DETECTION OF BETA GLOBIN GENE MUTATIONS IN THALASSEMIA PATIENTS IN BANGLADESH AND CORRELATION WITH HEMATOLOGICAL PARAMETERS

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MS IN BIOTECHNOLOGY

SUBMITTED BY

APARNA BISWAS
STUDENT ID: 16176003

DECEMBER, 2018

Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Dedicated to

My Parents

CERTIFICATE OF AUTHENTICITY

I, the undersigned, Aparna Biswas, declare that this dissertation titled "**DETECTION OF BETA GLOBIN GENE MUTATIONS IN THALASSEMIA PATIENTS IN BANGLADESH AND CORRELATION WITH HEMATOLOGICAL PARAMETERS"** is my original work, gathered and utilized specially to fulfill the purposes and objectives of this study and has not been previously submitted to any other university for a higher degree. I also declare that the publications cited in this work are authentic sources, responsibly required. It is further proclaimed this work has been carried out under the joint supervision of Dr. Md. Kaiissar Mannoor, Senior Scientist and Head at the institute for developing Science and Health initiatives (ideSHi), Mohakhali, Dhaka and Prof. Dr. Naiyyum Choudhury, Professor, Department of Mathematics and Natural Sciences, BRAC University, Dhaka; serving as internal and external supervisors, respectively.

Aparna Biswas
(MS Candidate)

Certified by

Prof. Dr. Naiyyum Chodhury
(Supervisor)
Chairman, Bangladesh Atomic Energy Regulatory Authority
Former Professor and Coordinator
Department of Mathematics and Natural Sciences
BRAC University

Email: naiyyum@gmail.com

Dr. Kaiissar Mannoor
(Supervisor)
Senior Scientist and Head, ideSHi
Institute for developing Science and Health initiatives (ideSHi),
Institute of Public Health (IPH), Mohakhali, Dhaka

Email: kaiissar@ideshi.org

Prof. Dr. Firdausi Qadri
(Supervisor)
Senior Scientist and Executive Director, ideSHi and
Emeritus Scientist and Acting Senior Director,
Enteric and Respiratory Infections, Infectious Disease Division, International Centre for Diarrheal Disease Research, Bangladesh, Mohakhali, Dhaka

Email: fqadri@icddrb.org

ACKNOWLEDGEMENTS

I would like to commence after expressing my earnest gratitude to the Almighty for endowing me with the opportunity of this research course and then supporting me throughout this journey to fulfill it successfully. I would like to take this time to thank the people who made it all possible. At first I would humbly extend my immense gratitude towards my professor and personal tutor, **Dr. Naiyyum Choudhury**; he has not only been an exemplary supervisor, but a pillar of support throughout this journey. Second only to him, I am indebted for the guidance of two of my professors at BRAC University, **Dr. Aparna Islam** and **Dr. Mahboob Hossain**; they have not only been fascinating instructors, but have undergone great lengths to imbibe their depth of knowledge, and push students like myself to complete their course. Most graciously of all, I am indebted to **Dr. Kaiissar Mannoor**. He personally took me under his wing and provided me numerous opportunities for gaining knowledge, constantly encouraging me for the development of qualities of an incredible researcher. **Dr. Firdausi Qadri**, one of the most enigmatic Emeritus scientists in our field, will always be in my fondest memories for honoring me with a place in her personal laboratory. She believed in my potential for which I am truly grateful for. Moreover, I could not have completed this project at all without the meticulous training and guidance by the incredibly talented Research Officers at ideSHi. Among them, **Suprovath Kumar Sark**er who always helped me to complete this project and encouraged me to work under pressure with positive attitude; I would also like to thank **Golam Sarower Bhuyan** and **Tarikul Islam** for supporting me always in the times of lab inquiries. A special token of appreciation should be given to **Dr. Nusrat Sultana** for the assistance during collection of specimen. In addition to the team, my further gratitude goes to lab friends

who have supported me throughout, including **Dr. Rosy Sultana**, **Noorjahan Begum**, **Farjana Akther Noor, Mohammad Al Mahmud-Un-Nabi**, **Kazi Sarjana Safain**, **Mohabbat Hossain**, **Fatema Ahmed Konica**, **Shagoofa Rakshanda**, **Pritha Pramita Biswas, Saad Hasan Hasib, Sadia Tasnim Tuba** and all of my lab friends. Lastly and most importantly I would like to declare my gratitude to my loving mother, father and sister for supporting me in every aspect of my life.

ABSTRACT

β-thalassemia and hemoglobin E (Hb E) diseases are highly prevalent autosomal recessive disorders characterized by the reduced or absent expression of the β globin gene. β-thalassemia is now regarded as the most common inherited single gene disorder in the world including Bangladesh. Like β-thalassemia, Hb E disorder is also supposed to be highly prevalent in Bangladesh. Around 4.1 % of total populations are carriers of beta thalassemia and 6.1% are carriers of Hemoglobin-E beta thalassemia (Hb-EBT) in Bangladesh. Around three hundred thousand infants are born with Thalassemia worldwide each year and more than 270 million people are carriers of Thalassemia. It has been estimated by Hardy-Weinberg equation that approximately 8990 beat-thalassemia babies are being born each year in Bangladesh. Individuals with beta-thalassemia major (BTM) suffer from severe anemia, growth retardation, splenomegaly, jaundice, expansion of bone-marrow, bone deformities and require blood transfusion to avoid complications. The aim of the present study is to identify the common mutations of beta-globin gene and to establish any possible correlation with hematological parameters and with blood transfusion interval of the patients which is the indicator of disease severity of the thalassemia patients. A total of 60 transfusion dependent thalassemia patients were enrolled for the study from Dhaka Shishu Hospital. Among these patients, 31 (51.7%) and 29 (48.3%) patients were males and females, respectively. Complete blood count (CBC) and Hb-electrophoresis were performed to identify the type of beta-thalassemia patients. The mutations of beta-globin gene were detected by amplification of selective region of beta-globin gene using polymerase chain reaction (PCR) followed by automated Sanger's DNA sequencing and BLAST method. Among 60 transfusion dependent thalassemia patients, 39 (65%) were suffering from Hb-EBT (EBT), whereas 21 (35%) patients had beta thalassemia major (BTM). Mutation analysis of beta-globin gene revealed 9 mutations, namely c.79 G>A, IVS-1-5 G>C, c.126-129 del_CTTT, IVS-I-130

G>C, c.47 G>A, c.33 delA, c.51delC, c. G92C and c.126delC. Further, we found lower level of RBC count, hematocrit, MCV and MCH compared to the respective reference values, whereas the mean RDW level and WBC count of the enrolled patients were higher than the reference range. The enrolled patients were divided into three groups based on their transfusion intervals, namely Group 1 (< 1 month), Group 2 (1 month to < 1.5 month) and Group 3 (> 1.5 months). One-way ANOVA test for hematocrit, Hb concentration, RBC count, MCV, MCH, RDW and WBC count among these three groups of patients had been performed. We found no statistically significant difference among these three groups in terms of percentages of hematocrit (ANOVA: p = 0.9973), MCHC (ANOVA: p = 0.0605), MCV (ANOVA: p = 0.0782), platelet count (ANOVA: p = 0.9710), RBC count (ANOVA: p = 0.3954) and WBC count (ANOVA: p = 0.7415) but there was statistically significant difference in terms of MCH (ANOVA: p = 0.0319) and RDW (ANOVA: p = 0.0020) value between Group 1 and Group 3 in our study. In conclusion, the data from our study clearly demonstrates that group 3 (less severe group) patients had higher MCH and RDW values compared to the Group 1 patients (most severe group).

Keywords: HBB gene mutation, Beta thalassemia major, Hb-E beta thalassemia, Splenomegaly, Complete Blood Count, Transfusion Interval.

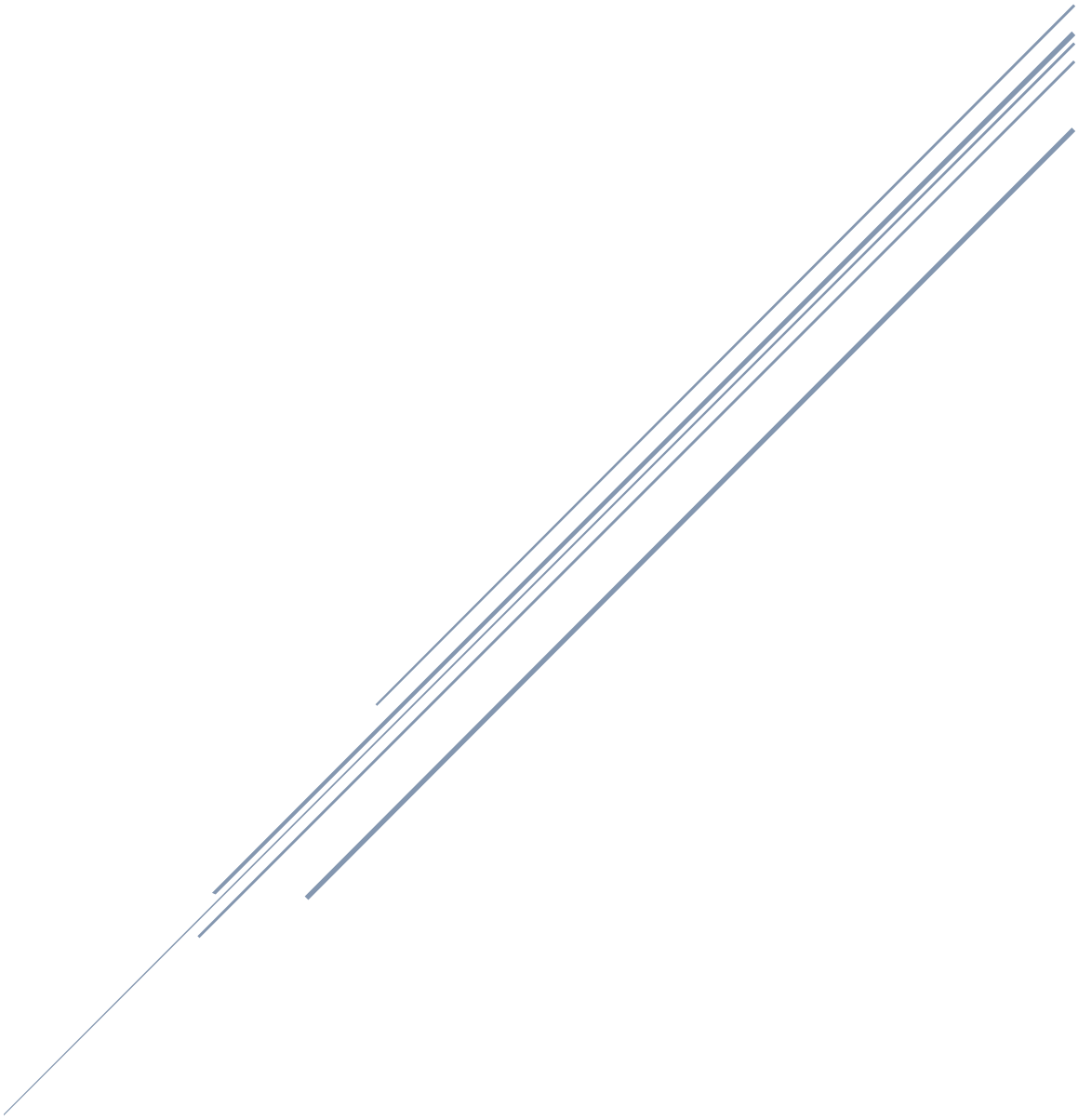
Contents

Tables

Figures

INTRODUCTION

Chapter 1



1. INTRODUCTION

1.1 Background of Thalassemia

The under-five mortality rate (U5MR) in Bangladesh has declined significantly from 144 to 38 per 1000 live births between 1990 and 2015 [1]. Much of this improvement can be attributed to successful prevention and treatment of communicable diseases such as acute respiratory tract infections, diarrheal diseases, and vaccine preventable infections. However, there are other barriers to further reduce child mortality, morbidity and disabilities in their life. This has been reflected by the formulation of the newly approved Sustainable Development Goals (SDG) and targets that have been approved by the United Nations General Assembly recently to address the problems in the increase of non-communicable diseases globally. The critical problems of birth defects and genetic disorders in surviving children have previously been ignored and failed to receive due attention in policy level in the south East Asian Region.

A genetic disorder (GD) is disease caused by one or more abnormalities in the genome. GD can be caused by a single error in one gene (monogenic disorder) or by errors in multiple genes (multifactorial inheritance disorder). Though genetic disorders (GD) are rare, collectively they comprise over 15,500 which affect millions of people worldwide [2]. Over 10,000 of human diseases are currently estimated to be monogenic. The global prevalence of all single gene diseases at birth is approximately 10/1000. According to WHO, the alpha and beta thalassemia are the most common inherited single-gene disorders in the world. Thalassemia are autosomal recessive disorders associated with hemoglobinopathies that are characterized by a decrease in the production of globin chains resulting in microcytic hypochromic anemia which is now recognized as the most common inherited single-gene disorder in the Bangladesh [3-5]. Almost 60,000 infants are born with β-thalassemia worldwide

each year and 270 million people are carriers of hemoglobinopathies [6, 7]. β-thalassemia is most commonly present among populations in all Mediterranean countries, as well as in Southeast Asia, Africa, Central America and the Middle East [6-8]. World Health Organization (WHO) estimates that at least 6.5% of the world populations are carries of different inherited disorders of hemoglobin [9]. Another WHO report estimates that 3% are carriers of β-thalassemia and 4% are carriers of HbE/β-thalassemia in Bangladesh. Khan WA *et al.* (2005) also showed that around 4.1 % of total populations are carriers of beta thalassemia and 6.1% are carriers of Hemoglobin-E beta thalassemia (Hb-EBT) in Bangladesh. In Bangladesh, more than 7000 children are born with thalassemia each year [10]. Majority are born in countries with limited resources where priority tends to be given to tackle high rates of infant and child mortality from infectious diseases and malnutrition [11]. Moreover, the patients suffering from β-thalassemia major and HbE/β-thalassemia do not survive for more than 5 years without blood transfusion [12].

Like β-thalassemia major, Hemoglobin E (Hb E) and Hb E/β-thalassemia hemoglobinopathies have been reported as an alarming public health issue in Bangladesh. The clinical importance of HbE trait arises when the β^E allele interacts with other β-thalassemia mutations leading to moderate-to-severe anemia known as HbE/β-thalassemia [13]. The HbE is a splice variant of normal β-globin protein. This cryptic splice site (related to HbE) is not normally used for mRNA processing. This new splice site competes with the normal splice site and thus produces a protein with a Lys instead of a Glu at position 26 [14]. This variant (HbE) is thus the protein, produced by the said mutation. HbE can be present in both heterozygous and homozygous state, but it can interact with other β-thalassemia types to produce HbE/β-thalassemia. The transmission patterns of beta-thalassemia and Hb E follows basic Mendelian inheritance. If

both parents carry the trait, there is a one in four chance that, in each pregnancy, their child will inherit two abnormal genes, resulting in beta-thalassemia major or Hb E hemoglobinopathies or Hb E/β-thalassemia disease. Patients of β-thalassemia major, Hb E disease, and Hb E/β-thalassemia suffer from life-threatening anemia and require treatment by regular blood transfusions, iron chelation and management of complications e.g. osteoporosis, cardiac dysfunction, endocrine problems, hepatitis B and C infection, HIV infection which pose a tremendous psychological and economical stress on the family, community and country as well.

1.1.1 Most common mutations of beta-globin gene in different countries

In South East Asia, genetic compounds for HbE and β-thalassemia are very common. Previous studies revealed that most of the beta-globin (HBB) gene mutations (>95%) in Bangladeshi thalassemia patients including three most common mutations, namely c.79G > A, c.92 + 5 G > C and c.126_129delCTTT account for ~85% of the beta-globin gene mutant alleles and these mutations are confined in the exon-1 (c.1 – c.92), the intron-1 (c.92 + 1 – c.92 + 130) and a portion of exon-2 (c.93 – c.217), thus constituting the hot-spot region (Table 1)[15]. The same hot-spot accounts for over 90% mutations in the HBB gene in India (West Bengal, Uttar Pradesh, Eastern India, and Southern India), Sri Lanka, and Malaysia and over 83%, 82% and 80% of the HBB gene mutations in Thailand, Pakistan, and Myanmar, respectively (Table 1) [16-23].

Table 1.1: The spectrum of HBB gene mutations in the regional hot-spot of South Asia and Southeast Asia [15].

Sl. No.	Countries	Mutations	Percentages of total mutation found in this region	References
1	Bangladesh	c.79G > A, c.92 + 5 G > C, c.126_129delCTTT, c.92 G > C, c.27_28insG, c.47G > A, c.92 G > A, c.46delT, c.92 + 130 G > C, and c.51delC	>95%	[16, 17]
2	India: West Bengal, Uttar Pradesh, Eastern India, Southern India	c.92 + 5 G > C, c.92 + 1G > C, c.27_28insG, c.47G > A, c.51delC, c.92 + 5 G > C, c.126_129delCTTT	> 90%	[18]
3	Sri Lanka	c.92 + 5 G > C, c.92 + 1G > C, c.79G > A, c.92 G > C, c.27_28insG, c.47G > A, c.51delC, c.126_129delCTTT	>90%	[19]
4	Malaysia	c.92 + 5 G > C, c.92 + 1 G > C, c.59A > G, c.126_129delCTTT, c.52A > T, c.27_28insG, c.216_217insA, c.92 + 1 G > C, c.79G > A	>90%	[20]
5	Thailand	c.126_129delCTTT, c.52A > T, c.59A > G, c.27_28insG, c.92 + 1 G > C, c.92 + 5 G > C, c.108C > A, c.47G > A	>83%	[22]
6	Pakistan	c.92 + 5 G > C, c.27_28insG, c.92 + 1G > C, c.126_129delCTTT, c.92 G > A, c.17_18delCT and c.47G > A	>82%	[21]
7	Myanmar	c.92 + 1 G > T, c.126_129delCTTT, c.92 + 5 G > C, c.53A > T, c.135delC, c.108C > A, c.47G > A, c.51delC, c.46delT, c.92 + 1 G > C, c.27_28insG, c.126delC	>80%	[23]

1.1.2 Structure of Human Hemoglobin

Hemoglobin is a 64.4 kd tetramer consisting of two pairs of globin polypeptide chains: one pair of alpha-like chains, and one pair of non-alpha chains. The chains are designated by Greek letters, which are used to describe the particular hemoglobin (eg, Hb A is alpha2/beta2). Two copies of the alpha-globin gene (HBA2, HBA1) are located on chromosome 16 along with the embryonic zeta genes (HBZ). There is no substitute for alpha globin in the formation of any of the normal hemoglobin (Hb) following birth (eg, Hb A, Hb A2, and Hb F) [24].

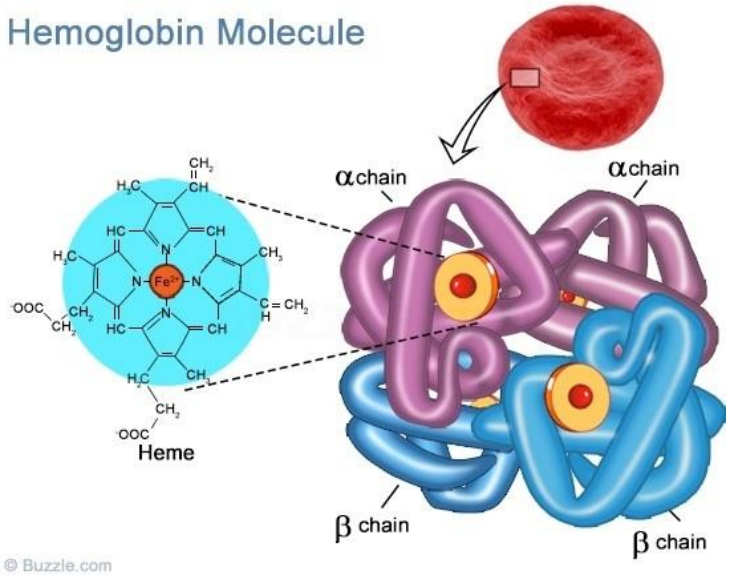


Figure 1.1: Structure of Hemoglobin (Source: http//:www.google.com/images).

A homotetramer of only alpha-globin chains is not thought to occur, but in the absence of alpha chains, beta and gamma homotetramers (HbH and Bart's hemoglobin, respectively) can be found, although they lack cooperativity and function poorly in oxygen transport. The single beta-globin gene (HBB) resides on chromosome 11, within a gene cluster consisting of an embryonic beta-like gene, the epsilon gene (HBE1), the duplicated and nearly identical fetal,

or gamma globin genes (HBG2, HBG1), and the poorly expressed delta-globin gene (HBD). [24].

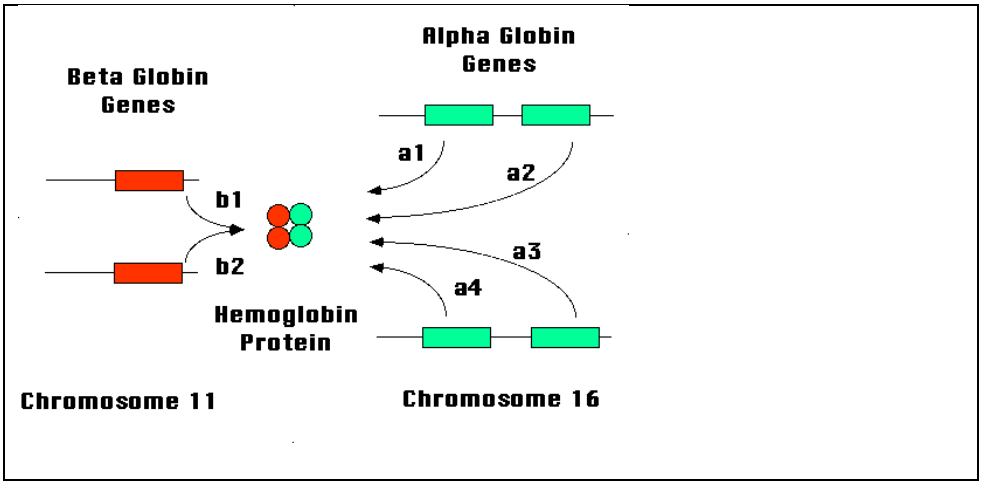


Figure 1.2: Formation of hemoglobin protein from the *α-globin* gene of chromosome 16 and the *β-globin* gene of chromosome 11. (Source: http//:www.google.com/images).

A heme group, consisting of a single molecule of protoporphyrin IX coordinately bound to a single ferrous (Fe^{2+}) ion, is linked covalently at a specific site to each globin chain. If the iron is oxidized to the ferric state (Fe^{3+}), the protein is called methemoglobin. [24].

Embryonic hemoglobin that contains zeta (HBZ)- or epsilon (HBE1)-globin chains are primarily the product of yolk sac erythroblasts and are detectable only during the very earliest stages of embryogenesis. In early development, between weeks 4 and 14 of gestation, the human embryo synthesizes three distinct hemoglobin molecules. These embryonic hemoglobin molecules are in the following according their order of formation [25].

Hb Gower I — zeta2/epsilon2

Hb Portland — zeta2/gamma2

Hb Gower II — alpha2/epsilon2

By approximately the 14th week of gestation, after establishment of erythropoiesis in the fetal liver and spleen, these embryonic hemoglobin molecules are replaced by HbF.

After the eighth week of gestation, HbF becomes the predominant hemoglobin of the fetus and its levels continue to increase until midway of gestation. The concentration of HbF increases up to early gestational age and is regulated developmentally. As an example, the concentration of HbF in an infant born at 28 weeks gestation is approximately 90 percent and decreases to approximately 60 percent at 10 weeks after birth, a value similar to that of full term infant born at 38 weeks [26].

The transition from HbF to HbA appears to be controlled via a multi-protein complex, which includes expression of BCL11A (B-cell lymphoma/leukemia 11A), a gene encoding a suppressor of HbF expression [27, 28]. Of interest, hereditary persistence of HbF (HPFH), in which persistent high levels of HbF continue into adulthood, has been attributed in one family of haploinsufficiency for KLF1 [29].

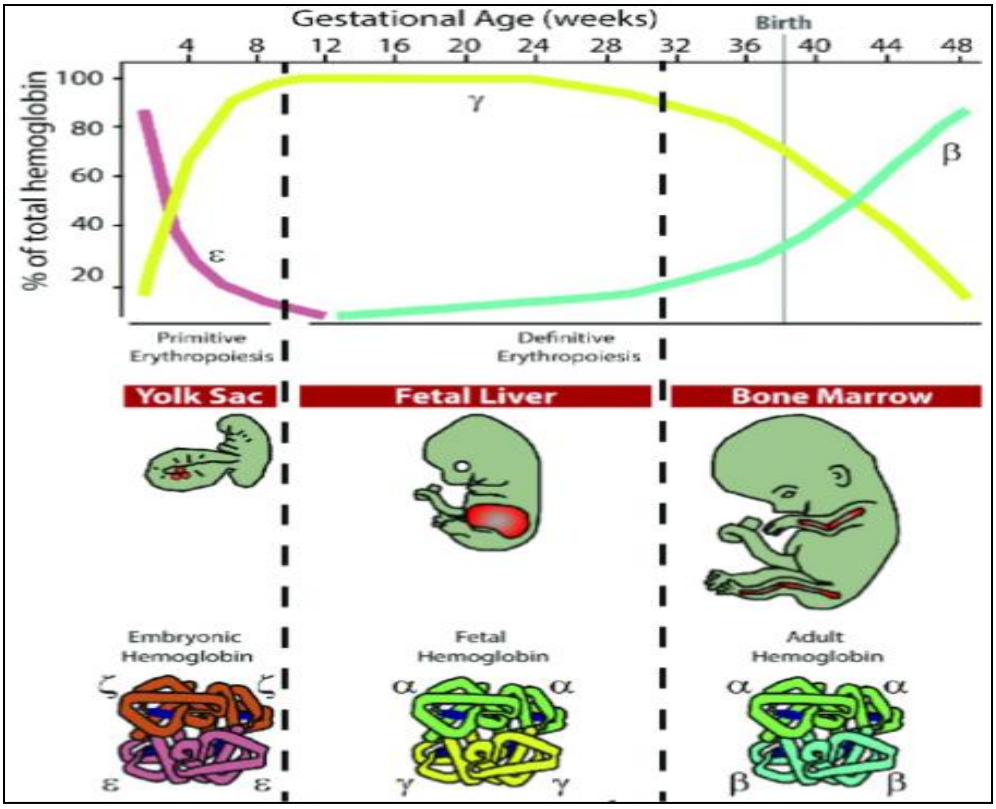


Figure 1.3: The developmental stage and specific regulation of different types of hemoglobin. (Source: http//:www.google.com/images)

1.1.3 Functions of Human Hemoglobin

1.1.3.1 Oxygenation and deoxygenation

Oxygenation and deoxygenation of hemoglobin occur at the heme iron. Upon deoxygenation, the hemoglobin molecule undergoes a marked change in conformation, as the beta chains rotate apart by approximately 0.7 nm. Deoxyhemoglobin is stabilized in a constrained or tense (T) configuration by inter- and intra-subunit salt bonds. These salt bonds are responsible for the Bohr effect and for the binding of 2,3-DPG, both of which modify oxygen affinity.

Upon the addition of oxygen, the salt bonds are sequentially broken and the fully oxygenated hemoglobin is in the relaxed (R) configuration. In this state, there is less bonding energy between the subunits and the oxygenated molecule is able to dissociate reversibly, forming two alpha-beta dimers. It is these dimers that are bound to haptoglobin and can be filtered at the glomerulus.

Each subunit in the tetramer is oriented toward the two unlike subunits in different ways (i.e., alpha1/beta1 and alpha2/beta2). There is stronger binding between alpha1 and beta1 subunits than between alpha2 and beta2 subunits; as a result, dissociation of the oxygenated tetramer into dimers occurs at the alpha2/beta2 interface. During oxygenation and deoxygenation, there is considerable movement of the alpha2/beta2 interface. Hemoglobin mutants with an amino acid substitution at this interface are likely to have markedly abnormal functional properties [26].

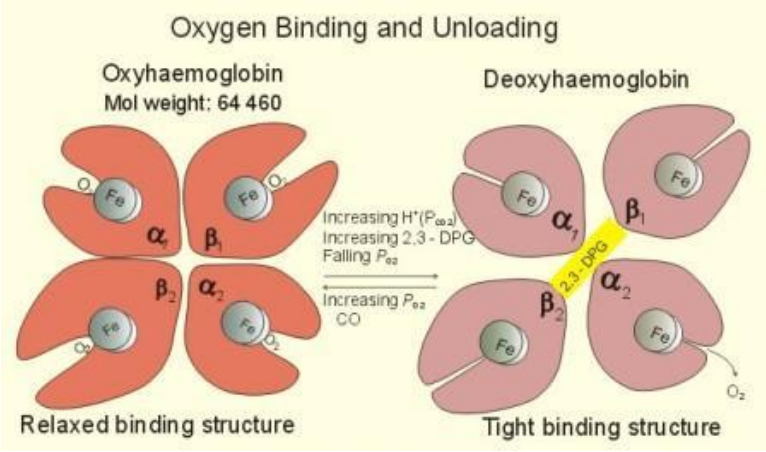


Figure 1.4: Oxygenation and deoxygenation of Hemoglobin. (Source: http//:www.google.com/images)

1.1.3.2 Cooperativity

The phenomenon of cooperativity is due to the fact that the T form of hemoglobin has a lower affinity for ligands such as oxygen and carbon monoxide (CO) than the R form. The heme-heme interaction, a function of the tetrameric nature of hemoglobin, implies that the four heme groups do not undergo simultaneous oxygenation or deoxygenation; instead, the state of each individual heme with oxygen influences the binding of oxygen to the other heme groups [26].

The totally deoxygenated hemoglobin tetramer is slow to bind the first oxygen molecule, but the reaction with oxygen accelerates as oxygenation proceeds. At some point during the sequential addition of oxygen to the four hemes of the molecule, a transition from the T to the R conformation occurs. At this point, the oxygen affinity of the partially liganded molecule increases markedly [30]. When two or three molecules of oxygen are bound, the alpha2/beta2 interface is sufficiently destabilized to flip the quaternary structure from T to R, thereby increasing the affinity of the remaining hemes for oxygen [26].

In Deoxyhemoglobin, the iron atoms lie outside the plane of the porphyrin ring by approximately 0.038 nm [31]. The smaller iron atom is now able to break into the plane of the porphyrin ring. This altered heme configuration is amplified by an intramolecular path that transduces this chemical signal to the alpha2/beta2 interface, resulting in increased ligand affinity [26].

1.1.4 Disorders associated with Hemoglobin

Hemoglobinopathies are genetic defect that results in abnormal structure of one of the globin chains of the hemoglobin molecule. Defects in these genes can produce abnormal hemoglobin

and anemia. Abnormal hemoglobin appears in one of three basic conditions; structural defects in the hemoglobin molecule, diminished production of one of the two subunits of the hemoglobin molecule (thalassemias), and abnormal association of otherwise normal subunits [32].

More than 700 hemoglobin variants have been described that involve genes both from alpha and beta gene clusters. Occasionally, someone inherit two different variants from the alpha-globin gene cluster or two different variants from beta-globin gene cluster. This condition is called compound heterozygosity. The nature of two genes inherited determines whether a clinically significant disease state develops or not. Among these the thalassemias comprise a diverse group of disorders and are broadly classified into α, β, δ-β and γ-δ-β thalassemias, depending on the globin chain(s), which are insufficiently synthesized [33].

Since humans have 4 α-globin genes on chromosome 16 and 2 β-globin genes on chromosome 11, symptomatic α-thalassemia is rarer than β -thalassemia. In addition to the transfusion-dependent form of β-thalassemia, β-thalassemia major, there are milder conditions that may escape detection until adulthood. Because of their high frequency and severity, the β-thalassemias pose the most important public health problem [34].

Beta thalassemia is the most frequent type of thalassemia which can be classified further into three subtypes; beta thalassemia major, beta thalassemia intermedia and beta thalassemia minor [35]. Individuals with beta thalassemia major inherit two mutant beta globin alleles (β^0); hence, synthesis of beta chain is completely absent with a consequence of the development of fatal anemia in early childhood, if untreated. Intermediate beta thalassemic individuals carry mutation in

one or both of the beta globin genes**,** whereas beta thalassemic trait bears mutation in only one of the two beta globin alleles.

Hb D/S trait is a heterozygotic condition where either one Hb D or S chain is combined with one normal β-chain. Both D and S are variants of beta chain, in which a single amino acid is replaced. In Hb S the beta subunit has the amino acid valine at position 6 instead of glutamic acid, whereas in Hb D glutamine replaces glutamic acid at 121 positions on beta chain. Sickle cell disease is most common in people of African ancestry and tribal people of India. The carrier frequency of sickle gene is cited 1 in 10 in the USA and may be higher in Canada where the black population is composed mostly of individuals of Caribbean and African origin [36].

Inherited disorders of hemoglobin synthesis are, therefore, an important cause of morbidity and mortality worldwide. They place a large burden to the patients, their families, and even their communities. They are generally not curable but can be prevented by population screening, genetic counseling, and prenatal diagnosis [34].

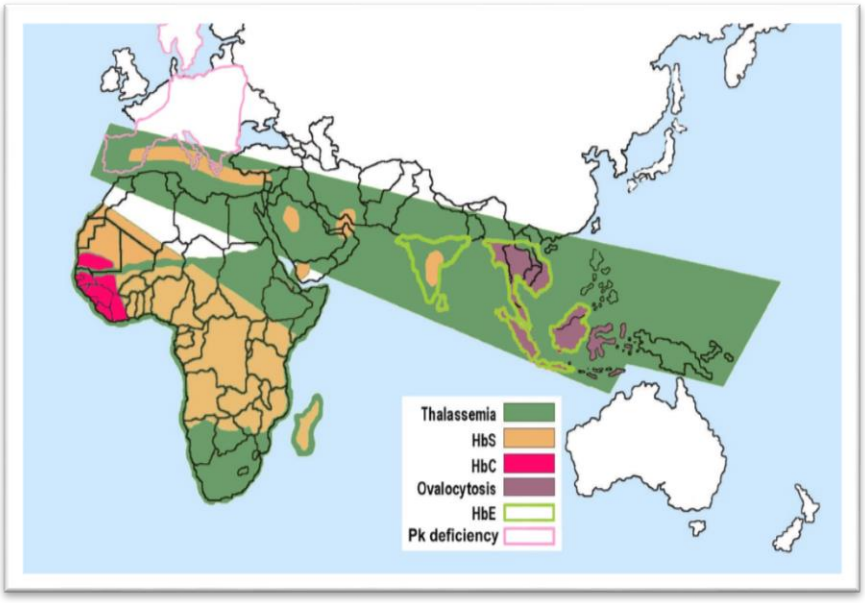


Figure 1.5: Global distribution of different Hemoglobin disorder.
(Source: http//:www.google.com/images)

1.1.5 Epidemiology of Hbβ & HbE/β-Thalassemia

Worldwide, patients with hemoglobin E-beta-thalassemia (Hb E/β-thalassemia) represent approximately 50 percent of those affected with severe beta thalassemia [37]. The highest frequencies are observed in India, Bangladesh and throughout Southeast Asia, particularly in Thailand, Laos and Cambodia, where it is common for individuals to inherit alleles for both hemoglobin E (Hb E) and beta-thalassemia [38]. Throughout these regions, Hb E/β-thalassemia has become an increasingly severe public health problem. In Thailand, about 3,000 affected children are born annually, and estimates of about 100,000 living patients have been provided [39]. In southern China, due to gene frequencies of about 4 per cent for β-thalassemia and for Hb E, thousands of patients are affected [40]. Hb E/β-thalassemia is also common in Indonesia and Sri Lanka, and while previously rarely diagnosed in North America or Europe,

has recently become the most common form of β-thalassemia identified by many newborn screening programs [41].

Figure 1.6: Distribution of Hbβ-thalassemia trait & HbE trait in Bangladesh [42].

1.1.6 Pathophysiology of beta-thalassemia

B-thalassemia occurs when there is a quantitative reduction of β globin chains that are usually structurally normal [11]. They are caused by mutations in the HBB gene and are extremely heterogeneous. Almost every possible defect affecting gene expression at transcription or post-transcriptional level, including translation, has been identified in β thalassemia. These genetic defects lead to a variable reduction in β globin output ranging from a minimal deficit (mild β^+ thalassemia alleles) to complete absence (β° thalassemia). Anemia in β thalassemia thus results from a combination of ineffective erythropoiesis, peripheral

hemolysis, and an overall reduction in hemoglobin synthesis. The severity of disease in β thalassemia correlates well with the degree of imbalance between α and non-α globin chains and the size of the free α chain pool.

1.1.7 Pathophysiology of HbE/β-Thalassemia

The most serious Hb E syndrome is Hb E/β-thalassemia. The compound heterozygote state of HbE/β-thalassemia results in a variable phenotype ranging from a complete lack of symptoms to transfusion dependency [11, 43]. The pathophysiology of HbE/β-thalassemia is complex. Ineffective erythropoiesis, apoptosis, and oxidative damage are central components of the disease and its shortened red cell survival [11, 44, 45]. The instability of Hb E is a minor factor in its pathophysiology, but in febrile patients, may account for accelerated hemolysis[46].

The interaction between HbE and β-thalassemia alleles is the main determinant in the pathophysiology [11]. The globin chain imbalance that results from these mutations correlates with the severity of the disease. However, the cause of the striking variability in individuals with E/β-thalassemia remains largely unknown. Patients with the same mutations within a family may show significant differences in clinical severity.

Hb E/β-thalassemia results from co-inheritance of a β-thalassemia allele from one parent and the structural variant Hemoglobin E from the other. Hemoglobin E results from a G→A substitution in codon 26 of the β globin gene, which produces a structurally abnormal hemoglobin as well as activates a cryptic splice site, resulting in abnormal messenger RNA (mRNA) processing. The level of normally spliced mRNA, β^{E}, is reduced [14] and, because a

new stop codon is generated, the abnormally spliced mRNA is nonfunctional. Hence, hemoglobin E is synthesized at a reduced rate, and behaves like a mild form of β-thalassemia.

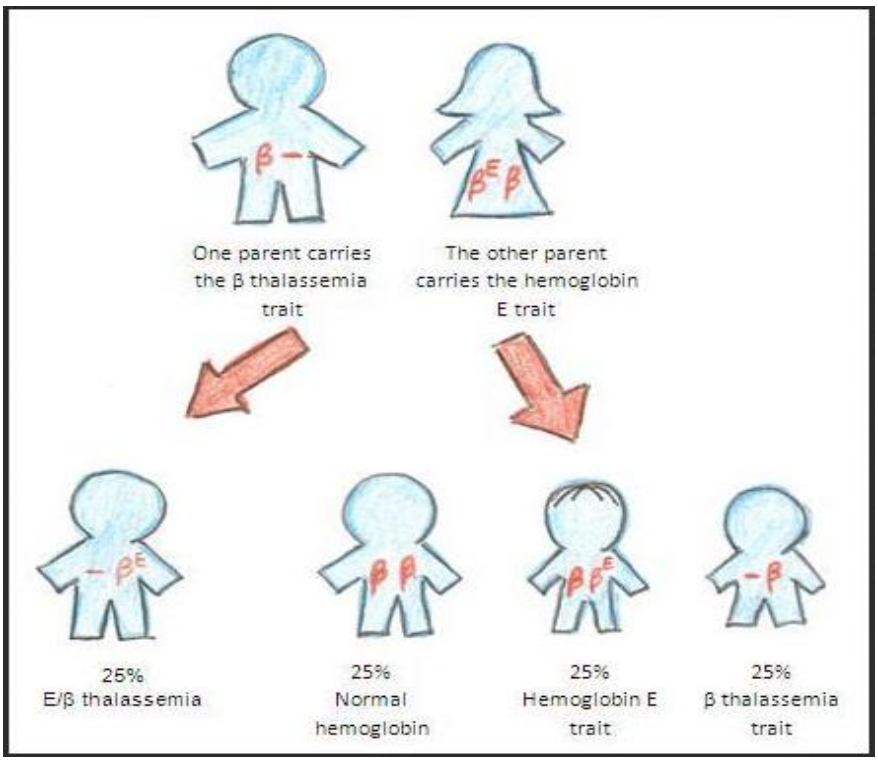


Figure 1.7: Genetic inheritance pattern of Hb E/β-thalassemia. (Source: http//:www.google.com/images).

1.1.8 Clinical manifestations of HbE/β-thalassemia

Clinical manifestations of HbE/β can be classified into three categories:

1) Mild HbE/β-thalassemia: According to Thalassemia International Federation mild form of HbE/β-thalassemia is observed in 15% of all thalassemia cases in Southeast Asia. Patients of this mild form of HbE/β-thalassemia maintain Hb levels between 9 and 12 g/dl. However, some patients suffer from growth failure, iron overload and other complications.

2) Moderately severe HbE/β-thalassemia: In case of most common form of HbE/β-thalassemia, patients usually maintain Hb levels at 6-7 g/dl. Clinical symptoms are similar to β-thalassemia intermedia and transfusions are not required unless infections precipitate further anemia.

3) Severe HbE/β-thalassemia: Patients in this group maintain Hb level at 4-5 g/dl and manifest symptoms similar to β-thalassemia major or transfusion dependent thalassemia.

1.1.9 Cost of thalassemia treatment in Bangladesh

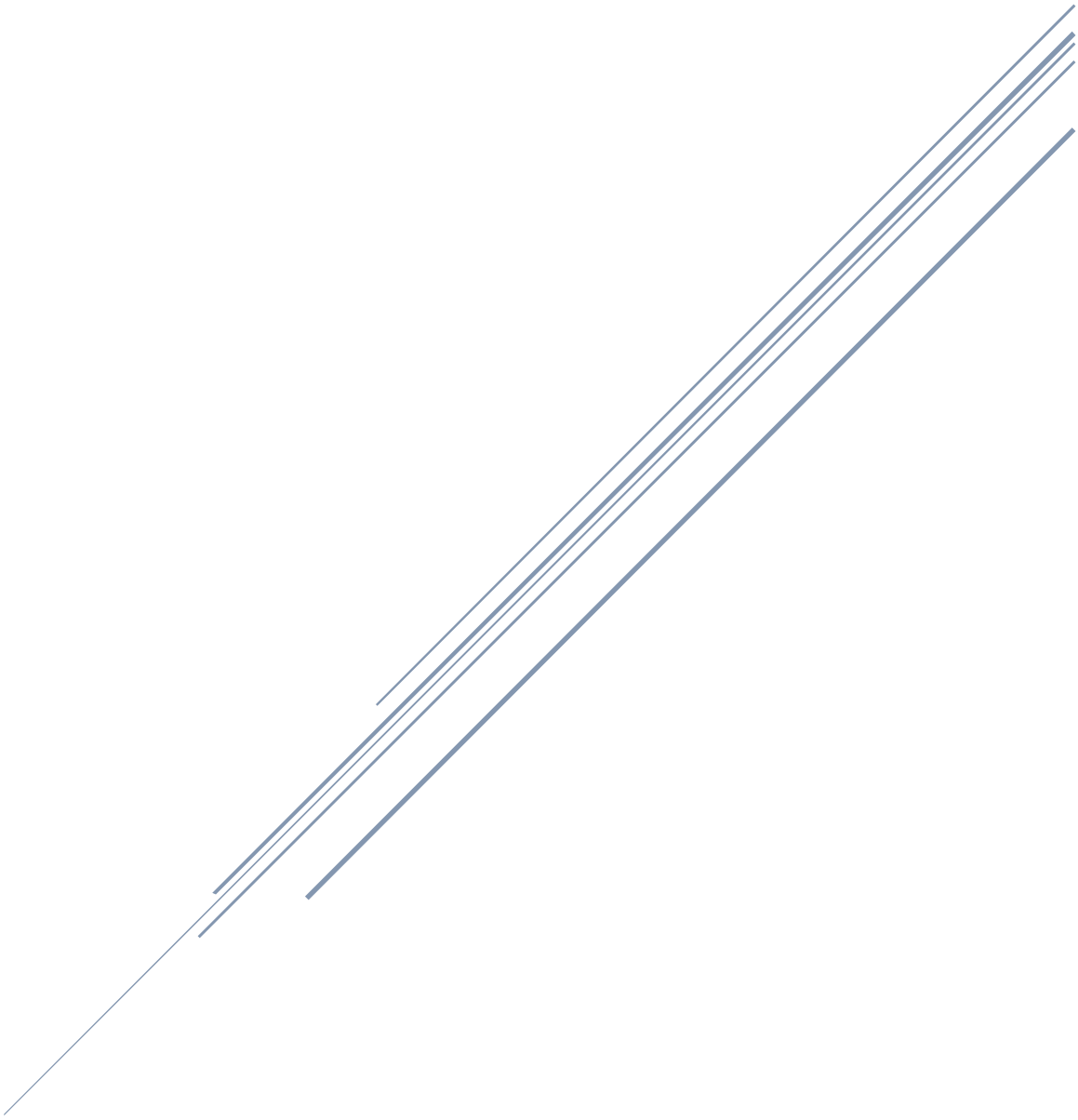
According to age, body weight and severity of the disease, the cost of the treatment varies. It is estimated that the most conservative medical cost ranges from BDT 127,000 (USD 1632; USD 1 = BDT 78) to BDT 309,000 (USD 3960) per year [47]. Hossain et al. (2017) reported in his study that over 72% of the patients' (n = 448) monthly household income was between BDT 10,000 (USD128) to BDT 20,000 (USD 256) which suggests that thalassemia patients' family in Bangladesh suffer from huge economic burden due to the costly treatment of this disorder [47].

1.2 Objectives of the study

1. Determination of mutation spectrum of beta-globin gene in Bangladeshi thalassemia patients.
2. Assessment of association between beta-globin gene mutations and hematological parameters

METHODS AND MATERIALS

Chapter 2



2. Methods and materials

2.1 Study site and ethical approval

The laboratory experiments including molecular, hematological and genetic studies were performed at the Biosafety level 2 (BSL-2) facility of Genetics and Genomics unit of institute for developing Science and Health initiatives (ideSHi). Ethical approval for this study was obtained from Bangladesh Medical Research Council (BMRC).

2.2 Study population

A total of 60 transfusion dependent beta-thalassemia major and E/Beta-thalassemia patients were enrolled for the study from Dhaka Shishu Hospital and Bangladesh Thalassemia Samity Hospital. A written informed consent was obtained from the participant or legal guardian. All the participants were clinically evaluated by physician and all the clinical information were collected from the attending physicians.

2.3 Specimen collection and storage

Before blood transfusion, approximately 5 ml of peripheral blood specimen was collected from study participants. A portion of the blood specimens (~ 2ml) were dispensed into a Ethylenediaminetetraacetic acid (EDTA) containing BD Vacutainer® (Becton Dickinson, Franklin Lakes, NJ, USA) for hematological analysis (CBC & Hb electrophoresis) and ~ 3 ml of blood specimens were kept into another EDTA containing BD Vacutainer® for genetic analysis. The tubes containing blood specimens were kept into a cool box (2-8 ˚C) and immediately transported to ideSHi laboratory for analysis. Complete blood count and Hb electrophoresis analysis was performed using one EDTA tube immediately after the

specimen's arrival. The other EDTA tube was stored in freezers (-70 ˚C) at the 'institute for developing Science and Health initiatives (ideSHi)' for genetic analysis.

2.4 Workflow of the study

The workflow of the study has been demonstrated in figure 2.1.

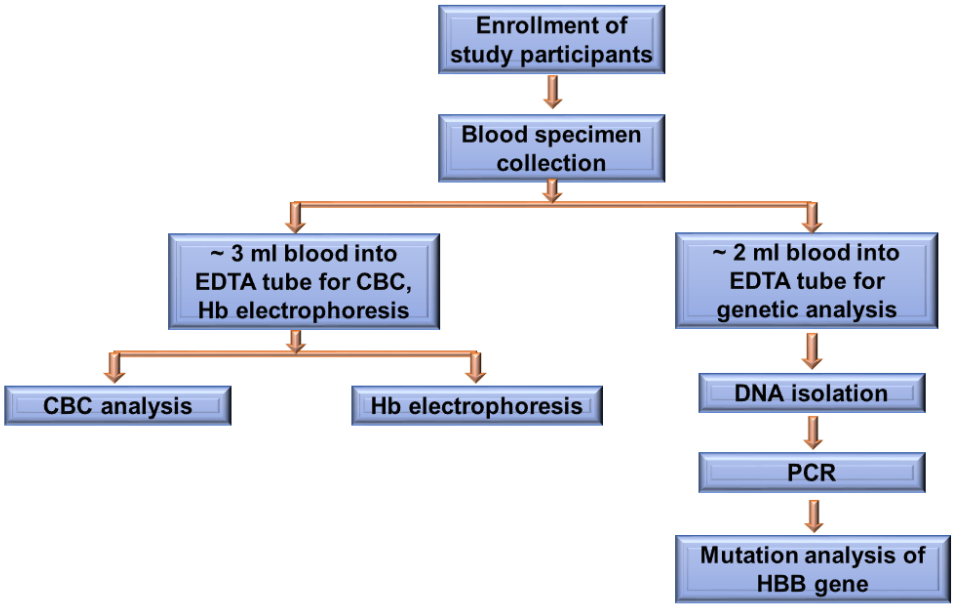


Figure 2.1: Workflow of the study.

2.5 Complete Blood Count (CBC) analysis

Automated hematology analyzed Sysmex kx-21 (Sysmex Corporation, Kobe, Japan) was used for complete blood count analysis from EDTA containing blood according to the manufacturer's instructions. Hematologic parameters including hemoglobin, WBC, RBC, PLT, HCT, MCV, MCH, MCHC, Lym%, Mon%, Gran%, RDW-SD, RDW-CV were analyzed. Among these parameters, level of blood hemoglobin was used for this current study.

2.6 DNA Extraction

Genomic DNA (gDNA) from whole blood was extracted according to guidelines of the QIAGEN flexigene1 DNA kit (QIAGEN, Hilden, Germany) manual.

2.6.1 Principle

Blood cells are lysed by the addition of lysis buffer and the cell nuclei and mitochondria are pelleted by centrifugation. The pellet was suspended in denaturation buffer, which contains a chaotropic salt, and QIAGEN Protease. The QIAGEN Protease and the chaotropic salt efficiently removes contaminants such as protein. The DNA is precipitated by addition of isopropanol and the DNA is washed with 70% ethanol. The DNA pellet is dried at room temperature and suspended in nuclease free water.

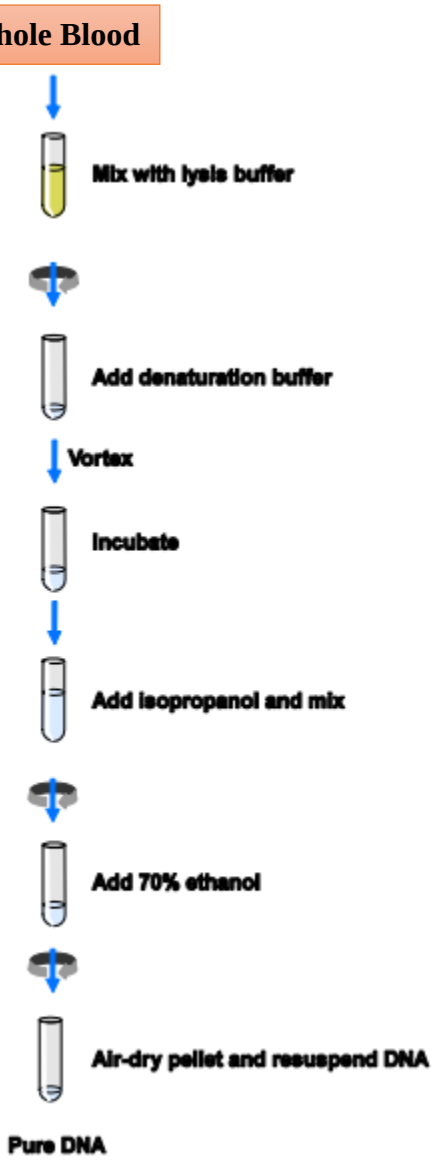


Figure 2.2: DNA isolation workflow using FlexiGene DNA isolation kit.

2.6.2 Equipment and Supplies

2.6.2.1 Equipment

1. Microcentrifuge
2. Water bath
3. Vortex machine
4. Biosafety cabinet (Level II)

5. Refrigerator (-70 °C& -20 °C)

2.6.2.2 Materials

1. BD Vacutainer (with EDTA)
2. 1 ml Pipettes
3. 100-200 µL Pipettes
4. 1-10 µL Pipettes

2.6.2.3 Reagents

1. 100% isopropanol
2. 70% ethanol
3. Buffer FG1 (FlexiGene DNA Kit)
4. Buffer FG2 (FlexiGene DNA Kit)
5. Nuclease Free Water (instead of Buffer FG3)
6. QIAGEN Protease (FlexiGene DNA Kit)

2.6.3 Starting material for isolation

Starting material for the DNA isolation was frozen blood and the blood specimen was quickly thawed at 37˚C water bath with mild agitation.

2.6.4 Isolation procedure

FG2/ QIAGEN Protease mixture (Denaturation buffer) was prepared by adding QIAGEN Protease with FG2 buffer in 1:100 ratios. This mixture was used within 1 hours of preparation. 500 µL of FG1 buffer was taken in a 1.5 mL micro-centrifuge tube and 200 µL whole blood was mixed with FG1buffer by inverting the tube 20 times. The tube was

centrifuged for 5 minutes at 10,000 x g using Heraeus™ Fresco™ 21 Microcentrifuge (Thermo Scientific™, 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. 100 µl FG2/QIAGEN Protease buffer was added and vortexed immediately to homogenize the pellet completely. The tube was then centrifuged for 3 – 5s and after centrifugation the tube was placed in a water bath at 65ºC for 5 min. After incubation, 100 µl isopropanol (100%) was added and mixed thoroughly by inversion (at least 20 times) until DNA becomes visible as threads or a clump. 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, making sure that the pellet is in the tube. 100 µl 75% ethanol was added and vortex for 5s. Then the tube was centrifuged for 3 minutes 10,000 x 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 until all the liquid has evaporated. 50 µl nuclease free water was added to dissolve the DNA pellet.

2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) technique was used to amplify the human beta- globin (HBB) gene using Qiagen HotStart Taq DNA Polymerase (Qiagen, USA). A pair of PCR primer was designed at ideSHi laboratory to amplify the 428 bp of HBB gene which covers the mutational hotspot of HBB gene.

2.7.1 Principle

Polymerase chain reaction (PCR) technique is applied in molecular biology laboratory for amplifying particular segments of DNA. This is an in vitro DNA amplification technique

where the enzyme DNA polymerase directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. First, a synthetic oligonucleotide (primer) is annealed to a longer template DNA and then the DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide. Thus, DNA polymerase is used to elongate its 3` end to generate an extended region of double stranded DNA. After completion of several cycle, a huge number of copies of particular gene is produced. Basic principle of PCR is illustrated in figure 2.3.

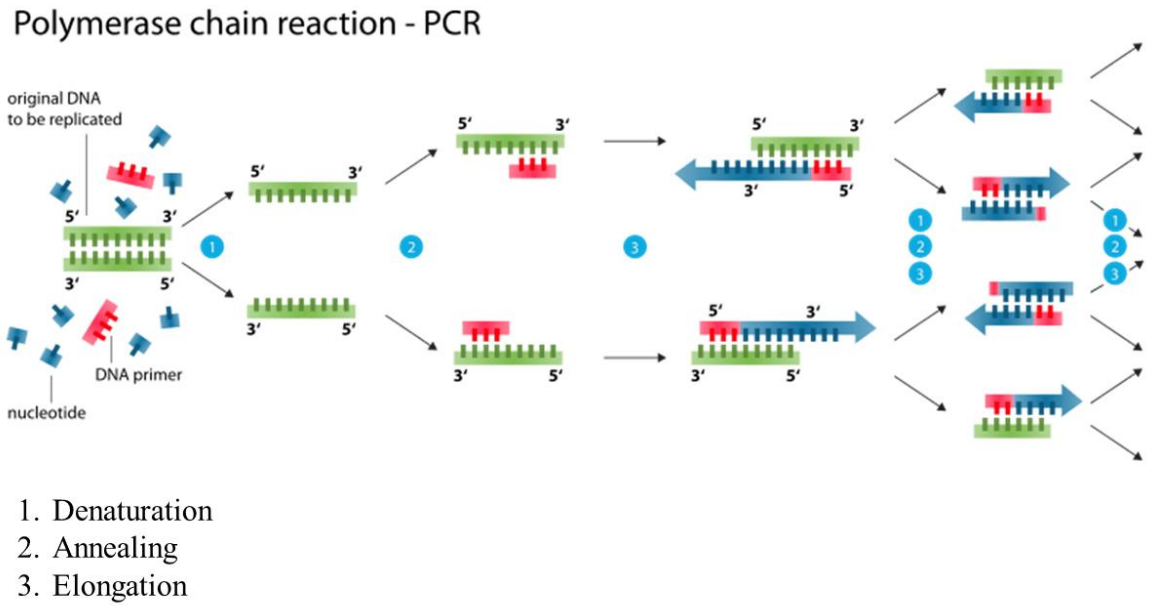


Figure 2.3: Basic principle of Polymerase Chain Reaction (PCR).

2.7.2 EQUIPMENTS AND SUPPLIES:

2.7.2.1 Equipment

1. Thermal cycler (Bio-Rad)
2. Microcentrifuge
3. Vortex machine

4. Spin machine
5. PCR cabinet
6. Refrigerator (-20 °C)

2.7.2.2 Materials

1. Microcentrifuge tube
2. 0.2 ml PCR tube
3. 20-200 µL Pipettes
4. 2-20 µL Pipettes
5. 0.1-10 µL Pipettes

2.7.2.3 Reagents

1. HotStart Taq DNA Polymerase (Qiagen; cat no. 203203) contains:
2. HotStart Taq DNA Polymerase
3. 10x PCR buffer
4. 25mM $MgCl_2$
5. 5x Q-solution
6. 2.5 mM dNTPs (Takara; cat no. 4030)
7. 10 mM Forward primer
8. 10 mM Reverse primer

2.7.3 Starting materials

Isolated genomic DNA from whole blood using FlexiGene DNA kit was used for PCR amplification.

2.7.4 Procedure

2.7.4.1 Primer designing

To design the primers for amplification of human beta-globin (HBB) gene, the DNA sequence of human HBB gene was retrieved from the nucleotide database of National Center for Biotechnology Information (NCBI). The primer pair is designed to amplify the 428 bp segment of HBB gene, which covers exon 1, intron 1 and exon 2 of *HBB* 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. Name of the primer, sequence, guanine-cytosine percentage (GC%) and Tm of the primer has been shows in figure 2.4.

Table 2.1: List of primers used for amplification of HB gene and properties of primer Sequence of primers, GC% and Tm

Primer Name	Sequence (5' – 3')	GC%	Tm	Product length (bp)
HBB_mut_F	GGCAGAGCCATCTATTGCTTAC	50.00	59.19	428
HBB_mut_R	CAGGCCATCACTAAAGGCACC	57.14	61.29	

2.7.4.2 PCR reaction

Human beta-globin gene specific PCR amplification was done using a BioRad T100™ thermal cycler (Bio-Rad, USA). Qiagen HotStart Taq DNA Polymerase (Qiagen, Hilden, Germany) was used for PCR amplification. Total reaction volume was 10 µL and PCR reaction components along with concentration has been given in Table 2.1.

Table 2.2: Composition of PCR reagent for amplification of HBB gene using HBB_mut_F and HBB_mut_R primers

Name of Reagent	Quantity (µL)
10X Qiagen PCR Buffer	1.0
25 mM $MgCl_2$	0.4
dNTPs (2.5 mM)	1.6
5X- Q-Solution (Qiagen)	2.0
HBB_mut_F primer	0.2
HBB_mut_R primer	0.2
Nuclease free water	3.55
DNA Template	1.0
HotStart Taq polymerase	0.05
Total Volume	10 µL

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 after PCR reaction the instrument will maintain 4°C until shutdown (Figure 2.4).

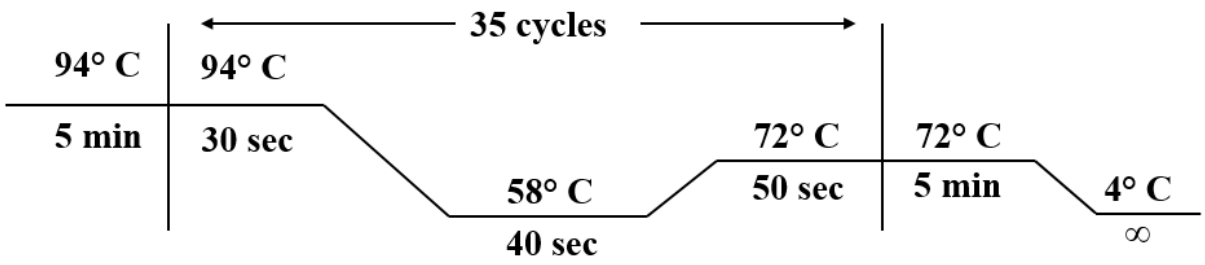


Figure 2.4: Thermal cycling conditions of PCR instruments for HBB gene amplification

2.8 PCR product purification

2.8.1 Principle

The MiniElute system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. MiniElute PCR purification columns are designed to give high end-concentrations of purified DNA fragments for subsequent reactions. Special buffers are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or Nuclease-free water.

2.8.2 EQUIPMENTS AND SUPPLIES:

2.8.2.1 Equipment

1. Microcentrifuge
2. Vortex machine
3. Spin machine
4. PCR cabinet
5. Refrigerator (-20 °C)

2.8.2.2 Materials

1. Microcentrifuge tube
2. 0.2 ml PCR tube
3. 20-200 µL Pipettes
4. 2-20 µL Pipettes
5. 0.1-10 µL Pipettes

2.8.2.3 Reagents

1. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)

2.8.3 Procedures

PCR product was transferred into the 1.5mL microcentrifuge tube and 5 volume PB buffer was added. After mixing with 10 µL 3 M sodium acetate (pH 5.0) the content was transferred to the MiniElute column along with 2mL collection tube. The tube was centrifuged at 17900xg for 1 min and the flow-through was discarded. The column was placed back into the collection tube and 750µL Buffer PE was added into the MiniElute Column. After centrifugation at 17900xg for 1 min the flow through was discarded. The column was again placed in a new collection tube and centrifuged at 17900g for 1 min to remove the residual ethanol. After centrifugation, the column was placed into a 1.5 mL microcentrifuge tube and 15-20 µL nuclease-free water was added. The column along with tube was centrifuged at maximum speed for 1 min to elute the DNA. This DNA containing solution was checked by agarose gel electrophoresis before sequencing. Work-flow of the PCR product purification procedure has been shown in figure 2.6.

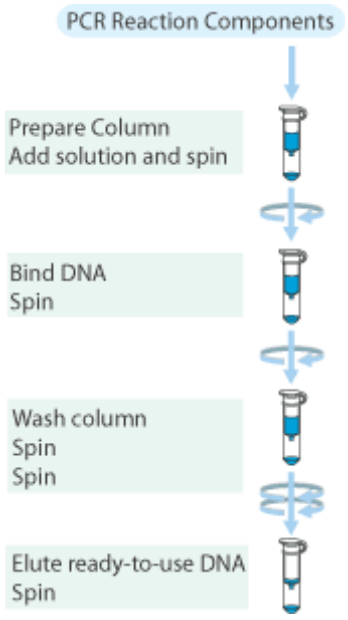


Figure 2.5: Work-flow of the PCR product purification

2.9 Agarose gel electrophoresis

2.9.1 Principle

Purified PCR products was checked by running the samples on a 1% agarose gel. At neutral or alkaline pH, the phosphate groups of DNA or PCR products give rise to a uniform negative charge. Agarose gel electrophoresis involves the use of a buffer to maintain the constant states of ionization since any change in pH would alter the charge on the DNA molecules to be separated or visualized. TBE buffer can be used as it gives good resolution and sharp band plus no microbes can grow if kept for a long time but at times the formation of precipitation can be its drawback. During electrophoresis, an electrical field is applied so that the negatively charged DNA fragments move towards the positively charged electrode (anode) through the pores in the gel. The velocity of movement is inversely proportional to the molecular weight of the DNA molecule. Therefore, the largest molecules will have most difficulty passing through the pores, whereas the smallest molecules are relatively unhindered,

moving the fastest. The principle of the agarose gel electrophoresis has been shown in figure 2.7.

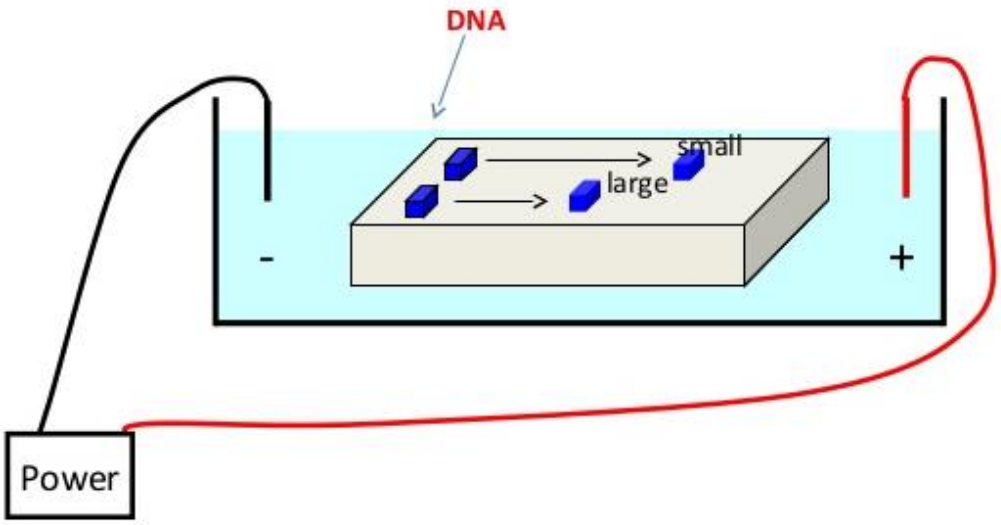


Figure 2.6: Principle of agarose gel electrophoresis

2.9.2 Equipment and reagents

2.9.2.1 Equipment

1. Electric Balance
2. Micropipettes
3. Gel Electrophoresis Apparatus
4. Gel Doc™ XR+ (BioRad, USA)

2.9.2.2 Reagents

1. Agarose powder
2. 1X TBE buffer

3. Gel Red (Intercalating nucleic acid fluorescent dye)
4. Bromophenol Blue (Loading dye)
5. DNA ladder (Marker DNA solution)

2.9.3 Procedure

2.9.3.1 1X TBE buffer preparation

To prepare 10X TBE buffer (pH-8.0), 108 g Tris-HCl (100 mM), 40 mL EDTA (0.5 M, pH-8.0) and 55 g Boric acid were dissolved in 1L deionized water. To prepare 1X TBE buffer, 100 mL of 10X TBE buffer was mixed with 900 mL deionized water.

2.9.3.2 Preparation of agarose gel

To prepare 1% (w/v) agarose gel, 1.0g agarose was measured into a 250 mL conical flask and 100 mL 1X TBE buffed was added. To dissolve the agarose, the mixture was heated for 2 minutes using a microwave oven. The solution was allowed to cool and 1.5 µL GelRed® Nucleic Acid Gel Stain solution (Biotium, USA) was added. The melted agarose gel was poured carefully into the gel tray that had a comb fixed in place near one end so that loading wells could be formed in the gel. Then the gel was allowed to solidified by placing it at the room temperature for at least about 15-20 mins. After the gel had solidified, the comb was removed gently, and the gel tray was placed within the electrophoresis tank. The tank was filled with 1X TBE buffer to submerge the gel. 1 volume of 6X Loading Dye bromophenol blue (dark blue color) was added to 5 volumes of purified PCR product on a small piece of Parafilm and mixed by pipetting up and down before loading into the gel wells. To estimate the size of the PCR products, 1 µL of 1 kb Plus DNA ladder (Invitrogen™, USA) that had a broad size range (75bp - 20kb). Then the gel was subjected to electrophoresis. After running the gel at

150 V for 1 hour, the bands of PCR products were subsequently visualized using the Gel Doc™ XR+ (BioRad, USA).

2.10 Sanger's DNA sequencing

2.10.1 Principle

DNA polymerases make copy of single-stranded DNA templates by adding nucleotides (A, T, C, G) at the 3′ end of a primer. The deoxynucleotide added to the extension product is selected by base-pair matching to the template. DNA polymerases can also incorporate into analogues of nucleotide bases. The dideoxy method of DNA sequencing developed by Sanger et al. (1977) takes advantage of this ability by using 2′,3′-dideoxynucleotides as substrates. When a deoxynucleotide is incorporated at the 3′ end of the growing chain, chain elongation is terminated selectively at A, C, G, or T because the chain lacks a 3′-hydroxyl group. However, when a ddNTP terminator (either ddA, ddC, ddG, or ddT each tagged with a different fluorescent dye) is added to the 3′ end, chain elongation is terminated, forming labeled extension products of various lengths. This step is called cycle sequencing. Principle of the cycle sequencing has been shown in figure 2.8. Applied Biosystems DNA sequencers detect fluorescence from four different dyes that are used to identify the A, C, G, and T extension reactions. Each dye emits light at a different wavelength when excited by an argon ion laser. All four colors and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection.

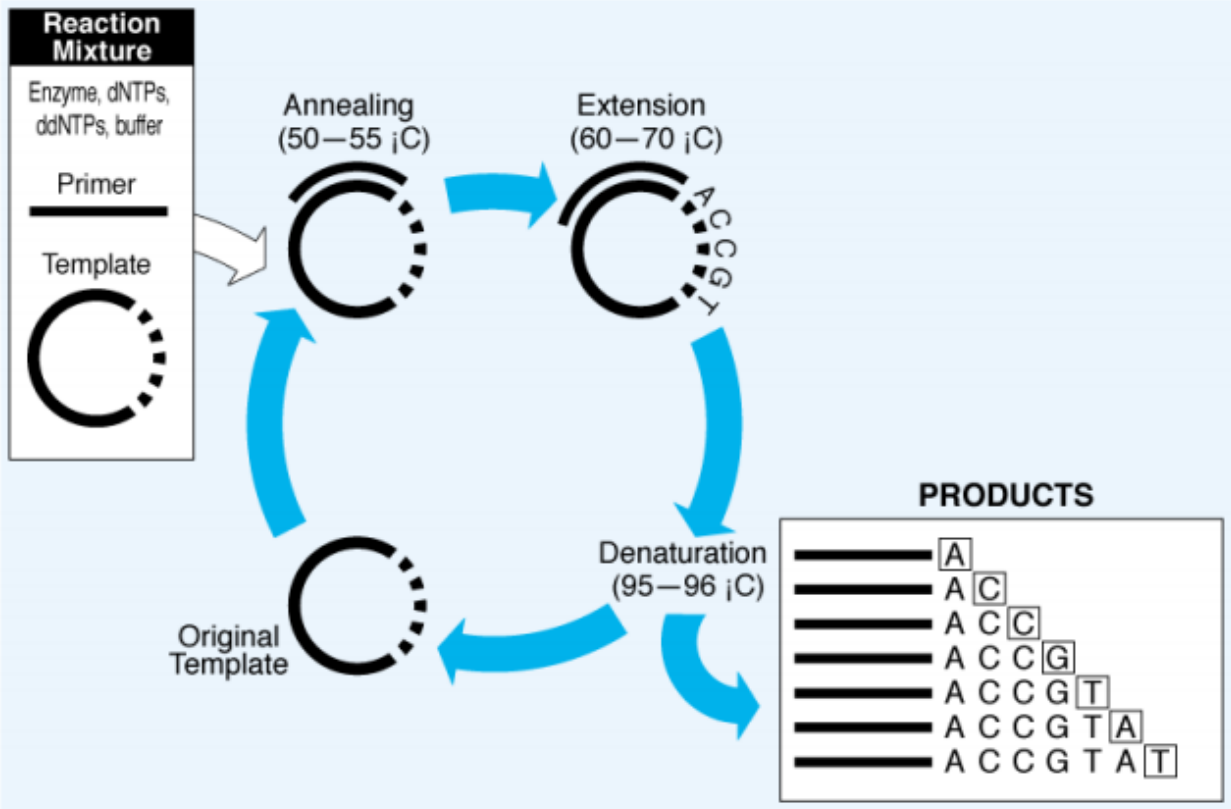


Figure 2.7: Principle of Cycle Sequencing

2.10.2 EQUIPMENTS AND SUPPLIES:

2.10.2.1 Equipment

1. ABI prism 310 genetic analyzer (Applied Biosystems, USA)
2. PCR machine
3. Microcentrifuge
4. Vortex machine
5. Spin machine
6. PCR cabinet
7. Refrigerator (-20 °C)

2.10.2.2 Materials

1. Microcentrifuge tube
2. 0.2 ml PCR tube
3. 20-200 µL Pipettes
4. 2-20 µL Pipettes
5. 0.1-10 µL Pipettes

2.10.2.3 Reagents

1. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA)
2. BigDye X Terminator Purification Kit (Applied Biosystems, USA)

2.10.3 Procedure

Reagent master mixture for the cycle sequencing step was prepared by adding the reagent including sequencing buffer, BigDye RR mix, sequencing primer (Forward or reverse), purified product and nuclease free water as specified in table 2.3.

Table 2.3: Master mix composition for cycle sequencing

Reagent	Concentration	Quantity/reaction (µl)
Sequencing Buffer	5x	2.0
Big Dye RR mix	2.5x	4.0
F Primer/ R primer	3.2pmol/5 pmol	1.0
Water	-	4.0
Template(Purified product)	10-50ng/µl	1.0 µl

Cycle sequencing was done using a thermal cycler (BioRad, USA) and thermal cycling condition has been shown in table 2.4.

Table 2.4: Thermal cycling condition for cycle sequencing

Stage	Step	Temperature (˚C)	Duration	Cycle
Stage-1	1(initial denaturation)	96	1min	1
Stage-2	1 (Denaturation)	96	10 Sec	25
			5 Sec	
	2 (Annealing)	50	4 min	
	3 (Extension)	60		
Stage-3	1 (Hold)	4	Hold	1

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

2.11 Sequencing data analysis and Statistical analysis

Sequencing data was retrieved from ABI Prism 310 genetic analyzer (Applied BioSystems, USA) as ab1 format and sequencing chromatogram was analyzed using Chromas Lite 2.4

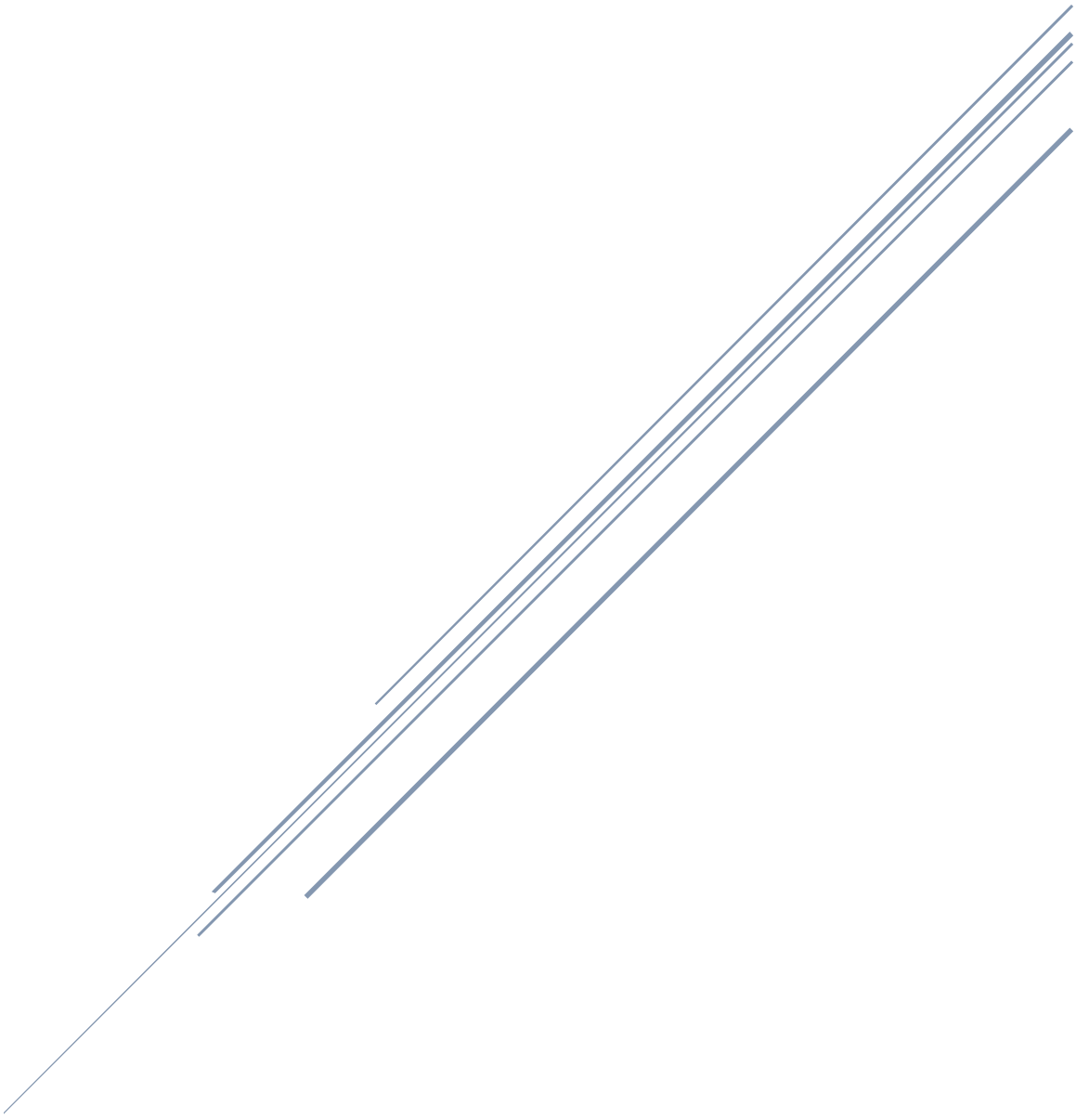
software to identify the sequences that are reliable and conclusive for mutational analysis and to eliminate sequences with noises that give inconclusive and unreliable data for mutational analysis. Sequence data was saved as FASTA format and CAP3 program (http://pbil.univ-lyon1.fr/cap3.php) was used to assemble the two sequences retrieved by two primers (Forward and reverse) of each pair for each sample on the basis of overlapping sequence to construct the contig. Sequence of nucleotides were used to perform alignment of the query sequence with wild-type sequence of mRNA or gene stored in NCBI database using BLAST (Basic Local Alignment Search Tool). From the BLAST result, the mismatched base was checked for the corresponding position of the mRNA and CDS (coding sequence) was retrieved from the sequence data. Sequence data was also analyzed for synonymous codon (different codon but coding same amino acid) or a codon that code for different amino acid in peptide.

2.12 Statistical analysis

Statistical analysis was done using the software GraphPad Prism version 7 (GraphPad Software, La Jolla California USA, www.graphpad.com). The variations between parameters were checked for significance via Unpaired Two tailed T test and Ordinary one way ANOVA. The difference was considered significant only when the probability (p) value was less than 0.05. Data were expressed as percentage (%), number of patients (N) and Mean ± standard deviation (SD).

RESULTS

Chapter 3



3. Results

3.1 Demographic information of study participants

A total of 60 transfusion-dependent thalassemia patients were enrolled for this study from Dhaka Shishu Hospital & Bangladesh Thalassemia Samity. The average age (± SD) of the patients was 6.94 ± 3.92 years and among them 31 (51.67 %) patients were male and 29 (48.33 %) patients were female (Table 3.1).

Table 3.1: Demographic information of study participants

Parameters	Patients (N = 60)
Average Age[a] (years)	6.94 ± 3.92
Male[a]	31 (51.67 %)
Female[b]	29 (48.33 %)
[a]Values denote mean ± standard deviation. [b]Data presented as No. or N (%)	

3.2 Type of thalassemia detection by hemoglobin electrophoresis

The patients had been previously diagnosed with beta-thalassemia by Hb electrophoresis and/or microscopic evaluation of thin blood film. Table 3.2 shows that 39 (65%) and 21 (35%) patients were diagnosed with HbE-beta thalassemia (EBT) and beta-thalassemia (BTM), respectively. The average (±SD) age of the patients diagnosed with HbE-beta thalassemia and beta-thalassemia major were 8.00 ± 4.3 years and 6.3 ± 3.8 years, respectively. Among EBT patients, 21 were male & 18 were female. Among BTM patients, 11

were male & 10 were female patients. The average age of 1st transfusion was 30.12 ± 30.03 months in case of EBT patients, whereas 10.1 ± 8.75 months in case of BTM patients.

Table 3.2: Type of enrolled thalassemia, patient's age, transfusion interval and average age of 1st transfusion.

Parameters	HbE-beta thalassemia	Beta-thalassemia major
Number of patients	39 (65%)	21 (35%)
Average Age of participants (years)	8.00 ± 4.3 (Lowest- 10m, Highest-16y)	6.3 ± 3.8 (Lowest-10m, Highest-14y)
Male/Female	21/18	11/10
Average age of 1st transfusion (months)	30.12 ± 30.03	10.1 ± 8.75
Transfusion interval (months)	2 ± 1.5	1.00 ± 0.4

3.3 Human beta globin (HBB) gene sequencing

Human beta globin (HBB) gene was amplified using polymerase chain reaction (PCR) method. HBB gene specific primer pair was designed to amplify 428 bp which covers exon 1, intron 1 and exon 2. This area covers approximately ~90% of beta-thalassemia mutations in several Asian countries. Figure 3.1 represents the agarose gel analysis of PCR amplified product of 5 sample.

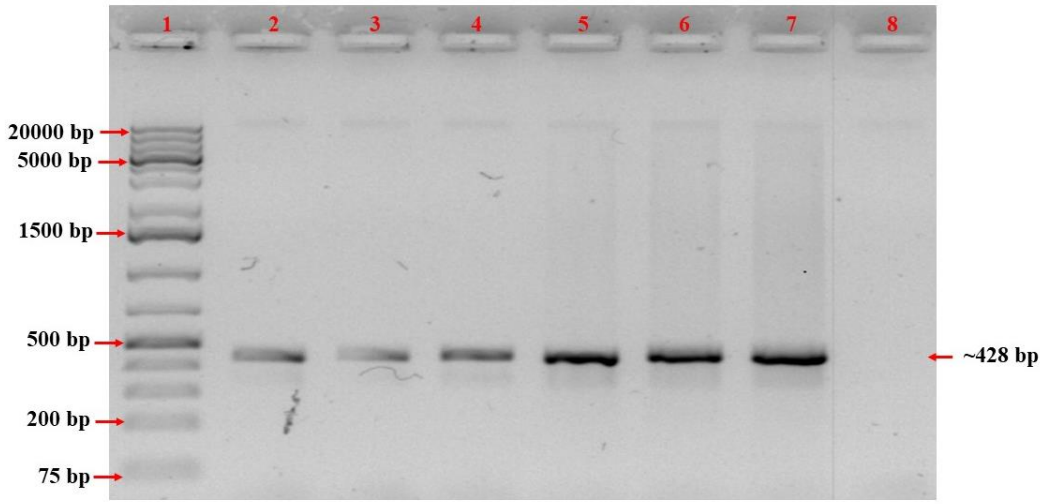


Figure 3.1: A representative figure of PCR product analysis using 1% agarose gel. Well number 1 contains 1 kb plus DNA ladder, 2 to 6 contains 5 samples, well number 7 contains positive control and 8 contains negative control.

3.4 Determination of HBB gene mutation

PCR products were purified using Qiagen PCR product purification kit and for the sanger sequencing both forward and reverse primers were used. CAP 3 online tool was used for contig preparation. This contig sequence was used for mutation identification using BLAST tool of the National Center for Biotechnology Information (NCBI). Step by step procedure for identification of mutation using BLAST has been described below.

3.4.1 Analysis of BLAST result of sequence data

HBB gene sequence data for all the patients were extracted form chromatogram as fasta format using Chromas Lite (version 2.1). A representative sequence of a patient's HBB gene has been shown in figure 3.2.

>Sample 5_Forward

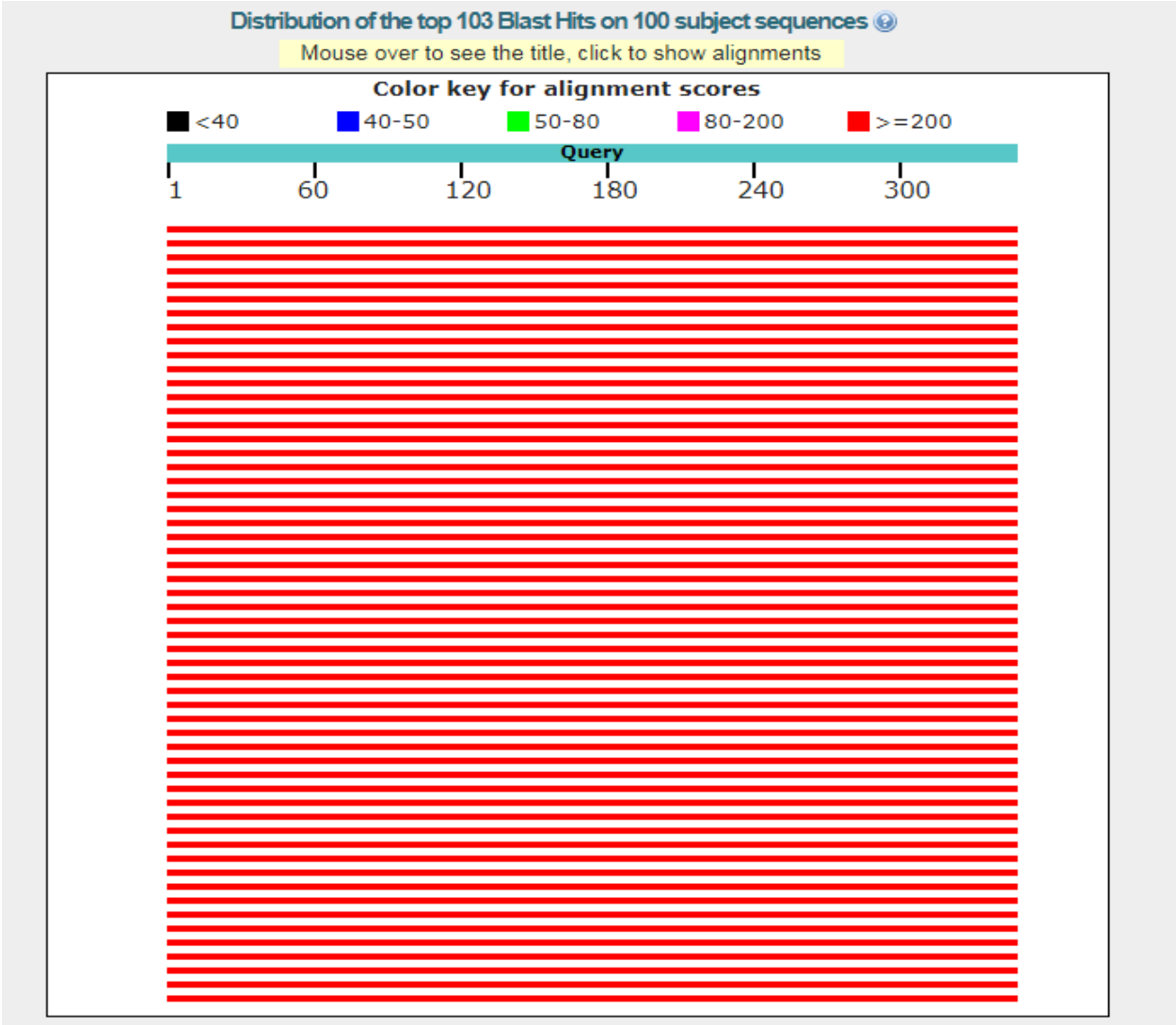
CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGT
TGGTGGTRAGGCCCTGGGCAGGTTGSTATCAAGGTTACAAGACAGGTTTAAGGAGA
CCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCAC
TGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTT
GGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGG
GCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGC

Figure 3.2: A representative HBB gene sequence of patient with thalassemia.

Sequence data was analyzed using BLAST tool of the National Center for Biotechnology Information (NCBI). Figure 3.2 (representative result of a patient's HBB gene) shows the BLAST result of sequence data. The sequence result matched with *Homo sapiens* beta-globin (*HBB*) gene. Red bad in figure 3.3 (A) indicates highly identical match with HBB gene of *Homo sapiens* and figure 3.3 (B) indicates very low E value, which indicates perfect match with deposited reference sequence of human HBB gene in NCBI. Figure 3.3 (C) indicates that 64th position and 82th position of the patient's HBB gene was mismatched with reference sequence of NCBI. Figure 3.3 (D) shows that at 64th position G was replaced by A (heterohygote) which is very common mutation of HBB gene. This mutation is known as codon 26 mutation (HbE variant) or c. G79A. Figure 3.3 (D) shows that, second mutation was G to C mutation at 5th nucleotide in intron 1, which is known as IVS-I-5(G>C) mutation. c.G79A mutation causes hemoglobin E variant and IVS-I-5(G>C) causes beta-thalassemia major if

present as homozygote form. Sequence data analysis reveals that this patient is suffering from HbE-beta thalassemia and mutation of HBB gene were c. G79A and IVS-I-5(G>C).

A



B

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Homo sapiens hemoglobin subunit beta (HBB) gene, complete cds	632	632	100%	2e-177	99%	MG675219.1
Homo sapiens hemoglobin subunit beta (HBB), RefSeqGene on chromosome 11	632	632	100%	2e-177	99%	NG_059281.1
Homo sapiens origin of replication at HBB (LOC107133510) on chromosome 11	632	632	100%	2e-177	99%	NG_046672.1
Homo sapiens HBB recombination region (LOC106099062) on chromosome 11	632	632	100%	2e-177	99%	NG_042296.1

C Homo sapiens hemoglobin subunit beta (HBB) gene, complete cds

Sequence ID: MG675219.1 **Length:** 1606 **Number of Matches:** 1

Range 1: 66 to 392 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
597 bits(323)	7e-167	325/327(99%)	0/327(0%)	Plus/Plus

```
Query  1    CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGT  125

Query  61   GGTRAGGCCCTGGGCAGGTTGSTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGA  120
            || |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  126  GGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGA  185

Query  121  AACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  AACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTG  245

Query  181  CCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  CCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTT  305

Query  241  CTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  CTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAA  365

Query  301  GGCTCATGGCAAGAAAGTGCTCGGTGC  327
            |||||||||||||||||||||||||||
Sbjct  366  GGCTCATGGCAAGAAAGTGCTCGGTGC  392
```

D

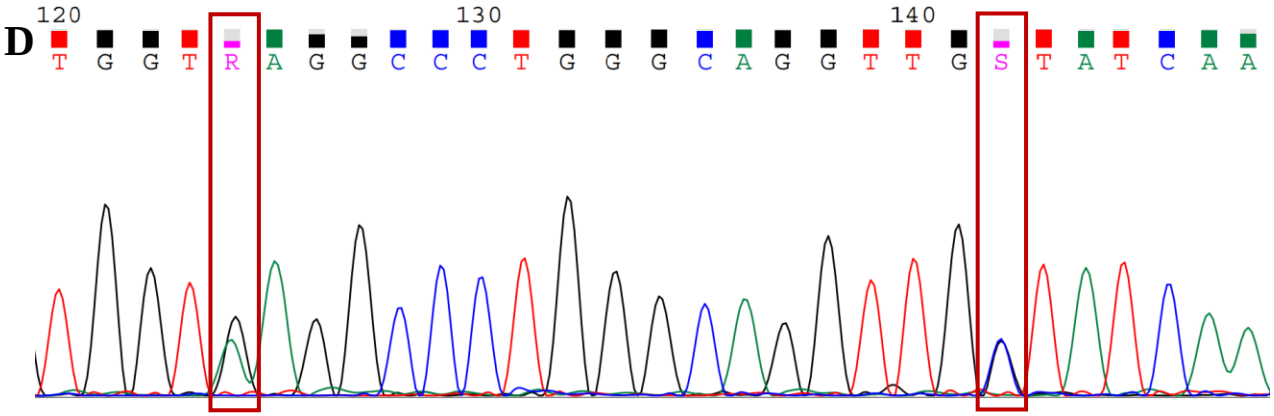
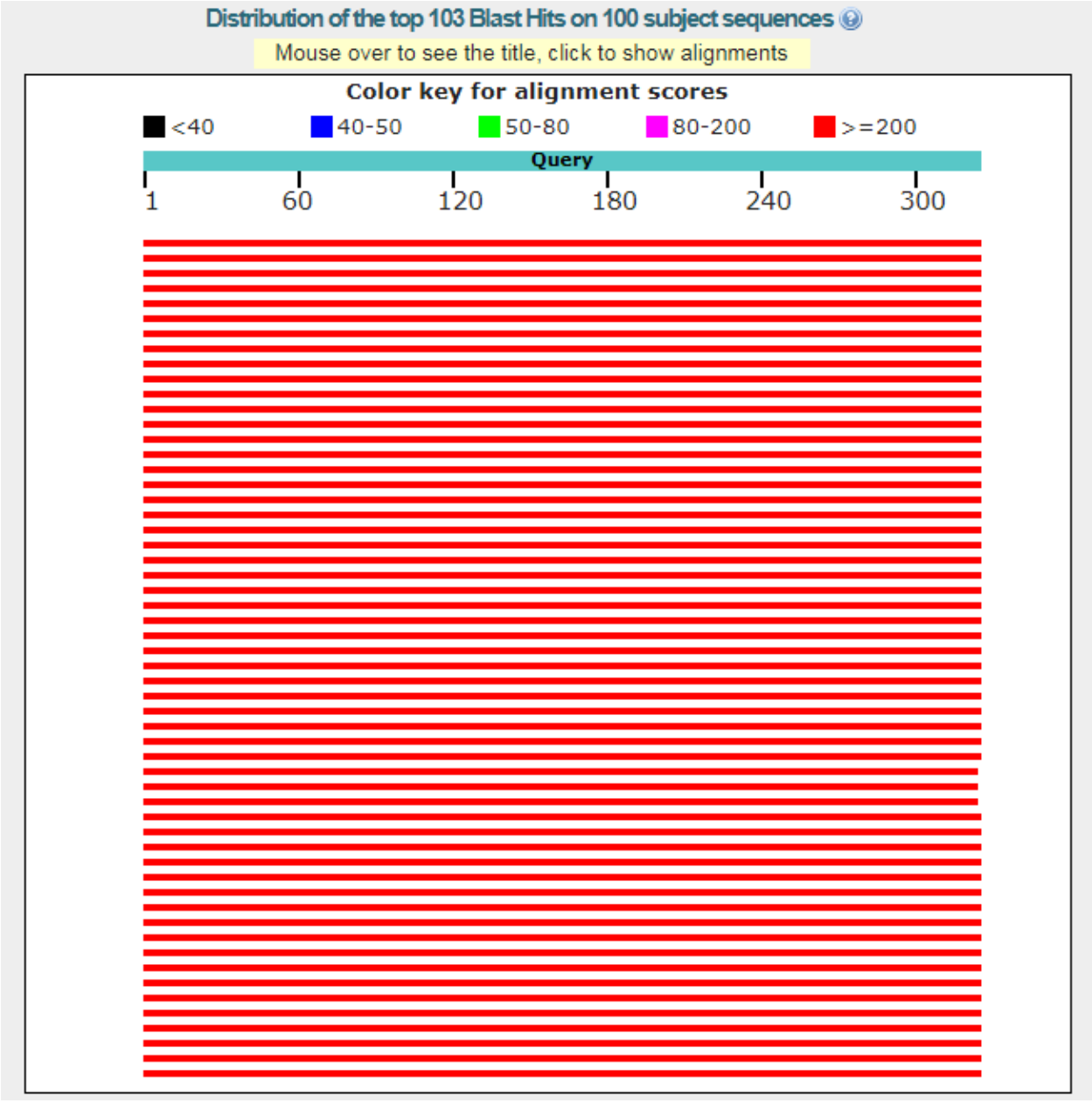


Figure 3.3: A representative figure of BLAST result of HBB gene of a patient. (A) Represents the alignment score of query (HBB gene sequence), (B) represents the matched sequence, query coverage, E value, identity and accession ID. (C) Indicates the mismatch nucleotide with reference sequence (wild type). (D) Sequence chromatogram of patient's HBB gene.

Figure 3.4 shows the BLAST analysis result of HBB gene sequence containing IVS-I-5(G→C) mutation in homozygous form. Alignment result shows perfect match with reference sequence (Red bar >= 200). Figure 3.4 (B) represents query coverage and identity was 100% with E-value of 7e-167, which indicates perfect match with reference sequence of NCBI. In case of homozygous mutation, sequence chromatogram shows single peak in sequence chromatogram (figure 3.4-D).

A



B **Sequences producing significant alignments:**

Select: All None Selected:0

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Homo sapiens isolate BT048A beta globin (HBB) gene, partial cds	597	597	100%	7e-167	100%	KP309825.1
Homo sapiens isolate BT046A beta globin (HBB) gene, partial cds	597	597	100%	7e-167	100%	KP309823.1
Homo sapiens isolate BT033A beta globin (HBB) gene, partial cds	597	597	100%	7e-167	100%	KP309821.1
Homo sapiens isolate BT032A beta globin (HBB) gene, partial cds	597	597	100%	7e-167	100%	KP309820.1

C Homo sapiens hemoglobin subunit beta (HBB) gene, complete cds

Sequence ID: MG675219.1 **Length:** 1606 **Number of Matches:** 1

Range 1: 43 to 365 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
592 bits(320)	3e-165	322/323(99%)	0/323(0%)	Plus/Plus

```
Query  1    CAGACACCATGGTGCATCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  43   CAGACACCATGGTGCATCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCA  102

Query  61   AGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGCTATCAAGGTTACAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103  AGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAG  162

Query  121  ACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  163  ACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTT  222

Query  181  CTGATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223  CTGATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTG  282

Query  241  GTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283  GTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCT  342

Query  301  GTTATGGGCAACCCTAAGGTGAA  323
            |||||||||||||||||||||||
Sbjct  343  GTTATGGGCAACCCTAAGGTGAA  365
```

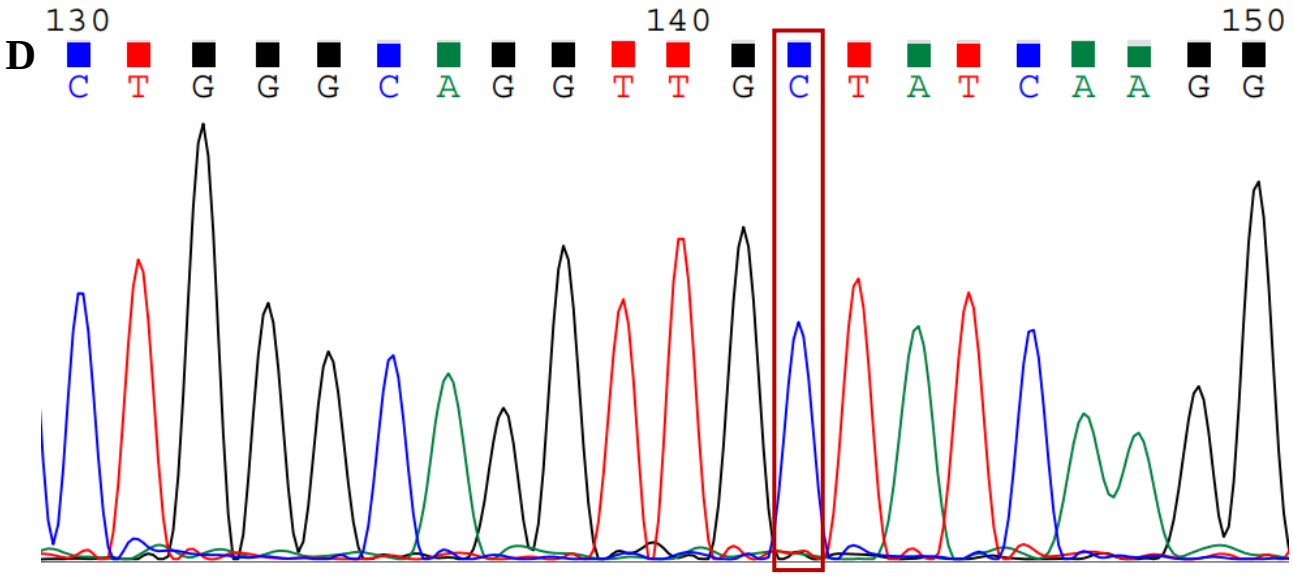


Figure 3.4: Representative BLAST analysis result of HBB gene sequence containing homozygous IVS-I-5(G>C) mutation. (A) Represents the alignment score of query (HBB gene sequence), (B) represents the matched sequence, query coverage, E value, identity and accession ID. (C) Indicates the mismatch nucleotide with reference sequence (wild type). (D) Sequence chromatogram of patient's HBB gene with IVS-I-5 (G>C) mutation in homozygous form.

3.4.2 Spectrum of HBB gene mutation of thalassemia patients

HBB gene mutation was identified for 60 patients enrolled for this study. Table 3.3 shows that combination of HBB gene mutation in thalassemia patients. Among these enrolled patients, most common mutations were c. G79A (codon 26) and IVS-1-5 (G>C). In case of heterozygote mutations, 31 (51.6%) patient's had c. G79A (codon 26) mutation along with IVS-1-5 (G>C) mutation and 11 (18.3%) patients had homozygous IVS-1-5 (G>C) mutation. In case of EBT patients, 03 (5%), 01 (1.6%), 01 (1.6%), 01 (1.6%) and 01 (1.6%) patients had c.126-129del_CTTT (codon 41/42), c. G92C, IVS-I-130 (G>C), c. G47A (Stop codon) and

c.126delC mutation respectively, along with c. G79A (codon 26) mutation in heterozygote form. In case of BTM patients, 02 (3.3%), 02 (3.3%), 02 (3.3%) and 01 (1.6%) patients had {c. G47A (stop codon) + IVS-I-5 G>C}, {c.C33A + c.51delC} and {IVS-I-5 (G>C) + IVS-I-130 (G>C)} mutations, respectively. There were 02 (3.3%) and 01 (1.6%) patients with IVS-I-130 G>C (homozygous) and c. G47A (stop codon) (homozygous) mutation, respectively.

Table 3.3: Spectrum of HBB gene mutations in thalassemia patient enrolled in this study.

Type of thalassemia	Combination of mutations	Sample No
EBT	1. c.G79A (codon 26) 2. IVS-1-5 (G>C)	31 (51.6%)
	1. c.G79A (codon 26) 2. c.126-129del_CTTT (codon 41/42)	03 (5%)
	1. c.G79A (codon 26) 2. c.G92C	01 (1.6%)
	1. c.G79A (codon 26) 2. IVS-I-130 (G>C)	01 (1.6%)
	1. c.G79A (codon 26) 2. c.G47A(Stop codon)	01 (1.6%)
	1. c.G79A (codon 26) (homozygous)	01(1.6%)
	1. c.G79A (codon 26) 2. c.126delC	01 (1.6%)
	1. IVS-I-5 (G>C) (homozygous)	11 (18.3%)

BTM	1. c.G47A (stop codon) 2. IVS-I-5 G>C	02 (3.3%)
	1. c.C33A 2. c.51delC	02 (3.3%)
	1. IVS-I-130 G>C (homozygous)	02 (3.3%)
	1. IVS-I-5 (G>C) 2. c.126-129del_CTTT (codon41/42)	02 (3.3%)
	1. c.G47A (stop codon) (homozygous)	01 (1.6%)
	2. IVS-I-5 (G>C) 3. IVS-I-130 (G>C)	01 (1.6%)
Total		**60**

3.5 Spleen related clinical complications of thalassemia patients

Majority of the participants for this study were suffering from spleen related complications such as Splenomegaly because these patients were transfusion dependent thalassemia patients. Table 3.4 shows that among 39 EBT patients, 18 (46.1%) and 06 (15.4%) had history of splenomegaly and splenectomy, respectively. On the other hand, among 21 BTM patients, 12 (57.1%) and 03 (14.3%) patients had history of splenomegaly and splenectomy, respectively. In case of 15 (38.5%) EBT patients and 06 (28.6%) BTM patients, there were no spleen related complication (Table 3.4).

Table 3.4: Spleen related complications of enrolled patients.

Complications	EBT	Percentage of total patients of EBT	BTM	Percentage of total patients of BTM
Splenomegaly	18	46.1%	12	57.1%
Splenectomy	06	15.4%	03	14.3%
No spleen related complication	15	38.5%	06	28.6%
Total	39		21	

3.6 Hematological parameters of enrolled transfusion dependent thalassemia patients

Before blood transfusion, patients venous blood was taken to measure various hematological parameters such as RBC count, Hemoglobin concentration, mean corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH), Red cell distribution width (RDW) and White blood cell (WBC) count. Table 3.5 shows that mean (±SD) RBC count and Hb concentration were lower in both EBT and BTM in compared to reference value (**RBC**: EBT - 2.93 ± 0.54 million/µl; BTM - 2.89±0.74 million/µl; reference - 5.4 ± 0.7; **Hb concentration**: EBT - 6.37±1.38 g/dL; BTM - 7.16±1.76 g/dL; reference - Men: 14.0-17.5 g/dL, Women: 12.3-15.3 g/dL). The mean (±SD) MCV and MCH were also lower in both EBT and BTM patients compared to reference value (**MCV**: EBT - 69.23 ± 6.82 fL/red cell, BTM - 74.76 ± 6.70, reference - 88±8 fL/red cell; **MCH**: EBT - 21.88 ± 3.49 fL/red cell, BTM - 24.83 ± 2.25 fL/red

cell, reference – 30 ± 3 fL/red cell). The mean (±SD) RDW and WBC count of the enrolled EBT and BTM patients were higher than reference range (**RDW**: EBT - 24.56 ± 4.16, BTM - 18.48±4.68, reference - 11.5-14.5; **WBC**: EBT - 14020 ± 9585.56 per mm^3, BTM - 16044 ± 17,348 per mm^3, reference - 7250 ± 2750 per mm^3) (Table 3.5).

Table 3.5: Comparison of hematological parameters of transfusion dependent thalassemia patients before transfusion.

Blood parameters	EBT	BTM	Reference Value
RBC count(M/µl)	2.93±0.54	2.89±0.74	5.4±0.7
Hb concentration (g/dL)	6.37±1.38	7.16±1.76	• Men: 14.0-17.5 (mean 15.7) g/dL • Women: 12.3-15.3 (mean 13.8) g/dL
MCV(fL/red cell)	69.23±6.82	74.76±6.70	88±8
MCH(pg/red cell)	21.88±3.49	24.83±2.25	30±3
RDW	24.56±4.16	18.48±4.68	11.5-14.5
WBC(/mm^3)	14020 ± 9585.56	16044 ± 17,348	7250 ± 2750

3.7 Disease severity according to frequency of blood transfusion requirement

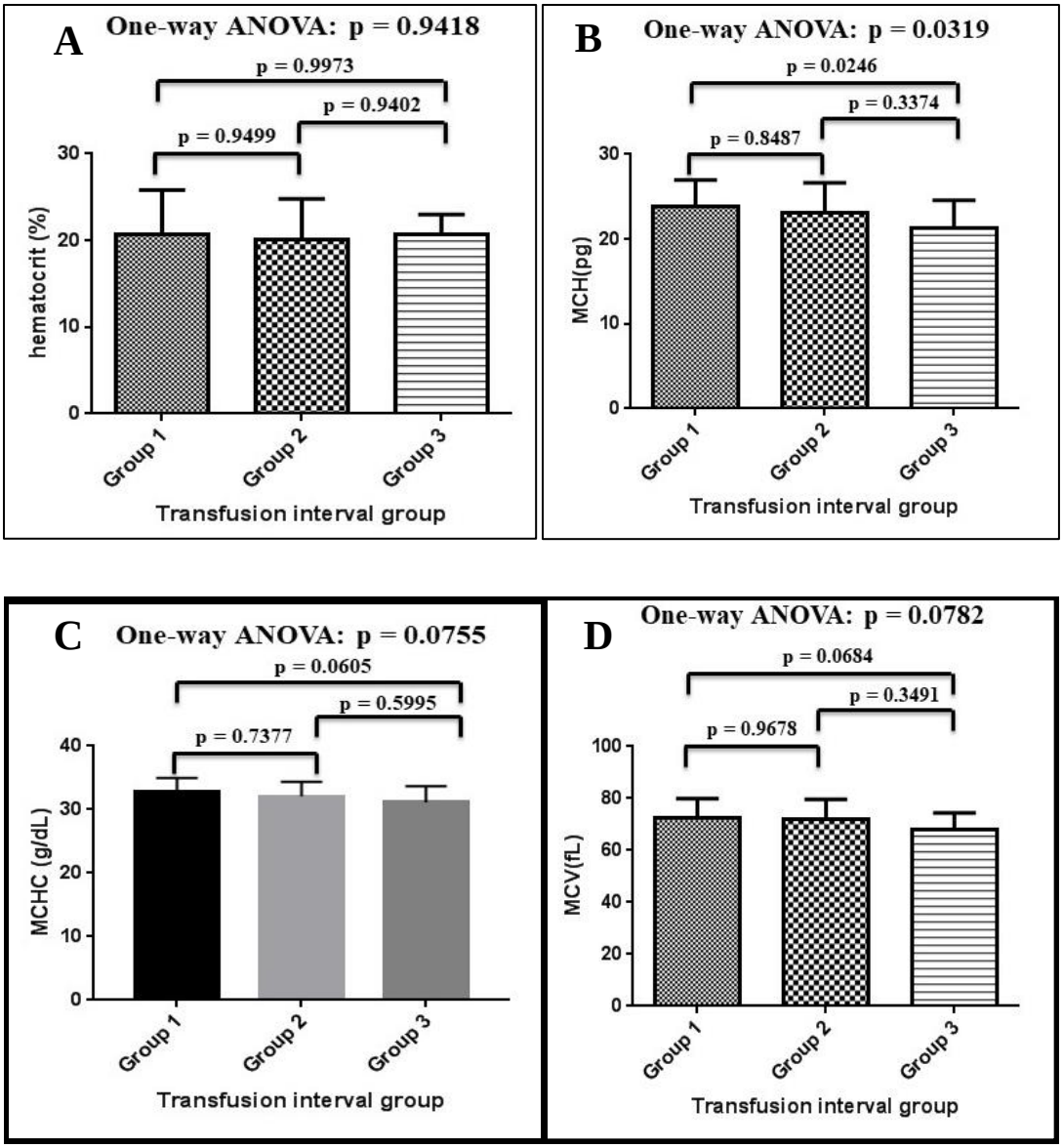
Disease severity can be determined form frequency of blood transfusion requirement. According to the frequency of blood transfusion, the enrolled patents were divided into three groups, namely, group 1 (transfusion interval less than 1 month), group 2 (transfusion interval between 1 month and 1.5 months) and group 3 (transfusion interval > 1.5 months). Table 3.6 shows the distribution of groups according to blood transfusion intervals and the type of thalassemia. Table 3.6 shows that Group 1 (most severe), Group 2 (moderate severe) and Group 3 (less severe) belongs to 32 (53.3%), 9 (15.0%) and 19 (31.7%) patients, respectively. Among Group 1 patients, 13 and 19 patients were EBT and BTM patients, respectively. In Group 2 and 3 majority of the patients were EBT patients (Group 2: EBT- 8, BTM – 1; Group 3: EBT – 18, BTM - 1).

Table 3.6: Classification of the enrolled patients according to the blood transfusion interval.

Thalassemia type	Group 1 (< 1 month)	Group 2 (>1 month < 1.5 months)	Group 3 (> 1.5 months)
EBT	13	8	18
BTM	19	1	1
Total	**32 (53.3%)**	**9 (15%)**	**19 (31.7%)**

3.8 Comparison of hematological parameters among patients' groups according to transfusion interval

Hematological parameters such as hematocrit, Hemoglobin concentration, RBC count, mean corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH), Red cell distribution width (RDW) and White blood cell (WBC) count were compared among three groups (Group 1, Group 2 and Group 3) using one-way ANOVA test. Figure 3.6 indicates that there was no statistically significant difference (p value less than 0.05 was considered as significant) among three group in terms of percentage of hematocrit (ANOVA: $p = 0.9973$), MCHC (ANOVA: $p = 0.0605$), MCV (ANOVA: $p = 0.0782$), platelet count (ANOVA: $p = 0.9710$), RBC count (ANOVA: $p = 0.3954$) and WBC count (ANOVA: $p = 0.7415$) but there was statistically significant difference in terms of MCH (ANOVA: $p = 0.0319$) and RDW (ANOVA: $p = 0.0020$) value between Group 1 and Group 3. The mean (±SD) MCH of Group 1 and Group 3 were 23.82 ± 3.16 pg and 21.27 ± 3.30 pg, respectively (Group 1 versus Group 3: $p = 0.0246$). On the other hand, the mean (±SD) RDW of Group 1 and Group 3 patients were 20.67 ± 5.31 and 25.75 ± 3.34, respectively (Group 1 versus Group 3: $p = 0.0014$). These data clearly demonstrate that group 3 (less severe group) patients had lower MCH and higher RDW value compared to the Group 1 patients (most severe group).


A
One-way ANOVA: p = 0.9418
p = 0.9973
p = 0.9402
p = 0.9499
hematocrit (%)
30
20
10
0
Group 1
Group 2
Group 3
Transfusion interval group
B
One-way ANOVA: p = 0.0319
p = 0.0246
p = 0.3374
p = 0.8487
MCH(pg)
30
20
10
0
Group 1
Group 2
Group 3
Transfusion interval group
C
One-way ANOVA: p = 0.0755
p = 0.0605
p = 0.5995
p = 0.7377
MCHC (g/dL)
40
30
20
10
0
Group 1
Group 2
Group 3
Transfusion interval group
D
One-way ANOVA: p = 0.0782
p = 0.0684
p = 0.3491
p = 0.9678
MCV(fL)
100
80
60
40
20
0
Group 1
Group 2
Group 3
Transfusion interval group

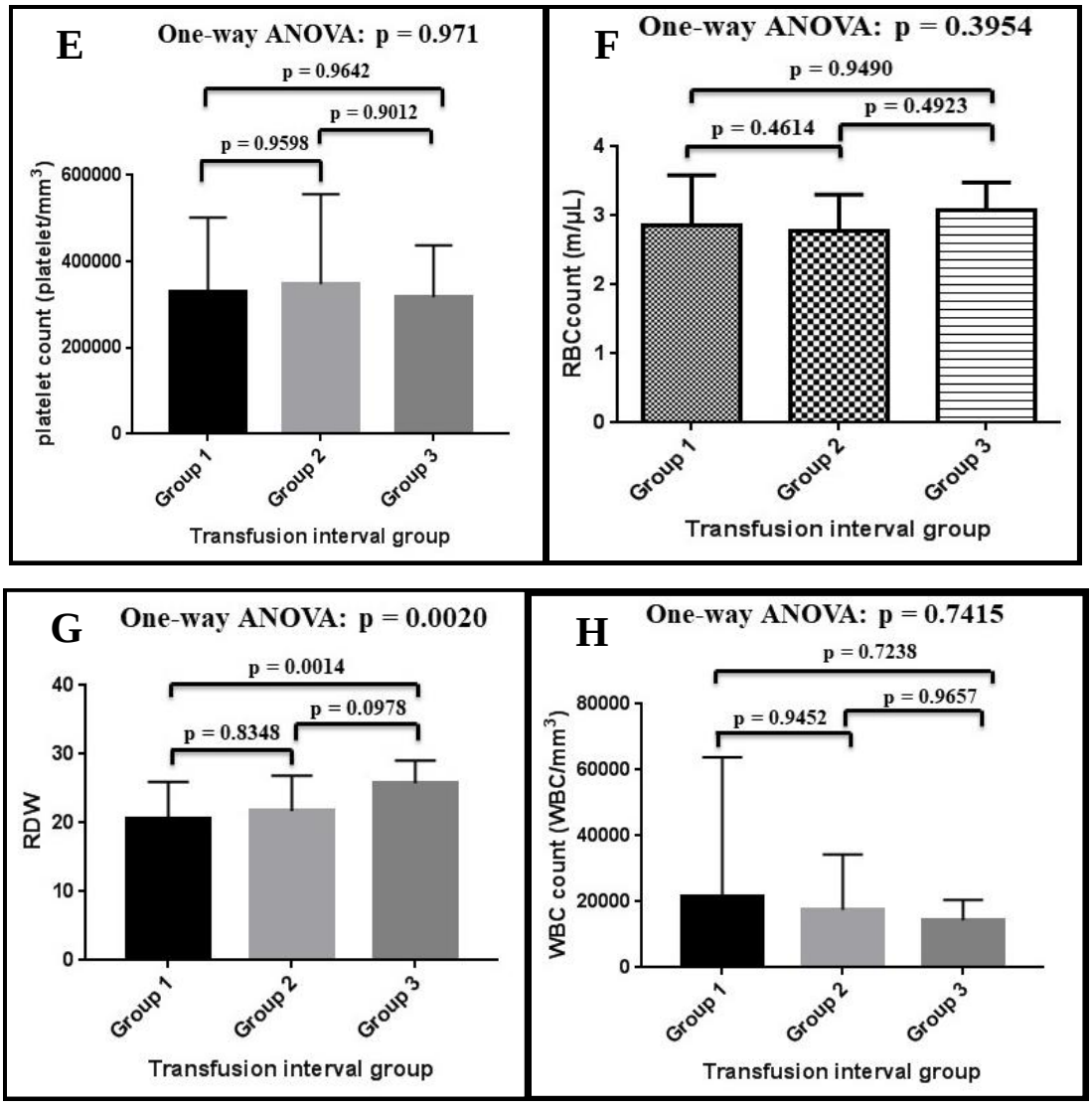
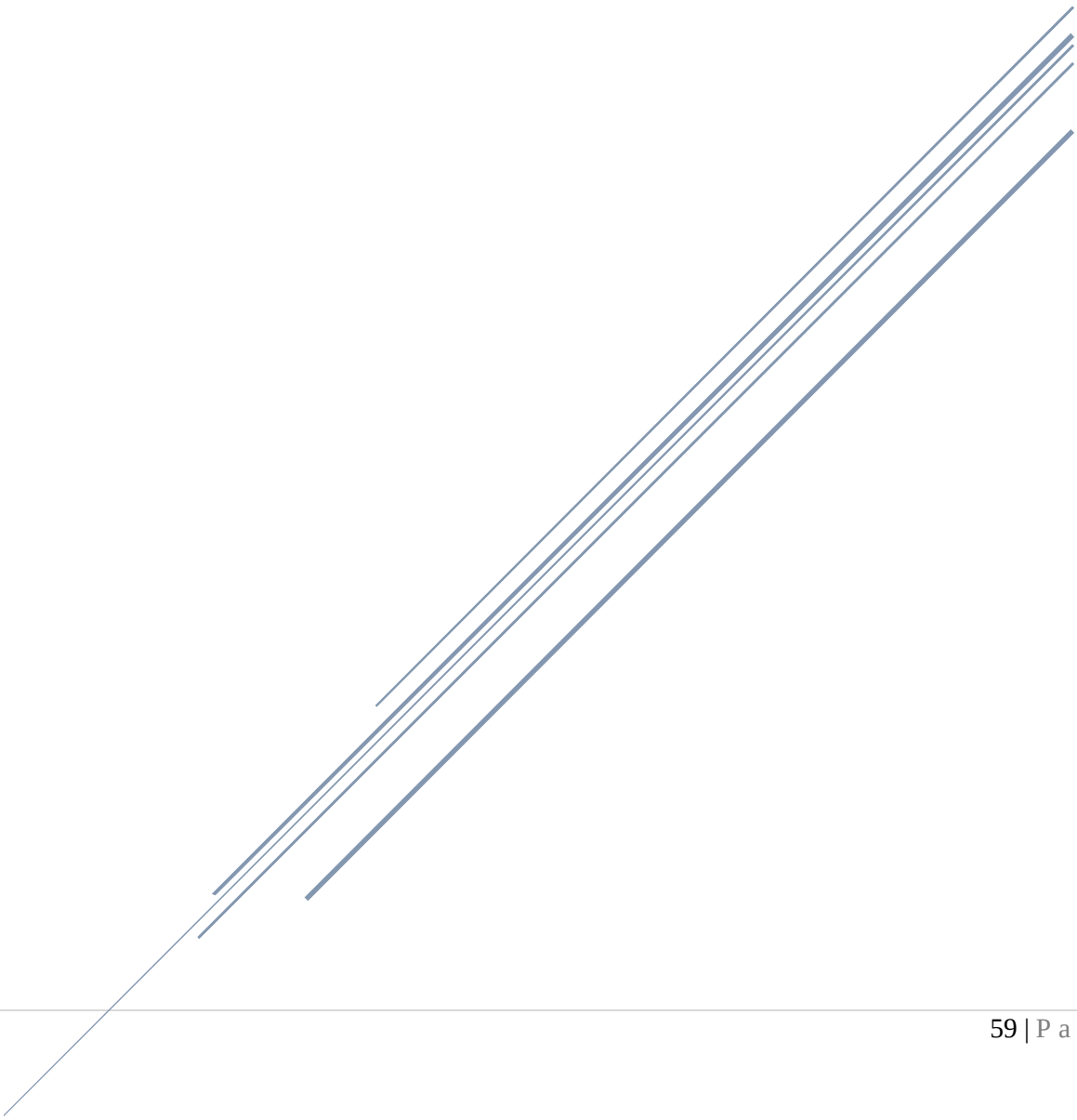


Figure 3.5: Comparison of hematological parameters among patients' groups according to transfusion interval using one-way ANOVA test. (A) Comparison of percentage of hematocrit among three groups, (B) Comparison of MCH among three groups, (C) Comparison of MCHC among three groups, (D) Comparison of MCV among three groups, (E) Comparison of platelet count among three groups, (F) Comparison of RBC count among three groups, (G) Comparison of RDW among three groups and (G) Comparison of WBC count among three groups.

DISCUSSION

Chapter 4



4. Discussion

Hemoglobinopathies are monogenic disorders of erythrocyte formation that has a widespread prevalence extending from Mediterranean zone, Middle East, Indian subcontinent, and parts of Southeast Asia [48]. This study provides a comprehensive data on the mutation pattern of *HBB* gene and association of disease severity with hematological parameters among the diagnosed patients with thalassemia in Bangladesh.

Information regarding mutation spectrum of thalassemia patient is still scarce in our country at national level. Moreover, investigation of mutation information is not a common practice in our country. Thalassemia patients are initially suspected based on common hematological parameter screening followed by hemoglobin electrophoresis. Through this study RDWs which were higher and MCH which were lower in the patients with both BTM and EBT could provide information about the existence of a wide range of microcytic RBC population that vary in size in the circulation of patients with BTM and EBT. However, no study has been conducted investigating any possible correlation between mutation in hemoglobin gene and hematological parameters among thalassemia patient. The results found in this study might help the clinicians to suspect the type of thalassemia based on the hematological parameter named RDW and MCH, although it requires further study using large number of thalassemia patient.

Present study was conducted among 60 transfusion dependent thalassemia patients. Among these patients 39 (65%) and 21 (35%) patients were diagnosed with HbE-beta thalassemia (EBT) and beta-thalassemia major (BTM), respectively using Hb electrophoresis. EBT was the most common type of thalassemia constituting of 65% of 60 cases. It was also detected as the

most common type of thalassemia in a similar study from Bangladesh by Uddin *et al.* (13%) and Khan *et al.* (10.87%) [34, 42]. Similar studies were conducted by Jain BB *et al.* [49], Mondal SK *et al.* [50] and Chaudhury SR *et al.* [51] from India. For identification of mutations of human beta globin (HBB) gene, 428 bp of *HBB* gene were amplified which covers exon 1, intron 1 and exon 2 using previously described PCR method. This region have been selected because this hot spot region harbors majority of *HBB* gene mutations in many other countries of South Asia and Southeast Asia [52].

This study had identified nine known variants (c.79 G>A, IVS-1-5 G>C, c.126-129 del_CTTT, IVS-I-130 G>C, c.47 G>A, c.33 delA, c.51delC, c. G92C & c.126delC) among EBT and BTM patients. Similar pattern of *HBB* gene mutations were observed by Sultana GNN *et al.* (2016), where they have also found nine mutations (-90 C>T, Codon 1 T>A, Codon 2C>A, Codon 2 T>C, HbE /Codon 26 G>A, Codon 30 G>C, IVS-I-5 G > C, IVS-2-16 G>C and IVS-2-81 C>T) of *HBB* gene among 70 Bangladeshi β-thalassemia patients. In this study, the most common mutations were IVS-1-5 (G>C) and c.79 (G>A), which was also reported as the most common mutations among Bangladeshi population by Sultana *et al.* (2016) [17]. In case of heterozygote mutations, 31 (51.6%) patients had c. G79A (codon 26) mutation along with IVS-1-5 (G>C) mutation and 11 (18.3%) patients had homozygous IVS-1-5 (G>C) mutation. In case of codon 26 (G>A) or Hb E {HBB c.79 G>A}[47], due to alternative splice site generation, a mutated beta-chain is produced which is associated with EBT [17]. In case of IVS-I-5 (G>C) mutation, very low amount of beta-globin chain synthesis occurred because this mutation inhibits the appropriate splicing of *HBB* gene. These mutations are reported as the most common mutation in Indian population by Panigrahi and Marwaha (2007) [18]. These mutations were also reported as the most common mutations in UAE (55%) and Oman (62%)

by Lahiry *et al.* and Khan *et al*., respectively [53, 54]. In Bangladesh, Hb-E (Codon 26 G>A) is the second most common mutation and most of the EBT patients had IVS-I-5 G> C mutation along with HbE codon 26 G>A [17]. Another study by Ibn Ayub *et al.* also observed that 62.5% patients had codon 26 (G>A) mutation among Bangladeshi population [54]. In case of c.126-129 del_CTTT (41/42) in exon 2, inhibition of synthesis of beta globin chain occurs due to frame shift[55] and this mutation was found among the EBT and BTM patients. For IVS-I-130 (G>C) mutation in intron 1, inhibition of splicing occurs which results the impaired synthesis of beta globin chain.

The c.47 G>A mutation of HBB gene causes premature termination which prevents the synthesis of beta-globin chain, present in both EBT and BTM patients. In case of c.C33A and c.51delC frame shift mutations in exon 1, inhibition of synthesis of beta globin chain occurs which was present in two type of BT patients among the enrolled patients for this study. Another frame shift mutation of *HBB* gene called c.126 delC prevents the synthesis of beta-globin chain and this mutation was found among the enrolled patients with EBT.

In this study it was also found that majority of the participants were suffering from spleen related complications and the spleen is most commonly affected in form of splenomegaly because of excessive destruction of abnormal RBCs, extra- medullary hematopoiesis and iron overload due to transfusion [56].

It was found that thalassemia patient's had lower level of RBC count, hematocrit, MCV and MCH compared to established reference value for the healthy individuals. These are consistent with the study performed by Galanello R *et al.*, which reported that the range of MCV, MCH and Hb level were 50-70 fl, 12-20 pg and < 7 gm/dl, respectively among EBT and BTM

patients [57]. The mean RDW level and WBC count of the enrolled EBT and BTM patients were higher than the reference range.

Blood transfusion was given to the patients after the Hb concentration drops below 7 g/dl. If the Hb level drops below 7 g/dl faster, the disease is more severe. Greater the disease severity is, the more frequent the patient requires blood transfusion, depending on the individual's consumption of the infused cells. In this study, group 1 (most severe), group 2 (moderate severe) and group 3 (less severe) belongs to 32(53.3%), 9(15.0%) and 19(31.7%) patients, respectively. The most severe disease group comprises of both EBT and BTM patients, whereas the less severe group comprises of mostly EBT patients. These findings show consistency with the findings of a study performed by Hossain *et al*. [47].

One-way ANOVA test was performed for some hematological parameters such as hematocrit, Hb concentration, RBC count, MCV, MCH, RDW and WBC count among three groups, namely, Group 1 (< 1 month), Group 2 (>1 month < 1.5 months) and Group 3 (> 1.5 months). No statistical significant difference was found (p value less than 0.05 was considered as significant) among three groups in terms of percentage of hematocrit (ANOVA: $p = 0.9973$), MCHC (ANOVA: $p = 0.0605$), MCV (ANOVA: $p = 0.0782$), platelet count (ANOVA: $p = 0.9710$), RBC count (ANOVA: $p = 0.3954$) and WBC count (ANOVA: $p = 0.7415$) but there was statistically significant difference in terms of MCH (ANOVA: $p = 0.0319$) and RDW (ANOVA: $p = 0.0020$) value between Group 1 and Group 3 in our study. The data from this study clearly demonstrates that group 3 (less severe group) patients had lower MCH and higher RDW value compared to the Group 1 patients (most severe group). Several studies reported that the Xmn I polymorphism, mutation or deletion in beta-globin locus control region (HBB-*LCR*), heterozygous distribution of HbF among RBCs etc. are the major factors that could

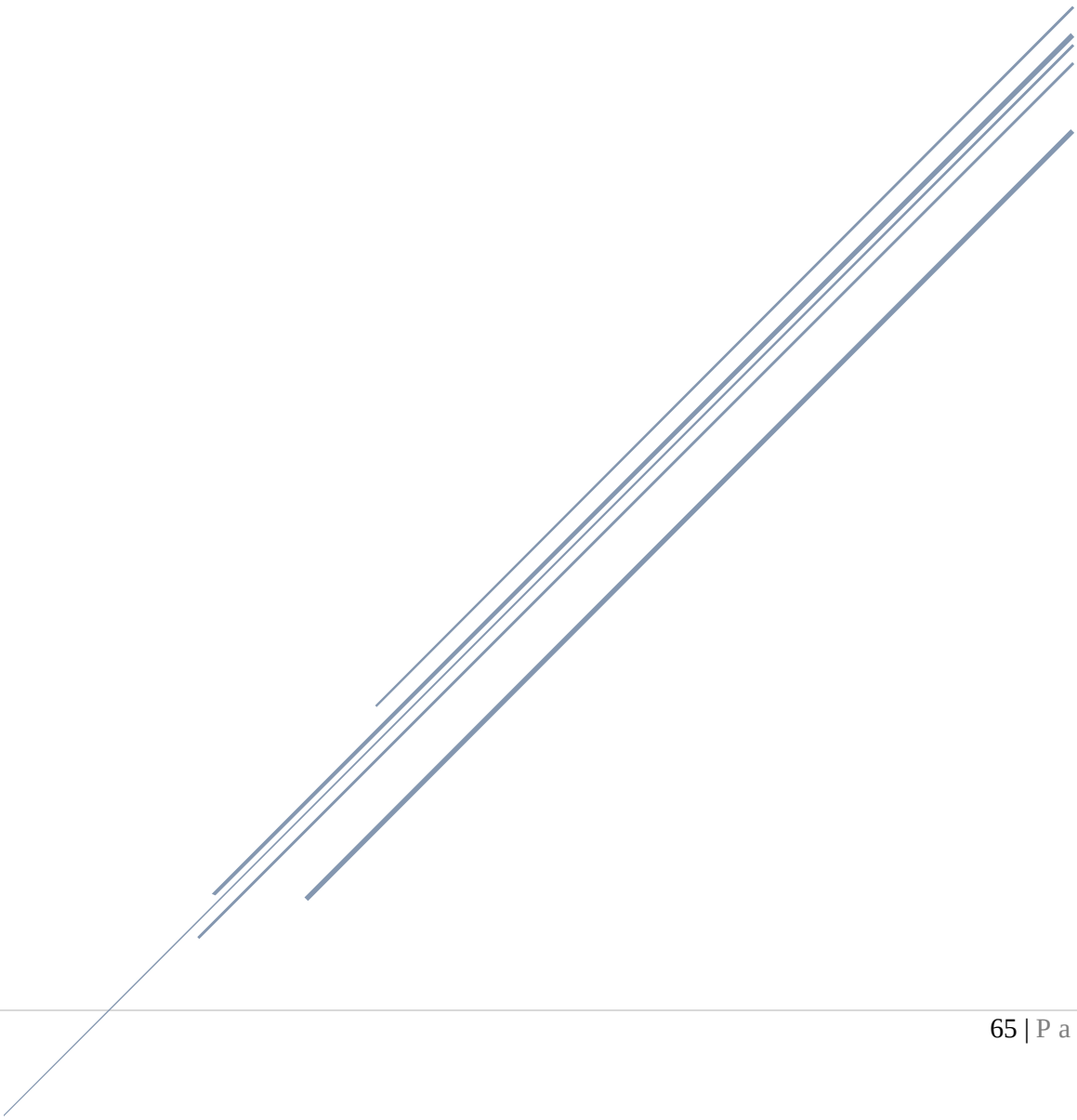
significantly contribute to the higher levels of RDW among the patients of less severe group compared to the patients of most sever group [58-63]. Spliced variants (Hb-E) are produced due to c. G79A (codon 26) mutation in *HBB* gene and majority of the patients were EBT among the less severe group in this study. For this reason, the much higher diversity in RDWs in the patients of less severe group (mostly with EBT patients) might be due to the presence of different levels of normal spliced variants in different RBCs, whereas such normal splice variants are absent in the patients with BTM (most sever group) [64].

Conclusion

In this study it has been demonstrated the mutation profile of limited number of EBT major patients and association of hematological parameters with disease severity among thalassemia patients. It was successfully found the most common mutations responsible for the disease and higher RDW level in less disease sever group compared to most disease severe group. This study will be helpful to understand the current mutation spectrum and association of disease severity with hematological parameters in Bangladesh. This finding in this study can be beneficial in future for effective molecular drug designing and other therapeutic approaches for EBT and BTM patients.

REFERENCES

Chapter 5



5. References

1. You D, Hug L, Ejdemyr S, Beise J. Levels and trends in child mortality. Estimates developed by the UN Inter-agency Group for Child Mortality Estimation (IGME). Report 2015. 2015.

2. McKusick VA. Mendelian inheritance in man: a catalog of human genes and genetic disorders. JHU Press; 1998.

3. Mennuti MT. Genetic screening in reproductive health care. Clinical obstetrics and gynecology. 2008;51(1):3-23.

4. Organization WH. Proposed international guidelines on ethical issues in medial genetics and genetic service. 1997.

5. Raffle AE. Information about screening–is it to achieve high uptake or to ensure informed choice? Health Expectations. 2001;4(2):92-8.

6. Abolghasemi H, Amid A, Zeinali S, Radfar MH, Eshghi P, Rahiminejad MS et al. Thalassemia in Iran: epidemiology, prevention, and management. Journal of Pediatric Hematology/Oncology. 2007;29(4):233-8.

7. Modell B, Khan M, Darlison M, King A, Layton M, Old J et al. A national register for surveillance of inherited disorders: beta thalassaemia in the United Kingdom. Bulletin of the World Health Organization. 2001;79:1006-13.

8. Birgens H, Ljung R. The thalassaemia syndromes. Scandinavian Journal of Clinical and Laboratory Investigation. 2007;67(1):11-26. doi:10.1080/00365510601046417.

9. Modell B. Update to epidemiology of hemoglobin disorders with special references to thalassemia. Thalassemia International Federation. Internet. Transfus Med. 1995;5:247-58.

10. Khan W. Prevention is the only way to combat thalassaemia. Daily Star. 2006;5(576):1-2.

11. Weatherall D, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization. 2001;79:704-12.

12. Shekhar H, Kabir Y. E-Jannat KK, Hossain S, et al. Blood transfusion-mediated viral infections in thalassemic children in Bangladesh J Med Sci. 2007;7:131-5.

13. Ong-Ajyooth S, Suthipark K, Shumnumsirivath D, Likidlilid A, Fucharoen S, Pootrakul P. Oxidative stress and antioxidants in beta-thalassaemia/haemoglobin E. Journal of the Medical Association of Thailand= Chotmaihet thangphaet. 1987;70(5):270.

14. Orkin SH, Kazazian Jr HH, Antonarakis SE, Ostrer H, Goff SC, Sexton JP. Abnormal RNA processing due to the exon mutation of βE-globin gene. Nature. 1982;300(5894):768.

15. Islam MT, Sarkar SK, Sultana N, Begum MN, Bhuyan GS, Talukder S et al. High resolution melting curve analysis targeting the HBB gene mutational hot-spot offers a reliable screening approach for all common as well as most of the rare beta-globin gene mutations in Bangladesh. BMC Genetics. 2018;19(1):1. doi:10.1186/s12863-017-0594-3.

16. Chatterjee T, Chakravarty A, Chakravarty S, Chowdhury MA, Sultana R. Mutation spectrum of β-thalassemia and other hemoglobinopathies in Chittagong, Southeast Bangladesh. Hemoglobin. 2015;39(6):389-92.

17. Sultana G, Begum R, Akhter H, Shamim Z, Rahim M, Chubey G. The complete Spectrum of beta (β) thalassemia mutations in Bangladeshi population. Austin Biomarkers Diagn. 2016;3:1-6.

18. Panigrahi I, Marwaha R. Mutational spectrum of thalassemias in India. Indian journal of human genetics. 2007;13(1):36.

19. Fisher CA, Premawardhena A, De Silva S, Perera G, Rajapaksa S, Olivieri NA et al. The molecular basis for the thalassaemias in Sri Lanka. British journal of haematology. 2003;121(4):662-71.

20. George E, Teh L, Rosli R, Lai M, Tan J. Beta Thalassaemia mutations in Malays: A simplified cost-effective strategy to identify the mutations. Malaysian Journal of Medicine and Health Sciences. 2012;8(1):1-8.

21. Ansari SH, Shamsi TS, Ashraf M, Bohray M, Farzana T, Khan MT et al. Molecular epidemiology of β-thalassemia in Pakistan: far reaching implications. International journal of molecular epidemiology and genetics. 2011;2(4):403.

22. Boonyawat B, Monsereenusorn C, Traivaree C. Molecular analysis of beta-globin gene mutations among Thai beta-thalassemia children: results from a single center study. The application of clinical genetics. 2014;7:253.

23. Harano T, Harano K, Okada S, Shimono K. A wider molecular spectrum of β-thalassaemia in Myanmar. British journal of haematology. 2002;117(4):988-92.

24. Steinberg MH, Forget BG, Higgs DR, Weatherall DJ. Disorders of hemoglobin: genetics, pathophysiology, and clinical management. Cambridge University Press; 2009.

25. Hofmann O, Mould R, Brittain T. Allosteric modulation of oxygen binding to the three human embryonic haemoglobins. Biochemical Journal. 1995;306(2):367-70.

26. Steinberg MH. In the clinic. Sickle cell disease. Annals of internal medicine. 2011;155(5):ITC31-15; quiz ITC316. doi:10.7326/0003-4819-155-5-201109060-01003.

27. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. British journal of haematology. 2010;149(2):181-94.

28. Sankaran VG, Menne TF, Xu J, Akie TE, Lettre G, Van Handel B et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. Science. 2008;322(5909):1839-42.

29. Borg J, Papadopoulos P, Georgitsi M, Gutiérrez L, Grech G, Fanis P et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nature genetics. 2010;42(9):801.

30. Monod J, Wyman J, Changeux J-P. On the nature of allosteric transitions: a plausible model. Biol. 1965;12:228-113.

31. Fermi G, Perutz M, Shulman R. Iron distances in hemoglobin: comparison of x-ray crystallographic and extended x-ray absorption fine structure studies. Proceedings of the National Academy of Sciences. 1987;84(17):6167-8.

32. Vidja PJ, Vachhani J, Sheikh S, Santwani P. Blood transfusion transmitted infections in multiple blood transfused patients of beta thalassaemia. Indian Journal of Hematology and Blood Transfusion. 2011;27(2):65-9.

33. Weatherall D, Clegg J. Thalassemia-a global health problem. Nat Med. 1996;2(8):847-9.

34. Uddin MM, Akteruzzaman S, Rahman T, Hasan A, Shekhar HU. Pattern of ISRN hematology. 2012;2012.

35. Safizadeh H, Farahmandinia Z. Quality of life in patients with thalassemia major and intermedia in kerman-iran (IR). Mediterranean journal of hematology and infectious diseases. 2012;4(1).

36. Grover R, Shahidi S, Fisher B, Goldberg D, Wethers D. Current sickle cell screening program for newborns in New York City, 1979-1980. American Journal of Public Health. 1983;73(3):249-52.

37. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bulletin of the World Health Organization. 2008;86:480-7.

38. Weathall D, Clegg J. Inherited Haemoglobin Disorders: an increasing global problem. Bull World Health Organ. 2001;79:704-12.

39. Flint J, Harding RM, Boyce AJ, Clegg JB. The Population-Genetics of the Hemoglobinopathies. BAILLIERES CLINICAL HAEMATOLOGY. 1993;6(1):215-62.

40. Angastiniotis M, Modell B. Global epidemiology of hemoglobin disorders. Annals of the New York Academy of Sciences. 1998;850(1):251-69.

41. Olivieri NF, Pakbaz Z, Vichinsky E. Hb E/beta-thalassaemia: a common & clinically diverse disorder. The Indian journal of medical research. 2011;134(4):522.

42. Khan W, Banu B, Amin S, Selimuzzaman M, Rahman M, Hossain B et al. Prevalence of beta thalassemia trait and Hb E trait in Bangladeshi school children and health burden of thalassemia in our population. DS (Child) HJ. 2005;21(1):1-7.

43. Fucharoen S, Ketvichit P, Pootrakul P, Siritanaratkul N, Piankijagum A, Wasi P. Clinical manifestation of β-thalassemia/hemoglobin E disease. Journal of Pediatric Hematology/Oncology. 2000;22(6):552-7.

44. Datta P, Basu S, Chakravarty SB, Chakravarty A, Banerjee D, Chandra S et al. Enhanced oxidative cross-linking of hemoglobin E with spectrin and loss of erythrocyte membrane asymmetry in hemoglobin Eβ-thalassemia. Blood Cells, Molecules, and Diseases. 2006;37(2):77-81.

45. Pootrakul P, Sirankapracha P, Hemsorach S, Moungsub W, Kumbunlue R, Piangitjagum A et al. A correlation of erythrokinetics, ineffective erythropoiesis, and erythroid precursor apoptosis in Thai patients with thalassemia: Presented in part at the XXI Congress of the

International Society of Hematology, Sydney, Australia, May 1985, and the Annual Meeting of the American Society of Hematology, San Diego, CA, December 1997. Blood. 2000;96(7):2606-12.

46. Jetsrisuparb A, Sanchaisuriya K, Fucharoen G, Fucharoen S, Wiangnon S, Jetsrisuparb C et al. Development of severe anemia during fever episodes in patients with hemoglobin E trait and hemoglobin H disease combinations. Journal of pediatric hematology/oncology. 2006;28(4):249-53.

47. Hossain MS, Raheem E, Sultana TA, Ferdous S, Nahar N, Islam S et al. Thalassemias in South Asia: clinical lessons learnt from Bangladesh. Orphanet journal of rare diseases. 2017;12(1):93.

48. Oliver N, Weatherall D. Thaseemias. Pediatrics Hematology. 1999:307-27.

49. Jain BB, Roy RN, Ghosh S, Ghosh T, Banerjee U, Bhattacharya SK. Screening for thalassemia and other hemoglobinopathies in a tertiary care hospital of West Bengal: implications for population screening. Indian journal of public health. 2012;56(4):297.

50. Mondal SK, Dasgupta S, Mondal S, Das N. Spectrum of thalassemias and hemoglobinopathies in West Bengal: A study of 90,210 cases by cation exchange high-performance liquid chromatography method over a period of 8 years. Journal of applied Hematology. 2014;5(3):91.

51. Chaudhury SR, Roy H, Bhattacharyya NK, Mondal P, Biswas P, Roy S. Spectrum of hemoglobin variants in Eastern Indian population; a study of 14,145 cases. Al Ameen J Med Sci. 2013;6(3):243-8.

52. Islam MT, Sarkar SK, Sultana N, Begum MN, Bhuyan GS, Talukder S et al. High resolution melting curve analysis targeting the HBB gene mutational hot-spot offers a reliable

screening approach for all common as well as most of the rare beta-globin gene mutations in Bangladesh. BMC genetics. 2018;19(1):1.

53. Lahiry P, Al-Attar S, Hegele R. Understanding beta-thalassemia with focus on the Indian subcontinent and the Middle East. The open hematology Journal. 2008;2(1).

54. Ayub MI, Moosa MM, Sarwardi G, Khan W, Khan H, Yeasmin S. Mutation Analysis of the HBB Gene in Selected Bangladeshi β-Thalassemic Individuals: Presence of Rare Mutations. Genetic testing and molecular biomarkers. 2010;14(3):299-302.

55. Kimura A, Matsunaga E, Takihara Y, Nakamura T, Takagi Y, Lin S et al. Structural analysis of a beta-thalassemia gene found in Taiwan. Journal of Biological Chemistry. 1983;258(5):2748-9.

56. Porecha M, Udani D, Mehta V, Gami A. Splenectomy in management of thalassemia major-A Boon for the little angel. The Internet Journal of Surgery. 2010;24(1).

57. Galanello R, Origa R. Beta-thalassemia. Orphanet journal of rare diseases. 2010;5(1):11.

58. Ansari SH, Shamsi TS, Munzir S, Khan MT, Erum S, Perveen K et al. Gγ-Xmn I polymorphism: a significant determinant of β-thalassemia treatment without blood transfusion. Journal of pediatric hematology/oncology. 2013;35(4):e153-e6.

59. Driscoll MC, Dobkin CS, Alter BP. Gamma delta beta-thalassemia due to a de novo mutation deleting the 5'beta-globin gene activation-region hypersensitive sites. Proceedings of the National Academy of Sciences. 1989;86(19):7470-4.

60. Aslan D, Gümrük F, Gürgey A, Altay C. Importance of RDW value in differential diagnosis of hypochrome anemias. American journal of hematology. 2002;69(1):31-3.

61. Panigrahi I, Agarwal S, Gupta T, Singhal P, Pradhan M. Hemoglobin E-beta thalassemia: factors affecting phenotype. 2005.

62. Khelil AH, Morinière M, Laradi S, Khelif A, Perrin P, Chibani JB et al. Xmn I polymorphism associated with concomitant activation of Gγ and Aγ globin gene transcription on a β0-thalassemia chromosome. Blood Cells, Molecules, and Diseases. 2011;46(2):133-8.

63. Fraser P, Pruzina S, Antoniou M, Grosveld F. Each hypersensitive site of the human beta-globin locus control region confers a different developmental pattern of expression on the globin genes. Genes & development. 1993;7(1):106-13.

64. Winichagoon P, Fucharoen S, Wilairat P, Chihara K, Fukumaki Y. Role of alternatively spliced beta E-globin mRNA on clinical severity of beta-thalassemia/hemoglobin E disease. The Southeast Asian journal of tropical medicine and public health. 1995;26:241-5.