COMPARISON OF MUTATION SPECTRUM, HEMOGLOBIN PROFILES AND HEMATOLOGICAL FEATURES BETWEEN TRANSFUSION DEPENDENT AND NON-DEPENDENT PATIENTS OF HB E/β THALASSEMIA

By

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A thesis submitted to the Department of Department of Mathematics and Natural Sciences
in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all of the main sources of help.

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Approval

The thesis titled "Comparison of Mutation Spectrum, Hemoglobin Profiles and Hematological features Between Transfusion Dependent and Non-dependent Patients of Hb E/β Thalassemia" submitted by Maliha Islam (15136003) of Spring 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor in Biotechnology on 03 October 2019.

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Ethics Statement

All the molecular, hematological and genetic laboratory experiments were conducted in the Biosafety Level 2 (BSL-2) Facilities of the Genetics and Genomics Unit of institute of developing Science and Health initiatives (ideSHi). The ethical approval had been provided by the Bangladesh Medical Research Council (BMRC). All the samples were collected from Thalassemia Samity Hospital with full consent from the patients and their legal guardians.

Abstract

Hemoglobin E/β Thalassemia is an inherited anemic genetic disorder that is highly prevalent in the Southern Asia part of the world. Bangladesh falls in the Thalassemia belt of the world. The clinical manifestation of this disease is widely heterogeneous. The patients are divided into three groups: severe, moderately severe and non-transfusion dependent. In Bangladesh, data about NTDT (Non-Transfusion Dependent Thalassemia) patients are scarce. However, studying NTDT patients is essential as the factors behind its widely variable clinical diversity are still unknown. Here, the mutation spectrum, hemoglobin profiles, and hematological features are compared between the transfusion-dependent and NTD patients to find a responsible factor behind the disease severity. Among the compared parameters, it had been found that Hb F and Hb E play a role in ameliorating the disease severity because the increase in gamma genes of Hb F decreases the α/β-chain imbalance and the high oxygen dissociation power of HbE. This study can be useful for the correct diagnosis of NTDT patients.

Keywords: Haemoglobin E/β Thalassemia; Non-Transfusion Dependent Thalassemia; Disease Severity; Haemoglobin Profiles; Haematological Features; Mutation Spectrum.

Dedicated to Kishwara Sultana and Dr. AKM Akhtar Murshed, my loving guardians

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List of Acronyms

BMRC	Bangladesh Medical Research Council
BSL 2	Biosafety Level 2
BTM	Beta Thalassemia
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
GDF15	Growth Differentiation Factor 15
Hb	Hemoglobin
HCT	Hematocrit
HGB	Haemoglobin Concentration
HRM	High-Resolution Melting
ideSHi	Institute Of Developmental Science And Health Initiatives
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
mRNA	Messenger RNA
NTDT	Non-Transfusion Dependent Thalassemia
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
RDW	Red Cell Distribution Width

Chapter 1: Introduction

1.1 Background

Thalassemia is an inherited hemoglobin genetic disorder. Among various types of Thalassemia, alpha (α) and Beta (β)-thalassemia are the most highlighted types. Alpha and Beta-thalassemia are the result of the malfunctioning production of α and β globin chains in Hemoglobin A variant. The inheritance of this disease involves a typical Mendelian-recessive pattern which implies that the inheritance of the defective genes each from one parent. Also, the parents need to be carriers or heterozygous in order to have the probability (50 %) of giving birth to a child with Thalassemia. Co-inheritance of Thalassemia with other variants of Hemoglobin can also occur in a patient depending on the inheritance pattern. The random inheritance pattern results in diversity in clinical severity among thalassemia patients, globally (Weatherall, 2001). The severity of Thalassemia can be characterized in different classes based on their transfusion intervals as this syndrome mainly includes erythropoiesis ineffectiveness, peripheral hemolysis, and anemia. A patient may need transfusion during the entire life span in order to survive. In these patients, the main issues are the increase in toxicity due to iron overloading which may lead to death at an early age (Rachmilewitz, 2011). However, some patients do not need transfusions regularly still survive a normal life; this classification is Non-Transfusion Dependent (NTD) patients. NTD patients can be of β Thalassemia or Hb E/β Thalassemia.

This study has been done on the patients of HbE/β Thalassemia. This disease occurs due to the substitution mutation of G to A in the codon 26 which results in the change in amino acid resulting in the mutated Hb E variant. The patients with HbE/β Thalassemia co-inherit the structural variant of Hb E from one parent and the allele of β-thalassemia allele from the other parent. The interactions of HbE with the β-globin gene make it a milder disease (Oliveri, 2010). However, the clinical diversity disease severity of E/β Thalassemia is wide as the phenotypic characteristics may alter overtime in one patient but the factors affecting this variation are still to be determined.

The patients suffering from Hb E/β Thalassemia are highly likely to suffer from other diseases such as Hepatitis B and C, HIV Infection, cardiac diseases, liver malfunctioning, spleen deformity, osteoporosis, growth retardation, brain strokes, etc. Altogether, families of Bangladesh who have a Thalassemia patient to nurture go through severe economic and psychological issues to provide the patient with proper treatment.

1.2 Global Epidemiology of Thalassemia

Bangladesh is a part of the global thalassemia belt where the number of β Thalassemia patients, Hb E/β Thalassemia patients and carriers are very high (Islam, 2018). The population with Thalassemia can be divided into halves with one half being suffering from E/β Thalassemia and the other half from β-Thalassemia. Among these, Hb E/β-Thalassemia is more observed in the South-East part of Asia including Bangladesh (Weatherall, 2001). According to a report by WHO (World Health Organization), 6.5 % of the world population are born with hemoglobin disorders among which Thalassemia is one of the most prevalent diseases (Biswas, 2018). Globally, 20,000 children are born with E/β Thalassemia (Rahman, 2011). Three thousand children are born in a year and more than one hundred thousand patients are in the population of Thailand, annually (Flint, 1998). Moreover, 4% is the estimated frequency for Hb E/β Thalassemia and β-Thalassemia gene in China (Angastiniotis, 1998). Also, in Indonesia and Srilanka, greater than half of the population suffers from Thalassemia (Olivieri, 2010).

It has been found out through surveys that Thalassemia is more prevalent in the economically less established countries where the mortality rate is higher along with increased rate of malnutrition, improper hygiene, communicable diseases, poor health care, not enough awareness campaigns, and consanguineous marriages (Weatherall, 2010). In India, Bangladesh, Thailand, Laos, and Cambodia, the largest frequencies of HbE thalassemia are found worldwide. Hb E/β thalassemia has become a major public health problem throughout these nations and through areas of China, Indonesia, and Sri Lanka. It has become the most common form of β-thalassemia found in many newborn screening programs in North America (Oliveri, 2012). In Bangladesh, there are in total over 10 million people born carriers and the number of patients with E/β Thalassemia is increasing with time (Rahman, 2011). The lack of centers for the statistical survey and the lack of awareness of the fact about the high prevalence of Thalassemia have made it impossible to estimate the correct number of patients in our country. However, there have been many studies on the Thalassemia carriers in Bangladesh through various screening programs. The demographic illustration of Thalassemia carriers are shown in the figure. Moreover, there is an emerging population of thalassemia disorders and carriers in the European countries in the last decade. In a survey carried out with the population of 12 different countries in Europe, it has been found out that

the carrier and the Thalassemia patient population is more prevalent within the immigrant population in these countries than the native people there. Overall, the prevalence of Thalassemia, both β and E/β, is high; however, the regions in Southeast Asia comprise the majority of the incidences.

Figure 1: Demographic Illustration of Thalassemia Carriers over Bangladesh (Tahura, 2016)

1.3 Hemoglobin E/β Thalassemia

Hemoglobin E/β Thalassemia is one of the very common hemolytic anemia in Southern Asia. A person is born with Hb E/β Thalassemia when the parents are carriers or patients of this disease. When one Hemoglobin β allele is inherited from one parent and the Hb E allele is inherited from the other parent, the child suffers from Hb E/β-Thalassemia. The inheritance pattern is provided below.

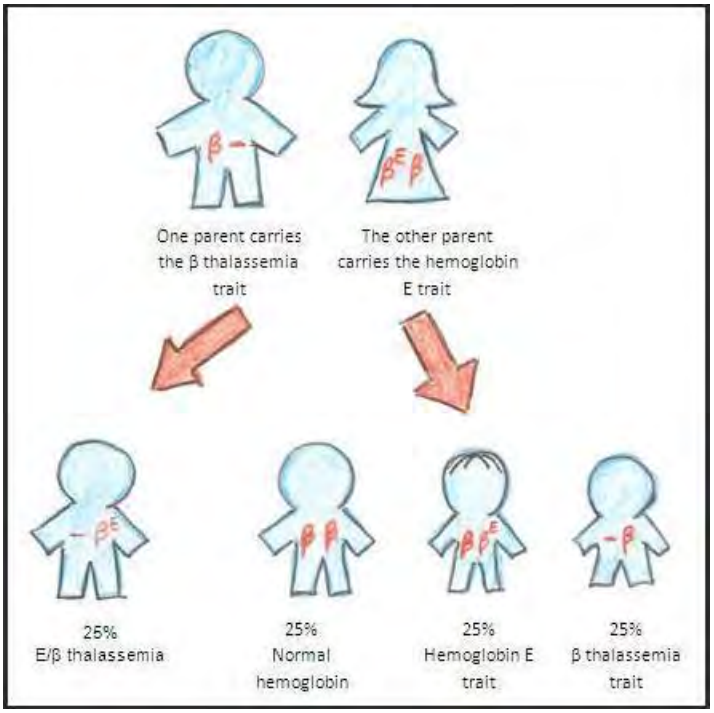

Figure 2: Inheritance pattern of E/β Thalassemia (Biswas, 2018)

1.4 History of Hb E/β Thalassemia

Hemoglobin E was the fourth to be discovered as an abnormal hemoglobin variant in 1954 and the first description of Hb E/β-Thalassemia was published in 1955 (Rahman, 2011). HbE/β-thalassemia in people may alter over time. The phenotypic variability, the way the later course of the disease cannot be assumed in the first few years of life, and the absence of understanding of the disease's natural history combine to make it particularly difficult to manage HbE/β-thalassemia (Weatherall, 1998).

1.5 Mutations involved in Hb E/β Thalassemia

Hb E/β-Thalassemia is caused by the substitution mutation from G>A in codon 26 of the β-globin gene so the alternate splicing gets induced that results in the change in the amino acid from Glutamic acid to Lysine. For this reason, β-globin E chains are synthesized at a reduced rate. The heterozygous condition (E/β^{o}) of this disease causes the diversified anemia ranging from extremely severe cases to transfusion independency (Sherva, 2010).

The mutation in codon 26 of the β-globin gene is also known as the c.79G>A mutation which indicates the nucleotide position in the coding sequence of the gene. The c.79G>A is a clinically silent mutation however, the combination of this mutation with a mutation in the HBB gene results in disease variation. This silent mutation can be successfully detected by Real-time High-Resolution Melting Curve analysis (PCR-HRM) (Islam, 2018).

High-Resolution Melting Analysis is a swift high-throughput method for screening genetic alterations in the sequences of nucleic acid utilizing the melting characteristics of double-stranded DNA. The HRM analysis works as an aid for researchers to categorize and detect mutations quickly. They can also identify new genetic variants through this method without sequencing. This technique is used to find out various types of genetic disorders including autosomal recessive, autosomal dominant, X-linked recessive disorders and somatic mutations. The HRM analysis was first done in ideSHi (institute of developmental Science and Health initiatives) before it was well established in Bangladesh. The technique can be used to detect the HBB gene mutations in the hot spot region of the gene where most of the mutations can be detected to determine which mutations are the most prevalent in Bangladesh (Islam, 2018; Krypuy, 2007).

In Bangladesh, the most common mutations found to be c.79G>A, IVS1_5G>C, c.92+5G>C and c.126_129delCTTT. Less common mutations are c.92G>C, c.27_28insG, c.47G>A, c.92G>A and many more. All the mutations reside in the hot spot region of the HBB gene (c.1-c.92 of exon1, c.92 + 1-c.92+130 intron -1 and c.93-c.217 of exon-2) of the population of our country. It has been found that this same hot spot region of the HBB gene harbors the mutations in Southern Asia. Therefore, it can be effectively used to detect HBB gene mutations and can act as a more accurate alternative to hematological and electrophoretic diagnosis as they have some limitations which can be mitigated by this technique (Islam, 2018).

1.6 Pathophysiology of Hb E/β Thalassemia

HbE thalassemia results from the co-inheritance of one parent's b-thalassemia allele and the other's structural variant, HbE. HbE is the result of a G>A substitution mutation in β-globin gene codon no. 26 that, in addition to generating structurally abnormal hemoglobin, activates a cryptic splice site that produces abnormal messenger RNA (mRNA) treatment. As the usual donor site has to compete with this new site, the level of normally spliced mRNA, beta E, is reduced and the abnormally spliced mRNA is nonfunctional as a result of the emergence of a new stop codon. As a consequence, HbE is synthesized at a slower pace and acts as a mild form of β-thalassemia. HbE/β-thalassemia's pathophysiology is associated with many variables including decreased β-chain synthesis resulting in globin chain imbalance, ineffective erythropoiesis, apoptosis, oxidative damage, and shortened red cell survival.

HbE's instability is a minor factor in the general pathophysiology of this disease except in febrile occurrences where this instability leads to acceleration (Orkin, 1982; Datta, 2006; Putrakul, 2000; Jetsrisuparb, 2006).

1.7 Diversity in Disease Severity of Hb E/β Thalassemia

Hemoglobin E/β-thalassemia can be classified into the following groups (Rahman, 2011):

a) Severe (hemoglobin level as low as 4-5 g/dL, transfusion-dependent, clinical symptoms similar to β-thalassemia major)
b) Moderate (hemoglobin levels between 6 and 7 g/dL, transfusion-independent, clinical symptoms similar to β-thalassemia intermedia).
c) Mild (hemoglobin levels between 9 and 12 g/dL, transfusion-independent, usually do not develop clinically significant problems) clinical forms.

A scoring system can be used to determine the severity of the groups. The latter 2 groups can be classified as Non-Transfusion Dependent (NTD) patients (Musallam, 2013).

1.8 Factors affecting Hb E/β Thalassemia

The factors behind the long transfusion intervals of Non-Transfusion dependent patients are still under research. However, studies have been carried out on the effect of Hemoglobin F on the severity of the patients. Recent studies suggest that a major but uncommon cause of clinical diversity of HbE /β-thalassemia is the severity of the β gene mutation. Very recently, in the population of Bangladesh, Sultana et al. conducted genotypic profiles of β thalassemia mutation and found great variability (heterogenicity) of β thalassemia in Bangladesh, identifying IVS1-5:G>C is the most common polymorphism for β thalassemia and a combination of IVS1-5:G>C and CD 26/E:G>A is the most common polymorphism for E-β thalassemia. In 1993, in 90 Thai patients with steady-state hemoglobin levels ranging from 4.2 to 12.6 g/dL, Winichagoon and peers researched the mutations. The same severe mutation of b-thalassemia was observed in both mild and severe anemia patients. There was a milder clinical phenotype in thirty-six of these patients. Nearly half (42 %) co-hosted a mutation that modified clinically. However, only one out of several genetic modifiers was a less severe b-thalassemia mutation. Other modifications included polymorphism Xmn1. Moreover, studies support the beneficial effect of a-thalassemia on Hb E/β-thalassemia but indicate multifactorial clinical severity. Studies provide proof of polymorphisms affecting HbF synthesis, including Xmn1 polymorphism, BCL11A, and several other genetic loci. In normal

HbF synthesis and HbE/β-thalassemia, XmnI polymorphism is a significant modifying factor. Xmn1 polymorphism accounts for about 9 % to 13 % of the genetic variability of HbF in Chinese β-thalassemia heterozygotes and healthy Europeans. Thai Hb E/β-thalassemia patients and Xmn1(+/+) genotype 27 patients had greater concentrations of complete hemoglobin (8.5 g/dL vs < 7.0 g/dL) and Hb F than patients with XmnI (-/-) genotype. These results were consistent with prior results indicating that Xmn1 polymorphism may require homozygosity to improve thalassemia severity. Other trials evaluated the connection of multiple SNPs with the severity of disease within the β-globin gene cluster. Although severity was demonstrated by SNPs in the locus control region and the gamma gene, the strongest association was with polymorphism Xmn1 (Olivieri, 2010).

Given Hb E/β-thalassemia's clinical heterogeneity, other genetic factors altering the seriousness of the disease are likely to await discovery. Association studies are underway to elucidate the function of genetic polymorphisms known to affect the expression of the globin gene and erythropoiesis as disease severity modifiers in this disorder. On chromosomes 6q23, 8q, HBBP1 gene, MYB gene, HBS1L gene, α-hemoglobin stabilizing protein and Xp22, other polymorphisms, QTL linked with enhanced fetal hemoglobin manufacturing, were defined (Olivieri, 2010).

1.9 Pathophysiology of Non-Transfusion Dependent Thalassemia Patients

The primary drivers behind NTDT's pathophysiology are chronic anemia; ineffective erythropoiesis and chronic hemolysis from peripheral RBC destruction; and compensatory physiological processes that try to rectify anemia, including marrow growth, extramedullary hematopoiesis, and enhanced gastrointestinal iron absorption. Because these mechanisms cannot overcome the genetic defect when fully functional RBCs are synthesized, they are constantly triggered, leading to various clinical problems.

Patients with NTDT have anemia that varies between 7–11 g/dL with Hb concentrations. Globin tetramers are unstable due to imbalances in α-and β-globin chains, leading to precipitation and degradation. This method releases free iron and causes reactive oxygen species to form, causing harm to the membrane and eventually premature death of cells in the bone marrow (ineffective erythropoiesis) or peripheral circulation (hemolysis). Hemolysis is connected with progressive splenomegaly and can also lead to a state of hyper coagulation experienced by many NTDT patients. Anemia can be serious and life-threatening in NTDT

patients with physiological challenges such as disease, trauma or pregnancy, as well as growth failure and delayed development.

Constant elevation of EPO concentrations leads to the expansion of erythroid marrow and the growth of extramedullary erythroid tissue in the neck, abdomen, and pelvis, causing skeletal deformities and osteopenia. Thalassaemia's inherently defective erythropoiesis inhibits early erythroid progenitor differentiation, leading in big numbers of these progenitor cells in the liver and spleen. This leads to hepatosplenomegaly along with the excessive quantities of damaged RBCs that are filtered by the spleen.

In NTDT, gastrointestinal tract (GIT) iron uptake is significantly greater than usual as the upregulated erythropoiesis rate sequesters big amounts of physiological iron. However, since the sequestered iron is not incorporated into fully functional RBCs, the level of transferrin becomes saturated and unbound physiological iron is deposited in organs, particularly the liver.

Also, Due to the decrease of hepcidin manufacturing in NTDT patients, iron absorption through the intestine can be improved. Hepcidin negatively controls the absorption of duodenal iron as well as the release of macrophage iron. Hepcidin binds ferroportin to intestinal duodenum cells, macrophages, and placenta cells, leading to internalization and degradation. In thalassemia, erythropoietic factor overexpression, growth differentiation factor 15 (GDF15) adds to iron overload by inhibiting hepcidin expression, thus improving iron absorption through the intestine.

Besides, in NTDT several molecular and cellular processes that play a role in regulating blood coagulability. Chronic platelet activation, dysregulation of adhesion molecules on vascular endothelial cells and abnormal RBC membranes lead to a hypercoagulability condition in these patients, which increases the danger of thromboembolic complications (Viprakasit, 2014).

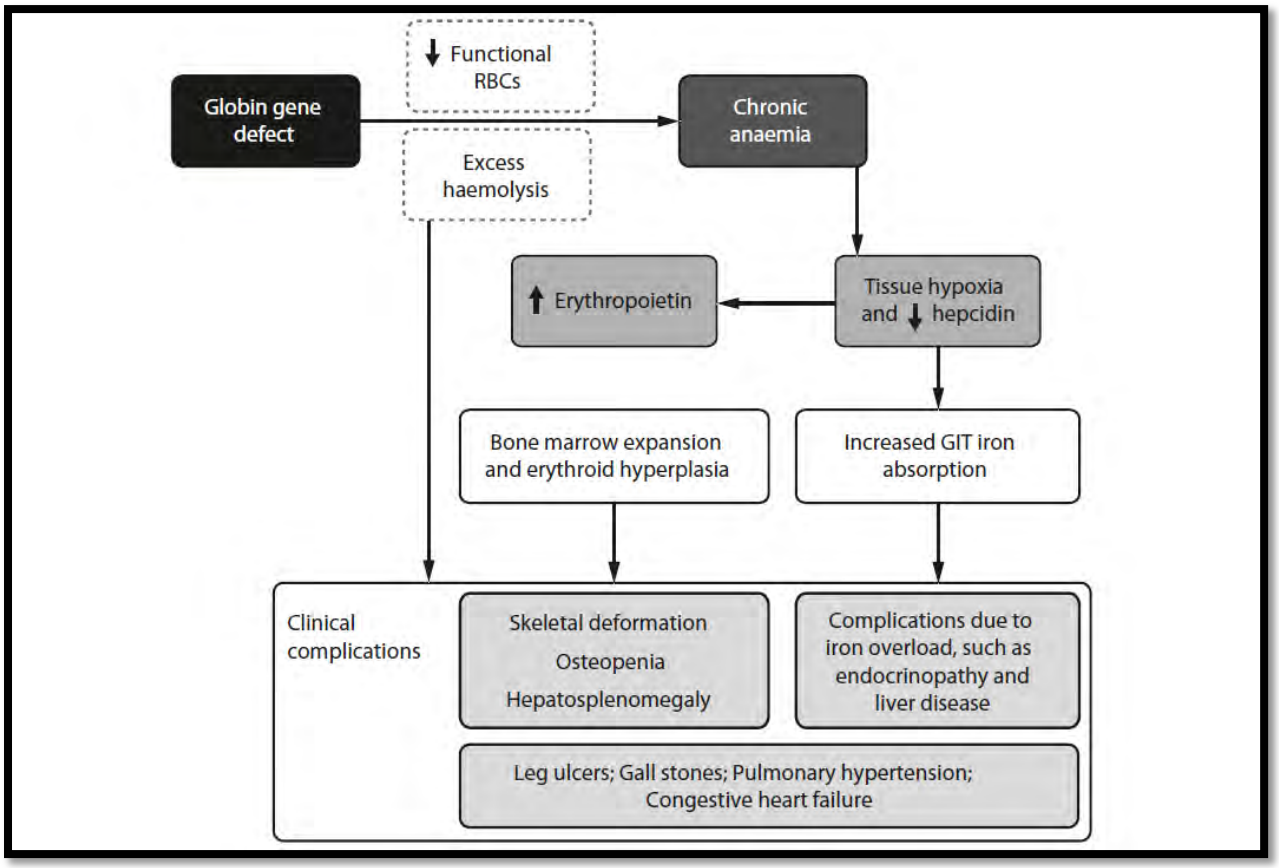

Figure 3: Pathophysiological mechanisms and clinical complications in NTD patients (Viprakasit, 2014)

1.10 Importance of early diagnosis of Non-transfusion Dependent patients

It is vital to thoroughly evaluate the patient in the first few months after the diagnosis has been developed and not to embark too quickly on any method of treatment, particularly transfusion therapy. Many non-transfusion dependent patients, who may not need periodic transfusion, embark on a lifetime of this type of unnecessary therapy, especially if they have an exceptionally low level of hemoglobin during an intercurrent infection period. Even if in the acute situation a few transfusions were administered, it is not recommended to commit immediately to a transfusion program. It is worth trying to evaluate the patient from the untransfused baseline in the non-emergency situation: i.e. to stop transfusions and carefully observe the situation. In reality, some non-transfusion dependent patients, specifically with hemoglobin E/β-thalassemia, have a notable capacity to adapt to low levels of hemoglobin. Instead, the variables to be taken into account are the well-being of the patient, especially concerning exercise, growth development, and the early appearance of skeletal modifications or other diseases (Musallam, 2013).

The primary problem with transfusion treatment is the risk of iron overload, particularly in NTD patients who have already accumulated a significant level of iron owing to enhanced bowel absorption. In minimally transfused and newly transfused patients, those at an old age at first transfusion, and splenectomized patients, the risk of alloimmunization should also be considered. The risk of alloimmunization is 1-1.6 % after one blood unit transfusion. This is particularly important during pregnancy when considering options to fully phenotype matched blood or transfusion. During infection, pregnancy, surgery, or any setting with expected acute blood loss, NTD patients may still require occasional blood transfusions. They may also require more frequent, but temporary, transfusions in the case of poor growth or development during childhood, or for the management of specific complications in adulthood, a setting in which the benefit of transfusion therapy has been established. Observational studies continue to confirm that patients receiving transfusions experience fewer leg ulcers, thrombotic events (Musallam, 2013; Taher, 2011; Taher, 2012; Oliveri, 2012; Karimi, 2011; Taher, 2010].

Furthermore, several organs and organ systems may be involved in the observed clinical morbidities with NTD patients. The incidence of these morbidities increases with advancing age if appropriate treatment is not provided. Moreover, the multiplicity of morbidity in NTD patients affects the patients‟ quality of life directly. This observation highlights the importance of timely management and prevention in this patient population. There are currently no available guidelines for the management of NTD patients; however, emerging data from recent studies alongside expert opinion usually help put forward a management framework for this group of patients. Morbidity is directly proportional to the severity of ineffective erythropoiesis and peripheral hemolysis in patients with NTDT, as these remain the characteristic of subsequent pathophysiological processes including iron overload and hypercoagulability (Musallam, 2013; Musallam, 2011; Pakbaz, 2008).

Many diseases are associated with the NTD patients which do not occur so readily in the transfusion-dependent patients. An illustration is shown below describing the types of diseases that are associated with non-transfusion dependent patients.

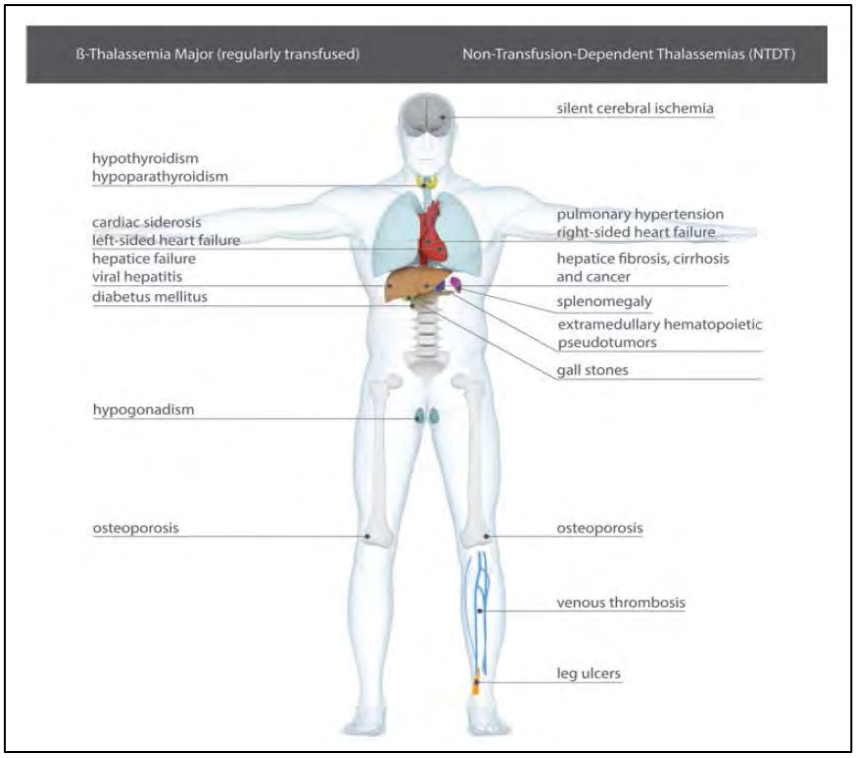

Figure 4: Diseases associated with Non Transfusion Dependent patients and severe patients (Olivieri, 2012)

1.11 Treatment Strategies

Patients of E/β-Thalassemia should be examined within the first 3 months of their life through Hemoglobin Electrophoresis and should be under supervision every three months throughout their life. Presentation age review, history of appetite and weight gain, power level, well-being and irritability, significant and minor diseases, and comparison of daily function relative to parents may assist identify a child's status. Physical examination at first and subsequent visits should include assessment of growth of the bone marrow (based on facial and skull examination, using a reproducible 4-point scale or pictures, and spleen and liver size), thorough assessment of heart status, and height and weight velocity. Laboratory diagnostic studies should include, where accessible, confirmation of the patient's genotype, both parents and any siblings. It is also necessary to determine the existence of α-thalassemia and polymorphisms connected with the enhanced manufacturing of Hb F. Each visit should evaluate complete blood counts, Hb concentration, and platelet count. The amount of serum EPO (acquired at least 3 months after a transfusion) and the saturation of serum ferritin or transferrin should be determined at first visit and frequently after. All findings should be recorded and, if possible, one copy should be provided in the local clinic chart to the guardian with another (Oliveri, 2012).

An important issue is when periodic transfusion treatment is indicated in the management of HbE thalassemia. The most frequently reported indication of transfusion at diagnosis in research in Sri Lanka was anemia, which is not particularly helpful as it occurs in all patients. Although severe or acute anemia may require transfusions and some patients may require regular transfusions thereafter, it is important to recognize that striking phenotypic heterogeneity between "mild" and "severe" patients was observed within a narrow range of steady-state hemoglobin values. The mean difference between the mildest and most severe groups in one study was only approximately 1.5–2.0 g/dL. Few patients with Hb E thalassemia should start periodic transfusions without, if possible, intercurrent disease observation of at least 6 months. Growth, sexual development, if appropriate, quality of life, anemia signs and symptoms, and spleen size changes should be closely tracked during the observation period. This conservative strategy can prevent a scenario where a kid with Hb E thalassemia has acute complications for which transfusions are indicated and is then unnecessarily administered periodic transfusion treatment for many years, which can lead to preventive complications. Over 15 years, marrow development appeared to stabilize in many patients after about 15 years of age in the Sri Lankan cohort. If ameliorated age-related EPO reactions are verified, this may have significant clinical leadership consequences. However, not much effect has been observed with this technique.

Splenectomy has been practiced when the spleen enlarges but it has been observed that the transfusion interval decreases after a few years or the operation. Given the multiplicity of adverse events associated with splenectomy, it is suggested that splenectomy should be reserved for instances of firstly, worsening anemia leading to poor growth and development when transfusion treatment is not feasible or iron chelation therapy is not available. Secondly, hypersplensim leads to worsening anemia, leukopenia or thrombocytopenia and resulting in recurrent bacterial infections. Lastly, splenomegaly is followed by symptoms such as pain in the upper left quadrant or early satiety or huge splenomegaly (biggest size > 20 cm) with concern for possible splenic rupture (Oliveri, 2012). Furthermore, patients receiving transfusions should be treated with iron chelation according to guidelines based on evidence. Quantitative body iron evaluation is now possible in developing nations and should promote suitable therapy. In some patients with HbE thalassemia, even in the lack of transfusions, chelation therapy may be essential due to excessive absorption of intestinal iron (Oliveri, 2010).

1.12 Objective of the Study

In this study, the concentrations of Hemoglobin E and Hemoglobin F of the three groups of patients (Severe, Moderate and NTD) had been compared. Also, the hematological features were measured and compared among the three groups. Moreover, the mutation spectrum of HBB gene was observed between the three groups of patients. Statistical Analysis had been done to find out the relation between the Hb E and F concentrations with disease severity. The study was done to determine the factors affecting the disease severity of non-transfusion dependent patients.

Chapter 2: Methods and Materials

2.1 Study Site and Ethical Approval:

All the molecular, hematological and genetic laboratory experiments were conducted in the Biosafety Level 2 (BSL-2) Facilities of the Genetics and Genomics Unit of institute of developing Science and Health initiatives (ideSHi). The ethical approval had been provided by the Bangladesh Medical Research Council (BMRC).

2.2 Sample Collection Site:

The samples were collected from the Thalassemia Samity Hospital with full consent of the hospital authority. All the patients or legal guardians provided their ethical consent by filling up a consent form before contributing to the study.

2.3 Study Population:

The target group in this study was the previously diagnosed Hemoglobin E/β Thalassemia patients by physicians at the Thalassemia Samity Hospital. The study is performed with 150 Transfusion Dependent and Transfusion Independent Hemoglobin E/β Thalassemia patients. The patients were divided into three groups: Group 1 patients had transfusion interval less than 30 days that More Severe patients, Group 2 comprises of patients with Transfusion Interval more than or equal to 30 days and Group 3 are the Non-Transfusion Dependent Patients whose age of first transfusion is more than 7 years and transfusion interval was at least greater than 80 days.

2.4 Sample Collection and Storage

Blood specimens were collected from every patient before transfusion in Ethyldiaminetetraacetic Acid (EDTA) containing BD Vacutainer® (Becton Dickinson, Franklin Lakes, NJ, USA) and transported to the ideSHi laboratory in a cool box with 2 to 4^{o}C temperature. 5 ml blood was used from the patients for Hematological Analysis (CBC and Hemoglobin Electrophoresis) from which 500 µl of blood was stored in the -70^{o}C for molecular and genetic analysis. Immediately after collection, complete blood count and Hemoglobin Electrophoresis were done at the institute of developing Science and Health initiatives (ideSHi) laboratory.

2.5 Workflow of the study

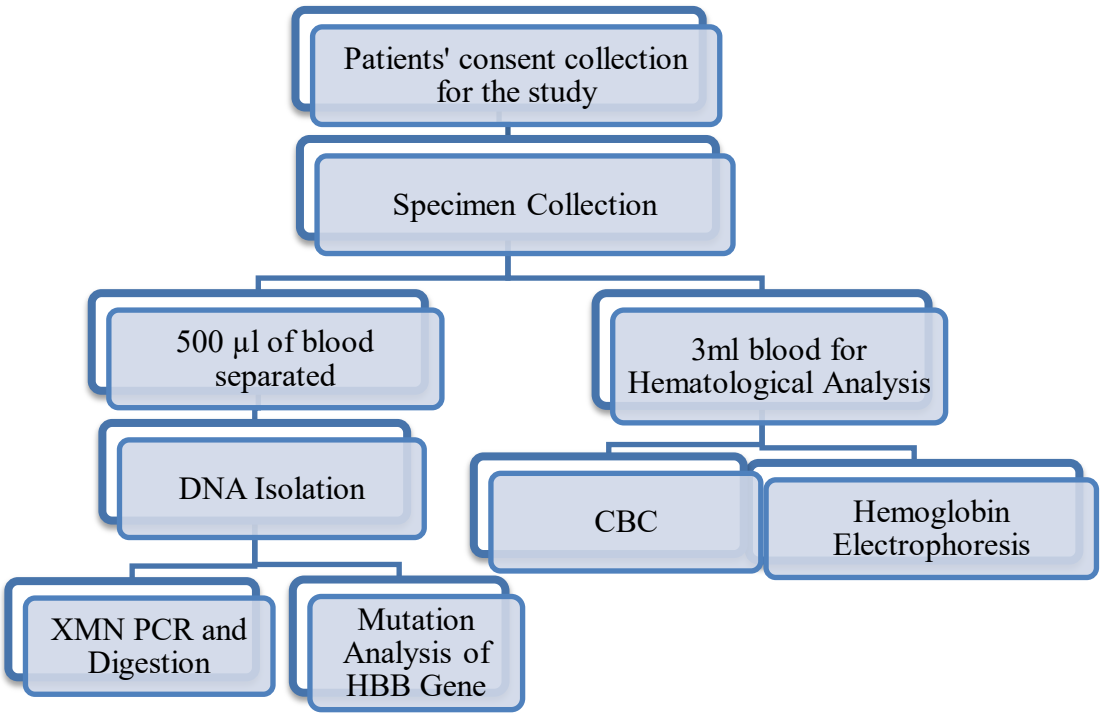

Figure 5: Workflow of Study

2.6 Complete Blood Count (CBC) Analysis:

CBC Analysis was carried out with the EDTA containing blood by following the instructions provided by the manufacturer using the automated hematology analyzed Sysmex kx-21 (Sysmex Corporation, Kobe, Japan). From this analysis, hematological parameters such as Hemoglobin level, MCV, MCH, MCHC, WBC, RBC, HCT, RDW, Lym%, Neut%, Platelets, etc were obtained from which Hemoglobin level; MCV, MCH, and RDW were used in this study.

2.7 Hemoglobin Electrophoresis:

Hemoglobin Electrophoresis of the blood specimens were done immediately after collection following the instructions of the manufacture of the CAPILLARYS 2 FLEX-PIERCING (Sebia, France) using the CAPILLARYS HEMOGLOBIN(E) kit. The percentage of different Hemoglobin variants are immediately measured through capillary electrophoresis and projected on screen. From this analysis, the percentage of Hemoglobin variants such as HbA, Hb A2, Hb E, and Hb F can be obtained, successfully.

2.8 DNA Extraction:

QIAGEN FlexiGene® kit (QIAGEN, Hilden, Germany) was used to isolate genomic DNA from the blood specimens collected from the patients following the instructions provided by the kit.

Principle:

Red Blood Cells (RBC) were lysed using the lysis buffer followed by centrifugation to pellet the nuclei and mitochondria. The resuspension of the pellet is carried out by adding the denaturing buffer which contained chaotropic salt and QIAGEN Protease which removes impurities such as proteins from the suspension. Furthermore, the DNA is concentrated and precipitated by using Isopropanol, 70 % Ethanol followed by air-drying and suspending the pellet in Nuclease Free Water.

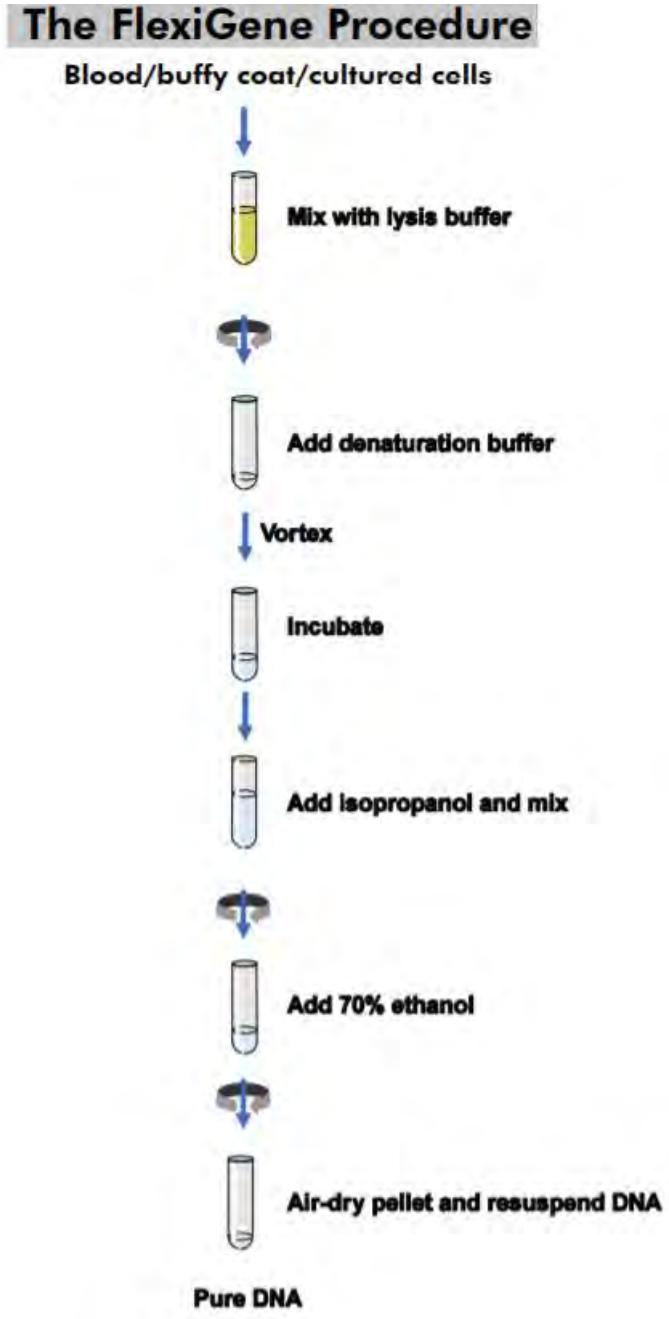

Figure 6: FlexiGene 1 Procedure of DNA Extraction (QIAGEN, Hilden, Germany)

Equipment and Supplies:

Equipment

1. Microcentrifuge
2. Vortex Machine
3. Water Bath
4. Biosafety Cabinet (BSL II)
5. Refrigerator (4^{o}C and -70^{o}C)

Materials

1. BD Vacutainer® containing EDTA
2. Pipettes (1 ml, 20-200 µl, 2-20 µl)

Reagents

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

Starting Materials

Frozen whole blood samples at -70°C were used which were thawed at 37°C gradually in the water bath.

Procedure

FG1 (500 µl) was added to a 1.5 ml microcentrifuge tube to which 200 µl of whole blood was added and the tube was inverted 20 times to carry out lysis. The mixture was then centrifuged in Heraeus™ Fresco™ 21 Microcentrifuge (Thermo Scientific™, USA) at 10,000 rpm for 20 seconds. The mixture of FG2 and QIAGEN Protease prepared in a 1:100 ratio was then added to resuspend the pellet followed by thorough vortex until the pellet resuspends completely. After centrifuging for a few seconds, the tubes were placed in the water bath at 65°C for 5 minutes. The olive green solution was then added with 100 % Isopropanol and thoroughly mixed by inverting multiple times unless a jelly-like or a thread-like appearance was seen in the solution which indicated the presence of DNA. Afterward, centrifugation was done at 10,000 rpm for 5 minutes. After decanting the supernatant, 70 % Ethanol was added to resuspend the DNA followed by centrifugation for 3 minutes at 10,000 rpm which resulted in pelleting the precipitated DNA which was air-dried and resuspended in nuclease-free water.

2.9 Xmn 1 Polymorphism Detection

Polymerase Chain Reaction (PCR)

PCR was done using the BioRad T100™ thermal cycler (Bio-Rad, USA) and Hot Start PCR reagents from Thermo Fisher Scientific. It was carried out to amplify the promoter region of the Gγ globin gene of 538 bp length. This region was amplified to detect the substitution mutation C to T at the upstream position, -158 by digesting the amplified region with the XMN1 enzyme. In this technique, Hot Start Taq Polymerase enzyme was used along with Hot Start Buffer (Thermo Fisher Scientific).

Principle

The basic principle of Polymerase Chain Reaction is the amplification of a specific region of a gene by using forward primer, reverse primer, Taq Polymerase, buffer, dNTPs $MgCl_2$, and nuclease-free water. The forward and reverse primers anneal to the 3' region of the denatured DNA strands on the sense and antisense strand respectively. After annealing, elongation is done by Hot Start Taq Polymerase to synthesize the target DNA region by joining the dNTPs complementary to the template strand. The longer template shortens after one cycle and the desired region is amplified for 35 cycles in this case. After 35 cycles the product size is measured using agarose gel electrophoresis.

Equipment

1. Thermo Cycler (Bio-Rad)
2. Centrifuge Machine
3. Vortex
4. Spin Machine
5. Refrigerator (4^oC and -20^oC)
6. PCR Cabinet

Materials

1. 0.2 ml PCR Tube
2. Microcentrifuge tube
3. 20-200 µl pipette
4. 0.1 to 2.5 µl pipette
5. 2 to 20 µl pipette

Reagents

1. 10X Hot Start Buffer (Thermo Fisher)
2. Hot Start Taq Polymerase
3. HBG2_Xmn1_Set2.1 F Primer
4. HBG2_Xmn1_Set2 R Primer
5. Nuclease Free Water
6. 2.5 mM dNTPs
7. 25 mM $MgCl_2$
8. Template DNA

Primers and Properties

The primers of XMN1 study used in this technique which was previously designed in ideSHi and is shown below:

Table 1: The primers of XMN1 study

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

The HBG2_Xmn1_Set2.1F and HBG2_Xmn1_Set2R primers were used as forward and reverse primer respectively which amplified the desired region of Gγ globin gene.

Table 2: Composition of Master Mix of XMN1 PCR for one sample

Reagent Name	Amount
10X Hot Start Buffer	1 µl
2.5 mM dNTPs	0.4 µl
5 mM $MgCl_2$	0.2 µl
HBG2_Xmn1_Set2.1F primer	0.2 µl
HBG2_Xmn1_Set2R primer	0.2 µl
Nuclease Free Water	6.9 µl
Hot Start Taq Polymerase	0.1 µl
Template	1 µl

The instrument was set to 94°C for 15 minutes for initial denaturation, 94°C for 15 seconds for denaturation, 62°C for 30 seconds for annealing, 72°C for 20 seconds for Extension and 72°C for 20 seconds for the final extension.

Table 3: Thermal cycling conditions of XMN1 PCR

Name of the step	Temperature	Time
Initial Denaturation	94°C	15 min
Denaturation	94°C	15 s
Annealing	62°C	30 s
Extension	72°C	20 s
Final Extension	72°C	5 min

The size of the amplified DNA was determined by Agarose Gel Electrophoresis (1% agarose gel) which is described later in this section.

2.10 PCR Product Purification

Principle

PCR product purification was done with the MiniElute system which is a combination of spin-column technology and a uniquely designed silica membrane with specific binding properties. This kit was designed to result in high concentrations of purified DNA for further sensitive reactions. The recovery of DNA and removal of contaminants were performed were ensured by specially prepared buffers. Attached to the silica membrane in the presence of high concentration salts, the DNA was washed with buffers multiple times to let the contaminants wash away through the column. At last, the DNA was eluted with Nuclease Free Water.

Equipment and Materials

1. Vortex Machine
2. Centrifuge
3. Pipettes
4. Microcentrifuge tubes
5. BSL II cabinet or PCR Cabinet
6. Refrigerator

Reagents

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

Procedure

At first, 5 µl volume of PB Buffer was added to the PCR Product in a 1.5 ml microcentrifuge tube followed by 10 µl 3 M Sodium Acetate (pH 5.0). The mixture was then transferred to the MiniElute spin column, attached to a 2 ml collection tube. Centrifugation was done at 17900 xg for 1 minute and the flow-through was decanted. Afterward, 750 µl Buffer PE was added to the column placed back in the collection tube followed by centrifugation at 17900 xg for 1 minute. The column was again centrifuged at 17900 xg for 1 minute to remove any remaining Ethanol. The column was then placed in a microcentrifuge tube and added with 15-20 µl of Nuclease free water followed by centrifugation at the maximum speed for 1 minute to elute the DNA. The size of the DNA was then determined by performing agarose gel electrophoresis which is described later in this chapter.

2.11 Xmn1 Restriction Digestion

Xmn1 Restriction Digestion was done using the BioRad T100™ thermal cycler (Bio-Rad, USA) to detect the C-T mutation in the previously amplified 538 bp long promoter region of the Gγ globin gene. This technique is regularly used in molecular biology to detect any single nucleotide polymorphism (SNP) in the desired portion of a gene by digesting the product using a restriction digestion enzyme, in this case, Xmn1 enzyme. The results can be viewed in agarose gel electrophoresis.

Principle

Restriction Digestion is a basic technique that needs a restriction digestion enzyme to detect any mutation present in the desired gene. The enzyme cuts the DNA if there is a mutation present in that specific portion. It can also be detected whether the gene is homozygous or heterozygous. If the gene is homozygous, the restriction enzyme will either cut all of the amplified genes resulting in two bands on an agarose gel or, it will not cut the gene resulting in just one band which would mean that the mutation is not present in the gene. To conclude that a gene is heterozygous, the enzyme will cut half of the amplified product resulting in three bands. Restriction Digestion is a two-step process which is carried out with specific buffers and commercially available restriction enzyme.

Equipment

7. Thermo Cycler (Bio-Rad)
8. Centrifuge Machine
9. Vortex
10. Spin Machine
11. Refrigerator (4^{o}C and -20^{o}C)
12. PCR Cabinet

Materials

6. 0.2 ml PCR Tube
7. Microcentrifuge tube
8. 20-200 µl pipette
9. 0.1 to 2.5 µl pipette
10. 2 to 20 µl pipette

Reagents

1. Xmn1 Restriction Enzyme (Thermo Fisher, Massachusetts, USA)
2. Cut Smart Buffer (Thermo Fisher Scientific, Massachusetts, USA)
3. Nuclease Free Water

Table 4: Composition of Master Mix for XMN1 digestion of one sample

Name of Reagent	Amount
Nuclease Free Water	7.75 µl
10X Cut Smart Buffer	1.0 µl
Xmn1 Restriction Enzyme	0.25 µl
Amplified PCR Product	1 µl
Total	10 µl

The instrument was set to the following incubation conditions to get successful restriction digestion products:

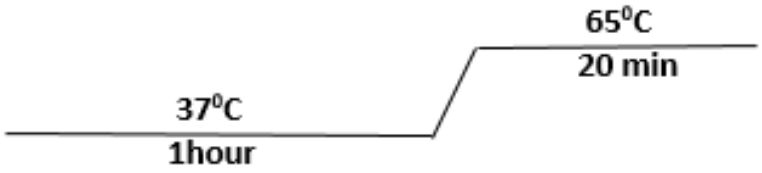

Table 5: Xmn1 digestion pattern

Xmn1 Genotype	Cutting Pattern	Length (bp)
CC (-/-) Wild Type Homozygous	Uncut-single band	583
CT (-/+) Heterozygous	Cut- Triple bands	583, 400, 183
TT (+/+) Homozygous	Cut- Two Bands	400, 183

After digestion, the product size is determined by Agarose Gel Electrophoresis (1.5% agarose gel).

2.12 Agarose Gel Electrophoresis

Principle

Agarose gel Electrophoresis uses a buffer that maintains uniform states of ionization. It is important because any slight change in pH will alter the charge on the DNA molecules resulting in inaccurate separation of DNA fragments. The phosphate groups of DNA or PCR products produce a constant negative charge at neutral or alkaline pH. To get sharp bands with good resolution TAE Buffer can be used. Additionally, this buffer prevents the growth of any microbes. Electrophoresis is done by applying an electric charge to make the negatively charged DNA fragments to migrate through the pores in the 1 % or 1.5 % agarose gel of the positively charged electrode known as anode. The largest molecules will travel slowly through the pores but the smallest ones will travel the fastest because the velocity of this movement and the molecular weight of the DNA is inversely proportional to each other, resulting in separated bands in the agarose gel.

Equipment

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

Reagents

1. Agarose powder
2. 1X TAE buffer
3. Gel Red (Intercalating nucleic acid fluorescent dye)
4. Bromophenol Blue (Loading dye)
5. DNA ladder (Marker DNA solution)

Preparation of Gel

To prepare 1 % and 1.5 % agarose gel, 0.4 g and 0.6 g of agarose powder was measured with an electric balance and added into a 250 ml conical flask with 40 ml 1X TAE Buffer. The mixture was heated for 1 minute in a microwave oven till all the grains of agarose dissolved and gave a transparent appearance to the solution. After cooling the solution for some time, Gel Red® Nucleic Acid Gel Stain (Biotium, USA) was added in 1 µl and swirled slowly. The

solution was poured on the gel preparation tray with the comb to form the desired number of wells on the gel and the gel was allowed to set for twenty minutes.

The comb was removed and the gel tray was placed in 1X TAE Buffer in the Gel Electrophoresis tank after the gel was solidified. After the TAE Buffer submerged the gel completely, 4 µl of Loading Dye and 4 µl of sample were carefully loaded in the wells after mixing by pipetting multiple times of a Parafilm. To measure the band size, 5 µl of 1 kb Plus DNA Ladder (Invitrogen^{TM}, USA) was loaded that had a broad size range (75 bp-20 kb). The electric field was applied to the gel at 100 V for 45 minutes and the bands were visualized using the Gel Doc^{TM} XR+ (BioRad, USA) under UV Light.

2.13 Real-time PCR High-Resolution Melting (HRM) Curve Analysis

Real-Time based HRM Analysis was carried out in a CFX96 Touch™ Real-Time PCR machine (BioRad). The protocol followed for this experiment was from a previously published research paper of ideSHi by Md. Tarikul Islam and colleagues.

Principle

Real-time PCR-HRM is a rapid throughput technique to detect different types of SNPs (single nucleotide polymorphisms) instead of sequencing. Here, the DNA is amplified using Real-Time PCR with a commercially available PCR mix along with previously designed forward and reverse primers. The steps are as follows: initial denaturation, denaturation, annealing, and elongation. After the completion of the PCR, the double-stranded amplified DNA is denatured, re-natured, then, melted to get different graphs for different mutated strands. The melting pattern is different for different mutations which result in successfully separated curves.

Equipment

1. Centrifuge machine
2. 96 well reaction plate
3. Pipettes
4. Vortex machine
5. CFX96 Touch™ Real-Time PCR machine (BioRad)

Reagents

1. 2X precision melt supermix (BioRad)
2. Forward Primer
3. Reverse Primer
4. DNA

The primers used were designed by the researchers for an earlier study aforementioned.

Table 6: Two sets of primers were used each with a different set of controls for Real-Time PCR HRM

Sl no,	Primer Name	Primer Sequence (5‟to3‟)	Primer Size
1.	P1	ATGGTGCATCTGACTCCTGAG	21
2.	R1	CCAATAGGCAGAGAGAGTCAGTG	23
3.	P2	CACTGACTCTCTCTGCCTATTGG	23
4.	R2	CAGGCCATCACTAAAGGCACC	21

Table 7: Composition of Master Mix for Real-time PCR HRM for one sample

Reagent Name	Amount
2X precision melt supermix (Bio-Rad)	5 µl
Forward Primer	0.2 µl
Reverse Primer	0.2 µl
Nuclease Free Water	4.6 µl
50 ng of DNA	1 µl
Total	10 µl

Procedure

A 96-well plate was taken to carry out the real-time PCR reactions with 10µl volume of reaction in every well. The 2X precision melt supermix (Bio-Rad). The aforementioned reagents were mixed in a microcentrifuge tube and added in equal volumes to all the wells followed by the addition of the DNA template. The Real-time PCR Machine (CFX96 Touch™ Real-Time PCR machine) was set to the following conditions for thermal cycling:

Table 8: Thermal Cycling temperatures for Real-time PCR HRM

Name of the step	Temperature	Time
Initial Denaturation	95°C	2 min
Denaturation	94°C	10 s
Annealing	60°C	15 s
Extension	72°C	15 s

PCR was done for 35 cycles in total. The thermal cycling conditions were the same for 2nd set of primers instead of the annealing temperature which was 58°C. Furthermore, the products were denatured and renatured followed by melting. The following conditions were followed:

Table 9: Thermal cycle temperatures for HRM

Name of the step	Temperature	Time
Denaturation	95°C	30 s
Renaturation	60°C	30 s
Melting	65°C to 95°C	Increment of 0.1°C per 5 s

When the real-time PCR-HRM was completed, the data were projected on the screen and analyzed with the Precision Melt Analysis™ Software (BioRad). To get accurate results, the curve shape sensitivity was set to 100 % for cluster detection and the difference in the Tm threshold was fixed to 0.1. Moreover, normalized and temperature shifted views were used for the HRM analysis.

2.14 Statistical Analysis

All the results were subjected to statistical analysis to find statistical significance. Two-Tailed T-test and one way ANOVA test was performed with the data set using the software GraphPad Prism version 7 (GraphPad Software, La Jolla California USA). The data were articulated as number (N), percentage, Mean ± Standard Deviation (SD) and suitable units depending on the variable mentioned. The Probability (P) Value less than 0.05 was considered significant when the data were compared.

2.15 Scoring System

The groups of patients were scored based on this Scoring system. The score from 0 to 3.5 indicates mild form of Thalassemia, from 4 to 7 indicates moderate form and from 7.5 to 10 indicates severe form of Hb E/β Thalassemia (Sripichai, 2008).

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

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

Figure 7: Scoring System of Hb E/β Thalassemia

Chapter 3: Results

3.1 Demographic Data of the Study

One thirty-four patients who were previously diagnosed Hemoglobin E/β Thalassemia participated in this study at The Thalassemia Samity Hospital. The patients" age ranged from 6 years to 60 years. Additionally, 82 were male patients and 68 were female patients which comprised 54.7 % and 45.3 % of the study population, respectively (Table 10).

Table 10: Demographic Data of the Study

Parameters	Number of Patients (N=150)
Age	6 years to 60 years
Male	74 (55.2 %)
Female	60 (44.8 %)

3.2 Groups of Patients

The patients were divided into three groups as follows. Group 1 comprised of severe patients whose mean age of the first transfusion was 20.78 ± 19.71 months and the mean transfusion interval was 18.0 ± 4.9. Moderately severe patients' transfusion interval was 37.34 ± 13.93 days and their mean age of the first transfusion was 45.16 ± 35.77 months which was Group 2. Non-transfusion dependent patients were classified as Group 3 had their mean transfusion 124.4 ± 86.90 days along with mean age of transfusion of 150.41 ± 121.40 months. The difference between the first age of transfusion and transfusion interval was statistically significant (P-value < 0.0001). This indicates that group three has the highest transfusion interval and the highest first age of transfusion; therefore, Group 3 can be entitled as Non-Transfusion Dependent Patients (Table 11).

Table 11: Groups of patients in the Study

Group Type	Average First age of transfusion (month)	Transfusion Interval (days)	Number of Patients
Group 1: Severe	20.78 ± 19.71	18.0 ± 4.90	46
Group 2: Moderate Severe	45.16 ± 35.77	37.34 ± 13.93	49
Group 3: Non-Transfusion Dependent (NTD)	150.41 ± 121.40	124.4 ± 86.90	39

3.3 Comparison of Hematological Parameters

The hematological parameters of every sample were recorded from their Complete Blood Count (CBC) report. Here the following Hematological Parameters were focused on: Haemoglobin concentration-HGB, Mean corpuscular Volume-MCV, Mean corpuscular Haemoglobin- MCH and Red Cell Distribution width -RDW. The mean ± SD value of the concentration of Hemoglobin of Group1, Group 2 and Group 3 were 7.78 ± 1.25 g/dl, 7.35 ± 1.33 g/dl and 7.35 ± 1.50 g/dl. In case of MCV, the mean ± SD of the three groups 1, 2 and 3 were 72.7 ± 5.11 fl/red cell, 69.10 ± 6.45 fl/red cell, and 64.38 ± 8.08 fl/red cell, respectively. MCH was also recorded which resulted in the following mean ± SD: 23.44 ± 2.08 pg/red cell (Group1), 21.39 ± 2.68 pg/red cell (Group 2) and 19.50 ± 2.52 pg/red cell (Group 3). Lastly, the mean ± SD values of RDW were 23.71 ± 5.36 %, 27.83 ± 6.12 % and 30.20 ± 2.62 % of group one, two and three, respectively. All the mean ± SD values of the three groups (Severe, Moderate Severe, and NTD) were compared using ANOVA and T-test. There was no statistically significant difference between the Hemoglobin concentrations between the three groups (ANOVA: p-value = 0.1497). However, statistical significance was found in the values of MCV (ANOVA: p-value < 0.0001) and MCH (ANOVA: p-value < 0.0001) between the three groups. Additionally, it could be concluded that severe patients (Group 1) had the highest levels of MCH and MCH which was supported by the T-test values between Group 1 and 2 (MCV: p-value = 0.0040, MCH: p-value = 0.00001), Group 2 and 3 (MCV: p-value = 0.0021, MCH: p-value = 0.0015). Moreover, RDW values were statistical-significantly different in the ANOVA test (ANOVA: p-value < 0.00001), however, the T-test values showed otherwise between groups 2 and 3 (p-value = 0.1220). Hence, it could be concluded that there was a significant difference between RDW values between severe

patients and moderately severe patients (p-value = 0.0013) but, no statistically significant difference was found between moderately severe and non-transfusion dependent patients (Table 12, Figure 8).

Table 12: Comparison of the Hematological Parameters (HGB concentration, MCV, MCH, and RDW) between three groups of E/β-Thalassemia Patients

Hematological Parameters	Group 1: High Severity	Group 2: Moderate Severity	Group 3: Non-Transfusion Dependent
HGB (g/dl)	7.78 ± 1.25	7.35 ± 1.33	7.35 ± 1.50
MCV (fl/red cell)	72.7 ± 5.11	69.10 ± 6.45	64.38 ± 8.08
MCH (pg/red cell)	23.44 ± 2.08	21.39 ± 2.68	19.50 ± 2.52
RDW (%)	23.71 ± 5.36	27.83 ± 6.12	30.20 ± 2.62

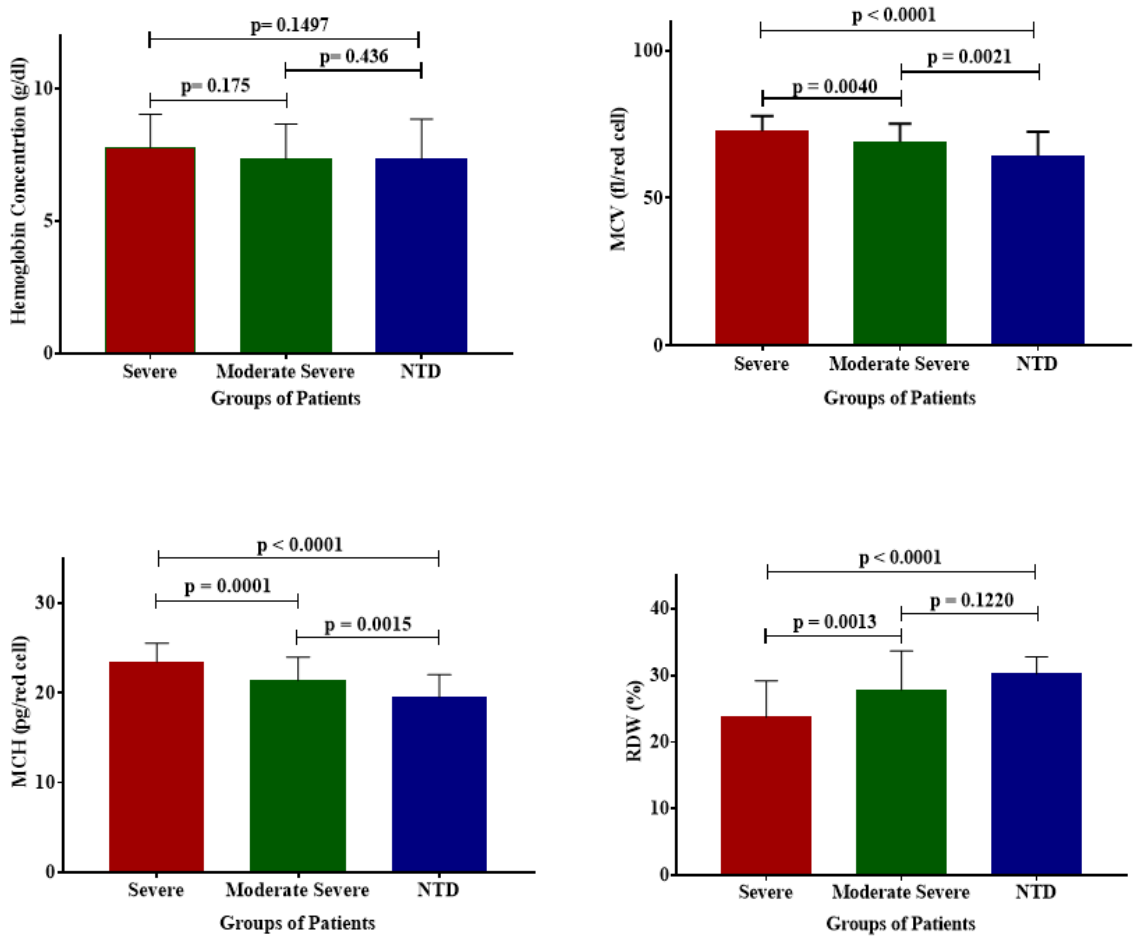

Figure 8: ANOVA test results and T-test results of comparing the Hematological parameters between the three patient groups of Hb E/ß Thalassemia

3.4 Comparison of Hemoglobin Variants in the three groups of patients

The Hemoglobin Variants of severe patients (group 1), moderately severe patients (group 2) and NTD patients (group 3) were measured in percentages in Hemoglobin Electrophoresis and then the concentrations were deduced from their Hemoglobin concentration levels. The HbA mean ± SD values came out as 5.50 ± 1.72 g/dl, 3.23 ± 2.10 g/dl and 1.10 ± 1.89 g/dl of group 1, 2 and 3, respectively. The mean ± SD values of HbA2 were 0.24 ± 0.06 g/dl (group 1), 0.31 ± 0.10 g/dl (group 2) and 0.34 ± 0.10 g/dl (group 3). Additionally, Hb F gave out mean ± SD values of group 1, 2 and 3 as 0.58 ± 0.76 g/dl, 1.11 ± 1.33 g/dl and 2.11 ± 1.65 g/dl, respectively. Lastly, the mean ± SD values of Hb E were 1.11 ± 0.72 g/dl (Group 1), 2.23 ± 1.24 g/dl (Group 2) and 3.11 ± 1.15 g/dl (Group 3). ANOVA test was performed in

every set of results to compare between the three groups of patients and it was found out that there were statistically significant differences between the values of HbA (ANOVA: p-value < 0.0001), Hb E (ANOVA: p-value < 0.0001) and Hb F (ANOVA: p-value < 0.0001) among the three groups. The T-test results between group 1 and 2 (HbA: p-value < 0.0001, Hb F p-value = 0.01 , Hb E: p-value < 0.00) also group 2 and 3 (HbA: p-value = 0.0006, Hb F p-value = 0.003, Hb E: p-value = 0.02) supported the statement that severe patients (group 1) had the highest level of Hb A, NTD patients (group 3) had the highest concentration of Hb F and HbE. Although, the ANOVA test gave statistically significant differences in Hb A2 levels (ANOVA: p-value < 0.0001) along with T-test value between groups 1 and 2 (p-value = 0.0017), the results T-test value between group 2 and 3 (p-value = 0.158) showed otherwise. Hence it could be concluded that severe patients had the significantly lowest concentration of Hb A2 and there was no statistically significant difference between group 2 and 3 which were moderately severe patients and non-transfusion dependent patients, respectively (Table 13, Figure 9).

Table 13: Comparison of the Level of Haemoglobin Variants i.e. Hb A, Hb A2, Hb E, Hb F

Hemoglobin Profiles	Group 1 : High Severity	Group 2 : Moderate Severity	Group 3: Non Transfusion Dependent
Hb A	5.50 ± 1.72	3.23 ± 2.10	1.10 ± 1.89
Hb A2	0.24 ± 0.06	0.31 ± 0.10	0.34 ± 0.10
Hb F	0.58 ± 0.76	1.11 ± 1.33	2.11 ± 1.65
Hb E	1.11 ± 0.72	2.23 ± 1.24	3.11 ± 1.15

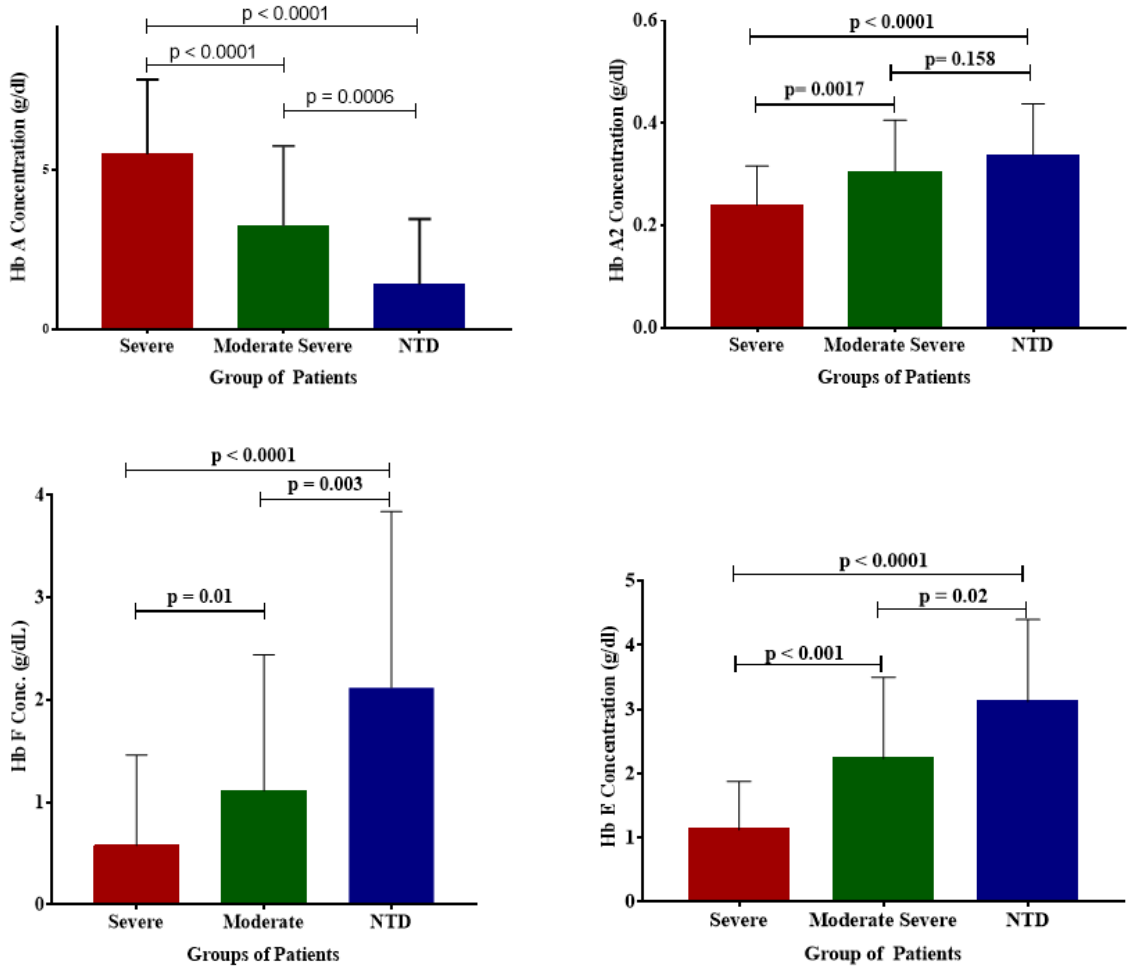

Figure 9: ANOVA test results and T-test results of comparing the Hemoglobin Variants (HbA, HbA2, HbF, and HbE) between the three patient groups of Hb E/ß Thalassemia

3.5 Different HBB Gene Mutation in the three groups

The mutations in the HBB gene hot spot were detected by Real-time PCR-High Resolution Melting (HRM) Curve Analysis and Xmn1 polymorphisms were detected by restriction digestion with Xmn1 enzyme. In the severe group of patients (group1), 74 % heterozygous polymorphism (CT), 10 % uncut i.e. homozygous wild type and no homozygous polymorphism were present. There were 83 % of heterozygous polymorphism, 11 % of wild type and 6.5 % of homozygous polymorphism in group 2 (moderately severe patients). Additionally, Non Transfusion Dependent patients (group 3) had 75 % of heterozygous polymorphism (CT), 17 % wild type and 2 % of homozygous polymorphism (TT). Therefore, it is evident that CT is the most prevalent among all the groups of patients with comparatively higher in number in moderately severe patients, also, non-transfusion-dependent patients had

diversified polymorphism rates in the case of Xmn1 polymorphism (shown in the figure). Most importantly, the substitution mutation from G to A in the codon 79 was present in all the samples showing that all the patients were Hb E/β Thalassemia patients. In addition, there were many mutations present in the HBB gene of the sample among which IVS1_5 G>C was highly prevalent in all the groups of patients (Group 1: 27, Group 2: 22, Group 3: 19). The most variety of mutations was observed in the moderately severe patients group (group2) where 8 types of mutation were found out, 2 patients had G>C mutation in codon 92, 1 patient had IVS_1_130 (G>C) substitution mutation (curve shown in the figure), 2 patients had a deletion mutation of T in codon 46, 1 patient with G>A substitution in codon 47, c.126_129delCTTT mutation (curve shown in the figure) was found in 2 patients and an insertion mutation was found in one patient in this group. Non-Transfusion dependent patients had one unique mutation which was a substitution mutation of G>T in codon 3 in 2 of the patients along with substitution of G>C in codon 92 in 1 patient, c.126_129delCTTT mutation in 1 patient and c.27_28 insertion G mutation in 2 patients. On the other hand, group 1 (severe patients) had only two types of mutations which were the substitution of G>C in 2 patients and IVS_1_130 (G>C) in 1 patient. It can be concluded that the mutations in the HBB gene can vary in the patients of Hb E/β Thalassemia patients in all types of cases (Table 14, Figure 10, 11, 12).

Table 14: HBB Gene Mutation in the three groups of patients of E/β Thalassemia

HBB Gene Mutation	Group 1: High Severity	Group 2: Moderate Severity	Group 3: Non-Transfusion Dependent
Xmn 1 CT (-/+)	29 (74 %)	38 (83 %)	27 (75 %)
Xmn 1 CC (-/-)	10 (26 %)	5 (11 %)	6 (17 %)
Xmn 1 TT (+/+)	0 (0 %)	3 (6.5 %)	2 (5.5 %)
c79 G>A	52	55	43
IVS1_5 G>C	28	22	19
c92 G>C	2	2	1
IVS _1_130 (G>C)	1	1	0
c.46delT	0	2	0
c47(G>A)	0	1	0
c.126_129delCTTT	0	2	1
c.27_28 ins G	0	1	2
c.3 G>T	0	0	2

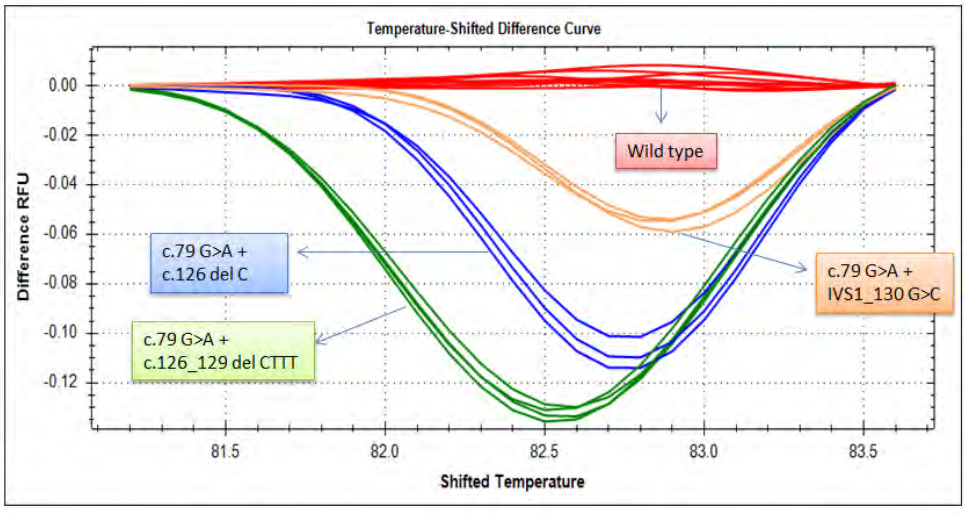

Figure 10: Real-time PCR-HRM Curve of a few β globin gene mutations

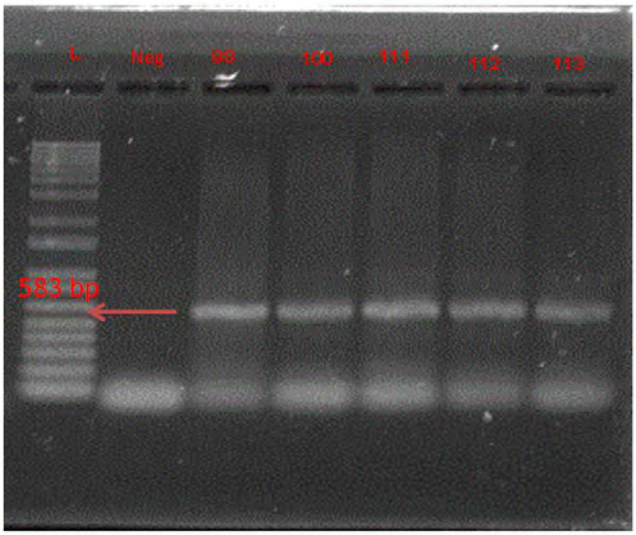

Figure 11: PCR image of XMN1 polymorphism

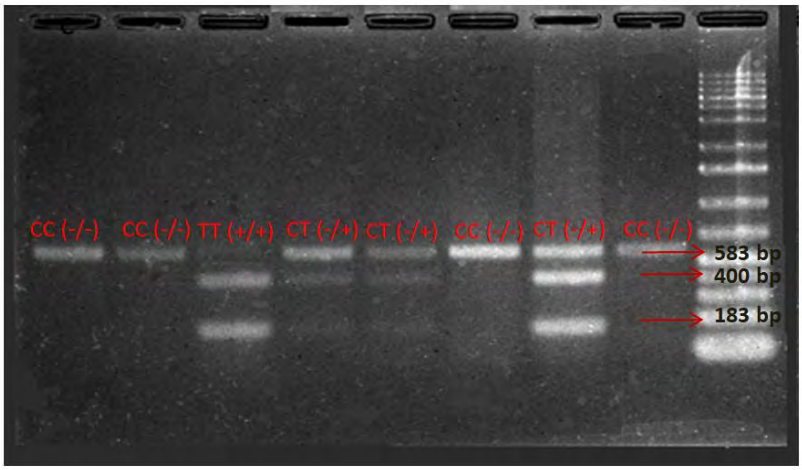

Figure 12: Xmn 1 polymorphism digestion image showing CC (wild type), CT (heterozygous) and TT (homozygous) polymorphisms and their band lengths

3.6 Statistical Analysis of Hb F, Hb E, Hemoglobin concentration level and disease severity

The Pearson correlation was done between the concentration of Hemoglobin E, Hemoglobin F, and concentration of Hemoglobin with disease severity by scoring the cases. Statistically significant negative correlation was found between the Hemoglobin concentration and disease severity (r = -0.5879, p = 0.0002). Also, it was deduced that there is a negative correlation between the concentration of Hemoglobin E and disease severity which is significant statistically (r = -0.411, p = 0.0142). Moreover, a statistically significant negative correlation had been seen between the disease severity and concentration of Hemoglobin F (r = -9.5393, p = 0.0010) (Figure 13).

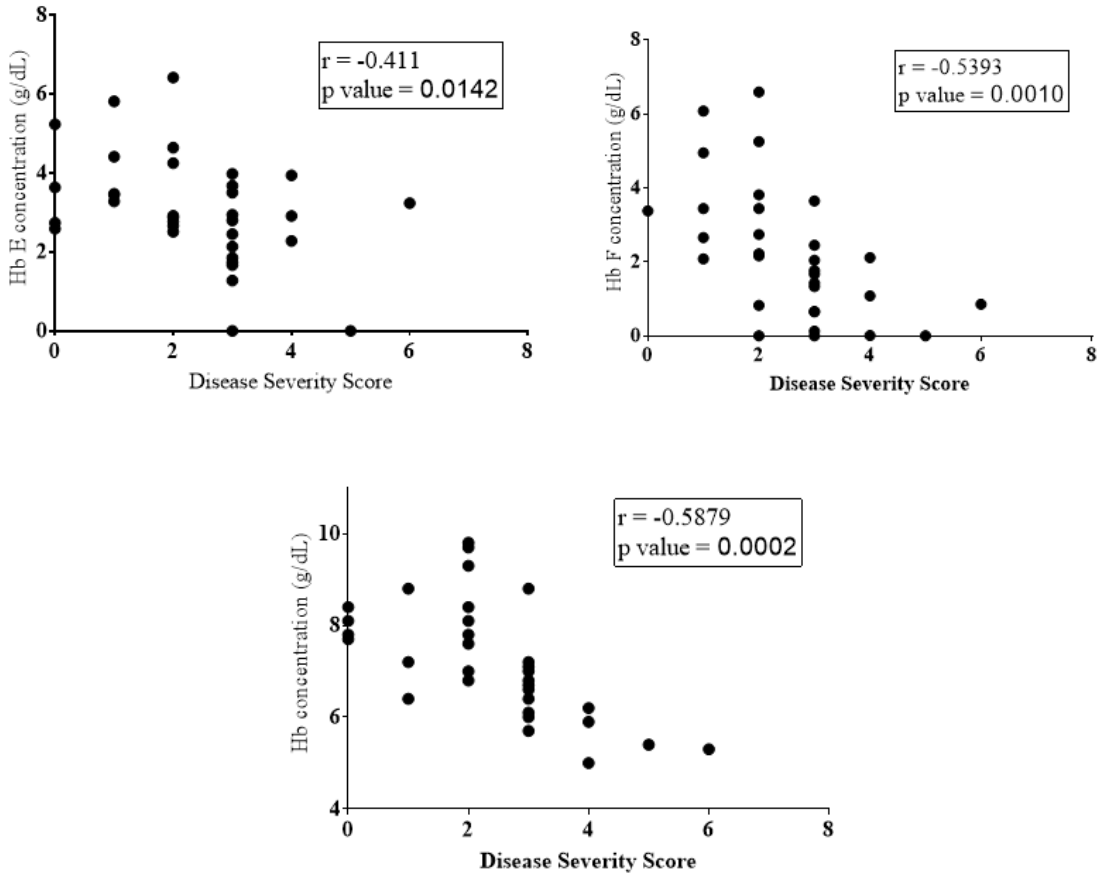

Figure 13: Pearson correlation test between disease severity and Hemoglobin concentrations

Chapter 4: Discussion

Thalassemia is an inherited anemic genetic disorder. Among different kinds of thalassemia, the most highlighted kinds are alpha (α) and beta (β)-thalassemia. In Hemoglobin A variant, alpha, and beta-thalassemia are the consequence of dysfunctional manufacturing of α and β globin chains. This disease's inheritance includes a typical Mendelian-recessive pattern that means that each parent inherits the faulty genes (Weatherall, 2001). Parents also need to be carriers of heterozygous to have the likelihood (50 %) of providing birth to a Thalassemia patient. Among different kinds of Thalassemia, this study was about Hemoglobin E/β Thalassemia which is a milder version of Beta Thalassemia with a heterozygous genotype. The clinical manifestations of Hb E/β thalassemia are highly heterogeneous. It had been observed during sample collection that the first age of transfusion of Hb E/β Thalassemia patients had a wide variation regardless of which group the patient belonged to. Especially, in the non-transfusion dependent group which comprises of patients with variable age of transfusion and very different transfusion intervals. This study was done to determine the factors responsible for the wide variation of the disease severity. Here, the concentrations of Hemoglobin E and Hemoglobin F of the three groups of patients (Severe, Moderate and NTD) had been compared. Also, the hematological features were measured and compared among the three groups. Moreover, the mutation spectrum of HBB gene was observed between the three groups. Statistical Analysis had been done to find out the relation between the Hb E and F concentrations with disease severity.

Globally, with Hb E/β Thalassemia, 20,000 babies are born (Rahman, 2011). The world's biggest frequencies of Hb E/β thalassemia are prevalent in India, Bangladesh, Thailand, Laos, and Cambodia. Moreover, Hb E/β thalassemia has become a significant public health issue across these countries and regions of China, Indonesia, and Sri Lanka. It has become the most prevalent type of β-thalassemia observed in numerous North American newborn screening programs (Olivieri, 2012). Over 10 million individuals are born carriers in Bangladesh, and with time the proportion of patients with E/β thalassemia is increasing (Rahman, 2011). In a recent study with a sample size of greater than one thousand, it has been found that the prevalence of Non-Transfusion Dependent Hb E/β Thalassemia patients was 28 % in Bangladesh (Hossain, 2017). However, no other report has shown the prevalence of this group of patients in our country. This study comprises 32 % of NTD patients out of 134 patients from Thalassemia Samity Hospital.

The non-transfusion dependent, the least severe form of thalassemia, patients of Hb E/β thalassemia usually start their transfusion later than 10 years and have their transfusion interval of more than 3 months or no transfusion at all (Sripichai, 2008). The disease severity of these patients cannot be determined by only a few phenotypic characteristics as it depends on many factors. The combination of the mutation in the HBB gene and the Hb E gene in NTDT patients result in the phenotypic heterogeneity. The factors responsible for this characteristic of non-transfusion dependent patients are still not determined. The NTDT patients may have to take blood transfusion rarely for growth development or not require blood transfusion at all.

It was found that the levels of absolute Hemoglobin F are greater in non-transfusion dependent patients as it may help in carrying the oxygen throughout the body because of its high oxygen affinity ability. In a study, it had been determined that Hb F plays a role in ameliorating the disease severity because the increase in gamma genes of Hb F decreases the α/β-chain imbalance. Also, Hb F is highly variable depending on the genetic allele so no absolute level or concentration of Hb F could be determined for NTDT patients (Panigrahi, 2005). This finding was supported by the results found in this study that was that the concentration of Hb F was higher in NTDT patients than severe and moderate patients. Also, in the group of NTD patients, a negative correlation was found between the Hb F concentration and the disease severity score [where the lower the score was the milder the form thalassemia it represented]. This implied that the well-proven theory that Hb F has a positive effect on decreasing the severity of Hb E/β thalassemia. On the contrary, in a few studies and also in this study, it had been found that some patients from the severe and moderate group of patients also had a high level of Hb F but relatively low levels in NTD patients (Fuchareon, 2012). This variation of the level of Hb F raised the doubt whether Hb F is the sole factor that is affecting the disease severity in NTD patients or not. Moreover, the finding that Hb F was not an effective oxygen carrier because of its poor dissociation ability contradicted the fact that it would be effective to increase the Hb F level as a treatment to decrease the disease severity. In another study, the treatment of Hydroxyurea to increase the Hb F did not work on the NTD patients (Hossain, 2017). Overall, it was implied that the Hb F may not be the only factor that decreases the disease severity of the Hb E/β Thalassemia patients, although, Hb F is needed for decreasing the α/β chain imbalance which is an important factor by itself.

The role of Hb E might be taken into consideration because of its high oxygen affinity (Fuchareon, 2012). It had been found in research that, the oxygen dissociation power of Hb E in the patients of Hb E disease where the Hb F was very low or not present was normal (Panigrahi, 2005). In this study, a negative correlation was found between the concentration of Hb E and the disease severity score [where the lower the score is the milder the form thalassemia it represents]. This supported the finding in a recent study that the high-level Hb E and the relatively low level of Hb F together help the NTD patients to adapt to the anemia (Fuchareon, 2012).

The mutation spectrum of HBB Gene did not show any significant difference between the three groups of patients in this study. The mutation IVS1_5_(G>C) was found to be the most prevalent form of mutation in the HBB gene of Hb E/β Thalassemia which supported the previous study done in the laboratory of ideSHi (Islam, 2018; Sultana, 2016). This non-distinguishable mutation spectrum implies that other factors also play a significant role in the heterogeneity of Hb E/β Thalassemia such as BCL11A, on chromosome 6q23, 8q, HBBP1 gene, MYB gene, HBS1L gene, α-hemoglobin stabilizing protein and other polymorphisms which may enhance fetal hemoglobin manufacturing (Olivieri, 2010). For further investigation, it is highly recommended that the three groups of patients are detected for the presence of the aforementioned genetic modifiers.

Furthermore, in NTDT patients absolute hemoglobin levels could not be deduced because there was not much difference between the Hemoglobin levels among the groups of patients of different levels of severity (Camaschella, 1995). This finding was supported by the results of this study which showed no statistically significant difference in the Hemoglobin levels between moderately severe group and NTDT group of patients because the collection point of time was when the patients needed the transfusion so their hemoglobin levels were already low. Although, there was no significant difference in the hemoglobin level of the groups of patients, it cannot be the sole factor on which proper diagnosis can be provided. Another study showed that hematological parameters and hemoglobin profiles are not sufficient for prescribing diagnosis for non-transfusion dependent patients. (Viprakasit, 2014).

It had been observed in children that they could adapt to a low hemoglobin level and survived without any complication. However, the clinical complications associated with NTDT are very alarming. Early identification of NTD patients could be useful because the severity of the condition could be observed based on the level of Hb F and Hb E. Treatment could be

done depending on the increase of severity and the results could be also keenly observed. Hence, the diagnosis of non-transfusion dependent patients is important along with proper treatment depending on the symptoms. The phenotypic characteristics were observed to change with age. Mostly, the condition deteriorated with a decrease in hemoglobin levels, an increase in iron, thrombosis and many more complications emerge with age (Taher, 2010). Moreover, to mitigate the effect of anemia, the body regulates a pathophysiological mechanism which includes bone marrow expansion, extra-medullary hematopoiesis and over absorption of iron from the gut (Viprakasit, 2014). These complications could be prevented if the diagnosis of NTDT patients could be done at an early age. Besides, it was observed that if the transfusions were continued from an early age, the patients depended on the transfusions and the transfusion interval decreased with time. Also, the frequency of the transfusion increased if regular transfusion therapy was provided because other complications such as iron overload, large spleen could be a consequence in NTD patients (Taher, 2011). Overall, all the factors associated with NTD must be considered before prescribing a treatment. In Bangladesh, lack of awareness and mistreatment causes the lives of these patients in more danger, therefore, the medical sector should be enriched with more research on the non-transfusion dependent patients of Hb E/β Thalassemia.

Chapter 5: Conclusion

The importance of studying with the non-transfusion dependent Hb E/β Thalassemia patients is immense as in our country the data about this group of patients is scarce. This study comprises the comparison of the different Hemoglobin profile, hematological features and mutation spectrum of the three groups of patients (severe, moderate and NTD). Further statistical analysis was done on the Hb F and Hb E concentrations and the disease severity of the NTDT patients. The findings in this study prove that all the factors should be taken in serious consideration before prescribing treatment for the non-transfusion dependent patients. The findings can act as an aid for the physicians to get a better understanding of the cases and help the patients to lead a better life.

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