Fellowship Trust
Ministry of Science and Technology, Govt. of Bangladesh

Certificate

This is to certify that Farjana Akther Noor (Registration No: 12, Session: 2017-2018) has conducted her thesis work entitled, 'Prevalence and Mutation Spectrum of Thalassemia Carriers and Study of Genetic Modifiers Affecting the Disease Severity of HbE/β-Thalassemia in Bangladeshi Population' under our supervision. This is to further certify that it is an original work and suitable for the fulfillment of the degree of 'Doctor of Philosophy in Biochemistry and Molecular Biology' from the University of Dhaka.

…….
(Supervisor)

Hossain Uddin Shekhar, PhD

Professor
Department of Biochemistry and
Molecular Biology
University of Dhaka, Dhaka-1000, Bangladesh
Phone: +8801715-059252
Email: hossainshekhar@du.ac.bd

…….
(Co-Supervisor)

Kaiissar Mannoor, PhD

Head Scientist
Institute for developing Science and Health initiative (ideSHi)
9, Mohakhali, Hosaf High Tower, Dhaka-1212
Phone: +8801797-440713
Email: mdkmannoor@gmail.com

Dedications

To

My Beloved Parents

Md. Abdul Majid Noor and Dr. Jahanara Majid

&

My Husband and my Daughter

Abstract

Background: As being a country of the global thalassemia belt, β-thalassemia, a hereditary hemolytic disorder caused by reduced or absent synthesis of the β-globin chain of the hemoglobin tetramer ($\alpha_2\beta_2$) is the most common congenital single gene disorder in Bangladesh. However, it is a matter of deep concern that thalassemia is not recognized as priorities in public health sectors of the country and there is no exact data representing the current prevalence of thalassemia in different regions Bangladesh. Also, molecular study for mutation profiling of the carriers is lacking. The only available remedy for thalassemia is an overly expensive allogeneic bone marrow transplant, otherwise lifelong regular blood transfusion that gradually damages the vital organs of the body due to excess iron accumulation. However, due to having autosomal recessive transmission this deadly incurable disease is completely preventable by carrier detection followed by genetic counselling and discouraging marriage between the carriers. For development of a proper prevention strategy, it is important to know the carrier frequency of thalassemia with complete mutational profiling involving participants from all over the country. To get an accurate prevalence data, a large-scale population survey, which should not be hospital-based, is mandatory using proper screening methods combining hematological and molecular approaches.

Another challenge is the clinical manifestations of thalassemia, particularly the Hb E/β-thalassemia thst is highly variable in terms of severity which might be influenced a number of genetic factors like types of β-globin mutations, polymorphisms in modifier genes of γ-globin expression, coinheritance of α-thalassemia etc. In Bangladesh, so far there is no any precise study on the genetic modifiers of the clinical variability of HbE/β-thalassemia patients. The proposed work will evaluate the genetic factors modulating the phenotypic heterogeneity of HbE/β-thalassemia in Bangladeshi patients.

Hypothesis: 1. Actual prevalence of thalassemia carriers is high in Bangladesh and molecular approaches will complement the conventional methods for carrier detection of thalassemia to provide an accurate data on frequency of the carriers in Bangladesh. 2. Specific mutations in the genetic modifiers affect the disease severity in HbE/β-thalassemia patients of Bangladesh.

Aim: One of the objectives of our study was to determine the nationwide (1) carrier frequency of hemoglobin (Hb) E (ETT) and β-thalassemia (BTT) and (2) mutation spectrum among the carriers using molecular, hematological and biochemical methods. Another objective was to study the effect of genetic modifiers influencing disease severity in HbE/β-thalassemia patients of Bangladesh that

includes types of β-globin mutation and coexistance of α-thalassemia along with fetal hemoglobin inducer 8 single nucleotide polymorphisms in 4 major modifier genes.

Methods: For prevalence determination, the study enrolled a total of 1877 individuals (60.1% male and 39.9% female) aged between 18-35 years. The total sample size and its division-wise breakdown were statistically calculated in proportion to national and division-wise population size. About 5.0 mL blood was collected and hematological indices were measured using Complete Blood Count (CBC) analysis and Hb-electrophoresis. Serum ferritin was measured to detect coexistence of IDA with thalassemia carrier. DNA-based High Resolution Melting curve analysis was performed for confirmation of carrier status and detection of mutations.

To determine the effect of genetic modifier of the HbE/β-thalassemia, a cross-sectional was performed on a total of 130 of HbE/β-thalassemia patients and 50 unrelated healthy individuals over a period of 11 months from September 2018 to August 2019. These patients, with the age range of 6 to 65 years (80 males, 50 females), were enrolled from the Bangladesh Thalassemia Samity Hospital located in Dhaka where they came for follow-up examination and blood transfusion. The patients were categorized into three severity groups – mild, moderate, and severe following previously reported Mahidol disease severity scoring system. The ethical clearance certificate and consent form regarding this research was approved by Bangladesh Medical Research Council (BMRC) of National Ethics Review Committee (NERC), Dhaka, Bangladesh. Upon obtaining written informed consent form along with a structured questionnaire of information about the age of onset, age of first transfusion, transfusion interval, and splenectomy status of the patients venous blood samples (~ 5 ml) were collected from the patients and subjected to CBC and Hb electrophoresis. After genomic DNA extraction, PCR-RFLP method was used to identify rs7482144 (-158 Xmn1-Gγ) in γ-globin promoter. As PCR-RFLP is a lengthy time consuming method requiring a number of steps, we established Real-time PCR followed by HRM curve analysis based SNP detection method for rest of the 7 SNPs namely, rs4895441, rs28384513, rs28384512, rs11886868, rs4671393, rs766432 and rs2071348. Conventional Gap-PCR was used for detection of $-\alpha^{3.7}$ deletion and $\alpha\alpha\alpha^{anti\ 3.7}$ triplication while $--^{SEA}$ deletion was detected using Real-time Gap-PCR followed by HRM curve analysis.

Result: Total carrier frequency of HbE plus β-thalassemia was 10.92%, where ETT had the highest frequency of 8.68% followed by BTT 2.24%. Among eight divisions, Rangpur had the highest carrier frequency of 27.1% (ETT-25%, BTT-2.1%), whereas Khulna had the lowest frequency of 4.2% (ETT-4.2% only). Moreover, α- thalassemia, HbD trait, HbE disease, hereditary persistence of HbF

were detected in 0.11, 0.16, 0.43 and 0.16% participants, respectively. HRM could identify two individuals with reported pathogenic mutations in both alleles who were erroneously interpreted as carriers by hematological indices. Finally, a total of nine different mutations including a novel mutation (c.151A > G) were detected in the β-globin gene.

Low levels of HbF and HbE were found to be significant determinants of high disease severity in patients with HbE/β-thalassemia as both were inversely related to the disease severity score as well as showing the higher was the level of HbF, the higher was the blood transfusion interval and age of 1st blood transfusion. 11 different causative mutations were found in the the β- globin allele trans to HbE allele in the patients with HbE/β-thalassemia in Bangladesh. c.79 G>A (E allele)+IVS1_5 G>C combination was found as most predominant mutation pair across all the 3 severity groups of patients covering 73.8% of HbE/β-thalassemia in the country. On the other hand, heterozygous silent carrier and homozygous trait of $\alpha^{3.7}$ deletion were found only in 1 moderate and 2 sever cases in the present study while α-globin triplication ($\alpha\alpha\alpha^{anti\ 3.7}/\alpha\alpha\alpha^{anti\ 3.7}$) was found in 4 moderate and 8 sever patients However, the $\alpha\text{--}^{SEA}$ thalassemia allele was not detected in our HbE/β-thalassemia patients.

Among the 8 SNPs, the highest MAF (minor allele frequency) was found for HBS1L-MYB rs28384513 with the frequency of 0.46 followed by -158 Xmn1-Gγ in HBG2 (MAF: 0.45), HBS1L-MYB rs28384512 (MAF: 0.45) and rs2071348 in HBBP1 gene (MAF: 0.28). The lowest MAF of 0.1 was found for the rs11886868 in BCL11A gene. Upon association study, only 4 SNPs, namely, -158 Xmn1-Gγ, rs4895441, rs28384513 and rs2071348 showed statistically significant association with elevated level of HbF in the study population and 3 SNPs namely -158 Xmn1, rs4895441 and rs2071348 showed significant association with the clinical scores of the patients with HbE/β-thalassemia. The strongest association in terms of both increasing HbF level and decreasing severity, was observed with SNPs in *HBG2* (-158Xmn1-Gγ) and *HBBP1* (rs2071348) gene in the β globin gene cluster followed by *HBS1L_MYB* rs4895441 and rs28384513. However, none of the 3 SNPs in BCL11A exhibited significant association with either HbF level or the severity score

Conclusion: Carrier frequencies of both HbE and β-thalassemia are alarmingly high in Bangladesh. A nationwide awareness and prevention program is in mandate to halt further aggravation of the current burden of thalassemia patients in Bangladesh. Detection of the HbF inducer SNPs for our population will help in planning appropriate management and treatment strategy revealing new therapeutic targets for increasing HbF levels in HbE/β-thalassemia patients in our country.

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List of Abbreviations

bp	Base pair
BTM	Beta thalassemia major
BCL11A	B-cell lymphoma/leukemia 11A
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
gDNA	Genomic DNA
Hb	Hemoglobin
HBA2	Hemoglobin subunit alpha 2 gene
HBB	Hemoglobin subunit beta gene
HBD	Hemoglobin subunit delta gene
HBG1	Hemoglobin subunit gamma 1 gene
HBG2	Hemoglobin subunit gamma 2 gene
HBS1L	HBS1 Like Translational GTPase
HCT	Hematocrit
HRM	High Resolution Melting
IVS	Intervening sequence
KLF1	Krupple like factor 1 gene
LCR	Locus control region
MCV	Mean cell volume
MCH	Mean corpuscular hemoglobin
MYB	Myeloblastosis oncogene
NCBI	National Center for Biotechnology Information
NTDT	Non-transfusion dependent thalassemia
PCR	Polymerase chain reaction
RDW	Red blood cell distribution width
RFLP	Restriction Fragment Length Polymorphism
SCD	Sickle cell disease
SNP	Single nucleotide polymorphism
UTR	Untranslated region
XmnI	Xanthomonas manihotis-I

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Introduction

CHAPTER 1

1.1 Background

Bangladesh has been fighting against communicable diseases for decades. In last few years, we have attained success in reducing child mortality and morbidity involving communicable diseases by effective interventions to control the infectious diseases through vaccination, vitamin A supplementation, oral rehydration therapy for diarrhoea, antibiotics usage, and increased breast feeding etc. (Khan Jahidur Rahman and Awan 2017, WHO 2011). However, due to unawareness about non-communicable diseases like inherited genetic disorders, the child mortality and morbidity is still high and according to UNICEF data on monitoring the situation of children and women, the under-5 child mortality rate was 3.08% for Bangladesh in 2019 (UNICEF 2020). Among the common genetically inherited disorders, hemoglobinopathies are predominant in Bangladesh affecting life of millions of people throughout the world as well (Weatherall David J and Clegg 2001). These disorders are inherited in autosomal recessive manner affecting the quality and quantity of hemoglobin (Hb) molecules within the red blood cells (RBCs). According to WHO, each year 300,000 – 400,000 babies with severe hemoglobin disorders are born due to ignorance of carrier screening for hemoglobinopathies like thalassemia and Sickle Cell Diseases, which simply can be avoided by performing premarital screening and/or prenatal screening (Weatherall David J 2010b). Premarital screening could help couples to be aware of their conditions regarding hemoglobinopathies and genetic counselling is recommended to carrier couples at risk of having affected child (Weatherall DJ 2010a).

In Bangladesh, more than 95% cases of hemoglobinopathies are related with β-globin (*HBB*) gene defect resulting in beta (β) and HbE/beta (β) thalassemia (Uddin et al. 2012). Bangladesh, with population of over 180 million, has no national data about the number of thalassemia patients in the country and information on the prevalence of thalassemia is scarce as well. According to a 15 years old report generated from a small sample study, annual thalassemic born is approximately 8990 (Khan WA et al. 2005). However, majority of this densely populated country remain unaware of the health risks associated with thalassemia. Moreover, most public hospitals and private clinics lack resources such as appropriate medication, expert professionals, and specialized medical equipment (Islam Anwar and Biswas 2014). The only available remedy for thalassemia is allogeneic bone marrow transplant (BMT) which is a sophisticated and an overly expensive procedure. Otherwise, patients with severe thalassemia (β – thalassemia major and E/β – thalassemia)

require life-long blood transfusions and highly expensive iron-chelation therapy to survive which causes endless sufferings to the patients and their family. However, this deadly incurable disease is preventable, and for development of a proper prevention program it is important to know the carrier frequency of thalassemia involving participants from all over the country.

Another challenge in the management of the disease is that the clinical manifestations of thalassemia, particularly the Hb E/β-thalassemia is highly variable in terms of severity (variation in transfusion interval, first age of transfusion, first onset of the disease, and splenomegaly etc) - from thalassemia intermedia like symptoms to severe transfusion-dependent thalassemia major. The reasons for this clinical variability is still poorly understood. The variable degree of clinical severity might be influenced by genetic factors either linked or unlinked to globin genes as well as environmental conditions and management. A number of genetic factors like types of β-thalassemia mutations, polymorphisms those influence the production of post-natal γ-globin expression, and thus elevates the fetal hemoglobin (HbF) level in adults, the coinheritance of α-thalassemia etc. and genetic modifiers unlinked to globin genes as well. Several strategies for thalassemia management can be developed by extensive research on the influence of these genetic modifiers. More than 900 mutations or variants have so far been reported in beta globin gene among which 535 mutations are associated with thalassemia (Giardine et al. 2021). The spectrum of mutations varies across different geographical regions and cultures. Hence, regional mutation profiling is essential to undertake any strategies (e.g. genetic counseling, prenatal diagnosis) to deal with thalassemias. In addition, for careful tailoring of treatment and management for each patient, understanding of genetic factors which modify the severity of HbE/β-thalassemia is required.

1.2 Hemoglobin

Hemoglobins are the highly specialized protein molecules which act as an oxygen transporter of our body. There are roughly 270 million hemoglobin molecules present in every red blood cell and thus constituting about 27 to 30 picogram per cell (Weatherall David J 2001). The matured form of Hb protein present in a healthy adult person contains 4 polypeptide chains of two different globin genes and all the subunits arranged like a thick-walled shell with a central cavity. The exceptionally characteristic structure of globin chains

makes them capable of rapid and efficient loading with oxygen in the lung alveoli following the gradual transport of the gas into different tissues throughout the body.

The globin genes

The specific genes coding the globin chains are present as two different gene clusters- the alpha globin gene cluster on chromosomes 16 comprising the zeta, ζ- and alpha, α-globin chains, and the second one is the β-globin cluster on chromosome 11, which is composed of several genes for different globin chains like epsilon (ε), gamma (γ), beta (β) and delta (δ). These chains are differentially produced, with different combinations, one after another during ontogeny; therefore, they are diverse at different stages of development (the embryonic, fetal, and adult stages) (El-Kamah and Amr 2015, Noor et al. 2019).

Regulation of Hemoglobin synthesis: Hemoglobin switching

Four major types of hemoglobin produced during the entire developmental process which requires two switches: the first switch occurs at 6 weeks of gestation from embryonic to fetal hemoglobin (Hb) and the second switch is from fetal Hb to adult Hb observed at birth.

Type-1: "Embryonic" hemoglobin, namely Hb Gower 1 (ζ2ε2) which is gradually replaced by α chains within 8 weeks; the other four embryonic hemoglobins are namely Hb Gower 2 (tetramers of α2ε2), Hb Portland 1 (tetramers of ζ2γ2) and Hb Portland 2 (ζ2β2 tetramers) which are present only between the third and 10th week of gestation.
Type-2: "Fetal" hemoglobin (HbF), a tetramer of α2γ2 globin chains and the main oxygen carrier molecule during pregnancy.

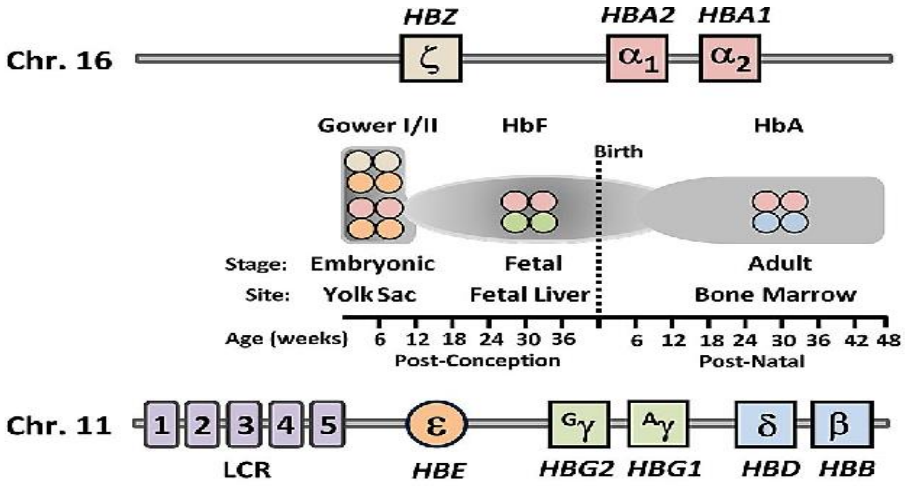


Figure 1.1: **The human globin genes and their patterns of expression throughout the development process** (Wilber et al. 2011).

Type-3: Hb A or "Adult" hemoglobin, a tetramer of two α and two β chains , replaces HbF within six to 12 months after birth; and

Type-4: HbA2 which is a minor adult hemoglobin component consisting two alpha and two delta chain (α2δ2).

In both the gene clusters, flanking upstream region (the 5' side of the gene sequence) and downstream region (the 3' side of the gene cluster) contain several distinguishing nucleotide sequences which regulate the gene expression level and also determine which gene is to be turned on and which is to be switched off. (Figure 1.2). The promoters of the genes interacts with the β Locus Control Region (β-LCR), an upstream regulatory element which is essential for transcribing the β-globin genes and is achieved through the formation of a chromatin loop (Noordermeer and de Laat 2008). The synthesis of human globin chains is tightly regulated by multiple fine and intricate mechanisms to assure the accurate and balanced assembly of globin peptide chains at every developmental stage by producing the equal amount of α- globin chains and β-globin chains throughout the ontogeny (Cao and Moi 2000).

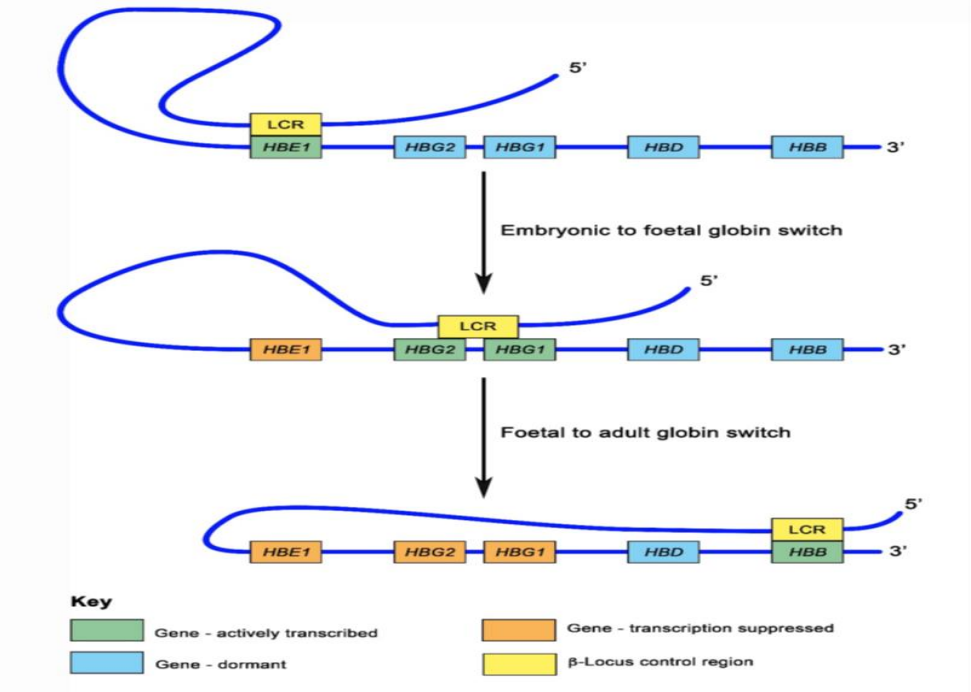


Figure 1.2: **Globin gene switching mechanism**. The HBE1 gene is the first gene in the locus to be expressed during embrionic stage. Autonomous suppression of the HBE1 gene permits the HBG1 and HBG2 genes to access the β-LCR leading to a switch from embryonic to fetal hemoglobin. β-LCR binds to the HBB gene promoters with the help of Krupple Like Factor 1 (KLF1) allows the HBB gene access to the β-LCR resulting in the switch from fetal to adult hemoglobin (Paikari and Sheehan 2018).

1.3 Hemoglobinopathies: A group of monogenic disorders

A monogenic disorder is an abnormality in an individual's genome which caused by a single error in one gene. More than 7,000 genetic disorders have been well recognized among which around 6,000 are caused by single gene defect according to the Online Mendelian Inheritance in Man database (OMIM), as of June, 2022 (OMIM 2022). Groups of inherited monogenic disorders of the hemoglobin molecule located in red blood cells are termed as 'Hemoglobinopathies' which are now predominant in almost all countries across the world (Modell and Darlison 2008, Vichinsky Elliott P 2005). Approximately 320,000 babies are born every year with a clinically significant hemoglobin disorder among which, about 80% of the babies are from the developing countries. Highly regarded estimates show that over 360 million people constituting approximately 5.2% of the world population carry genes of hemoglobin variant (Modell and Darlison 2008).

1.3.1 Classification of Hemoglobinopathies

More than 1000 disorders of hemoglobin structure and/or synthesis have been identified and characterized (Forget and Bunn 2013). These are categorized into two main groups: 1. structural variants of hemoglobin variants (abnormal Hb) and 2. Thalassemia syndromes. There are many subtypes and combined types in each group (Lee et al. 2019).

Structural variants of hemoglobin are caused by single alteration of amino acid sequence in the α- or β-globin chains leads to the structural change (primary, secondary, tertiary, and/or quaternary) of the Hb molecule resulting in qualitative defect. The majority of Hb variants do not cause disease and are most commonly discovered either incidentally or through newborn screening. However, a subset of Hb variants can cause severe disease when inherited in the homozygous or compound heterozygous state in combination with another structural variant or a thalassemia mutation (Kohne 2011). The common variants are-

– HbS is the most dangerous of all hemoglobinopathies.

– HbC disease course is similar to progression of sickle cell disease but is less fatal.

– HbE diseases often produces phenotypes like β-thalassemias.

Thalassemia syndromes causes quantitative defects with decreased levels of one of the two globin chains (α- and β), which leads to an imbalance in the ratio of α-chains to β-chains.

Table 1.1: Classification of major Hemoglobinopathies (Lee et al. 2019).

Type	Diagnosis		Gene type
Thalassemia	α-thalassemias	Heterozygous α^{+}-thalassemia	$-\alpha/\alpha\alpha$
		Homozygous α^{+}-thalassemia	$-\alpha/-\alpha$
		Heterozygous α^{0}-thalassemia	$--/\alpha\alpha$
		Mixed heterozygosity, α^{+}/α^{0}-thalassemia	$--/-\alpha$
		Homozygous α^{0}-thalassemia	$--/--$
	β-thalassemias	Heterozygous β-thalassemia	β^{++}/β, β^{+}/β, β^{0}/β
		Mild homozygous or compound heterozygous β-thalassemia	β^{+}/β^{+}, β^{+}/β^{++}, β^{+}/β^{0}, β^{0}/β^{0}
		Homozygous β-thalassemia	β^{+}/β^{+}, β^{0}/β^{0}
		Compound heterozygous β-thalassemia	β^{+}/β^{0}
Structural variants	HbS	HbS heterozygosity	HbAS
		Sicklecell disease	HbSS
	HbC	HbC heterozygosity	HbAC
		HbC disease	HbCC
	HbE	HbE heterozygosity	HbAE
		HbE disease	HbEE
Mixed variants	β-thalassemias + HbS or HbE	Sickle cell β^{+}-thalassemia	HbS β^{+}-thalassemia
		Sicklecell β^{0}-thalassemia	HbS β^{0}-thalassemia
		HbE β^{+}-thalassemia	HbE β^{+}-thalassemia
		HbE β^{0}-thalassemia	HbE β^{0}-thalassemia
	HbS + HbC	HbSC	HbSC disease

1.4 Thalassemia: Genotypic and Phenotypic Classification

The term "Thalassaemia" or "Thalassemia" stands for a group of blood disorders characterized by declined or an absent synthesis of typical globin chains with functional structure. They are inheritable hemolytic congenital disorder where globin gene defects are inherited as recessive trait (Weatherall David J 2001). Based on the genotypes, the thalassemias are classified according to the impaired chain whose synthesis or structure is affected due to the corresponding defected gene and named as α-, β-, γ-, δ -, δβ-, or εγδβ-thalassemias accordingly (Viprakasit and Ekwattanakit 2018). Co-inheritance of structural hemoglobin variants such as hemoglobin S, C, and E with thalassemia trait are also quite common which results in several forms of compound heterozygous thalassemia like hemoglobin E/ β -thalassemia, S/ β -thalassemia, and hemoglobin C/ β –thalassemia etc (Steinberg et al. 2009). However, generally the thalassemia syndrome is broadly characterized as α or β-thalassemia.

The clinical manifestations of thalassemia are highly variable, ranging from no symptom to critically severe even life threatening (Danjou et al. 2011). The terminology used to describe the different clinical presentations of the disease is explained in Table 1.2.

Table 1.2: Clinical terms used to indicate the severity of thalassemia**.**

Clinical term	Description
Silent thalassaemia trait	No apparent effect on phenotype (Weatherall David J 2001).
Thalassaemia minor also known as Thalassaemia carrier or trait	Individuals are largely asymptomatic, but have mild hematological abnormalities, including subclinical anaemia and a reduced mean red cell volume (MCV) (Brancaleoni et al. 2016) .
Non-transfusion dependent thalassaemia (NTDT), or Thalassaemia intermedia	Individuals with NTDT are symptomatic and may occasionally require blood transfusions, but not totally transfusion dependent (Saliba & Taher 2016).
Thalassaemia major	Severely affected individuals who are dependent on blood transfusions to maintain a hemoglobin level compatible with life (Weatherall David J 2001).

1.4.1 The α-Thalassemia

Alpha thalassemia is the result of deficient or absent synthesis of α-globin chains, leading to excess β-globin chains. In human, there are 4 genes for α-globin chains- two copies inherited from each of the parents. Inadequate production of α-globin chains is generally caused by a deletion of at least one of these 4 genes.

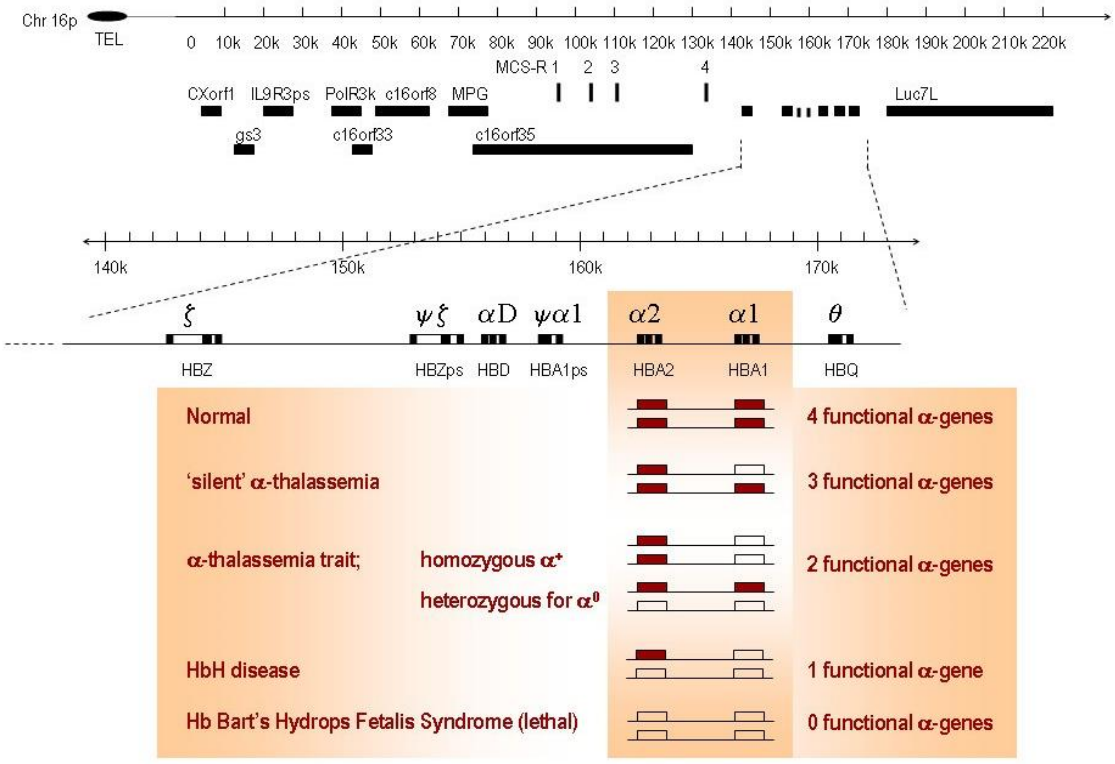


Figure 1.3: The classification of gene defects and phenotypic expression in α-thalassemia (Harteveld and Higgs 2010).

A single gene deletion brings about alpha thalassemia silent carrier state. The two genes deletion causes alpha thalassemia trait (minor). The three gene deletion leads to excessive production of hemoglobin H (HbH), which is defined as alpha thalassemia intermedia, or HbH illness with moderate to severe hemolytic anemia, modest degree of ineffective erythropoiesis, spelomegaly, variable bone changes. The four-genes deletion results in significant production of hemoglobin Bart's (Hb Bart's), which has four gamma chains and normally leads to deadly hydrops fetalis syndrome (Muncie and Campbell, 2009). Thus, four clinical conditions of phenotypic severity are recognized in α-thalassemia (Figure 1.3). *α+-thalassemia* usually caused by the deletion or dysfunction of one of the four normal alpha globin genes (Asymtomatic). *α°-thalassemia* resulting from deletion or dysfunction of two alpha genes (Asymtomatic) (Galanello Renzo and Cao 2011).

1.4.2 The β-Thalassemia

β -thalassemias are heterogeneous autosomal recessive hereditary anemias characterized by reduced (β^+) or absent (β^0) β-globin chain synthesis of the hemoglobin tetramer. The resulting relative excess of unbound α-globin chains precipitate in erythroid precursors in the bone marrow, leading to their premature death and, hence, to ineffective erythropoiesis (Figure 1.4). β-thalassemia phenotypes are variable, ranging from the severe transfusion dependent thalassemia major to the mild form of thalassemia intermedia. Patients with the major form of the disease have severe anemia, microcytic and hypochromic anemia, hepatosplenomegaly, and usually come to medical attention within the first two years of life (Danjou, Anni and Galanello, 2011).

Based on clinical phenotypes, β-thalassemia can be classified into:

• *β-Thalassemia major (BTM, Cooley´s Anemia):* refers to a severe clinical phenotype that occurs when patients are homozygous or compound heterozygous for more severe β chain mutations, e.g. severe β^+/β^+ mutations, β^+/β^0, β^0/β^0.

• *β-Thalassemia intermedia (BTI):* An in between clinical phenotype with heterogeneous genetic mutations that still allow for some β chain production (e.g. β+/ β0, β+/ β+). Some rare cases also exist in which both β and α mutations coexist.

• *β-Thalassemia minor (BTT, carrier/trait):* a mild clinical phenotype when one normal copy of the β globulin gene is present (β^+/β, β^0/β)(Forget and Bunn 2013).

The basic pathogenesis of all kinds of thalassemia is a consequence of the synthesis of leftover unsteady globin chains which ultimately precipitates inside the cell and totally

distorts the structure of RBCs making them unstable and fragile. However, the precipitation rate and pattern of the unsteady hemoglobin chains on the RBC membrane are variable and thus the effects also fluctuate in different forms of thalassemia, even in patients with the same disease (Shinar and Rachmilewitz 1990).

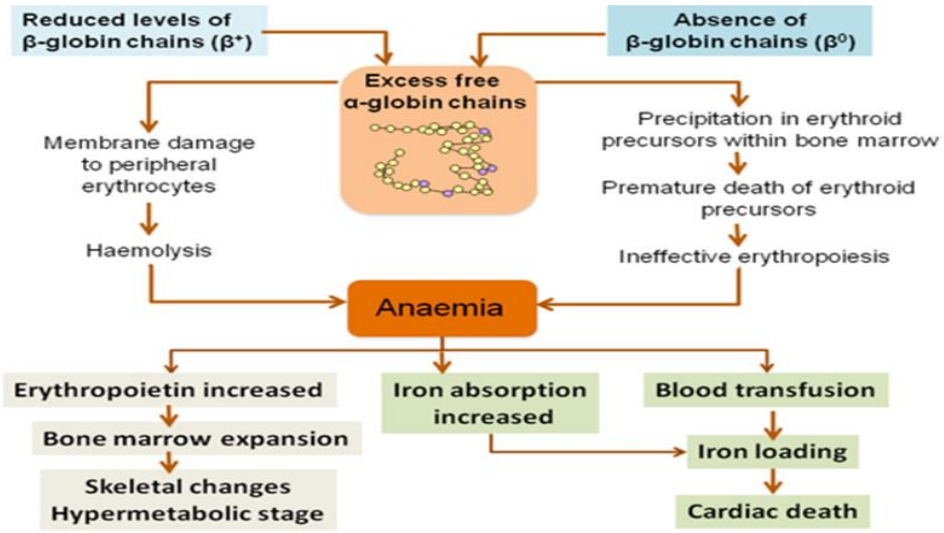


Figure 1.4: Pathophysiology of β-thalassemia (Cappellini et al. 2014).

Thalassemia major patients have a severe anemia, with low mean corpuscular volume (MCV) and mean corpuscular Hb (MCH). Peripheral blood smear shows, microcytosis (smaller in size) and hypochromia, anisocytosis (unequal sized RBCs), poikilocytosis that refers to the variation in cell shape (tear-drop and elongated cells). The other forms can also be defined by specific hematological and clinical features (Brancaleoni et al. 2016).

Table 1.3: Hematological and clinical feature of β-thalassemia syndromes.

	BTT	**BTM**		**BTI**
Hb levels	Normal/ slightly reduced(<12g/dL)	<7 g/dL		~7–10 g/dL
Hemoglobin study	HbF may be increased to 10% HbA2 >3.5%	β^0/β^0	β^0/β^+	HbF 10–50% HbA2 >5%
		HbF up to 95% HbA2 >5%	HbF 70–90% HbA upto 30%	
RBC indices	MCV<80 fl MCH<27 pg	MCV<60 fl MCH<20 pg		Variable.
Transfusion requirements	Not required	Transfusion dependent		NTDT or rare/occasional/ intermittent transfusions depending on the clinical situations like infections and pregnancy.
Clinical presentation	Asymptomatic	Severe microcytic hypochromic anemia, mild to moderate jaundice, and hepatosplenomegaly		

1.4.3 HbE/β-thalassemia

It results from co-inheritance of a β-thalassemia allele from one parent and the structural variant Hemoglobin E from the other. Haemoglobin E (HbE) is the common most structural variant causing attributes particularly when co-inherit with mutant copy of gene in another allele. The structural variant HbE is formed due to a single nucleotide substitution mutation from G to A at the 79th position of the β-globin genes resulting in the amino acid substitution by lysine for glutamic acid at position 26 of the β-globin chain. This missense mutation generates a hidden splice site between codon 24 and 25, leading to a different splicing pattern resulting in less production of the variant haemoglobin (HbE). It is the underlying reason of 25-30% HbE of total haemoglobin in individuals with HbE traits instead of projected 50% (Kawthalkar 2012). These phenomenon suggest that the codon 26 G→A mutation results in not only a qualitative faulty gene by producing a hemoglobin variant but also a quantitative β-globin gene defects affecting the expression level of HbE protein (Fucharoen and Weatherall 2012) resulting in highly variable degree of severity among the patients. HbE trait and homozugous Hb E diseases are mild disorders while HbE/β-thalassemia is the most serious form of HbE syndromes, affects a million people worldwide (Vichinsky Elliott 2007).

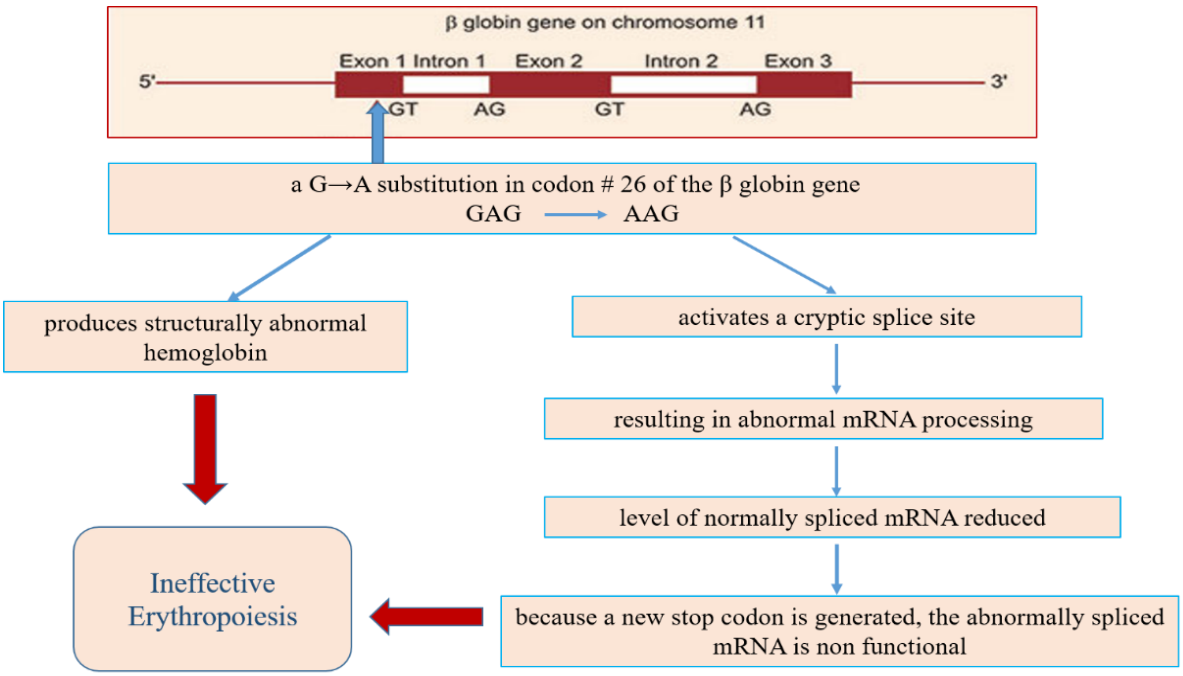


Figure 1.5: Pathophysiology of HbE/β-thalassemia.

1.5. Epidemiology

1.5.1. Worldwide distribution

According to the World Health Organization (WHO), around 6.5% of the world populations are currently carriers of different hemoglobinopathies (Modell and Darlison 2008). Among these, β-thalassemia is the most prevalent non communicable disease of blood worldwide with global annual incidence of one in 100,000 (Galanello Renzo and Origa 2010). Around sixty countries with highest occurrence of thalassemia in Mediterranean, part of North and West Africa, the Indian subcontinent, Middle East and Southeast Asia together constitute the "Thalassemia Belt" (Figure 1.6). The distribution of the disease is not uniform, even in the countries or regions residing in thalassemia belt. These variations in frequency of beta thalassemia mainly depend on the ethnic population. β-thalassemia is particularly widespread Cyprus with the highest carrier frequency (14%), Greece (5-15%), Sardinia (10.4%) and Iran (4-10%) (Flint et al. 1998).

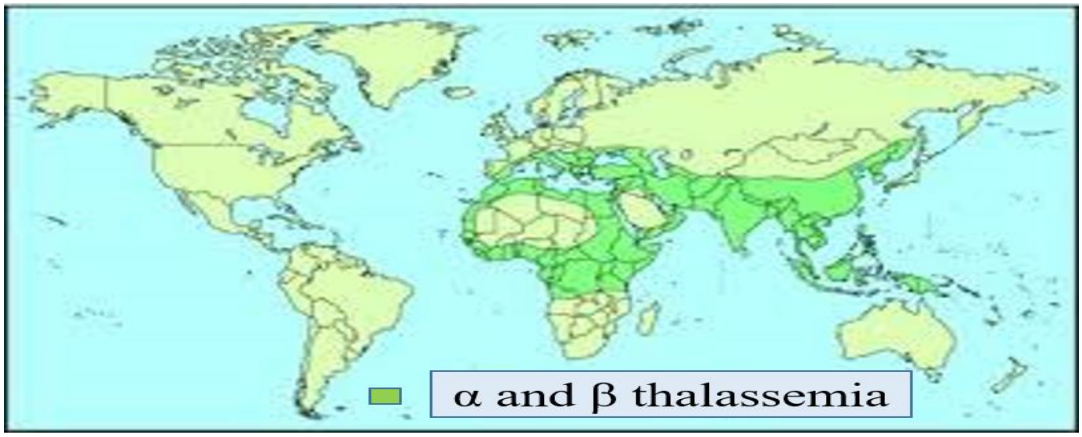


Figure 1.6: Global Thalassemia Belt (*Figure source: internet***).**

However, thalassemia have introduced in almost every country of the world due to increased rate of population immigration and intermarriage between different customs and cultures. In Northern Europe thalassemia was previously absent but now the country have been reported having patients with thalassemia (Vichinsky Elliott P 2005). According to WHO, the overall estimated β-thalassemia carriers present worldwide are approximately 80–90 million which is about 1.5% of the total global population and predicts that around 68,000 homozygous symptomatic borns annually with thaassemia. Incidence of thalassemic baby birth rate has been reported as 1 in 10,000 in Europe (Vichinsky Elliott P 2005). Middle East countries are also highly prevalent for β-thalassemia particularly in Iraq, Lebanon, Egypt and Morocco with moderate to high carrier rate. In Iraq, β-thalassemia is an

evident health problem (Kadhim et al. 2017) and the most common genetic disorder in Lebanon with a carrier frequency of nearly 2.3% (Abi Saad et al. 2014).

Both β-thalassemia and compound heterozygote HbE/β-thalassemia are very prevalent in South Asian and South East Asian countries. HbE is the hallmark for South East Asia and, in some regions, the carrier frequency of HbE variants is as high as around 50%. HbE-beta thalassemia accounts for above half of the cases of severe form of thalassemia in Indonesia and Bangladesh and is also very frequently seen in India, in Vietnam, Sri Lanka, Cambodia, Laos, Maldives and Malaysia (Fucharoen and Winichagoon 2011). The carrier frequency of thalassemia gene is significantly high in Thailand, as a result around 3,000 babies are born with thalassemia per year (Fucharoen and Winichagoon 1997, 2011). Beta thalassemia is also highly widespread in the Indian subcontinent, particularly in many parts of India, Pakistan, Maldives, Bangladesh, Nepal and Sri Lanka.

1.5.2. Prevalence of β-thalassemia and HbE/β-thalassemia in Bangladesh

To date very few of the thalassemia prevalence studies conducted over the last couple of decade in Bangladesh, most of which were retrospective studies on patient samples obtained from tertiary level hospitals. The only published data obtainable on the prevalence of thalassemia was a small scale study among (n = 735) school children in Bangladesh which showed the prevalence of the β-thalassemia trait (BTT) and the HbE trait were 4.1% and 6.1% respectively. The study also showed the regional variation in prevalence rate in different divisions of the country (Khan WA et al. 2005). Notably, in the hill track, the frequency of the HbE carriers was considerably higher (41.7%) and another study with a small sample repoted the similar rate of HbE trait of 39–47% in the tribal population in Bangladesh (Shannon et al. 2015).

According to a study of 2011, among 600 anaemic patients, β-thalassemia minor (21.3%) was the commonest hemoglobinopathy followed by HbE/β-thalassemia (13.5%), HbE trait (12.1%), and HbE disease (9.2%) (Uddin et al. 2012) while other two retrospective studies shown the high prevalent cases of homozygotes β-thalassemia (4% and 31% respectively) and compound heterozygotes HbE/β-thalassemia (10.9% and 68.5% respectively)(Khan Waqar A et al. 2017, Tahura 2017). Nevertheless, all of these studies represent too much variation form each other in the frequency of different hemoglobinopathies prevalent in the country (Noor et al. 2019).

1.6 Molecular basis of beta thalassemia in South Asian population

South Asia consists of eight countries - Bangladesh, India, Pakistan, Bhutan, Maldives, Nepal, Sri Lanka and Afghanistan. Although a great numbers of mutations in the β-globin gene have been described for in the β-thalassemia gene carriers all over the world, a few number of mutations are found as most frequent in the Indian subcontinents as well as other South Asian countries shown in Table 1.4 (Thein SL et al. 1988). For example, above 90% of the beta thalassemia causing mutations in India included only five common mutations (Varawalla et al. 1991). The particular region of the gene containing all these common mutations together constitute the mutational hot-spot (c.1 to c.92 of exon 1, c.92 + 1 to c.92 + 130 intron-1 and c.93 – c.217 of exon-2) in the HBB gene for most of the countries of South Asia and Southeast Asia including Bangladesh. Three β-globin (HBB) gene mutations, c.79 G>A, IVS1-5 G>C and c.126_129delCTTT are most common in Bangladeshi thalassemia. Some other mutations like Codon8/9(+G), Codon16(-C), -90 (C>T) and IVS 1-1G>T occurrs in small extent including the rare mutations, -29 (G-A) & -92C>G (Chatterjee et al. 2015, Islam Md Tarikul et al. 2018).

Table 1.4: The most common HBB gene mutations in the regional hot-spot of South Asia and Southeast Asia (Noor et al. 2019).

Countries	Common Mutations	References
India	IVSI-5G>C, 619-bp deletion, Codon 26 G>A, IVSI-1 G>T, Codon41/42(−TCCT), Codon8/9(+G), Codon15G>A, Codon 30G>C, Cap site+1(A>C), Codon5(−CT) and Codon 16(−C)	(Panigrahi Inusha and Marwaha 2007, Sinha et al. 2009)
Bangladesh	Codon26 G>A , IVS1-5 G>C, Codon 41/42delCTTT, Codon 30 G>C, Codon 8/9(+G), Codon 15 G>A , Codon 30G>A, c.46delT, IVS1-130 G > C, and c.51delC	(Banu et al. 2018, Chatterjee et al. 2015, Islam Md Tarikul et al. 2018, Sultana et al. 2016)
Pakistan	IVS1-5 G >C, c.27_28insG, IVS1-1 G >C, Codon 30 G>A, c.126_129delCTTT, Codon 5(−CT) and Codon 15 G>A	(Ansari et al. 2011)
Sri Lanka	IVS1-5G>C, IVS1-1 G >C, Codon 26 G>A, Codon 30 G>C, c.27_28insG, Codon 15 G>A, c.51delC, c.126_129delCTTT	(Fisher et al. 2003)
Malaysia	IVS1-5 G >C, IVS1-1 G>C, c.59 A > G, c.79G>A, c.52A>T, c.126_129delCTTT, c.27_28insG, c.216_217insA, IVS1-1G>C,	(George et al. 2012)
Thiland	c.126_129delCTTT, c.52A > T, c.59A > G, c.27_28insG, IVS1-1 G >C, IVS1-5 G >C, c.108C > A, c.47G > A	(Boonyawat et al. 2014)
Mayanmar	IVS1-1 G > T, c.126_129delCTTT, IVS1-5 G >C, c.53A > T, c.135delC, c.108C > A, c.47G > A, c.51delC, c.46delT, c.126delC, IVS1-1 G >C, c.27_28insG,	(Harano et al. 2002)

1.6.1 Clinical Burden of beta and HbE/β-thalassemia

The most severe form of thalassemia is referred as thalassemia major, clinical management of which is highly expensive and troublesome including lifelong regular blood transfusion and iron chelation therapy to remove excess iron introduced and deposited with every transfusion (Poggiali et al. 2012).

Multiple clinical complications are associated with both beta thalassemia and HbE/beta thalassemia including severe anemia, growth retardation, splenomegaly, jaundice, cardiac and liver dysfunctions, expansion of bone-marrow, endocrine disorders, bone deformities and require lifelong blood transfusions in regular intervals to avoid complications. Without blood transfusion the β-thalassemia major and HbE/β-thalassemia patients cannot survive for more than 5 years (Modell and Darlison 2008), and the average life expectancy of such patients is about 30 years in spite of taking regular blood transfusions (Mandal et al. 2014), particularly in the countries with extremely low resources. In addition to the transfusion-dependent form of β-thalassemia, there are also some milder conditions which might not be detected till adulthood.

On the other hand, maintaining an Hb concentration of not less than 9.5 g/dL by a regular and adequate amount of blood transfusion program following proper guideline helps the transfusion dependent major thalassemia patients to thrive with normal growth and development until the age of 10–11 years. However, after the age of 10 to 11 years, affected individuals are at risk of developing severe complications related to post transfusional iron overload, development of allo-immunization and various infections (Noor et al. 2019). The β-thalassemias cause the significant public health problems because of their high incidence rate and severity and are an important catalyst of morbidity and mortality worldwide putting immense burden not only on the patients but also on their families, their communities and ultimately on the whole country.

Iron Overload

An unavoidable fate of the patients of thalassaemia major after starting regular blood transfusion is the excess deposition of iron inside different vital organs of their bodies. The underlying reason behind this consequence is that the human body has no mechanism to excrete left-over iron from the system. A unit of donor blood which is usually processed from 420 mL contains nearly 200 milligram of iron constituting around 0.47 mg/mL of

whole donor blood (Zimmermann et al. 2008). Iron is very reactive and easily undergo the processes of electron loss and gain resulting in generation of harmful free radicals which in turn destroy the lipid membranes of organelles as well as causes DNA damage, thus leading to cell death and the generation of fibrosis. Therefore iron overload following deposition is highly toxic to many tissues, causing heart failure, liver cirrhosis and carcinoma, growth retardation and multiple endocrine abnormalities (De Sanctis et al. 1998, Galanello R et al. 2001, Taher et al. 2008). Several studies done in Bangladesh revealed the same degree of clinical situations of almost all kinds exist in both beta and HbE/β-thalassemia major patients (Bhuyan et al. 2021, Ferdaus et al. 2010, Palit et al. 2012, Shazia et al. 2012). Without proper management of this iron overloading, several fatal complications occur, leading to low quality of life and, finally, pre-death at young age.

Hypersplenism

Splenomegaly, together with pooling of red cells and their increased rate of destruction, is extremely common in the patients with beta and HbE/β-thalassemia. In the more severe phenotypes, it often progresses rapidly from the first few years of life and some requires splenectomy. In the milder phenotypes, although the spleen is palpable, it usually does not attain a size greater than 5–6 cm below the costal margin. Much less common, and usually in the milder phenotypes, splenomegaly may slowly increase over 10–20 years and only become a problem later in life.

Heart disease

Cardiovascular complications are common and appeared to be one of the major causes of death in about 35% of cases. This is associated with failure of other organs, growth and sexual retardation, hepatomegaly, and endocrinopathies. Organ failure results from iron deposition in the heart and other tissues. In homozygous β-thalassemia, however, patients still develop organ failure, despite appropriate transfusion and chelation therapy and low ferritin levels. In hemoglobin E/β-thalassemia, myocardial iron deposition occurred primarily as small granules in perinuclear areas, with later accumulation throughout the fibers, predominantly in subepicardial and occasionally subendocardial region. Cardiomegaly was proportional to the severity of anemia and systolic murmurs were frequently present (Fucharoen and Winichagoon, 2000).

1.7 Importance of prevalence determination and carrier screening for HbE and β-thalassemia

Thalassemia is a deadly hereditary hemolytic diseases which is mostly an incurable disease with only treatment option of life-long blood transfusion which gradually damages the vital organs of the body due to excess iron accumulation and therefore highly expensive iron-chelation therapy is needed for survival of the patients. However, thalassemia is an autosomal recessive disorder that is both parents must be carriers for a child to be affected (Weatherall David J and Clegg 2008). If both parents carry a thalassemia trait, the risk is 25% for each pregnancy for an affected child.

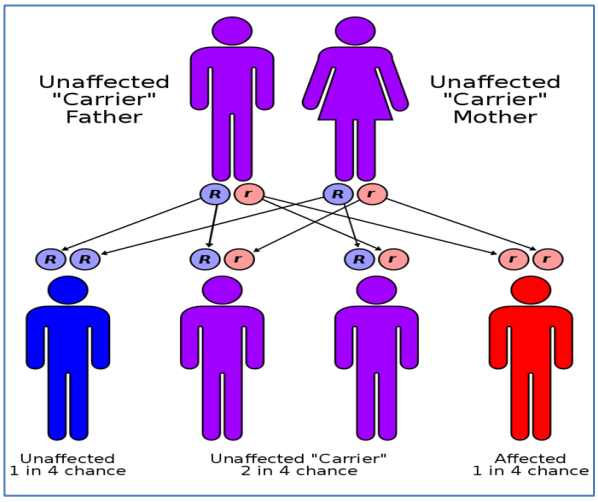


Figure 1.7: Inheritance of genes for β-thalassemia and HbE from parents to descendants.
A married couple with β-thalassemia or HbE trait may transmit the β-thalassemia or HbE/β-thalassemia disease to 25% of their progenies and 50% chance of having a carrier child in each pregnancy.

Therefore, both β-thalassemia and HbE/ β-thalassemia are completely preventable trough carrier-screening followed by awareness raising to avoid marriage between carriers and prenatal diagnosis (Colah et al. 2010). Cyprus started a premarital screening program in 1973,which caused the reduction of affected births from 51 in 1974 to 8 in 1979 (Cousens et al. 2010). The rates continued to decrease after the voluntary screening program was made mandatory in early 1980s, resulting in only five affected births between 1991 and 2001 and there were no affected births between 2002 and 2007. However, a successful preventive approach is not possible without exact data on the prevalence spectrum of the disease and the carrier frequency in a country.

1.8 Techniques used in detection of β and HbE/β-thalassemia and importance of using molecular based detection

Diagnosis of β and HbE/β-thalassemia can be performed using hematological, biochemical and molecular approaches. The conventional laboratory tests for diagnosis of β-thalassemia and HbE include an initial screening tests followed by confirmatory tests which are highly specific and comparatively expensive (Tatu 2020).

1.8.1 Screening tests for HbE and β-thalassemia

a) Hematological test measures RBC indices MCV, MCH, RDW etc. Normal ranges of MCV and MCH are 85.5±6.8 fL and 27.1±3.1 pg, respectively. MCV<80fL and MCH<27pg the two most widely recommended *Cut-off* points for preliminary screening of β-thalassemia carriers (Tatu 2020). However, MCV and MCH levels in HbE carriers are just slightly lower than reference values and therefore, not much effective in screening for HbE carriers (Ittarat et al. 2000, Tatu 2020, Yeo et al. 1994).

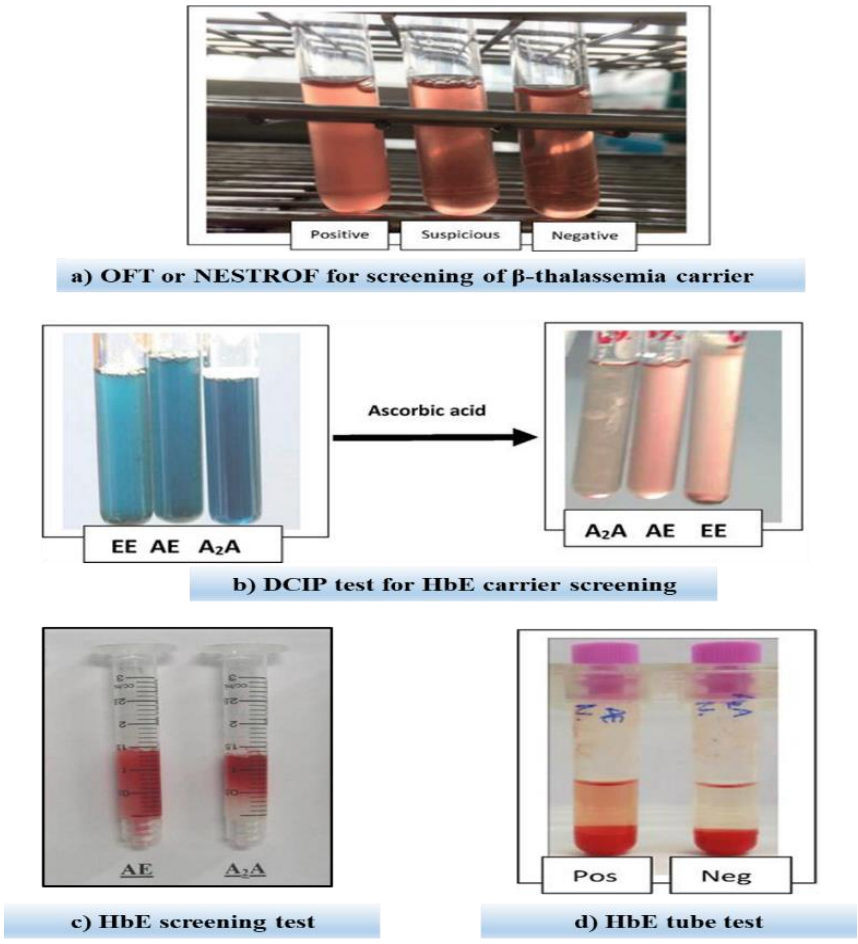


Figure 1.8: Laboratory tests for β-thalassemia and HbE carriers screening (Tatu 2020).

b) **Screening tests for HbE carrier** include dichlorophenolindophenol precipitation (DCIP) test, HbE-tube test, and HbE screening test (Tatu 2020) (figure 1.8). Several studies reported these tests almost 100% sensitive and specific for HbE screening and not suggested for detection of β-thalassemia carrier.

b) One-tube osmotic fragility test (OFT), also known as **NESTROF (Naked Eye Single Tube Red Cell Osmotic Fragility)** tests the osmotic resistance of the RBC. In a hypotonic condition, higher salt concentration inside the red blood cell than that of the outside results in net water drive into the cell followed by lysis of normal RBCs which makes the mixture reddish and clear. RBCs of the β-thalassemia and HbE carriers possess higher osmotic resistance leads to slower rupture rate which makes the mixture turbid (Tatu 2020). However, this test is found to be sensitive and specific only for screening the β-thalassemia carrier (Chow et al. 2005), while fails to detect HbE carriers producing 40% false negative results in several studies.

1.8.2 Confirmatory tests for HbE and β-thalassemia

a) Studies of Hemoglobin fractions

It includes the estimation of the percentage of different Hb like HbA, HbA2, HbF as well as the common variants HbE, HbC, HbD, HbS, Hb Barts etc. Hemoglobin study is considered as the gold standard for thalassemia diagnosis and performed worldwide using various techniques like cellulose acetate electrophoresis (CAE), capillary zone electrophoresis (CZE), cation-exchange high performance liquid chromatography (HPLC), micro column chromatography, cation-exchange low pressure liquid chromatography (LPLC) etc. are used to perform Hb study where HbA2>3.5% is most widely used cut-off for detection of β-thalassemia trait.

b) Molecular methods: implication for accurate detection of β-thalassemia carrier

Although HbA2 quantification is widely used for carrier screening, β-globin gene mutations which result in only minimal elevation in HbA2 levels could be missed. Moreover, external conditions like co-existence of α-thalassemia and iron deficiency anemia that influence HbA2 level are known to complicate the interpretation of β-thalassemia screening results. Supplemental DNA-based approach would help to overcome these limitations (Tatu et al. 2012). Several molecular method used for scanning mutation in β-globin gene includes

multiplex Sanger nucleotide sequencing, allele-specific polymerase chain reaction (MAS-PCR), amplification refractory mutation system (ARMS)-PCR (Old et al. 1990), and high resolution melting curve (HRM) analysis (He et al. 2010, Lin et al. 2014) .

1.9 Variable degree of disease severity in HbE/β-thalassemia

Clinical manifestations of HbE/β-thalassemia is highly heterogeneous in terms of severity that is, variation in transfusion interval, first age of transfusion, rate of splenomegaly and splenectomy, degree of growth retardation etc. HbE/beta thalassemia is clinically characterized by marked variability, ranging from non-transfusion dependent asymptomatic anemia to a fatal condition requiring regular blood transfusions from the very early ages until death (Olivieri et al. 2011, Weatherall David J and Clegg 2008).

1) Mild form of HbE/β-thalassemia: Around 15% of HbE/beta thalassemia in Southeast Asia are of this category. Such patients always maintain hemoglobin levels at 9-12 g/dl and typically does not develop any significant clinical complications in early ages. Nevertheless, few of the patients can suffer from improper growth, excess iron deposition in the vital organs due to increased iron absorption from food (Zimmermann et al. 2008) and other problems similar to those of non-transfusion dependent thalassemia patients.

2) Moderately severe HbE/β-thalassemia: The Hb levels usually stay at 6-7 g/dL and the clinical symptoms are similar to that of β-thalassemia intermedia whose blood transfusion interval is much longer than the severe groups. Iron overload and the associated long term complications may occur.

3) Severe HbE/β-thalassemia: In the patients with severe form of compound heterozygous HbE/beta thalassemia, hemoglobin level can hit as low as 4-5 g/dL. They show the clinical symptoms similar to major β-thalassemia patients and are treated as transfusion dependent thalassemia patients.

The phenotype of Hb E/β-thalassaemia may be unstable as well. A study on Sri Lankan HbE/β-thalassaemic children highlighted the fluctuating pattern of anaemia and erythroid enlargement during the first ten years of life. The phenotype became steadier later in development in many patients, and it was frequently possible to stop blood transfusion in a proportion of older patients with no apparent subsequent effects on quality of life (Premawardhena et al. 2005). Conversely, the limited data available indicate that many

adults appear to develop worsening anemia with age. The reasons for such inconsistency in the severity pattern of the HbE/beta thalassemia have only partly been outlined so far. Also, there is a widely disparate range of clinical and hematological parameters in patients with HbE/β-thalassaemia (Fucharoen et al. 2000). Moreover, an emerging consciousness is that, its phenotype may evolve over time along with phenotypic variability and this phenomenon combines to the limited understanding of its natural history make the management of Hb E/β-thalassaemia mostly challenging.

1.10 Genetic modifiers affecting the disease severity of HbE/β-thalassemia

The reasons for this clinical variability is still poorly understood. However, the severity of the disease is related mainly to the degree of α-globin chain excess, which precipitates in the red blood cell precursors, causing both mechanic and oxidative damage (ineffective erythropoiesis). Thus, any mechanism that diminishes the amount of unbound α-globin chains in the red cells may ameliorate the detrimental effects of excess α-globin chains. Grossly, the modifiers of the degree of severity in HbE/β-thalassemia act at 3 levels-

1. Primary level modifiyers: The broad diversity of causative mutaion in β-thalassaemia allele trans to haemoglobin E in HbE/β-thalassemia patients are the primary modifier of clinical phenotype (Rujito and Sasongko 2018).

2. Secondary level modifiers: Severity of HbE/β-thalassemia can also be modified by genes other than the β-globin gene which are the secondary genetic factors and modifies the clinical phenotype by repairing the α/β-globin chain disproportion. Two important secondary modifiers are co-inheritance of α-thalassemia and effective synthesis of γ-chains in adult life (Galanello Renzo 2012), though all the clinical heterogeneity could not be explained by them. The genome-wide association studies (GWASs) has revealed some other genetic modiffers in such complex traits which could enhance γ-chain production and ameliorate disease severity (Raychaudhuri 2011). These genes are encoded either in the HBB gene cluster, such as- $\delta\beta^0$-thalassemia, point mutations at HBBP1 gene, A^{γ} or G^{γ} promoters etc; while others are unlinked globin genes present on different chromosomes like BCL11A, KLF1, HBS1L-MYB (Rujito and Sasongko 2018).

3. Tertiary level modifiers: Variations in genes affecting the phenotype of β-thalassemia syndrome complications. These are polymorphisms unrelated to globin chain expression but

having an modulating effect on specific complications such as iron absorption, bilirubin and bone metabolism, cardiovascular disease and infeciton susceptibility (Galanello R et al. 2001, Hentze et al. 2004, Weatherall David 2004).

1.10.1 Types of β- globin gene mutations co-inherited with HbE: *The primary determinant of severity in HbE/β-thalassemia*

Functionally, β-thalassemia alleles are considered as β^0 when no β globin is produced, or β+ in which β-globin is produced partially, but less than normal. The least severe forms are designated β++ with the nominal deficit in production of β-chain. Patient severity and the amount of produced globin protein are directly associated with a mutation on the β globin gene. More than 400 thalassemia causing point mutations in β-globin gene have been identified and deletion is very rare(Giardine et al. 2021). A complete updated list of β-thalassemia mutations is available through the Globin Gene Server Web Site (http://www.globin.cse.psu.edu).

Single nucleotide polymorphisms (SNPs) impairing the synthesis of beta globin chains in terms of either the expression level or the structure, belong to 4 major mutants of β-thalassemia: 1. **promoter mutant**, 2. **mutation in splice site of intron-exon junction**, 3. **mutant for the region of RNA capping/poly-A tailing**, and 4. **translation mutant** (Thein Swee Lay 2013). Another causes are frameshift mutation transforming the open reading frame results in a fragmented unstable polypeptide or introduction of a stop codon leading to a premature termination of translation (Galanello Renzo and Origa 2010). The majority of mutations are found in front of the β gene covering the upstream TATA box, 5' UTR including start codon ATG, Exon 1, Exon 2 and Intron 1 (IVS/intervening sequences) (Rujito and Sasongko 2018).

β^0-thalassemias are mainly the consequences of deletional mutations, alteration in start codon or in the splice sites, especially at the splice-site junction in addition to some nonsense and frameshift mutations. However, mutations in the regulatory sites like promoter area, the polyadenylation signal, and the 5' UTR or 3' UTR result in the moderate to severe reduction in beta chain synthesis or abnormalities in the splicing process leading to ***β^+-thalassemia***. Several previous studies suggested that patients co-inheriting a mild β-thalassaemia allele **(β^{++})** with Hb E might have very mild symptoms, while those who co-inherited severe β+ or β°-thalassaemia alleles might be more severely affected

(Winichagoon et al. 1993). A study in 2000 conducted on Thai population showed, co-inheritance of Hb E and the SNP at -28 position in the ATA box of the β-globin gene is related to the mild phenotype in 6 Hb E/β-thalassemia patients (Winichagoon et al. 2000). However, it was also observed that, both mild and severely anemic patients might have the same severe β-thalassemia mutation. On the contrary, a less severe β-thalassemia mutation might not be the only modifier. Winichagoon *et al* reported that 42% of 36 patients having a "milder" clinical phenotype co-inherited a modifier other than less severe β-thalassemia allele, like the *XmnI* polymorphism, α^+ thalassemia or hemoglobin H-Constant Spring. The finding has also been confirmed in several recent studies imposing the β mutation as an crucial severity modifier but inconsistently influence the clinical heterogeneity of HbE/β-thalassemia. Studies on populations from India and Sri Lanka also suggested that β-thalassemia mutation has more limited effect on the clinical severity of Hb E/β-thalassemia than previously supposed (Kalantri et al. 2020, Panigrahi I et al. 2005, Premawardhena et al. 2005).

In summary, β-globin gene mutations seem not to be the only responsible factor for the wide phenotypic deviation found in Hb E/β-thalassaemia and evidently other modifying genetic aspects should be considered.

1.10.2 Co-inheritance of α-thalassemia: *α/β Globin Ratio Modifier in HbE/β-thalassemia*

Co-inheritance of the deletional allele of α-globin gene seems to be a vital genetic factor modulating clinical phenotypes of HbE/β-thalassaemia. Accoding to several early studies, a substantial number of patients with HbE/β-thalassaemia co-inheriting α-thalassaemia genotypes found to be diagnosed later in life and mostly presented with a mild severity. In particular, the patients with an α^+ thalassaemia allele demonstrated higher steady-state Hb level than those without α-thalassaemia (Winichagoon et al. 1985, Winichagoon et al. 2000). The mechanism of this modification effect is that, a reduced number of unbound excess α-globin chains leads to more balanced hemoglobin synthesis and a milder clinical phenotype. Studies from Thailand reported that coinheritance of α^0-thalassemia lessens the degree of disease severity in Hb E/β-thalassemia with the findings of requiring regular blood transfusions for the patients with a normal α-globin gene while the patients with α thalassaemia were not transfusion dependent (Charoenkwan et al. 2005, Sripichai et al. 2008).

Recent studies also supported the beneficial consequence of α-thalassaemia on Hb E/β-thalassaemia showing effects of the co-inheritance of different copy numbers of the α globin genes. The diversity in the disease severity mainly depends on the mutation allele type of the β gene and the copy number of functional α gene. The concomitant inheritance from a single α gene deletion has a minimum impact on HbE/β-thalassemia while the deletion of 2 α genes shows a milder phenotype (Guvenc et al. 2012). The patients with triplicated alpha genes (ααα / ααα) were reported to have a more severe phenotype with more frequent blood transfusion (Sharma and Saxena 2009, Sripichai et al. 2008). On the other hand, quadriplication of α globin genes may produce symptomatic thalassemia syndromr in carriers of suspected silent β-thalassemia (Sollaino et al. 2009). The recent reports also indicates that this kind of genotype make-up among the HbE/β-thalassemia patients is very frequent in case of thalassemia intermedia (Origa et al. 2014).

1.10.3 High fetal hemoglobin level as disease modifier in HbE/β-thalassemia

Another potential modifier of the α-chain/non- α chain ratio on HbE/β-thalassemia includes a characteristic tendency to continue γ-globin gene expression in adulthood. The consequence is high HbF percentage than normal which is able to reduce the amount of excess alpha globin chains as well as compensate the shortage of β-globin chains and the function of HbA in some extent, thus lessen the disease severity (Rujito and Sasongko 2018). A normal adult has less than 1% of HbF, present at F-cells which is 3 to 7% of total erythrocyte. In the case of β and HbE/β- thalassemia, HbF is reasonably increased due to the selective survival from the erythroid precursor expressing the γ-chain as they are secured from the damaging effect of excess α globin precipitate and premature death (Rees et al. 1999). High HbF levels are correlated with reduced morbidity and mortality in both diseases.(Galanello Renzo and Origa 2010).

In adults, the HbF level can be enhanced by as high as 30% of total Hb in a conditions called ‘Hereditary Persistence of Fetal Hemoglobin’ (HPFH) caused by large deletions of the HBB locus or point mutations in the promoters of the γ-globin genes (Jouini et al. 2012, Wood 2001). Another potential HbF modulators are the Quantitative Traits Locus (QTL). The three major QTLs, found to dominate the expression of HbF are HBG2 promoter and HBBP1 gene on the chromosome 11p15.4, BCL11A on Ch.2p16.1 and HBS1L-MYB intergenic region on Ch.6q23.3 (Hanafi et al. 2016). A number of SNPs present in these main QTLs were found to be associated with elevated production of fetal hemoglobin and

with a milder clinical phenotype of Hb E/β-thalassaemia in diverse population groups (Galanello Renzo et al. 2009, Nuinoon et al. 2010, Olivieri et al. 2011).

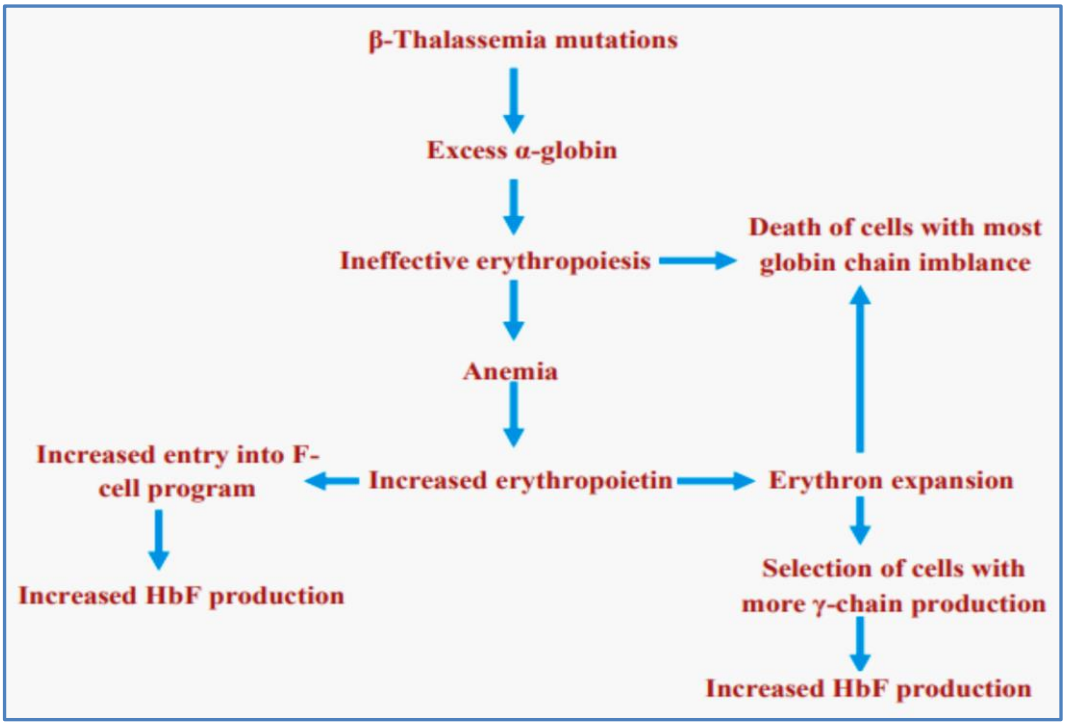


Figure 1.9 : Proposed mechanism for increased HbF production in β-thalassaemia syndromes (Rees et al. 1999).

1.10.4 HBG2-*XmnI* (*Xanthomonas manihotis-I*) polymorphism in HbF induction

Xmn1-HBG2 (or, Xmn1-Gγ) SNP (rs7482144) is a C→T substitution at the -158 position of the promoter of $^{G}\gamma$ gene (HBG2) that introduces a restriction site for the restriction enzyme Xmn1 shown in **figure 1.10**. The mutant allele T is quite common in all population groups with an average frequency of 0.32 to 0.35 (Garner et al. 2000, Gilman and Huisman 1985).

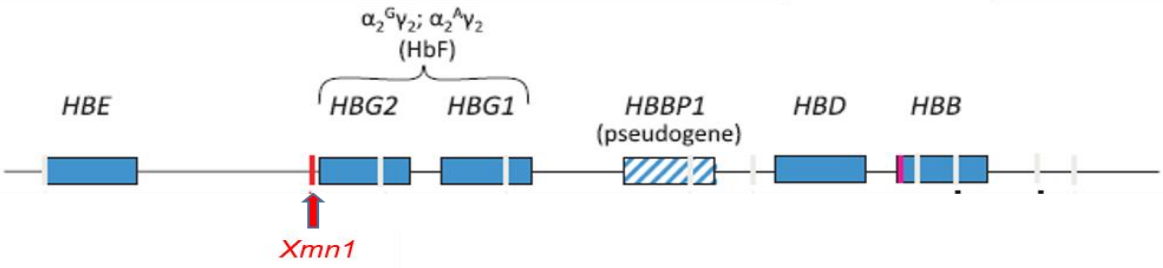


Figure 1.10: Xmn1 polymorphic Site in the β-Globin Gene Cluster.

The *XmnI* polymorphism is a *cis* acting element located at the upstream regulatory region of HBG2 gene of HBB locus. It directly influences the binding of transcription factors and the repressors, thus promote the expression $^{G}\gamma$ gene resulting in elevated synthesis of HbF (Garner et al. 2000). In several studies from China, Thailand, Sri Lanka and India, it has been reported that the Hb E/β-thalassemia patients with *Xmn1* TT or +/+ genotype had higher HbF concentrations than those with a *XmnI* CC or -/- genotype (Gibney et al. 2008, Kalantri et al. 2020, Panigrahi I et al. 2005, Premawardhena et al. 2005, Winichagoon et al. 1993). In addition, patients having homozygous Gγ-158(C→T) polymorphic allele were identified as mildly affected accompanying with high HbF; while HbE/β-thalassemia major patients with early onset of disease and high transfusion frequency, were more likely to have homozygous wild -/- genotype (Liu et al. 2010). These findings implies that homozygosity for the *Xmn1* polymorphism is responsible for ameliorating the disease severity. However, a positive correlation was also found between the heterozygous mutant allele (+/-) and HbF concentration with findings that the patients with *Xmn1* -/- presented at a younger age with higher transfusion rate, compared to those with *Xmn1* (+/-) (Ma et al. 2007). However, rises in HbF and F cells are only associated with Xmn1-Gγ polymorphism under anemic stress conditions while the effect is negligible or undetectable in healthy adults (Ho et al. 1998).

1.10.5 SNPs in *BCL11A* (B-cell lymphoma/leukemia 11A) gene on HbF production

BCL11A gene is located on the chromosome 2p16. It encodes a repressor of $^{G}\gamma$ and $^{A}\gamma$ gene expession which is a C2H2-type zinc finger protein. It binds to the locus control region (LCR) and an intergenic region in the HBB gene cluster, promotes interaction between LCR and beta globin gene (Fanis et al. 2014, Sedgewick et al. 2008) and inhibits γ-globin synthesis leads to switching from fetal to adult hemoglobin (Uda et al. 2008) by interacting with many other trancriptional factors like GATA1, FOG1, SOX6 etc along with participation of other chromatin regulators and transcriptional corepressors (Bauer and Orkin 2015). Genetic association studies have identified sequence variants in the gene BCL11A that influence HbF levels (Sankaran et al. 2008). Recently, six SNPs in the *BCL11A* gene were described to be associated with F-cell numbers in a study of 179 unrelated normal subjects from British population (Menzel et al. 2007). In addition, several correlation studies suggest that *BCL11A* polymorphisms may be important modulators of fetal haemoglobin in HbE/β-thalassaemia patients from Hongkong, China, Thailand,

African American as well as South Asian countries (Gibney et al. 2008, Nuinoon et al. 2010, Olivieri et al. 2011, Sedgewick et al. 2008, Sripichai et al. 2005).

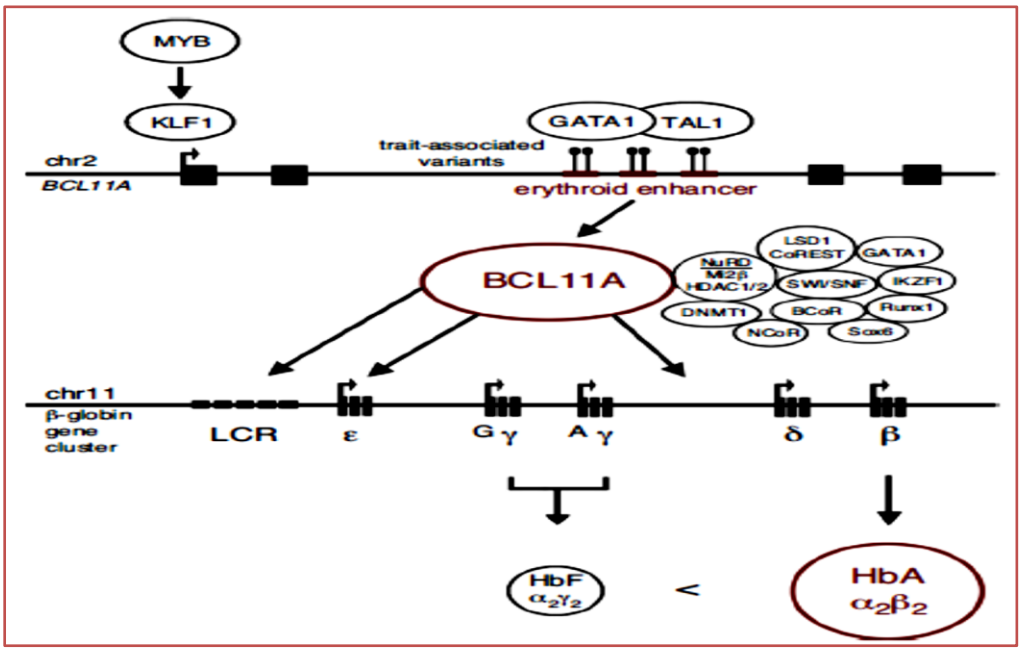


Figure 1.11 : HbF repression by BCL11A (Bauer and Orkin 2015).

However, a few SNPs in particular, rs11886868, rs4671393 and rs766432 in BCL11A displayed in **figure 1.12** , are mostly shown to be associated with an up-regulation of fetal hemoglobin synthesis which are targeted in our study (Chaouch et al. 2016, Rujito et al. 2016).

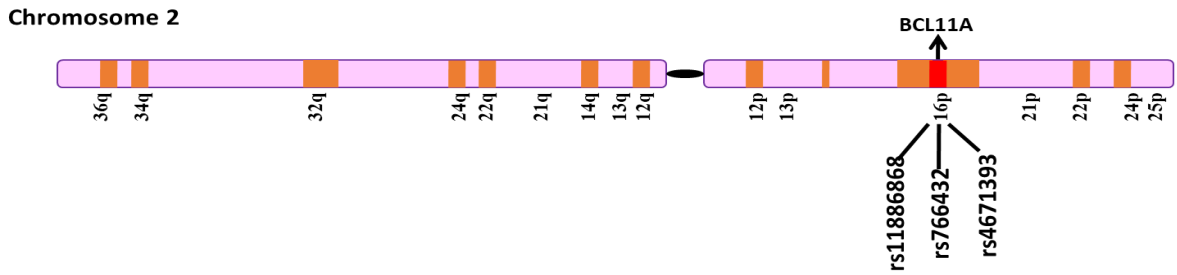


Figure 1.12 : Three SNPs rs11886868, rs4671393 and rs766432 on BCL11A gene.

1.10.6 SNPs in HBS1L-MYB intergenic region on Chromosome 6

MyB (myeloblastosis oncogene) is a proto-oncogene which encodes a transcription factor playing a critical role in erythroid cell proliferation and differentiation in hematopoiesis, while also controlling HbF levels through an unknown mechanism (Fanis et al. 2014) and HBS1L is the human ortholog of Saccharomyces cerevisiae which encodes a GTP-binding elongation factor, involved in the regulation of a variety of critical cellular processes (Thein Swee Lay et al. 2007).

HBS1L-MYB intergenic region contains a cluster of erythroid-specific enhancers controlling the expression of MYB. Several HBS1L-MYB intergenic variants might affect binding site for the key erythroid transcription factors within this region; thus affects long-range interactions between MYB and other transcription factor by reducing their binding and MYB expression levels also altered (Stadhouders et al. 2014). MYB represses the fetal globin gene by upregulating both the DRED and KLF1/BCL11A pathways in human erythroid cells, but upon the downregulation of MYB, both the TR2/TR4 and KLF1/ BCL11A pathways are suppressed, and this activates the fetal globin gene expression (Suzuki et al. 2013). Common variants within the intergenic region (HBS1L-MYB) between HBS1L and, MYB on chromosome on 6q23.3 have identified which are related with elevated fetal hemoglobin (HbF) levels and alterations of other clinically important human erythroid traits.

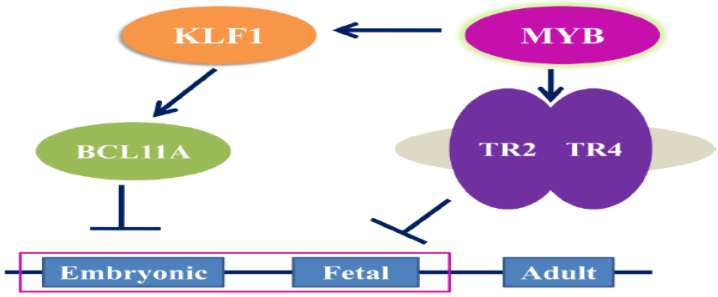


Figure 1.13: A model for repression of fetal hemoglobin gene expression by MYB.

Earlier studies repoted the increased number of F-cells regulated by HBS1L-MYB intergenic polymorphism (HMIP) (Gorji et al. 2011; Wonkam et al. 2014). A number of SNPs namely rs4895441, rs28384513, and rs9399137 in the same region found to enhance HbF yield improving disease symptoms among the beta and HbE/β-thalassemia patients (Hashemi-Gorji et al. 2011). Some other polymorphisms like C32T and SNP7 in the exon of HBS1L were also reported to up-regulate HbF production in thalassemic patients polymorphism (Pandit et al. 2008, Uda et al. 2008).

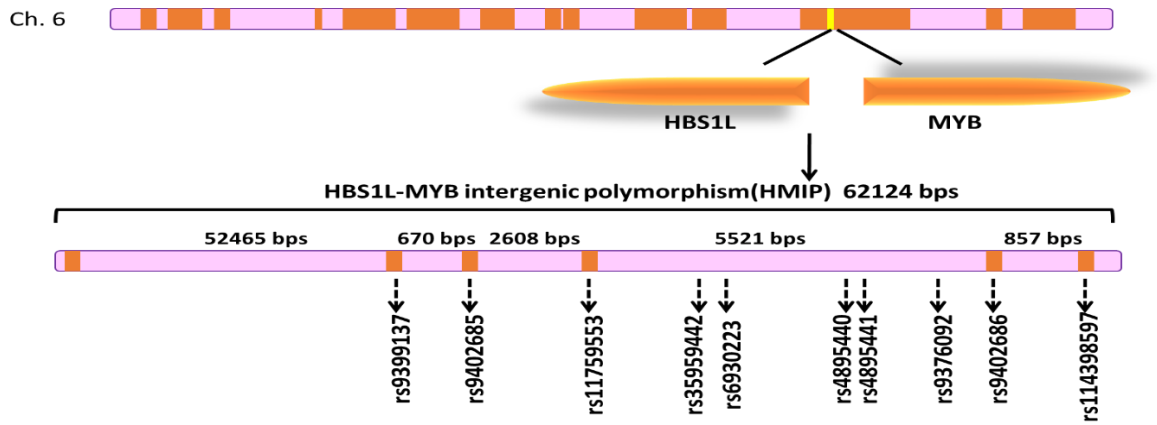


Figure 1.14: HBS1L-MYB intergenic polymorphisms (HMIP) on 6q23.3 . (Mohammdai-Asl et al. 2015).

1.10.7 Mutation in HBBP1 gene of HBB locus

HBBP1 is a pseudogene located on chromosome 11 and is a member of HBB gene cluster. Although the pseudogene does not have a protein product, it transcribes regulatory RNAs, primarily which were associated with the regulation of HBE1 gene and also associated to multiple chromatin sites throughout the cluster HBB locus (Tomkins 2013).

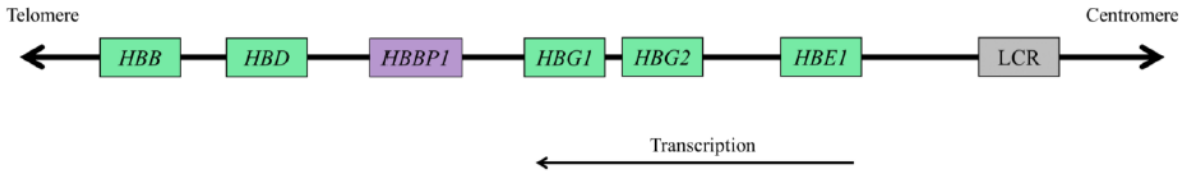


Figure 1.15 : Graphical representation of the β-globin gene cluster showing the arrangement of the HBBP1 pseudogene in the HBB locus.

Therefore, it can be inferred that transcriptional products of HBBP1 gene have regulatory functions and it may also be assumed that any mutation on the pseudogene can affect these regulations. The rs2071348 polymorphism on this pseudogene has shown strong association with elevated HbF levels and hence comparatively milder disease severity in Thai and Indonesian populations of HbE/β-thalassemia patients (Giannopoulou et al. 2012, Ma et al. 2007, Nuinoon et al. 2010).

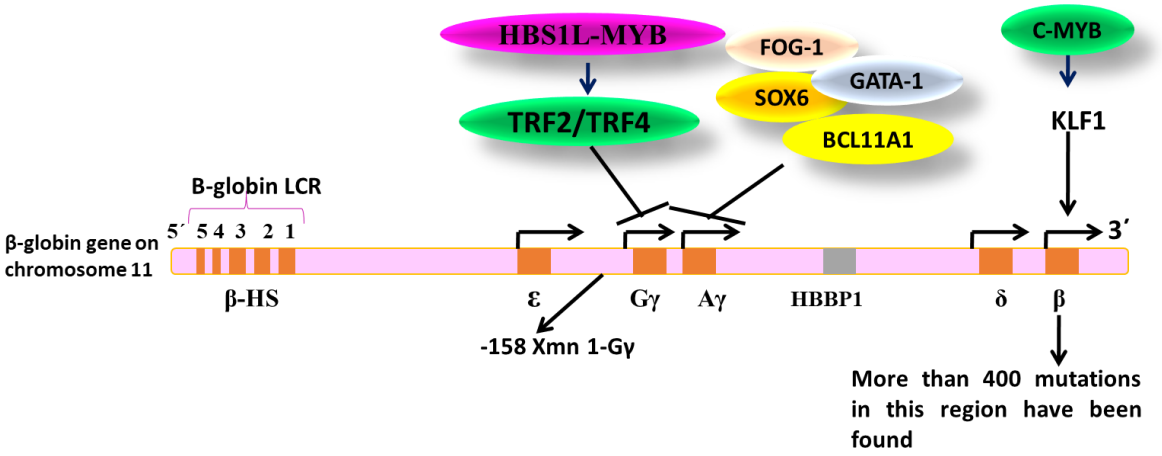


Figure 1.16: Single nucleotide polymorphisms in transcription factors and their effect on γ-globin gene expression (Mohammdai-Asl et al. 2015).

In summary, proof from different studies indicate that several mutations that account for increased fetal hemoglobin production, have effects on the phenotype of HbE/β-

thalassemia. Several research have correlated milder phenotypes of this disease with fetal hemoglobin modifiers including Xmn1, HBBP1 SNP and polymorphisms in transcription factor genes such as BCL11A, HBS1L-MYB, and GATA-1 that play a role in the expression of beta and gamma chains are observed in patients with HbE/β-thalassemia and affect HbF production rate and change patient's symptoms (Figure 1.16).

1.10.8 Potential therapeutic targets for induction of HbF in β-thalassemia patients

It is evident that HbF is one of the key modifier of the disease severity in β-thalassemia and thus has been using as potential therapeutic target for ameliorating the clinical symptoms.

Pharmacological reactivation of gamma globin gene

Several Drugs have been used targeting chromatin regulators. Azacitidine and Decitabine acts on reducing DNA methylation and as histone deacetylase inhibitors. Hydroxyuria acts as ribonuclease reductase inhibitor and nitric oxide (NO) donor (Algiraigri and Kassam 2017, Fard et al. 2013); others like Sodiumbutyrate and Arginine butyrate affect DNA-binding transcription factors (Fard et al. 2013).

HbF activation by genome editing

Gene therapy approaches based on hemoglobin-switching comprise a patient's hematopoietic stem cells modification ex-vivo followed by autologous transplantation for reconstitution. Several trials of gene editing for inducing gamma-globin expression are under investigation. Potentially three transcription factors – BCL11A, KLF1 and MYB – can be considered for HbF induction.

BCL11A is a validated therapeutic target for reactivation of the γ-globin gene in the β-thalassemia patients provided that down modulation or genetic deletion of BCL11A relieves γ-globin repression (Sankaran et al. 2008). BCL11A expression can be disrupted selectively in erythroid cells by genome editing the intronic BCL11A erythroid enhancer, targeting BCL11A mRNA for degradation using an erythroid selective shRNA lentiviral transduction (Guda et al. 2015) or CRISPR-Cas 9 technology (Canver et al. 2015), by genome editing the HBG proximal promoters to mimic HPFH mutations and Forced

looping of the LCR to the HBG promoters by lentiviral transduction of an LDB1-zinc finger fusion protein (Breda et al. 2016).

Universal expression of MYB and its essential role in hematopoiesis, raises concerns on the ability to achieve adequate therapeutic window while the erythroid-specific KLF1 has pleiotropic effects on erythropoiesis (Thein Swee Lay 2018). High expression of miR-15a, miR-16-1-1, miR-486-3p, miR-23a, and miR 27a154 were suggested to down-regulation of repressors of γ-globin genes (MYB, BCL11A, KLF3, Sp1) and increased HbF production (Finotti et al. 2015).

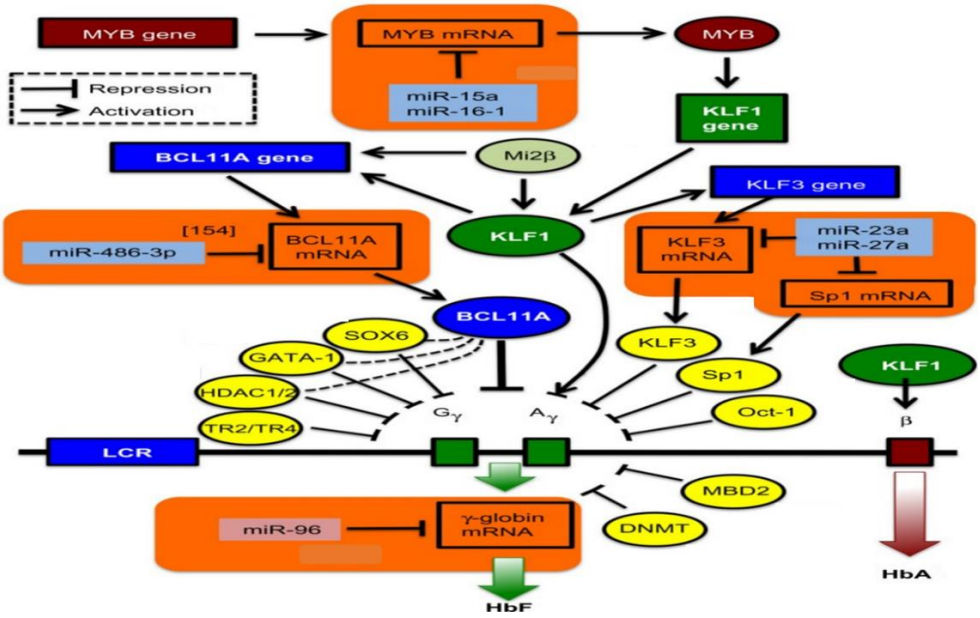


Figure 1.17: Novel therapeutic genome editing targets for γ-globin gene transcriptional activation (Finotti et al. 2015)

1.11 Rationality and expected outcome of the study

Bangladesh lies in the global thalassemia belt and thalassemia is the most common congenital disorder in Bangladesh. However, it is a matter of deep concern that thalassaemia is not recognized as priorities in public health sectors of the country and there is no exact data representing the current prevalence of thalassemia in Bangladesh. A few studies are available which were either on hospital-based patients or small groups of population. Prevalence data preferably require large-scale population surveys with appropriately calculated sample size and should not be hospital-based. It is essential to have a more accurate assessment of the gene frequency of BTT and HbE trait in our population for planning a national thalassemia prevention program.

Secondly, Bangladesh is a small country, but with considerable regional heterogeneity. It is important to know the regional prevalence to identify the endemic region and thus to target services where it is most needed.

Thirdly, the study aims to identify the common mutations in β-globin gene in the carriers with the thalassemia traits detected in the study population. The spectrum of mutations varies across different geographical regions and cultures provided that different mutations are associated with different types of thalassemias that influence the severity of the diseases. Hence, the complete mutational profiling for Bangladesh is essential to undertake any strategies (e.g. genetic counseling, prenatal diagnosis) to deal with thalassemia. Any novel mutations if present can also be identified and will be beneficial for prenatal diagnosis.

Fourthly, effect of the consanguinity in increased thalassemia carrier frequency in the country will be investigated. Also, the level of knowledge on thalassemia among the young educated adults is the another research question of the study. It will grasp the gravity of the situation by identifying the contributing factors to increase the disease burden and emphasize the necessity of mass awareness programs.

Next, as there is no documented registry of thalassemia patients in our country, the current study will estimate the expected annual new births with thalassemia disease. Calculated value using the Hardy-Weinberg rule from the carrier status can predict the disease burden.

Furthermore, in Bangladesh, around 70% transfusion dependent thalassemia patients are HbE/β-thalassemia, management of which is quite difficult due to its widely disparate range of clinical and hematological parameters in patients leading to highly variable degree of disease severity in terms of age of first transfusion and transfusion interval; and phenotype may evolve over time. The reasons for such extraordinary clinical variability or heterogeneity of Hb E/β-thalassemia are poorly understood. For careful tailoring of the treatment and management for each patient, understanding of genetic modifiers of the severity of HbE/β-thalassemia is required. In Bangladesh, there is no study on the genetic factors responsible for modifications of disease severity of HbE/β-thalassemia till date.

Therefore, the study on disease modifying genes and the associated SNPs responsible for the clinical variability of HbE/β-thalassemia in Bangladeshi patients will help in proper diagnosis and prognosis of different severe groups and planning appropriate management as well as to reveal new therapeutic targets for reducing the disease severity.

1.12 Hypothesis of the study

There are two hypotheses of the study-

1. Actual prevalence of thalassemia carriers is high in Bangladesh and molecular approaches will complement the conventional methods for carrier detection of Thalassemia to provide accurate data on frequency of the carriers in Bangladesh.
2. Specific mutations in the genetic modifiers affect the disease severity in HbE/β-thalassemia patients of Bangladesh.

1.13 Objective of the study

General Objectives

1. To determine the prevalence and mutation spectrum of thalassemia carriers in Bangladeshi population.
2. To study the genetic modifiers influencing disease severity in HbE/β-thalassemia patients of Bangladesh.

Specific Objectives

1. To determine the prevalence of β-thalassemia and HbE carriers in Bangladesh by a) using appropriate sample size representing the eight administrative divisions, b) applying combination of molecular, hematological and biochemical methods.
2. Identification of common mutations in beta globin gene in the thalassemia traits detected in the study population.
3. To detect the following genetic modifiers among HbE/β-thalassemia patients in Bangladesh-
    a. Mutations in β-globin gene
    b. Deletions of α-globin genes
    c. SNPs in HbF associated QTLs including
        i. *XmnI* polymorphism on Gγ promoter,
        ii. 3 SNPs (rs28384512, rs4895441 and rs28384513) in HBS1L-MYB intergenic region
        iii. 3 SNPs (rs11886868, rs4671393 and rs766432) in BCL11A gene
        iv. rs2071348 SNP in HBBP1 gene
4. To establish the correlation between different polymorphisms and disease severity among HbE/β-thalassemia patients.

Study population & Methods

CHAPTER 2

2.1 Determination of prevalence and mutation spectrum of β-thalassemia and HbE carriers in Bangladeshi population

2.1.1 Ethical approval

The study protocol for the Determination of prevalence and mutation spectrum of β-thalassemia and HbE carriers in Bangladeshi population was study was approved by the National Ethics Research Committee (NERC) of Bangladesh Medical Research Council (BMRC). Registration no # 102 03 04 2018.

2.1.2 Study design and place of study

***Sampling method*:** Random quota sampling

Type of study: Cross sectional study

***Study participants*:** A total of 1877 unmarried participants (irrespective of sex) aged between 18-35 years was enrolled in the study with the intent to screen students who have come from across the country to study in the universities and medical colleges in Dhaka city. The consideration of participant recruitment sites are based on the fact that these public universities and medical colleges are attended by the students who come from all 8 divisions of Bangladesh.

Duration of the study: from March, 2018 to June, 2019

***Sample collection sites*:** 4 universities, 4 medical colleges, 1 College and 2 business organizations

Place of study: Dept. Biochemistry and Molecular Biology, University of Dhaka and the institute for developing Science and Health initiatives (ideSHi). Laboratory analyses, both hematological and molecular, were carried out at the BSL–2 laboratory facility of the institute for developing Science and Health initiatives (ideSHi), Mohakhali, Dhaka-1212.

2.1.3 Sample size calculation

Total sample: Size of the total sample was statistically calculated to be 1875. From the following formula total sample size was calculated based on the study performed in 2005 by Dr. Waqar and groups (Khan et al. 2005) in which the overall prevalence of beta-thalassemia trait and HbE trait was 10.2% (4.1% + 6.1%).

$$n = \frac{(\alpha+\beta)^2\{p_1(1-p_1)+p_2(1-p_2)\}}{(p_2-p_1)^2} \text{ X design effect}$$

n= Sample size
α= the probability of type I error
β= the probability of type II error (power of the test)
p_1= 10.2% current prevalence
p_2= 15% expected prevalence

Considering 95% confidence interval, power 80% and a design effect of 2.5, the estimated sample size was 1875 (Noor et al. 2020). To eliminate bias, the participants was enrolled by simple random sampling from each sites representing all eight divisions of Bangladesh.

Division-wise sample size: Statistical enrollment was made with a view to proper covering of all regions of Bangladesh encompassing Bangladeshi Bengali population. The division wise sample size were calculated according to Bangladesh Bureau of Statistics and samples were collected proportionately to the population size of that division by quota sampling method (http://203.112.218.65:8008/WebTestApplication/userfiles/Image/PopMonographs/Volume-6_PDIM.pdf 2015).

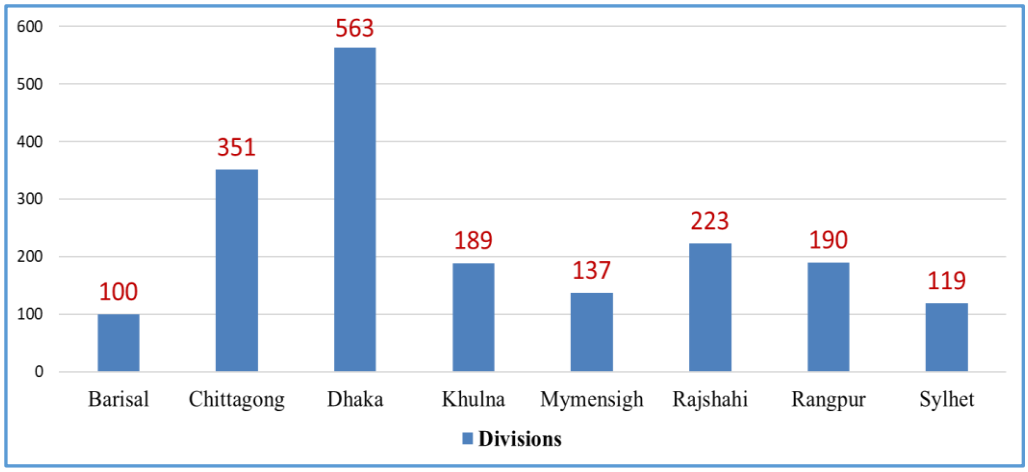


Figure 2.1: Division wise sample size recruited in this study.

2.1.4 Specimen collection

Field setting

The following list of sample collection sites (Table 2.1) in the public sphere were chosen for conducting awareness campaign and collecting specimen for the screening program. After giving awareness speech, each participant was given an information collection sheet and a consent form. Tribal people of non-Bengali ethnicity and thalassemia patients were excluded from the study.

Table 2.1: Sample recruitment sites.

Serial No	Field Site	No of participants
1	Jagannath University	272
2	Dept. of Biochemistry and Molecular Biology, University of Dhaka	156
3	Mugda Medical College	78
4	TSC, Dhaka University	116
5	Salimullah Medical College	77
6	Dhaka Medical College	78
7	Shaheed Suhrawardy Medical College	86
8	Jahangirnagar University	106
9	Banking & Finance Dept, DU	112
10	Dept of Microbiobiology, DU	56
11	Energypac Power Generation Ltd	97
12	Tiger IT	99
13	Jagannath University	160
14	BRAC University	207
15	Tejgaon College	177
Total participant		1877

Sample collection

Approximately 5.0 mL of venous blood was collected from the each participant via standard venipuncture method by a certified phlebotomist. The specimens collected was then de-identified and made anonymous with ID numbers of study subjects. Privacy and confidentially of the patients was maintained strictly. Collected blood was kept in the following tubes:

1. One K_3 EDTA vacutainer for CBC test, Hb electrophoresis and DNA-based analysis
2. One serum vacutainer for serum ferritin assay.

Immediately after blood collection, the vacutainers containing the blood specimen transported to institute for developing Science and Health initiatives (ideSHi) laboratory within 4 hours of collection maintaining 2-8° C using cool box. Utmost care was taken to avoid transportation-related hemolysis.

Sample processing

Immediately after transportation of blood specimens to ideSHi, a portion of the collected blood specimen (approximately 100 µL) was subjected to analysis for complete blood count (CBC) by an automated cell counter. Three aliquots were made from the K_3 EDTA vacutainer; one for hemoglobin electrophoresis, another one for DNA extraction for Sanger

sequencing, and an additional one aliquot with the left-over blood specimen for plasma collection. From the serum tube, blood serum was separated after centrifugation at 3000rpm for 10 minutes and then aliquot in 1.5ml micro centrifuge tube. The serum and plasma samples were stored at -80 °C for future use.

2.1.5 Overview of Laboratory Analysis

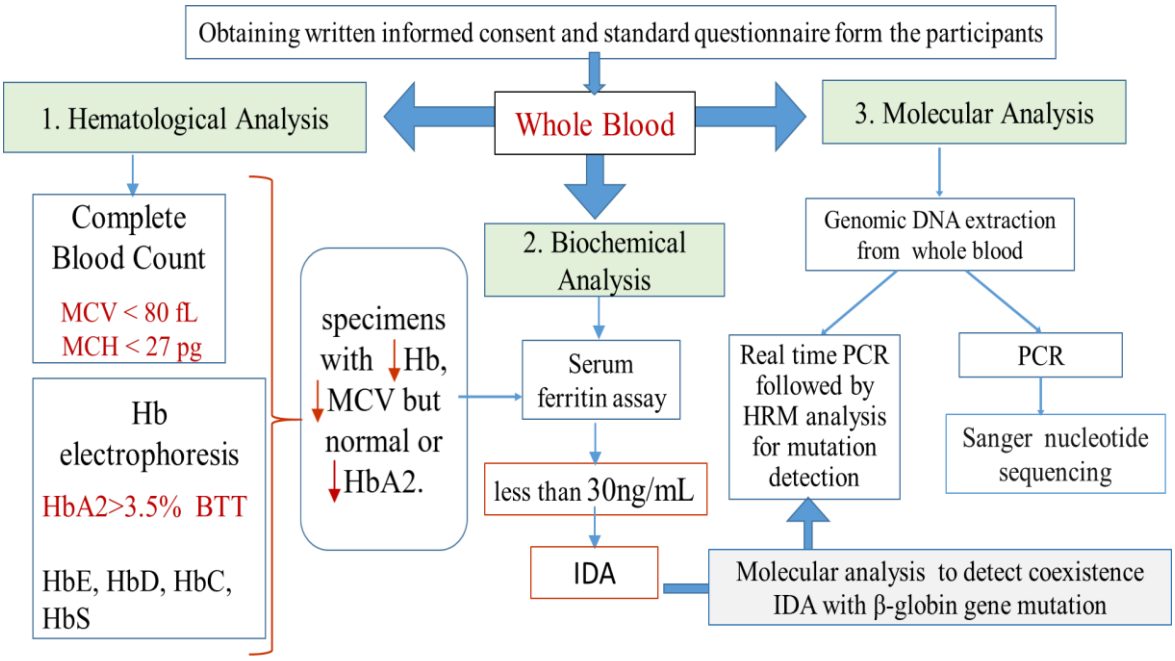


Figure 2.2: Overview of the work flow of the study.

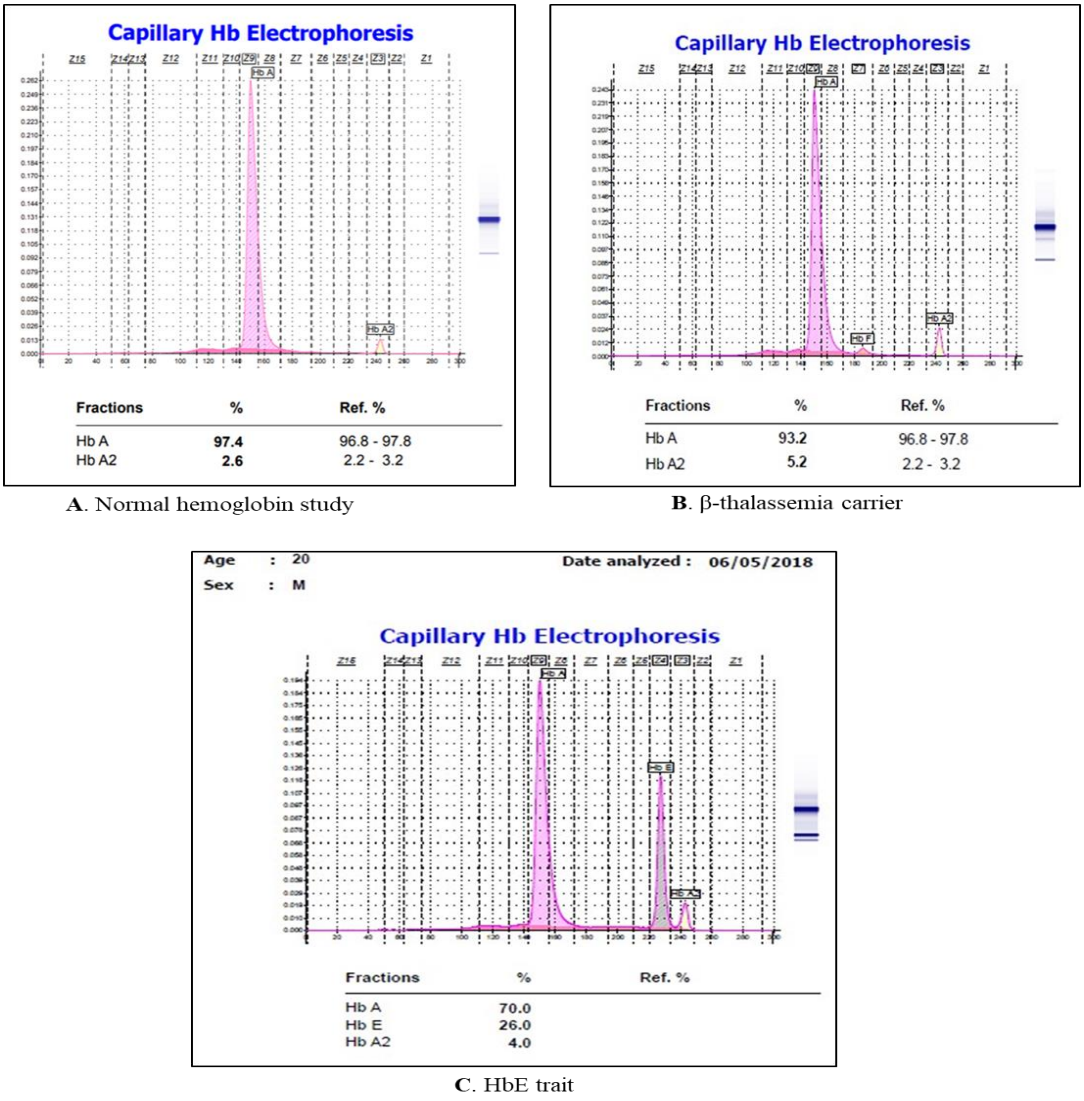
The blood sample analysis was done to confirm whether a participant is a carrier for beta-thalassemia or HbE trait and to identify the respective mutation in beta globin chain. A total of 3 different technological approaches was used. These includes (1) Hematological analysis (2) Biochemical analysis and (3) DNA-based molecular analysis for mutation scanning.

2.1.6 Analysis of hematological parameter

2ml of whole blood collected from vein in anticoagulant (EDTA) coated BD vacutainer tubes were used for hematological analysis. A complete blood count (CBC) was performed to analyze hematological parameters, namely, hemoglobin concentration (Hb; g/dL), mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), hematocrit (HCT; %) and red cell distribution width (RDW) using an automated hematology analyzer, Sysmex kx-21 (Sysmex Corporation, Kobe, Japan) according to manufacturer's instruction.

2.1.7 Hemoglobin electrophoresis

Hemoglobin electrophoresis is the gold standard for thalassemia carrier detection and diagnosis.



A. Normal hemoglobin study

B. β-thalassemia carrier

C. HbE trait

Figure 2.3: Electrophoretic pattern of A) healthy subject, B) carrier of β-thalassemia and C) carrier of HbE

Hemoglobin electrophoresis was done according to manufacturer's instructions using Sebia CAPILLARYS 2 Flex Piercing (Sebia, Lisses, France) automated hemoglobin analyzer. This instrument uses the principle of capillary electrophoresis in free solution. Charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electro-osmotic flow. A high voltage protein separation is performed and direct detection of the hemoglobins is made at 415 nm at the cathodic end of the capillary. The electrophoregrams are evaluated visually for pattern abnormalities. By using alkaline pH buffer, normal and abnormal (or variant)

hemoglobins are detected in the following order, from cathode to anode: δA'2 (A2 variant), C, A2/O-Arab, E, S, D, G-Philadelphia, F, A, Hope, Bart's, J, N-Baltimore and H.

2.1.8 Estimation of Serum Ferritin

Serum ferritin was measured for the samples suspected for iron deficiency amaemia. Enzyme Linked Fluorescent Assay technique was employed for serum ferritin assay on the miniVIDAS® Immunoassay Analyzer (bioMérieux, Inc. North USA) using VIDAS® FERRITIN kit (bioMérieux SA, Chemin de l'Orme, Marcy-l'Etoile, France) according to manufacturer's instruction.

2.1.9 Molecular Analysis

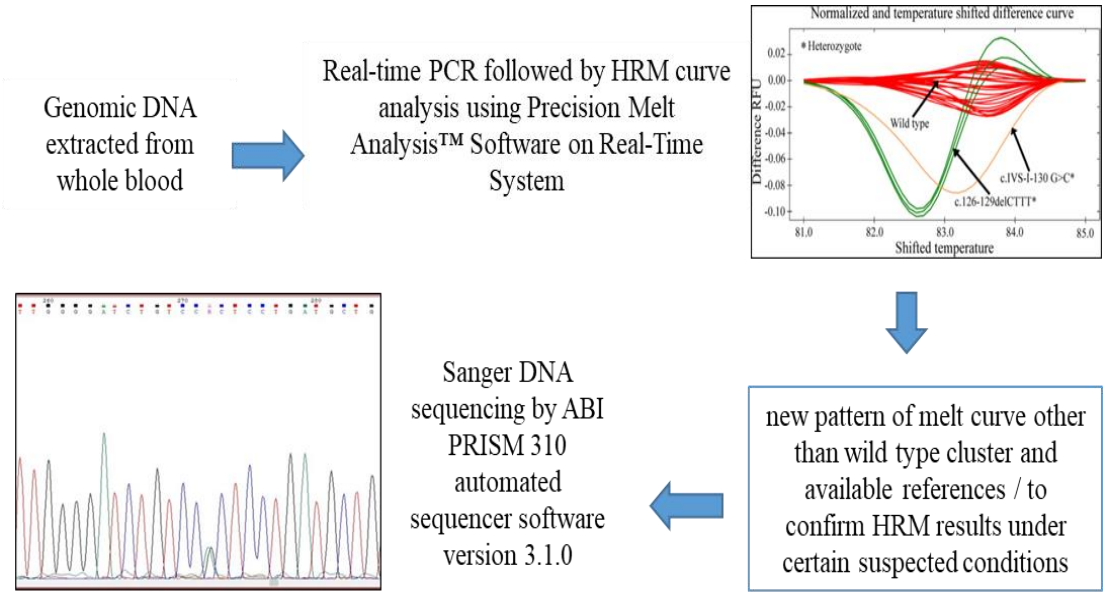


Figure 2.4: Overview of molecular analysis of β-globin gene in the study population.

2.1.9.1 DNA extraction

Genomic DNA was extracted from the whole blood using QIAGEN flexigene® DNA kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

Steps of DNA isolation procedure

500µL of FG1 buffer was taken in a 1.5 mL micro-centrifuge tube and 200 µL of whole blood was mixed with FG1 buffer by inverting the tube 5 times. The tube was centrifuged for 20 seconds at 10,000 x g using 'HeraeusTM FrescoTM 21 Microcentrifuge (Thermo ScientificTM, USA)'. The supernatant was discarded and the tube was kept inverted on a clean tissue paper for 2 minutes, taking care that the pellet remains in the tube. FG2/QIAGEN

Protease mixture (Denaturation buffer) was prepared by adding QIAGEN Protease with FG2 buffer in 1:100 ratios and must be used within 1 hours of preparation. 100 µL FG2/QIAGEN Protease buffer was added and vortexed immediately to homogenize the pellet completely. After centrifugation for 3-5s, the tube was placed in a water bath at 65^0 C for 5 min. After incubation, 100 µL isopropanol (100%) was added and mixed thoroughly by inversion at least 20 times until jelly-like or a thread-like DNA became visible. After then, the tube was centrifuged for 3 minutes at 10,000 x g. The supernatant was discarded and the tube was inverted on a clean tissue paper for 5 minutes. 100µL 70% ethanol was added and vortexed for 5s and centrifuged for 3 minutes at 10,000x g. The supernatant was discarded and the tube was inverted on a clean piece of tissue paper for at least 5 minute, taking care that the pellet remains in the tube. The pellet was air dried at least for 5 minutes until all the liquid had been evaporated. 'Nuclease free water' was added to dissolve the DNA pellet and was kept at 4^0 C overnight. The sample was stored at -20^0 C for long time.

2.1.9.2 DNA Quantification

DNA concentration was measured with (Nanodrop 2000). 2µl of nuclease-free water was used as blank and 2µl of DNA sample was loaded on the sensor. After that, the optical density (OD) was measured. Thus, the concentration (ng/µl) and the purity of the DNA (260/280 nm OD) were obtained and stored at -20°C until analysis.

2.1.9.3 Mutation detection using HRM curve analysis method

Real-time PCR was followed by HRM analysis was used for mutation detection in the samples with β-thalassemia trait. The method followed was as per the previously developed directions used for screening Bangladeshi and regional population of thalassemic endemic countries (Islam et al. 2018).

Basic principle and procedure of HRM curve analysis

Firstly, the DNA is amplified using Real-Time PCR. The steps are as follows for real-time PCR - Initial denaturation, denaturation, annealing, and elongation. After the completion of the real-time PCR, the amplicon DNA is melted by warming up from around 50^0 C up to around 95^0 C to get different graphs for different mutated strands. For different mutations, the melting pattern is different resulting separated curves is seen in real time using fluorescence dye. The real-time PCR reactions were carried out in a 96-well plate with 10µL volume of reaction in every well. Real-time PCR was performed on BioRad CFX96 Touch Real-Time System with a master mix consisting the following reagents: Nuclease Free water,

SYBR® Green PCR Master Mix (Applied Biosystems).The reagents were mixed for preparing master-mix in a microcentrifuge tube and added in equal volumes (9µL) to all the wells and the template DNA was added after then for 10 µL volume. The Real-time PCR Machine (CFX96 TouchTM Real-Time PCR machine) was set to the protocol for thermal cycling mentioned by Islam et al. 2018. The data was analyzed using Precision Melt Analysis™ Software (BioRad) after projecting the data on the screen when the real-time PCR followed by HRM was completed. To get accurate results for cluster detection, the curve shape sensitivity was fixed to 100% and the difference in the Tm threshold was fixed to 0.1. Furthermore, normalized and temperature shifted views were used for successfully detecting SNPs in HRM analysis.

2.1.9.4 Sanger DNA sequencing

In this study, once the mutation positive specimens with a new HRM patterns other than the references were identified, nucleotide sequencing was carried out for those samples to identify the mutation. Basic steps are as follows:

a) Conventional polymerase chain reactions (PCR)

At first PCR was performed targeting the mutational hot-spot region of HBB gene for Bangladesh (exon1, intron 1 and a portion of exon 2 of beta-globin gene) using previously designed primers and developed protocol for screening Bangladeshi population (Islam et al. 2018). PCR amplification was done using a BioRad T100^{TM} thermal cycler (Bio-Rad, USA) and Qiagen HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Total reaction volume was 10 µL. PCR instrument was programmed to maintain the thermal cycling condition describes by Islam et al. 2018.

b) Agarose Gel Electrophoresis for PCR product visualization

The basic principle is that, as DNA is negatively charged due to its phosphate groups and at neutral or alkaline pH, PCR products give rise to a uniform negative charge, when an electrical field is applied during electrophoresis, the negatively charged DNA fragments move towards the positively charged electrode (anode) through the pores of 1/1.5% gel. The molecular weight of the DNA molecule is inversely proportional to the velocity of movement. Therefore, the smallest molecules move faster than the largest molecules.

***Preparation of 1% gel*:** 1 gm of agarose (ultrapure, Invitrogen, USA) was dissolved in 100 ml 1XTAE buffer (Tris-Acetate EDTA) by heating in oven for 1/2 minutes. After cooling

down a little at room temperature, 2µl of Gel red was added in the gel mixture. When the liquid gel was lukewarm, this gel mixture was poured on a casting tray and a comb was set carefully, bubbles forming should be avoided while pouring the gel. And after that, the gel was allowed to solidify at room temperature.

Gel Run: After the gel was solid, it was placed electrophoresis chamber filled with 1X TAE buffer. 2µL of loading dye was mixed with 3µL of PCR products and loaded into the well gently. At last, a well was filled with 1KB+ ladder to determine the band size of PCR amplicons and run at 110 volts for 30-45 minutes. Then, the gel was observed on the Gel documentation system (Bio-Rad, USA) under Ultraviolet light and separated DNA bands were recognized.

c) PCR product purification

Then the PCR products were purified using the MinElute® PCR purification kit (Qiagen) following the manufacturer's instructions.

Procedure

All PCR products were spun and transferred to Eppendorf tube. 5x volumes of binding buffer (PB) were added to each PCR product in each tube. The mixture was transferred to the spin column; placed on a collection tube and waited for 1-2 minutes for binding, after that, centrifuged for 1 min at 13,000 rpm. The flow-through was discarded from the MinElute column and was again placed into the same collection tube. 750µL wash buffer (PE) was added to wash each column and was again centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the column was again placed back on the same collection tube for additional centrifugation for the complete removal of residual ethanol for 1min at 13,000 rpm. The columns were transferred on 1.5 mL micro-centrifuge tubes. For elution of the PCR product, 15µL of nuclease-free water was applied to the center of the membrane of each MinElute column, allowed to stand at room temperature for 1 min and centrifuged at 14,000 rpm for 2 mins to elute and discarded the column. Concentration and purity of the purified DNA were measured using NanoDrop and then stored at -20 C for further genetic analysis.

d) Cycle sequencing of purified PCR product

Purified PCR products were sequenced at ideSHi and Center for Medical Biotechnology (CMBT) by using BigDye Chain Terminator version 3.1 Cycle sequencing Kit (Applied Biosystems, USA) and ABI PRISM 310 automated sequencer (Applied Biosystem, USA)

sequencing of the specific region of PCR purified products was done according to manufacturer's instruction.

Master-mix preparation: For each sample 1.75µl 5x sequencing buffer, 0.5µl 2.5x BigDye Chain Terminator version 3.1 ready reaction mix, 0.32µl 10 µM forward or reverse primer were added. After that, the master mix was spun and pipetted equally to each of the 8-tube PCR strips and nuclease-free water was added up to 9µL.

Cycle sequencing PCR run: 1 The cycle sequencing reaction was performed in a final reaction volume of 10.0 µL containing 4.0 µL of BigDye™ Terminator 3.1 Ready Reaction mix, 1 µL of forward (3.2 µM) or reverse primer (3.2 µM), template DNA (10-50 ng), and nuclease-free water (up to 10 µL). Cycle sequencing was done using a thermal cycler (BioRad, USA) and the thermal cycling profile as follows: initial incubation at 96˚C for 1 minute; 35 cycles of denaturation at 96˚C for 10 seconds, annealing at 58˚C for 5 seconds and extension at 60˚C for 4 minutes; and finally hold at 4˚C until purification.

e) Purification of cycle sequencing products

To purify the products after cycle sequencing, 45 µl SAM solution was added into each well for 10 µl cycle sequencing PCR product. Xterminator solution (10 µL) was added in each well and the PCR tube was vortexed at 2500 rpm for 30 minutes. The tube was centrifuged at 1000 x g for 2 minutes and the supernatant was kept in an 8 well strip which was then placed on the ABI Prism 310 capillary (Applied BioSystems, USA) automated sequencer for DNA sequencing.

f) Sequencing data collection and analysis

Sequencing data was collected using an ABI PRISM 310 data collection software version 3.1.0 (Applied Biosystems). The collected FASTA format of the sequencing data was used to identify substituted base(s) by aligning query sequence with wild type sequence (NC_000011.10) retrieved from NCBI database by using the Basic Local Alignment Search Tool (BLAST).

2.1.10 Statistical analysis

The comparison of sensitivity and specificity between traditional methods and molecular approach were performed using a web tool named https://www.openepi.com/DiagnosticTest/ DiagnosticTest.htm with 95% CI. The CI for an observed proportion was calculated using

Stata software (version 14.2). With the known genotype frequency, the number of expected newborns with thalassemia was calculated by Hardy-Weinberg equation (Noor et al. 2020).

2.2 Study of Genetic modifiers affecting the disease severity in HbE/β-thalassemia patients of Bangladesh

2.2.1 Sample size calculation

Sample size was calculated using the following formula (Hulley et al. 2001):

$$N = [(Z\alpha+Z\beta)/C]\ 2 + 3$$

$$C = 0.5 * \ln [(1+r)/(1-r)] = 0.549$$

N = Sample size
α = Type I error rate (Threshold probability for rejecting the null hypothesis)
β = Type II error rate (Probability of failing to reject the null hypothesis under the alternative hypothesis)
r = the expected correlation coefficient

Considering 95% (α = 0.05) confidence interval, for power 95% (β = 0.05) and the expected correlation (r = 0.5), the estimated sample size would be 44 per group.

Thus, total sample size for 3 groups of patient (mild, moderate and severe) = 132.

2.2.2 Study subjects

It was a Cross-sectional study using the purposive sampling method which recruited a total of 130 patients with genotyping of HbE/β-thalassemia over a period of 11 months from September 2018 to August 2019. Initially 157 thalassemia patients were enrolled for this study. But 138 patients were diagnosed as HbE/β-thalassemia. However, 130 patients with the age range of 6 to 65 years (80 males and 50 females), were finally enrolled upon the availability of all the clinical history including age of first transfusion and transfusion interval data, splenomegaly, hematological parameters, hemoglobin electrophoresis data etc. A total of 50 unrelated healthy individuals with age range of 25-35 years were also recruited who had apparently normal hematological parameters and hemoglobin study.

2.2.3 Sample collection site

These patients, were enrolled from the Bangladesh Thalassemia Samity Hospital located in Dhaka where they came for follow-up examination and blood transfusion. A written informed consent form along with a structured questionnaire of information about the age of

onset, age of first transfusion, transfusion interval, and splenectomy status of the patients was obtained from the patients or their legal guardians prior to the enrollment in the study.

2.2.4 Ethical approval

The ethical clearance certificate and consent form regarding this research was approved by Bangladesh Medical Research Council (BMRC) of National Ethics Review Committee (NERC), Dhaka, Bangladesh. All the patients or legal guardians provided their ethical compliance by filling up consent form before contributing to the study.

2.2.5 Place of study

All the hematological, molecular and genetic laboratory experiments were conducted in Biosafety Level 2 (BSL-2) facilities of the Genetics and Genomics Laboratory at Institute for Developing Science and Health Initiatives (ideSHi).

2.2.6 Overview of the study design

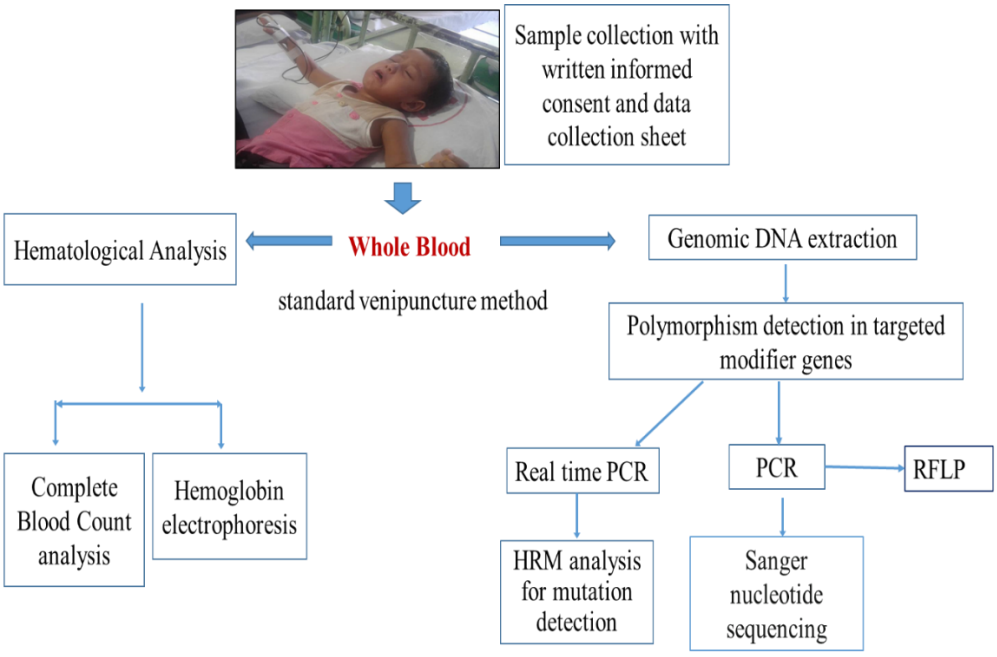


Figure 2.5: Overview of the laboratory work flow for Study of Genetic modifiers affecting the disease severity in HbE/β-thalassemia patients of Bangladesh

2.2.7 The severity scoring of the HbE/β-thalassemia patients

All the patients with HbE/β-thalassemia were classified into three groups –mild/NTD, moderate and severe based on six clinical criteria following the Mahidol scoring system showed in Table 2.2 (Sripichai et al. 2008).

Table 2.2: Scoring System of Hemoglobin E/β-Thalassemia (Sripichai et al. 2008).

	Total severity score	Points scored[a] 0	0.5	1	2
Clinical criteria					
Hemoglobin at steady state (g/dl)		>7.5		6–7.5	<6
Age at receiving first blood transfusion (year)		>10		5–10	<5
Requirement for blood transfusion		None/rare		Occasional	Regular
Size of spleen (cm)		<3		3–10	>10 or splenectomized
Age at thalassemia presentation (year)		>10	3–10	<3	
Growth and development[b]		>25th percentile	3rd–25th percentile	<3rd percentile	
Severity category					
Mild	0–3.5				
Moderate	4–7				
Severe	7.5–10				

The scoring system consisting of 6 clinical criteria scored as 0, 0.5, 1 or 2, accordingly to clinical presentation. β-Thalassemia patients with total score ranging from 0–3.5, 4–7, and 7.5–10 are grouped as mild, moderate, and severe cases, respectively.
[a]The weighted scores are obtained by dividing the coefficients of the selected significant parameters by the smallest significant coefficient and rounding the resulting number to the nearest integer.
[b]Percentile of growth development was assessed based on weight and height measurements plotted on a Thai standard growth chart.

2.2.8 Specimen collection

Venous blood samples (~ 5 ml) were collected from the patients in the standard venipuncture method before blood transfusion. A vacutainer was used to store each sample along with EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) as an anticoagulant. The collected samples were immediately transported to ideSHi laboratory facility in an ice box (2 -8˚C). Blood samples were then stored in freezer (-70˚C) for long time preservation.

2.2.9 Hematological analysis and Hemoglobin study

A complete blood count (CBC) was performed to analyze hematological parameters, hemoglobin concentration (Hb; g/dL), MCV (fL), MCH (pg), HCT (%) and RDW (%) and Hb electrophoresis was done to evaluate the percentage of HbE, HbF, HbA and HbA2. Same procedures were followed descried in section 2.1.6 & 2.1.7.

2.2.10 Detection of -158 Xmn1-Gγ polymorphism using PCR-RFLP

Genomic DNA was extracted from the whole blood of patients according to section 2.1.9.1.

a. Primer designing

To design the primers for amplification of human gamma-globin (**HBG2**) gene, the DNA sequence of human HBG2 gene was retrieved from the nucleotide database of National Center

for Biotechnology Information (NCBI). A set of primer pair was designed to amplify the 583 bp segment of the promoter site of HBG2 gene. The properties of primers such as Tm, GC%, self-dimer and heterodimer was checked by oligoanalyzer tool of IDT (Integrated DNA Technologies, USA). Specificity and product length of the amplified products of primers were checked by NCBI primer BLAST tool.

Table 2.3: Forward and reverse primer for detection of -158 Xmn1-Gγ polymorphism on human gamma-globin (HBG2) gene

Primer Name	Sequence (5' – 3')	GC %	Tm	Product length (bp)
HBG2_Xmn1_Set2.1F	GAG ATA ATG GCC TAA AAC CAC AG	43.48	57.24	583
HBG2_Xmn1_Set2R	AGA AGC GAG TGT GTG GAA CTG	52.38	60.54	

b. Conventional PCR

PCR was performed in a final reaction volume of 20.0 µL containing 2.0 µL of 10X PCR buffer (with 15.0 mM $MgCl_2$), 0.5 µL $MgCl_2$ (25 mM), 4.0 µL Q-solution (Qiagen), 3.2 µL dNTPs mixture (2.5 mM), 0.4 µL forward (10 mM) and 0.4 µL reverse primers (10 mM), 0.2 µL of HotStarTaq DNA polymerase (Qiagen) and 200.0 ng of genomic DNA finally total volume was made to 20.0 µL with nuclease-free water. PCR instrument was programmed to maintain the thermal cycling condition as follows: pre-denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 40 seconds and extension at 72°C for 50 seconds; final extension at 72°C for 5 minutes and hold at 4°C. Then the PCR product was checked using gel electrophoresis as described in 2.1.9.4 b.

c. Purification of PCR product

PCR product was purified following the same procedure described in section 2.1.9.4 c.

d. Restriction Digestion using *Xmn* I restriction enzyme

A restriction digestion is a molecular technique which is used for fragmentation of the DNA at a specific site. This fragmentation pattern can be used to detect a specific DNA sequence because a specific restriction enzyme cut the DNA only at specific site.

Procedure of restriction digestion

At first, a master mix was prepared for restriction digestion containing 0.25µL *XmnI* Restriction Enzyme (NEB, UK), 1µL 10X cut smart buffer (NEB, UK), 1µL DNA (PCR product) and 7.75 µL Nuclease free water. Total reaction volume was 10.0 µL.

Then the master mix was incubated using a thermal cycler and the thermal cycler profile was as follows- at 37^0 C for 1hour of digestion step, 65^0 C for 20 min as enzyme inactivation step and hold at 4°C. 1.5% gel was used to visualize or detect restriction digested PCR products following the procedure described in the section 2.1.9.4b. XmnI restriction digestion pattern was as follows-

XmnI genotype	**Cutting pattern**	**Length (base pair)**
CC (Wild type homozygous)	Uncut (single bands)	583
CT (Heterozygous)	Cut (triple bands)	583, 400, 183
TT (Mutant homozygous)	Cut (double bands)	400, 183

2.2.11 Establishment of HRM curve based SNP detection method for other HbF associated SNPs in BCL11A, HBS1L_MYB and HBBP1 gene

As PCR-RFLP is a lengthy time consuming method requiring a number of steps which includes conventional PCR followed by gel run and PCR product purification, then Restriction digestion of the PCR product and finally detection of the restriction fragments using Gel electrophoresis, here we established Real-time PCR followed by HRM curve analysis based SNP detection method for rest of the 7 SNPs namely, rs4895441, rs28384513, rs28384512, rs11886868, rs4671393, rs766432 and rs2071348.

2.2.11.1 Preparation of reference sample by Sanger Sequencing for HRM curve analysis

For identifying the target SNPs from unknown samples, reference samples were prepared for each of the SNPs. To get the reference samples, a certain number of samples were randomly selected for conventional PCR to amplify the specific PCR products containing the region of polymorphism and Sanger sequencing was done to identify the polymorphic alleles.

Primer designing

A total of 14 set primers were designed for 7 SNPs following the same method described in section 2.2.10.a. The DNA sequence of human BCL11A gene, HBBP1 gene and HBS1L_MYB intergenic region was retrieved from the nucleotide database of National Center for Biotechnology Information (NCBI) to design the primers. Two pairs were for each SNPs, 1 pair for conventional PCR followed by sequencing and 1 pair for Real-time PCR followed by HRM curve analysis (Table 2.4).

Table 2.4: List of primers used for amplification of the targeted SNP regions in the study.

Primers	**Sequence 5′-3′**
HBBP1_ rs2071348_HRM_F	GAGCTATCAAATGGTAAGTGGCC
HBBP1_ rs2071348_HRM_R	GGGATATGATATTTCAGCAGTGGG
HBBP1_ rs2071348_SEQ_F	CCAGGACTATGCAGAAAAGTGAC
HBBP1_ rs2071348_SEQ_R	GACTGTGCAATAATGGGCAACC
HBS1L-MYB_rs4895441_SEQ_F	GTGTTGGGATATAGGCCATAGAC
HBS1L-MYB_rs4895441_SEQ_R	GGTCTACAAAGCCCTACAGGATC
HBS1L-MYB_rs4895441_HRM_F	ATGGGGGTAAGAAGGAAACCAG
HBS1L-MYB_rs4895441_HRM_R	CTCCCTGTCCCCAGATACTTAC
HBS1L-MYB_rs28384513_SEQ_F	CGGCAATGCCTCAGGGTCACTG
HBS1L-MYB_rs28384513_SEQ_R	TATGTTGCTCAGGCTGGTCTCG
HBS1L-MYB_rs28384513_HRM_F	TTGGACTAAATGTTGCAAGCGG
HBS1L-MYB_rs28384513_HRM_R	ACTGAGCGCATAGCTTTCTCAG
BCL11A_rs4671393_SEQ_F	CCTTCTGCTTCCTGTTCACCTC
BCL11A_rs4671393_SEQ_R	GGGCATTCATTTGAATCAGGCC
BCL11A_rs4671393_HRM_F	ATGGCCAAGCTGATGAGGATG
BCL11A_rs4671393_HRM_R	AGGTCTCACACAACACTCCAGG
BCL11A_rs11886868_SEQ_F	GTGGTCACAACCCACATGGCAA
BCL11A_rs11886868_SEQ_R	AGTTGTATACAGCTGTGTGGCC
BCL11A_rs11886868_HRM_F	CCACACCATGGATGAATCCCAG
BCL11A_rs11886868_HRM_R	CCACCTACCACCACAGTGTTGA
BCL11A_rs766432_Seq_F	TGGGGGTTCAGTGGTTAGAAGG
BCL11A_rs766432_Seq_R	CCAAATGCTCTGCTTATGGTGG
BCL11A_rs766432_HRM_F	GGCCACACAGCTGTATACAACT
BCL11A_rs766432_HRM_R	AGACTTGGTTCCACTCCAGTGG

PCR reaction

Reaction mix was prepared in same composition for all the modifier genes as described in 2.2.10.b but different reaction protocols were used for each after optimization. Thermal cycling conditions for PCR of 3 SNPs in BCL11A were pre-denaturation at 94°C for 15 minutes; 35 cycles of denaturation at 94°C for 15 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 20 seconds; final extension at 72°C for 5 minutes and hold at 12°C. Thermal cycling profile was same for amplification of the HBS1L-MYB SNPs (rs28384513, rs28384512 and rs4895441) except the annealing step which was 60°C.

HBBP1 (rs2071348) polymorphic region was amplified using 35 cycles of denaturation at 94°C for 30 seconds and annealing at 58°C for 30 seconds, rest of the steps were same.

Agarose Gel electrophoresis

1% and 1.5% gel were used to detect the PCR products following the procedure described in the section 2.1.9.4b. Gel pictures of PCR products are given in figure 2.6.

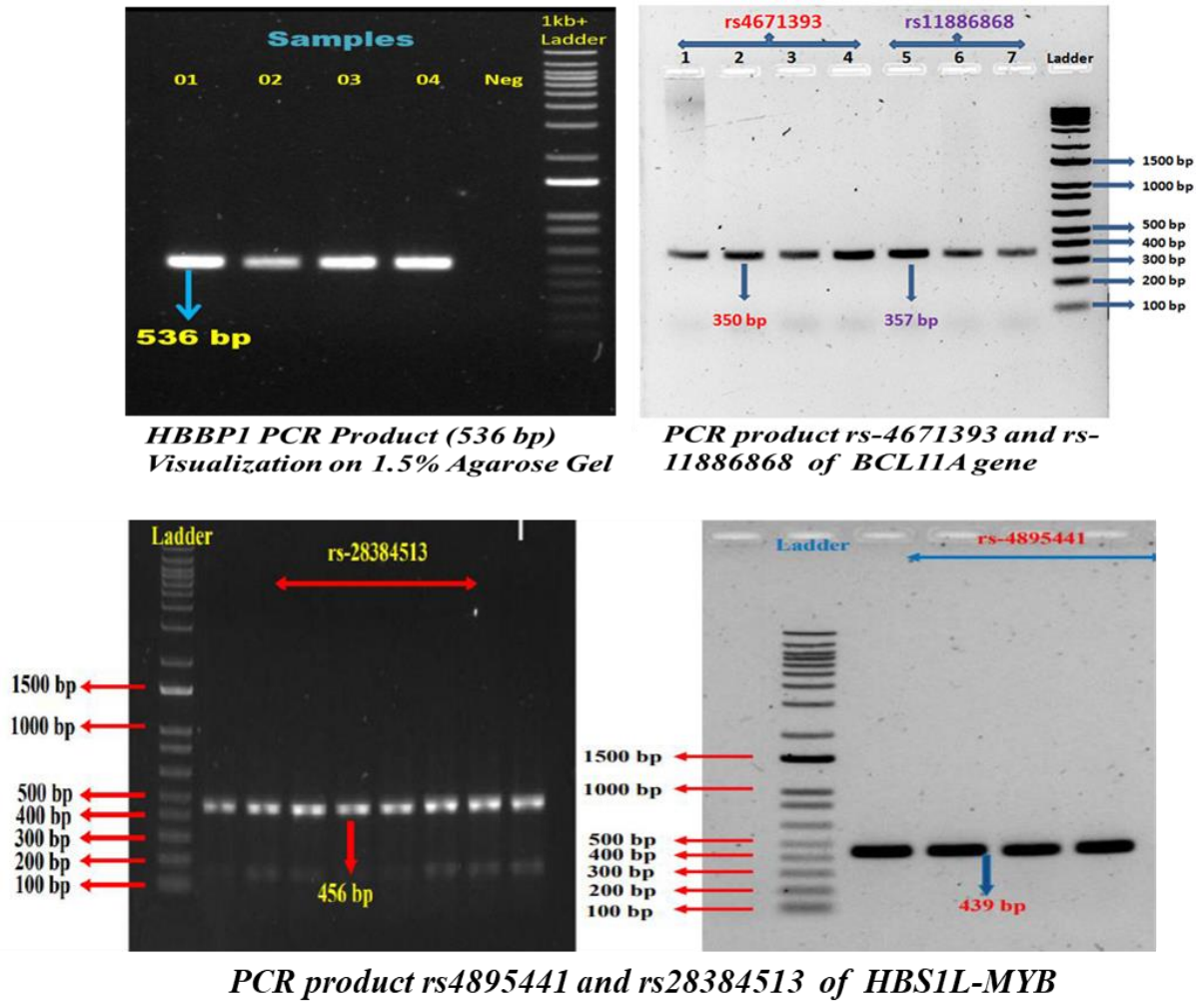


Figure 2.6 Gel Electrophoresis of conventional PCR product of polymorphic region on BCL11A, HBS1L_MYB and HBBP1 gene.

Purification of PCR product

PCR product obtained from conventional PCR for each of the seven SNPs were purified following the same procedure described in section 2.1.9.4 c.

Sanger DNA sequencing

All the steps required for Sanger nucleotide sequencing were done including cycle sequencing, purification of cycle sequencing product followed by capillary electrophoresis

using POP-6 (Applied Biosystems, USA) on an ABI PRISM 310 Automated Sequencer (Applied Biosystems, USA) according to section 2.1.9.4.

2.2.11.2 High Resolution Melt Curve Analysis

Real-time PCR was performed on BioRad CFX96 Touch Real-Time System with a master mix consisting the following reagents: Nuclease Free water, SYBR® Green PCR Master Mix (Applied Biosystems), forward and reverse primer pair for each of the 7 SNPs and DNA samples of the study subjects. The procedure details was the same as described in 2.1.9.3. The thermal cycling profile for the real-time PCR was as follows: initial denaturation at 95°C for 3 minutes; 40 cycles of PCR amplification with denaturation at 94°C for 10 seconds followed by primer annealing for 30 seconds at 63°C for HBBP1 and 60°C for BCL11A and HBS1L_MYB, and then extension at 72°C for 30 seconds. The final extension of PCR product was performed at 72°C for 5 minutes. After completion of PCR, the subsequent melt curve program had the following steps: denaturation at 95°C for 1 min, renaturation at 60°C for 1 min, and then melting at 60°C to 95°C with an increment of 0.1°C per 5 seconds.

2.2.12 Detection of α-globin gene deletions

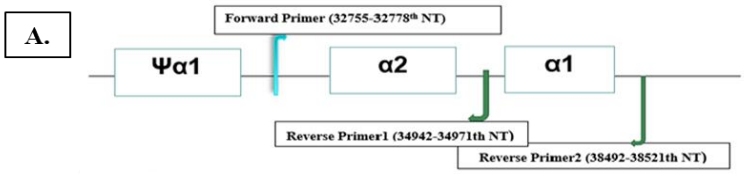
Here we examined two most frequent mutations in α-globin gene, namely, ($--^{SEA}$) deletion and $-\alpha^{3.7}$ deletion along with gene triplication ($\alpha\alpha\alpha^{anti\ 3.7}$).

Conventional Gap-PCR for detection of -α3.7 deletion and $\alpha\alpha\alpha^{anti\ 3.7}$ triplication

Gap-PCR detects deletion that might be missed by DNA sequencing. Specific primers are designed to flank a known deletion. The principle of gap-PCR is based upon the inability of the primers to generate a PCR product unless a deletion joins the flanking sequences together. If a deletion is present, PCR amplification will occur and the product is examined by electrophoresis. In order to detect -α3.7 deletions and $\alpha\alpha\alpha^{anti\ 3.7}$ triplication conventional Gap-PCR was applied by using the primer pairs obtained from (Liu et al. 2000).

Procedure

PCR reaction mix was prepared at final volume of 10µL as described in previous section using deletion specific primer sets shown in figure 2.7. Genomic DNA extracted from whole blood was then amplification with an initial heat activation step of 95°C for 15 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 66°C for 1 min and extension at 72°C for 2 min 30 sec; final extension at 72°C for 10 min and after PCR reaction and hold at 4°C. PCR products was checked by running the samples on a 2% agarose gel.



Genotype	Primer	Sequence	Location	Product size(bp)
α α	alpha_3.7_F	AAG TCC ACC CCT TCC TTC CTC ACC	Z84721: 32755±32778	2217
	alpha_3.7_R1	ATG AGA GAA ATG TTC TGG CAC CTG CAC TTG	Z84721: 34942±34971	
$-\alpha^{3.7}$	alpha_3.7_F	AAG TCC ACC CCT TCC TTC CTC ACC	Z84721: 32755±32778	1963
	alpha_3.7_R2	TCC ATC CCC TCC TCC CGC CCC TGC CTT TTC	Z84721: 38492±38521	
$\alpha\alpha\alpha^{anti3.7}$	alpha_3.7_F	AAG TCC ACC CCT TCC TTC CTC ACC	Z84721: 32755±32778	2440
	alpha_3.7_R1	ATG AGA GAA ATG TTC TGG CAC CTG CAC TTG	Z84721: 34942±34971	

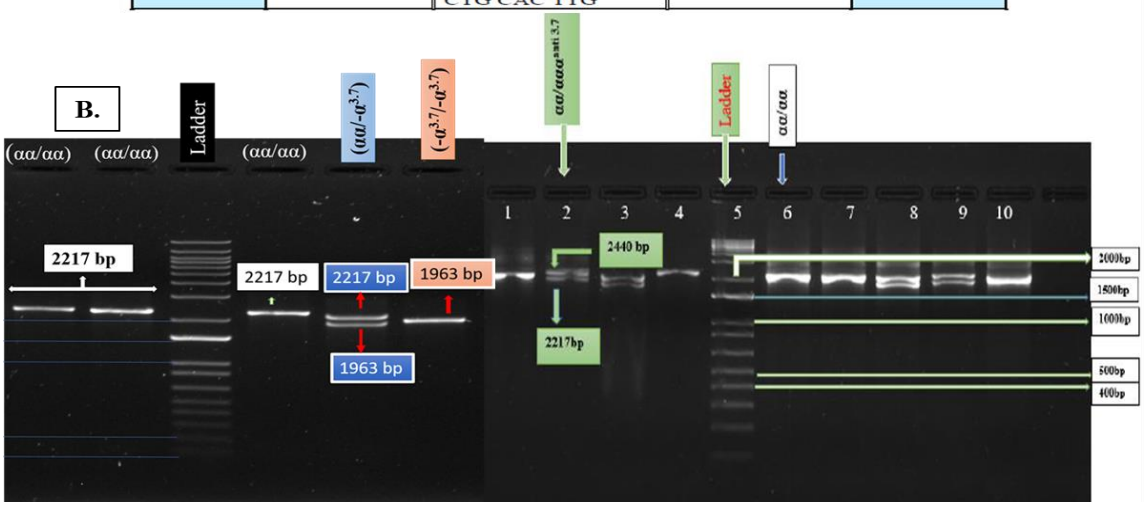
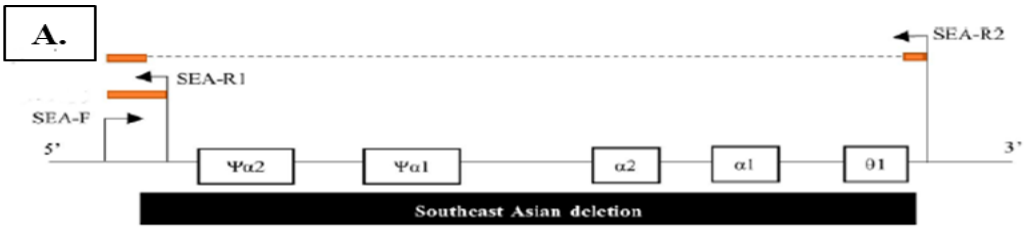


Figure 2.7: A) Primer pairs flanking deletion points according to the NCBI Reference Sequence: NG_000006.1 and Hbvar ID 1076 and the sequences retrieved from (Liu et al. 2000). **B)** Gel-Red stained gel showing representative results from gap- PCR assay for $-\alpha^{3.7}$ deletion and triplicated heterozygous genotype ($\alpha\alpha/\alpha\alpha\alpha^{anti\ 3.7}$).

Detection of ($--^{SEA}$) deletion using real-time Gap-PCR and HRM analysis

Real-time Gap-PCR follows the same principle to conventional Gap-PCR, but the reaction is based on faster real-time detection methods avoiding multiple steps using HRM analysis. Isolated genomic DNA from whole blood was used for PCR amplification using one forward and two reverse primer sequences taken from the reference paper (Pornprasert et al. 2008). Based on rapid detection and appropriation for HRM analysis, three oligonucleotide primers amplify small DNA fragments, 134 and 196 bp from the α-thalassemia $--^{SEA}$ allele and wild type α-globin gene allele, respectively which could be detected after HRM temperature analysis based on peak heights of Tm specific for each fragment. Reaction protocol was: 95°C for 2 min and then the PCR was cycled 39 times at 95°C for 15s, 63°C for 25s, and 72°C for

20s, followed by HRM cycle of 95°C for 1min, 60°C for 1 min, then from 60°C to 95°C at a rate of 0.1°C per 5 second.



Genotype	Primer	Primer Sequence (5'>3')	Location	Product size(bp)
αα	alpha_SEA_F	AGAAGCTGAGTTGATGGGTCCG	Z84721	196
	alpha_SEA_R1	ACAAACGCCCGTCCGACTCAA	Z84721	
-αSEA	alpha_SEA_F	AGAAGCTGAGTTGATGGGTCCG	Z84721	134
	alpha_SEA_R2	TGGACTTAAGTGATCCTCCTGCCC	Z69706	

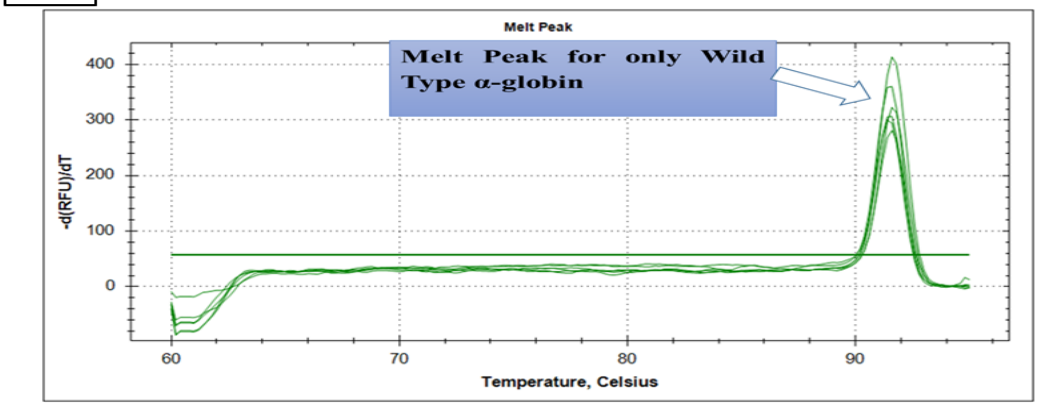


Figure 2.8: A) The position and sequences of forward (SEA-F) and reverse primers (SEA-R1 and SEA-R2) in the α-globin gene complex. The black box indicates Southeast Asian deletion (__SEA) **B)** High resolution melting plot showing dissociation curve of wild type (--^{SEA}) allele.

2.2.13 Statistical Analysis

All of the data were expressed as mean ± standard deviation (SD). Two – tailed unpaired t – test, one – way ANOVA test and Pearson's correlation coefficient analysis and Tukey HSD post hoc test were performed using GraphPad Prism® (GraphPad Prism V.5.02; GraphPad Softwares, Inc.), where $P<0.05$ was considered statistically significant. Genotypes (combination of alleles of a given SNP) and allele frequencies were analyzed using chi-square (χ^2) test and Fisher's exact test with Yates correction. Hardy-Weinberg equilibrium was analyzed by chi-square (χ^2) test. Odds ratios (ORs) were calculated using a dominant model due to the low frequency of polymorphic homozygous variants in case of some of the SNPs.

Results

CHAPTER 3

3.1 Determination of prevalence and mutation spectrum of β-thalassemia and HbE carriers in Bangladeshi Population

The result chapter has been divided into two sections. The first section (section 3.1) of the chapter describes the result of the part of the study which was conducted to determine the nationwide carrier frequency of β-thalassemia and HbE variants in Bangladesh.

3.1.1 Socio-Demographic information of the participants in the study

A total of 1877 participants were enrolled in this study and several demographic information in terms of the numbers of male and female participants along with their percentages among total participants, age and consanguinity, knowledge regarding thalassemia etc. were recorded. The male to female ratio was 1.5:1 with the age range of 18 to 35 years **(Table 3.1).** The participants were from both rural (32.4%) and urban (67.6%) origins. About 4.32% of the participants had consanguineous parents. Moreover, although all the participants had general education at the university level, only 68.14% of them knew the term 'thalassemia', whereas 62.3% had no prior knowledge of the disease etiology, severity and risk factors etc. before attending the awareness program, which was arranged as a part of this study.

Table 3.1 Participants' information regarding gender, parental consanguinity, residence and their knowledge on thalassemia.

Characteristic	Parameters	No. of Participants, n (%)
Gender	Male	1138 (60.1)
	Female	739 (39.9)
Consanguineous parents	Yes	81 (4.32)
	No	1796 (95.68)
Residence	Urban	1268 (67.6)
	Rural	609 (32.4)
Knowledge regarding thalassemia	Prior knowledge about thalassemia	1279 (68.14)
	Knowledge about how thalassemia is acquired	707 (37.66)
Presence of patients or Carriers in the participants family	Yes	50 (2.66)
	No	459 (24.45)
	Not known	1368 (72.88)

3.1.2 Preliminary screening of the study participants based on hematological parameters

The red blood cell count (RBC) and the hematological indices are extremely important in the diagnosis of asymptomatic carriers as almost all kinds of thalassemia carriers show microcytic hypochromic parameters with apparently normal hemoglobin level. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are the most widely used RBC indices for detecting microcytic hypochromic anemias. In the present study, MCV value of less than 80fL and/or MCH of less than 27pg were used as cutoff levels to initially suspect the participants as thalassemia carriers (Viprakasit et al. 2014). Accordingly, the study participants (n=1877) were divided into four categories, namely category A, category B, category C and category D. The category A participants had apparently normal RBC indices having MCV greater than or equal to 80fl and MCH greater than or equal to 27pg and they constituted 53% (995 out of 1877) of the study samples. Then 612 participants (32.6% of total samples) having MCV and MCH values less than cutoff ranges (<80fL and <27pg, respectively) had been suspected to have microcytic hypochromic phenotype which are tha hallmark of β-thalassemia and HbE carriers and were categorized as B. A total of 13 samples of mixed criteria having MCV value less than 80fL but MCH higher than cutoff (>27pg) were categorized as group C and the remaining samples (257 out of 1877, 13.7%) which had normal MCV (>80 fl) but MCH less than 27 pg were categorized as D (**Table 3.2**).

Table 3.2: Categories of the study participants based on MCV and MCH information after performing preliminary screening by CBC analyzer.

Categories	No. of Participants, N (%)
Category A (MCV ≥80 and MCH ≥27)	995 (53 %)
Category B (MCV< 80 and MCH <27)	612 (32.6 %)
Category C (MCV< 80 and MCH ≥27)	13 (0.7 %)
Category D (MCV> 80 and MCH <27)	257 (13.7 %)
** MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin	

3.1.3 Hemoglobin electrophoresis result

Hemoglobin electrophoresis was done for all the samples as it is the gold standard for thalassemia carrier detection. Sebia capillary electrophoresis is able to separate distinctly HbA2 from HbE. HbA2 level of >3.5% was used as a cutoff for diagnosis of β-thalassemia carriers (Giordano 2013). Upon electrophoresis, total study participants were divided into seven groups (Group I, II, III, IV, V, VI, VII) based on the percentages of HbA2 and HbE in total hemoglobin, and presence of other hemoglobin variants. A total of 1563 out of 1877 participants had HbA2 levels within the normal range of 2.2% to 3.2% and no HbE or other variants and thus these samples which constitute largest group were confirmed as not carriers according to hemoglobin electrophoresis result and were designated as Group II. The second largest group was Group V constituting 165 participants who showed the presence of HbE (25% to 30% of total hemoglobin) along with normal or slightly increased level of HbA2 and were thus suspected as carriers of HbE, whereas 46 participants having HbA2 > 3.5% with no HbE fraction were placed in Group IV and were assumed to be β thalassemia carriers. The participants having HbA2 level less than 2.2%, which is the lower limit of normal range, were designated as Group-I. On the other hand, Group III was denoted for the borderline suspected samples showing HbA2 values between 3.3% and 3.5%. The numbers of participants for Group-I and Group-III were 64 and 23, respectively. In group VI, there were 8 participants having greater than 90% HbE with absence of HbA along with normal or slightly increased HbA2 and thus these participants had the characteristic of HbE disease. The group VII constituted eight participants showing the presence of other hemoglobin variants namely hereditary persistent fetal hemoglobin, HPFH, HbD trait and α-thalassemia trait etc.

Table 3.3: Post-screening categories of the participants using Hemoglobin electrophoresis.

Parameters	**Range of HbA2 (%)**						**Other Hb variants**
	< 2.2	**2.2-3.2**	**3.3-3.5**	**> 3.5**	**Normal or slightly increased**		
HbE (%)	0	0	0	0	25-30	>90 %	
Number of Participants N	64	1563	23	46	165	08	08
Groups	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
** Hb, Hemoglobin; Other Hb variants includes hereditary persistent fetal hemoglobin, HPFH, HbD trait and α-thalassemia trait							

3.1.4 Molecular analysis using high resolution melt curve for confirmation of the carrier status of β-thalassemia and HbE traits

Beta thalassemia carriers have generally mild anemia, low MCV and MCH and elevated HbA2 level. However, there may be considerable variability in hematological phenotype resulting from coexistence with iron deficiency anemia (IDA) and/or coinheritance with alpha thalassemia or delta-globin gene mutations, and presence of silent mutations in HBB gene. These individuals may have milder hematological findings with minimal abnormalities in Hb, MCV, MCH, and HbA2 which may confound the correct diagnosis of β-thalassemia carriers. Considering these facts, we identified a total of 89 samples with misperceive hematological parameters and divided them into two groups (group 1 & 2) based on the HbA2 percentage, hemoglobin levels and distinctive MCV values **(Table 3.4)**. Group 1(n=64) had reduced HbA2 percentage along with low hemoglobin concentration (less than 10g/dl) and very low MCV and the presence of β thalassemia trait in this group might be masked by the coexistence of IDA and/or α-thalassemia. In Group 2, there were 25 samples having HbA2 level in the borderline range (3.3% -3.5%) but low MCV and normal Hb concentration which might result from coinheritance of β gene mutations with α or δ thalassemia traits that usually lower the level of HbA2 to normal or borderline range in β-thalassemia carriers. To overcome these shortcomings, all of the samples as described in **Table 3.4** along with the suspected cases of being β thalassemia and HbE carrier / homozygous HbE (group 3, 4 &5), were subjected to further analysis for serum ferritin and/or mutation in the HBB gene.

Table 3.4: Molecular analysis of the selected participants for confirmation of the carrier status

Groups	Selection parameters	Total, N	No. of participants detected with β globin gene mutation		
			No mutation	Heterozygous	Homozygous*/compound heterozygous
Group 1	HbA2<2.2% and Hb< 10g/dl	64	64	0	0
Group 2	Hb A2: 3.3-3.5%	25	24	1	0
Group 3	HbA2 > 3.5%	46	05	41	0
Group 4	HbE: 25-30%	165	0	163	02
Group 5	HbE >90%	08	0	0	08*

For the Group-1 in Table 3.4, serum ferritin was analyzed to exclude IDA followed by HRM analysis to detect any coexistence of β mutation with IDA. No mutation was detected in this

group. Thus, it was confirmed that none of the cases of β thalassemia traits were overlooked because of low HbA2 level which might be the result of IDA or alpha thalassemia co-existence with β globin gene mutation.

For Group-2, one sample with 3.5% HbA2, 77.6 fl MCV and 25.2 pg MCH was identified with a mutation in the β globin gene. A distinguished pattern for each mutation was seen in the HRM curve analysis from our previously established settings of HRM curve based diagnosis of β-thalassemia for Bangladeshi population, targeting the mutational hot-spot region of HBB gene (Islam et al. 2018). But the pattern did not match to the reference controls available in the lab. Then the mutation was confirmed and identified by Sanger sequencing at the position c.151G>A (Thr>Ala) which was a novel mutation (**Figure 3.1B**).

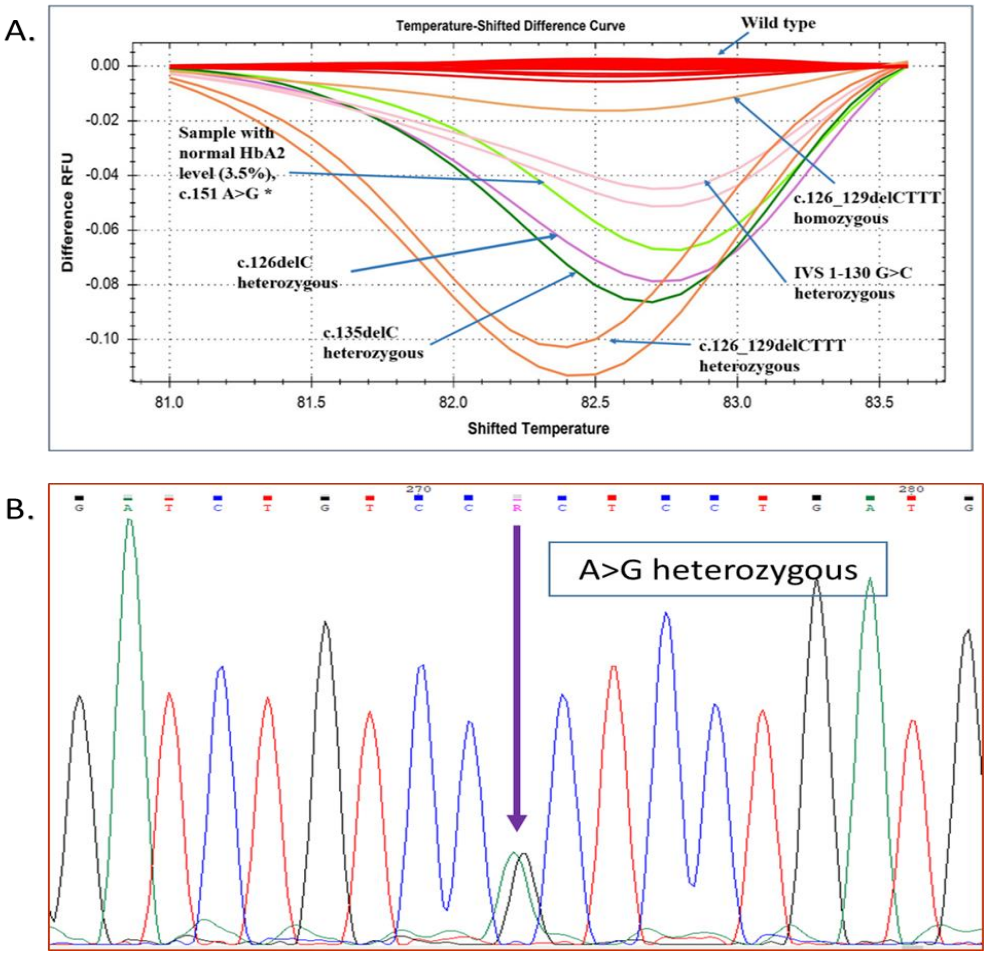


Figure 3.1: A) HRM curve analysis for mutation detection in β-globin gene targeting the hot-spot region produced a new pattern**.** RFU, Relative Fluorescence Unit; * indicates novel mutation. **B)** Sanger sequencing identified and confirmed the suspected mutation as c.151A> G (ACT> GCT; Thr> Ala)

From group-3, three samples having 3.7% HbA2, one with 4% and one having 3.6% HbA2 but MCV > 78 turned out to be normal in HRM curve analysis which were further confirmed by Sanger sequencing. In the group 4 which included the HbE carriers based on Hb Electrophoresis, 2 with relatively high level of fetal hemoglobin (HbF of 2.8% and 11.4% respectively) were identified having compound heterozygous mutation which represents HbE-β thalassemia. Hemoglobin indices of the two samples have been summarized in **Table 3.5**. This condition indicates transfusion independent thalassemia.

Table 3.5: Hemoglobin indices of the two participants containing compound heterozygous mutation

Serial no	Age of the participant (Years)	Hb (g/dl)	MCV (fl)	MCH (pg)	RDW (%)	HbA (%)	HbF (%)	HbE (%)	HbA2 (%)
1	20	9.7	69.6	20.8	19	63.5	11.4	22.1	3
2	21	9.9	66	19	19.3	79.3	2.8	14.3	3.6

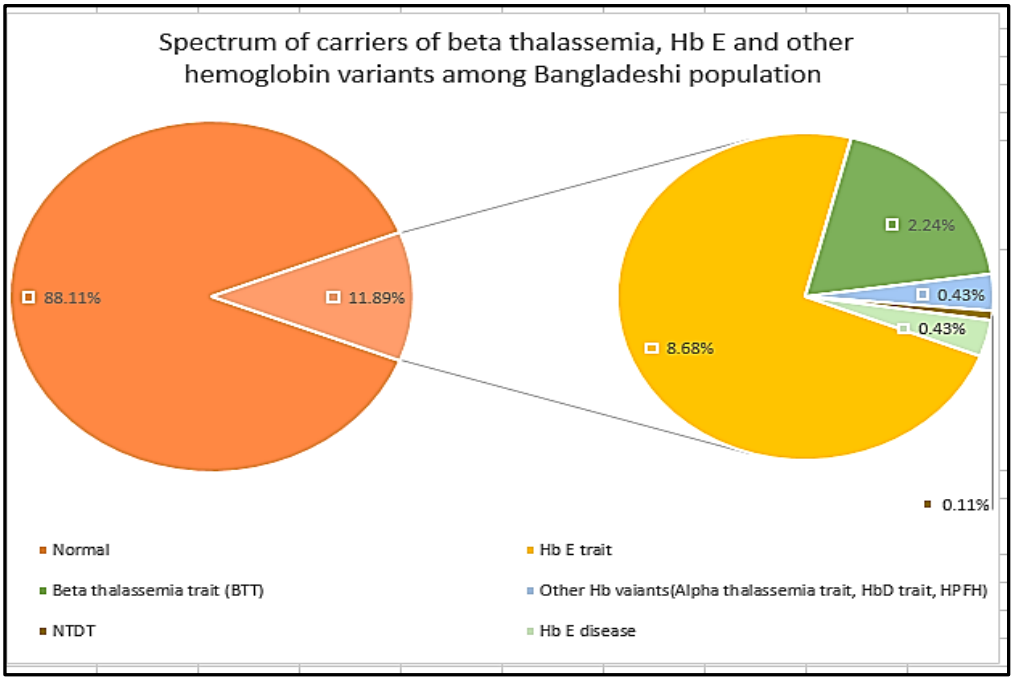
3.1.5 Prevalence pattern of β thalassemia traits, carriers of HbE and other hemoglobin variants among Bangladeshi population

After hematological and electrophoretic indices study in combination with molecular analysis, the most prevalent thalassemia trait found in this present study was HbE trait (ETT) comprising 8.68% (163/1877) of the study participants. The 2nd most prevalent trait belonged to the β-thalassemia trait (BTT) group and 2.24% (42/1877) of the study participants were BTT **(Table 3.6).**

Table 3.6: Distribution of thalassemia carriers and other Hb variants among the study participants

Types of thalassemia carriers and other Hb variants	Number of participants, n	Frequency %, (95% CI)
HbE Trait (ETT)	163	8.68 (7.41–9.95)
β-thalassemia trait (BTT)	42	2.24 (1.57–2.91)
HbE disease	08	0.43 (0.13–0.73)
α-thalassemia trait	02	0.11 (0.04–0.26)
HbD trait	03	0.16 (0.02–0.34)
NTD HbE/β-thalassemia	02	0.11 (0.04–0.26)
HPFH	03	0.16 (0.02–0.34)
Total carriers of mutations in one or both alleles of globin genes	223	11.89 (10.43–13.35)

However, 0.43% (8/1877) of the study participants had asymptomatic HbE-disease, 0.11% (2/1877) were identified to carry variants of alpha-thalassemia and 3 participants (0.16%) were HbD trait. Moreover, 3 with hereditary persistence of fetal hemoglobin, HPFH (0.16%) and two non-transfusion dependent HbE/β-thalassemia (0.11%) were found. Altogether, 11.89% (223/1877) of the study participants were carrier of mutations in one or more of the globin genes. The pie chart given bellow represents the complete spectrum of the carriers for thalassemia and other Hb gene mutations among Banglashi population found in the present study.



3.1.6 Mutation spectrum in the HBB gene of the study participants with thalassemia traits

Upon HRM curve analysis of the carriers of β thalassemia and HbE traits, seven types of mutation were identified in our study population by comparing with the reference samples of known mutations previously confirmed through Sanger sequencing which have been listed in **Table 3.7**. The most common mutations were CD 26/ HbE: c.79 G>A (73.42%) and IVS1_5 G> C or, c.92+5G>C (14.41%). The other mutations found in Bangladeshi population were c.126_129delCTTT (1.35%), c.47G>A (1.35%), c.27_28insG (0.9%), c.92+130G>C (0.45%), and a novel mutation c.151 A>G. Homozygous condition of c.79G>A+ c.79 G>A was found in 8 samples (3.61%) and c.79G>A + c.92+5G>C was found in 2 samples.

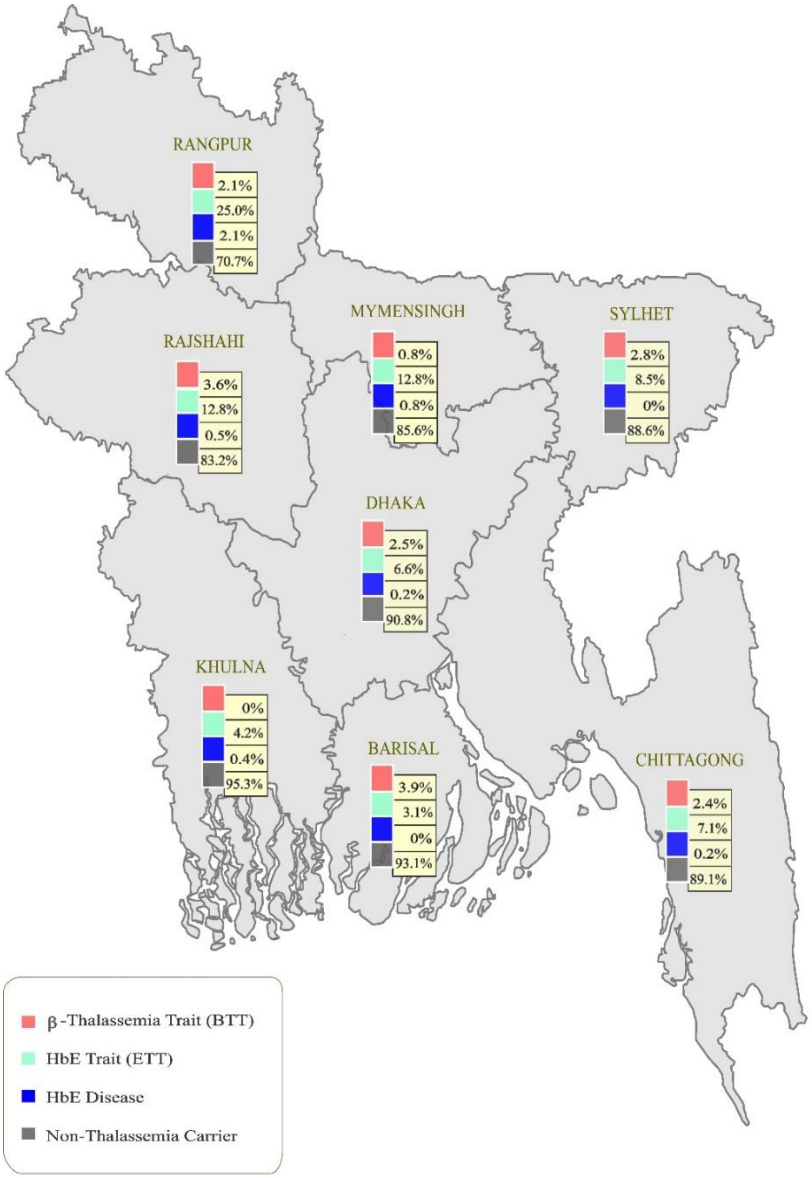
Table 3.7: Mutation spectrum of β-globin gene detected in the β thalassemia and HbE carriers in Bangladeshi population.

SL No.	Mutation Pattern	Number of samples, n (%; 95% CI)
1	c.79G>A (HbE)	163 (73.42; 67.62–79.22)
2	c.92+5G>C	32 (14.41; 9.8–19.02)
3	c.79G>A + c.79G>A	8 (3.61; 1.16–6.06)
4	c.92+130G>C	01 (0.45; 0.43–1.33)
5	c.151A>G *	01 (0.45; 0.43–1.33)
6	c.126_129delCTTT	03 (1.35; 0.16–2.86)
7	c.27_28insG	02 (0.90; 0.34–2.14)
8	c.47G>A	03 (1.35; 0.16–2.86)
9	c.79G>A + c.92+5G>C	02 (0.90; 0.34–2.14)

*novel mutation; not reported in Bangladeshi population and also globally (Patrinos et al. 2004).

3.1.7 Frequency of β thalassemia and HbE carriers in eight administrative divisions of Bangladesh

Our data suggests that carrier frequency for both the HbE and β thalassemia are significantly high in all the eight administrative divisions of Bangladesh. Although the situation is not uniform throughout the country, the most prevalent thalassemia trait found in almost all divisions except Sylhet was HbE trait (ETT) which varied from 3.1% (Barishal) to as high as 25% (Rangpur) across eight divisions of Bangladesh. The overall frequency of β-thalassemia trait (BTT) was 2.24% and it varied from 0% (Khulna) to 3.9% (Barishal) across different divisions. The highest frequency of thalassemia carrier (ETT+BTT) was found in Rangpur division (27.1%) followed by Rajshahi division (16.4%). Our study found a total of 8.68% (163/1877) HbE trait in Bangladesh comprising 7.1% (18/256) in Chittagong, 6.6% in Dhaka, 4.2% in Khulna, 12.8% in Mymensingh, 8.5% in Sylhet, and 12.8% in Rajshahi. In Chittagong, β-thalassemia trait was found in 2.4%, 2.5% in Dhaka, 0.8% in Mymensingh, 2.1% in Rangpur, and 3.6% in Rajshahi, with a total prevalence of 2.24% (42/1877) in all over the country (**Figure 3.2**).



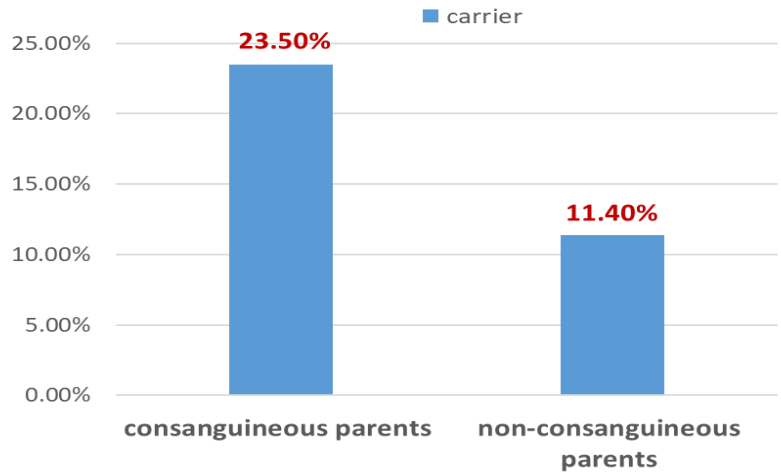
Divisions	BTT + HbE Trait
Rangpur	27.1 %
Rajshahi	16.4 %
Mymensing	13.6 %
Sylhet	11.3 %
Chittagong	9.5 %
Dhaka	9.1%
Barisal	7.0 %
Khulna	4.2 %
Average carrier rate	10.92%

Figure 3.2: Frequency of β thalassemia and HbE carriers in eight divisions of Bangladesh

3.1.8 Contribution of consanguinity to increase thalassemia carrier frequency in the country

There were 81 (4.32%; 95% CI, 3.4–5.24) participants of consanguineous parents. The carrier frequency among the participants with history of consanguinity was 23.5% (19/81), whereas it was almost half (11.4%, 204/1796) among the children of non-consanguineous parents (**Figure 3.3**). The highest consanguinity was observed in Rangpur division (8/140; 5.7%), which also had the highest carrier frequency among the eight administrative divisions of Bangladesh. The findings suggest that consanguinity contributes significantly to the increased rate of thalassemia in Bangladesh.

highest consanguinity was observed in **Rangpur** division- 5.7% (8/140),



.Figure 3.3: Frequency of thalassemia carriers among the participants from consanguineous and non-consanguineous parents

3.2 Study of Genetic modifiers affecting the disease severity in HbE/β-thalassemia patients of Bangladesh

This section describes the result of the part of the study which was conducted to determine the potential genetic modifiers responsible for the variable degree of disease severity of the HbE/β-thalassemia patients in Bangladesh. Although the underlying causes of extraordinary clinical heterogeneity of the disease are yet to be understood, scientists have been able to associate some genetic and environmental factors that may result in diverse clinical manifestations of HbE/β-thalassemia (Olivieri et al. 2011). In the current study, we have investigated the effect of primary genetic modifiers which include HbE level and co-inheritance of β-thalassemia mutation along with HbE as well as the secondary modifiers including-a) level of fetal hemoglobin, HbF, b) single nucleotide polymorphisms (SNPs) that are associated with increased production of HbF than normal percentage and c) co-inheritance of a determinant of α-thalassemia along with HbE/β-thalassemia.

3.2.1 Demographic and baseline characteristics of the different severity groups of the patients

Demographic information of the study participants has been elaborated in **Table 3.8**. The study enrolled a total of 130 HbE/β-thalassemia patients in the age range of 6 to 65 years of

age. Among the enrolled patients, there were 80 males and 50 females with mean age of 20.85 and 18.13 years, respectively. In addition, 50 healthy controls aged between 16 to 38 years were also enrolled in the study and all of them had normal haematological parameters. These patients were registered as HbE/β-thalassemia patients at the Thalassemia Samity Hospital in Dhaka city and came to visit the hospital for follow-up examinations, blood transfusion and iron chelation therapy. Further confirmation of the disease was confirmed by blood hemoglobin electrophoresis before enrolment of the patients for the study.

Table 3.8: Demographic data of the study.

Parameters		**Healthy controls**	**HbE/β-thalassemia Patients**
Number of participants, N		50	130
Age (Years)	Range	16 to 38	6 to 65
	Mean ± SD	21.63 ± 3.37	19.66 ± 10.22
Male	Numbers (Percentage)	27 (54%)	80 (61.5%)
	Mean ± SD (Age)	21.8 ± 3.2	20.85 ± 11.27
Female	Numbers (percentage)	23 (46%)	50(38.5%)
	Mean ± SD (Age)	21.1 ± 1.9	18.13 ± 8.53

Since HbE/β-thalassemia patients are clinically heterogeneous, baseline information has been provided for different groups of patients in **Table 3.9**. As per Mahidol severity criteria, the patients were classified as (1) mild with ≤ 3 severity score (N=39 including 23 (59%) males and 16 (41%) females, Age: 24.38±11.83 years), (2) moderately severe with 4-7 score (N=35 including 21(60%) males and 14 (40%) females, Age:17.30 ± 9.94 years), and (3) severe with>7 score (N=56 including 36 (64%) males and 20 (36%) females, Age: 17.61 ± 7.49 years). These 3 clinically heterogeneous groups of HbE/β-thalassemia patients differed in ages (months) of First Transfusion which were 171.90 ± 119.21, 57.90 ± 44.21, and 22.04 ± 21.14 months for mildly severe, moderately severe and severe groups. They had also different transfusion intervals (days) including 125.37 ± 90.40, 38.27 ± 17.87, and 21.16 ± 8.44 days for mild, moderate, and severe groups. Thus, First blood Transfusion and transfusion interval data together show that clinically less severe patients need First blood transfusion in earlier age of life than the more severe patients and also the former group of patients had higher transfusion intervals than the latter groups of patients.

Table 3.9: Groups of enrolled patients based on phenotypic severity classified according to Mahidol severity score* for HbE/β-thalassemia patients and baseline information of the different groups of patients.

Severity Groups	Mild / Non-transfusion dependent (score ≤ 3)	Moderate (score= 4-7)	Severe (score > 7)
Number of Patients, n (%); N= 130	39 (30%)	35 (26.92%)	56 (43.08%)
Male, n (%)	23 (59%)	21 (60%)	36 (64%)
Female, n (%)	16 (41%)	14 (40%)	20 (36%)
Age (Years) Mean ± SD	24.38 ± 11.83	17.30 ± 9.94	17.61 ± 7.49
Age of 1st Transfusion (Months), Mean ± SD	171.90 ± 119.21	57.90 ± 44.21	22.04 ± 21.14
Transfusion Interval (Days), Mean ± SD	125.37 ± 90.40	38.27 ± 17.87	21.16 ± 8.44
Splenomegaly	28.2% (11/39)	34.28% (12/35)	41.07% (23/56)
Splenectomized	2.5% (1/39)	11.4% (4/35)	17.8% (10/56)

* Mahidol severity score includes 6 phenotypic clinical criteria, ranged between 0 (asymptomatic) to 10 (most severe).

In terms of splenomegaly and splenectomy, the percentages (%) of patients with splenomegaly for mildly severe, moderately severe, and severe groups were 28.2% (11/29), 34.28% (12/35), and 41.07% (23/56), whereas the % of patients with splenectomy for these 3 groups were 2.5% (1/39), 11.4% (4/35), and 17.8% (10/56), respectively.

3.2.2 Hematological parameters and the Hemoglobin variants of the study participants

The Hemoglobin Variants of severe patients, moderately severe patients and the mild/NTD patients were measured in percentages in Hemoglobin Electrophoresis and then the concentrations were deduced from their Hemoglobin concentration levels. We compared the levels of different haemoglobin variants including HbA, HbE, HbF and HbA2 along with total Hb, MCV, MCH, and RDW among 3 different severity groups. **Table 3.10** shows that HbE levels (g/dL) of mild/NTD, moderate, and severe patients were 2.88±1.47, 1.36±1.1, and

1.35±0.97 with a significant p-value of <0.0001; indicating HbE could render protection to patients with HbE /β thalassemia through oxygen transport in the tissue.

Table 3.10: Comparison of Hematological Parameters and Hemoglobin Variants among different severity groups of HbE/β-thalassemia patients in Bangladesh

Parameters	**NTD / Mild** N=39	**Moderate** N=35	**Severe** N=56	**ANOVA Test (*p value*)**	**Healthy controls** N=50
Total Hb (g/dl)	7.78±1.23	7.08±1.44	6.92±1.03	0.0238	13.87±2.27
RDW (%)	28.05±8.5	24.24±8.87	22.97±7.59	0.0124	13.95±3.18
MCV(fL)	64.11±7.24	69.46±6.85	73.26±3.82	0.0001	81.72±4.57
MCH(pg)	20.21±2.9	21.99±3.08	23.28±2.27	0.0013	26.59±2.20
Hb A (g/dL)	2.27±2.81	3.89±2.42	5.33±2.61	<0.0001	12.97±3.15
HbE (g/dL)	2.88±1.47	1.36±1.1	1.35±0.97	<0.0001	0
Hb F (g/dL)	2.03±2.05	0.84±1.27	0.58±0.78	<0.0001	0.15 ± 0.08
Hb A2 (g/dL)	0.33±0.1	0.27±0.1	0.23±0.07	0.0106	0.45 ± 0.46

*Hb, Hemoglobin; MCV, Mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; NTD, Non-transfusion dependent.
**results have been shown as mean ± SD

Similarly, HbF level (g/dL) was highest (2.03±2.05) in the mild patients and lowest (0.58±0.78) in the severe patients and in the moderate patients it was in-between the levels of mild and moderate patients (0.58±0.78). Like HbE, ANOVA test demonstrated a significant p-value of <0.0001 for the differences in HbF among the 3 groups, indicating a protective role of HbF in thalassemia. In case of regularly transfused patients, the actual levels of MCV, MCH, RDW, and total Hb, HbA, and HbA2 levels of the patients are affected by transfused blood from healthy donors and therefore, these are not the parameters of consideration for this study. Only HbE and HbF levels are exclusively of patient origin as HbE is produced only in the patients with HbE/β-thalassemia without exception. Older children/adults rarely produce significant levels of HbF indicating that the healthy donors cannot be the source of HbE and HbF in the patients with HbE/β-thalassemia. However, among the RBC indices RDW (%) was highest in the mildly sever/NTD groups with the mean value of 28.05±8.5 implies the possible role of RDW on reducing the clinical phenotype in HbE/β- thalassemia.

Figure 3.4 demonstrates the result of the comparative ANOVA test performed for these parameters across the patient categories

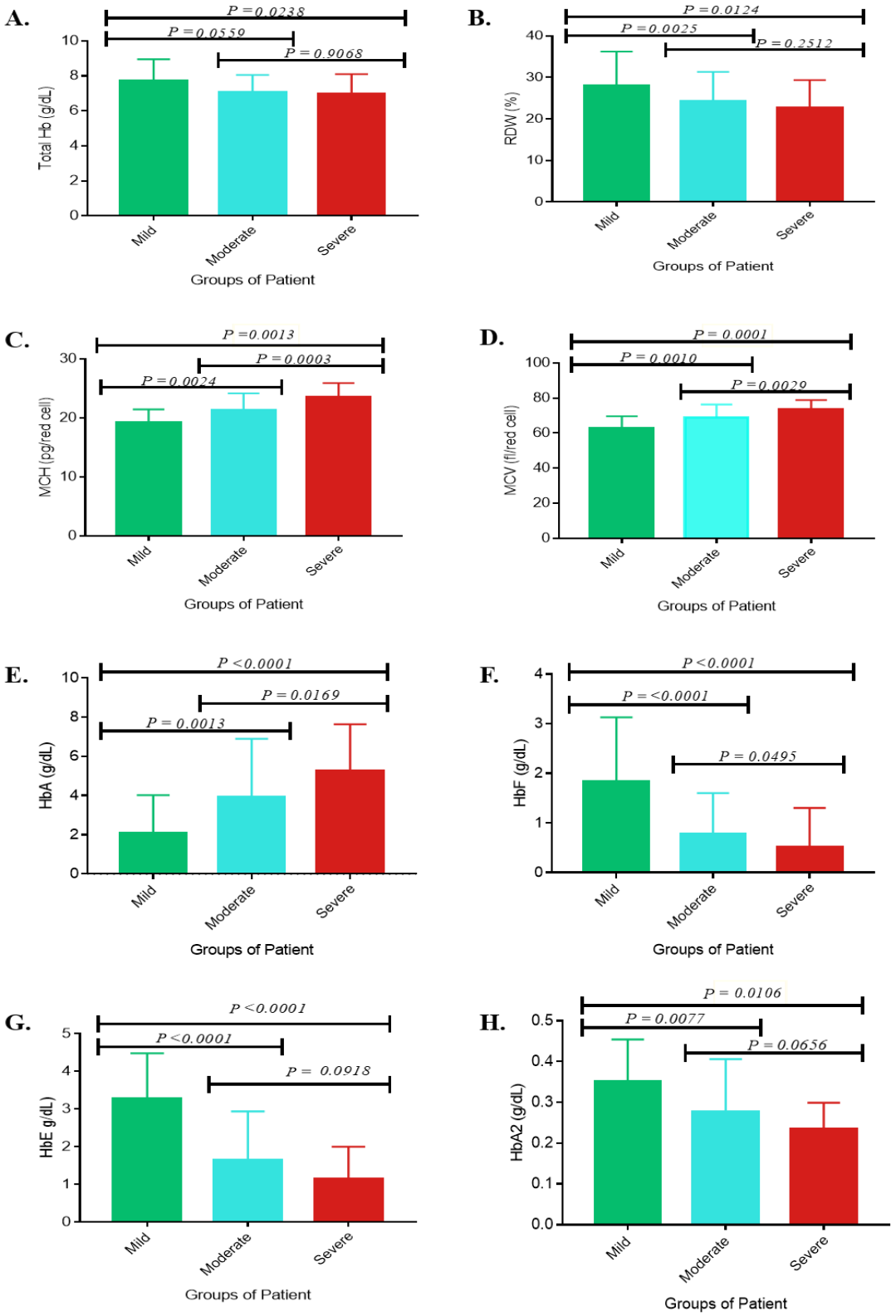


Figure 3.4: *ANOVA* and *t - test* Results for Hematological Parameters (section A to D) and Hemoglobin variants (E to H) across the different severity groups of patients with HbE/β-thalassemia.

Firstly, all the hematological parameters resulted in a significant ANOVA tests among the patient categories (p <0.05). However, t-test between mild and moderate, and moderate and severe groups of patients exhibited both significant and non-significant results. For MCV, a *P* value of 0.0010 was found when the data were compared between the mild and moderate groups; while the moderate and severe group showed a significant *p-value of* 0.0029. ANOVA tests among the patient categories displayed significant values for MCH (p <0.001), RDW (p = 0.0124) and total Hb level (p = 0.0238). Similar to obtained values of t- test of MCV, in case of MCH, p values were significant between mild and moderate (p = 0.0024) and between moderate and severe groups (p = 0.0003). For RDW, t–test between mild and moderate displayed a significant p value of 0.0025 while the case was not the same between severe and moderate groups (P = 0.2512). Lastly, none of the t – tests exhibited significant values for the total hemoglobin level, (P = 0.0559 and P = 0.9098) and this finding should be accepted as usual because at least the moderately severe and severe patients had circulation of donor blood.

Similarly, the comparison of Mean ± SD of hemoglobin variants in these three groups (NTD/Mild, Moderate & Severe) was done by ANOVA and T-test and the results were shown in **Figure 3.4**. There were statistically significant differences in HbA, HbA2, HbF and HbE levels while compared among all the three groups (ANOVA: P = <0.0001, 0.0106, <0.0001 and <0.0001 respectively). But no significant differences were found while compared individually between moderate and severe groups for HbA2 and HbE. However, t-test of all the variants were statistically significant between the patients of NTD/mild and the moderate group.

3.2.3 Correlation study of fetal hemoglobin (HbF) level with the disease severity in the patients with HbE/β-thalassemia

To study the correlation of fetal hemoglobin (HbF) concentration and disease severity among the HbE/β-thalassemia patients, *Pearson Correlation* test was conducted for HbF level against three conditions- 1) severity score, 2) transfusion interval and 3) age of first blood transfusion. **Figure 3.5** shows that there was highly significant positive correlation of HbF concentration with both age of first blood transfusion (months) and transfusion interval (days). Pearson's correlation coefficient analysis exhibited p <0.001 for both cases. However, for HbF versus AFT correlation study, Pearson r = 0.51 and for HbF versus TI correlation

study, r = 0.56. On the other hand, concentration of HbF displayed a strong negative correlation with the clinical score of the studied patients with a *p* value of <0.0001 and *r* value of 0.55.

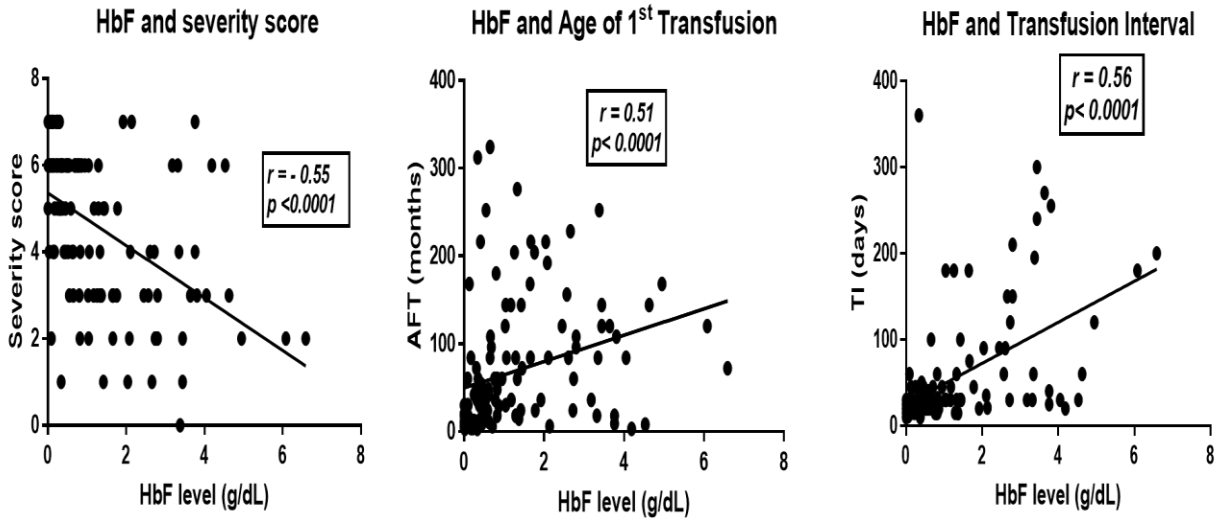


Figure 3.5: Pearson correlation analysis between fetal hemoglobin (HbF) level and disease severity in patients with HbE/β-thalassemia in terms of severity score, age of first transfusion and transfusion intervals.

3.2.4 Role of hemoglobin E (HbE) level in the disease manifestation of the HbE/β-thalassemia patients

Our next study aimed to find out the correlation of HbE levels (g/dL) with modifications of disease manifestations of HbE/β-thalassemia in terms of severity score (mild, moderate, and severe), age of first blood transfusion (months), and blood transfusion interval (days). To achieve the goal, Pearson Correlation Test was performed.

Figure 3.6 demonstrates that HbE had moderately positive correlations with age of first transfusion (AFT, middle panel) and transfusion interval (TI, right-hand panel)) with r= 0.28 and p-value of 0.0013 for the former and r=0.29 and p=0.001 for the latter, indicating that higher HbE levels has moderate level of positive impact to ameliorate disease conditions. That is, higher the HbE levels, the higher is the AFT/TI. On the other hand, HbE concentration (g/dL) was negatively correlated with severity score of HbE/β-thalassemia disease with r=-3.24 and p-value= 0.0045. That is, the higher is the concentration of HbE, the higher is severity score.

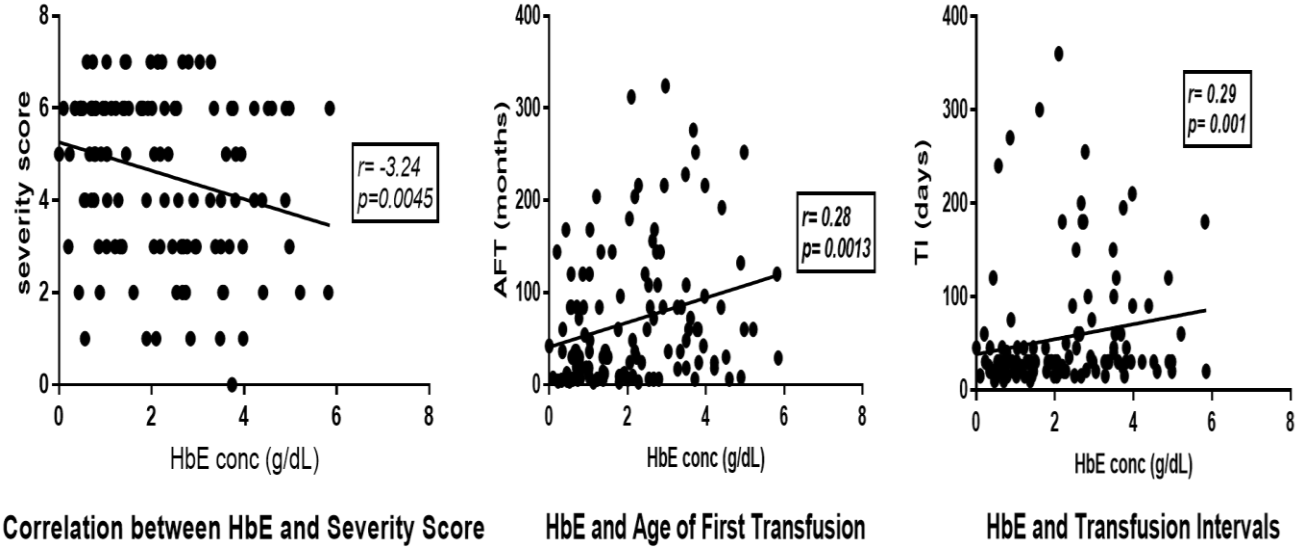


Figure 3.6: Disease modifying effect of hemoglobin E (HbE) in patients with HbE/β-thalassemia. HbE level (g/dl) showed significant correlation with clinical score, Transfusion Interval (Days) and Age of first blood Transfusion (Months) in the Pearson correlation test.

3.2.5 Study of causative mutations in the β- globin gene allele trans to HbE among HbE/β-thalassemia patients in Bangladesh

A total of 11 different mutations were detected in the the β- globin gene allele trans to HbE among HbE/β-thalassemia patients in Bangladesh. **Table 3.11** shows that IVS1_5 G>C was the most common β- globin gene mutation which occur with c.79 G>A mutation in the HbE allele and thus c.79 G>A + IVS1_5 G>C combination could cover 73.8% of HbE/β-thalassemia. Additional β- globin gene mutations/combination of mutations included c.27_28 ins G, c.126_129delCTTT, c.3G>T, c.51delC + c.33C>A, c.92 G>C, 126delC, IVS1_1 G>A, IVS1_130(G>C), c.46delT, and c.47(G>A) with frequencies of 1.5%, 5.4%, 2.3%, 2.3%, 3.1%, 1.5%, 3.8%, 2.3%, 2.3%, and 1.5%, respectively to cause HbE/β-thalassemia provided that HbE allele had c.79 G>A mutation. The mutation patterns and corresponding distributions of patients among 3 different severity groups of HbE/β-thalassemia patients including mild/NTD, moderate and severe could not provide exclusive information to indicate whether certain mutation/combination of mutations in the β- globin allele were responsible for distinct clinical severity. For example, with c.79 G>A + IVS1_5 G>C pattern, there were 23 mild, 24 moderate, and 49 severe patients, whereas with c.79 G>A + c.27_28insG pattern we saw 1 patient in the mild group, 1 in the moderate group and none in the severe group. On

the other hand, with c.79 G>A + c.126_129delCTTT mutations, the ratio of mild to moderate to severe patients were 3:3:1. Similarly, with other mutation combinations to cause HbE/β-thalassemia, we could not exclusively say that certain mutation/mutations in the β- globin allele would cause certain type of clinical severity except c. 79 G>A + c.3G>T and c.79G>A + c.51delC + c.33C>A genotypes, for which all the patients were in mild group. However, still we could not say that c. 79 G>A + c.3G>T and c.79G>A + c.51delC + c.33C>A genotypes were responsible for mild clinical manifestations of the patients because the sample size was very low (N=3 for each group).

Table 3.11: Causative mutations in the β- globin gene allele Trans to HbE among HbE/β-thalassemia patients in Bangladesh.

β- genotype		**% (n); N=130**	**Phenotypic severity**		
			Mild/NTD (n); N= 39	**Moderate (n); N=35**	**Severe (n); N=56**
1	c.79 G>A + IVS1_5 G>C	73.8 (96)	23	24	49
2	c.79 G>A + c.27_28 ins G	1.5 (2)	1	1	0
3	c.79 G>A + c.126_129delCTTT	5.4 (7)	3	3	1
4	c. 79(G>A) + c.3G>T	2.3 (3)	3	0	0
5	c.79G>A + c.51delC + c.33C>A	2.3 (3)	3	0	0
6	c.79(G>A) + c.92 G>C	3.1 (4)	2	1	1
7	c.79(G>A) + 126delC	1.5 (2)	1	1	0
8	c79(G>A) + IVS1_1 G>A	3.8 (5)	3	0	2
9	c.79 (G>A) + IVS1_130(G>C)	2.3 (3)	0	2	1
10	c.79(G>A) + c.46delT	2.3 (3)	0	2	1
11	c79(G>A) + c.47(G>A)	1.5 (2)	0	1	1

3.2.6 Study of single nucleotide polymorphisms (SNPs) that are associated with increased production of HbF

Several studies provide evidence that polymorphisms associated with increased synthesis of fetal hemoglobin, such as- the presence of the *Xmn1* polymorphism, single-nucleotide polymorphisms in the gene *BCL11A*, and other genetic loci like HBS1L-MYB, HBBP1 etc. may be associated with a milder clinical phenotype in HbE/β-thalassemia. A genome-wide

association study conducted in 618 patients with HbE/β-thalassemia analyzed 23 SNPs in three independent genes/regions identified as being significantly associated with disease severity (Nuinoon et al. 2010). The strongest association was observed with SNPs in the *HBG2* and *HBBP1* gene in the HBB locus (β globin gene cluster, chr.11p15) followed by the association with SNPs identified in the intergenic region between the *HBS1L* and *MYB* genes (chr.6q23) and the third potentially important region was located in the BCL11A gene. These findings have been replicated in a cohort of Indonesian patients (Rujito et al. 2016) suggesting that several genetic loci act in concert to influence fetal hemoglobin levels of HbE/β-thalassemia patients, with three reported loci.

Based on these information, we have targeted the following 8 SNPs in four different genes to detect the potential genetic modifiers of the disease severity among HbE/β-thalassemia patients in Bangladesh. The basic information of the SNPs has been presented in **Table 3.12**.

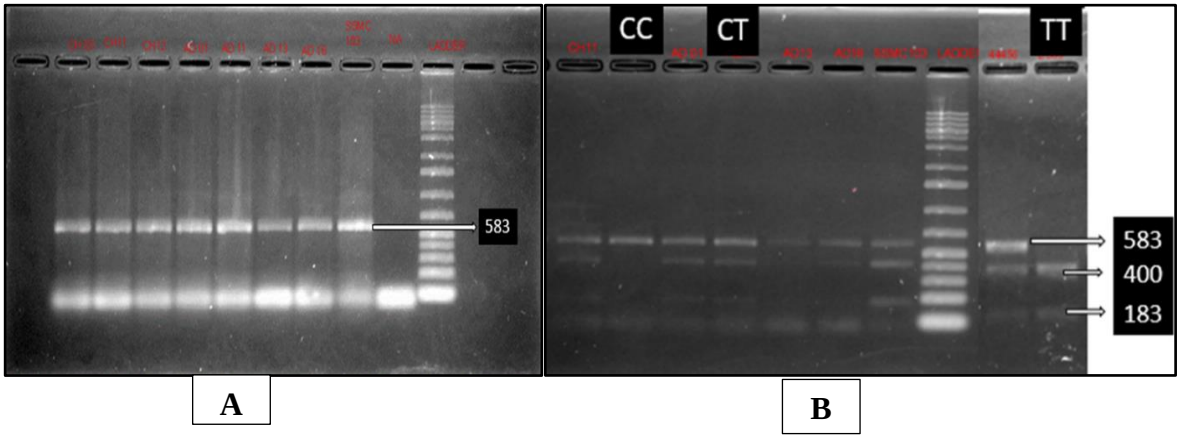
Table 3.12: The basic information of the 8 SNPs of our study.

Modifier gene	SNPs	Chromosomal Location	Function	Mutation
HBG2	rs7482144 (Xmn1)	Chr11: 5,276,169	Promoter	C>T
HBS1L-MYB	rs28384513	Chr6: 135,376,209	Intergenic region	T>G
	rs4895441	Chr6: 135,426,573	Intergenic region	A>G
	rs28384512	Chr6: 135,376,232	Intergenic region	T>C
BCL11A	rs11886868	Chr2: 60,493,111	intron	C>T
	rs4671393	Chr2: 60,493,816	intron	G>A
	rs766432	Chr2: 60,720,951	intron	A>C
HBBP1	rs2071348	Chr11:5,242,916	pseudogene	A>C

3.2.6.1 Detection of -158 Xmn1-Gγ (C>T) polymorphism in the promoter region of gamma globin gene (HBG2) in the HBB locus

A number of studies on various populations suggests that *XmnI* polymorphism is an important key regulator of disease severity, which increases fetal hemoglobin (HbF) level in thalassemia patients thereby reducing severity of the disease. This study aimed to determine the frequency of XmnI polymorphism among HbE/β-thalassemia patients in Bangladesh and to examine its

relationship with genotypes and variable clinical phenotypes of HbE/β-thalassemia. PCR-RFLP method was used to detect the XmnI C→T polymorphism at -158th position of $^{G}\gamma$-globin gene promoter. The PCR amplification products and digested product by Xmn1 restriction enzyme to detect the presence of Xmn1 polymorphism in $^{G}\gamma$ gene are shown in **Figure 3.7**.



C XmnI genotype	Cutting pattern	Length (bp)
CC (-/-) (Wild type)	Uncut (single bands)	583
CT (-/+) (Heterogygous)	Cut (triple bands)	583, 400, 183
TT (Varient homogygous)	Cut (double bands)	400, 183

Figure 3.7: Agarose gel electrophoresis of A) PCR amplified DNA using specific primer, B) After digestion with XmnI for detection of the Gγ-globin promoter -158 (C>T) polymorphism alleles in E/β -thalassemia patients, C) XmnI restriction digestion pattern.

Frequency Distribution of -158 Xmn1-Gγ (C>T) polymorphism (rs7482144) among the healthy controls and the HbE/β– thalassemia Patients in Bangladesh

The most common genotype of the Gγ Xmn1 polymorphism observed in the Bangladeshi population was the presence of both the Xmn I (+) and (-) sites that is the heterozygous allele-CT and this was confirmed at a frequency of 77.67% and 52% in the HbE/β– thalassemia patients and the healthy controls respectively (**Table 3.13**). Among the patients, homozygosity for the Xmn I (-/-) genotype was observed in 16.18% while the presence of the Xmn I cleavage site in both chromosomes (+/+) was observed in only 6.15% of the patients

which was found in higher frequency (18%) among the healthy participants. Of the 260 alleles studied in the thalassemia patients of Bangladesh, 101+16=117 possessed the Xmn I restriction site (T allele) while 42+101= 143 did not, thus, the ratio of the (+) allele to the (-) allele was 0.81. Although the frequency of CC and TT genotype was found to be higher and that of CT was lower among the healthy population compared to the patient groups, the ratio of the (+) allele to the (–) allele is nearly similar observed at 0.78.

Table 3.13 also shows that, in the homozygous Xmn1 genotype CC (-/-) group, there were 5.13% (2/39) mild, 14.28% (5/35) moderate, and 25% (14/56) severe HbE/β-thalassemia patients which was significantly different from the frequency distributions of heterozygous Xmn1 genotype CT (-/+) and homozygous genotype TT (+/+) among mild, moderate, and severe HbE/β – thalassemia patients . Overall, the data suggests that CT (-/+) heterozygosity is the most common Xmn1 genotype among Bangladeshi population and it is found in noticeable numbers among all severity groups of patients. Nevertheless, T allele is significantly associated with the milder severity of the disease (OR= 4.88, 95% CI= 1.1 to 22.1, p= 0.019).

Table 3.13: Frequency of G^{γ} Xmn 1 polymorphism in the study population.

Xmn1 genotypes	Healthy controls, % (n)	All patients. % (n)	Frequency in the HbE/β – thalassemia Patients, % (n)				
			Mild/ NTD	Moderate	Severe	Fisher Exact test, *p*	OR (95% CI); p value
CC (-/-)	30.00 (15/50)	16.18 (21/130)	5.13 (2/39)	14.28 (5/35)	25.0 (14/56)	*0.032*	Reference
CT (-/+)	52.00 (26/50)	77.67 (101/130)	82.05 (32/39)	80.00 (28/35)	73.22 (41/56)		4.88 (1.1 - 22.1), *P= 0.019*
TT (+/+)	18.00 (09/50)	6.15 (08/130)	12.82 (5/39)	5.71 (2/35)	1.78 (1/56)		

Correlation of Xmn1 polymorphism with age of first blood transfusion and transfusion intervals

Table 3.14 depicted that the average (±SD) age of first blood transfusion for CC (-/-) and CT (-/+) were 23.27 ± 36.93 months and 50.50 ± 46.99 months, respectively. The average (±SD) intervals of blood transfusion were 31.12 ± 18.53 days and 50.88 ± 44.98 for CC (-/-) and CT (-/+) genotype, respectively. In case of TT (+/+) genotype, both the age of first blood transfusion (67.2 ± 52.20) and the transfusion intervals (77.5 ± 69.46) are much higher than

both CC and CT. These data clearly demonstrate that the patients with CC (-/-) genotype are more severe than the CT (-/+) and TT (+/+) genotypes in terms of 1st transfusion and blood transfusion intervals. Out of 130 patients, XmnI polymorphism was absent (- / -) in 21 patients (16.18), where 109 patients (83.82%) showed polymorphism either in one loci or both loci (Table 3.13). Age of first blood transfusion was significantly higher (p = 0.0012) in patients who had Xmn1 polymorphism in comparison with the patient group who had no polymorphism. Even though the mean value (±SD) of transfusion interval demonstrates that the participants with CT (-/+) and TT (+/+) genotypes had less disease severity, there is marginally statistical significant difference found in transfusion interval (p = 0.056).

Table 3.14: Comparison of age of first blood transfusion and Blood transfusion interval among the different Xmn1 genotype groups.

Xmn1 genotype	Age of first blood transfusion (months) (Mean ± SD)	Blood transfusion interval (days) (Mean ± SD)
CC (-/-)	23.27 ± 36.93	31.12 ± 18.53
CT (-/+)	50.50 ± 46.99	50.88 ± 44.98
TT (+/+)	67.2 ± 52.23	77.5 ± 69.46
T test (CC vs CT+TT)	p = 0.0012	p = 0.056

Correlation of the Xmn1 polymorphism with the Clinical Severity and HbF level in the HbE/β – thalassemia Patients

To observe the differences of HbF concentration along with clinical scores among the three identified genotypes of *Xmn1 polymorphism*, ANOVA– tests were performed. **Figure 3.8** depicted the significant differences in HbF level among the patients with CC, CT and TT genotypes. The mean concentration of HbF was 0.49± 0.37g/dL for the CC genotype inherited patients while it was 1.35 ± 1.07 for the patients who inherited CT genotype. TT genotyped group showed the highest HbF level with the mean value of 1.84± 1.07.

On the other hand, the clinical score was highest in the patient group with homozygous genotype CC compared to the heterogenic genotype CT and homozygous mutant genotype TT imposing less severity in the patients having polymorphic allele T. The clinical scores of the three genotypes were compared and a significant difference was obtained (p = 0.009) in the ANOVA test which was illustrated in the **Figure 3.8**.

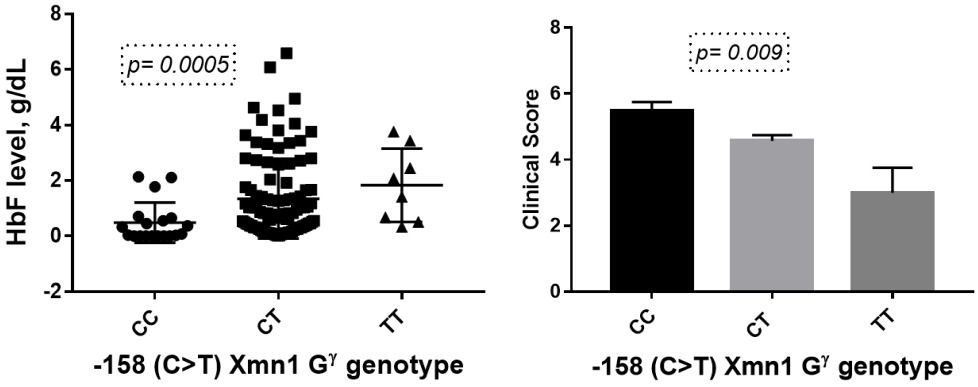


Figure 3.8: Comparison of fetal hemoglobin level and the clinical severity score among HbE/β-Thalassemia patients with different Xmn1 genotype.

3.2.6.2 Study of three SNPs- rs28384513, rs28384512 & rs4895441 in HBS1L-MYB intergenic region

Several GWAS have identified a cluster of common variants in the interval between HBS1L and MYB that modulate a broad spectrum of hematological traits, in particular erythroid phenotypes, suggesting that this locus may have a key role in the regulation of erythropoiesis. Here we have studied the 3 SNPs of HBS1L-MYB intergenic region-- rs28384513, rs28384512 & rs4895441, which were previously reported as HbF inducer in the Asian patients with β-thalassemia and sickle cell anemia.

Detection of HBS1L-MYB polymorphisms (rs28384513, rs28384512 & rs4895441) among the study population

As PCR-RFLP is a lengthy time consuming method requiring a number of steps which includes conventional PCR followed by gel run and PCR product purification, then Restriction digestion of the PCR product and finally detection of the restriction fragments using Gel electrophoresis, here we used Real-time PCR followed by HRM curve analysis method to detect the above mentioned *HBS1L-MYB* SNPs in the healthy controls and the patients of HbE/β-Thalassemia. Firstly, a certain number of samples were randomly selected to get the reference samples. Conventional PCR was done using newly designed 3 pairs of primer sets for sequencing the regions of the 3 SNPs. Agarose Gel Electrophoresis was done to amplify and detect the specific PCR products and Sanger sequencing was done to identify

the polymorphic alleles. **Figure 3.9** shows the Sanger chromatogram of the 3 SNPs of HBS1L-MYB in the study population.

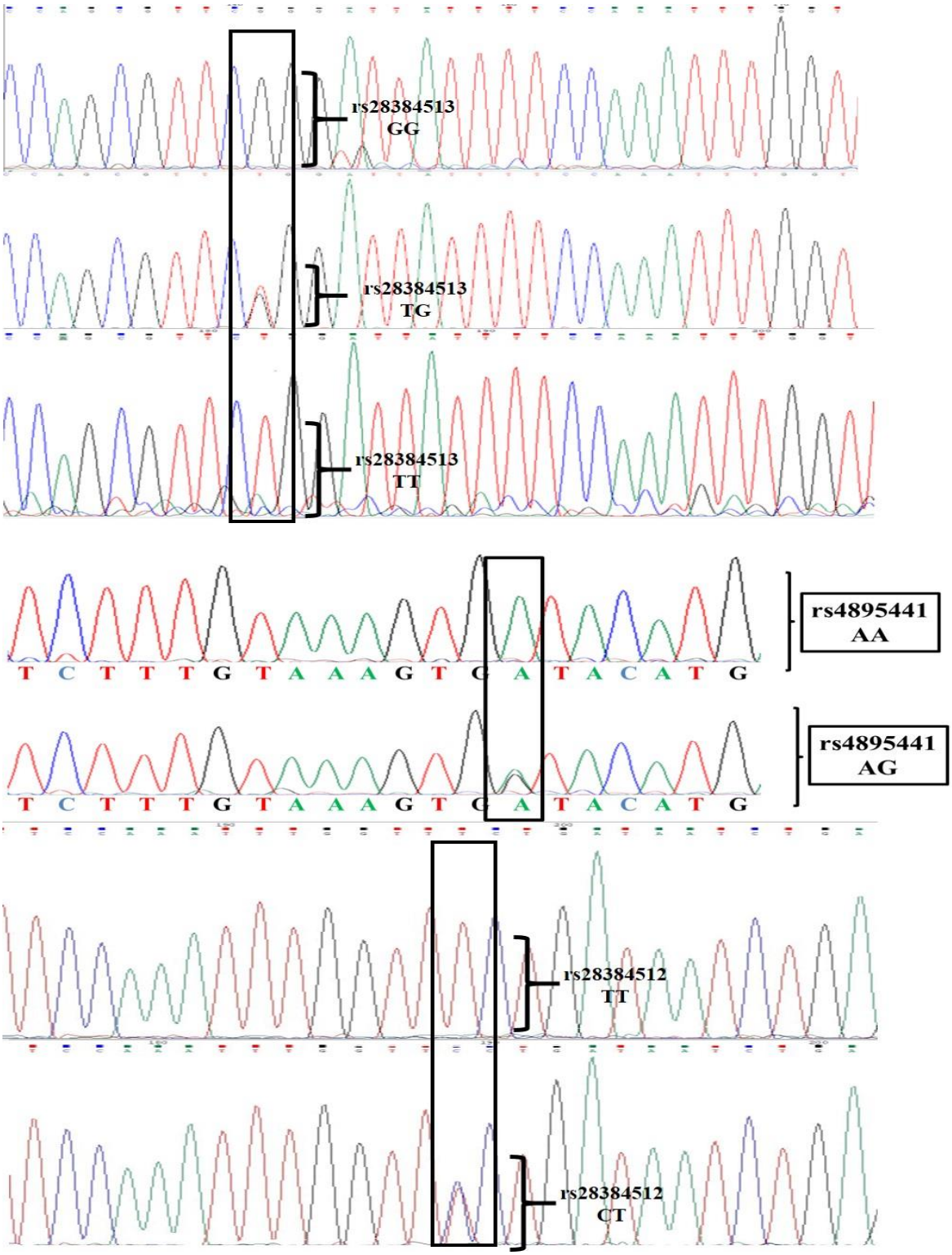
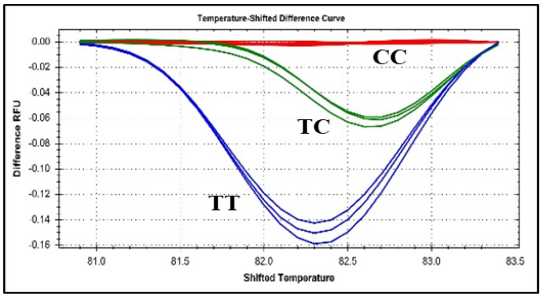
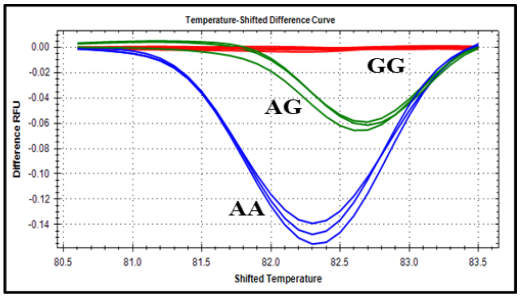


Figure 3.9: Chromatogram showing Sanger Nucleotide Sequencing to prepare Reference Samples for HBS1L-MYB SNPs (rs28384513, rs28384512 & rs4895441) among the study population.

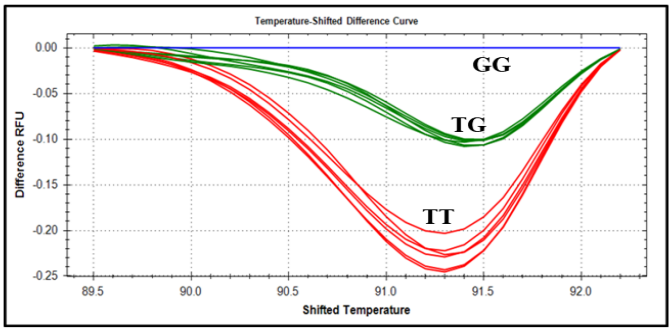
In this study, major allele and minor allele had been detected in all the three status for all the 3 SNPs of HBS1L-MYB in both the healthy controls and the HbE/β-Thalassemia patients. For the rs28384513, homozygous major allele was found to be 'TT', heterozygous 'TG' and homozygous mutant allele was 'GG'. In case of SNP rs4895441 of HBS1L-MYB, homozygous mutant allele 'GG', heterozygous 'AG' and homozygous major allele 'AA' had been found. Similarly, for rs28384512, homozygous mutant, heterozygous and homozygous major allele were 'CC', 'CT' and 'TT' respectively. Then the sequenced samples were used as reference samples with known genotypes and rest of the samples were tested for the presence of the 3 SNPs (rs28384512, rs28384513 & rs4895441) by Real-time PCR followed by High Resolution Melting (HRM) Curve Analysis among the study population (**Fig 3.10**).



***(A)** HRM curves for rs28384512(T>C)*



***(B)** HRM curves for rs4895441 (A>G)*



***(C)** HRM curves for rs28384513(T>G)*

Figure 3.10: Detection of three HBS1L-MYB SNPs (A) rs28384512, (B) rs4895441 and (C) rs28384513 through High Resolution Melt Curve (HRM) analysis

Frequency distribution of HBS1L-MYB polymorphisms (rs28384513, rs28384512 & rs4895441) among the study population

The major SNPs that describe HBS1L-MYB polymorphisms include rs28384513 (T>G), rs4895441 (A>G), and rs28384512 (T>C). As per **Table 3.15**, out of 50 healthy controls, highest number of participants (n=38) had rs4895441 (A>G) SNP having AA genotype followed by 34 participants having rs28384513 (T>G) SNP with TG+GG genotype, and 33

participants having rs28384512 (T>C) SNP with CT+CC genotype etc. Distribution frequencies of rs28384513 (T>G) SNPs for TT and TG+GG genotypes among mild, moderate, and severe patients with HbE/β-Thalassemia were 19.5% (8/39) and 79.5% (31/39), 20% (7/35) and 80% (28/35), and 32.15% (18/56) and 67.85% (38/56) respectively. On the other hand, the frequencies of rs4895441 (A>G) SNPs for AA and AG+GG genotypes among mild, moderate, and severe patients were 61.52% (24/39) and 39.48% (15/39), 68.57% (24/35) and 31.43% (11/35), and 89.28% (50/56) and 10.72% (6/56) respectively, where 98 (75.4%) patients had AA genotype and 32 patients had AG+GG genotype among a total of 130 patients. Finally, the frequencies of rs28384513 (T>C) SNPs for TT and CT+CC genotypes among mild, moderate, and severe patients were 35.9% (14/39) and 64.1% (25/39), 28.9% (9/35) and 71.4% (25/35), and 28.58% (16/56) and 71.42% (40/56) respectively, where 39 (30%) patients had TT genotype and 91(70%) patients had CT+CC genotype among a total of 130 patients. Significantly high frequency was found in mildly severe patients for 2 SNPs rs4895441 (OR= 2.72, p=0.009) and rs28384513 (OR= 1.86, p=0.06) while genotypes of only the first one shows significant variation among mild, moderate, and severe patients (p= 0.004).

Table 3.15: Frequency of HBS1L-MYB polymorphisms in the healthy controls and the patients with HbE/β-Thalassemia.

SNP ID	Genotype	Healthy controls, N=50 n, (%)	All patients N=130 n, (%)	Frequency in the HbE/β – thalassemia Patients, n (%)				
				Mild/NTD N=39	Moderate N=35	Severe N=56	χ^2 test, *p*	Odd Ratio (95%CI), *p* value
rs28384513 (T>G)	TT	16 (32%)	16 (32%)	08 (19.5%)	07 (20%)	18 (32.15%)	*0.124*	Reference
	TG+GG	34 (68%)	34 (68%)	31 (79.5%)	28 (80%)	38 (67.85%)		1.86 (0.84-4.13), *P = 0.06*
rs4895441 (A>G)	AA	38 (76%)	38 (76%)	24 (61.52%)	24 (68.57%)	50 (89.28%)	*0.004*	Reference
	AG+GG	12 (25%)	12 (25%)	15 (39.48%)	11 (31.43%)	06 (10.72%)		2.72 (1.18-6.26), *P* = 0.009
rs28384512 (T>C)	TT	17 (34%)	17 (34%)	14 (35.9%)	09 (28.9%)	16 (28.58%)	*0.64*	Reference
	CT+CC	33 (66%)	33 (66%)	25 (64.1%)	25 (71.4%)	40 (71.42%)		0.68 (0.31-1.53), *P* = 0.13

Association of SNPs in HBS1L-MYB with Disease Severity of HbE/β-Thalassemia patients

To investigate how the 3 SNPs of *HBS1L-MYB* intergenic regions affect the clinical heterogeneity in the HbE/β-Thalassemia patients of Bangladesh, we compared the severity score and the HbF level among the patients with different SNP genotypes.

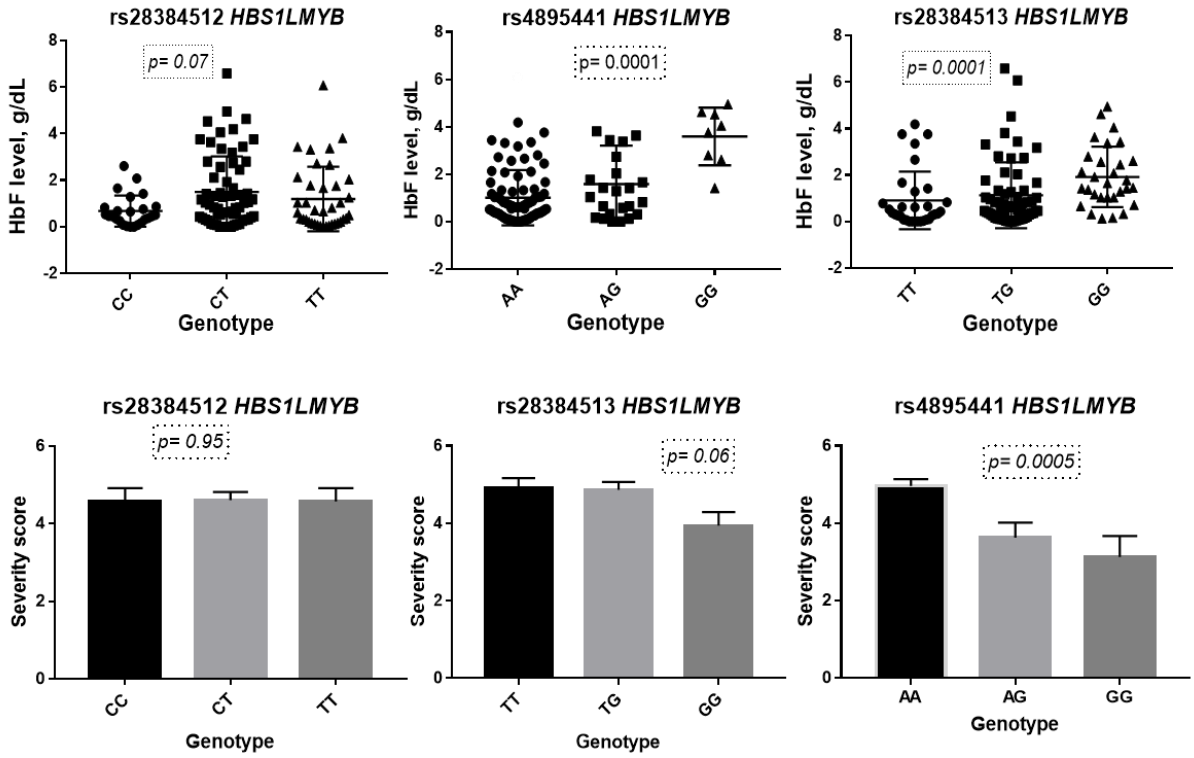


Figure 3.11: Comparison of HbF level (g/dL) and severity score through ANOVA -test among the homozygous major, heterozygous and homozygous mutant genotyped patients of rs28384512, rs4895441 & rs28384513 in the HBS1L-MYB intergenic region. P<0.5 is considered as significant result.

Among the 3 SNPs, rs4895441 & rs28384513 provided the signiant differences in the mean HbF concentrations among the different SNP genotypes with p value of 0.0001 showed in **Figure 3.11**. In case of rs-28384513, 'GG' alleles showed the highest HbF values as expected with mean ± SD of 1.92 ± 1.63 g/dl HbF while the mean HbF level in the patients with 'TG' and 'TT' alleles were 1.13g/dL and 0.93g/dL respectively. Similarly, in rs-4895441, both 'AA', 'AG' and 'GG' genotyped patients showed expected HbF level with mean values of 2.58, 1.58 and 1.01 g/dL respectively and thus statistically significant higher HbF was present among the patients having at least one copy polymorphic allele compared to the patients lacking the SNP allele. However, in case of rs28384512, though the average HbF

concentration was higher in the patients having one (CT, HbF: 1.5g/dL) or both polymorphic allele (TT,HbF: 1.2g/dL) compared to the patient groups with homozygous wild genotype CC (HbF: 0.98g/dL), these differences were not found to be statistically significant (p=0.07) in our study population.

To study the effect of the 3 *HBS1L-MYB* SNPs on reducing the clinical severity in the HbE/β – thalassemia patients of Bangladesh, the severity score of the patients were compared among the different SNP genotypes including homozygous and heterozygous version and the result has been illustrated in **Figure 3.11**. Although we could not find significant differences in severity score for rs28384512 SNP for the genotypes CC/CT/TT (p=0.95) and rs28384513 for the genotypes TT/TG/GG (p=0.06), significant reductions of severity scores with a p-value of 0.0005 were found for the genotypes AG and GG compared to AA for the HBS1L-MYB SNP rs4895441. Notably, when compared to AA genotype, most reduction of severity score was observed for GG genotype followed by AG genotype.

3.2.6.3 Determining the effect of SNPS in BCL11A gene on the clinical heterogeneity of the patients with HbE/β-thalassemia in Bangladesh

Transcription regulator BCL11A gene encodes a zinc-finger repressor protein of gamma globin gene, thus a major contributor to HbF and F cell variability. In several genome-wide association study, at least 12 SNPs of the BCL11A gene have been described to be associated with high level of HbF in patients with β-thalassemia. This study aimed to evaluate the disease modifying influence of 3 SNPs (rs11886868, rs766432 & rs4671393) in the BCL11A gene among HbE/β-thalassemia patients in Bangladeshi population.

Detection of BCL11A polymorphisms (rs11886868, rs766432 & rs4671393) among the study population

The same procedure described earlier for detection of HBS1L_MYB SNPs was followed to determine the 3 SNP targets in the BCL11A gene. Chromatogram of the respective sequences are shown in **Figure 3.12**. In this study, for rs766432 & rs4671393, only homozygous major allele (AA and GG respectively) and heterozygous mutant allele (AC and AG) found but no homozygous mutant allele (CC and AA respectively) had been found. However, in case of SNP rs-11886868 of BCL11A, major allele ‘C’ and minor allele ‘T’ had been detected in all

the three status i.e homozygous CC, Heterozygous CT and Homozygous TT in the study population (Figure 3.12).

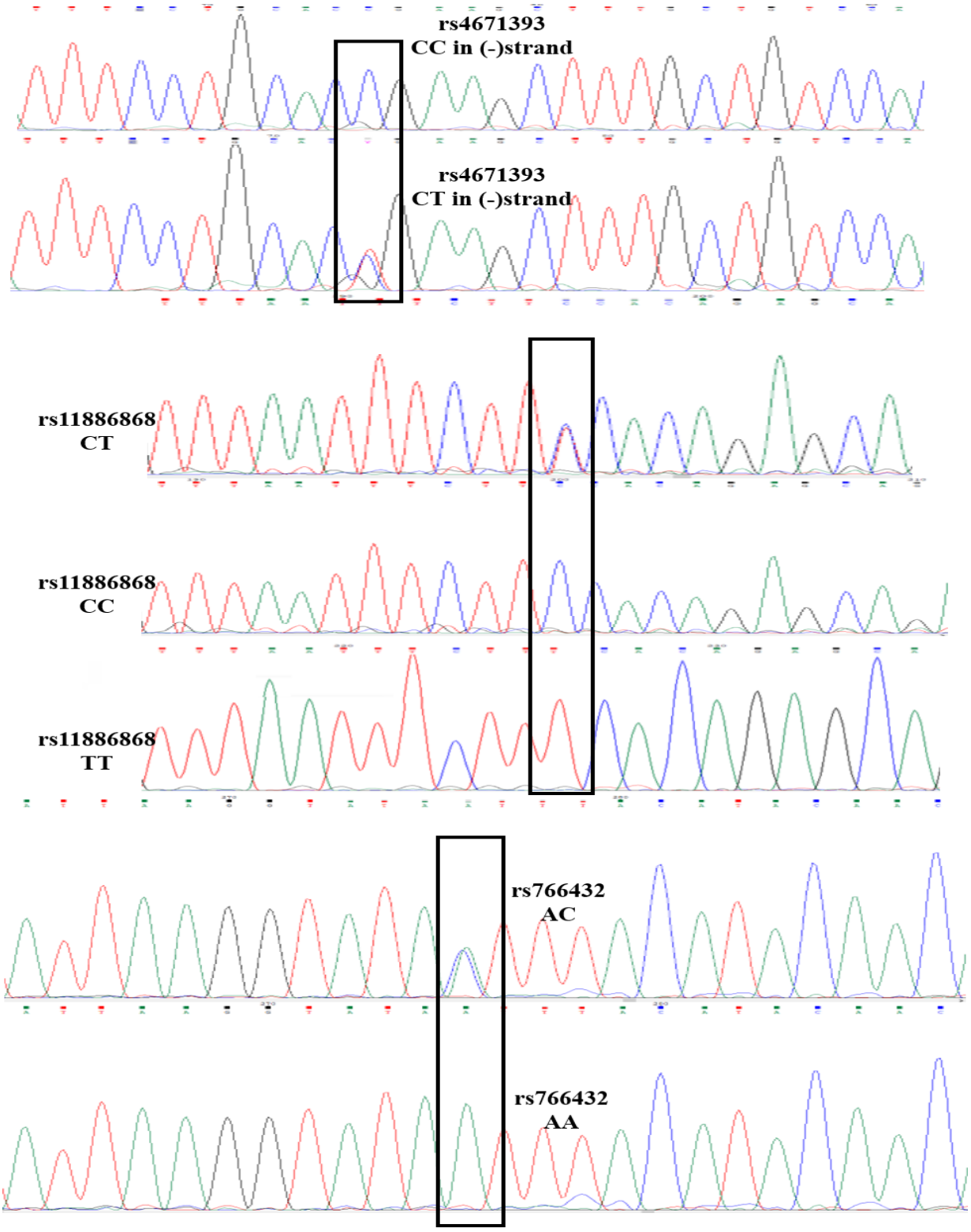
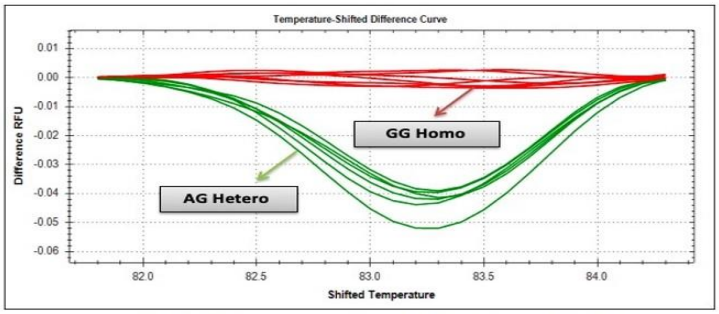
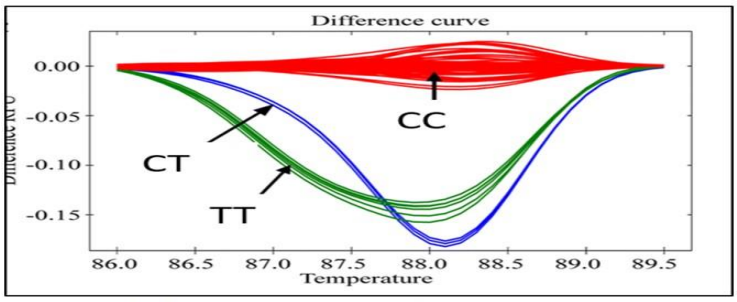


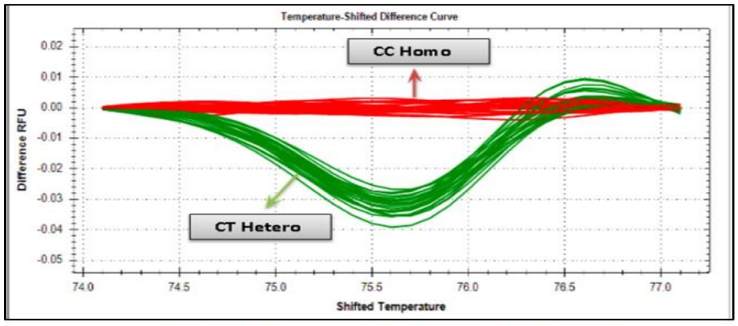
Figure 3.12: Chromatogram showing Sanger Nucleotide Sequencing results for three BCL11A SNPs (rs11886868, rs766432 & rs4671393) among the study population.



(A) HRM curves for BCL11A rs4671393



(B) HRM curves for BCL11A rs11886868



(C) HRM curves for BCL11A 766432

Figure 3.13: Detection of three BCL11A SNPs (A) rs4671393, (B) rs11886868 and (C) rs766432 through High Resolution Melt Curve (HRM) analysis

Secondly, the sequenced samples were used as reference samples with known genotypes and rest of the samples were tested for the presence of the three SNPs (rs11886868, rs766432 & rs4671393) by HRM Curve Analysis in both the healthy control and the patients of HbE/β-thalassemia. In HRM curve analysis, the temperature shifted curves showed the differences of melting temperatures in presence of the polymorphic alleles. Red curves in the **Figure 3.13**a showed homozygous 'GG' alleles and Green showed heterozygous AG' alleles of rs-4671393 SNP of BCL11A gene. **Figure 3.13b** showed the differences among melting temperatures of homozygous 'CC' allele (Red), heterozygous 'CT' allele (blue) and heterozygous 'TT' (green) and for SNP (rs-11886868) of BCL11A gene. Red curves in the **Figure 3.13**c showed homozygous 'CC' alleles and Green colored cluster is for the heterozygous 'AC' alleles of rs-766432 SNP of BCL11A gene.

Frequency distribution of three BCL11A SNPs (rs11886868, rs766432 & rs4671393) among the study population

The present study analysed the frequency of 3 SNPs including rs11886868, rs766432 & rs4671393 at BCL11A gene in the patients as well as in healthy control. As per **Table 3.16**, rs11886868 SNP with genotypes CC and TC + TT was distributed as 62% (31 out of 50) and 38% (n=19 out of 50), respectively, whereas distributions of these genotypes among mild, moderate, and severe patients were 43.58% (17 out of 39) vs 56.42 (22 out of 39), 54.28% (19 out of 35 vs 45.72% (16 out of 35), and 53.57% (30 out of 56) vs 46.43 (26 out of 56), respectively. On the other hand, frequency distributions of GG vs AG genotypes of rs4671393 SNP among healthy controls and mild, moderate, and severe patients were 66% (n=33) vs 34% (n=17), 74.35% (29) vs 25.65% (n=10), 85.7% (n=30) vs 14.3% (n=5), and 75% (n=42) and 25% (n=14), respectively. Finally, for AA vs AC genotypes of rs766432 (A>C) at BCL11 locus, there were 64% (n=32) vs 36% (n=18), 64.1% (n=25) vs 35.9% (n=14), 85.7% (n=30) vs 14.3 (n=05) , and 76.78% (n=43) vs 23.22% (n=13) among the healthy controls, and patients with mild, moderate, and severe groups, respectively.

Table 3.16: Genotype frequency of rs11886868, rs766432 & rs4671393 in BCL11A gene among the healthy controls and the patients with HbE/β-Thalassemia.

SNP ID	Genotype	Healthy controls, N=50 n, (%)	All patients N=130 n, (%)	Frequency in the HbE/β – thalassemia Patients, n (%)				
				Mild/NTD N=39	Moderate N=35	Severe N=56	χ^2 test, *p*	Odd Ratio (95%CI), *p* value
rs11886868	CC	31 (62%)	66 (48.46%)	17 (43.58%)	19 (54.28%)	30 (53.57%)	*0.56*	Reference
	TC+ TT	19 (38%)	64 (51.54%)	22 (56.42%)	16 (45.72 %)	26 (46.43%)		1.51 (0.71-3.2), *P = 0.14*
rs4671393	GG	33 (66%)	101 (77.7%)	29 (74.35%)	30 (85.7%)	42 (75%)	*0.409*	Reference
	AG	17 (34%)	29 (22.30%)	10 (25.65%)	05 (14.3%)	14 (25%)		1.31 (0.54-3.15), *P* = 0.2
rs766432	AA	32 (64%)	98 (75.38%)	25 (64.1%)	30 (85.7%)	43 (76.78%)	*0.09*	Reference
	AC	18 (36%)	32 (24.62%)	14 (35.9%)	05 (14.3%)	13 (23.22%)		2.27 (0.98-5.2), P = 0.026

However, no significant variation was found for any of the genotypes of 3 SNPs among mild, moderate, and severe patients (p> 0.05 in χ 2 test,) and only rs766432 showed significant association found with less severe group (OR= 2.27, 95% CI 0.98-5.2, P = 0.026).

Effect of SNPs in BCL11A on HbF level and Disease Severity of HbE/β-Thalassemia patients

Once we could know the frequency distributions of rs11886868, rs766432 & rs4671393 SNPs at the BCL11A gene locus, we wanted to investigate the effect of these SNPs in the induction of HbF levels, which in turn, was expected to influence the disease spectrum of patients with HbE/β-Thalassemia. As we can see in **Figure 3.14**, although HbF levels of the homozygous major genotypes of all the three SNPs, namely GG, CC, and AA of rs11886868, rs766432 & rs4671393 SNPs at the BCL11 locus were higher than their respective mutant counterpart genotypes, namely AG, CT+TT, and CA, respectively, the results were not statistically significant (p-values were 0.461, 0.221, and 0.563, respectively).

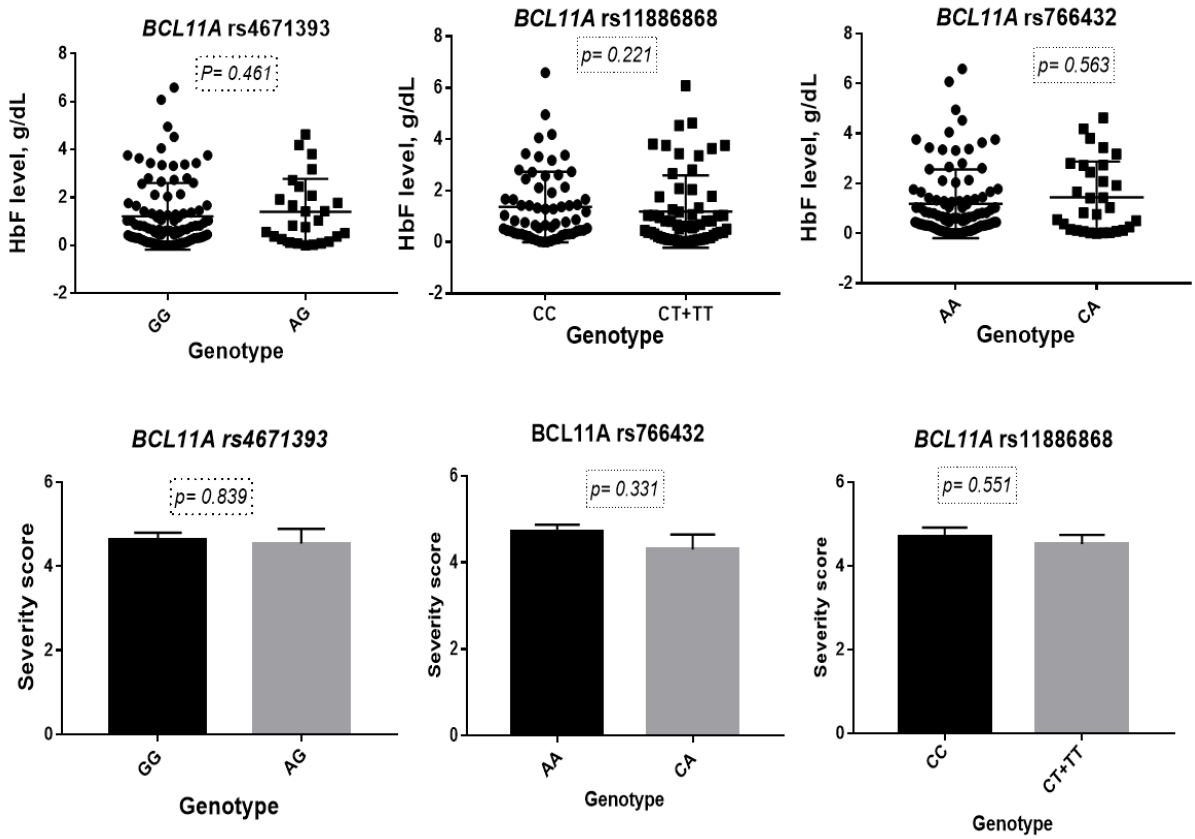


Figure 3.14: Unpaired *t*-test results for HbF level (g/dL) and the severity score across the groups of patients with HbE/β-thalassemia having different SNP genotypes for rs4671393, rs766432 & rs11886868 in BCL11A gene. P<0.5 is considered as significant result.

Next we observed the effects of HbF levels on disease severity scores of patients with HbE/β-Thalassemia who had rs4671393, rs11886868, & rs766432 SNPs at the BCL11A gene locus. As it was expected from HbF levels associated with s4671393, rs11886868, & rs766432 SNPs, there were no significant changes in differences of disease severity scores between the two allelic versions of the genotypes of each SNP and these findings are supported by non-significant p-values - 0.839, 0.331, and 0.551 for rs4671393, rs11886868, & rs766432 SNPs, respectively (**Figure 3.14**).

3.2.6.4 Study on the association of HBBP1 rs2071348 (A>C) Polymorphism with HbF level and disease severity among Bangladeshi HbE/β-thalassemia Patients

HBBP1, a member of HBB locus, is another QTL of the gamma-globin gene found to be linked with high expression of fetal hemoglobin in genome wide association study. Several studies have concluded that the HBBP1 gene has a wide network of interactions with functional transcription sites ranging across its gene cluster. Therefore, it can be inferred that transcriptional products of HBBP1 gene have regulatory functions and it may also be assumed that any mutation on the pseudogene can affect these regulations.

In current study, firstly we aimed to determine the frequency of HBBP1 rs2071348 polymorphism among Bangladeshi population which includes both healthy control and patents with HbE/β-thalassemia. For detection of the rs2071348 SNP, the same procedure was followed for SNPs in BCL11A and HBS1L_MYB (described in earlier section **3.2.6.2** and **3.2.6.3**) using real-time PCR followed by HRM Curve Analysis method comparing with the reference samples prepared earlier by Sanger DNA sequencing. It was observed that the reference samples contained wild type homozygous (TT) and mutant heterozygous (TG) and mutant homozygous (GG) genotypes **(Figure 3.15**). In the figure, the representations are on the 5' – 3' strand. However, as the β – globin gene cluster is present on the antisense strand, the mutations were interpreted accordingly (AA for wild type homozygous, AC for mutant heterozygous and mutant homozygous CC).

In **figure 3.16**, the controls and samples containing the wild type homozygous genotype (AA) displayed melt curves as the blue cluster while the green cluster represented controls and samples containing the mutant heterozygous genotype (AC) and the red cluster was for the samples containing the homozygous mutant genotype GG.

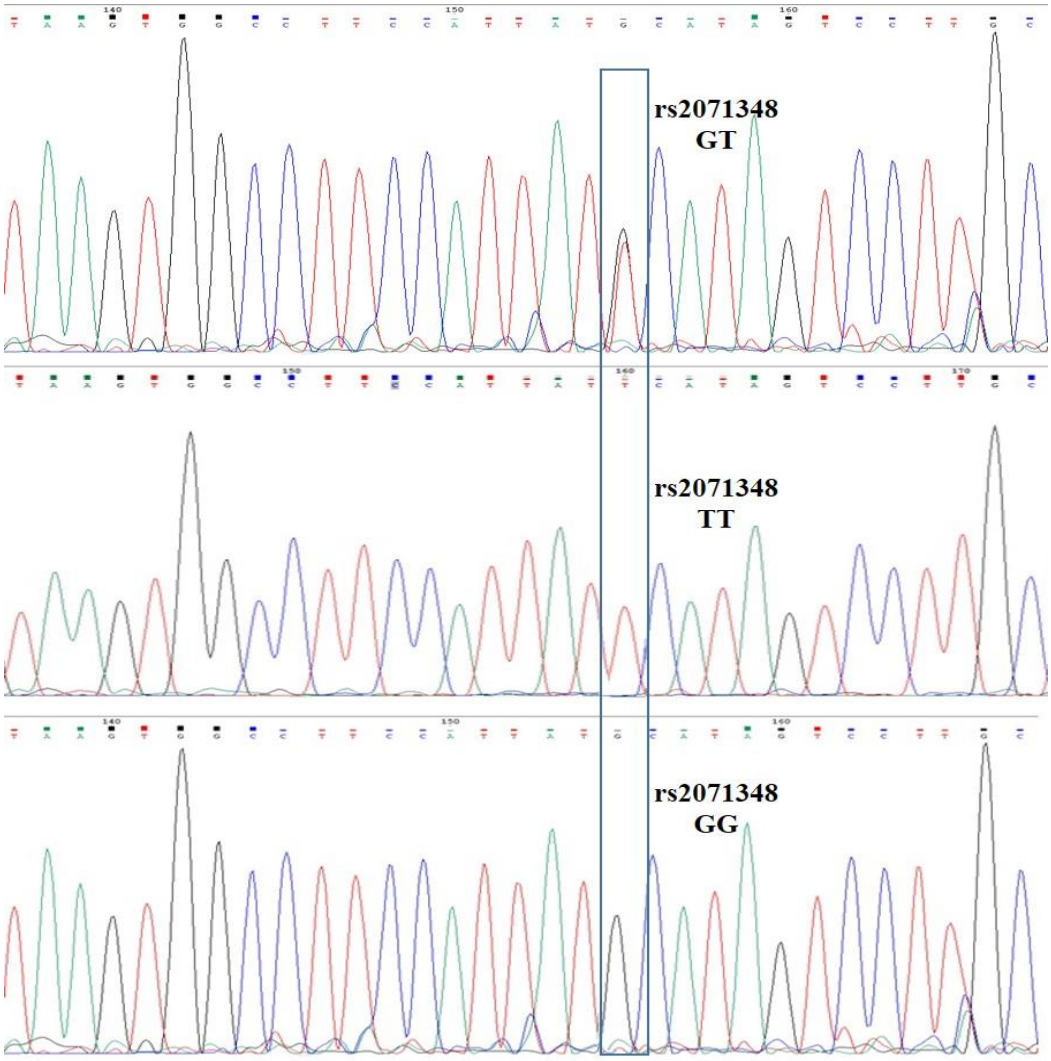


Figure 3.15: Chromatogram showing Sanger Nucleotide Sequencing results for rs2071348 SNP in HBBP1 gene among the study population.

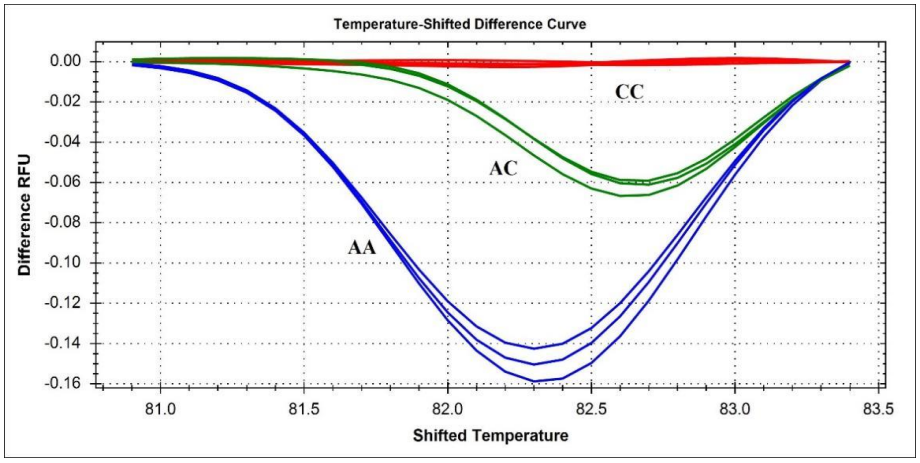


Figure 3.16: **High Resolution Melt Curve (HRM) for HBBP1 rs2071348 (A>C) Polymorphism**

Frequency distribution of SNP rs2071348 in HBBP1 gene among the study population

In addition to the already mentioned SNPs, we analysed another HbF-inducing SNP which is designated as rs2071348 and is located at the HBBP1 gene locus.

Table 3.17: Genotype frequency of SNP rs2071348 in HBBP1 gene among the healthy controls and the patients with HbE/β-Thalassemia.

SNP Genotype	Healthy controls, n, (%) (N=50)	All patients n, (%) (N=130)	Frequency in different severity groups, n (%)				
			Mild/NTD N=39	Moderate N=35	Severe N=56	Fisher Exact test, *p*	Odd Ratio (95% CI); p value
AA	26 (52%)	69 (53.07%)	10 (25.64)	17 (48.57%)	40 (71.43%)	0.0005	Reference
AC	21 (42%)	44 (33.82%)	20 (51.28%)	13 (37.14)	12 (21.43%)		4.35 (2.1- 9.2); *P*<0.0001
CC	03 (6%)	17 (13.1%)	09 (23.08%)	05 (14.29%)	04 (7.14%)		

Table 3.17 shows that frequency distributions of 3 versions of genotypes of this SNP among healthy controls, and mild, moderate, and severe patients were 52%, (n= 26), 25.64% (n=10), 48.57% (n=17), and 71.43% (n=40), respectively for genotype AA, followed by 42% (n=21), 51.28% (n=20), 37.14% (n=13), and 21.42% (n=12) , respectively for genotype AC; and 6% (n=3), 28.03% (n=9), 14.29% (n=5) and 7.14% (n=4), respectively for genotype CC. The genotype distributions of the SNPs significantly differed between mildly, moderately and severely affected patients (p=0.0005). AA genotype predominantly present (71.43% vs. 25.64%) in severely affected patients with HbE/β-Thalassemia compared to mild form and reached statistical significance (P< 0.0001, OR = 4.35, 95% CI = 2.1- 9.2).

Correlation of rs2071348 in HBBP1 gene on HbF level and Disease Severity of HbE/β-Thalassemia patients

To observe the differences of HbF concentration along with clinical scores among the three identified genotypes of rs2071348 in HBBP1, a one way ANOVA – tests were performed. **Figure 3.17** showed that the comparison among the genotypes for their respective HbF values displayed a P value of 0.0044 which is statistically significant. Similarly, the clinical scores

of the three genotypes were compared and a significant p< 0.0001 was obtained indicating that the SNP causes highly significant differences on clinical phenotypes in HbE/β-Thalassemia patients provided that the patients having the polymorphic allele C had milder disease severity.

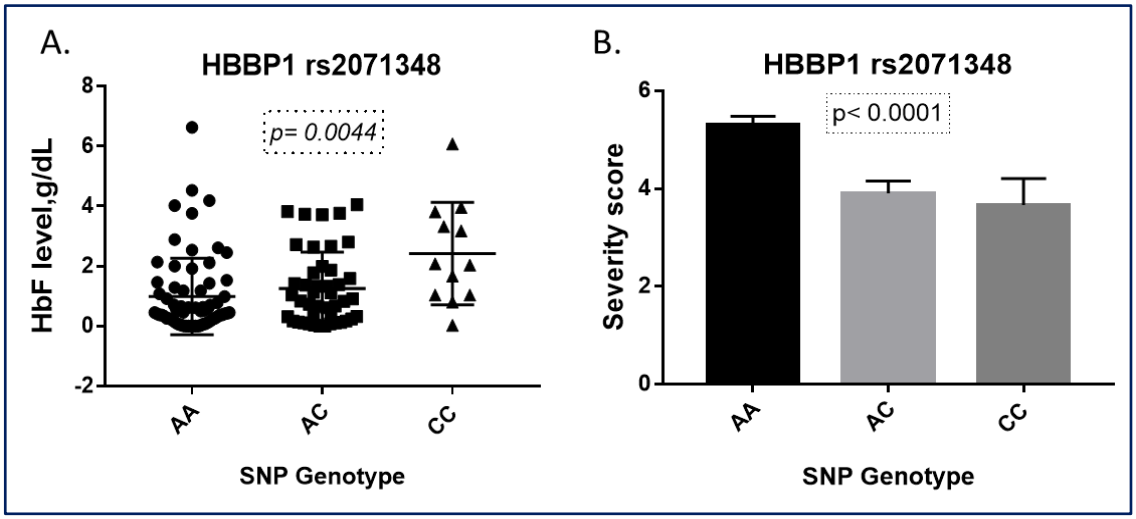


Figure 3.17: ANOVA- test results for A) HbF level in g/dL and B) severity score across the groups of patients with HbE/β-thalassemia having different SNP genotypes for rs2071348 in HBBP1 gene. P<0.5 is considered as significant result.

3.2.6.5 Summary of the modifying effect of the 8 SNPs in 4 genes on HbF level and the clinical severity score of HbE/β-thalassemia patients.

In conclusion, the study demonstrates that out of 8 SNPs which had been subjected to analysis, only 4 SNPs, namely Xmn1 at the HBG2 gene, rs4895441 and rs28384513 at the HBS1L-MYB intergenic region, and rs2071348 at the HBBP1 gene loci could show significant association with elevated levels of HbF in the study patients with haemoglobin E/β-thalassemia, generating p-values of 0.005, 0.0001, 0.0001, and 0.004, respectively.

Furthermore, 3 out of these 4 SNPs, namely Xmn1, rs4895441 in HBS1L-MYB intergenic region, and rs2071348 in HBBP1 gene which possessed significant HbF-inducing effects were also associated with significantly reduced clinical severity scores with p-values of 0.009, 0.0005, and 0.0001, respectively (**Table 3.18**). Unusually, only 3 out of 12 SNPs at the BCL11 gene locus had been analysed and none could show significant correlation with induction of HbF levels and clinical severity scores.

Table 3.18: Association of the 8 SNPs present in four genes with fetal hemoglobin level and the clinical severity score of HbE/β-thalassemia patients.

Gene	SNP locus	Genotype (n)	Mean value		*p* value	
			HbF (g/dL)	clinical score	HbF	Clinical score
HBG2	Xmn1 (C>T)	CC (21)	0.49	5.5	**0.0005**	**0.009**
		CT (101)	1.35	4.6		
		TT (08)	1.84	3.0		
HBS1L-MYB	rs28384512 (C>T)	CC (25)	0.98	4.6	0.07	0.95
		CT (67)	1.5	4.3		
		TT (38)	1.2	4.4		
	rs4895441 (A>G)	AA (95)	1.01	5.0	**0.0001**	**0.0005**
		AG (25)	1.58	3.6		
		GG (10)	2.58	3.1		
	rs28384513 (T>G)	TT (32)	0.91	4.9	**0.0001**	0.06
		TG (68)	1.13	4.7		
		GG (30)	1.92	3.9		
BCL11A	rs11886868 (C>T)	CC (63)	1.37	4.6	0.22	0.55
		CT+TT (67)	1.19	4.4		
	rs4671393 (G>A)	GG (101)	1.22	4.7	0.46	0.84
		AG (29)	1.4	4.5		
	rs766432 (A>C)	AA (98)	1.19	4.7	0.56	0.33
		AC (32)	1.45	4.3		
HBBP1	rs2071348 (A>C)	AA (69)	0.99	5.9	**0.0044**	**<0.0001**
		AC (44)	1.26	3.9		
		CC (17)	2.42	3.7		

3.2.6.6 Combined effect of the 4 HbF associated SNPs on raising HbF level in the HbE/β-thalassemia patients

In our study, we found that XmnI polymorphism and SNPs in HBS1L_MYB and HBBP1 genes are associated with HbF level that affects the patients' disease severity. Next, we wanted to analyze whether the combined presence of HBS1L_MYB and HBBP1 polymorphisms along with XmnI polymorphisms can affect the level of HbF among these thalassemia patients. First, we have categorized the patients as XmnI (+) and (-) polymorphism. These patients have further sorted according to the presence/absence of other polymorphisms in HBS1L_MYB or HBBP1 gene. HbF levels were compared among these groups of patients using Tukey's test (**Figure 3.18**).

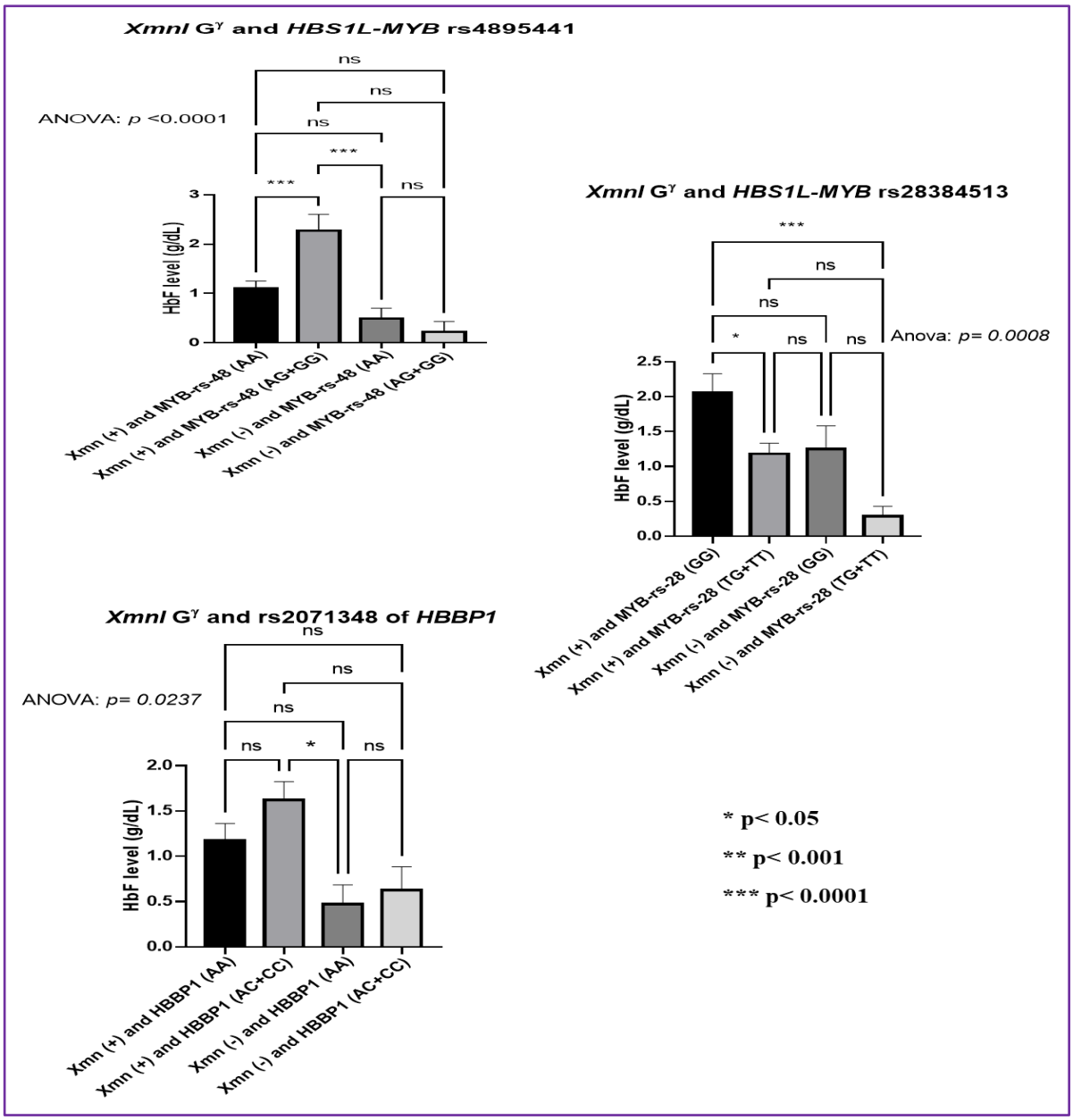


Figure 3.18: HbF level for each combination of XmnI-$^{G}\gamma$ polymorphism with other 3 HbF associated SNPs- rs4895441, rs28384513 of HBS1L-MYB and rs2071348 of HBBP1 gene.

Figure 3.18 shows that the level of HbF among Xmn I (+) and (-) along with MYB-rs48 (A>G) is statistically significantly different between the groups as well as with MYB-rs28 (T>G). For the rs28 in HBS1L_MYB, the XmnI (+) + GG combination shows the highest HbF whereas XmnI + (TG+TT) has intermediate and Xmn (-) + (TG+TT) has the least value. Similarly, the co-presence of MYB rs48 and the XmnI (+) polymorphism shows the highest HbF among other combination. In case of rs2071348 in HBBP1, the XmnI (+) + (AC+CC) group shows significantly highest HbF while the combination of XmnI (-) + (AA) has the least HBF value. Overall, the Tukey's test shows the statistically significant difference

presents in HbF levels of patients in all the three SNP (MYB rs48, MYB rs28 and HBBP1 rs20) combination with Xmn1 polymorphism.

3.2.6.7 Hardy–Weinberg equilibrium test for the allele frequency of the 8 SNPs studied in our population

Among the 8 SNPs namely, -158 Xmn1-Gγ, rs4895441, rs28384513, rs28384512, rs11886868, rs4671393, rs766432 and rs2071348 in four gene, the highest MAF (minor allele frequency) was found for HBS1L-MYB rs28384513 with the frequency of 0.46 followed by -158 Xmn1-Gγ in HBG2 (MAF: 0.45), HBS1L-MYB rs28384512 (MAF: 0.45) and rs2071348 in HBBP1 gene (MAF: 0.28).

Table 3.19: Frequency distribution of 8 SNPs among the study participants.

Gene	**SNP locus**	**Allele frequency**		**Hardy–Weinberg equilibrium**	
		Type	Frequency (%)	$\chi 2$	p^*
HBG2 (11.p15.5)	Xmn1 (C>T)	C (-)	55	41.71	<0.0001
		T(+)	**45**		
HBS1L-MYB (6.q23.3)	rs28384513 (T>G)	T	54.72	0.36	**0.54**
		G	**46.28**		
	rs4895441 (A>G)	A	82.17	8.61	0.03
		G	17.83		
	rs28384512 (T>C)	T	55	0.04	**0.83**
		C	**45**		
BCL11A (2.p16.2)	rs11886868 (C>T)	C	89.9	6.13	0.01
		T	10.1		
	rs4671393 (G>A)	G	87	1.65	**0.19**
		A	13		
	rs766432 (A>C)	A	74	0.65	**0.42**
		C	26		
HBBP1 (11.p15.4)	rs2071348 (A>C)	A	72	2.22	**0.13**
		C	28		

* $p > 0.05$ = alleles in the equilibrium.

However, when it was tested whether the SNP alleles are in equilibrium, it was found three SNP locus namely Xmn1, MYB rs4895441 and BCL11A rs11886868 were not in Hardy–Weinberg equilibrium (HWE) distribution in Bangladeshi population and the rest 5 SNP alleles are in equilibrium as each locus showed HWE over 0.05 (**Table 3.19**).

3.2.7 Effect of co-existence of α-globin gene deletions in HbE/β-thalassemia

As the co-existence of α-thalassemia is one of the key genetic modifier of the disease severity in β-thalassemia, here we examined two most frequent mutations in α-globin gene, namely, α--^{SEA} deletion and -α$^{3.7}$ deletion along with gene triplication (ααα$^{anti\ 3.7}$) to investigate their effects on disease scores among the different severity groups of patient with HbE/β-thalassemia. Real-time GAP-PCR followed by HRM curve analysis was used to detect α--^{SEA} deletion and a conventional multiplex Gap-PCR method was used for detection of -α$^{3.7}$ deletion and α-gene triplication (ααα$^{anti\ 3.7}$). However, (_ _^{SEA}) deletion was not found in our study population. As per **Table 3.20**, there was only one patient who had silent carrier status (αα/-α$^{3.7}$) genotype with mild level clinical score. Both 2 patients with α-thalassemia trait (α$^{3.7}$/-α$^{3.7}$) were in clinically severe group. Among 10 patients with hhomozygous α-globin triplication (ααα$^{anti\ 3.7}$/ααα$^{anti\ 3.7}$), 7 patients were in the severe group and 3 patients were in mildly severe group. 1 out of 2 patients with heterozygous α-globin triplication (αα/ααα$^{anti\ 3.7}$) had moderate clinical score, whereas another one had severe clinical score.

Table 3.20: Frequency of -α$^{3.7}$ deletion and α-gene triplication (ααα$^{anti\ 3.7}$) among the different severity groups of patient with HbE/β-thalassemia.

-α$^{3.7}$ deletion genotype	**Phenotypic severity groups**		
	Mild N= 39	Moderate N=35	Severe N=56
Silent carrier status (αα/-α$^{3.7}$)	0	1	0
α-thalassemia trait (-α$^{3.7}$/-α$^{3.7}$)	0	0	2
Heterozygous α-globin triplication (αα/ααα$^{anti\ 3.7}$)	0	1	1
Homozygous α-globin triplication (ααα$^{anti\ 3.7}$/ααα$^{anti\ 3.7}$)	0	3	7
Wild type (αα/αα)	39	30	46

Discussion

CHAPTER 4

4.1 Current nationwide prevalence of β-thalassemia and HbE carriers in Bangladeshi population

This is the first study of thalassemia carrier screening in Bangladesh on large samples from marriageable age which aimed to determine (1) nationwide carrier frequencies of β-thalassemia (BTT) and HbE traits (ETT) more precisely using molecular approach along with conventional methods (2) distribution of division-wise carrier frequencies, and (3) the mutation spectrum in the HBB gene of the carriers.

In the present study, total carrier frequency of HbE plus β-thalassemia was 10.92%, where ETT had the highest frequency of 8.68% followed by BTT 2.24%, supported by the previous study conducted in 2005 (Khan et al. 2005). Neighboring India and Sri Lanka reported the comparable BTT frequencies of from 2.2% to 4.05% and carrier frequency of HbE is 10.9% in Eastern and Far Eastern part with highest frequency in Assam, Meghalaya, Arunchal Pradesh, West Bengal, Manipur and Nagaland. (de Silva et al. 2000, Madan et al. 2010, Nagar et al. 2015). Among 8 administrative divisions, the highest frequency of thalassemia carrier (ETT+BTT) was found in Rangpur division (27.1%) followed by Rajshahi division (16.4%).

The current disease burden in the country was estimated to be 9,176 expected born with thalassemia each year (according to Hardy-Weinberg equation), thereby further worsening the situations by creating severe health and economic burden to the nation (Noor et al., 2020). Thus, adoption of a national thalassemia prevention strategy is a demand of time. Several thalassemia endemic countries have set up comprehensive national prevention programs, which include public awareness, carrier screening, genetic counseling and prenatal diagnosis (De Sanctis et al., 2017, Cao and Kan, 2013). Effectiveness of such prevention program in Sardinia is evidenced by a reduction in thalassemia major birth from 1:250 to 1:4000 and such success is also found in Cyprus, Iran, and Turkey (Cao and Kan, 2013, Cao et al., 2007, Bozkurt, 2007). Some countries like UAE, Saudi Arabia, Jordan, Cyprus, Iran and Turkey are performing compulsory premarital screening for thalassemia to discourage marriage between two carriers (Cao and Kan, 2013). These strategies may help guide health policy makers of Bangladesh to adopt an appropriate thalassemia prevention strategy considering the available resources, religious values and social culture (Noor et al., 2020).

4.2 Consanguinity and lack of knowledge contributes to high thalassemia carrier rates

The number of population with thalassemia carriers has been gradually increasing in the country over time and become almost 1.5 times higher within 40 years. It was 7% in 1980,

10.2% in 2005 (Khan et al., 2005) and has raised to 10.92% BTT+ETT along with 11.89% overall frequency of abnormal hemoglobin gene according to our study. It might be due to marriages between the thalassemia carriers as a consequence of lack of knowledge about the disease as well as due to vertical transmission of mutant beta globin allele(s) to the offspring from consanguineous parents (Denic et al., 2013a).

Our study found that the thalassemia carrier frequency was almost double among the children of consanguineous parents, a common socio-culture of this region and thus consanguinity contributes to increased burden of thalassemia. The highest consanguinity was observed in Rangpur division (8/140; 5.7%), which also had the highest carrier frequency, in particular the highest ETT was 25.2%, among the eight divisions of Bangladesh. It also justifies the higher incidences of HbE disease in the region (3 participants with HbE disease out of 8 were from Rangpur Division). It indicates the alarming situation in future with rapid increase of both the carrier frequency and the homozygous status of HbE. Even though majority of the participants were university/college going students, 62% of them did not know the disease etiology and about 32% did not have any knowledge about the disease prior to enrolment suggesting that the knowledge regarding thalassemia is quite insufficient among the mass population (Noor et al., 2020).

4.3 Appropriate sample size and molecular approach used as supplementation to conventional methods provided an accurate data on actual frequency of β-thalassemia and HbE carriers in the country

To get an accurate prevalence data, a large-scale population survey, which should not be hospital-based, is mandatory on an appropriately calculated sample size using proper screening methods. Thalassemia screening so far conducted by different groups during the last 2/3 decades gives different views and wide variation in carrier status which vary from less than 7% to 26%. Such discrepancies among the surveys performed by different groups might be due to -sampling error or improper selection of technology for carrier detection.

The sample size of this study was 1877 which is larger than that of the previous studies which might have helped to reflect the true frequency of thalassemia carriers in the country. The number of total participants was calculated using proper statistics as well as division-wise enrollment was enumerated statistically in proportion to the population size of each division for a precise information on distribution of carrier frequency in each division.

It is the first thalassemia carrier screening study done in combination with hematological, biochemical and DNA-based assays considering all possible suspected cases to make more accurate interpretation during detection of carrier status. Conventional screening methods use only hematological parameters and/or Hb electrophoresis indices which is not sufficient enough to overcome the problems associated with false positive and false negative cases. The conventional MCV-based thalassemia carrier screening strategy might miss a significant number of E-traits (Insiripong et al., 2012). For example, although the parameters MCV ≥80 fL and MCH ≥27pg are usually considered as non-carrier status, our study had identified 4 participants with MCV≥80fL and MCH≥27 as Hb-E carriers, indicating shortcomings of hematological indices in screening of HbE carriers. Similarly, 5 participants having HbA2> 3.5%, which should indicate beta trait, found to be non-carrier by HRM curve analysis and Sanger sequencing, thereby demonstrating shortfalls of Hb electrophoresis in thalassemia carrier detection. The higher HbA2 might be caused by mutations in the KLF1 gene leading to borderline high HbA2 and thus may result in false positive findings in Hb electrophoresis (Perseu et al., 2011). On the other hand, the study could identify 1 β-thalassemia carrier with borderline HbA2 of 3.5% and low MCV in molecular analysis which implies that specimens with HbA2 level in the borderline range (3.3%-3.5%) with low MCV must be subjected to DNA-based analysis. Notably, coinheritance of β-globin gene mutations with α or δ thalassemia traits that usually lower the level of HbA2 to normal or borderline range in the β-thalassemia carriers (Denic et al., 2013b).Thus the DNA-based approaches like high resolution melt curve (HRM) analysis and Sanger sequencing had been proved to be very useful to avoid false positive and false negative results by detecting mutations in the β-globin gene, and thereby confirming the true thalassemia carrier status of the participants. More importantly, 2 participants detected as HbE carriers by traditional methods were identified with HbE/β-thalassemia genotype by HRM method, and might be at risk of developing non-transfusion dependent thalassemia (NTDT) in future. Identification of NTDT patients is vital for prognosis because increased intestinal iron absorption in such patients increases the risk of sudden cardiac arrest (Sleiman et al., 2018, Musallam et al., 2013) and a timely treatment intervention will curtail the progression of disease severity and thus prevent an untimely death.

All these aspects emphasize on the requirement of molecular-based carrier screening which is the ultimate confirmation of a carrier status. In this study, all the specimens with abnormal hematological indices were tested with HRM approach to confirm the mutation in β-globin

gene and if the molecular tests were not performed, about 5 in 1000 of the carriers would have been missed and 1.8% cases could have been misinterpreted. In this study, we used Real-time PCR based HRM curve analysis for mutation detection in beta globin gene because of its advantages and cost-effective nature other DNA-based screening methods like Denaturing High Performance Liquid Chromatography (DHPLC), Single Strand Conformational Polymorphism (SSCP) and Denaturing Gradient Gel Electrophoresis (DGGE), Amplification Refractory Mutation System (ARMS) PCR, Sanger nucleotide sequencing etc. (Islam et al., 2018b) as this real time PCR-based high throughput HRM curve analysis is easy to perform and time-saving as there is no need of post PCR amplification processes like PCR product purification, gel electrophoresis etc.

4.4 Mutation spectrum in the HBB gene of the carriers detected in the study

A total of 9 different types of mutant genotype in the beta globin gene including a novel mutation were identified in the study populations which would help in making a regional mutation profile for Bangladeshi population. The spectrum of mutations varies across different geographical regions and cultures. Hence, regional mutation profiling is essential to undertake any strategies e.g., genetic counseling, prenatal diagnosis, to deal with thalassemia. Different mutations are associated with different types of thalassemias that influence the severity of the diseases. A complete mutational profile prior to initiation of treatment will be helpful to determine the prognosis, appropriate therapy and family counseling. Majority of the mutations identified in the study had been found in the mutation list of our previous study (Islam et al., 2018b). However, we could identify a novel mutation, namely c.151G>A (Thr>Ala) in the HBB gene of a thalassemia carrier. A change in amino acid from Thr to Ala in the beta globin protein is expected to confer pathological manifestations, which had not been investigated yet in thalassemia patients.

4.5 Variability in degree of clinical phenotypes and hematological parameter found in HbE/β-thalassemia patients of Bangladesh

As a country on thalassemia belt, thalassemia has been found as a very concerning public health problem in Bangladesh in our study with 10.92 % carrier frequency and based on current HbE trait of 8.68%, the expected annual birth of Hb E/β-thalassemia patients in Bangladesh is 7,193. In Bangladesh, more than 70% thalassemia patents suffer from hemoglobin E/β thalassemia (Hossain et al., 2017, Uddin et al., 2012), whereas the rest are β-thalassemia major (BTM) known as the most serious form of the disease requiring 1st blood

transfusion by 1/2 year after birth and then continuing regular blood transfusion by an interval between 1 week to 3/4 weeks along with iron chelation therapy as frequent blood transmission causes iron deposition in the liver, different glands, and heart. On the other hand, unlike BTM, the clinical manifestations of E/β-thalassemia are highly heterogeneous which may be in the form of (1) mild with occasional blood transfusion or non-transfusion-dependent (NTD) form, (2) moderately severe- with blood transfusion frequency of more than the NTD group and less than the severe group, and (3) severe group- clinical manifestations are usually like BTM requiring frequent blood transfusion. The present study demonstrates differential clinical criteria, investigational findings, and the mechanism of genetic basis of clinical heterogeneity of hemoglobin E/β thalassemia.

The findings showed that all 3 forms of the disease were in consistent with the above-mentioned defined criteria of HbE/β thalassemia in terms of 1st blood transfusion and transfusion intervals. Our data showed that clinically less severe patients needed First blood transfusion in earlier age of life than the more severe patients and also the former group of patients had higher transfusion intervals than the latter groups of patients. In addition, the study also showed that the rate of splenomegaly and splenectomy was higher in the severe patients, compared to moderately severe and less severe groups. Our findings in terms of 1st blood transfusion, transfusion intervals, and splenomegaly/splenectomy supported the findings of previous studies (George, 2013, Winichagoon et al., 1993, Viprakasit and Ekwattanakit, 2018, Sripichai et al., 2008a).

Among the hematological parameters, RDW percentage significantly varied across the severity groups of patients and Mild/ NTD patients had higher RDW (> 28%) compared to moderately severe (~24%) and severe groups (~22%) which is similar to the previous studies reporting significantly higher RDW in NTDT patients (Piriyakhuntorn et al., 2018). Red cell distribution width values (RDW), one of the RBC indices reflects the degree of anisocytosis of red blood cells. This value was also reported as an indicator to differentiate between patients with IDA and a thalassemia trait or δβ thalassemia for decades (Velasco-Rodríguez et al., 2014, Bessman et al., 1983, Aslan et al., 2002) .

4.6 Disease modifying role of HbF and HbE in Bangladeshi HbE/β-thalassemia patients

Our study found low levels of HbF and HbE to be significant determinants of high disease severity in patients with HbE/β-thalassemia as both were inversely related to the disease severity score. HbF have protective role in thalassemia patients by neutralization of excess

alpha-chain which, in unbound form, may aggregate in the nucleated erythrocytes and cause apoptosis of the cells before maturation to reticulocytes/erythrocytes and this is the main pathological consequence of thalassemia. (Lim et al. 2015, Paikari and Sheehan 2018, Rees et al. 1999). In our study, the amounts of HbE and HbF produced were highest in the mild group of patients with HbE/β thalassemia followed by moderately severe and severe groups of HbE/β thalassemia. Again, the study showed that the higher was the level of HbF, the higher was the blood transfusion interval and and age of 1st blood transfusion. On the contrary, in a few studies and also in this study, it had been found that some patients from the severe and moderate group of patients also had a high level of HbF but relatively low levels in NTD patients (Fucharoen and Weatherall 2012) (Fuchareon, 2012). This variation of the level of HbF raised the doubt whether it is the sole factor that is affecting the disease severity in NTD patients or not. Moreover, the finding that Hb F was not an effective oxygen carrier because of its poor dissociation ability contradicted the fact that it would be effective to increase the HbF level as a treatment to decrease the disease severity. Overall, it was implied that the HbF may not be the only factor that decreases the disease severity of the HbE/β Thalassemia patients, although, HbF is needed for decreasing the α/β chain imbalance which is an important factor by itself. On the other hand, the role of HbE might be taken into consideration as oxygen saturation curve produced by HbE is either like normal hemoglobin or slightly red-shifted, meaning HbE can carry oxygen to some extent in the tissues and thus protects thalassemia patients. (Bhattacharya et al. 2010). It had been found in research that, the oxygen dissociation power of Hb E in the patients of Hb E disease was normal where the HbF was very low or not present (Panigrahi et al. 2005). Similar to HbF, our study found HbE showing protective actions against thalassemia by extending transfusion intervals and age of 1st blood transfusion. This supported the finding in a recent study that the high-level HbE and the relatively low level of Hb F together help the NTD patients to adapt to the anemia (Fucharoen and Weatherall 2012).

Together, these results suggest that differential levels of expression of HbE and HbF are the basis of clinical heterogeneity of HbE/β thalassemia. However, as per our study, it seemed that the effect of HbF in the protection to thalassemia was stronger than that of HbE.

4.7 Genetic modifiers affecting the severity in HbE/β-thalassemia patients of Bangladesh

Types of mutations in HBB gene are the primary determinant of the disease severity in β-thalassemia and our study identified a total of 11 different causative mutations in the the β-

globin allele trans to HbE allele in the patients with HbE/β-thalassemia in Bangladesh. IVS1_5 G>C was the most common mutation in the β- globin allele and thus c.79 G>A (E allele)+IVS1_5 G>C combination was found as most predominant mutation pair across all the 3 severity groups of patients covering 73.8% of HbE/β-thalassemia in the country. Additional mutation pairs including c.27_28 ins G, c.126_129delCTTT, c.3G>T, c.51delC + c.33C>A, c.92 G>C, 126delC, IVS1_1 G>A, IVS1_130(G>C), c.46delT, and c.47(G>A) were identified with frequencies of 1.5%, 5.4%, 2.3%, 2.3%, 3.1%, 1.5%, 3.8%, 2.3%, 2.3%, and 1.5%, respectively to cause HbE/β-thalassemia provided. The mutation patterns and corresponding distributions of patients among 3 different severity groups of HbE/β-thalassemia patients including mild/NTD, moderate and severe could not provide exclusive information to indicate whether certain mutation/combination of mutations in the β- globin allele were responsible for distinct clinical severity. That is, the causative mutations could not explain the basis of clinical heterogeneity of HbE/β-thalassemia. To explain the mechanistic basis of clinical heterogeneity of HbE/β-thalassemia, at least in partial term; the role of secondary modifiers like SNPs in HbF associated QTLs and co-existence of α-thalassemia must be considered (Rujito and Sasongko, 2018, Olivieri et al., 2011).

Several studies provide evidence that SNPs in the 3 major QTLs- 1) HBG2 and HBBP1 gene in the HBB locus, 2) HBS1L_MYB intergenic region, 3) repressor gene BCL11A are associated with increased synthesis of fetal hemoglobin and the presence of the respective polymorphic alleles are associated with a milder clinical phenotype in HbE/β-thalassemia (Nuinoon et al., 2010). With the view in mind, the study was designed to explore the effects of these modifier genes to favor disease manifestations in the direction of amelioration through induction of HbF. To achieve the goal, the study aimed to identify the frequency and effect of 8 SNPs in the namely rs7482144 (-158 Xmn1-Gγ) in HBG2, rs4895441, rs28384513 rs28384512 in HBS1L-MYB region, , rs11886868, rs4671393, rs766432 in BCL11A and rs2071348 in HBBP1 which had been reported to cause elevation of HbF (Rujito et al., 2016). Among the 8 SNPs namely, the highest MAF (minor allele frequency) was found for HBS1L-MYB rs28384513 with the frequency of 0.46 followed by -158 Xmn1-Gγ in HBG2 (MAF: 0.45), HBS1L-MYB rs28384512 (MAF: 0.45) and rs2071348 in HBBP1 gene (MAF: 0.28). The lowest MAF of 0.1 was found for the rs11886868 in BCL11A gene. For Xmn1-Gγ polymorphism, nearly similar frequency was similar to West Bengal (76.6% of +/- genotype) and Malaysian population (0.66) (Wong et al., 2006, Bandyopadhyay et al., 2001). However,

frequency pattern observed for BCL11A and HBS1L_MYB SNPs was different from other previous studies (Chalerm et al., 2017).

Among these 8 SNPs, only 4 SNPs- Xmn1, rs4895441, rs28384513 and rs2071348 showed significant association with elevated level of HbF in the study population and 3 SNPs namely Xmn1, rs4895441 and rs2071348 showed significant association with the clinical scores of the patients with HbE/β-thalassemia. In our study, the strongest association in terms of both increasing HbF level and decreasing severity score, was observed with SNPs in the *HBG2* (-158 Xmn1-Gγ) and *HBBP1* (rs2071348) gene in the HBB locus (β globin gene cluster, chr.11p15) followed by rs4895441 and rs28384513 SNPs identified in the intergenic region between the *HBS1L* and *MYB* genes (chr.6q23) which was in line with previous findings of the study in India, Thailand and Indonesia(Nuinoon et al., 2010, Nuinoon et al., 2022, Rujito et al., 2016, Kalantri et al., 2020). Previous reports demonstrated that XmnI polymorphism could significantly increase Gγ % and Gγ to Aγ ratio with concomitant clinical improvement of splenomegaly and bone marrow expansion (Mannoor et al., 2019). This is in agreement with our findings which showed that homozygosity of Xmn 1 polymorphism could reduce the number of HbE/β thalassemia patients with splenomegaly and splenectomy, although the study did not investigate the status of bone marrow expansion. However, none of the 3 SNPs in BCL11A exhibited significant association with either HbF level or the severity score which contradicts several previous reports (Chalerm et al., 2017, Fanis et al., 2014, Sedgewick et al., 2008). Our study analysed only 3 out of 12 reported HbF inducer SNPs at BCL11A locus. This is one of the limitations of the study. Further studies on other SNPs are expected to make a panel of sets of SNPs which might be used as a prognostic markers because the associated information will deliver more detailed picture on the disease spectrum of HbE/β-thalassemia patients. Although the effect of individual SNP had been studied to observe the effect on HbF induction and associated disease manifestations, very few studies had focused on the effects for the simultaneous presence of multiple SNPs in the same patient. In this sense, the present study is unique. We investigated whether the combined presence of HBS1L_MYB and HBBP1 polymorphisms along with Xmn I polymorphisms can affect the level of HbF among these thalassemia patients and found that the statistically significant increment in HbF levels of patients in all the three SNP (MYB rs48, MYB rs28 and HBBP1 rs20) combination with Xmn1 polymorphism.

Coinheritance of α-thalassemia in HbE/β-thalassemia patient is one of the ameliorating factors producing more balanced globin chain synthesis. Heterozygous silent carrier ($\alpha\alpha/-\alpha^{3.7}$)

and homozygous α-thalassemia trait (-α3.7/-α3.7)were found only in 1 moderate and 2 sever cases in the present study while α-globin triplication ($\alpha\alpha\alpha^{anti\ 3.7}/\alpha\alpha\alpha^{anti\ 3.7}$) was found in 4 moderate and 8 sever patients which was consistent with other previous studies (Sripichai et al., 2008b, Nuinoon et al., 2022). The $\alpha\text{--}^{SEA}$ thalassemia allele was not detected in our HbE/β-thalassemia patients. A possible reason is that the coinheritance of deletional α-thalassemia leads to mild β-thalassemia; thus, these patients were not found in a hospital-based sample collection (Winichagoon et al., 1985).

Conclusion

This is the first thalassemia carrier screening study in Bangladesh which demonstrates an in-depth and thorough screening of thalassemia carriers in the country involving all the eight administrative divisions by applying hematological, biochemical and DNA-based approaches. As a country on thalassemia belt, thalassemia has been revealed as a very concerning public health problem in Bangladesh with 10.92% carrier frequency which is in agreement with the hypothesis of the study. Also, this work has generated the national data of the carrier frequency for beta thalassemia and HbE variants for Bangladeshi population.

The information of this study will be helpful in several ways, such as measuring current nationwide carrier frequency with accuracy and grasp the gravity of the situation, identifying the at-risk population and thus prioritizing them, and necessities and benefits of molecular-based carrier screening. Moreover, the study shows that the cost-effective HRM approach can be used effectively in other countries of thalassemia-belt for detection of HBB gene mutations and confirmation of the carrier status. The present study is expected to motivate those who are involved in thalassemia carrier screening to include DNA technology, at least as a supplemental approach, provided that resources are available.

In conclusion, as prevention of thalassemia is far cheaper and better than treatment and currently no affordable cure is available, an immediate and concerted action on thalassemia prevention should be made mandatory in Bangladesh. Discouraging marriages between the thalassemia carriers through counseling should be the main target for raising awareness. In addition, since children of consanguineous marriage had the highest carrier frequency compared to that of the non-consanguineous family and since consanguineous marriage is quite common in Bangladesh, raising awareness to discourage consanguineous marriages

should also be included in "thalassemia prevention and control strategy". A massive awareness program targeting general population and an intensive educational program for health personnel including physicians, nurses, health and family planning workers should be carried out nationwide promptly. Also, appropriate screening method associated with genetic counseling should be required in existing hospitals and health facilities.

Another concluding remark of our study was the alarmingly high (8.68%) carrier frequency of HbE in our population with 7,193 expected annual birth of HbE/β-thalassemia in Bangladesh. It infers that the HbE/β-thalassemia is highly prevalent in our population, management of which is very challenging due to its extreme phenotypic variability and limited understanding of its natural history. These findings motivated us for further research on genetic modifiers of clinical variation in HbE/β-thalassemia patients in our country.

Remarkably, this is the first study on the disease modifiers of HbE/beta-thalassemia in Bangladesh considering to examine the effect of both primary and secondary level genetic modifiers of the disease severity in thalassemia that includes coinheritance of α-thalassemia and the HbF inducing SNPs in modifier genes. Also, faster and easier HRM analysis based methods have been established for detection of 7 SNPs in three major HbF associated QTLs. The influence of SNPs can be different among different regions, reflecting a link between ethnicity and other genetic susceptibility factors. The findings of our study will help in proper diagnosis and prognosis of different severe groups of patient along with planning appropriate management like time of starting with appropriate regime of blood transfusion and planning effective treatment strategy monitoring the complications. Detection of the HbF inducer SNPs for our population will reveal new therapeutic targets for increasing HbF levels in HbE/β-thalassemia patients in our country which plays a key role in reducing the degree of severity in HbE/β-thalassemia.

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CHAPTER 5

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Appendices

Appendix 1	Published paper based on this work
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Nationwide carrier detection and molecular characterization of β-thalassemia and hemoglobin E variants in Bangladeshi population

Farjana Akther Noor[1,2†], Nusrat Sultana[1,3†], Golam Sarower Bhuyan[1], Md Tarikul Islam[1], Mohabbat Hossain[1], Suprovath Kumar Sarker[1], Khaleda Islam[4], Waqar Ahmed Khan[5], Mujahida Rahman[6], Syeda Kashfi Qadri[7], Hossain Uddin Shekhar[2], Firdausi Qadri[1,8], Syed Saleheen Qadri[1] and Kaiissar Mannoor[1*]

Abstract

Background: ß-thalassemia is one of the most common inherited blood disorders in the world and a major deterrent to the public health of Bangladesh. The management of thalassemia patients requires lifelong frequent blood transfusion and the available treatment options are unsatisfactory. A national policy on thalassemia prevention is mandatory in Bangladesh. However, precise and up-to-date information on the frequency of ß-thalassemia carriers are missing due to lack of accurate diagnostic approaches, limited access to information and absence of national screening program. This study aims to determine the nationwide carrier frequency of hemoglobin E (HbE) and β- thalassemia and mutation spectrum among the carriers using molecular, hematological and biochemical methods.

Methods: The study enrolled a total of 1877 individuals (60.1% male and 39.9% female) aged between 18 and 35 years. Total sample size and its division-wise breakdown were calculated in proportion to national and division-wise population. Venous blood was collected and subjected to CBC analysis and Hb-electrophoresis for each participant. Serum ferritin was measured to detect coexistence of iron deficiency anemia with thalassemia carrier. DNA-based High Resolution Melting (HRM) curve analysis was performed for confirmation of carrier status by mutation detection.

Results: Of 11.89% (95% CI, 10.43–13.35) carriers of β-globin gene mutations, 8.68% (95% CI, 7.41–9.95) had HbE trait (ETT) and 2.24% (95% CI, 1.57–2.91) had beta-thalassemia trait (BTT). Among eight divisions, Rangpur had the highest carrier frequency of 27.1% (ETT-25%, BTT-2.1%), whereas Khulna had the lowest frequency of 4.2% (ETT-4.2% only). Moreover, α- thalassemia, HbD trait, HbE disease, hereditary persistence of HbF were detected in 0.11, 0.16, 0.43 and 0.16% participants, respectively. HRM could identify two individuals with reported pathogenic mutations in both alleles who were erroneously interpreted as carriers by hematological indices. Finally, a total of nine different mutations including a novel mutation (c.151A > G) were detected in the β-globin gene.

Conclusions: Since carrier frequency for both HbE and β-thalassemia is alarmingly high in Bangladesh, a nationwide awareness and prevention program should be made mandatory to halt the current deteriorating situations. Mutation-based confirmation is highly recommended for the inconclusive cases with conventional carrier screening methods to avoid any faulty detection of thalassemia carriers.

Keywords: ß-thalassemia, Carrier frequency, HRM curve analysis, Detection accuracy, Novel mutation, National policy

* Correspondence: kaiissar@ideshi.org
†Farjana Akther Noor and Nusrat Sultana contributed equally to this work.
[1]Laboratory of Genetics and Genomics, Institute for Developing Science and Health Initiatives, Mohakhali, Dhaka 1212, Bangladesh
Full list of author information is available at the end of the article

Background

β-thalassemia, characterized by reduced or absent β-globin chain synthesis, is one of the most common inherited blood disorders in the world and hence a major deterrent to public health. Although widespread, the major at-risk populations are mainly from Mediterranean, Middle East and Southeast Asian countries including Bangladesh [1, 2]. WHO reported that approximately 1.5% of global population are carriers of β-thalassemia and 68,000 children are born each year with various thalassemia syndromes [3]. However, precise and up-to-date data on the frequency of β-thalassemia carriers and patients are missing due to lack of accurate diagnostic approach, limited access to information and absence of national screening program in many of the thalassemia-inflicted countries.

Although thalassemia carriers are usually healthy, the patients with β-thalassemia major and HbE/β-thalassemia require lifelong blood transfusion and iron chelation therapy for survival [4]. Cardiac failure due to iron overload and transfusion-related infections have remained the leading causes of deaths of patients with thalassemia [5, 6]. Still, there is no cure for thalassemia except allogeneic bone marrow transplantation (BMT). Also, BMT therapy is too expensive to afford for majority of the world population and the outcome is often unsatisfactory. Due to lack of regular treatment and optimal health care facilities, most thalassemia patients die before adulthood in developing countries [7]. However, many at-risk populations in Cyprus, Greece, Sardinia, Iran etc. have successfully reduced the births of β-thalassemia children by adopting and implementing various preventive measures including nationwide awareness, carrier screening and prenatal diagnosis combined with genetic counseling [8–11].

Although Bangladesh has gained tremendous success in fulfilling Millennium Development Goal-4 by reducing under-5 mortality, there has been an increasingly rapid transition in the burden of disease from primarily communicable to non-communicable diseases. Thalassemia is the single most inherited monogenic blood disorder in Bangladesh and it causes not only substantial morbidity and deaths but also inflicts emotional and financial burden on the family and society [12]. An estimation using limited prevalence data has shown that roughly 33/10,000 newborns are being born each year with thalassemia in Bangladesh [12]. However, thalassemia patient-care and support facilities are barely available in most public and private hospitals. Moreover, health awareness on thalassemia remains highly inadequate among mass population. So, in addition to adoption of proper patient management strategy, prevention by premarital screening and/or prenatal diagnosis should be a useful approach for reducing the risk of thalassemia [7–9, 11]. However, considering socio-religious and financial issues as well as health risk associated with prenatal diagnosis and abortion of the affected fetus, pre-marital screening followed by genetic counseling is arguably the best approach to prevent thalassemia in Bangladesh. In order to weigh the future situation and implement an appropriate policy to tackle thalassemia, precise and up-to-date information on current carrier status is required. The last thalassemia carrier screening was conducted in 2005 on 735 school-going children in Bangladesh [13]. Moreover, the hematological and biochemical methods which are commonly used for screening purposes have limitations as these often end up in false positive and false negative results [14, 15]. In this regard, supplemental molecular methods have been in wide use for their accuracy in carrier screening and predicting severity of the thalassemia patients including their treatment, prognosis and overall management [4, 11]. Recently, the Government of Bangladesh has launched a National Thalassemia Prevention Program. As a part of that strategy, this study was conducted using funds from Non Communicable Disease Control (NCDC) Program, Director General of Health Services, MOHFW, Government of Bangladesh and Rotary Club of Dhaka North. In addition to conventional methods, the study applied DNA-based approaches to determine the accurate status of carriers and also incidence of the at-risk cases with both mutant alleles of HBB gene, which might be responsible for transition from asymptomatic to symptomatic non-transfusion dependent thalassemia in future. Accordingly, the study was conducted on a total of 1877 marriageable-aged participants. Also, a methodical approach was used to accurately determine the division-wise distribution of thalassemia carriers across Bangladesh.

Methodology

Study population

This cross-sectional study enrolled a total of 1877 participants (aged between18–35 years) from March 2018 to February 2019 from 10 different (4 universities, 4 medical colleges, and 2 business organization) institutions of Dhaka city with the intent to conduct awareness campaign and screen young unmarried adults. In addition, these institutes are attended by the students and employees from all 8 administrative divisions across Bangladesh. Upon obtaining written informed consent, approximately 5.0 mL of venous blood was collected from each participant via standard venipuncture in EDTA tube. The study was ethically approved by Bangladesh Medical Research Council (BMRC). After completion of the thalassemia screening tests, each participant received the report through email. Those who were found to be carriers of mutations in β- globin gene, were further given an opportunity to receive additional information and counseling.

Sample size calculation

Total sample size was calculated using the following formula and division-wise sample size was calculated in proportion to national and division-wise population size according to Bangladesh Bureau of Statistics [16].

$$n = \frac{(\alpha+\beta)^2\{p_1(1-p_1)+p_2(1-p_2)\}}{(p_2-p_1)^2} \times \text{design effect}$$

Where, α = the probability of type I error
β = the probability of type II error (power of the test)
p_1 = 10.2% previously estimated prevalence [13]
p_2 = 15% expected prevalence

Considering 95% confidence interval (CI), power 80% and a design effect of 2.5, the estimated sample size was 1875. To eliminate bias and for proper representation of each administrative division, samples were collected proportionately to the population size of that division by quota sampling method.

Analysis of hematological parameters

About 2.0 ml of collected whole blood were used for CBC (Complete Blood Count) analysis to determine RBC indices including hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and Red cell distribution width (RDW) using automated Hematology analyzer (Sysmex kx-21, Sysmex Corporation, Kobe, Japan).

Hemoglobin electrophoresis

Hemoglobin electrophoresis was performed on Sebia CAPILLARYS-2 Flex Piercing (Sebia, Lisses, France) using Capillarys Hemoglobin (E) kit to measure HbA, HbA2, HbF and other abnormal Hb variants following manufacturer's instructions.

Serum ferritin assay

Serum ferritin was assayed on miniVIDAS® Immunoassay Analyzer (bioMérieux, USA) using VIDAS® FERRITIN kit (bioMérieux SA, Marcy, France) following manufacturer's instructions.

Molecular analysis

Real-time PCR-based high resolution melting (HRM) curve analysis

Genomic DNA was extracted from whole blood using QIAGEN flexigene® DNA kit (Qiagen, Hilden, Germany) according to manufacturer's guidelines. Real-time PCR and HRM curve analysis using Precision Melt Analysis™ Software (BioRad) were performed on Bio-Rad CFX96 Real-Time System. This HRM method was previously developed for mutation screening in the β-globin gene of Bangladeshi and regional population of thalassemic endemic countries [2, 17, 18]. This high throughput approach enables to screen mutation(s) in unknown specimens in the presence of reference samples without nucleotide sequencing as well as to screen a large number of samples in a quick and cost-effective manner.

Sanger DNA sequencing

In this study, once the mutation positive specimens with a new HRM patterns other than the references were identified, nucleotide sequencing was carried out for those samples to identify the mutation. Sanger DNA sequencing using ABI PRISM-310 software version 3.1.0 (Applied Biosystems) was performed following the polymerase chain reactions (PCR) targeting the mutational hot-spot region of HBB gene for Bangladesh (exon1, intron 1 and a portion of exon 2 of beta-globin gene) and the PCR products purification using the MinElute® PCR purification kit (Qiagen) following the manufacturer's instructions. Then the retrieved sequence results were compared with the reference sequences (NC_000011.10) for confirmation of the mutation.

Statistical analysis

The comparison of sensitivity and specificity between traditional methods and molecular approach were performed using https://www.openepi.com/DiagnosticTest/DiagnosticTest.htm with 95% CI. The CI for an observed proportion was calculated using Stata software (version 14.2). With the known genotype frequency, the number of expected newborns with thalassemia was calculated by Hardy-Weinberg equation [19].

Results

Of the 1877 participants, male to female ratio was 1.5:1 and their average age was 23.4 ± 5.02 (mean ± SD) years (Table 1). The participants were from both rural and urban origins. About 4.32% of the participants had consanguineous parents. Moreover, although all the participants had general education, only 68.14% of them knew the term 'thalassemia', whereas 62.3% had no prior knowledge of the disease etiology, severity and risk factors etc. before attending the awareness program, which was arranged as a part of this study.

Screening for thalassemia carriers based on MCV, MCH and hemoglobin electrophoresis

The red blood cell count (RBC) and the hematological indices are important in the diagnosis of asymptomatic carriers as almost all kinds of thalassemia carriers show microcytic hypochromic parameters with apparently normal hemoglobin level. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are the two most widely used RBC indices for detecting microcytic hypochromic anemia. In the present study,

Table 1 Participants' information regarding gender, parental consanguinity, residence and their knowledge on thalassemia

Characteristic	Parameters	No. of Participants, *n* (%)
Gender	Male	1138 (60.1)
	Female	739 (39.9)
Consanguineous parents	Yes	81 (4.32)
	No	1796 (95.68)
Residence	Urban	1268 (67.6)
	Rural	609 (32.4)
Knowledge regarding thalassemia	Prior knowledge about thalassemia	1279 (68.14)
	Knowledge about how thalassemia is acquired	707 (37.66)
Presence of patients or carriers in the participants family	Yes	50 (2.66)
	No	459 (24.45)
	Not known	1368 (72.88)

MCV value of less than 80 fL and/or MCH of less than 27 pg were used as cutoff levels to initially suspect the participants as thalassemia carriers as these are the widely recommended RBC indices for the preliminary screening [20]. Based on these cutoff levels, the study participants (n = 1877) were divided into four categories, namely category A, category B, category C and category D. The category A participants had apparently normal RBC indices having MCV greater than or equal to 80 fL and MCH greater than or equal to 27 pg and they constituted 53% (995 out of 1877) of the study samples. Then 612 participants (32.6% of total samples) having MCV and MCH values less than cutoff ranges (< 80 fL and < 27 pg, respectively) had been suspected to have microcytic hypochromic phenotype and were categorized as B. A total of 13 samples with mixed criteria having MCV value less than 80 fL but MCH higher than the cutoff (> 27 pg) were categorized as group C and the remaining samples (257 out of 1877, 15.8%) which had normal MCV (> 80 fL) but MCH less than 27 pg were categorized as D (Table 2).

Second to MCV and MCH, hemoglobin electrophoresis using Sebia capillary electrophoresis was performed for all the samples as it is the gold standard for thalassemia carrier detections. HbA2 level of > 3.5% was used as a cutoff for screening of β-thalassemia carriers [21, 22]. Since Sebia capillary electrophoresis was able to separate HbA2 distinctly from HbE and other Hb variants like HbD, HbC, HbS and Hb Barts or HbH, the presence of HbE fraction or other hemoglobin variants could indicate the carriers of respective hemoglobin gene mutation.

Table 2 summarizes the results of hematological and electrophoresis analysis of the study participants. About 35% (215/612) of Group B participants had abnormal Hb electrophoresis results compare to 0.9% (9/995), 0% (0/13) and 1.6% (4/257) participants of Group A, C and D, respectively, with abnormal Hb electrophoresis results.

However, in Group-A having apparently normal RBC indices, there were 3 participants with BTT, 2 with ETT and 4 with other Hb variants. In addition, there were two BTT and two ETT carriers among Group-D participants. Finally, all the suspected cases based on MCV, MCH and Hb electrophoresis were subjected to DNA analysis for β-globin gene mutation.

Second-tier tests using high resolution melt curve analysis and sanger DNA sequencing for detection of β-globin gene mutations

β-thalassemia carriers have generally mild anemia, low MCV and MCH and elevated HbA2 levels. However, there may be considerable variability in hematological phenotype resulting from coexistence with iron deficiency anemia (IDA) and/or coinheritance with alpha thalassemia or delta-globin gene mutations, and presence of silent mutations in HBB gene. These individuals may have milder hematological findings with minimal abnormalities in Hb, MCV, MCH, and HbA2 which may confound the correct diagnosis of β-thalassemia carriers [14]. Considering these facts, a total of 89 samples (Group 1 plus 2 in Table 3) along with samples of BTT, ETT and HbE diseases which had been detected by Hb electrophoresis (Group 3, 4 and 5 in Table 3) were subjected to molecular analysis using HRM curve analysis followed by DNA sequencing. Molecular analysis aimed to (1) avoid faulty detection and confirm that the suspected cases were not left undetected, (2) determine the mutational spectrum of all β-thalassemia and HbE carriers and (3) identify any participants with non-transfusion dependent thalassemia (NTDT). The findings of molecular analysis have been summarized in Table 3.

For Group 1 samples, serum ferritin was measured to confirm IDA and HRM was then performed to detect any coexistence of β-globin gene mutation with IDA. Absence of β-globin gene mutation confirmed that there was no carrier in this group and thus none of the β-thalassemia traits was overlooked because of low level of HbA2.

From the borderline suspected Group 2, a participant with 3.5% HbA2 generated a HRM curve pattern different from the wild type cluster (without mutation in HBB gene) and also did not match with any of the HRM curves previously established for all the reported mutations in Bangladesh [2]. Sanger sequencing identified and confirmed the suspected mutation as c.151A > G

Table 2 Hemoglobin electrophoresis information of the study participants categorized based on MCV and MCH parameters

Groups	No. of participants, n (%) Total, *N* = 1877	Hb electrophoresis results					
		BTT suspects, n (%) (HbA2 > 3.5%)	ETT Suspects, n (%) (HbE = 25–30%)	HbE disease, n (%) (HbE > 90%HbA = 0%)	Others Hb Variants, n (%)	Total participants with abnormal Hb-electrophoresis, n (%)	Normal Hb electrophoresis results, n (%)
Group A (MCV ≥ 80 fL and MCH ≥ 27 pg)	995 (53.0)	3 (0.3)	2 (0.2)	0	4 (0.4)	9 (0.9)	986 (99.1)
Group B (MCV < 80 fL and MCH < 27 pg)	612 (32.6)	41 (6.6)	161 (26.3)	8 (1.3)	5 (0.8)	215 (35.0)	397 (65.0)
Group C (MCV < 80 fL and MCH ≥ 27 pg)	13 (0.7)	0	0	0	0	0	13 (100.0)
Group D (MCV ≥ 80 fL and MCH < 27 pg)	257 (13.7)	2 (0.8)	2 (0.8)	0	0	4 (1.6)	253 (98.4)

MCV Mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *BTT* β-thalassemia traits, *ETT, HbE traits* HbE disease refers to the homozygous states of HbE, whereas non-carrier status is defined as "Normal"

(ACT>GCT; Thr > Ala) in the HBB gene and upon BLAST with databases it was found to be a novel mutation, thereby confirming the carrier status of this participant (Fig. 1).

Identification of a single β-globin gene mutation in 41 out of 46 participants of group-3 could confirm their carrier status. However, of the rest five samples, three having 3.7% HbA2, one with 4% and one having 3.6% HbA2 turned out to be normal in HRM curve analysis and the HRM results were further confirmed by Sanger sequencing. All of these five participants had lower MCH values than the cutoff value (< 27 pg) and three of them showed MCV within normal range i.e. higher than the cutoff value (> 80 fL). Table S1 (Additional file 1: Table S1) shows the hematological features of these 5 participants having HbA2 > 3.5% without mutation in HBB gene.

Molecular analysis was also able to identify 2 participants (out of 165 HbE carriers based on Hb electrophoresis) with compound heterozygous mutations (c.79G > A + c.92 + 5G > C); one in Hb-E allele and another one in the trans-allele to HbE. These participants had relatively high levels of fetal hemoglobin (HbF of 2.8 and 11.4%), which also could justify the presence of mutations in both alleles because presence of a mutation in the allele which is trans to HbE allele (c.79G > A mutation) induces higher levels of HbF production. Hemoglobin indices of the two samples have been summarized in Table 4. However, homozygous c.79G > A was identified in all 8 participants of Group 5, thus confirming their HbE disease status. Therefore, the study identified a total of 163 HbE carriers, 42 β-thalassemia carriers and 8 participants with HbE disease.

Next we wanted to compare the sensitivity and specificity between traditional hematological analysis and HRM-based molecular approach (Table 5). Compared to the molecular method with 100% sensitivity and 100% specificity, combination of CBC and Hb electrophoresis tests showed 99.55 (95% CI, 97.51–99.92) sensitivity and 99.82 (95% CI, 99.47–99.94) specificity. Furthermore, only Hb electrophoresis gave 5 false positive and one false negative results, whereas the combination of CBC and Hb electrophoresis resulted in faulty detection in 4 cases.

Mutation spectrum in the HBB gene of the study participants with thalassemia traits

As shown in Table 6, out of nine different mutations including a novel mutation, the most common mutation was c.79G > A (CD 26/ HbE) (73.42%) followed by c.92 + 5G > C (14.41%).

Frequency of thalassemia carriers and other hemoglobinopathies among Bangladeshi population

ETT was found to be the most prevalent trait with a frequency of 8.68% (163/1877) followed by 2.24% BTT

Table 3 Molecular analysis of the selected participants for confirmation of the carrier status

Groups	Selection parameters	Total, n	Mutation Absent, n	Number of participants having mutation in β-globin gene, n	
				Heterozygous	Homozygous[a]/compound heterozygous[b]
Group 1	HbA2 < 2.2% Hb < 10 g/dl	64	64	0	0
Group 2	Hb A2: 3.3–3.5% (borderline suspected)	25	24	01	0
Group 3	HbA2 > 3.5%	46	05	41	0
Group 4	HbE: 25–40%	165	0	163	2[b]
Group 5	HbE > 90% HbA = 0%	08	0	0	8[a]

Hb Hemoglobin; [a] indicates the presence of two mutations in homozygous condition and [b] indicates the presence of compound heterozygous mutation

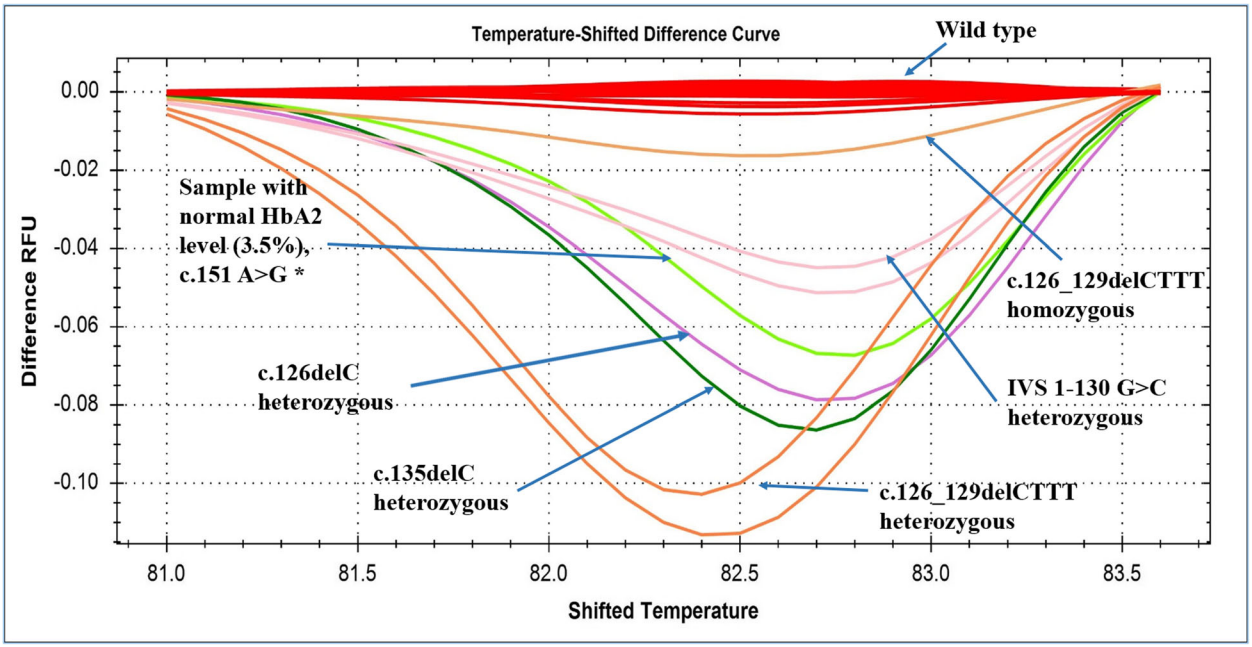

Fig. 1 HRM curve analysis for mutation detection in β-globin gene targeting the hot-spot region. The temperature shifted difference curves generated by the mutant alleles of unknown samples could be easily distinguished from the wild type samples and identified by comparing to the controls with known mutations based on differences in the melting curve shapes. RFU, Relative Fluorescence Unit; * indicates novel mutation

(42/1877) and; thus ETT and BTT together comprised of a total frequency of 10.92% (205/1877). Moreover, participants with HbD trait, asymptomatic HbE disease, suspected NTD HbE-β-thalassemia, hereditary persistence of fetal hemoglobin (HPFH) and α-thalassemia trait were also identified (Table 7). Altogether, 11.89% (223/1877) participants were carriers of abnormal hemoglobin genes.

Contribution of consanguinity to increase thalassemia carrier frequency in the country

There were 81 (4.32%; 95% CI, 3.4–5.24) participants of consanguineous parents. The carrier frequency among the participants with history of consanguinity was 23.5% (19/81), whereas it was almost half (11.4%, 204/1796) among the children of non-consanguineous parents. The highest consanguinity was observed in Rangpur division (8/140; 5.7%), which also had the highest carrier frequency among the eight administrative divisions of Bangladesh. The findings suggest that consanguinity contributes significantly to the increased rate of thalassemia in Bangladesh.

Distribution of β-thalassemia and HbE carriers across eight divisions of Bangladesh

We found that the frequency of ETT was higher than that of the BTT across all divisions except Barisal (Fig. 2). The ETT frequency varied from as low as 4.2% (95% CI, 1.65–6.75) in Khulna Division to as high as 25% (95% CI, 17.83–32.17) in Rangpur. Conversely, the highest BTT frequency was found in Barisal Division (3.9%; 95% CI, 0.57–7.23). Unexpectedly, we could not detect any participants with BTT in Khulna Division. The highest frequency of BTT plus ETT was found in Rangpur division (27.1%; 95% CI, 19.74–34.46) followed by Rajshahi Division (16.4%; 95% CI, 11.22–21.58).

Discussion

This is the first thalassemia carrier screening study conducted among young individuals of marriageable-age in Bangladesh. The study aimed to determine (1) nationwide carrier frequencies of β-thalassemia and HbE traits more precisely using molecular approach as supplementation to hematological and electrophoretic indices for rectification of the false positive or false negative cases (2) distribution

Table 4 Hemoglobin indices of the two participants containing compound heterozygous mutation

Participant No.	Age (Years)	Hb (g/dl)	MCV (fL)	MCH (pg)	RDW (%)	HbA (%)	HbF (%)	HbE (%)	HbA2 (%)
1	20	9.7	69.6	20.8	19	63.5	11.4	22.1	3
2	21	9.9	66	19	19.3	79.3	2.8	14.3	3.6

Table 5 Comparison of sensitivity and specificity among the conventional hematological-analysis based approaches for thalassemia carrier detection

Screening method	Mean sensitivity, % (95% CI)	Mean specificity, % (95% CI)	Positive predictive value, % (95% CI)	Negative predictive value,% (95% CI)	Diagnostic accuracy, % (95% CI)	True +ve (n)	False + ve (n)	True –ve (n)	False -ve(n)
MCV + MCH only	96.04 (92.64–97.9)	59.76 (57.37–62.17)	24.72 (21.98–27.67)	99.1 (98.29–99.52)	64.14 (61.95–66.28)	218	664	986	9
Hb electrophoresis	99.55 (97.51–99.92)	99.7 (99.29–99.87)	97.81 (94.97–99.06)	99.94 (99.66–99.99)	99.68 (99.3–99.85)	223	05	1648	01
MCV+ MCH and Hb Electrophoresis	99.55 (97.51–99.92)	99.82 (99.47–99.94)	98.67 (96.17–99.55)	99.94 (99.52–99.96)	99.79 (99.66–99.99)	223	03	1650	01

CI Confidence Interval, *MCV* Mean corpuscular volume, *MCH* Mean corpuscular hemoglobin

Table 6 Mutation spectrum of β-globin gene in the β-thalassemia and HbE carriers in Bangladeshi population

SL No.	Mutation Pattern	Number of samples, n (%; 95% CI)
1	c.79G > A (HbE)	163 (73.42; 67.62–79.22)
2	c.92 + 5G > C	32 (14.41; 9.8–19.02)
3	c.79G > A + c.79G > A	8 (3.61; 1.16–6.06)
4	c.92 + 130G > C	01 (0.45; 0.43–1.33)
5	c.151A > G [a]	01 (0.45; 0.43–1.33)
6	c.126_129delCTTT	03 (1.35; 0.16–2.86)
7	c.27_28insG	02 (0.90; 0.34–2.14)
8	c.47G > A	03 (1.35; 0.16–2.86)
9	c.79G > A + c.92 + 5G > C	02 (0.90; 0.34–2.14)

[a]novel mutation; not reported in Bangladeshi population and also globally [23]

of division-wise carrier frequencies, and (3) the mutation spectrum in the HBB gene of the carriers.

Carrier frequency of ETT plus BTT was 10.92% (95% CI, 9.51–12.33), where ETT had the highest frequency (8.68%; 95% CI, 7.41–9.95) followed by BTT (2.24%; 95% CI, 1.57–2.91). A previous study conducted in 2005 by Khan et al. reported an average frequency of 10.2% in Bangladeshi population with 6.1% ETT and 4.1% BTT [13]. This difference in frequencies between these two studies can be explained by the fact that previous studies used only conventional hematological approaches which often give false positive and false negative results [14, 15], whereas the present study applied molecular approach to avoid faulty detection of any carriers. Moreover, this study was conducted on a larger number of participants and the number of total participants as well as division-wise enrollment was enumerated statistically in proportion to the population size of each division for a precise apprehension of distribution of carrier frequency. Other studies conducted in neighboring India and Sri Lanka reported the comparable BTT frequencies of 2.68–4.05% and 2·2%, respectively, where the ETT frequencies were 3.4 and 0.5%, respectively [24–26]. However, our study showing an alarmingly high ETT frequency (8.68%) is supported by the previous study in Bangladesh [13]. Among eight administrative divisions, the highest carrier frequency was detected in Rangpur Division, where the HbE carrier frequency was 25%. Previous study showed the highest thalassemia carrier frequency in Rajshahi Division because the newly created administrative region of Rangpur was the part of Rajshahi Division during that study.

Table 7 Distribution of thalassemia carriers and other Hb variants among the study participants

Types of thalassemia carriers and other Hb variants	Number of participants, n	Frequency %, (95% CI)
HbE Trait (ETT)	163	8.68 (7.41–9.95)
β-thalassemia trait (BTT)	42	2.24 (1.57–2.91)
HbE disease	08	0.43 (0.13–0.73)
α-thalassemia trait	02	0.11 (0.04–0.26)
HbD trait	03	0.16 (0.02–0.34)
Suspected NTD HbE/β-thalassemia	02	0.11 (0.04–0.26)
HPFH	03	0.16 (0.02–0.34)
Total carriers of mutations in one or both alleles of globin genes	223	11.89 (10.43–13.35)

CI Confidence Interval, *Hb* Hemoglobin, *HPFH* Hereditary persistence of fetal hemoglobin, *NTD* Non-transfusion dependent

The first line of screening for identification of thalassemia carriers is a complete blood count followed by a measurement of HbA2 and HbF proportions. Since screening by hematological indices may result in misdetection of carriers due to factors like co-inheritance of α-thalassemia, mutations in β- and δ-globin genes, mutations in KLF1 gene and iron deficiency anemia [15, 27, 28], this study applied a comparatively cheaper and easy-to-perform DNA-based HRM curve analysis method to confirm and characterize mutations in the β-globin gene. This method supplements the conventional hematological and electrophoretic parameter based approaches for identification of the thalassemia carriers and patients [2]. For example, although the parameters MCV ≥ 80 fL and MCH ≥ 27 pg are usually considered as a negative indicator for HbE trait, our study using electrophoresis had identified two HbE carriers with MCV ≥ 80 fL and MCH ≥ 27 pg and a case having MCV > 80 fL and MCH < 27 pg as HbE carrier, indicating the shortcomings of hematological indices in screening of HbE carriers. Similarly, five participants with HbA2 > 3.5%, which is a widely used indicator of beta thalassemia trait, turned out to be normal by HRM curve analysis and Sanger sequencing, thereby further demonstrating shortfalls of Hb electrophoresis in detection of thalassemia carriers. The higher HbA2 might be caused by mutations in the KLF1 gene leading to borderline high HbA2 and thus may result in false positive findings in Hb electrophoresis [15]. Moreover, a number of studies reported frequent detections of high HbA2 levels in healthy individuals without any mutation in the HBB gene [29, 30]. On the other hand, specimens with HbA2 level in the borderline range (3.3–3.5%) with low MCV and/or low MCH must be subjected to DNA-based analysis to determine the carrier status of the participants. Notably, borderline HbA2 level might result from coinheritance of β-globin gene mutations with iron deficiency anemia and α thalassemia traits that usually lower the level of HbA2 to normal or borderline range in the β-thalassemia carriers [31]. In this study, all the specimens with abnormal hematological indices were tested using high resolution melt (HRM) curve analysis to confirm the

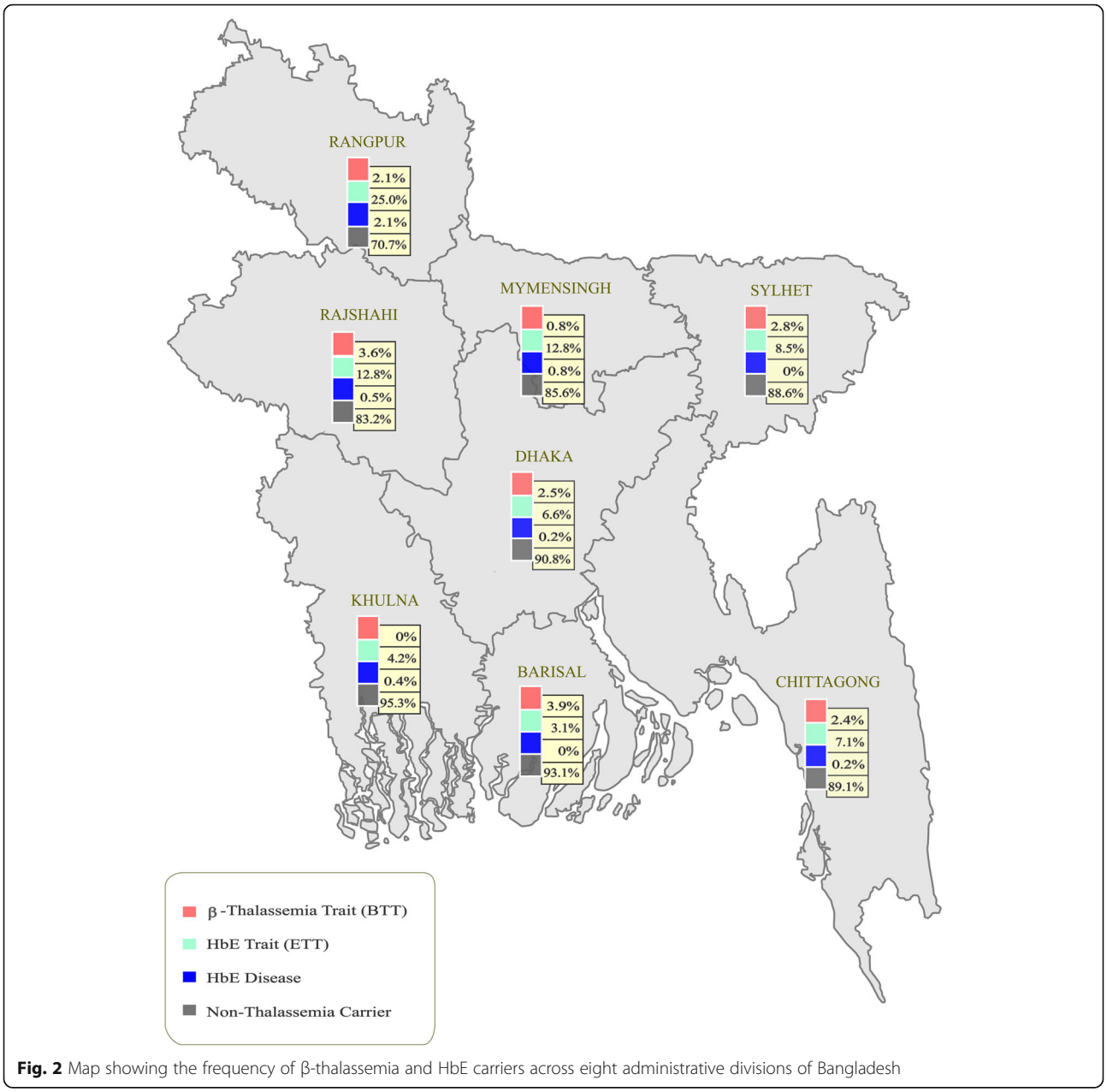

Fig. 2 Map showing the frequency of β-thalassemia and HbE carriers across eight administrative divisions of Bangladesh

presence of mutation in β-globin gene and if the molecular tests were not performed, about 5 in every 1000 carriers of the β-thalassemia and HbE variants would have been missed and around 1.8% cases could have been interpreted erroneously. Therefore, although the combination of MCV, MCH and Hb electrophoresis resulted in high sensitivity and specificity, the DNA-based approaches like HRM curve analysis and Sanger sequencing had been proved to be very useful to avoid false positive and false negative results by detecting mutations in the β-globin gene, and thereby confirming the true thalassemia carrier status of the participants. In a previous study, we described the advantages and cost-effective nature of this approach over other DNA-based screening methods like Denaturing High Performance Liquid Chromatography (DHPLC), Single Strand Conformational Polymorphism (SSCP) and Denaturing Gradient Gel Electrophoresis (DGGE), Amplification Refractory Mutation System (ARMS) PCR, Sanger nucleotide sequencing etc. [2]. Notably, this real time PCR-based high throughput HRM curve analysis is easy to perform and time-saving as there is no need of post PCR amplification processes like PCR product purification, gel electrophoresis etc. All these advantages offered by the HRM-based techniques make it an ideal candidate for molecular screening of thalassemia in countries of thalassemia belt and resource limitation.

More importantly, this study identified two clinically asymptomatic individuals with pathogenic mutations in both alleles of HBB gene using HRM method, who were detected as carriers by Hb electrophoresis. Although the association between genotype and phenotype is established for both α- and β-thalassemia, differentiation into various phenotypes of thalassemia is mostly based on clinical signs and symptoms. However, the same mutations i.e. c.79 G > A + c.92 + 5G > C have been reported as pathogenic in patients with mild to severe form of HbE-beta thalassemia [32, 33]. Hence, although currently these two individuals who seem to be carriers without any clinical manifestations, they might be at risk of developing non-transfusion dependent thalassemia (NTDT) in future. In our ongoing study on the role of various genetic modifiers on clinical heterogeneity of thalassemia patients in Bangladesh, the age of first transfusion of NTDT patients ranged from 13 to 60 years (unpublished data). It should also be mentioned that patients with hemoglobin E/β-thalassemia show different phenotypic variability at different stages of development [34]. Since numerous factors have been identified to be associated with disease severity of the NTDT patients, identification of NTDT patients is vital for prognosis because increased intestinal iron absorption in such patients increases the risk of thrombotic disease, pulmonary hypertension, sudden cardiac arrest, and liver damages etc. [34–36]. Thus the significance of diagnosis of NTDT is crucial because a timely treatment intervention will curtail the progression of disease severity and thus prevent an untimely death. All these aspects emphasize on the requirement of molecular-based carrier screening which is the ultimate confirmation of a carrier status. Furthermore, the study identified 9 different mutations including a novel mutation (c.151A > G) in the beta-globin gene of the carriers. Further studies are needed to know the pathogenesis of this novel mutation.

With the current ETT plus BTT carrier frequency of 10.92%, 9176 babies are born with thalassemia each year (according to Hardy-Weinberg equation), thereby further worsening the situations of thalassemia patients [13, 19]. Moreover, the study found that the thalassemia carrier frequency was almost double among the children with history of consanguineous marriage, a common socio-culture of this region and thus consanguinity contributes to increased burden of thalassemia. Even though majority of the participants were university/college going students, 62% of them did not know the disease etiology and about 32% did not have any knowledge about the disease prior to enrolment suggesting that the knowledge regarding thalassemia is quite insufficient among the mass population.

At present, the yearly medical cost required for thalassemia patient ranges from $1632 to $3960 in Bangladesh and there is neither a national insurance facility nor a subsidized or free treatment system from the government [12], suggesting a severe health, economic and emotional burden to the nation and thus adoption of a national thalassemia prevention strategy is a demand of time. Several thalassemia endemic countries have set up comprehensive national prevention programs, which include public awareness and education; carrier screening using molecular diagnostics, genetic counseling and prenatal diagnosis [37, 38]. Effectiveness of such prevention program in Sardinia is evidenced by a reduction in the birth rate with thalassemia major from 1:250 live births to 1:4000 and such success is also achieved by other countries including Cyprus, Iran, and Turkey [10, 38, 39]. Although carrier screening and counseling are being performed on a voluntary basis in some countries, countries like UAE, Saudi Arabia, Jordan, Cyprus, Iran and Turkey are performing compulsory premarital screening for thalassemia to discourage marriage between two carriers [38]. These strategies may help guide health policy makers of Bangladesh to adopt an appropriate thalassemia prevention strategy considering the available resources, religious values and social culture.

In summary, as prevention of thalassemia is far cheaper and better than treatment and currently no affordable cure is available, an immediate and concerted action on thalassemia prevention should be made mandatory in Bangladesh. A massive awareness program targeting general population and an intensive educational program for health personnel including physicians, nurses, health and family planning workers should be carried out nationwide promptly. Also, appropriate screening methods combining hematological, electrophoretic and molecular approaches associated with genetic counseling should be required in existing hospitals and health facilities. The information of this study will be helpful in several ways, such as measuring nationwide carrier frequency with accuracy and grasp the gravity of the situation, identifying the at-risk population and thus prioritizing them, and necessities and benefits of molecular-based carrier screening. Moreover, this study demonstrated the feasibility and usefulness of cost-effective HRM approach in resource limited settings which can be followed in other countries of thalassemia-belt for detection of HBB gene mutations and confirmation of the carrier status.

Conclusion

This study highlights that adoption of a molecular screening method for detection of mutations in the HBB gene could overcome the shortcomings of the conventional methods, in particular, for prenatal and newborn screening and for confirmation of the inconclusive cases by the traditional approaches. With the current carrier frequency, HbE/β-thalassemia will be posing a tremendous threat to the public health of Bangladesh if necessary measures like awareness program for mass population and medical personnel and; establishment of

carrier screening facilities aligned with genetic counselling in health centers and hospitals across the country, are not implemented immediately. Lastly, the HRM-based cost-effective molecular methods can be initiated in other thalassemia-prone countries and help in fighting these non-curable and life-threatening disorders.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13023-020-1294-z.

Additional file 1: Table S1. Hematological features of the participants having high HbA2 level (HbA2 > 3.5%) without mutation in beta globin gene of hemoglobin.

Abbreviations

BMT: Bone Marrow Transplantation; BTT: Beta-thalassemia trait; CBC: Complete Blood Count; CI: Confidence Interval; EDTA: Ethylenediamine tetra acetic acid; ETT: HbE Trait; fl: Femtolitre; Hb: Hemoglobin; HBB: Hemoglobin beta subunit gene; HPFH: Hereditary Persistence of Fetal Hemoglobin; HRM: High Resolution Melting; IDA: Iron Deficiency Anemia; KLF1: Krueppel-like factor 1; MCH: Mean Corpuscular Hemoglobin; MCV: Mean Corpuscular Volume; NTDT: Non Transfusion Dependent Thalassemia; PCR: Polymerase Chain Reaction; pg: Pictogram; WHO: World Health Organization

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Authors' contributions

FAN, GSB, MTI and MH conducted the laboratory work, data analysis and manuscript draft writing. NS, SKS, KI, WAH, MR and SKQ assisted in clinical information collection, interpretation and to improve the manuscript. KM, FQ, SSQ, HUS designed the study plan and supervised the overall project. All authors read and approved the final manuscript.

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Availability of data and materials

All relevant data are within the paper. Further information is available from the authors on request.

Ethics approval and consent to participate

This study was approved by the National Ethics Research Committee (NERC) of Bangladesh Medical Research Council (BMRC). Registration no # 102 03 04 2018.

Consent for publication

All the participants described in this article provided written informed consent for participation in the study and for publication of the results. All the authors have read and approved the paper for publication.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Laboratory of Genetics and Genomics, Institute for Developing Science and Health Initiatives, Mohakhali, Dhaka 1212, Bangladesh. [2]Department of Biochemistry and Molecular Biology, University of Dhaka, Shahbagh, Dhaka 1000, Bangladesh. [3]Department of Virology, Dhaka Medical College Hospital, Shahbagh, Dhaka 1000, Bangladesh. [4]Directorate General of Health Services, MoHFW, Government of Bangladesh, Mohakhali, Dhaka 1212, Bangladesh. [5]Department of Biochemistry and Molecular Biology, Dhaka Shishu Hospital, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh. [6]Department of Hematology, Bangabandhu Sheikh Mujib Medical University, Shahbagh, Dhaka 1000, Bangladesh. [7]Department of Pediatric Medicine, KK Women's and Children's Hospital, 100 Bukit Timah Road, Singapore, Singapore. [8]Department of Enteric and Respiratory Infectious Diseases, Infectious Diseases Division, International Centre for Diarrhoeal Disease Research, Mohakhali, Dhaka 1212, Bangladesh.

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Publisher's Note

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Bangladesh Medical Research Council
BMRC

Ref: BMRC/NREC/2016-2019/612 Date: 23/04/2018

National Research Ethics Committee

Registration Number: 102 04 03 2018.

Principal Investigator:
Md. Kaiissar Mannoor, PhD
Head and Senior Scientist
Institute for Developing Science and
Health Initiatives (ideSHi), IPH Building (2nd Floor)
Mohakhali, Dhaka 1212.

Title of the Project:
"Determination of prevalence of Thalassemia"

Duration of Project: 6 Months

Budget: BDT- 44,95,300/-
In words: Forty Four Lac Ninety Five Thousand Three Hundred Taka Only.

Subject: Ethical Clearance

With reference to your application on the above subject, this is to inform you that above mentioned Research Title has been registered and approved by the National Research Ethics Committee (NREC).

(Dr. Mahmood-uz-jahan)
Director

N.B: You are requested to follow the guidelines as mentioned at page two.

THE ETHICAL GUIDELINES TO BE FOLLOWED BY THE PRINCIPAL / CO-INVESTIGATORS

- ☐ The rights and welfare of individual volunteers are adequately protected.
- ☐ The methods to secure informed consent are fully appropriate and adequately safeguard the rights of the subjects (in the case of minors, consent is obtained from parents or guardians).
- ☐ The Investigator(s) assume the responsibility of notifying the National Research Ethics Committee (NREC) if there is any change in the methodology of the protocol involving a risk to the individual volunteers.
- ☐ To report immediately to the NREC if any evidence of unexpected or adverse reaction is noted in the subjects under study.
- ☐ Principal Investigator will facilitate supervision of the project by the BMRC authority time to time.
- ☐ This approval is subject to Principal Investigator's reading and accepting the BMRC ethical principles and guidelines currently in operation.
- ☐ You are requested to submit a report to the BMRC half yearly and after completion of the research work.

Checked by: GV

Informed Consent and Questionnaire form (Page 1)

গোপনীয়, শুধু গবেষনার কাজে ব্যাবহৃত হবে Sample Identification Number: **TPD** | | | | | |

Determination of Prevalence of Thalassemia

Conducted for: *Non Communicable Diseases Control (NCDC), Directorate of General Health Services (DGHS), Ministry of Health and Family Welfare (MOHFW), Bangladesh*

Conducted by: Institute for developing Science and Health Initiatives (ideSHi) Foundation

SURVEY QUESTIONNAIRE

সাক্ষাৎকার গ্রহনের তারিখঃ							সময়ঃ	ঘণ্টা			মিনিট		

অংশগ্রহনকারীর বয়সঃ ১৮-২৫ বছর বয়সের পুরুষ ও মহিলা

সমীক্ষা ও গবেষনার উদ্দেশ্যঃ

বর্তমানে আমরা থ্যালাসেমিয়া নামক এক মরণঘাতি বংশগত রক্তরোগের বিস্তৃতি জানার জন্য বাংলাদেশ সরকারের স্বাস্থ্য অধিদপ্তরের আহ্বানে একটি সমীক্ষা ও গবেষণা করছি। ইতোপূর্বে বিচ্ছিন্নভাবে বাংলাদেশে এই রোগের উপর গবেষনা হয়েছে। থ্যালাসেমিয়া একটি মারাত্মক বংশগত রক্তরোগ। বাবা মা উভয়েই যদি এ রোগের বাহক হয়ে থাকেন তবে প্রতি গর্ভকালে শিশুর থ্যালাসেমিয়া নিয়ে জন্মগ্রহন করার আশংকা ২৫%। উল্লেখযোগ্য যে এ রোগের বাহকেরা সুস্থ জীবন যাপন করেন। ফলে নিজের অজান্তে তাঁরা তাঁদের সন্তানদের এই ভয়াবহ রোগের উৎস ও কারন হয়ে থাকেন। এতে করে থ্যালাসেমিয়া রুগীর সংখ্যা দিনে দিনে বেড়েই চলেছে।একটি সমীক্ষায় দেখা গেছে আমাদের দেশে ১০% জনগণ থ্যালাসেমিয়া রোগের বাহক।একসময় মনে করা হতো শুধুমাত্র রক্তসম্পর্কীয় আত্মীয়ের সাথে বিয়ে হলেই সন্তানের থ্যালাসেমিয়া রোগহয়। কিন্তু আমাদের দেশে বাহকের সংখ্যা অনেক বেড়ে যাওয়ায় অনাত্মীয়ের মধ্যে বিয়ের ফলে ও শিশু থ্যালাসেমিয়া রোগ নিয়ে জন্মগ্রহন করে।বলতে গেলে আমরা একটা ভীতিকর অবস্থানে আছি। আরেকটা উল্লেখযোগ্য ব্যপার হলো, শুধু থায়ালাসেমিয়াই নয়, এখানে যথেষ্ট হিমগ্লোবিন ই এর বাহক রয়েছে এবং এর সংখ্যা থ্যালাসেমিয়ার বাহকের চেয়ে অনেক বেশি।থ্যালাসেমিয়া বাহকের সাথে হিমগ্লোবিন ই এর বাহকের বিয়ে হলে ই-বিটা থ্যালাসেমিয়া হয় যা বিটা থ্যালাসেমিয়া মেজরের মতই ভয়ংকর। থ্যালাসেমিয়া রোগের একমাত্র স্থায়ী চিকিৎসা অস্থি মজ্জা সংস্থাপণ (Bone marrow transplantation) যা অত্যন্ত ব্যায়বহুল। বেঁচে থাকার জন্য সারাজনম নির্দিষ্ট সময় পরপর এদের রক্ত নিতে হয়। তা সত্ত্বে ও এদের বারবার রক্ত গ্রহণের ফলে নানাবিধ সমস্যা এবং উপসর্গ দেখা দেয় যার চিকিৎসা ব্যয়বহুল ও কষ্টদায়ক। সুচিকিৎসা করালেও এঁদের আয়ুষ্কাল স্বাভাবিক অপেক্ষা অনেক কমে যায় এবং অকাল মৃত্যু হয়। থ্যালাসেমিয়ার প্রতিকার করা যায় না। শুধু বাহকের সাথে বাহকের বিয়ে বন্ধ করেই এই রোগ প্রতিরোধ করা সম্ভব। তাই আপনি বাহক কি না তা জানা আপনার ভবিষ্যতের জন্য খুব ই জরুরী। আসুন আমরা এখন সচেতন হই এবং স্বেচ্ছায় রক্ত পরীক্ষা করে নিজেরা বাহক কিনা তা জেনে নেই। আপনার কাছ থেকে যে তথ্য নিবো তা গোপন রাখা হবে এবং শুধুমাত্র গবেষনার কাজে ব্যবহার করা হবে। উল্লেখ্য, রক্ত পরীক্ষা করে আপনি থ্যালাসেমিয়ার বাহক/ বাহক নয় তার রিপোর্ট আপনার ব্যক্তিগত ই-মেইলে যথাসময়ে পাঠিয়ে দেয়া হবে। এই কাজে অংশগ্রহণের জন্য আপনাকে কোন পারিশ্রমিক বা অন্যকোন কিছু দেয়া হবে না। এই সমীক্ষায় অংশগ্রহণ করা বা না করা সম্পূর্ণ আপনার নিজ ইচ্ছা। স্বেচ্ছায় অংশগ্রহণকরেও আপনার ইচ্ছা হলে আপনি কোন প্রশ্নের উত্তর নাও দিতে পারেন।

অংশগ্রহণকারীর সাক্ষাৎকার

১	অংশগ্রহণকারীর নাম	পুরুষ
২	লিঙ্গ	নারী ☐ পুরুষ ☐
৩	বয়স	

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৪	পিতার নাম	
৫	মাতার নাম	
৬	ফোন নম্বর	
৭	ই-মেইল (পরিষ্কারভাবে লিখুন)	
৮	ঠিকানা	
৯	বিভাগ	
১০	বসবাসের স্থান	শহরাঞ্চল ☐ গ্রামাঞ্চল ☐
১১	জাতিগত অবস্থা	অ-উপজাতীয় ☐ উপজাতীয় ☐
১২	আপনি থ্যালাসেমিয়া রোগটির নাম কখনও শুনেছেন?	হ্যাঁ ☐ না ☐
	যদি শুনে থাকেন, তাহলে কিভাবে ছড়ায় তা কি জানেন?	হ্যাঁ ☐ না ☐
১৩	অংশগ্রহণকারীর পিতা মাতার মধ্যে নিকট আত্মীয়তার সম্পর্ক আছে কি না-	হ্যাঁ ☐ না ☐
	যদি হয়ে থাকে তাহলে আত্মীয়তার ধরন	
১৪	পরিবারে থ্যালাসেমিয়ার রুগী বা বাহক আছে কি না -	হ্যাঁ ☐ না ☐ জানা নেই ☐
	যদি থাকে,	বাহক ☐ রোগী ☐

সম্মতির স্বীকারোক্তি-

আমি বুঝেছি যে, এ সমীক্ষা ও গবেষনায় অংশগ্রহণ স্বেচ্ছামূলক এবং আমি যে কোন সময় কোন বাধ্যবাধকতা ছাড়াই গবেষনা থেকে নিজেকে বিরত রাখতে পারবো। আমি এই সমীক্ষা ও গবেষণার উদ্দেশ্য ও শর্ত গুলো পড়েছি এবং আমি স্বেচ্ছায় এতে অংশগ্রহন করতে সম্মতি জানাচ্ছি।

অংশগ্রহনকারীর নাম ও স্বাক্ষরঃ তারিখঃ

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	সুপারভাইজার	স্বাক্ষী ১	স্বাক্ষী ২
নাম			
স্বাক্ষর			
তারিখ			

PART-H

সম্মতিপত্র

(অংশগ্রহণকারীর বয়স : ৫ থকে <৬৫ বছর)

১। প্রকল্পের শিরোনামঃ

বাংলাদেশের ই-বিটা থ্যালাসেমিয়াতে আক্রান্ত রোগির রোগের তীব্রতার সাথে জিনগত পরিবর্তনের পারস্পরিক সম্পর্ক স্থাপন।

২। গবেষকঃ ১) ফারজানা আক্তার নূর, রিসার্সফেলো, ইন্সটিটিউট ফর ডেভেলপিং সায়েন্স অ্যান্ড হেলথ ইনিসিয়েটিভস (আইদেশি), ঢাকা-১২১২, বাংলাদেশ।

৩। ভূমিকা ও গবেষণার উদ্দেশ্যঃ

হিমোগ্লোবিন-ইজিনগত প্রচ্ছন রোগটি হয়ে থাকে বিটা-গ্লোবিন জিন এ বংশগত পরিবর্তন আসার ফলে, যার ফলে কম পরিমাণ স্বাভাবিক হিমোগ্লোবিন প্রোটিনতৈরী হয়। হিমোগ্লোবিন-ই রোগটির মত বিটা-থ্যালাসেমিয়াও হয়ে থাকে বিটা-গ্লোবিন জিন এ জন্মগত সমস্যা হওয়ার কারণে, তবে এই রোগে আক্রান্ত ব্যক্তির মধ্যে খুব কম পরিমাণ বিটা-গ্লোবিন তৈরী হয় অথবা কোন বিটা-গ্লোবিন তৈরী হয় না। সমষ্টিগতভাবে এই রোগ দুইটি দিয়ে পৃথিবীর সবচেয়ে বেশি সংখ্যক মানুষ আক্রান্ত। যদিও বিটা-থ্যালাসেমিয়াতে আক্রান্ত রোগীরা বারবার রক্তশূন্যতাজনিত গুরুত্বর অবস্থার সম্মুখীন হন, হিমোগ্লোবিন-ই তে আক্রান্ত রোগীরা সাধারনত সামান্যই রক্তাল্পতাজনিত সমস্যারমুখোমুখি হয় থাকেন। তবে হিমোগ্লোবিন-ই রোগে আক্রান্ত ব্যক্তির রোগের তীব্রতা অল্প থেকে তীব্র হতে পারে যদি হিমোগ্লোবিন-ই রোগের পাশাপাশি বিটা-গ্লোবিন জিনে বাড়তি আরও পরিবর্তন থেকে থাকে, যা হিমোগ্লোবিন-ই/বিটা-থ্যালাসেমিয়া নামে পরিচিত। বাংলাদেশে হিমোগ্লোবিন-ই এবং বিটা-থ্যালাসেমিয়া দুইটি রোগই খুব দেখতে পাওয়া যায়, এই রোগ দুটোর বাহকদের ব্যাপ্তি যথাক্রমে ৩% এবং ৪%।

হিমোগ্লোবিন-ই রোগটির ফলে রক্তস্বল্পতা সচরাচর দেখতে পাওয়া যায় না এবং রোগীর বারবার রক্তগ্রহণের প্রয়োজন হয় না। কিন্তু হিমোগ্লোবিন-ই/বিটা-থ্যালাসেমিয়া রোগে আক্রান্ত রোগীদের রক্তগ্রহণের মধ্যবর্তী সময় এক রোগী থেকে অন্যরোগীতে আলাদা হতে দেখতে পাওয়া যায় এবং এর সঙ্গে বিটা-গ্লোবিন জিনের পরিবর্তনগুলোর সম্পর্ক থাকতে পারে। এই পরিপ্রেক্ষিতে আমরা থ্যালাসেমিয়াতে আক্রান্ত রোগিদের রোগের তীব্রতার সাথে জিনগত পরিবর্তনের পারস্পরিক সম্পর্ক পর্যালোচনা করতে চাই। গবেষণার ফলাফল আমদের হিমোগ্লোবিন-ই/বিটা-থ্যালাসেমিয়ায় আক্রান্ত রোগীদের রক্তগ্রহণের মধ্যবর্তী সময় আলাদা হওয়ার কারণ অবগত করবে। এছাড়াও গবেষণায় প্রাপ্ত ফলাফল সঠিক সময়ে জীবনের প্রথমদিনগুলোতেই থ্যালাসেমিয়া রোগের চিকিৎসা এবং ব্যবস্থাপনা সংক্রান্ত সিদ্ধান্ত নিতে সহায়ক হতে পারে যা এই রোগের সাথে জড়িত সমস্যা যেমনঃ স্প্লেনোমেগালি, বিভিন্ন অঙ্গে আয়রন জমে যাওয়া এই সকল সমস্যা এড়িয়ে যেতে অথবা এগুলোর সূত্রপাতকে বিলম্বত করবে।

৪। গবেষণায় অংশগ্রহণের জন্য আপনাকে অনুরোধের কারণঃ

আমাদের এই গবেষণাটি ০ থকে ১৮ বছরবয়সের থ্যালাসেমিয়ায় আক্রান্ত রোগিদের উপর পরিচালিত হবে। আমাদের পর্যালোচনায় আপনার শিশু গবেষণায় অংশগ্রহণের যোগ্য হলে, আমরা আপনাকে এই প্রচেষ্টায় অংশগ্রহণের জন্য অনুরোধ করবো।

৫। গবেষণা পদ্ধতিঃ

আপনি যদি এই গবেষণায় অংশগ্রহণ করতে চান তবে গবেষকগণ আপনাকে নিম্নলিখিত কাজসমূহ করার জন্য আহবান করবে।

৫.১ আপনাকে এই সম্মতি পত্রে স্বাক্ষর করতে হবে এবং আপনি এই গবেষণার অংশীদার হবেন। যদি আপনি লিখতে বা পড়তে না পারেন তবে আমরা আপনাকে এই সম্মতি পত্রটি পড়ে শোনাব। আপনি এই গবেষণায় অংশগ্রহণ করতে সম্মতএর প্রমাণ স্বরূপ এই সম্মতি পত্রে আপনাকে আপনার বৃদ্ধাঙ্গুলির ছাপ দিতে হবে। আপনি এই গবেষণার উদ্দেশ্য বুঝতে পেরেছেন তার প্রমাণ হিসেবে আমরা একজন সাক্ষীর সাক্ষ্য রাখব।

৫.২.আপনাকে আমাদের অভিজ্ঞ চিকিৎসক প্রশ্নাবলী জিজ্ঞাসাবাদ করবেন।

৫.৩. এই গবেষণায়অন্তর্ভুক্ত হওয়ার সময় আপনার শিশুররক্তের নমুনা সংগ্রহ করা হবে। আপনার শিশু নবজাতক হলে ১মিঃ লিঃ (১/৫ চা চামচ পরিমাণ) এবং তদুর্ধ হলে ২.৫ মিঃ লিঃ (১/২ চা চামচ পরিমাণ) রক্ত বাহুর শিরা থেকে সংগ্রহ করব।

৫.৪. যদি পরীক্ষার ফলাফলে কোন অস্বাভাবিকতা ধরা পরে তবে আমরা আপনার শিশুর আরও কিছু পরীক্ষা করার জন্য আপনার সাথে যোগাযোগ করতে পারি।

৬. ঝুঁকি ও উপকারিতাঃ

৬.১.ঝুঁকি

আপনার সন্তান রক্ত সংগ্রহ কালে সূঁচ ফুটানোর সময় সামান্য খোঁচা লাগার মত ব্যথা অনুভব করবে যা ঝুঁকিপূর্ণ নয় এবং সামান্য রক্তপাতের কারণে রক্ত সংগ্রহের স্থানের চামড়ার চারপাশ হালকা নীলচে বর্ণ ধারণ করতে পারে। এই সমস্যাগুলো প্রতিরোধের জন্য আমরা একবার ব্যবহারযোগ্য সিরিঞ্জ এবং সূঁচ ব্যবহার করবএবং কোন সমস্যা দেখা দিলে আমরা আমাদের খরচে উপযুক্ত চিকিৎসা সেবা এবং ঔষধাদি প্রদান করব।

৬.২. উপকারিতা

এই গবেষণায় অংশগ্রহণকরলে আপনি আপনার শিশুর হিমোগ্লোবিন-ই রোগ, হিমোগ্লোবিন-ই/বিটা থ্যালাসেমিয়ার এবং বিটা থ্যালাসেমিয়ার সম্পর্কিত জন্মগত অবস্থা জানতে পারবেন এবং ফলস্বরূপ আপনি একজন বিশেষজ্ঞ ডাক্তারের পরামর্শ নিতে পারবেন।

৭। আর্থিক ব্যয়ঃ

আপনাকে এই গবেষণায় অংশগ্রহনের জন্য কোন খরচ দিতে হবে না এবং আপনাকে কোন পারিশ্রমিক প্রদান করা হবে না।

৮। গবেষণায় অংশগ্রহণকারীর অধিকারঃ

আপনার শিশু এই গবেষণায় অংশগ্রহণ করবেন বা না করবেন তা সম্পূর্ণভাবে আপনার ইচ্ছাধীন। আপনি গবেষণা চলাকালীন যে কোন সময়ে কোন প্রকার কারণ প্রদর্শন ছাড়াই আপনার শিশুকে এই কার্যক্রম থেকে প্রত্যাহার করে নিতে পারেনঅথবা নতুন পরীক্ষার জন্য যে কোন ল্যাবরেটরি নমুনা প্রদান নাও করতে পারেন। আপনার শিশু গবেষণায় অংশ না নিলে আপনার শিশুর কোন নমুনা সংগ্রহ করা হবে না।

৯। গোপনীয়তাঃ

গবেষনাগারে প্রাপ্ত ফলাফল , আপনার ও আপনার শিশুর ব্যক্তিগত তথ্য গোপনীয় স্থানে আলমিরায় তালাবদ্ধ করে রাখা হবে। গবেষণার গবেষকগণ, অংশগ্রহণকারী কর্মকর্তা, ইন্সটিটিউট ফর ডেভেলপিং সায়েন্স অ্যান্ড হেলথ ইনিসিয়েটিভস (আইদেশী) এর নীতি পর্যালোচনা কমিটি এবং তথ্য নিরাপত্তা পর্যবেক্ষণ কমিটি (এই দুটি কমিটি ফলাফল পর্যালোচনা এবং অংশগ্রহণকারীদের নিরাপত্তায় দায়িত্বপ্রাপ্ত) ছাড়া কেউ এই সব তথ্য দেখতে পারবে না। বাংলাদেশের তথ্য অধিকার আইন দ্বারা আপনার তথ্য সংরক্ষিত থাকবে। প্রতিবেদন প্রকাশ জন্য ফলাফল বিশ্লেষণের সময় অথবা চিকিৎসাবিদ্যা সম্পর্কিত পত্রিকায় গবেষণা পত্র প্রকাশের সময় আপনার নাম পরিচয় ব্যবহার করা হবে না।

আপনার কাছ থেকে সংগ্রহীত নমুনার অবশিষ্টাংশসমূহ আইদেশী ল্যাবরেটরিতে -৮০ ডিগ্রি সেলসিয়াস তাপমাত্রার ফ্রিজারে সংরক্ষণ করা হবে যা ভবিষ্যতে গবেষণায় ব্যবহার করা হতে পারে। কিন্তু নমুনার গায়ে আপনার নাম সংযুক্ত করা হবে না এবং ভবিষ্যতে কোনভাবেই তা থেকে আপনাকেসনাক্ত করা যাবে না। পরবর্তীতে যে কোন গবেষণায় আপনার কাছ থেকে সংগ্রহকৃত নামবিহীন নমুনা ব্যবহারের পূর্বে প্রথমে এই কেন্দ্রের নীতি পর্যালোচনা কমিটির কাছ থেকে সম্মতি নেয়া হবে। ভবিষ্যতে এই নামবিহীন নমুনা ব্যবহারের জন্যও আমরা আপনার অনুমতি চাচ্ছি। নামহীন অথবা সংকলিত তথ্য এবং উপাত্ত অন্যান্য গবেষকদেরকে হস্তান্তর করা যাবে যা অংশগ্রহনকারীর গোপনীয়তা রক্ষার সাথে সাংঘর্ষিক নয়। এই সম্মতিপত্রে স্বাক্ষরের মাধ্যমে আপনি উল্লেখিত ব্যক্তি এবং প্রতিষ্ঠানকে তথ্য ব্যবহারের অনুমতি দিচ্ছেন।

আপনি যদি আমাদের গবেষণা সম্পর্কে আরো কিছু জানতে চান এখনই বলতে পারেন বা পরে প্রধান গবেষক সুপ্রভাত কুমার সরকার এর কাছে আপনি ব্যক্তিগতভাবে বা টেলিফোনের মাধ্যমে জানতে পারেন নিম্ন লিখিত
ঠিকানায়ঃ

ফারজানা আক্তার নূর
ইন্সটিটিউট ফর ডেভেলপিং সায়েন্স অ্যান্ড হেল্‌থ ইনিসিয়েটিভস (আইদেশি),
আই পি এইচ, মহাখালী, ঢাকা-১২১২, বাংলাদেশ।

১০। অংশগ্রহণকারী শিশুর বৈধ অভিভাবকের ঘোষণাঃ

এই গবেষণার গবেষক/গবেষণাকর্মীগণ গবেষণার উদ্দেশ্য, পদ্ধতি, ঝুঁকি এবং উপকারিতা, গবেষণায় অংশগ্রহণকারী, ব্যক্তিগত তথ্যসহ গবেষণার ফলাফলের গোপনীয়তা ও তা সংরক্ষণের ব্যাপারে আমাকে বলেছেন এবং আমি তা বুঝেছি। আমি বুঝতে পেরেছিযে, কোন প্রকার কারণ প্রর্দশনব্যতীত আমি ইচ্ছে করলে যেকোন সময়ে গবেষণা কাজে অংশগ্রহণে অপারগতা প্রকাশ করতে পারি। আমাকে এও অবহিত করা হয়েছে যে, আমি চাইলে আমাকে ভবিষ্যতেও তথ্যাদিপ্রদান করা হবে এবং আমার নাম বা পরিচয় গবেষণার ফলাফল/গবেষণা পত্রে প্রকাশ করা হবে না। উপরোক্ত তথ্যের ভিত্তিতে আমি আমার এই গবেষণায় অংশগ্রহণে স্বেচ্ছায় সম্মতি প্রদান করছি।

১১। অংশগ্রহণকারীর বয়সঃ ০থকে ১০ বছর

অংশগ্রহনকারীর নাম _____

অংশগ্রহনকারীর অভিভাবকের স্বাক্ষর/বামহাতের বৃদ্ধাঙ্গুঁলির ছাপ_____তারিখঃ_____

অপ্রাপ্তবয়স্কদের সম্মতি(শিশুর বয়স ১১-৬৫ বছরের জন্য)

তদন্তকারীগণ অপ্রাপ্তবয়স্কদের যারা গবেষণায় সম্মতি দিয়েছেন তাদের সঙ্গে এই গবেষণায় অন্তর্ভুক্তির বিষয়ে আলোচনা করেছেন। এছাড়াও সন্তানের বাবা-মা / অভিভাবকের অনুমতি নথিভুক্ত করা হয়।

অংশগ্রহনকারীর নাম ----------

অপ্রাপ্তবয়স্ক অংশগ্রহনকারীর স্বাক্ষর/বামহাতের বৃদ্ধাঙ্গুঁলির ছাপ_____তারিখঃ_____

অপ্রাপ্তবয়স্ক অংশগ্রহনকারীর পিতা/মাতা বা অভিভাবকের নাম_____তারিখঃ _____

অপ্রাপ্তবয়স্ক অংশগ্রহনকারীর অভিভাবকের স্বাক্ষর/বামহাতের বৃদ্ধাঙ্গুঁলিরছাপ _____

তারিখঃ _____

স্বাক্ষীর নাম _____

স্বাক্ষীর স্বাক্ষর ---------- তারিখঃ_____

গবেষনায় সম্মতিপত্র গ্রহনকারীর নাম _____

গবেষনায় সম্মতিপত্র গ্রহনকারীর স্বাক্ষর _____তারিখঃ _____

_____ _____

প্রধান গবেষক অথবা তার প্রতিনিধির স্বাক্ষর তারিখঃ

বি.দ্র: আপনাকে স্বাক্ষর ও তারিখ সহকারে এই দলিলের একটি কপি দেওয়া হবে।

PART H

তথ্য সংগ্রহের পত্র

রোগীর নামঃ নমুনা নাম্বারঃ

রোগীর বাবার নামঃ

রোগীর মায়ের নামঃ

রোগীর বয়সঃ লিঙ্গঃ পুরুষ ☐ মহিলা ☐

উচ্চতাঃ ওজনঃ বিএমআইঃ

রক্তের গ্রুপঃ

স্থায়ী ঠিকানাঃ

মোবাইল নাম্বারঃ

জাতিগত গোষ্ঠীঃ অ- উপজাতীয় ☐ উপজাতীয় ☐

প্রাসঙ্গিক পারিবারিক ইতিহাস:

নিকটাত্মীয়ের মধ্যে বাবা মায়ের বিবাহ হয়েছে কিনা?

ভাইবোন আছে কিনা? হ্যাঁ ☐ না ☐

যদি হ্যাঁ হয় তাহলে,

ভাইবোনের সংখ্যা:

তাদের মধ্যে কতজন থ্যালাসেমিয়াতে আক্রান্তঃ

থ্যালাসেমিয়ার বাহকঃ

থ্যালাসেমিয়ার ধরনঃ

পরীক্ষাগারের ফলাফলঃ

CBC:	Total RBC:	Hb:	HCT:
MCV:	MCH:	MCHC:	Reticulocyte:
Hb Electrophoresis:			

রক্ত গ্রহনের ইতিহাসঃ

প্রথমবার রক্ত গ্রহণের বয়সঃ

রক্ত গ্রহণের মধ্যবর্তী সময়ঃ

শেষবার রক্তগ্রহণের সময়ঃ

ঔষধ সেবনের ইতিহাসঃ

Chelation therapy:

Hydroxyurea:

Folison:

Fe studies:

Fe: TIBC: % saturation: Ferritin:

কোন জটিলতা:

স্প্লেনোমেগালি: ক্ষুদ্র ☐ মধ্যম ☐ বৃহৎ ☐

হাইপোথাইরয়ডিজমঃ ☐ ডায়াবেটিসঃ ☐ অস্থিক্ষয়ঃ ☐ অন্যান্যঃ ☐

প্লীহা অপসারণ করা হয়েছে কিনাঃ

. . . .

তদন্তকারীর স্বাক্ষর রোগীর স্বাক্ষর