MUTATION PATTERNS IN THE *HBB* GENE DETERMINE THE FREQUENCY AND ONSET OF BLOOD TRANSFUSION IN PATIENTS WITH BETA-THALASSEMIA MAJOR AND HBE-BETA THALASSEMIA

By

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

Department of Mathematics and Natural Sciences
BRAC University
November 2019

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing 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 main sources of help.

Raisa Tasnim

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Approval

The thesis titled "Mutation patterns in the *HBB* gene determine the frequency and onset of blood transfusion in patients with beta-thalassemia major and HbE-beta thalassemia" submitted by **Raisa Tasnim (**Student ID: 17376001) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science on November 14, 2019.

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Ethics Statement

This is to certify that the thesis titled “Mutation patterns in the *HBB* gene determine the frequency and onset of blood transfusion in patients with beta-thalassemia major and HbE-beta thalassemia” has been reviewed for and has been approved by the Biosafety, Biosecurity and Ethical Committee of Bangladesh Medical Research Council (BMRC).

Abstract

Thalassemia is a blood disorder passed down through families (inherited) in which the body makes an abnormal form or inadequate amount of hemoglobin. Co-inheritance of beta-thalassemia alleles from two parents results in beta thalassemia major. Again co-inheritance of a beta-thalassemia allele from one parent and a structural variant of hemoglobin E from the other parent results in HbE-beta-thalassemia disorder. Patients with HbE/beta-thalassemia represents approximately 50 percent of severely affected beta thalassemia worldwide. Clinical manifestations of HbE-beta thalassemia are highly variable in terms of severity- from thalassemia intermedia like symptoms to severe transfusion-dependent thalassemia major. The reasons for this clinical variability is still poorly understood.

In this study, a total seventeen patients with HbE-beta thalassemia and Beta thalassemia major were included. DNA was isolated from the patients' blood samples and PCR was performed targeting a 428 bp as the mutational hot spot region of *HBB* gene. Sanger Nucleotide Sequencing was performed from the purified PCR products. BLAST and HbVar data base were used to identify the mutations of *HBB* gene responsible for the disease manifestations of the listed patients. Hemoglobin Electrophoresis was also performed to detect and quantify the Hb variants presented in the patients.

Total five types of mutations were identified in the patients of group-1 with HbE-beta thalassemia and beta thalassemia major (early-onset) namely: c.79G>A, IVS-1_5 G>C, c.126_129del_CTTT, c.33 C>A, c.47 G>A and total four types of mutations were found in the patients of group-2 with HbE-beta thalassemia (late-onset) namely: c.9 T>C, c.79 G>A, IVS-1_130 G>C and c.126_129 del_CTTT. Group-1 patients needed blood transfusions from an early age as they had mutation IVS-1_5 G>C (homozygous) or combination of mutations c.79G>A and IVS-1_5 G>C which were highly pathogenic. Group-2 patients needed blood transfusion after 18 years as their mutations were less pathogenic or severe.

The findings from this study will be beneficial for careful tailoring of therapeutic approaches and management of the complications associated with the treatments of Hb E/beta-thalassemia and beta thalassemia major patients.

Keywords: HbE-beta thalassemia, Beta thalassemia major, Sanger Nucleotide Sequencing, HbVar database.

Dedication

This work is especially dedicated to my loving mother, who has always encouraged and inspired me throughout her life to ensure I reach the height of my potential.

Acknowledgement

First of all, all praise to the almighty Allah who has given me the ability to complete my thesis work. Most respectfully I would like to convey my best regards towards Dr. Firdausi Qadri and Dr. Sayed Saleheen Qadri for giving me the opportunity to conduct my thesis work at the Institute for Developing Science & Health Initiatives (ideSHi).

In that vein, I would humbly extend my immense gratitude towards my professor and personal tutor, Dr. Naiyyum Choudhury; he was not only being an exemplary supervisor, but a pillar of support throughout this journey.

I would like to extend my deepest gratitude to Md. Kaiissar Mannoor PhD, the Head and Senior Scientist of Institute for Developing Science & Health Initiatives (ideSHi), for his wise advice, affectionate guidance and for allowing me to use laboratory facilities for some experiments through my thesis work.

I place on record, my sincere thanks to Dr. Mahboob Hossain, Professor of microbiology discipline at MNS Department of BRAC University for giving me the opportunity to enroll my thesis under his kind supervision.

Moreover, I could not have completed this project at all without the training and guidance by the talented Research Officer Tarikul Islam at ideSHi. I would also like to convey my thanks to my supportive and helpful seniors; Mohabbat Hossain and Golam Sarower Bhuyan who made this work easy for me by their unconditional assistance.

I would like to express my feeling to my parents; Shahjahan Bhuiyan and Joshna Shahnaz for the unceasing encouragement, and my friend Shahreaz Sarker for his help and support.
I am also thankful to every parson, who directly or indirectly, have lent their hand in this venture.

Author,
Raisa Tasnim (November, 2019)

Contents

INTRODUCTION
CHAPTER ONE

1.1 Background

Thalassemia is an inherited hematologic disorder. It is a genetic disorder that is caused by defects in the synthesis of one or more of the hemoglobin chains. Hemoglobin is a protein molecule in red blood cells that carries oxygen to tissues throughout the body. This disorder results in hemolytic anemia because of the decreased or absent synthesis of a globin chain. Alpha thalassemia is caused by reduced or absent synthesis of alpha-globin chains, and beta-thalassemia is caused by reduced or absent synthesis of beta-globin chains (Campbell, 2009). Imbalances of globin chains cause hemolysis and impair erythropoiesis.

People with mild thalassemia may be asymptomatic whereas people with moderate to severe thalassemia show various abnormalities. Affected children will require regular lifelong blood transfusions. Transfusion-dependent patients develop iron overload and require chelation therapy to remove excess iron. Persons with thalassemia carrier status should be referred for preconception genetic counseling to avoid thalassemia baby or babies in a family.

1.2 HBB gene

Beta-thalassemia occurs due to the mutation or deletion of bases in the HBB gene. The HBB gene provides instructions for making a protein called beta-globin. Beta-globin is a component (subunit) of a larger protein called hemoglobin, which is located inside red blood cells. In adults, hemoglobin normally consists of four protein subunits: two subunits of beta-globin and two subunits of another protein called alpha-globin, which is produced from another gene called HBA. Each of these protein subunits is attached (bound) to an iron-containing molecule called heme; each heme contains an iron molecule in its center that can bind to one oxygen molecule. Hemoglobin within red blood cells binds to oxygen molecules in the lungs. These cells then travel through the bloodstream and deliver oxygen to tissues throughout the body. Any defects or mutations in the gene results in some genetic disorders such as beta-thalassemia, sickle cell anemia.

Nearly 400 mutations in the HBB gene have been found to cause beta-thalassemia. Most of the mutations involve a change in a single DNA building block (nucleotide) within or near the HBB gene. Other mutations insert or delete a small/large number of nucleotides in the HBB gene.

HBB gene mutations that decrease beta-globin production result in a type of condition called beta-plus (B+) thalassemia. Mutations that prevent cells from producing any beta-globin result in beta-zero (B0) thalassemia (Cao & Galanello, 2010)

Problems with the subunits that make up hemoglobin, including low levels of beta-globin, reduce or eliminate the production of this molecule. A lack of hemoglobin disrupts the normal development of red blood cells. A shortage of mature red blood cells can reduce the amount of oxygen that is delivered to tissues below what is needed to satisfy the body's energy needs. A lack of oxygen in the body's tissues can lead to poor growth, organ damage, and other health problems associated with beta-thalassemia.

1.3 Epidemiology

Approximately 5 percent of the world's population has a globin variant, but only 1.7 percent has an alpha or beta thalassemia trait (Rund & Rachmilewitz, 2005). Men and women can be equally affected by thalassemia and occur in approximately 4.4 of every 10,000 live births. Alpha thalassemia occurs most often in persons of African and Southeast Asian descent, and beta-thalassemia is most common in persons of Mediterranean, African, and Southeast Asian descent. Thalassemia trait affects 5 to 30 percent of persons in these ethnic groups (Rund & Rachmilewitz, 2005).

1.4 Inheritance of thalassemia

Thalassemia is a group of inherited blood diseases that affect a person's ability to produce hemoglobin in red blood cells. Thalassemia is inherited when the mutated thalassemia gene is passed from parents to their child. Worldwide, approximately 100,000 babies are born with severe forms of inherited thalassemia each year. If only one of the parents is a carrier for thalassemia, the offspring may develop a form of the disease known as thalassemia minor and the person may won't show any symptoms but he or she will be the carrier of the disease. But if both are carriers of defected HBB gene, the risk is 25% that the child will be born with severe thalassemia (Wilkie et al., 1990).

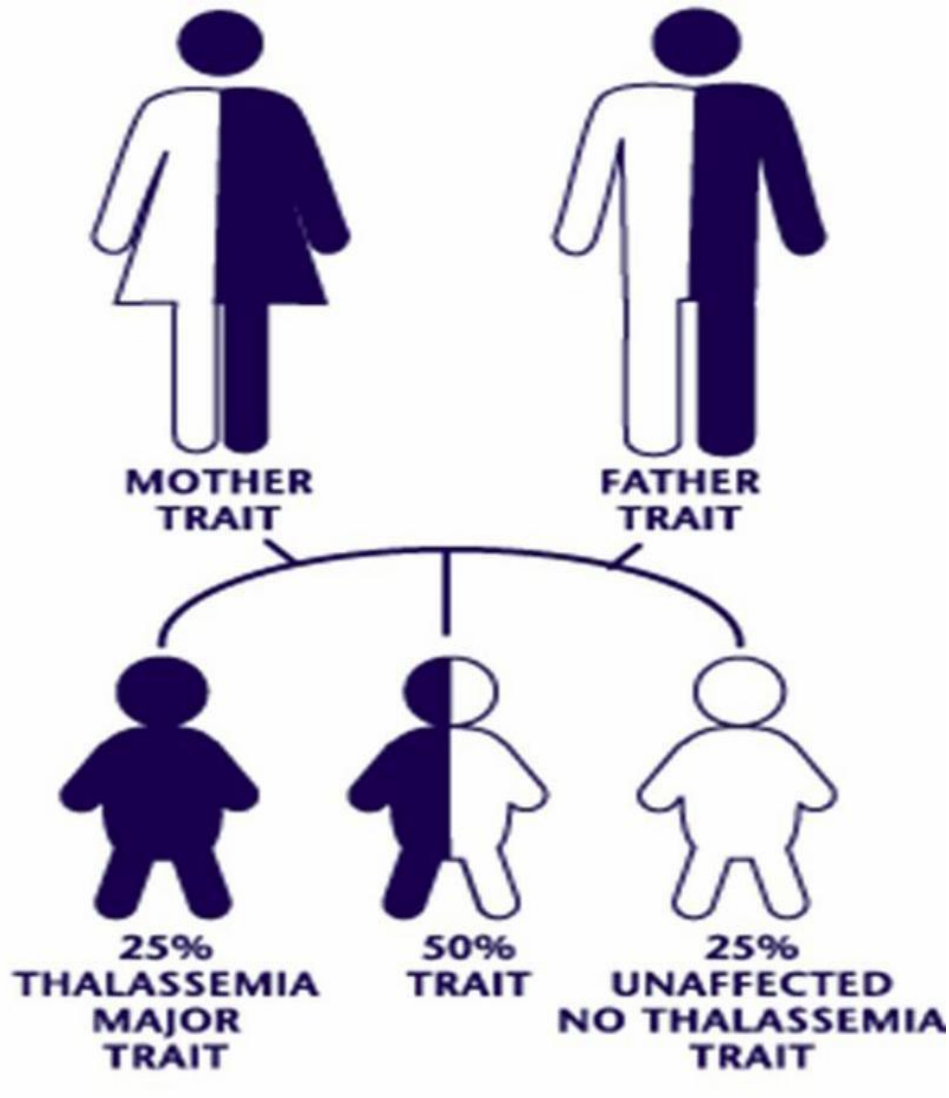

Figure 1.1: Genetic inheritance of thalassemia

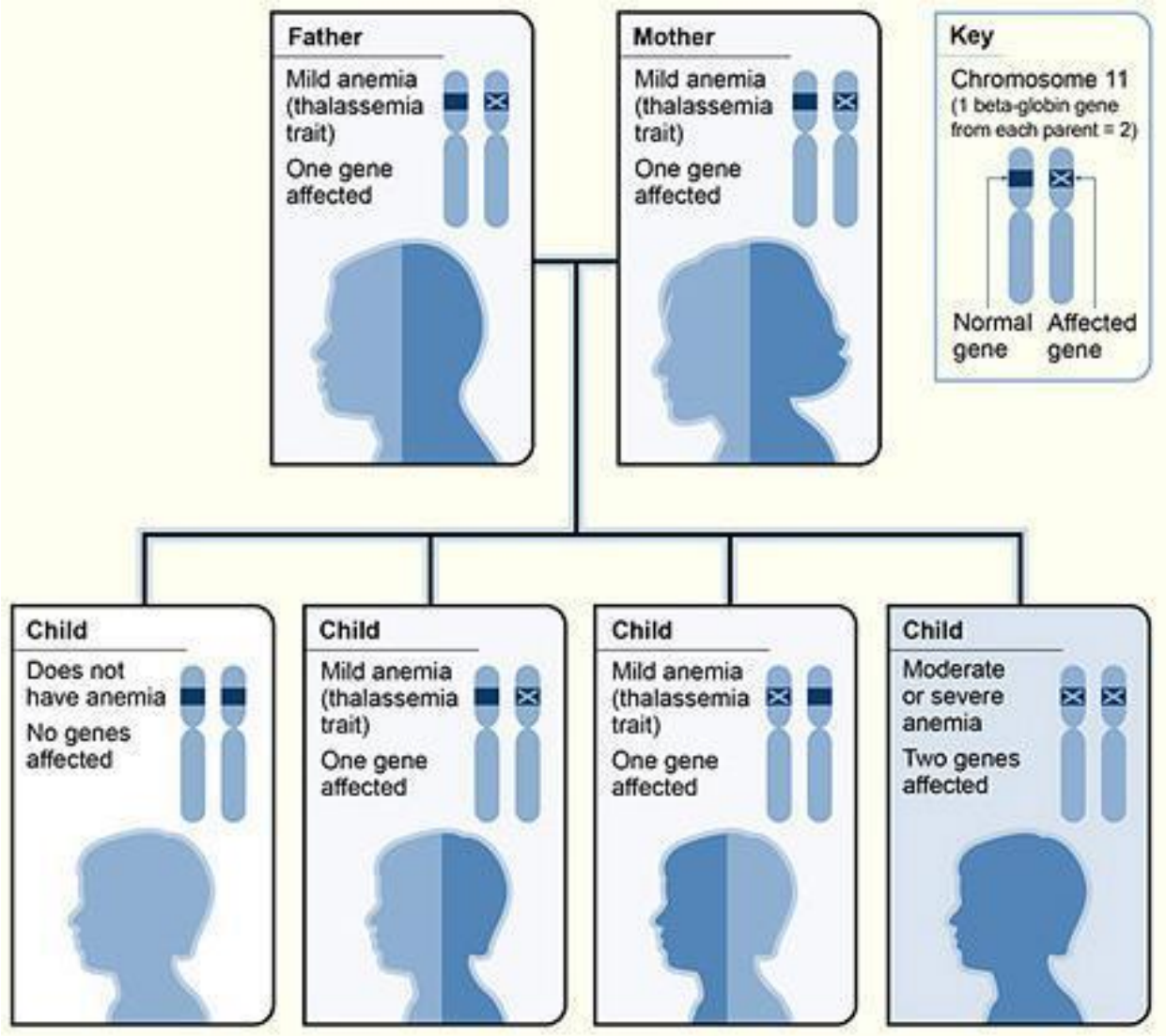

Figure 1.2: Inheritance pattern of beta-thalassemia

1.5 Pathophysiology

Hemoglobin composed of an iron-containing heme ring and four globular chains: two alpha-globin and two non-alpha globin chains (Wilkie et al., 1990). The composition of the four globin chains determines the hemoglobin type. They are arranged into a hetero-tetramer form. Fetal hemoglobin (HbF) has two alpha and two gamma chains (alpha2 gamma2). Adult hemoglobin A (HbA) has two alpha and two beta chains (alpha2 beta2), whereas hemoglobin A2 (HbA2) has two alpha and two delta chains (alpha2 delta2). At birth, HbF accounts for approximately 80 percent of hemoglobin and HbA account for 20 percent (Wilkie et al., 1990). The transition from gamma-globin synthesis (HbF) to beta-globin synthesis (HbA) begins before birth. By approximately six

months of age, healthy infants will have transitioned to mostly HbA, a small amount of HbA2, and negligible HbF. Figure 1.3 shows normal and abnormal hemoglobin's.

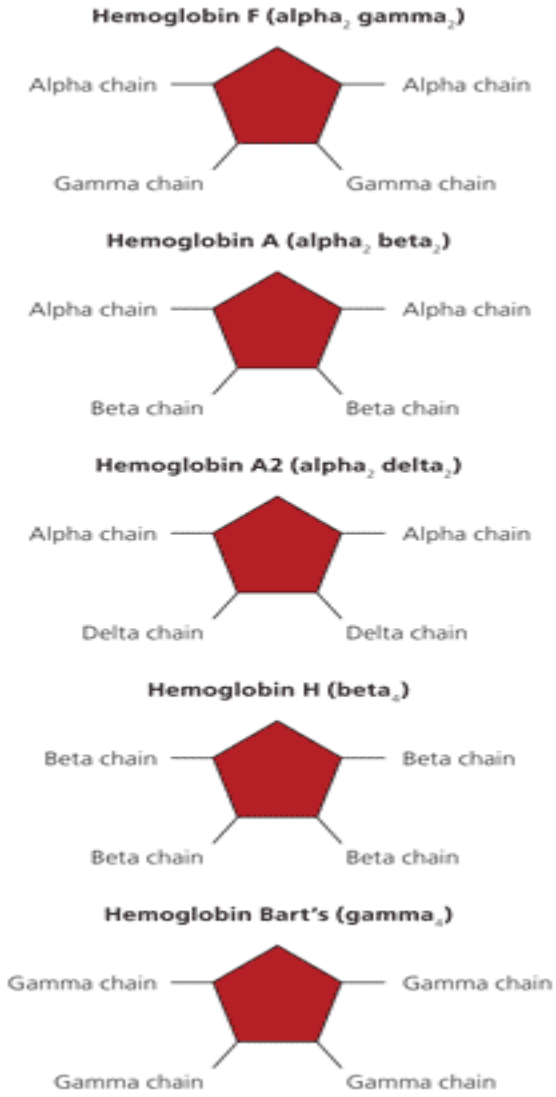

Figure 1.3: Normal (hemoglobin F, A, and A2) and abnormal (hemoglobin H and Bart's) hemoglobin's. Hemoglobin consists of an iron-containing heme ring and four globin chains: two alpha and two non-alpha. The composition of the four globin chains determines the hemoglobin type.

In thalassemia affected patients, defects are observed in either alpha or beta globin chains which is one of the main reasons for the production of abnormal red blood cells. Thus the classifications of thalassemia are based upon which chain of the hemoglobin molecule is affected. There are two main types of thalassemia present including four subtypes:

- Beta thalassemia; subtypes major and intermedia

- Alpha thalassemia; subtypes hemoglobin H and hydrops fetalis

1.6 Beta Thalassemia

β thalassemia is an autosomal recessive disorder that is caused by the reduced (beta+) or absent (beta0) synthesis of the beta-globin chains of the hemoglobin (Hb) tetramer and is made up of two alpha-globin and two beta-globin chains (Weatherall & Clegg, 2001). The severity of beta-thalassemia depends on the nature of the mutation or deletion and the presence of mutations in one or both alleles. Depending on the clinical and hematological conditions of increasing severity; beta-thalassemia is recognized into three subtypes: the beta-thalassemia major, thalassemia intermedia, and thalassemia carrier state or minor.

1.7 Beta thalassemia major

Thalassemia major is the most severe form of transfusion-dependent anemia. In the major type of thalassemia, β0/ β0 genotype is observed and no functional β chains in hemoglobin are produced. Individuals with thalassemia major usually come to medical attention within the first 2 years and required regular blood transfusion to survive. Affected infants with thalassemia major fail to thrive and become progressively pale along with feeding problems, diarrhea, and irritability, recurrent bouts of fever, and enlargement of the abdomen may occur. If a regular transfusion program that maintains a minimum Hb concentration of 95–105 g/L is initiated, then growth and development are normal until the age of 10–11 years. After the age of 10–11 years, affected individuals are at risk of developing severe complications related to post-transfusional iron overload, depending on their compliance with chelation therapy.

1.8 Beta thalassemia intermedia

It is a less severe form of thalassemia which is caused by β+/ β^0 or β+/ β+ genotypes. Patients with thalassemia intermediate present later in life with moderate anemia and do not require regular transfusions. Main clinical features in these patients are hypertrophy of erythroid marrow with medullary and extramedullary hematopoiesis and its complications (osteoporosis, masses of erythropoietic tissue that primarily affect the spleen, liver, lymph nodes, chest and spine, and bone deformities and typical facial changes), gallstones, painful leg ulcers and increased predisposition to thrombosis**.**

1.9 Beta thalassemia minor/carrier

People having β/ β^0 or β/ β+ genotype are carrier or minor thalassemia affected. Individuals with beta-thalassemia minor do not develop any symptoms of the disorder or only very mild anemia. When the disorder develops later during life, a diagnosis of beta-thalassemia intermedia is given; individuals may only require blood transfusions on rare, specific instances. Thalassemia minor is clinically asymptomatic but some people may have moderate anemia. When both parents are carriers there is a 25% risk at each pregnancy of having children with homozygous thalassemia.

1.10 Differences between thalassemia major and minor

The individual with thalassemia minor has only one copy of the beta-thalassemia gene (together with one perfectly normal beta-chain gene). The person is said to be heterozygous for beta-thalassemia. Persons with thalassemia minor have (at most) mild anemia (with a slight lowering of the hemoglobin level in the blood). This situation can very closely resemble mild iron-deficiency anemia. However, persons with thalassemia minor have a normal blood iron level (unless they are iron deficient for other reasons). People born with minor thalassemia can show symptoms at adulthood.

The child born with thalassemia major has two genes for beta-thalassemia and no normal beta-chain gene. The child is homozygous for beta-thalassemia. This causes a striking deficiency in beta chain production and in the production of Hb A. Thalassemia major is, therefore, a serious disease. At birth, the baby with thalassemia major seems entirely normal. This is because the predominant hemoglobin at birth is still fetal hemoglobin (Hb F). Hb F has two alpha chains (like Hb A) and two gamma chains (unlike Hb A). It has no beta chains so the baby is protected at birth from the effects of thalassemia major.

Anemia begins to develop within the first months after birth. It becomes progressively more and more severe. The infant fails to thrive (to grow normally) and often has problems feeding (due to easy fatigue from lack of oxygen with the profound anemia), bouts of fever (due to infections to which the severe anemia predisposes the child) and diarrhea and other intestinal problems.

1.11 Alpha thalassemia

Alpha thalassemia is the result of the deficient or absent synthesis of alpha-globin chains, leading to excess beta-globin chains. Alpha globin chain production is controlled by two genes on each chromosome 16.

Prototypical Forms of Alpha Thalassemia is shown in table 1.1 below (Chui, Fucharoen, & Chan, 2003; Chui & Waye, 1998)

VARIANT	CHROMOSOME 16	SIGNS AND SYMPTOMS
Alpha thalassemia silent carrier	One of four gene deletions	Asymptomatic
Alpha thalassemia trait	Two of four gene deletions	Asymptomatic
Hemoglobin Constant Spring	Reduced output of alpha-globin	Silent or mildly symptomatic
Alpha thalassemia intermedia with significant hemoglobin H (hemoglobin H disease)	Three of four gene deletions	Moderate to severe hemolytic anemia, modest degree of ineffective erythropoiesis, splenomegaly, variable bone changes
Alpha thalassemia major with significant hemoglobin Bart's	Four of four gene deletions	Causes nonimmune hydrops fetalis, usually fatal

Deficient production is usually caused by a deletion of one or more of these genes. A single gene deletion results in alpha thalassemia silent carrier status, which is asymptomatic with normal hematologic findings. The two-gene deletion causes alpha thalassemia trait (minor) with microcytosis and usually no anemia. The three-gene deletion results in significant production of hemoglobin H (HbH), which has four beta chains (beta4). Alpha thalassemia intermedia, or HbH disease, causes microcytic anemia, hemolysis, and splenomegaly. The four-gene deletion results in significant production of hemoglobin Bart's (Hb Bart's), which has four gamma chains (gamma4). Alpha thalassemia major with Hb Bart's usually results in fatal hydrops fetalis.

1.12 Symptoms of the disease

The signs and symptoms of a thalassemia affected patient can be observed within the first two years of birth of a child. The signs and symptoms can vary from person to person depending on the severity of thalassemia. People affected with milder forms of thalassemia can be asymptomatic or may develop mild anemia. Some common signs and symptoms are observed in people affected with moderate to severe thalassemia are-

- Fatigue
- Weakness
- Pale or yellowish skin
- Slow growth
- Shortness of breath
- Facial bone deformities
- Abdominal swelling
- Dark urine

1.13 Complications

Some complications are also detected in the patients affected with severe thalassemia

- Iron overload
- Infection
- Enlarged spleen
- Slowed growth rate
- Heart problems
- Bone deformities

1.14 Aims and objectives of the study

1. To determine the mutation spectrum in HbE-beta-thalassemia and Hb beta-thalassemia major patients.
2. To check the mutational status among patients who manifested disease in childhood or early life and patients manifested disease at adult life

MATERIALS AND METHODS
CHAPTER TWO

The whole working method is showing in a figure below

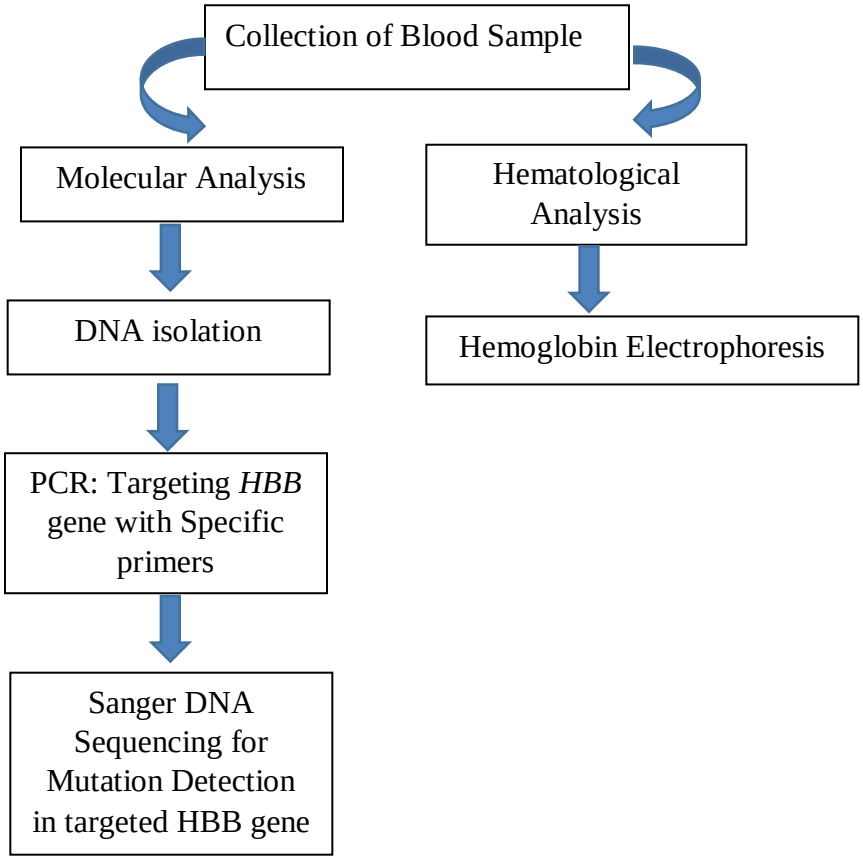

Figure 2.1: Work methods

2.1 Study place

The study was conducted in Genetics and Genomic laboratory of the institute of developing Science and Health initiatives (ideSHi), Mohakhali, Dhaka-1212.

2.2 Sample collection

Blood samples were collected from 12 children and 5 adults diagnosed as thalassemia earlier. Written consent was taken prior to the enrollment of study participants.

2.3 Hematological Analysis

2.3.1 Hemoglobin Electrophoresis

Hemoglobin electrophoresis is a well-established technique routinely used in clinical laboratories to screen samples for hemoglobinopathies (hemoglobin variants and thalassemias). The

CAPILLARYS HEMOGLOBIN(E) kit enables, on CAPILLARYS instruments, the efficient separation of hemoglobin fractions and the detection of a large number of hemoglobin variants and thalassemias patterns. CAPILLARYS instruments allow high-resolution separation of the major hemoglobin variants (Hb S, Hb C, Hb D and Hb E) and accurate quantification of the Hb A2 and Hb F. Hb H and Bart's, as seen in alpha-thalassemia, are clearly separated and easily quantified. The CAPILLARYS HEMOGLOBIN(E) kit allows hemoglobin analysis on adult and newborn cord blood samples.

The CAPILLARYS HEMOGLOBIN(E) assay is based on the principle of capillary electrophoresis in free solution. Hemoglobin fractions are separated in silica capillaries, by their electrophoretic mobility and electroosmotic flow at a high voltage in an alkaline buffer. Hemoglobin fractions are directly detected at the specific absorbance of 415 nm. The pattern is divided into 15 zones (12 zones for cord blood). Each zone displays a drop-down library of possible variants migrating within this zone. More than 300 variants are listed.

2.3.2 Capillarys Hemoglobin(E) kit content:

- Buffer
- Hemolyzing solution
- Wash solution
- Dilution segments
- Filters

Related accessories:

- Sebia Capillarys 2 (France) machine
- Cassettes
- Segments
- Plasma separated blood sample

Procedure:

1. Plasma was separated from the collected blood samples by centrifugation at 3000rpm for 10 minutes.
2. Plasma separated blood sample tubes were transferred in cassettes.
3. A green segment with a hemolyzing solution was placed in the cassette.

4. The cassette was placed in the Sebia Capillarys 2 machine.
5. The machine was operated by using specific software.
6. After analyzing, the result was generated.

2.4 Molecular Analysis

2.4.1 DNA extraction Procedure

For DNA extraction GeneJet Genomic DNA Purification Kit was used.

1. Four hundred microliter of Lysis Solution and twenty microliter of Proteinase K Solution was added to two hundred microliter of whole blood and was mixed thoroughly by vortexing to obtain a uniform suspension.
2. The sample was incubated on the sample at 56 °C after vortexing in a water bath until the cells were completely lysed (10 min).
3. Then two hundred microliter of ethanol was added and mixed by vortexing.
4. The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube.
5. The column was centrifuged for 1 min at 6000 rpm.
6. The collection tube containing the flow-through solution was discarded and the GeneJET Genomic DNA Purification Column was placed into a new 2 mL collection tube.
7. Five hundred microliter of Wash Buffer I was added. Centrifuge was done for 1 min at 8000 rpm.the flow-through was discarded and the purification column was placed back into the collection tube.
8. Five hundred microliter of Wash Buffer II was added next to the GeneJET Genomic DNA Purification Column.
9. Centrifugation for 3 min was done at maximum speed (≥12000 rpm). If the residual solution was seen in the purification column, the collection tube was emptied and the column was re-spinned for 1 minute at maximum speed.
10. The collection tube containing the flow-through solution was discarded the GeneJET Genomic DNA Purification Column was transferred into a sterile 1.5 mL microcentrifuge tube.

11. Two hundred microliter of Elution Buffer was added to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA.
12. Incubation for 2 min at room temperature and centrifugation for 1 min at 8000 rpm was done.
13. Finally, the purification column was discarded and the purified DNA was used immediately in or was stored at -20 °C.

2.4.2 Measurement of DNA concentration and purity

DNA concentration was measured with (Nanodrop 2000). One drop or nearly, 2µl of nuclease-free water was used as blank and 2µl of DNA sample was loaded on the sensor. The optical density (OD) was measured. The concentration (in ng/µl) and the purity of the DNA (from the OD ratio of 260nm/280nm) were checked.

2.4.3 Polymerase Chain Reaction

2.4.3.1 PCR Principle

Polymerase chain reaction (PCR) is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA. PCR can use the smallest sample of the DNA to be cloned and amplify it to millions of copies in just a few hours. The PCR involves the primer mediated enzymatic amplification of DNA. PCR is based on using the ability of DNA polymerase to synthesize a new strand of DNA complementary to the offered template strand. Primer is needed because DNA polymerase can add a nucleotide only onto a preexisting 3′-OH group to add the first nucleotide. The DNA polymerase then elongates its 3 end by adding more nucleotides to generate an extended region of double-stranded DNA.

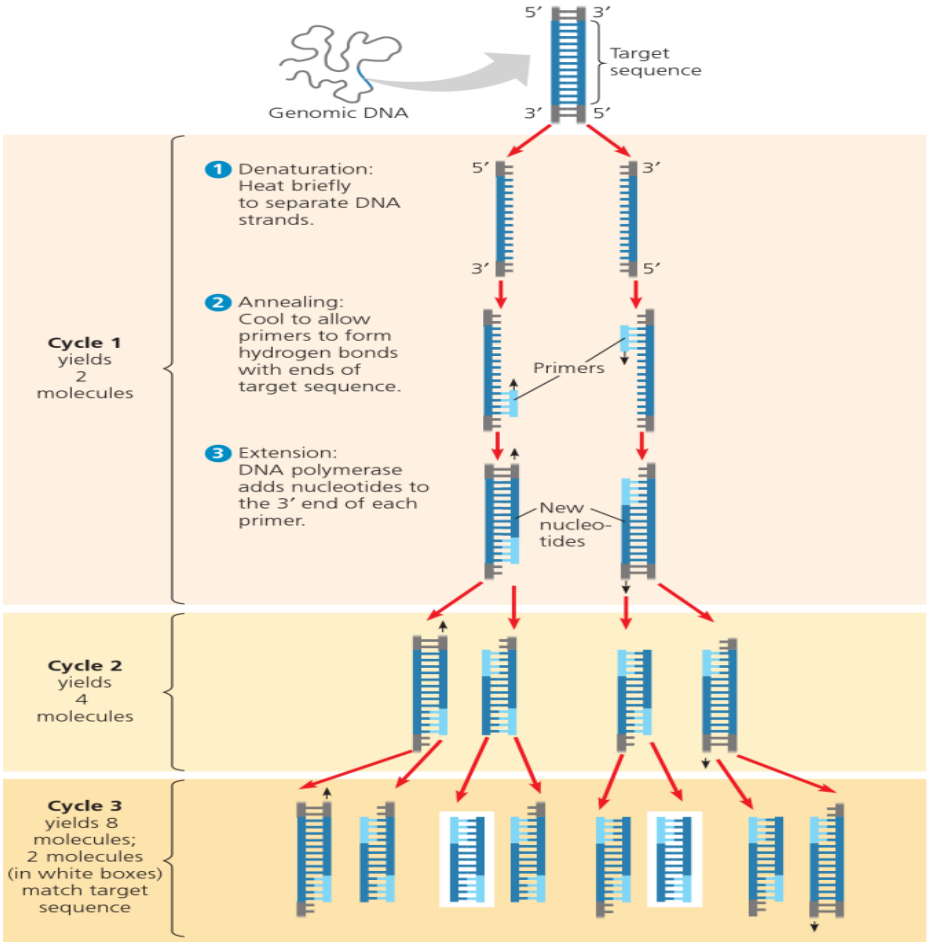

Figure 2.2: Principle of PCR

2.4.3.2 Components of PCR

In this study, conventional PCR was performed. PCR was conducted using specific primers using T100^{TM} thermal cycler (Bio-Rad, USA). The PCR reaction required the following components: 10X buffer. $MgCl_2$, 2.5 nM dNTPs, forward primer, reverse primer, Q solution, nuclease-free water, and Taq polymerase. Ten microliter of the master mix was prepared with all these components with a specific amount.

2.4.3.3 Procedure of PCR

All the PCR components were mixed together and are taken through a series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine.

1. Denaturation:

   This step involved heating the reaction mixture at 94°C for 15-30 seconds. During this, the

double-stranded DNA was denatured to single strands due to breakage in weak hydrogen bonds.

2. Annealing:
   The reaction temperature was rapidly lowered at 54-60°C for 20-40 seconds. This allowed the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Elongation:
   Also known as extension, this step usually occurs at 72-80°C (most commonly 72°C). In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction. Under optimal conditions, DNA polymerase added about 1,000 bp/minute.

With one cycle, a single segment of double-stranded DNA template was amplified into two separate pieces of double-stranded DNA. These two pieces were then available for amplification in the next cycle. As the cycles are repeated, more and more copies are generated and the number of copies of the template was increased exponentially.

2.4.3.4 Agarose gel preparation for gel electrophoresis

Agarose gel electrophoresis uses the negative charge property of DNA to separate the DNA strands according to their size and molecular weight. Shorter DNA fragments have low molecular weight and thus migrate faster than the larger DNA fragments.

For preparing 1% gel for gel electrophoresis, 1 gm of agarose (ultrapure, Invitrogen, USA) was dissolved in 10 ml 1X TBE buffer (Tris-borate EDTA) by heating in oven for 1 minute. The mixture was allowed to cool down at room temperature and after some time 2µl of Gel red was added. When the liquid gel was cooled, it was poured on the gel casting tray and a comb was set gently. During pouring the gel, bubbles can form which was avoided by taken care. Then the gel was allowed to solidify at room temperature.

2.4.3.5 Gel run of PCR product using gel electrophoresis

After the gel was solid, it was placed into the gel electrophoresis chamber filled with 1X TBE buffer. 3µl of PCR products were mixed with 2µl of loading dye and loaded into the well of the gel. 1KB plus ladder was also added in one of the well to determine the band size of PCR amplicons. The PCR products were run at 130 volts for 30-45 minutes in the gel. Finally, the gel

was observed for a separated DNA band on the Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

2.4.4 PCR Product Purification

2.4.4.1 Principle

PCR purification is a method of purifying DNA from other mixed reagents. Firstly, DNA is absorbed onto the spin column through binding buffer in acidic solution as the buffer contains chaotropic salts which remove all DNA binding proteins and contaminants from PCR products through disrupting protein structure by destabilizing hydrophobic interactions. High salt existence decreases the negativity of DNA and increases binding affinity and stronger interaction with the column membrane. Ethanol added with washing buffer efficiently removes dNTPs, primer dimers, salt, buffer or anything except DNA from the column. For complete removal of residual ethanol, extra centrifugation is done. Finally, DNA is eluted utilizing nuclease-free water as binding affinity of DNA with nuclease-free water is high and for having neutral pH.

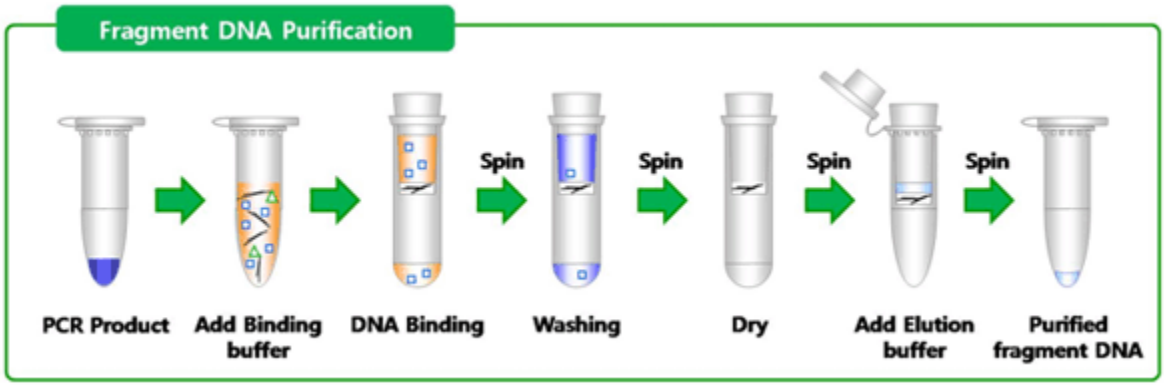

Figure 2.3: **Purification of PCR products**

2.4.4.2 Equipment and reagents for purification of PCR products

Equipment

- Biosafety cabinet
- Micro-centrifuge tubes

- Water bath
- NanoDrop DNA quantitation system
- Refrigerator (-70ºC)

Materials

- MinElute Column
- Pipettes

Reagents

- MinElute PCR purification Kit
- Buffer PB
- Buffer PE
- 3M Sodium acetate
- Nuclease-free water

2.4.4.3 Procedure

1. All PCR products were spun and transferred to Eppendorf tubes.
2. 5 volumes of buffer PB was added to each PCR product in each Eppendorf tube.
3. After mixing, the reaction mixture did not yellow (similar to buffer PB). So 10µL 3M sodium acetate was added, mixed well and inverted several times. Then the color had been changed to yellow.
4. The mixture was transferred to the spin column; placed on a collection tube and centrifuged for 1 min at 17900xg.
5. The flow-through was discarded from the MinElute column and was again placed into the same collection tube.
6. 750µL buffer PE was added to wash each column and was again centrifuged for 1 min at 17900xg.
7. The flow-through was discarded and the column was again placed back on the same collection tube for additional centrifugation for the complete removal of residual ethanol for 1min at 17900xg.
8. The columns were transferred on 1.5 ml micro-centrifuge tubes.

9. For elution of the PCR product, 20µL of nuclease-free water was applied to the center of each MinElute column, allowed to stand at room temperature for 1 min and centrifuged at 17900xg for 1 min to elute.
10. Concentration and purity of the purified DNA were measured using NanoDrop.
11. For further analysis, purified DNA was stored at -70ºC for further genetic analysis.

2.4.5 Sanger Sequencing

2.4.5.1 Principle

DNA sequencing entails template DNA, primer (forward or reverse), DNA polymerase, dNTPs, sequencing buffer, ddNTPs (2', 3'-dideoxynucleotide triphosphates). After mixing all these components, the reaction mixtures are subjected to thermal cycling protocol through denaturation, annealing, and extension. When polymerase initiates its amplification, the dNTPs or ddNTPs bind with complementary bases on the relative concentration of both molecules. When dNTPs are added continuously, the chain extension continues but after the addition of ddNTPs, the chain extension is terminated for the absence of 3'-hydroxyl group as ddNTPs work as a chain-terminating inhibitor. These ddNTPs are tagged with fluorescently labeled and labeled extension products of various lengths that are detected through these chain terminator ddNTPs.

For the detection of the fragmented pieces of various product lengths of template DNA, they are subjected to capillary gel electrophoresis. When high voltage is applied in buffered sequencing reaction, the fragments are forced to run from negative to positive electrode as DNA is negatively charged. The smaller fragments can run through a porous channel easily than relatively bigger fragments. Before reaching the electrode, the fragments are separated by their molecular weight and size, move across the path of the laser beam. When they reach the laser beam, it will excite, fluorescently labeled ddNTPs on the fragments will emit light at unique wavelength respective to all four colors and will be detected in one capillary injection through optical detection device. The emitted fluorescence signal is converted to digital data and analyzed by data collection software.

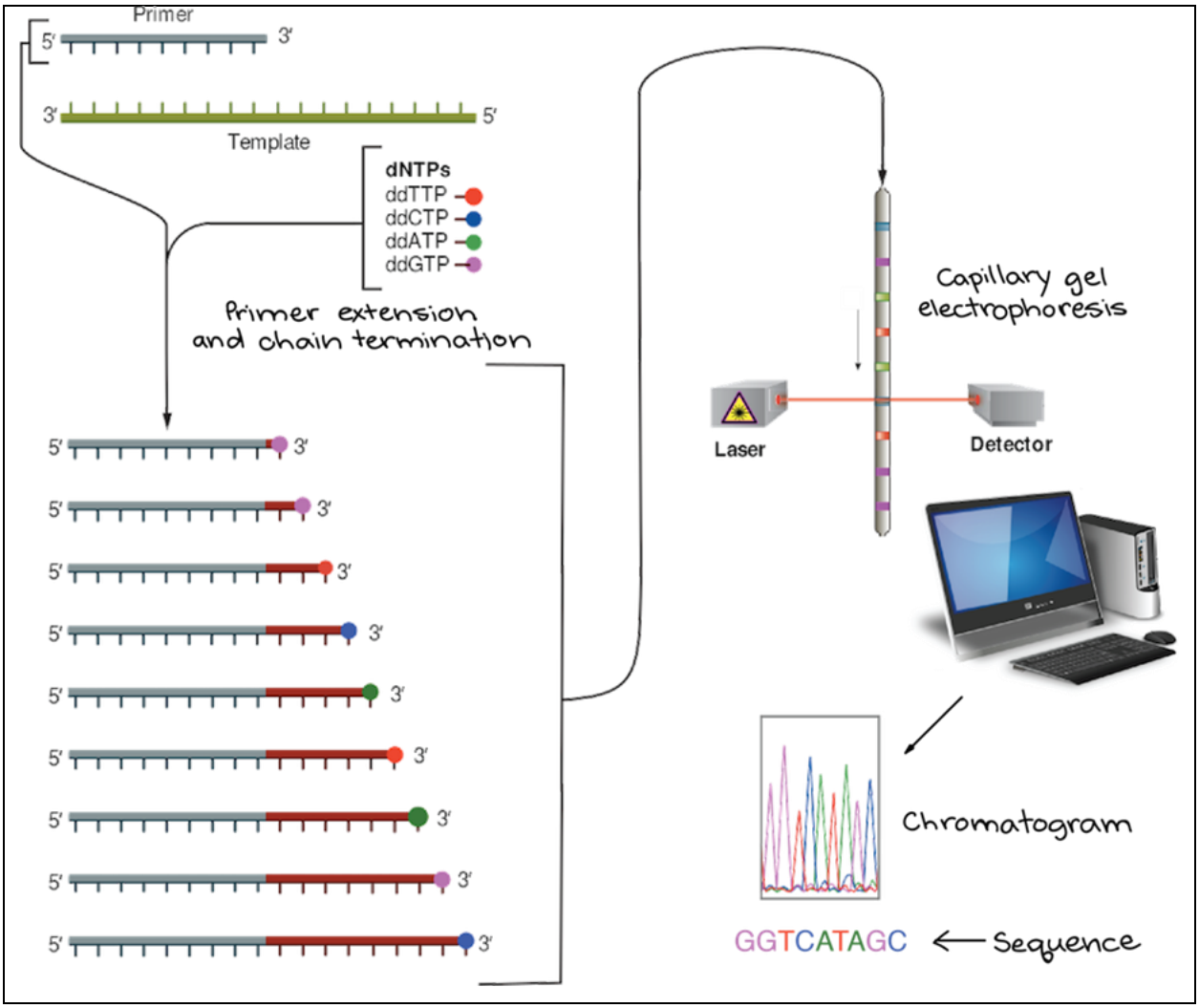

Figure 2.4: Sanger Sequencing

2.4.5.2 Reagents and equipment for sequencing

Equipment

- Applied Biosystems 310 Genetic Analyzer
  - No. of capillaries: 1
  - Capillary Array Length: 47 or 61 cm
  - Sample capacity: 48 or 96 sample tubes
- Cycle sequencing machine Mastercycler® gradient

- Centrifuge machine
- 8-tube PCR strip

Reagents

Reagents needed for sequencing are mentioned below:

- 5X Sequencing buffer
- BigDye Chain Terminator v3.1 Ready Reaction(RR) Mix
- Forward primer or Reverse primer
- SAM solution (Applied Biosystems, USA)
- X-terminator solution (Applied Biosystems, USA)

2.4.5.3 Cycle Sequencing Master-mix preparation

Purified PCR products were sent to the Institute of Epidemiology Disease Control and Research for sequencing. Sequencing of the specific region of PCR purified products was done using BigDye Chain Terminator version 3.1 Cycle sequencing Kit (Applied Biosystems, USA) and ABI PRISM 310 automated sequencer (Applied Biosystem, USA) according to manufacturer's instruction. Mastermix was prepared for multiple samples where for each sample 2µl 5x sequencing buffer, 0.2 µl 2.5x BigDye Chain Terminator version 3.1 ready reaction mix, 0.2 µl 10 µM forward or reverse primer were added. After that, the master mix was spun and pipetted equally to each of the 8-tube PCR strips. 10ng/µl PCR purified products were added to each well of the strip. Total reaction volume 10 µl was optimized by adding nuclease-free water.

Table 2.1: Reagents and their amounts used for Cycle Sequencing Master-mix preparation

Reagent	Concentration	Amount of single reaction
Sequencing buffer	5x	2 µl
BigDye chain terminator RRmix	2.5x	0.2 µl
Forward or reverse primer	0.2 µM	0.2µl
Templates	10ng/µl	1 µl
Nuclease free water	Not applicable	Up to 10 µl

The tubes containing reaction mixture for cycle sequencing were vortexed and centrifuged for 4000xg for 3min. then the tubes were placed in cycle sequencing machine Mastercycler gradient thermal cycler. Following thermal cycling profile(Table.2.2) was used for PCR: initial denaturation at 94ºC for 1 minute; 25 cycles of denaturation at 94 ºC for 10 seconds, annealing at 58 ºC for 5 seconds and extension at 60 ºC for 4 minutes; and a final elongation at 60 ºC for 10 minutes (Table 2.2)

Table 2.2: Cycle sequencing thermal condition cycle

Steps	Temperature	Duration	Cycle
Pre denaturation	94 ºC	1min	1
Denaturation	94 ºC	10s	25
Annealing	58 ºC	5s	25
Elongation	60 ºC	4min	25
Final elongation	60 ºC	10min	1

After completion of cycle sequencing, products were centrifuged at 4100xg for 2 min. SAM solution an X-terminator solution were used to purify cycle sequencing products. Before using, X-terminator solution, a container carrying it was vortexed at minimum speed for at least 30 seconds for complete homogenization as it was highly denser. In the case of SAM solution, it was heated at 37ºC, mixed and cooled to room temperature before using as any particulate might present. 45µl SAM solution and 10 µl X-terminator solution were mixed to cycle sequencing products, vortexed on an automatic shaker for 30 min at 3000 rpm. After centrifugation at 4000xg for 2 minutes, 10 µl supernatant from each well was transferred to a new 0.2ml new PCR tube strip and solutions were subjected to capillary electrophoresis through POP-6TM polymer in an automated sequencing machine ABI PRISM 310 Genetic Analyzer.

2.4.5.4 Sequencing analysis

Sequencing results were analyzed by Chromas Lite 2.44 software to detect reliable and conclusive data for mutational analysis and eliminate sequences with noises that gave inconclusive and unreliable data. Reliable and conclusive sequences were analyzed to locate any mutation using the Basic Local Alignment Search Tool from NCBI. To detect any mismatched bases, the FASTA

format of the sequence was aligned with wild type sequences stored in the NCBI database. If any mismatched was found in the BLAST result, the mismatch bases were checked in Chromas Lite Tool to detect whether any significant noise or misreading existed there. If there is no significant noise or any evidence of misreading and the quality value goes above 15, the base certainly results in a base change or mutation. Then the bases were checked for the corresponding position in mRNA and position in coding sequences was recorded. The CDS position of the mutated base would be divided by three to detect the amino acid position in the peptide. It was also checked whether base substitution or deletion caused any changes in coding sequences as well as open reading frames. Sometimes base substitution does not alter the coding to a new amino acid.

RESULTS
CHAPTER THREE

In this study we have divided the thalassemia patients into two groups:

Group-1: Patients below 18 years (Early Onset), 12 children.

Group-2: Patients above 18 years (Late Onset), 5 adults.

3.1 Group 1

3.1.1 Patient 1

Patient 1 was diagnosed as Hb E /beta-thalassemia at the age of 10 months old. Now he is 3 years 11-month old and takes regular blood transfusion at two months' interval and chelation therapy with the medication deferoxamine (50 mg/kg). He did not develop splenomegaly till now and no other complications history has been found. His parents had no previous relationship and they do not belong to any tribe. Genetic analysis of the patient 1 specimen revealed 2 point mutations in the compound heterozygous state namely; c.79G>A and IVS-1_5 G>C (Fig. 3.1).

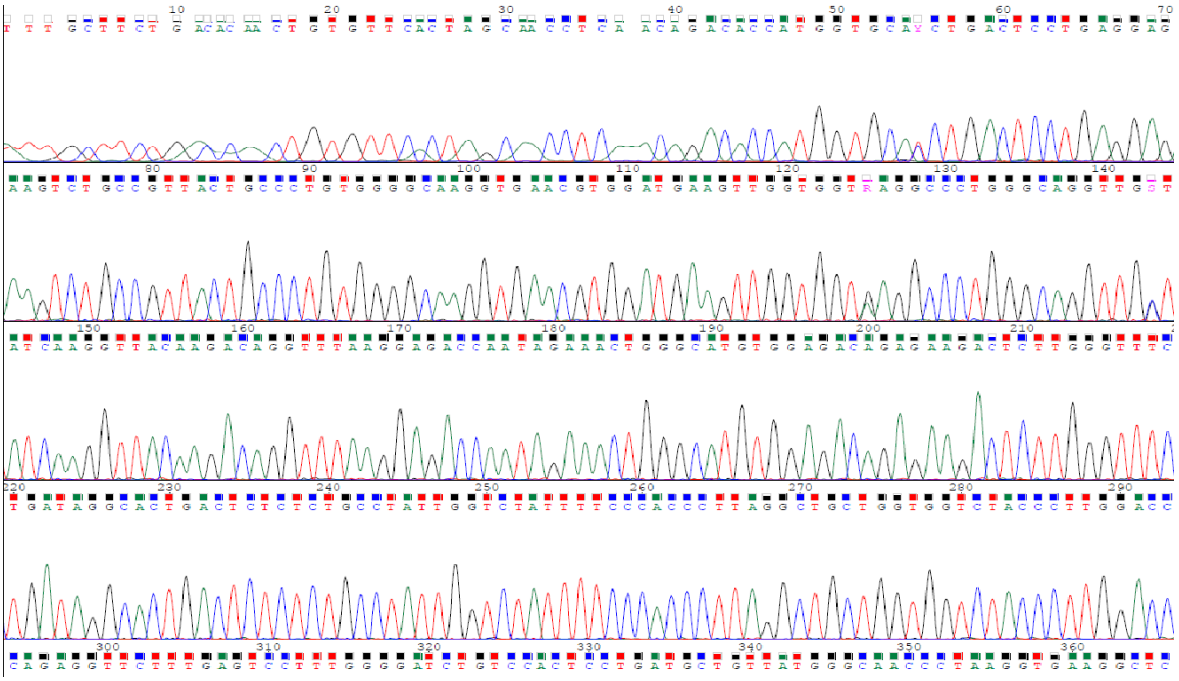

Figure 3.1: The chromatogram of patient 1 is showing compound heterozygous point mutations c.79G>A and IVS-1_5 G>C.

3.1.2 Patient 2

Patient 2; a female child who was diagnosed as HBB beta-thalassemia at the age of 3 months old and the patient's age is now 6 years 1-month. The patient takes regular blood transfusion at a 1-month interval and chelation therapy. The patient has splenomegaly but shows no other complications during chelation therapy till now. Her parents are first cousins but they do not belong to any tribe. Genetic analysis of the patient 2 specimen revealed homozygous mutation namely; IVS-1_5 G>C (homozygous).

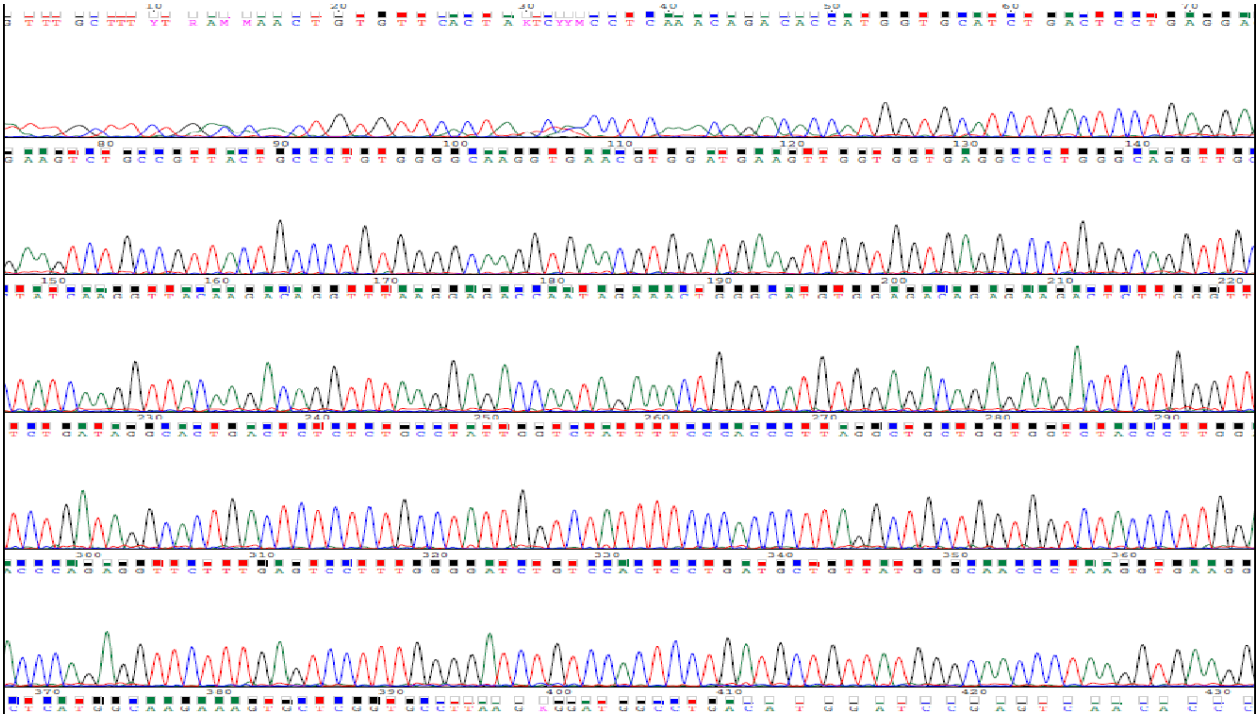

Figure 3.2: The chromatogram of patient 2 is showing homozygous mutation namely; IVS-1_5 G>C (homozygous).

3.1.3 Patient 3

Patient 3 was a male child who was diagnosed as E-beta thalassemia at the age of 2.5 years and now the patient is 8-year-old. The patient needs to take regular blood transfusion at 1 to 1.5 months' interval along with chelation therapy. The patient did not develop splenomegaly till now and shows no other complications during transfusion. The patient's parents had no previous relationship and they do not belong to any tribe. Genetic analysis of the patient's specimen revealed a 2-point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C.

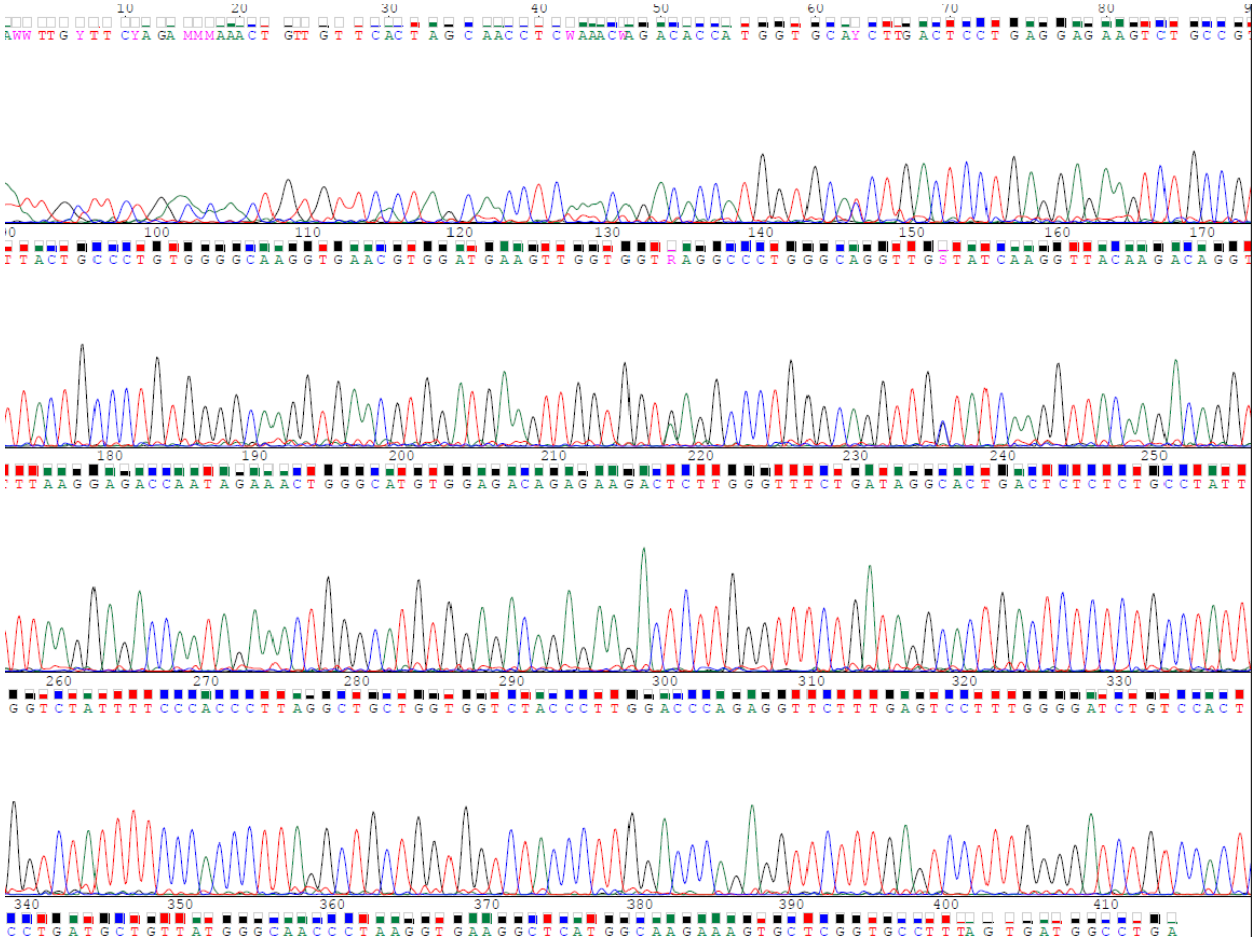

Figure 3.3: The chromatogram of patient 3 is showing 2 point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C

3.1.4 Patient 4

Patient 4 is a 6-year-old female child and was diagnosed as E-beta thalassemia at the age of 2 years. The patient takes blood transfusion every 2.5 months with chelation therapy. The patient did not have splenomegaly and shows no other complications during transfusions. Her parents are not previously related and they do not belong to any tribe. Genetic analysis of the patient's specimen revealed 2 point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C

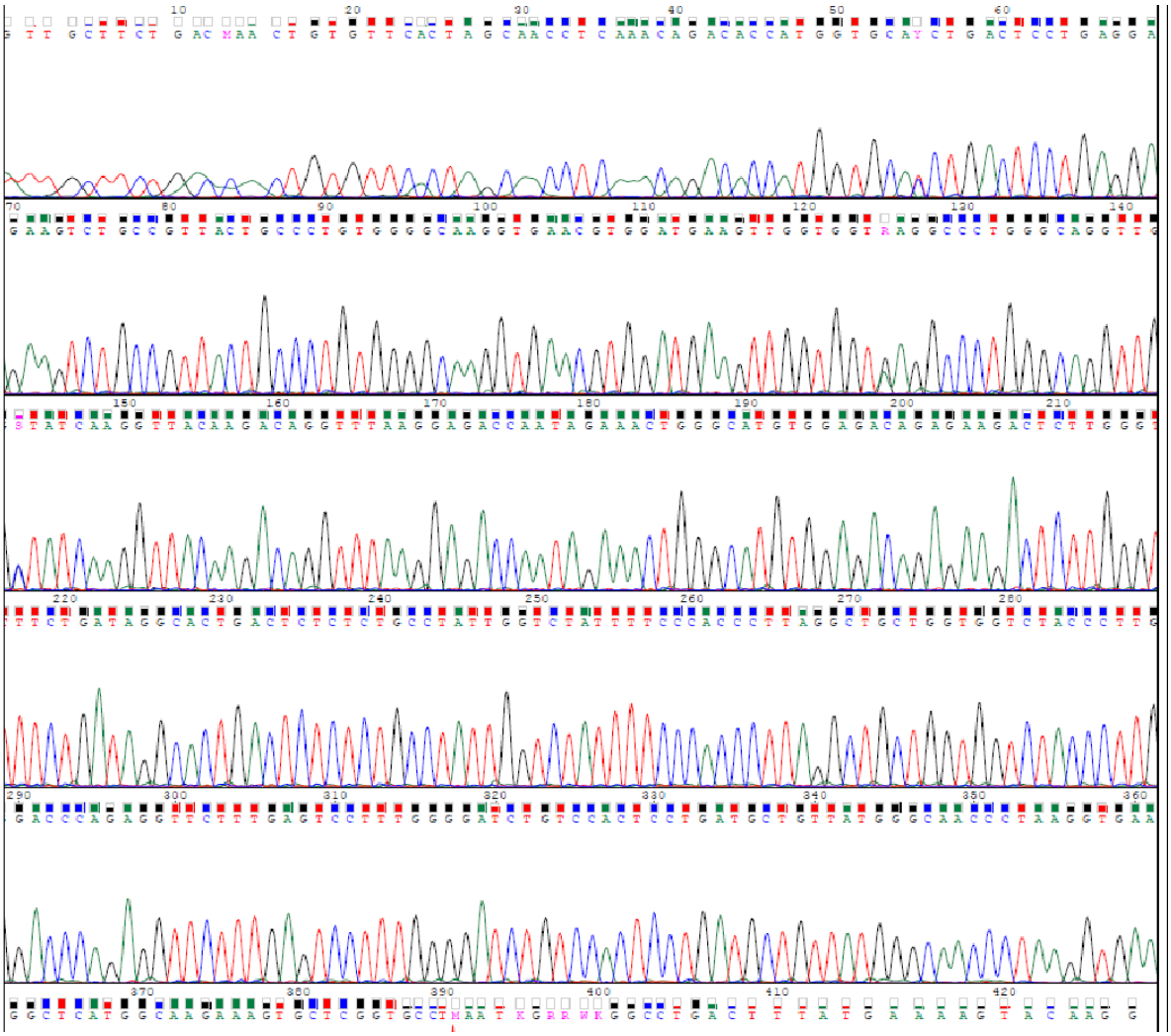

Figure 3.4: The chromatogram of patient 4 is showing 2-point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C

3.1.5 Patient 5

Patient 5 is a 10-year-old female child who was diagnosed as E-beta thalassemia at the age of 4.3 years. Her transfusion interval is after every 1 to 1.5 months. The patient also takes chelation therapy with blood transfusions. The patient shows no complications during her chelation therapy but the patient had done splenectomy before. The patient does not belong to any tribe and her parents also do not have any previous relationship. The genetic analysis of the patient's specimen shown 2-point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C

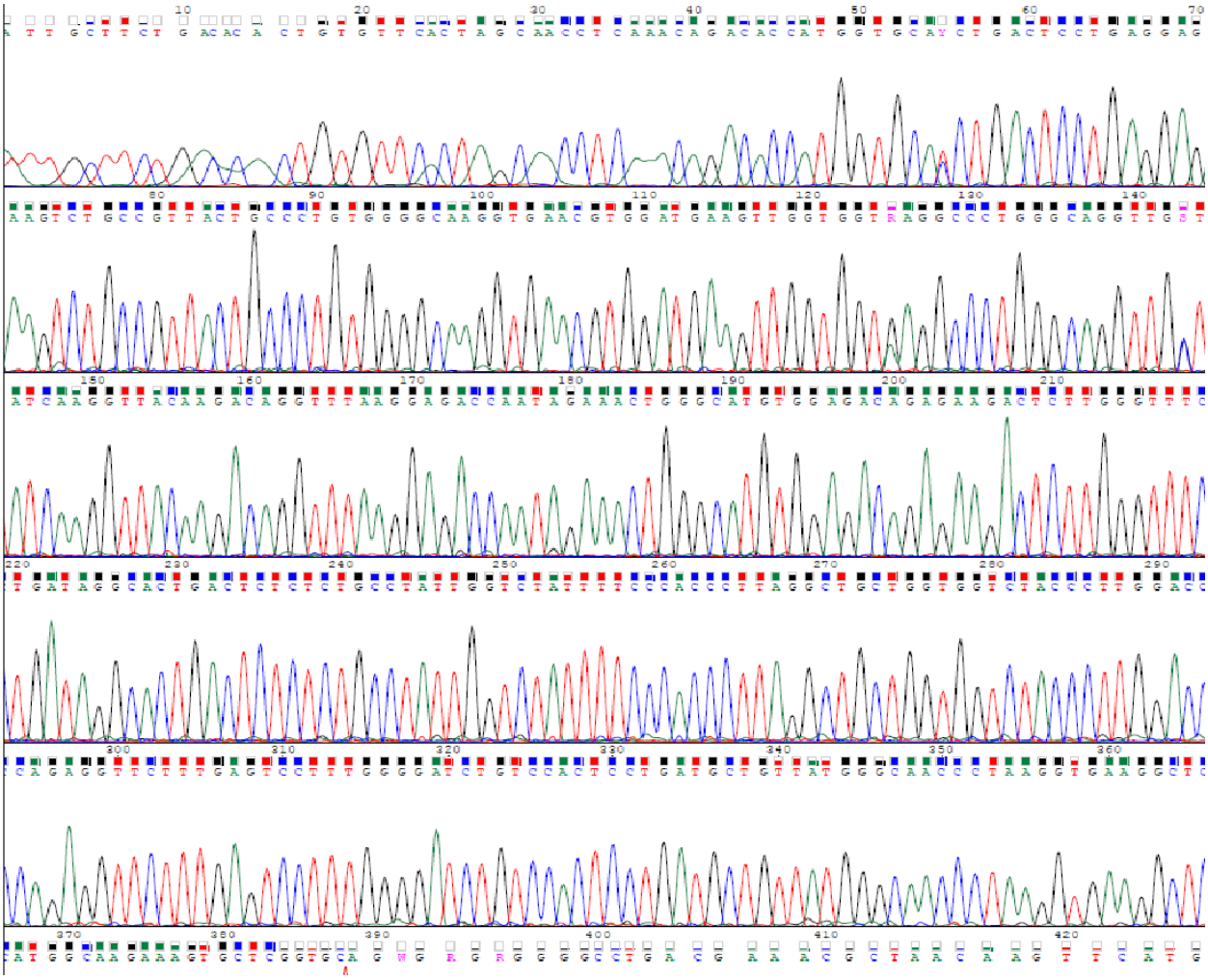

Figure 3.5: The chromatogram of patient 5 is showing 2-point mutation in compound heterozygous states: c.79G>A and IVS-1_5 G>C

3.1.6 Patient 6

Patient 6 was a male patient who was diagnosed as beta-thalassemia at the age of 3 months. The patient is now 10 years 2 months old and the patient needs blood transfusion with chelation therapy at every 1-month interval. The patient had splenectomy done before. The patient's parents are first cousins but they do not belong to any tribe. Genetic analysis of the patient specimen revealed homozygous mutation namely; IVS-1_5 G>C (homozygous)

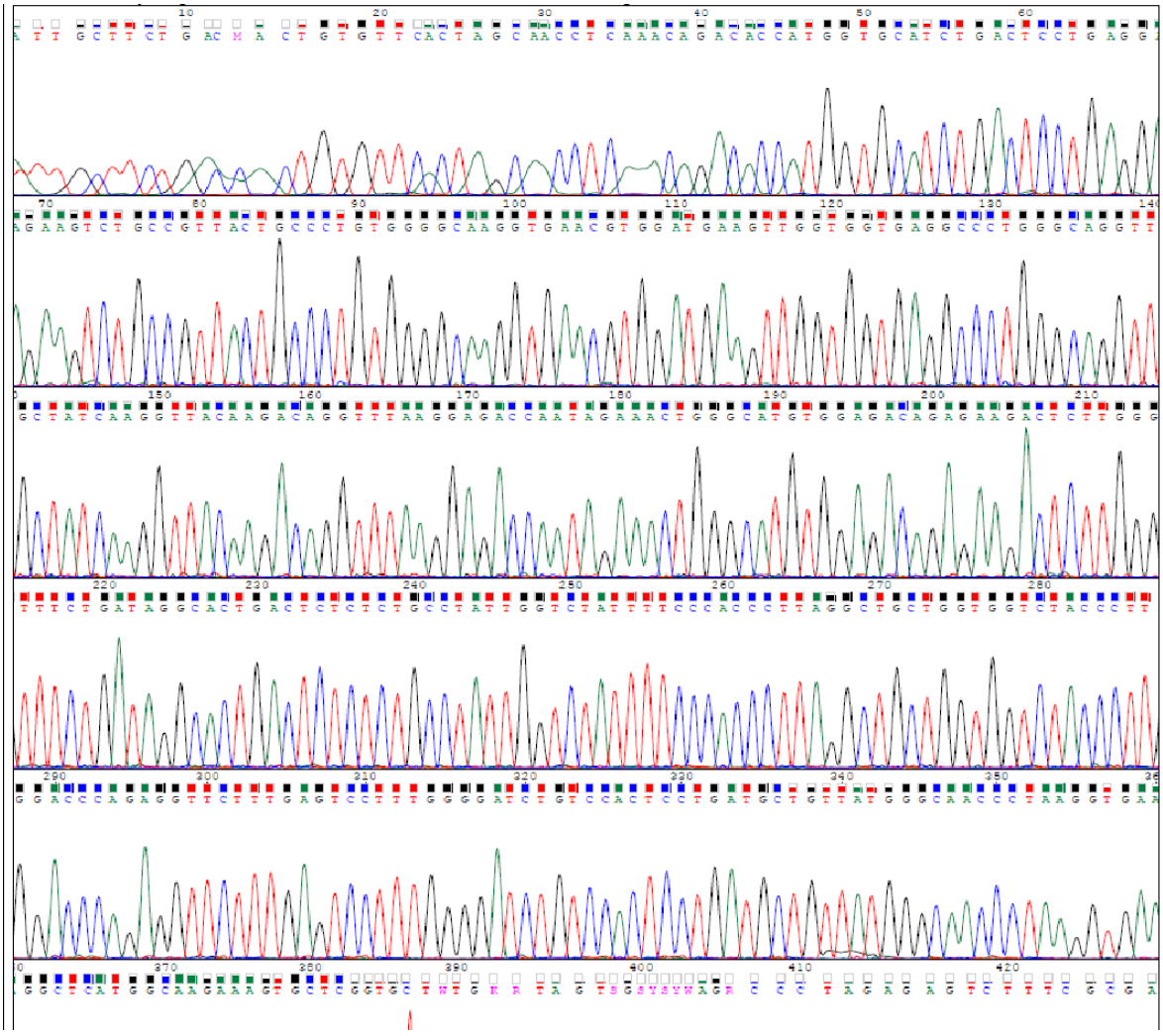

Figure 3.6: The chromatogram of patient 6 is showing homozygous mutation namely; IVS-1_5 G>C (homozygous)

3.1.7 Patient 7

Patient 7 is 2 years 1-month old male child who was diagnosed by E-beta thalassemia at the age of 1 year. The patient takes regular blood transfusion at 1 month 15 days' interval along with chelation therapy. The patient shows no complication during chelation therapy and the patient has splenomegaly. His family does not belong to any tribe and his parents have no previous relationship.

Genetic analysis of the patient's specimen revealed is 2-point mutation in compound heterozygous states- c.79G>A and IVS-1_5 G>C

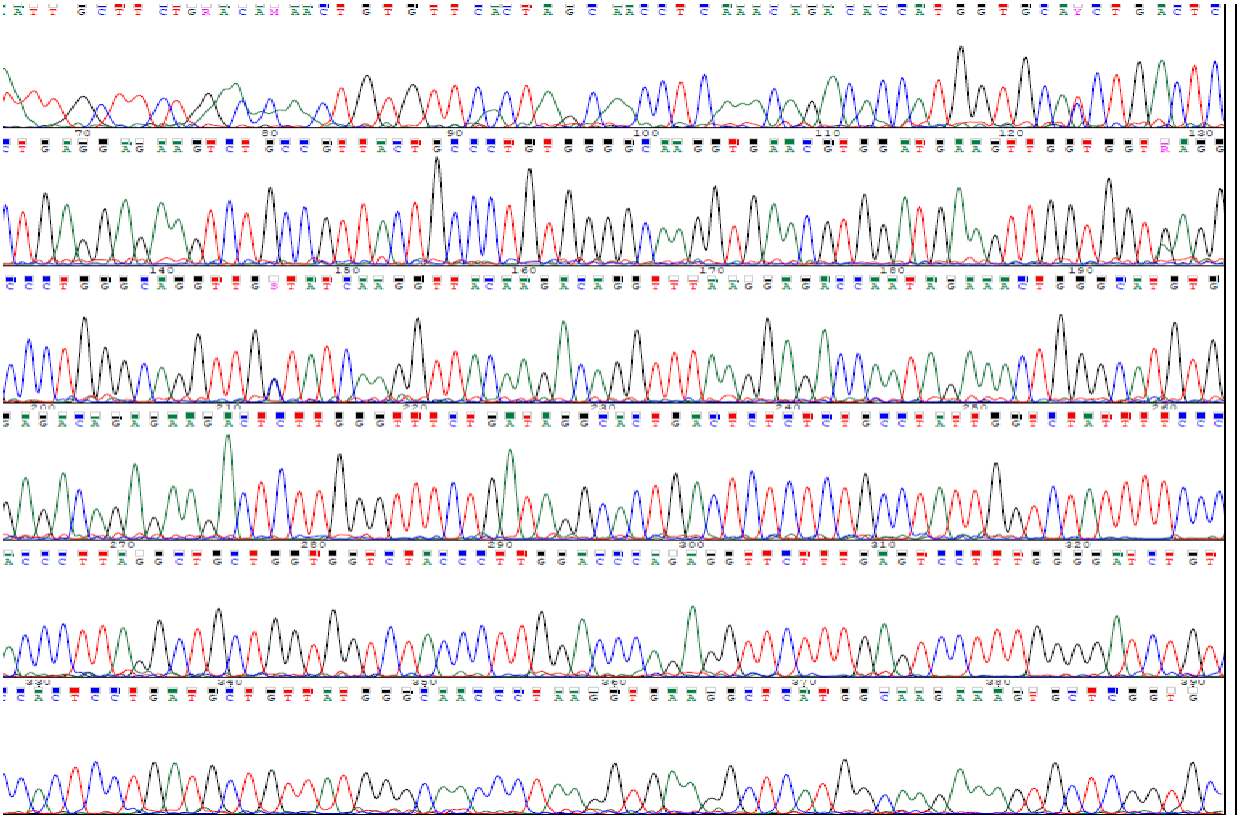

Figure 3.7: The chromatogram of patient 7 is showing 2-point mutation in compound heterozygous states- c.79G>A and IVS-1_5 G>C

3.1.8 Patient 8

Patient 8 is 18 months old, non-tribal male patient. His parents were not related to any blood relation. The patient was diagnosed as beta-thalassemia at the age of 3 months. The patient takes regular blood transfusion at every 1 and half month interval along with chelation therapy. The patient does not show any other complications during the transfusion and chelation therapy and did not develop splenomegaly till now. Genetic analysis of the patient's specimen revealed mutation in compound heterozygous states: IVS-1_5 G>C and c.126-129 and del_CTTT (codon 41/42) (heterozygous)

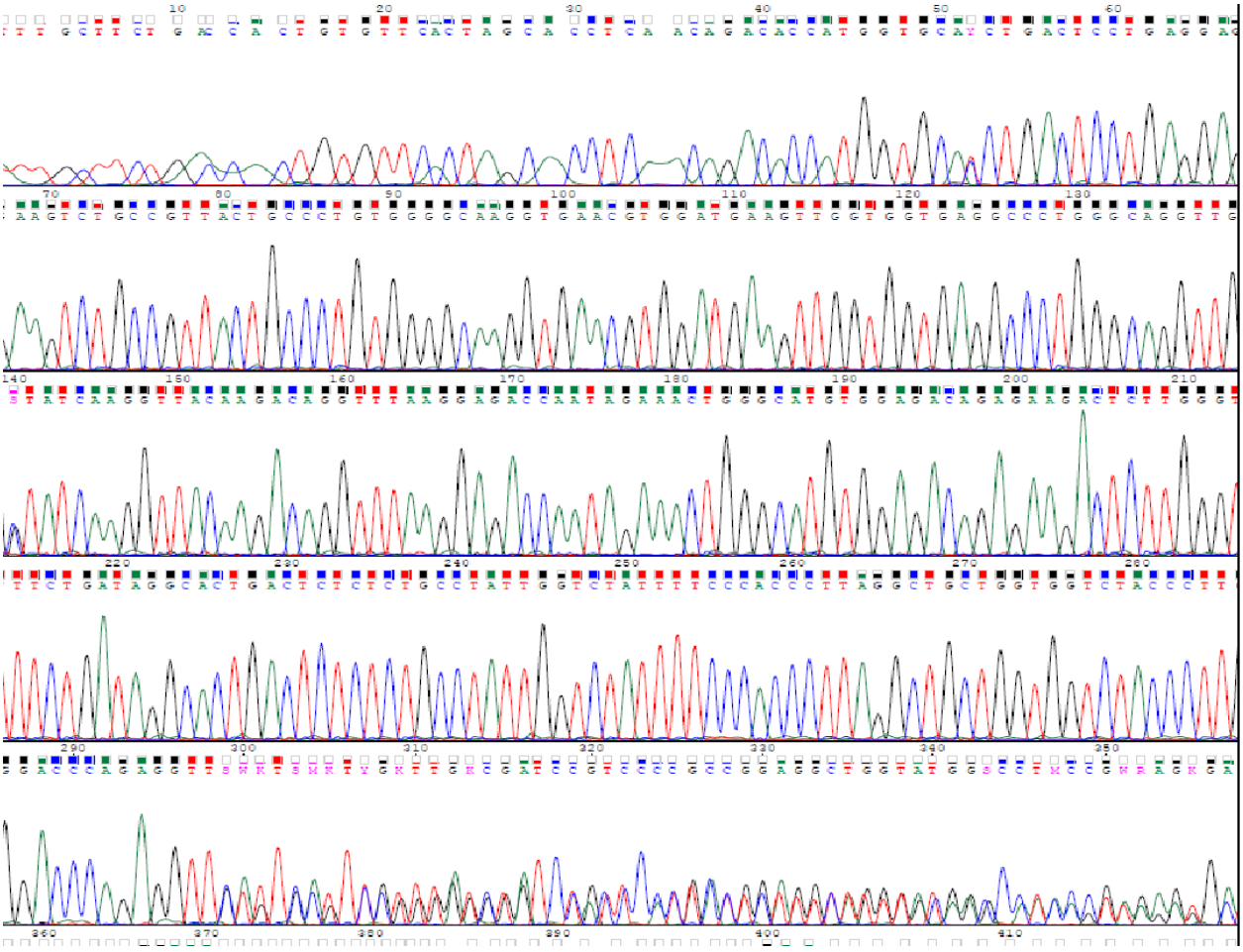

Figure 3.8: The chromatogram of patient 8 is showing 2 point mutation in compound heterozygous states- IVS-1_5 G>C and c.126-129 and del_CTTT (codon 41/42) (heterozygous)

3.1.9 Patient 9

Patient 9 is a 3 and half-year-old male patient whose parents do not have any previous relationship and the do not belong to any tribe. He was diagnosed as beta-thalassemia at the age of 1 year and now takes regular blood transfusion at every 1-month interval along with chelation therapy. The patient needs to take desferrioxamine during his chelation therapy and has splenomegaly or enlarged spleen. Genetic analysis of the patient's specimen revealed 2-point mutation in compound heterozygous states: IVS-1_5 G>C and IVS1_130 G>C

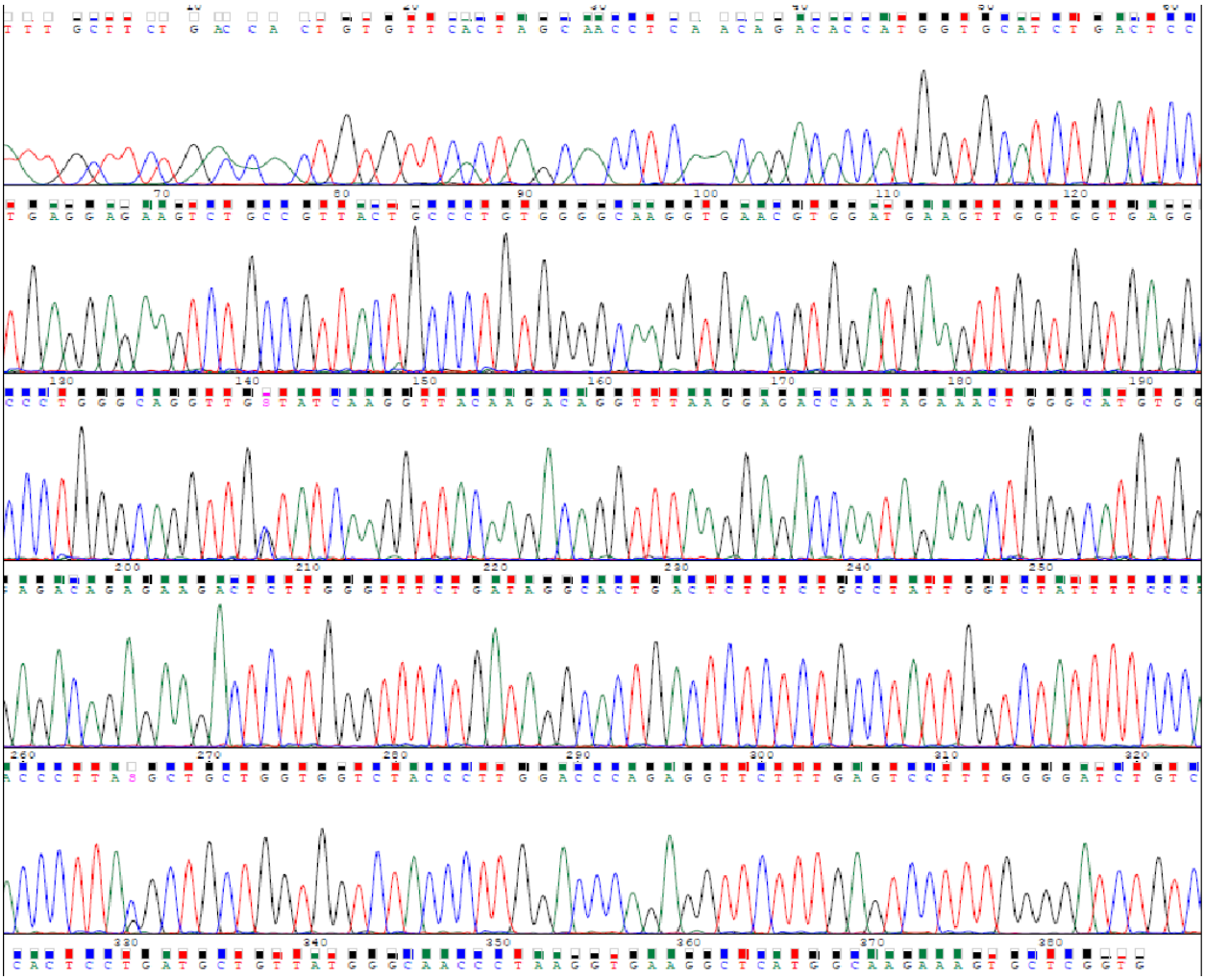

Figure 3.9: The chromatogram of patient 9 is showing 2-point mutation in compound heterozygous states: IVS-1_5 G>C and IVS1_130 G>C

3.1.10 Patient 10

Patient 10 is a female child who was diagnosed as beta-thalassemia at the age of 3 years old. Now the patient is 12 years old and needs to take regular blood transfusion and chelation therapy at every 22 to 23 days' interval. The patient needs to take hydronix medicine during chelation therapy and the patient has splenomegaly. The patient's parents are not related previously and the do not belong to any tribe. The genetic analysis of the collected specimen from the patient showed heterozygous mutation states: c.33C>A and IVS-1_5 G>C (heterozygous)

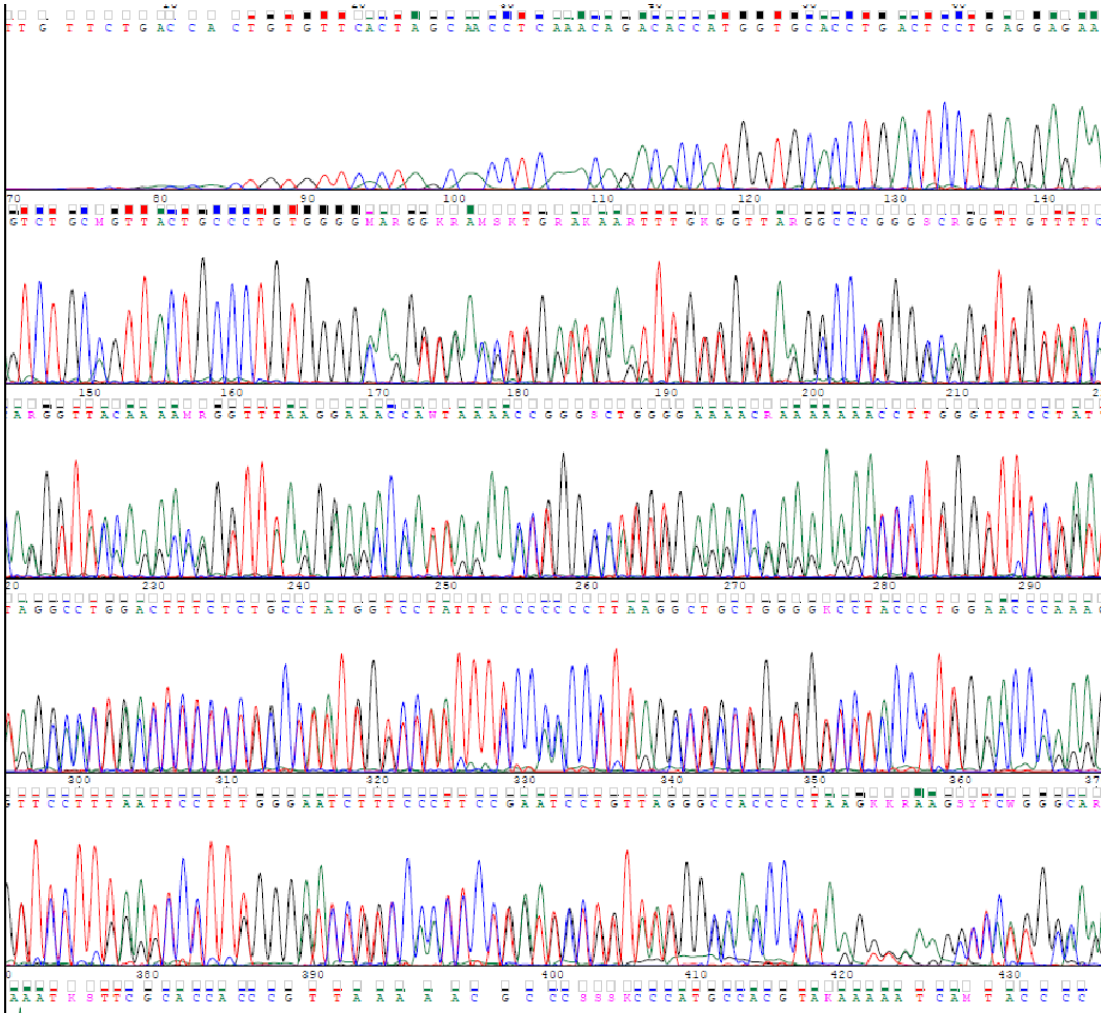

Figure 3.10: The chromatogram of patient 10 is showing heterozygous mutation states: c.33C>A and IVS-1_5 G>C (heterozygous)

3.1.11 Patient 11

Patient 11 is a female child who was diagnosed as E-beta thalassemia at the age of one and a half years. Now the patient is four years old and needs to take regular blood transfusion and chelation therapy at every 45 days' interval. The patient needs to take Deferasirox medicine during chelation therapy and the patient has splenomegaly. The patient's parents are first cousins but do not belong to any tribe. The genetic analysis of the collected specimen from the patient showed compound heterozygous mutation states: c.47G>A and c.79G>A

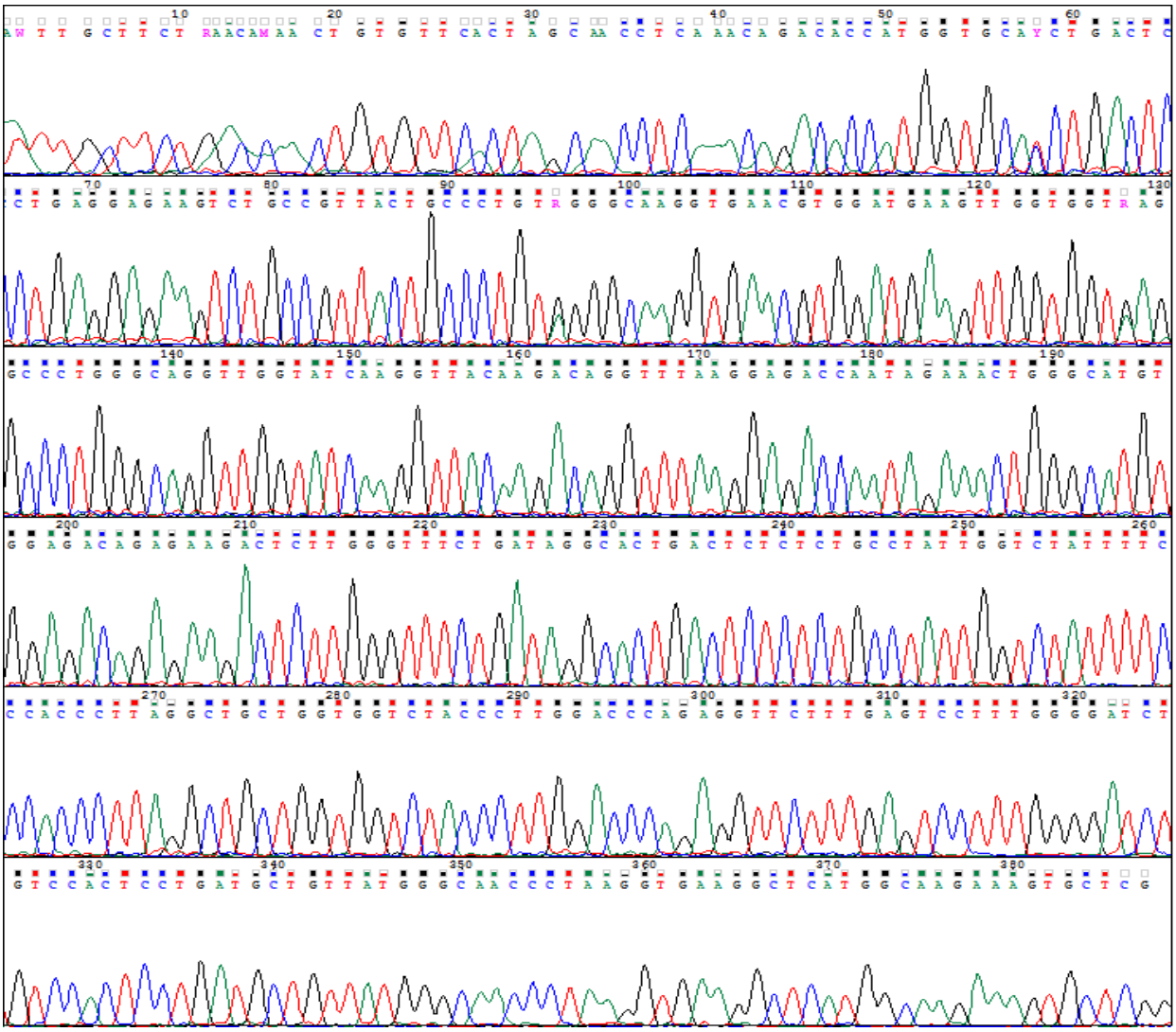

Figure 3.11: The chromatogram of patient 11 is showing compound heterozygous mutation states: c.47G>A and c.79G>A

3.1.12 Patient 12

Patient 12 is a 5-year-old male patient whose parents did not have any previous relationship and the do not belong to any tribe. He was diagnosed as E-beta thalassemia at the age of 2-year-old and now takes regular blood transfusion at every 1 and half month interval along with chelation therapy. The patient needs to take folison during his chelation therapy and has splenomegaly or enlarged spleen. Genetic analysis of the patient's specimen revealed point mutation in heterozygous states**:** c.79G>A, c.126-129del_CTTT (codon 41/42) (Heterozygous)

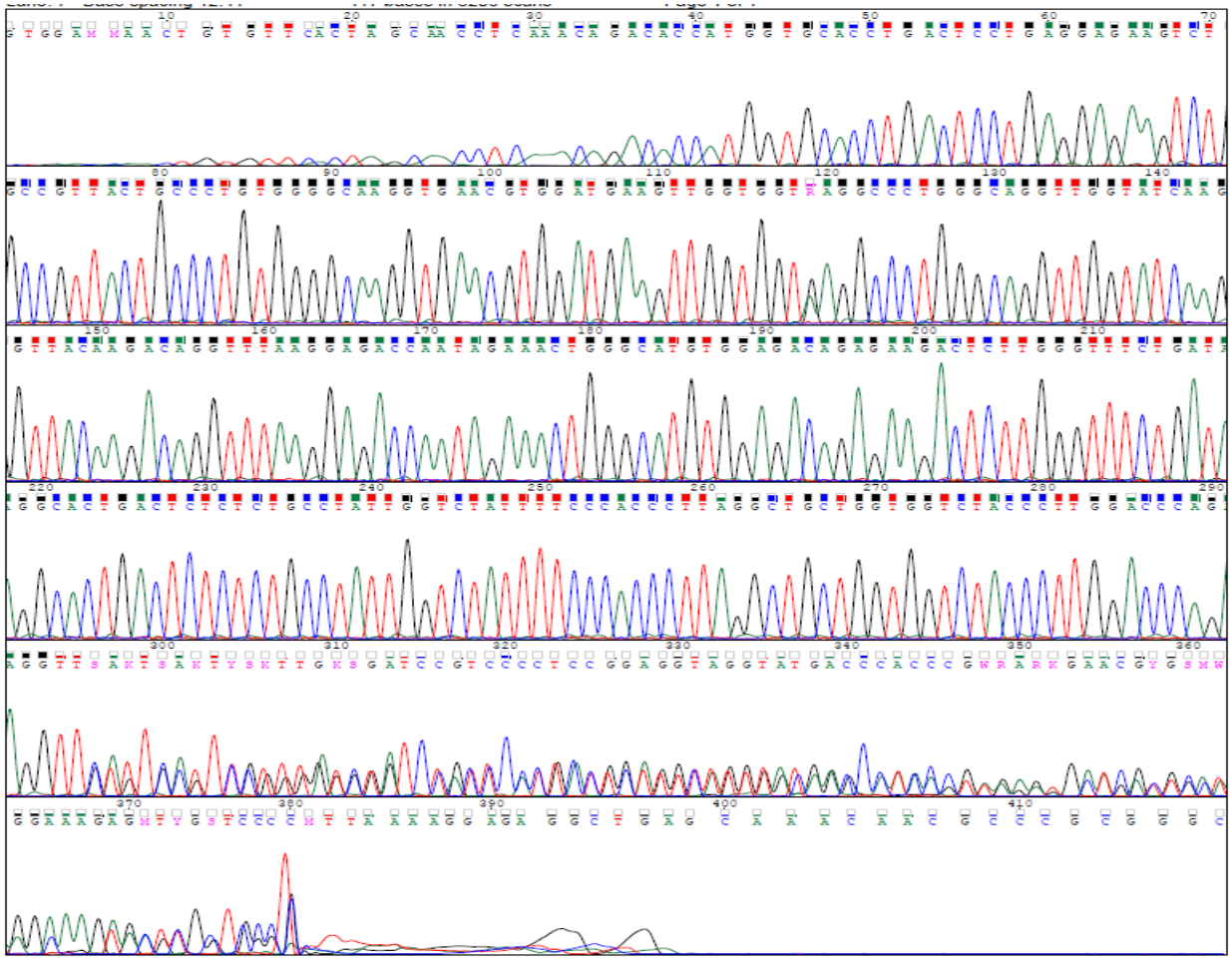

Figure 3.12: The chromatogram of patient 12 is showing point mutation in heterozygous states: c.79G>A and c.126-129del_CTTT (codon 41/42)

Table 3.1: The table shows the list of patients who manifested the disease at an early age

Patient ID	Patient type	HBB gene mutation	Gender	Age of first transfusion	Transfusion Interval	Other Complications	Hb Electrophoresis Result
1	Group-1: P1	c.79 G>A IVS-1_5 G>C	Male	10 m	2 m	NIL	EBT
2	Group-1: P2	IVS-1_5 G>C (Homozygous)	Female	3m	1m	Splenomegaly	BTM
3	Group-1: P3	c.79 G>A IVS-1_5 G>C	Male	2.5 y	1-1.5 m	NIL	EBT
4	Group-1: P4	c.79 G>A IVS-1_5 G>C	Female	2Y	2.5m	NIL	EBT
5	Group-1: P5	c.79 G>A IVS-1_5 G>C	Female	4.3y	1-1.5 m	Splenectomy	EBT
6	Group-1: P6	IVS-1_5 G>C	Male	3m	1m	Splenectomy	BTM
7	Group-1: P7	c.79 G>A IVS-1_5 G>C	Male	1y	1m 15days	Splenomegaly	EBT
8	Group-1: P8	IVS-1_5 G>C c.126-129 del_CTTT (codon 41/42)	Male	3m	1.5 m	NIL	BTM
9	Group-1: P9	IVS-1_5 G>C IVS1_130 G>C	Male	1y	1m	Splenomegaly	BTM
10	Group-1: P10	IVS-1_5 G>C c.33C/A (heterozygous)	Female	3y	3m(prev), now: 22-23 days	Splenomegaly	BTM
11	Group-1: P11	c.47G>A c.79 G>A	Female	1 and a half y	1.5 m	splenomegaly	EBT
12	Group-1: P12	IVS-1_5 G>C c.126-129 del_CTTT (codon 41/42) (heterozygous)	male	2y	1.5 m	splenomegaly	EBT

3.2 Group 2

3.2.1 Patient 1

Patient 1 in group 2 was diagnosed recently as E-beta thalassemia. The patient's age is above 18 years. Genetic analysis of the patient's specimen revealed 2-point mutation in compound heterozygous states: c.79 G>A and IVS-1_130 G>C

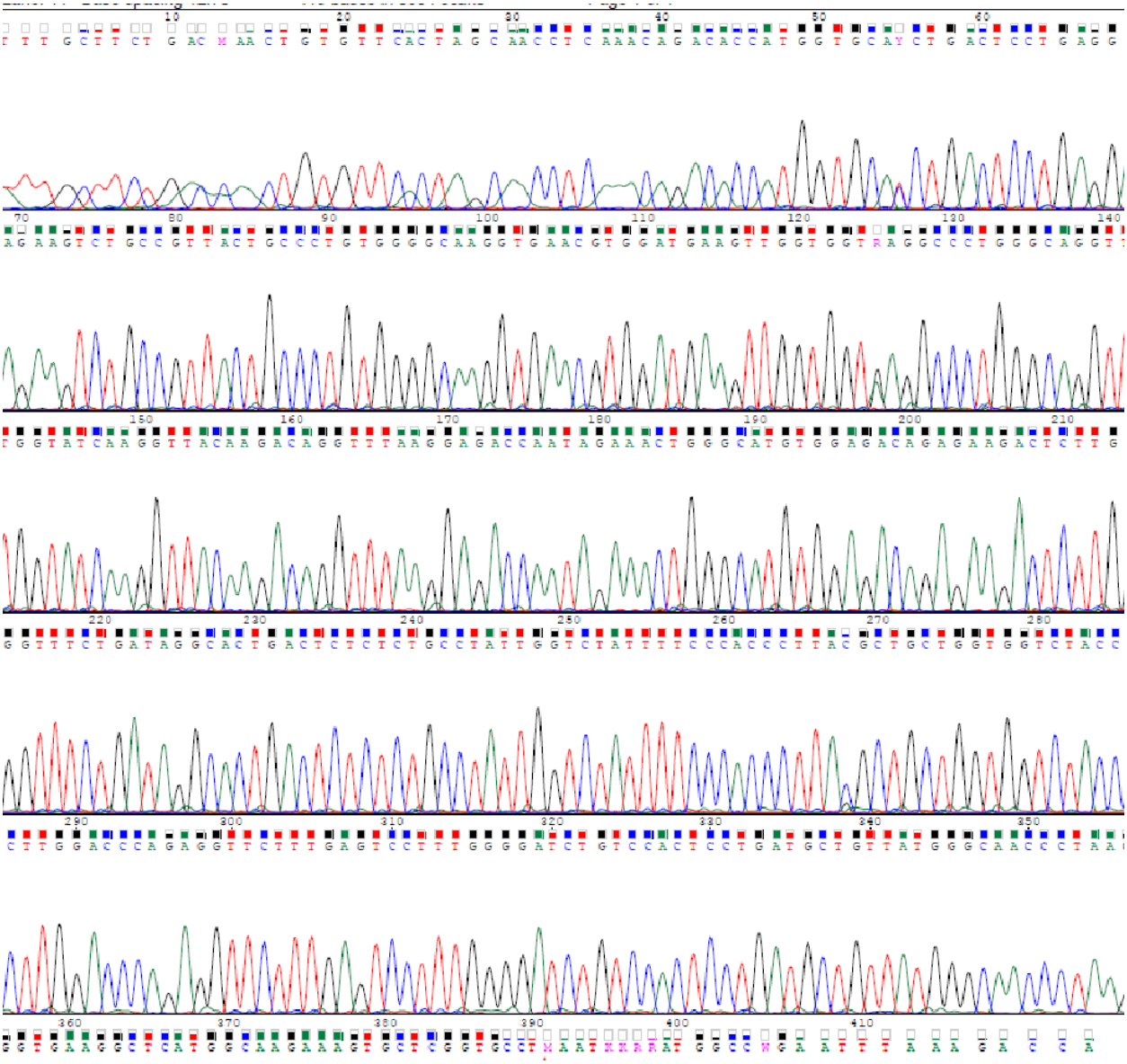

Figure 3.1: **The chromatogram of patient 1 is showing 2-point mutation in compound heterozygous states: c.79 G>A and IVS-1_130 G>C**

3.2.2 Patient 2

Patient 2 is an adult patient whose age is above 18 years and is diagnosed as E-beta thalassemia recently. Genetic analysis of the patient's specimen revealed 2-point mutation in compound heterozygous states: c.9 T>C, c.79 G>A, and IVS-1_5 G>C

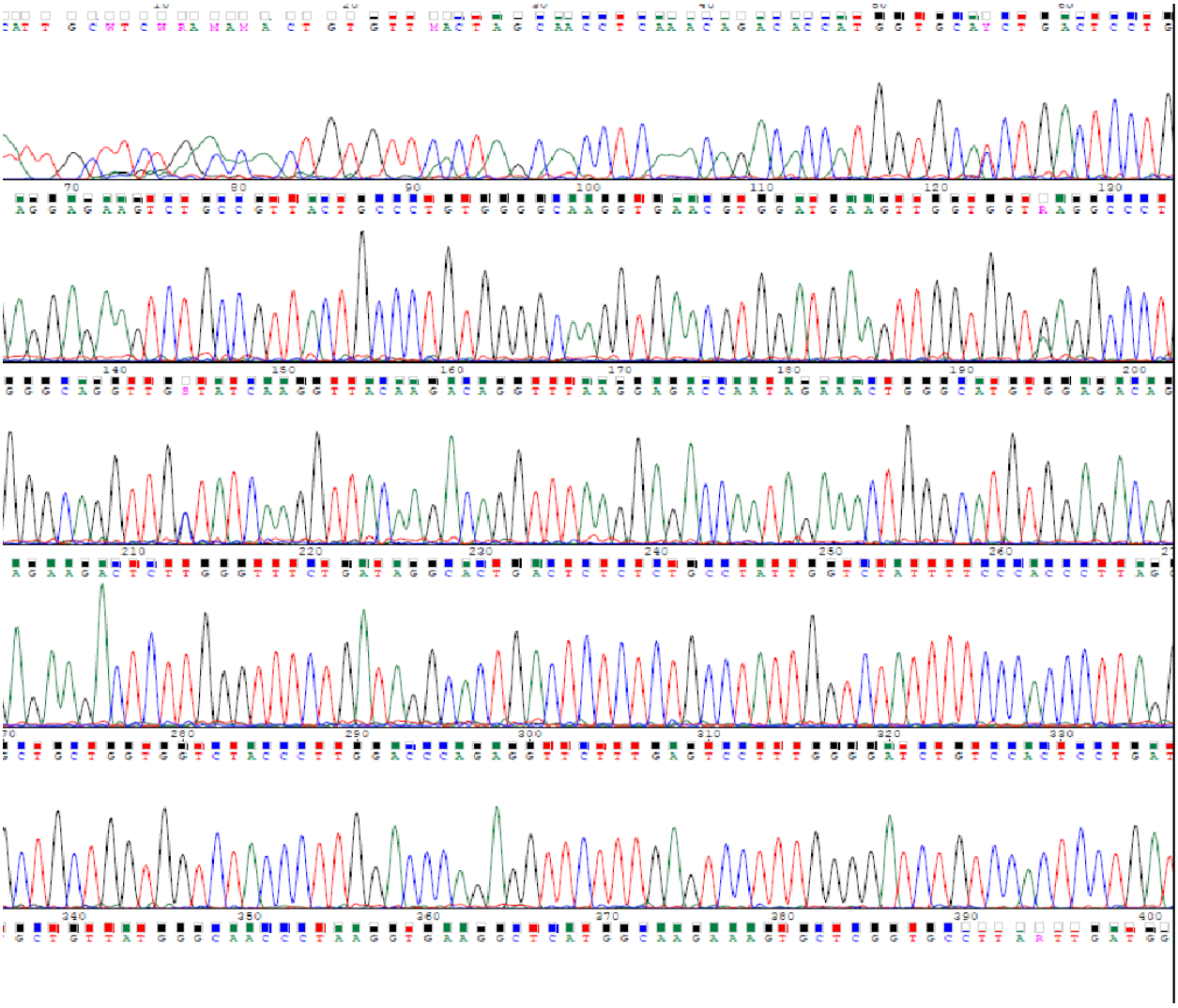

Figure 3.2: The chromatogram of patient 2 is showing 2-point mutation in compound heterozygous states: c.9 T>C, c.79 G>A, and IVS-1_5 G>C

3.2.3 Patient 3

Patient 3 in group 2 was diagnosed recently as E-beta thalassemia. The patient's age is above 18 years. Genetic analysis of the patient's specimen revealed mutation in compound heterozygous namely: c.79 G>A and c.126-129del_CTTT (codon 41/42) (heterozygous)

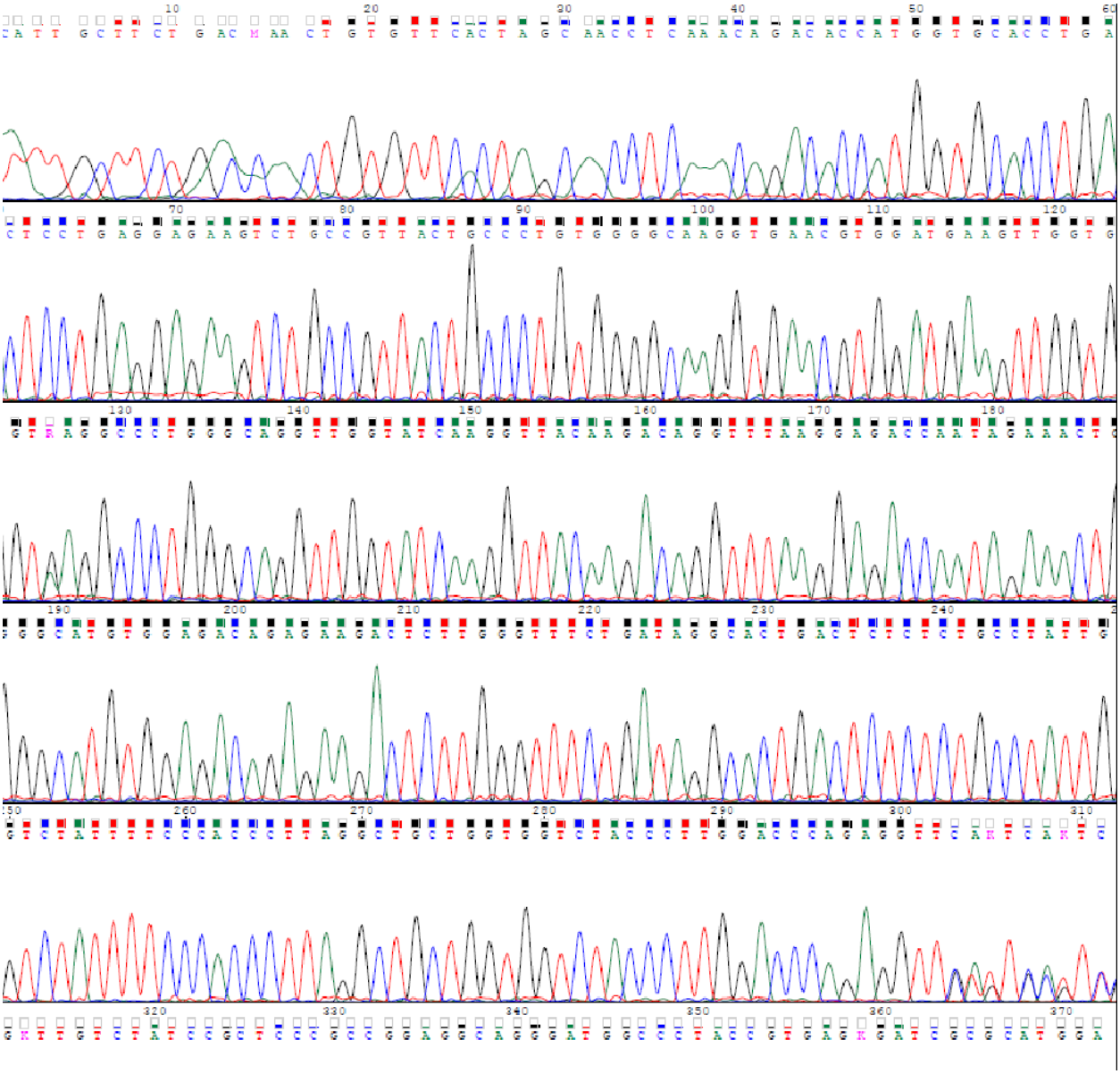

Figure 3.3: The chromatogram of patient 3 is showing mutation in compound heterozygous namely: c.79 G>A and c.126-129del_CTTT (codon 41/42) (heterozygous)

3.2.4 Patient 4

Patient 4 is an adult, recently diagnosed as E-beta thalassemia affected patient and the patient's age is 18+ years. Genetic analysis of the patient's specimen revealed 2-point mutation in compound heterozygous states: c.9 T>C, c.79 G>A, and IVS-1_5 G>C

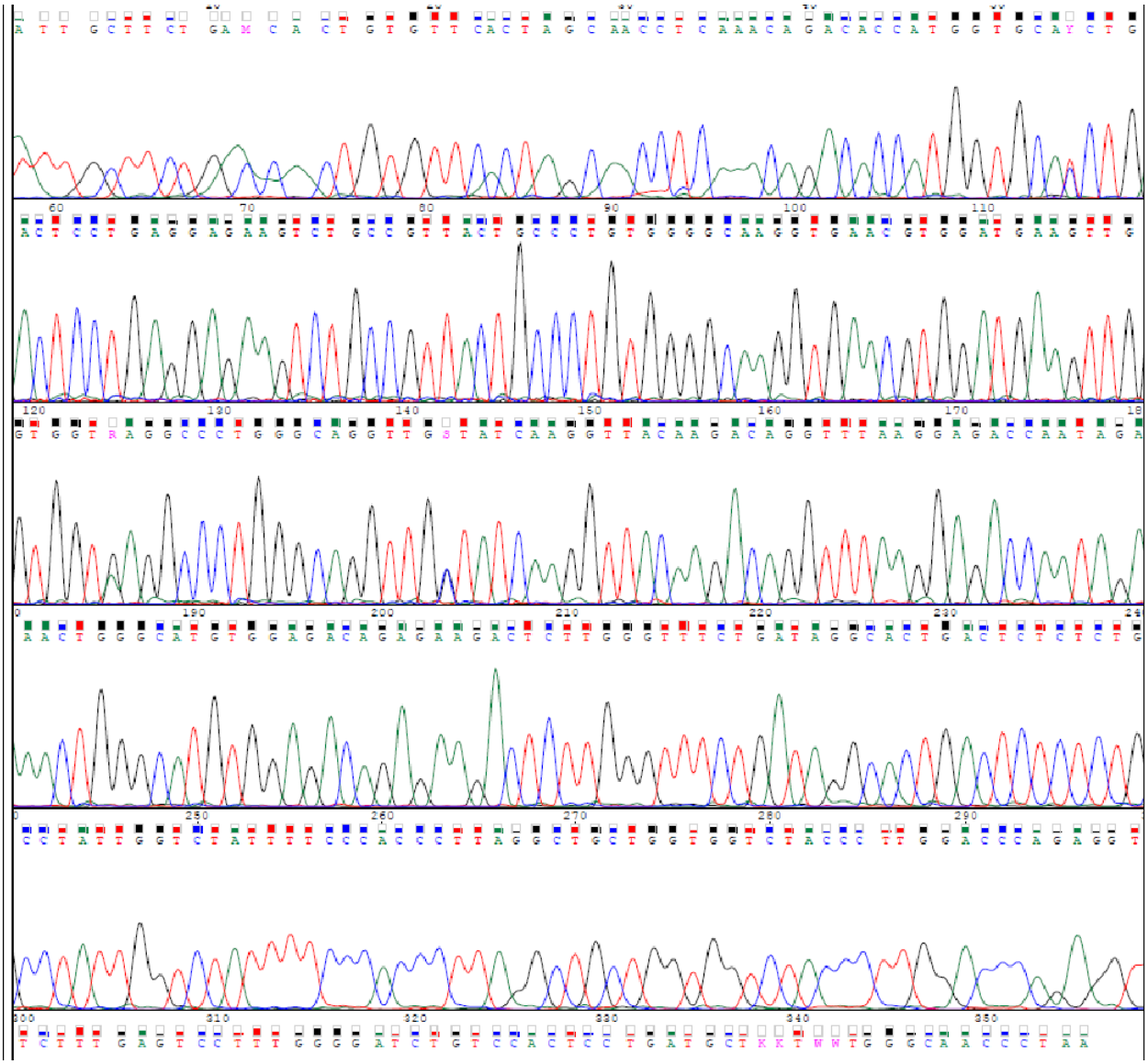

Figure 3.4: The chromatogram of patient 4 is showing mutation in compound heterozygous states: c.9 T>C, c.79 G>A, and IVS-1_5 G>C

3.2.5 Patient 5

Patient 5 recently established E-beta thalassemia and the patient's age is above 18 years. Genetic analysis of the patient's specimen revealed heterozygous mutation namely: c.79 G>A and c.126-129del_CTTT (codon 41/42) (heterozygous)

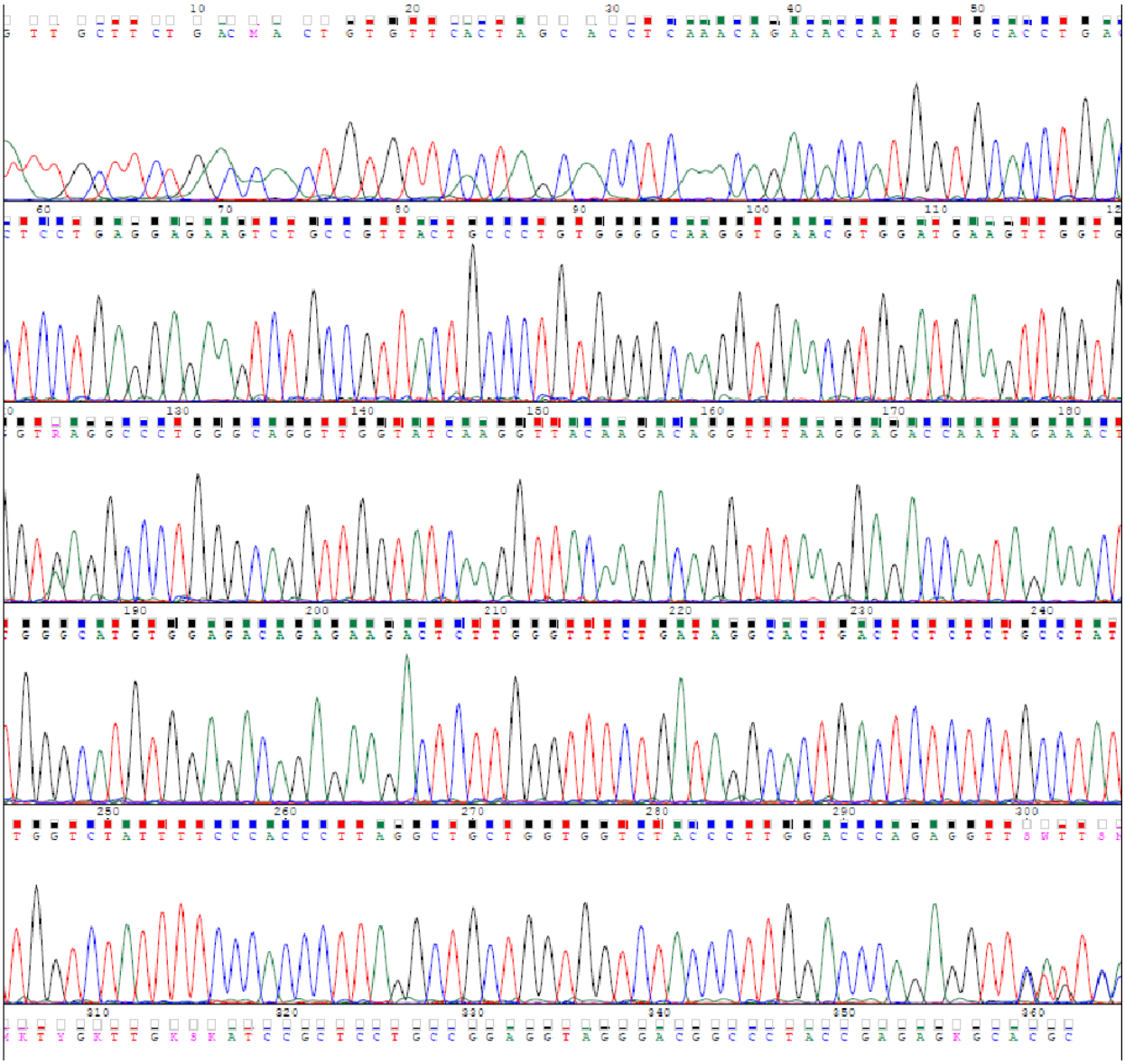

Figure 3.5: The chromatogram of patient 5 is showing revealed heterozygous mutation namely: c.79 G>A and c.126-129del_CTTT (codon 41/42) (heterozygous)

Table 3.2: List of patients who manifested the disease at adulthood.

Patient ID	**Patient type**	***HBB* Gene Mutation**	**Age of first transfusion**	**Hb Electrophoresis result**
1	Group 2: P1	c.9 T>C c.79 G>A IVS-1_130 G>C	18 years+	EBT
2	Group 2: P2	c.9 T>C c.79 G>A IVS-1_130 G>C	18 years+	EBT
3	Group 2: P3	c.79G>A c.126-129del_CTTT (codon 41/42) (heterozygous)	18 years+	EBT
4	Group 2: P4	c.9 T>C c.79 G>A IVS-1_130 G>C	18 years+	EBT
5	Group 2: P5	c.G79(A+G) c.126-129del_CTTT (codon 41/42) (Heterozygous)	18 years+	EBT

Table 3.3: Mutations in group-1 and group-2 patients

Patient type	HBB gene mutation	Type of Thalassemia	Age of first blood transfusion
Group-1: P1	c.79 G>A IVS-1_5 G>C	EBT	10 m
Group-1: P2	IVS-1_5 G>C (Homozygous)	BTM	3m
Group-1: P3	c.79 G>A IVS-1_5 G>C	EBT	2.5 y
Group-1: P4	c.79 G>A IVS-1_5 G>C	EBT	2Y
Group-1: P5	c.79 G>A IVS-1_5 G>C	EBT	4.3y
Group-1: P6	IVS-1_5 G>C (Homozygous)	BTM	3m
Group-1: P7	c.79 G>A IVS-1_5 G>C	EBT	1y
Group-1: P8	IVS-1_5 G>C c.126-129del_CTTT (codon 41/42) (Heterozygous)	BTM	3m
Group-1: P9	IVS-1_5 G>C IVS1_130 G>C	BTM	1y
Group-1: P10	IVS-1_5 G>C c.33C/A (Heterozygous)	BTM	3y
Group-1: P11	c.47 G>A c.79 G>A	EBT	1 and a half y
Group-1: P12	c.79 G>A c.126-129del_CTTT (codon 41/42) (Heterozygous)	EBT	2y
Group-2: P1	c.79 G>A IVS-1_130 G>C	EBT	18 years+
Group-2: P2	c.79 G>A IVS-1_130 G>C	EBT	18 years+
Group-2: P3	c.79 G>A c.126-129del_CTTT (codon 41/42) (Heterozygous)	EBT	18 years+
Group-2: P4	c.79 G>A IVS-1_130 G>C	EBT	18 years+
Group-2: P5	c.79 G>A, c.126-129del_CTTT (codon 41/42) (Heterozygous)	HbE-beta thalassemia	18 years+

All group 1 patients are below 18 years old and manifested the disease at an early age. Seven of the patients from group 1 were diagnosed as HbE-beta-thalassemia and five of the patients are diagnosed as beta-thalassemia major. The genetic analysis result of HbE-beta-thalassemia affected patient's specimens revealed mutation states: c.9 C>T, c.79 G>A, IVS-1_5 G>C, c.126-129del_CTTT (codon 41/42) (heterozygous) and IVS1_130 G>C.

The common mutation of HbE patients is c. 79 G>A. which makes the disease condition less severe than HBB as it acts as a backup for other mutations.

The genetic analysis of beta-thalassemia patient's specimen revealed mutation namely: IVS-1_5 G>C (homozygous) in two patients, IVS-1_5 G>C and c.126-129del_CTTT (codon 41/42) (heterozygous) in 1 patient and IVS-1_5 G>C and IVS1_130 G>C.

All the patients of group 2 were adult and all of them manifested the disease after 18 years' age. There were five patients in group 2 and all of them are diagnosed as HbE-beta-thalassemia. Genetic analysis of group 2 patient's specimen revealed mutation states: c.79 G>A, IVS-1_130 G>C and c.126-129del_CTTT (codon 41/42) (Heterozygous).

DISCUSSION
CHAPTER FOUR

Mutations or deletion of bases or defects in the synthesis of one or more of the hemoglobin chains causes an inherited hematologic disorder; thalassemia. Thalassemia affects men and women equally and occurs in approximately 4.4 of every 10,000 live births. Approximately 5 percent of the world's population has a globin variant, but only 1.7 percent has alpha or beta thalassemia trait (Rund & Rachmilewitz, 2005).

Between two major types of thalassemia, alpha thalassemia is caused by reduced or absent synthesis of alpha-globin chains, and beta-thalassemia is caused by reduced or absent synthesis of beta-globin chains. Imbalances of globin chains cause hemolysis and impair erythropoiesis.

Beta-thalassemia is one of the most common autosomal recessive disorders worldwide. High prevalence of beta-thalassemia is present in populations in the Mediterranean, Middle-East, Trans Caucasus, Central Asia, Indian subcontinent, and Far East (Weatherall & Clegg, 2001) (Cao & Galanello, 2010b). In our country's perspective, beta-thalassemia affected patient's rate is higher than alpha-thalassemia. This study focused on two groups of young and adult people who were diagnosed with beta-thalassemia.

Beta-thalassemia patients resulting in reduced Hb in red blood cells (RBC), decreased RBC production and anemia. Homozygotes for beta-thalassemia may develop either thalassemia major or thalassemia intermedia. Clinical presentation of thalassemia major occurs between 6 and 24 months. Affected infants face several problems like feeding problems, diarrhea, and irritability, recurrent bouts of fever, and progressive enlargement of the abdomen caused by spleen and liver enlargement. In some developing countries, where due to the lack of resources patients are untreated or poorly transfused, the clinical picture of thalassemia major is characterized by growth retardation, pallor, jaundice, poor musculature, genu valgum, hepatosplenomegaly, leg ulcers, development of masses from extramedullary hematopoiesis, and skeletal changes resulting from expansion of the bone marrow. Skeletal changes include deformities in the long bones of the legs and typical craniofacial changes (bossing of the skull, prominent malar eminence, depression of the bridge of the nose, tendency to a mongoloid slant of the eye, and hypertrophy of the maxillae, which tends to expose the upper teeth) (Galanello & Origa, 2010).

Hemoglobin E beta-thalassemia is the commonest form of severe thalassemia. Globally, the intermediate forms of beta-thalassemia do not cause a major public health problem (Toumba, Kanaris, Simamonian, & Skordis, 2008), except for the case of hemoglobin Hb E/beta-thalassemia

(Weatherall et al., 1985). Worldwide, Hb E/beta-thalassemia is one of the most important varieties of thalassemia (World Health Organization (WHO), 1983; Chen, 1996; Weatherall & Clegg, 2001b). The condition results from co-inheritance of a beta-thalassemia allele from one parent, and the structural variant hemoglobin E from the other (Weatherall, 1965). Haemoglobin E results from a G to A substitution in beta codon 26 which activates a cryptic splice site that causes abnormal messenger RNA processing (15-20%) as well as produces a structurally abnormal haemoglobin Hb E (75-80%), because the usual donor site has to compete with this new site, the level of normally spliced, that is Hb E, mRNA is slightly (20%) reduced (Orkin et al, 1982). The abnormally spliced mRNA is non-functional because a new stop codon is generated. As a result, hemoglobin E is synthesized at a reduced rate and behaves like a mild form of beta-thalassemia.

Genetic factors influencing the severity of this disorder include the type of beta-thalassemia mutation, the co-inheritance of alpha-thalassemia, and polymorphisms associated with increased production of fetal hemoglobin. Other factors, including a variable increase in serum erythropoietin in response to anemia, previous or ongoing infection with malaria, previous splenectomy, and other environmental influences, may be involved. The remarkable variation, and the instability, of the clinical phenotype of Hb E beta-thalassemia, suggests that careful tailoring of treatment is required for each patient, and that therapeutic approaches should be re-assessed over-time.

The present study aimed to analyze the mutational status of two groups of patients with beta-thalassemia, (a) the first group consists of patients with beta-thalassemia who were diagnosed as beta-thalassemia patients either with beta-thalassemia major or Hb E/beta-thalassemia in early life just within one to few years' age, and (b) the second group consist of beta-thalassemia patients who were diagnosed with Hb E/beta-thalassemia in adult life.

Both β-thalassemia major and Hb E/β-thalassemia can be transfusion dependent. Patients need to take regular transfusion along with chelation therapy at a specific interval. It has been recently reported that some patients diagnosed or established as Hb E/β-thalassemia are above 18 years old, whereas beta-thalassemia major and some Hb E/β-thalassemia affected patients shows the disease symptoms and manifest the disease at an early age after their birth. In this study a group of children who were previously diagnosed as beta-thalassemia major and Hb E/β-thalassemia; blood samples were taken from them for genetic analysis. Another group of adult people where all of them were

anemic patients; blood sample was taken from them and Hb electrophoresis was done and the result of Hb electrophoresis showed that all the five people from the adult group are affected as Hb E/β-thalassemia. The adult group of people did not show any signs or symptoms of thalassemia since their childhood except they developed anemia recently.

In this study all the patients of group 1 were children and their age were below 18 years old and group 2 patients were adult and their age was above 18 years. All the patients' sample specimen was collected and genetic analysis of the samples was done to detect the mutations in young and adult patients. Genetic analysis of young group patients showed that 5 children were diagnosed as beta-thalassemia major and 5 children were diagnosed as Hb E-beta thalassemia. The five beta-thalassemia major affected young groups patient's mutational status: IVS-1_5 G>C (homozygous) in two patients, IVS-1_5 G>C and c.126-129del_CTTT (codon 41/42) mutations were in compound heterozygous state in 1 patient, IVS-1_5 G>C and IVS1_130 G>C mutations were in compound heterozygous state in 1 patient, and c.33C>A and c.51delC mutations were in compound heterozygous state in 1 patient.

The mutation found in E-beta thalassemia affected group 1 patients are: c.79 G>A and IVS-1_5 G>C in five number of patients, c.47 G>A and c.79 G>A in one patient, c.79 G>A and c.126-129del_CTTT (codon 41/42) (Heterozygous) in two patients.

Genetic analysis of Hb E/beta-thalassemia affected adult group people's specimen were done and the revealed compound heterozygous mutational states in all patients including the following mutations: c.79 G>A and IVS-1_5 G>C in 2 patients, c.79 G>A and IVS-1_130 G>C in 1 patient, c.79 G>A and c.126-129del_CTTT (codon 41/42) in 2 adults.

The point mutation c.79 G>A could result in two phenomena: a) a normally spliced mRNA is produced that result in a one amino acid change in beta-globin chain which is glutamate to lysine in coding position 26 and b) alternative splicing which result in premature stop codon and consequently non-functional small unstable peptide is formed from alternatively spliced mRNA. Due to c.79 G>A mutation only 10-20% abnormally spliced mRNA is produced and the remaining 80-90% mRNA is normal. In normally spliced mRNA only 1 amino acid is changed and this does

not create any major functional changes in the beta-chain. But in alternatively spliced mRNA cannot produce any stable protein. Thus mild effect is observed due to c.79 G>A. mutation.

The point mutation IVS-1_5 G>C creates alternate splicing sites and no normal splicing happens or only 2%-5% normal splicing occurs and most of the splicing for this point mutation is abnormal. As most of the splicing part is abnormal; the effect of this mutation is severe.

The point mutation c.126-129del_CTTT (codon 41/42) results in the deletion of four bases and subsequently frameshifts occurs in the coding sequence and this phenomenon leads to abnormal unstable peptide production form the altered mRNA. However, the point mutation IVS1_130 G>C creates an alternate splicing site which results in a higher level of abnormally spliced mRNA. Only 7-10% normal splicing happens and the effect for this mutation is severe. Point mutation c.33C>A results in a change of 1 amino acid in the beta-globin chain which causes a severe effect on the patient. The mutation c.47G>A occurs in codon no. 15 and it translate tryptophan into a stop codon. This change of one nucleotide results in the termination of translation at codon 15. Altogether it appears that except the point mutation c.79 G>A all other point mutations found in this study have a severe impact on the beta-globin chain production. Thus the severe outcome in beta-thalassemia major could be easily explained by the inheritance of mutations that are damaging to the beta-globin chain.

In group 1 patients whose manifestation of disease is in early life they were either affected with BTM or EBT. However, the transfusion interval varies between the two groups. For BTM patients the transfusion interval was short (15-30 days) than the patients with EBT (30 days to several months). Implying that manifestation of EBT could be much more diverse than BTM. However, all the patients in group 2 who started to manifest the disease in adult life were affected by EBT. These huge diversities in EBT is really staggering and requires more study to understand it so that it could become possible to make better treatment and management strategies.

There are several genetic factors that have shown association with disease diversity in beta-thalassemia which includes Xmn I polymorphism, coinheritance of interacting α-thalassemia,

mutation or deletion in the beta-globin locus control region (HBB-LCR), heterozygous distribution of HbF among RBCs, etc.

However, in the Hb E/beta-thalassemia patients, the c.79G>A is a common mutation in both young (group 1) and adult (group 2) patients. All other mutations that are observed with the c.79G>A mutations have severe effects. Yet two different groups namely group 1(young group) and group 1 (adult group) manifesting the disease at a different stage of their life. This proves hemoglobin E (HbE)/b-thalassemia has a wide spectrum of clinical manifestations that cannot be explained purely by its genetic background only and requires consideration of metabolic factors as well. Several studies have proved changes in the metabolites and amino acid profiles in beta-thalassemia affected patients. Changes in the carnitine and acylcarnitine profile has been observed in beta-thalassemia affected patients. In a recent study, it has been observed that changes occur in plasma vesicles and their protein composition in EBT patients and that has shown association with different disease manifestation in EBT affected patients (Musharraf et al., 2017)

Significant alterations occur in the whole blood metabolome of patients with thalassemia (Panetsos, Livadara, Dimopoulou, Tzivaras, & Voskaridou, 2013). The role of the metabolic profile may differ in adult and young thalassemia patients. In adult patients; during their childhood the red blood cell count, hemoglobin level was normal. However, not all of their RBCs are normal. So abnormal RBCs undergo destruction and contribute to the iron level in serum or plasma. As the patients don't manifest disease they don't take chelation therapy. Thus iron level gradually increases than starts to contribute to organ damage such as liver, kidney, heart damage as well as contribute to oxidative stress. Thus gradually RBCs destruction increases which contribute more to organ damage. As these organs are associated with the synthesis of important molecules such as carnitine, several proteins, and other metabolites thus the metabolite profile becomes altered and contributes to disease manifestation. Consequently, they manifest disease at a later stage of their life.

Similar to metabolite profile plasma vesicles and protein composition becomes affected in those group 2 patients in the later stage of life. These vesicles and their protein are important to work against oxidative stress. As the plasma vesicle and proteins of the vesicles become affected and could not work against oxidative stress thus the patients could not live a normal life anymore starts to manifest the disease in adult life.

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