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Genetic Polymorphisms in β-Thalassemia Major in Misan

A thesis

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By

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Dedication

To..... the master of humanity, the messenger of mercy dedicated to our master Muhammad and his good and pure family and his faithful companions

To ……. my country with everything in it

To …… those who stayed up nights and drowned me with their kindness and tenderness and taught me honesty, patience, perseveranc, giving and loyalty my mother and father

To …… my support in my life my brothers and sisters For everyone who taught me

To …… my friends and colleagues and to everyone who stood with me, even with a glimmer of hope I dedicate the result of my effort

To …… my husband is more wonderful than the body of love with all its meanings, so the bond and giving gave me a lot in pictures of patience, hope and love. I will not say thank you, but I will always live thanks to you (Marwan)

To …… my children, the eyes from which I draw strength and continuity, the sweetest of my life (Hassan, Hayder).

To …… my friends (Israa,Ohood) who accompanied me and encouraged my steps when the days overwhelmed me. You have my love and gratitude

Zahraa

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I am pleased as I put the last fingerprints of my thesis to express my heartfelt thanks and gratitude to my esteemed Assis. Prof. Dr. **Maytham Abdul Kadhim Dragh**, who kindly supervised my thesis and suggested the subject of the thesis, and his continued encouragement to me in all stages of the research work, I wish him all success and continued .health, and may God reward him on my behalf

I also extend my heartfelt thanks to the esteemed Head of the department of Biology, my distinguished teachers, and to the Deanship of the College of Science, especially the esteemed Dean.

I extend my deep thanks and gratitude to my family, especially my father, mother, brothers and sisters, may God reward them with the best reward , praying to God to bless them with health, wellness and healing

Zahraa

Summary

Summary

The current study was conducted to know the genetic polymorphism of samples of people with thalassemia and their comparison with the genetic polymorphisms of healthy people and prediction of thalassemia severity.

The Blood samples are collected from the Center of Hereditary Hematology of the Maysan Health Directorate/ AL-Sader Teaching Hospital/ in Maysan province, Southern of Iraq, November 2021. The study group consisted of 140 samples, 100 samples are patients with thalassemia and 40 samples are a control group with no family history of thalassemia. A blood smear and complete blood count are performed on patient and control samples. Whole blood DNA is extracted to study genetic polymorphism in thalassemia. The genes studied in this experiment are *BCL11A* and *HBB* genes:

The results of *BCL11A* gene show the presence of two alleles A and C as well as three genotypes AA, AC, and CC in β-thalassemia. ARMS-PCR using with frequencies 0.3, 0.6, 0.1, respectively, and in control 0.1, 0.8, 0.1, respectively. The frequency of the two alleles A and C in patients A (0.56), C (0.44). As for the control A (0.52), C (0.48), no significant differences are found between the genotype distribution of β- thalassemia major patients and controls under the $p<0.05$ probability level whereas it reaches P-value (0.220).

Also PCR-RAPD-PCR is used, four primers (APAA 11, APU15, APAA17, and APD18) to detect genetic polymorphism of *BCL11A* gene. The results show that the number of bands in β-thalassemia major patients are more than the control group, there is also a difference in their molecular weights according to the type of primer used. The results of the current study for the unique, monomorphic and polymorphism bands of *BCL11A* gene in patient samples show that the total bands are 10, 17 and 6 bands respectively. In the control group, it is 13, 13 and 5 respectively.

Noting that *HBB* primer gives bands in patients and does not give bands in controls. In other words, the result of PCR amplification is positive in patients and negative in controls. This result can be used as a method for diagnosing thalassemia.

Finally, nucleotides sequencing was done for the two genes. Using bioinformatics programs, nucleotide sequencing was analyzed. The National Center for Biotechnology Information or what is known as NCBI Blast was used to compare our sequences with the standard sequences published in this center.

Nucleotide sequencing analysis showed 2 SNPs mutations in the *BCL11A* gene and 21 mutations in *HBB* gene.

The BCL11A gene polymorphism rs766432A > C is found in all patients at the (A65664C) site, according to the sequencing results.

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List of Abbreviations

No.	Title
%	Percent
+	Positive
<	Less than
>	Greater than
3 D	Three Dimension
A	Adenine
AA	Amino acid
ARMS	Amplification refractory mutation system
BCL11A	β-cell lymphoma/leukemia 11A
BFU-E	Brust - forming unit erythroid
BM	Bone marrow
bp	base pair
C	Cytosine
Cas9	CRISPR associated protein 9
CFU-E	Colony- forming unit erythroid
CIs	Confidence intervals
CRISPR	Clustered regularly interspaced short palindromic repeats
ddNTPs	Dideoxynucleotide phosphate(ddNTPs)
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EPO	Erythropoietin
Et al.	et alia (and others)
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal

gm	gram
gr/dL	Grams per deciliter
H.W.E	Hardy Weinberg Equilibrium
Hb	Hemoglobin
Hb F	Hemoglobin fetal
HbA	Hemoglobin adult
HBB	Hemoglobin beta
HSCT	Hematopoietic stem cell transplantation
IE	Ineffective erythropoiesis
Indel	Insertion / Deletion
KLF1	Krueppel-like factor 1
LCR	Locus control region
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
miRNA	Micro RNA
ml	milliliter
MPV	Mean platelet volume
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
ng	Nanogram
NMD	Nonsense-mediated RNA decay
No.	Number
^{o}C	Degree Celsius
OR	Odds ratio
P	Probare (P-value)
PCR	Polymerase chain reaction
PRP	PCR-RAPD-PCR

PTCs	Premature stop codons
QTL	Quantitative trait loci
RAPD	Random Amplified Polymorphic DNA
RBCs	Red Blood cells
RNA	Ribonucleic acid
Sec	Second
SNPs	Single nucleotide polymorphisms
T	Thymine
TBE	Tris Borate EDTA
U	Uracil
UTR	Un-translated region
UTR	Untranslated region
UV	Ultraviolet
X_2	Chi Square
α	Alpha
β	Beta
β^+	Beta plus
β^o	Beta zero
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
θ	Theta
μg	Microgram
μl	Microliter
ψ	Psi

Introduction

Introduction

Thalassemia is an autosomal recessive disorder. It only occurs when both parents are either carriers of this disorder or are impacted by it. So that, they can transmit it on to the following generation (Aksu and Ünal, 2021).

Around the world, 0.3–0.4 million children are thought to be born each year with hemoglobin abnormalities (Amjad *et al*., 2020). There are 60,000 symptomatic persons born each year and 80–90 million people worldwide 1.5% are β-thalassemia carriers. Additionally, it has been calculated that the overall annual incidence of people with symptoms is 1 in 10,000 in the European Union and 1 in 100,000 worldwide. The effects of β-thalassemia are equal for both female and male (Hossain *et al*., 2017).

The classifications of thalassemia include β, α, δγ, δβ, as well as γδβ, according to the affected globin chain. Two major types of thalassemia are α- and β-thalassemia, and their occurrence are determined by four and two genes respectively (Sirachainan *et al*., 2016).

The β-thalassemia results from base substitutions on the β-globin genes, whereas the α-thalassemia results from deletions that remove α-gene. (Stauder *et al*., 2018). It is further classification including to absent (β^0) or decreased (β^+) globin chain synthesis which result microcytic and hypochromic anemia like a wide range of syndromic forms (Lei *et al*., 2019).

There are three types of β-thalassemia: β-thalassemia major, β-thalassemia intermedia and β-thalassemia minor (Ali *et al*., 2021).

Based on the history of the family, phenotype, and pertinent laboratory screening test results, thalassemia/hemoglobinopathy is initially suspected with anemic patients. The diagnosis is established by molecular genetic validation through the search for pathogenic mutations. In the past, the mutations were

missed by conventional sequence analyses and genetic heterogeneity of thalassemia hindered molecular genetic diagnosis. However, the development of novel molecular genetic technologies allowed for a more accurate molecular diagnosis of hereditary hemolytic anemia and a better comprehension for the genetic and genomic mechanisms underlying the disease (Ferrão *et al*., 2017; Russo *et al*., 2018).

It is commonly acknowledged that higher fetal hemoglobin (HbF) synthesis and increased gamma (γ) -globin gene expression are linked to a less severe or even asymptomatic condition in β-thalassemia patients. The Patients with β-thalassemia who express high levels of HbF frequently do not require chelation therapy or a transfusion regimen (Breveglieri *et al*., 2017). This widely known observation has motivated researchers to create effective Hb fetal (HbF) inducers for treating individuals with β-thalassemia who exhibit low quantities of HbF (Finotti *et al*., 2015).

Increased level of Hb fetal is associated with polymorphism of some genes like the *BCL11A* (Bianchi *et al*., 2016).

Genetic polymorphism is a variation in deoxyribonucleic acid (DNA) sequence that occurs more frequently than 1 % in the general population (Funnell, 2012). Polymorphism may be has related with a single nucleotide change, known as SNP (a single-nucleotide polymorphism), or with variation in a number of repetitive DNA sequences, like microsatellites or minisatellites, called length polymorphism. In most cases, genetic polymorphism may act as a predisposing factor rather than directly causing them mutation is a permanent change to a gene's DNA sequence that occurs in less than 1% of the population and almost certainly results in disease. Mutations existing in a coding sequence, at the exon-intron boundaries of a gene, or in the regulatory elements, may impact transcription and / or translation and may generate in alteration of the protein structure and function. (Somberg, 2002).

Despite the relatively high rate of the β-thalassemia gene carriers, there is no preventive program in Iraq (Hassan *et al*., 2003; Al-Allawi and Al-Dousky, 2010).

1-1- Aims of the Study

According to the previously mentioned information about the genetic polymorphism of genes that related to β-thalassemia, the present study attempts to illuminate the following axes:

1. Identification of *BCL11A* and *HBB* gene which are related to β-thalassemia.
2. Dedication of genetic polymorphisms in the identified genes.
3. Use a modern molecular techniques in the diagnosis of beta thalassemia

Chapter Two

Literature Review

2- Literature Review

2-1- Erythropoiesis in Humans

Red blood cells (RBCs), the most prevalent form of cell in the human body, can be divided into two categories: primitive and definitive as shown in figure 2-1. During fetal and postnatal life, definitive RBCs circulate as anucleate cells, smaller, whereas primitive RBCs circulate briefly in the early embryo as big, nucleated cells before ultimately enucleating (Palis, 2014).

Both cell types develop from lineage-committed progenitors, which produce a succession of morphologically distinct precursors that enucleate to give rise to mature RBCs. While definitive erythroid precursors matured extravascular in the postnatal marrow and fetal liver in relation with macrophage cells and primitive erythroid precursors (Minetti *et al*., 2020).

2-2- Definitive Erythropoiesis

Definitive Erythropoiesis is distinguished by the transition of lineage-committed cells through progenitor-precursor-mature RBC compartments and occur in the fetal liver and postnatal BM (bone marrow), the progenitor and precursor Compartments happen in safe extravascular spaces and are related to cellular amplification and maturation. The RBCs that circulate within the vascular network maintain the third functional compartment (Moras *et al*., 2017).

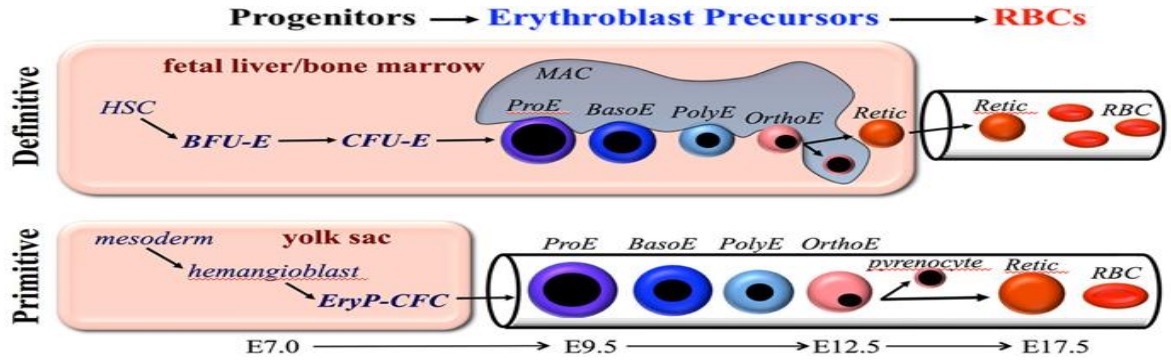


Figure 2-1: The changes in human globin chains synthesis (Palis, 2014).

2-2-1- Definitive Erythroid Progenitors

Burst-forming unit erythroid (BFU-E) and Colony-forming unit erythroid (CFU-E), which are lineage-committed definite erythroid progenitors, make up the most immature erythroid compartment. In human systems, BFU-E-derived colonies need 14 days to develop into mature colonies, which typically comprise more than a thousand erythroid cells. In comparison, the more developed CFU-E progenitors only need seven days in the human body to create mature colonies with just 16–32 cells. Therefore, CFU-E are just 4-5 cell divisions preceding mature RBC (Valent *et al*., 2018).

The ability of CFU-E to produce colonies in vitro depends on the cytokine erythropoietin (EPO). EPO is very necessary for the survival of CFU-E (Weiss *et al*., 2020).

Hypoxia is considered to control EPO levels in the blood, which affect the quantity of CFU-E and hence control the production of definitive RBCs (Weiss *et al*., 2020).

Recent research has shown that BFU-E and CFU-E have a limited capacity for self-renewal, particularly in response to acute stressors like anemia, and that CFU-capacity for self-renewal is reliant on the activity of EPO (Dulmovits *et al*., 2017).

2-2-2- Definitive Erythroid Precursors

The second erythroid compartment is made up of morphologically distinct, nucleated precursors that develop from proerythroblast to basophilic, polychromatophilic, and orthochromatic forms (Wu *et al*., 2021).

Erythroid precursor maturation is distinguished by progressive erythroblast expansion through a limited set of symmetric cell divisions, accumulation of hemoglobin, and decrease in cell size, nuclear pyknosis, and decrease in RNA

content, are where definitive erythroblasts mature in the fetal liver as well as postnatal BM (Liggett and Sankaran, 2020).

Reticulocyte maturation is a complicated process that results in the loss of all remaining cytoplasmic organelles, such as mitochondria and ribosomes, as well as a 20 percent reduction in plasma membrane surface area, a reduction in cell volume, a stronger association of the cytoskeleton with the outer plasma membrane, and a reduction in cell volume ,organelle clearance happens through both autophagy and exocytosis (Griffiths *et al*., 2012).

A multilobulated immature reticulocyte undergoes membrane modifications to become a biconcave disc with enhanced viscoelasticity. All of these modifications get the reticulocyte ready for its demanding 120-day stay in adult humans' blood. In adults, this steady-state production causes the bloodstream to receive more than 2 million reticulocytes every second (Franco, 2012). As shown in figure 2-2.

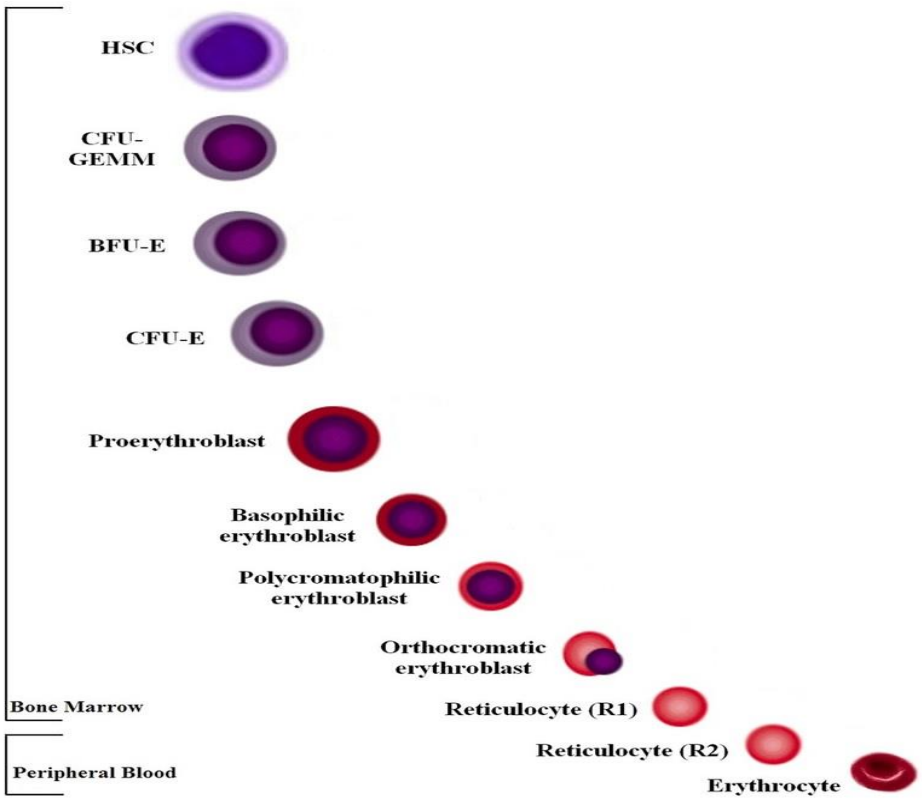


Figure 2-2: The erythropoiesis (Macrì *et al*., 2015).

2-3- Hemoglobin

Hemoglobin is consisted of heme (iron) and globin (protein). Hemoglobin transports oxygen from the lungs to the tissues (Harewood and Azevedo, 2021). Including of four chains of proteins, two α- chains and two other (mostly beta) chains. Each chain is covalently linked to a heme molecule to form what is known as hemoglobin, which is binds to four oxygen molecules in the form of O2. (Ouellette and Rawn, 2018).

2-3-1- Ontogeny of Human Hemoglobins

The individual globin subunits are expressed at precise times and in a particular order in a process known as ontogeny (Higgs *et al*., 2012). During the second month of pregnancy, hemoglobin synthesis begins in the yolk sac, Hb Gower1 is the name of the earliest embryonic hemoglobin tetramer, which composed of 2 β-like (2ε) and 2 α like (2ζ) chains (Taher *et al*., 2021).

After the tenth week of pregnancy, hemoglobin becomes synthesized in the liver. During this period of pregnancy, embryonic hemoglobin decreases and fetal hemoglobin (Hb F) increases. Eventually, throughout the embryonic life, becomes the dominant (Elizabeth and Ann, 2010).

2-3-2- Fetal to Adult Hemoglobin Switch

The switch Hb from Hb fetal to Hb Adult Depends on silencing or repression gamma (γ) globin gene (Wang and Thein, 2018). This molecule is encoded by two duplicated γ-globin genes found within the β-globin gene cluster. The γ-globin chains combine with adult α-globin chains into a stable tetramer forming HbF. This remains the predominant hemoglobin for much of gestation. Shortly after the time of birth there is a switch from predominant expression of HbF to adult hemoglobin (HbA), which is mediated by a transcriptional switch in definitive erythroid progenitors from γ- to β-globin. However, the Hb switch is

not complete or irreversible, all adults maintain the ability to contain a little amount of HbF. (Khan *et al*., 2021; Orkin, 2015a). As shown in figure 2-3.

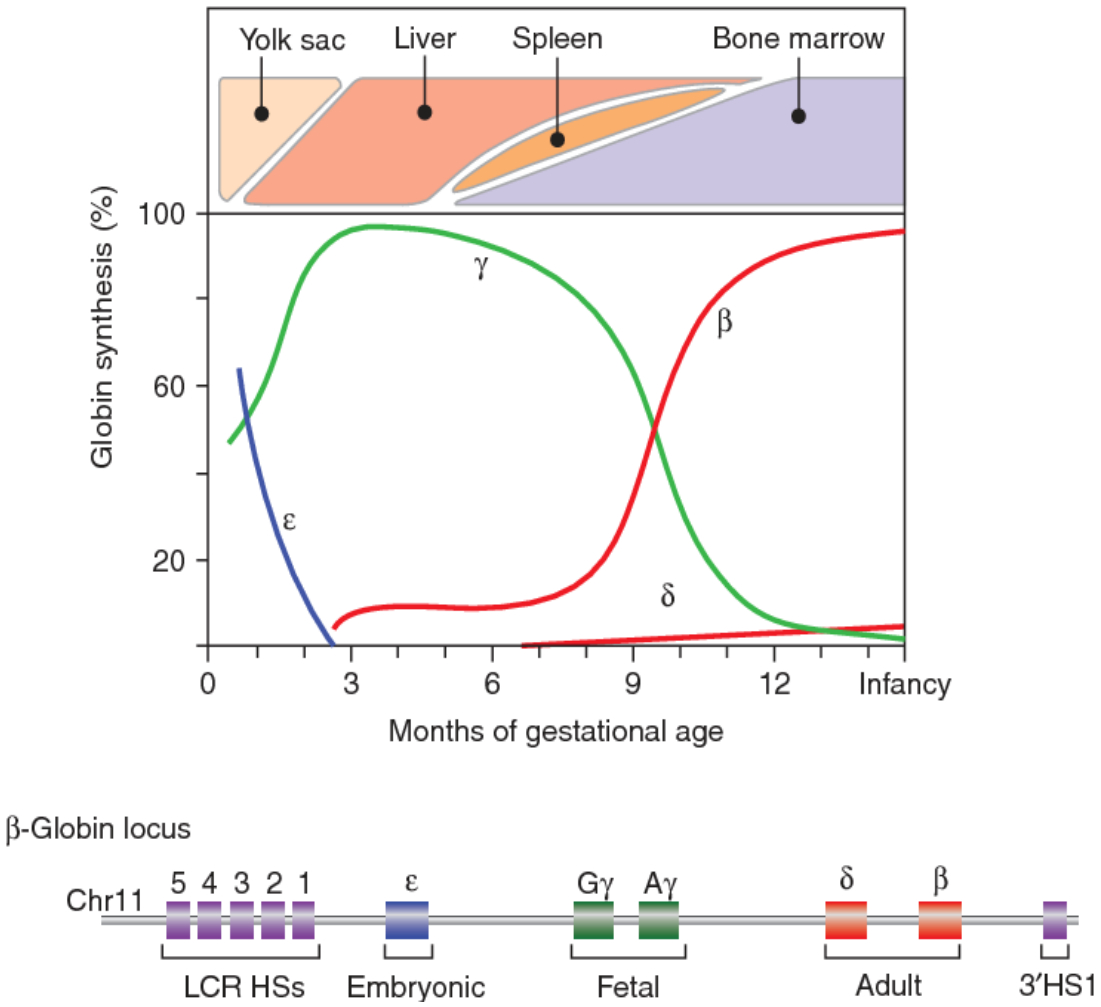


Figure 2-3: The changes in human globin chains synthesis during developmental stages of life (Sankaran and Orkin, 2013).

2-3-3- Genetic Control of Human Hemoglobins

Two gene clusters drive the formation of different types of human globin chains: the β-like genes and the α-like genes cluster.(Elizabeth and Ann, 2010).

2-3-3-1- Globin Gene Clusters

All globin strings are encoded by two distinct gene clusters (Asadov *et al*., 2018). Synthesis and structure of the various globins is regulated by genes arranged in two separate clusters:

Alpha-like Globin Genes Cluster

Alpha-like globin genes cluster found in the following order 5' - ζ - ψζ - $\psi\alpha_2$ - $\psi\alpha_1$ - α_2 - α_1-θ - 3' which are encoded on short arm chromosome 16 and are responsible on the synthesis of alpha-type polypeptide chains (Thein, 2005).

The α-like gene cluster is located close to the telomere of chromosome 16 (16p13.3) including an embryonic gene (ζ) and two fetal/adult genes arranged along the chromosome in the order telomere-ζ-α2-α1-centromere, surrounded by widely expressed genes (Aliyeva *et al*., 2019). As shown in figure 2-4.

A. Beta-like Globin Gene Cluster

The β-globin gene cluster located at 11p15. 5 comprises one pseudogene and five genes whose expression undergoes two critical switches: the embryonic-to-fetal and fetal-to-adult transition. There are five functional genes in this cluster (HBE, HBG2, HBG1, HBBP1, HBD, and HBB). Hb tetramers are produced by the developmental expression of these genes along the chromosome as follows:

Embryonic (Hb Gower-1, Hb Gower-2, and Hb Portland), fetal (α2γ2), adult (HbA1 (α2β2) and HbA2 (α2δ2) (Asadov *et al*., 2018).

βeta globin gene expression is systematized by the locus control region (LCR), (Lee *et al*., 2021). As shown in figure 2-4.

In an adult human, there are two types of hemoglobin, they are; HbA and HBA2. HbA is the most common type of hemoglobin.It consists of alpha and beta globin (α2β2) that begins to be produced after birth. It constitutes 95-98% of the normal hemoglobin. HbA2 consists of alpha and delta globin (α2δ2) and constitutes 1-3% of normal hemoglobin (Taher *et al*., 2021).

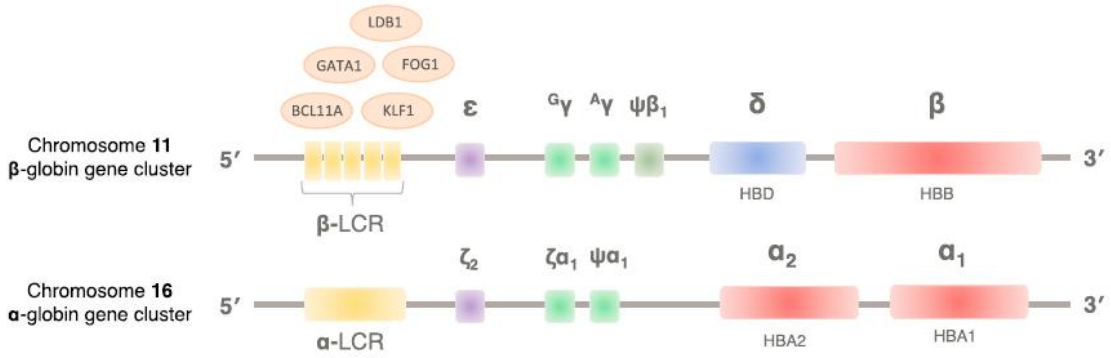


Figure 2-4: Schematic presentation of the chromosomal position of the alpha- and β-globin gene clusters (Code and Project, 2018).

2-4- Gene Expression

The process that uses the data provided by DNA to creating a functional gene product is called gene expression (Perdew *et al*., 2007; Singh *et al*., 2018). Ribonucleic acid RNA is generated from DNA by transcription, and it is a section of the process of gene expression (Koleck and Conley, 2016; Singh *et al*., 2018). The final product of gene expression are that determine biological processes and phenotypes (Narrandes and Xu, 2018).

The gene expression protein is formed through four essential steps (Transcription, RNA splicing, translation, post translational modification) (Watson *et al*., 2014). Transcription is controlle by epigenetic marks, chromatin topology and transcription factors (Koch, 2015).

mRNA processing is dominated by modifications, splicing and poly adenylation, while transport and degradation are regulate by non-coding RNAs and RNA-binding proteins (Hentze *et al*., 2018).

The organism responds to changes in its cells, and the differences among populations could be explain by small changes in the regulatory mechanisms associate with alterations in gene expression (Koleck and Conley, 2016; Singh et al., 2018).

2-5- Thalassemia

Thalassemia is a genetic blood disorder in which the body produces an abnormal form of hemoglobin. There are two types of thalassemia, depending on which type of globin is mutated: α-thalassemia and β-thalassemia. α-thalassemia occurs when one or more of the four α-globin genes are deleted, while β-thalassemia forms when both β-globin genes mutants occur (Kim and Tridane, 2017).

When treating thalassemia with blood transfusions to maintain hemoglobin levels in their body, iron levels rise and overload affects endocrine failure and liver functions as well as heart failure and other organs, or may Treatment is to increase HbF by increasing the expression of the γ-globin gene. Currently, allogeneic haematopoietic Stem Cell Transplantation (HSCT) or the lentiglobin vector as a gene therapy with no mortality and clonal dominance issues or graft rejection, is treated with good treatment. Another treatment is genome editing at specific sites using a precise tool called CRISPR/Cas9 (Amjad *et al*., 2020).

2-5-1- Alpha-Thalassemia

It is an inherited disorder leading to microcytic hypochromic anemia (Code and Project, 2018). There are studies that indicated that the number of α-thalassemia mutations amounted to 120 deletion mutations where different lengths of α-globin gene are deleted. α-globin genes are four distributed on chromosomes 16 (αα/αα). The α- thalassemia is caused by the deletion of one of these genes or a pair of them, and the four genes may be deleted, (Mettananda and Higgs, 2018). For this reason there is variation in the clinical status of the disease (Taher *et al*., 2018).

2-5-2 Beta -Thalassemia

It occurs due to mutations in the β-globin gene that lead to a decrease or absence of β-globin chains, which is the opposite of α-thalassemia 95% of them are point mutations that lead to the replacement of one nucleotide or deletions within the β-globin gene in rare cases. The occurrence of β-thalassemia with a relative decrease in the synthesis of the β-globin chain due to mild mutations is denoted by the symbol β^{+}-thalassemia, but the complete absence of β-globin chains due to severe mutations is characterized by β^{0}-thalassemia (Sabath, 2017; Steinberg *et al*., 2009).

Mostly, there is an excess and accumulation of α-globin tetramers in erythroid cells due to an imbalance between the synthesis of α-globin and β-globin chains. Accumulation of free α-globin protein causes cellular precipitates and generates cytotoxic reactive oxidant species that impair the maturation and viability of red-cell precursors, resulting in ineffective erythropoiesis and pre-mature hemolysis of circulating red cells (Khandros *et al*., 2012).

2-5-2-1- Genetics of β-thalassemia

The number of mutations in the β thalassemia gene has reached more than 350 (Jaing *et al*., 2021). Note that only 40 mutations are responsible for 90% or more of β-thalassemia worldwide (Kountouris *et al*., 2014).

2-5-2-1-1- Non-Deletion Mutations

Mutations that occur due to a single nucleotide substitution or oligonucleotide insertion/deletion that affect β-globin gene expression are known as point mutations. They are common in β-thalassemia and range from single base substitution to instant flanking sequence. There are three types of point mutations depending on their effect on β-globin expression:

- ❖ Transcriptional mutations: mutations that result in defective β-globin expression and occur in the promoter (100 bp upstream to the site of the initiation of transcription) as well as mutations in the 5 untrastated region (UTR) that cause β^{++} thalassemia or silent mutations (Cao and Galanello, 2010; Kumari *et al*., 2013).
- ❖ Mutations affecting messenger RNA processing: During primary mRNA transcript processing, a group of mutations occurs that affect invariant dinucleotide GT or AG sequences at exon-intron splice junctions and completely prevent normal splicing. This type of mutation leads to β^0 thalassemia. These mutations allow normal splicing to varying degrees, producing a phenotype ranging from mild to severe (Jaing *et al*., 2021; Thein *et al*., 2009).

Translational mutations: About 50% of thalassemia alleles completely inactivate the gene through single base substitution to a nonsense codon, premature stop codons (PTCs) or through a frameshift mutation. Due to a nonsense-mediated RNA decay (NMD) phenomenon that is part of the surveillance mechanism that monitors the quality of the processed mRNA. mRNA containing PTCs is destroyed and not transported to the cytoplasm to prevent the accumulation of mutant mRNAs coding for truncated peptides (Kurosaki and Maquat, 2016; Schoenberg and Maquat, 2012).

In addition to the above types of mutations, there are studies that indicated the occurrence of β-thalassemia due to frameshift deletion mutations, but it is rare (Chauhan *et al*., 2021).

2-5-2-2- Clinical Classification of Beta Thalassemia

β-thalassemia is widespread in the Mediterranean region, as well as in the regions of Africa and Asia. Also, in areas where malaria is endemic, thalassemia is common (Kariuki and Williams, 2020). Where thalassemia carriers are more

resistant to malaria, thalassemia is found all over the world due to global migration and ethnic interactions (Guvenc *et al*., 2012). Clinically classified into:

2-5-2-2- 1- Beta Thalassemia Major

Also known as Cooley's anemia and Mediterranean anemia, it's the most severe form of thalassemia because both β-globin alleles are mutated. In other words, this type occurs when the parent compounds are heterozygous or homozygous for mutations in the β-chain (Ali *et al*., 2021). Homozygous leads to β^0-thalassemia and results in severe anemia in the third or fourth month after birth due to a lack of Hb A synthesis (Orkin, 2015b).

Inclusion bodies are formed due to the accumulation of excess unpaired α-globin chains, which leads to damage of RBCs membranes and thus Intravascular hemolysis (Ibrahimi *et al*., 2022). Also, precursors of RBCs are damaged and destroyed early, causing ineffective erythropoiesis, compromised oxygen transport, and severe anemia (Uda *et al*., 2008).

Thalassemia causes hepatosplenomegaly, hypersplenism, growth retardation, thalassemia face (maxillary hyperplasia, frontal bossing, depressed nasal bridge), and bone changes due to bone marrow enlargement, which rapidly progress when blood transfusion therapy is delayed (Orkin, 2015b). The body acquires iron from transfusion-dependent thalassemia, where its absorption increases in the digestive system and accumulates in certain areas of the body, such as the liver, endocrine organs, and the heart, causing growth hormone deficiency, hypoparathyroidism, hypothyroidism, hypogonadotropic hypogonadism, and diabetes mellitus. To iron overload, which causes morbidity and mortality (Bilgin *et al*., 2020). These complications can be prevented or delayed by treating them with chelation therapy (Fernandes, 2018).

There are treatments for thalassemia major, such as hematopoietic stem cell transplantation (HSCT) which require screening for family donors (Yesilipek *et*

al., 2012). Thalassemia major patients who do not have HSCT donors have other options, such as gene therapy (Kunz and Kulozik, 2020).

2-5-2-2-2- Beta Thalassemia Intermedia

This type of depend on blood transfusion is not always required, but they need blood transfusions at certain times, such as pregnancy, infections, and infections due to increased hemolysis (Weatherall, 2012). In this type of thalassemia, Hb levels range between 6-10 gr/dL and are characterized by hypochromia and microcytic anemia. Hepatosplenomegaly, hypersplenism, extramedullary hematopoiesis, medullary expansion in bone marrow, pulmonary hypertension, leg ulcers, thrombosis, and growth retardation can be seen in patients with β-thalassemia intermedia (Asadov *et al*., 2018).

2-5-2-2-3- Thalassemia Minor

Usually, people with this type of thalassemia are asymptomatic (Basut *et al*., 2018). It is formed due to heterozygous resulting from a specific hemoglobin allele of thalassemia with a normal hemoglobin allele (Caocci *et al*., 2017). And because they do not suffer from diseases, they are usually detected by routine blood tests as they have mild anemia and small RBCs, but the risk factor here is the possibility of giving birth to a generation with thalassemia when they are infected with thalassemia. Marriage to a carrier of the disease or suffering from it (Fathi *et al*., 2019).

2-6- Genetic Polymorphism

It is astounding to know that 99.9% of every individuals genome between persons is the same with only 0.1% having chromosome differences, This variation is responsible for the diversity of phenotypes and their susceptibility to environmental influences DNA variations occur in a variety of formulas. Mutations might be distinct as order variants which occur in less than 1% of the

populace, whereas the extra prevalent differing is described as polymorphisms (Somberg, 2002).

The inheritance of a trait controlled by a single genetic locus with two alleles is referred to as genetic polymorphism. Single nucleotide polymorphisms (SNPs), sequence repeats, insertions, deletions, and recombination are all potential sources. Genetic polymorphisms can occur as a result of random processes or as a result of external agents such as viruses or radiation. If a difference in DNA sequence between individuals has been shown to be linked to disease, it is usually referred to as a genetic mutation. changes in DNA sequence that have been proven to be caused by external agents are referred to as "mutations" rather than "polymorphisms". DNA polymorphisms are classified into three types: tandem repeat polymorphism, copy number variations, and single nucleotide polymorphisms (SNPs) (Ismail and Essawi, 2012).

The most common polymorphism in human:

2-6-1- A Single Nucleotide Polymorphism (SNP)

In humans, SNPs are the most common type of genetic variation. Understanding the functions of SNPs can help us greatly understand the genetics of human phenotype variation, particularly the genetic basis of complex human diseases (Ismail and Essawi, 2012).

A single base pair at one position in the genome is genetically changed by an SNP. SNPs are a common phenomenon and affect the expression of mRNA. To distinguish between genetic expression and protein synthesis, they have been thoroughly researched (Ghosh *et al*., 2021). As shown in figure 2-5.

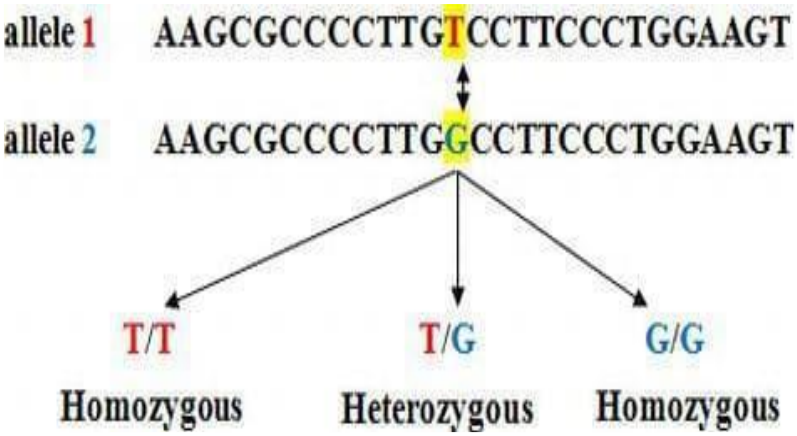


Figure 2-5: Single nucleotide polymorphism (Somberg, 2002).

2-6-2- Methods for Determination of Genetic Polymorphism

2-6-2-1- The Amplification Refractory Mutation System (ARMS)

A variety of polymerase chain reaction (PCR) methods are commonly used to identify genetic polymorphisms in the β-globin gene. Among these PCR methods are the amplification refractory mutation system and the amplification refractory mutation system (ARMS) (Rujito *et al*., 2016).

ARMS analysis is based on primer-specific polymerase amplification with a set of primers complementary to the most common polymorphism in a population under study (Yang *et al*., 2018).

ARMS analysis, also known as the allele-specific PCR method, employs two PCR reactions, one containing primers specific for the wild allele and the other containing primers specific for the mutant allele. A band from the normal reaction correspond to the wild allele, bands from the mutant reaction correspond to the mutant allele, and bands from both reactions correspond to the heterozygous allele when gel electrophoresis is performed (Chen *et al*., 2000; Simsek *et al*., 1999).

2-6-2-2- Random Amplified Polymorphic DNA (RAPD)

Molecular marker technologies are important biotechnologies that are widely used for the characterization, identification, and variety authentication of various living organisms. Random amplified polymorphic DNA (RAPD) is a widely used technique that was developed for genetic characterization of organisms three decades ago(Cheng *et al*., 2015; Desai *et al*., 2015; Fu *et al*., 2013; Long *et al*., 2015).

In RAPD PCR, a single arbitrary primer is used in a PCR reaction, resulting in the amplification of many distinct DNA products. The standard RAPD technique employs short synthetic oligonucleotides 10 bases in length of random sequences as arbitrary primers to amplify nanogram amounts of total DNA by polymerase chain reaction (PCR) at low annealing temperatures (Rahiman *et al*., 2015).

2-6-2-3- DNA Sequencing

The process of determining the number and order of nucleotides in DNA strands is known as sequencing (Bamanga *et al*., 2018). Maxam Gilbert sequencing and Sanger sequencing are two methods for detecting:

- DNA sequencing: is a chemical method that first chemically modifies DNA nucleotides before cleaving the DNA backbone at sites adjacent to the modified nucleotides. Because of the technical difficulty and the use of hazardous chemicals As a result, it is not currently used in standard molecular biology (Haldar, 2019).
- Sanger sequencing is a type of DNA sequencing that uses dideoxynucleotide phosphates (ddNTPs), are incorporated by DNA polymerase during in vitro DNA replication. This modified ddNTPs terminate DNA strand elongation because they lack the 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, this technique is known as

dye terminator sequencing. Each of the four ddNTPs (where N = A/T/G/C) is labeled with fluorescent dyes that emit light at different wavelengths, and thus can be captured in the form of colored peaks known as chromatograms (Haldar, 2019).

Chain termination method is more efficient than the Maxam and Gilbert method because it uses low-toxicity and low-radiative chemicals. To apply this method, it requires one chain of DNA strand, primer and DNA polymerase, fluorescent or radioactive nucleotides and nucleotides Modified to terminate (Fernandez-Cadenas *et al*., 2003).

2-7- Bioinformatics Methods

Bioinformatics is the field that deals with the collection and storage of biological information, it includes everything related to biological databases (Thapar, 2018). Bioinformatics was created as a science to address the computational challenges associated with analyzing large amounts of data generated by the DNA sequencing technique (Harris *et al*., 2017).

It is also the use of the latest techniques in applied mathematics (Informatics) and statistics and computer sciences to solve vital problems, including sequence alignment, protein structural alignment, protein structure prediction, gene expression prediction, and other applications (Kumar and Chauhan, 2017).

2-7-1- Genetic Prediction

Gene prediction describes the process of determining detailed regions of genomic DNA that involve genes. These genes are probably of different varieties like protein-coding genes, RNA genes, and may also involve genes implementing other functions like regulatory genes (Mathé *et al*., 2002).

It is a complex process involving mathematical modeling of positions instrumental in classifying genes in a DNA sequence (SHAH *et al*., 2013)

Predicting genes from DNA sequences is a difficult task in genomics, but it adds many insightful hints about genes, such as deducing the protein product code, detecting genetic malfunctioning, and tracing hereditary (SHAH *et al*., 2013).

2-7-2- Three Dimensional Protein (3 D)

The proteins exist in all living systems proceeding a variety of essential functions. The quality of a function achieved by a certain protein is closely related to its folding or adopted conformation. The folding of an amino acid sequence is restricted by a lot of types of non-covalent bonds produced by interactions among different parts of the amino acid chain, these forces contain atoms in the polypeptide backbone beside atoms placed in the amino acid side chains (Corrêa *et al*., 2018).

The prediction of the three-dimensional (3D) structure of polypeptides or proteins is one of the most essential and difficult problems in structural bioinformatics (Dorn *et al*., 2014).

Each protein is determined by a unique sequence of chained amino acids which under little physiological conditions fold into a specific three dimensional shape (3 D) (LaPelusa and Kaushik, 2021).

2-8- Beta Globin Gene

The β-globin gene maps clustered with another β-like genes on chromosome eleven (11 p 15.5). (Reading *et al*., 2016).

Mutations in the β- globin gene, are responsible for many of serious hemoglobinopathies like sickle cell anemia and β-thalassemia. Hemoglobinopathies are a group of hereditary diseases because of the insufficient production or abnormal structure of hemoglobin (Carlice-dos-Reis *et al*., 2017). the β-globin gene large number of mutations (Jarjour *et al*., 2014).

Which could occur anywhere within the (1,600 bp) DNA segment consists of the three coding exons , splicing sites , and another regulatory elements, Patients with mutations in both β-globin alleles that significantly decrease the β-globin protein production experience skeletal abnormalities, severe anemia, and have a high level of mortality, a quantitative decrease in β-globin and accumulation of α- globin chains are responsible for the pathophysiology of β-thalassemia (Cai *et al*., 2018).

Downregulation of the β-globin gene can be caused by a spectrum of molecular lesions ranging from point mutations to small deletions limited to the β-globin gene to extensive deletions of the whole β-globin cluster (Moleirinho *et al*., 2013).

Mutations in exon 3 of the β-globin gene include premature chain termination (nonsense) mutations, frameshifts, and complex rearrangements that synthesize truncated or elongated and volatile β-globin gene products. The resulting β-chain variants are very unstable and in many cases, the dominantly inherited β-thalassemia is not detectable (Munkongdee *et al*., 2020). As shown in figure 2-6.

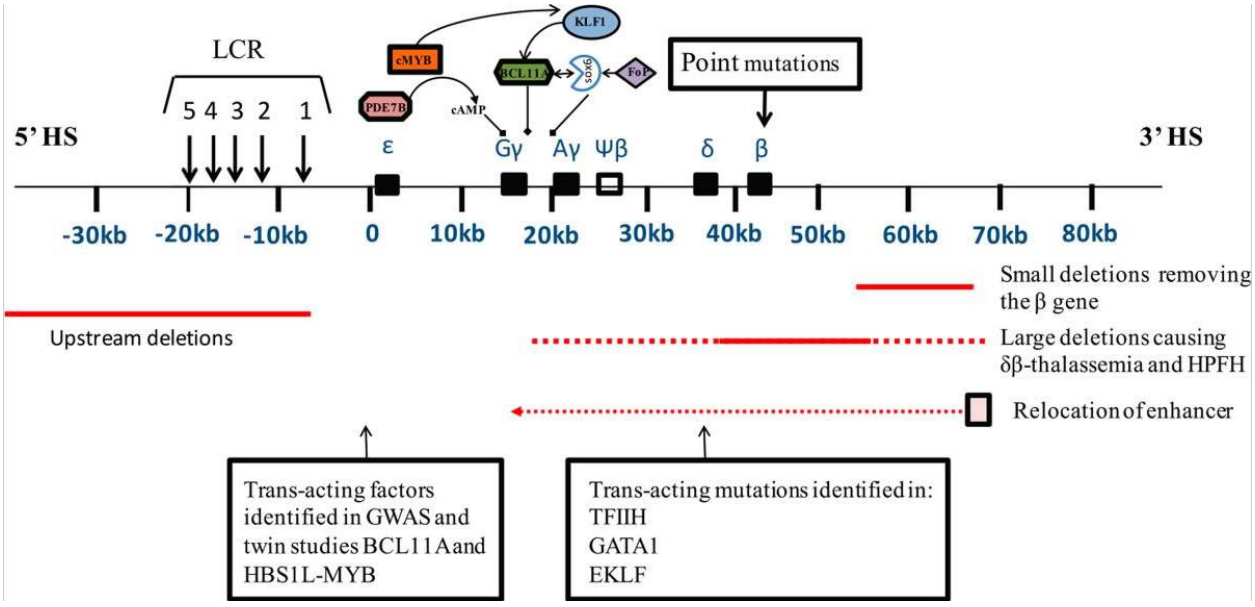


Figure 2-6: Schematic presentation of β- globin gene.

2-9- ß-thalassemia Genetic Modifiers

β-thalassemia genetic modifiers are genes or loci that interact epistatically with the ß-globin gene to induce changes in the β^0-thalassemia phenotype (Thein, 2008). In general, these genetic modifiers can be divided into two categories:

2-9-1- Primary Modifiers

These are genetic variants in the α-globin gene and β-globin gene clusters they are the coinheritance:

- ❖ Compound heterozygous alleles or homozygous for β-thalassemia.
- ❖ Genetic factors in order to maintain continuous synthesis of γ-globin subunits in adults.
- ❖ Heterozygous mutations in the β-globin gene cause unstable β-globin subunits to be synthesized (Galanello *et al*., 2011).

2-9-2- Secondary Modifiers

There are genetic variants or variables that affect some of the clinical problems associated with the β-thalassemia phenotypes but are not mapped to the β-globin cluster. These comprise; variations at the hemoglobin fetal (Hb F) related quantitative trait loci (QTL) like the *BCL11A* gene (Menzel *et al*., 2007; Thein *et al*., 2007). As shown in figure 2-7.

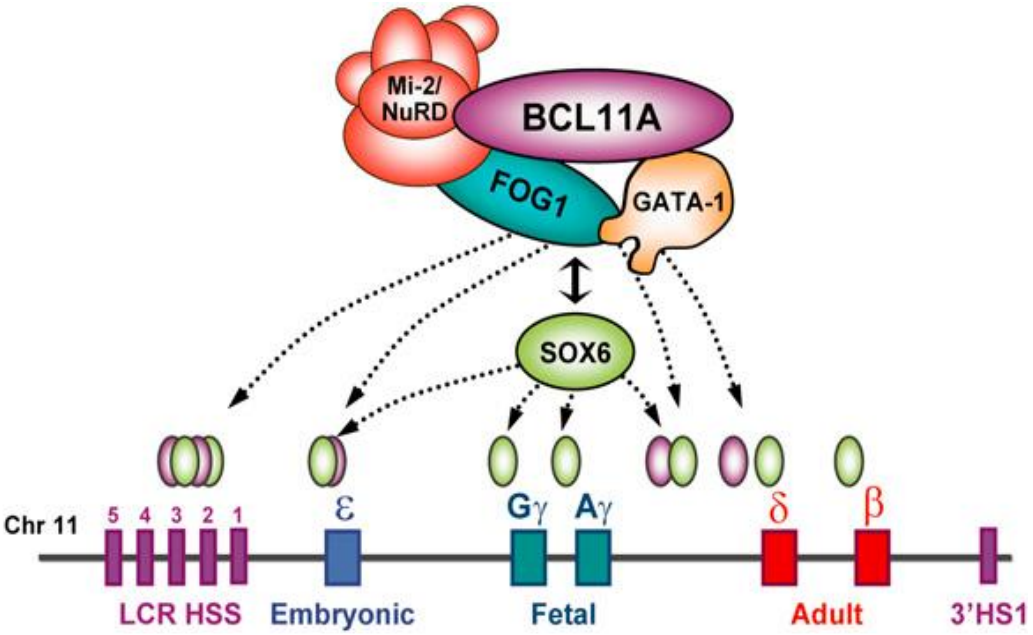


Figure 2-7: Molecular interaction of *BCL11A* gene (Xu *et al*., 2010).

So we will explain *BCL11A* in more details:

2-9-2-1 β-Cell Lymphoma/Leukemia 11A (*BCL11A*)

BCL11A position on chromosome (2p16.1) encodes its transcription factor (Luc *et al*., 2016).

The expression of *BCL11A* is mainly regulated by ways:

- ❖ miRNAs regulation. (Lee *et al*., 2013; Li *et al*., 2018).
- ❖ Transcription factor regulation (Shariati *et al*., 2016). Some *BCL11A* SNPs, primarily rs1427407 and rs11886868, can decrease *BCL11A* expression (Chaouch *et al*., 2016) .So, disrupting the enhancer of the *BCL11A* gene can decrease its expression (Psatha *et al*., 2018).

BCL11A performs its function in fetal to adult hemoglobin switching, *BCL11A* indirectly or directly regulates the downstream targets' expression. Because of its critical function in the fetal to adult hemoglobin switching in erythroid biology, *BCL11A* has recently gained increased research. In human erythroid cells, it was found that *BCL11A* acts as a crucial factor for the silence of the γ-globin gene and

can decrease fetal hemoglobin (HbF) while promoting adult hemoglobin (HbA) (Xu *et al*., 2010).

Additional research demonstrated that krueppel-like factor 1 (KLF1) directly stimulates the expression of β-globin and indirectly suppresses β-globin by acting on *BCL11A* (Esteghamat *et al*., 2013).

It has been discovered that KLF1 can influence the expression of *BCL11A* in a way that indirectly regulates γ-globin, adding greater support for the development of clinical therapeutic medicines leveraging this regulatory network (Amaya *et al*., 2013).

Recently, target genes can be safely and accurately edited thanks to the advantages of CRISPR-Cas9 technology (Canver *et al*., 2015).

These findings provide patients with beta-hemoglobinopathies a therapeutic approach using autologous stem cell editing and transplantation. All of the aforementioned data points to *BCL11A* as a potential treatment gene for beta-hemoglobinopathies (Chang *et al*., 2017).

Chapter Three
Materials and Methods

3- Materials and Methods

3-1- Materials

3-1-1- Kits

Table 3-1: Show the type of kits that used in experiment.

Name of kit	**Manufacture /country of origin**	**Purpose of use**
Taq G2® green master mix	Promega / USA	For amplified DNA template by PCR and for amplified PCR product in PCR – RAPD – PCR
AccuPower® ProFi Taq PCR PreMix & Master Mix	Bioneer / Korea	For amplified DNA by PCR for sequence DNA
Taq 2X master mix red	Ampliqon / Denmark	ARMS PCR
gSYNC™ DNA Extraction	Genaid / Taiwan	Extraction of blood genomic DNA
Wright-Giemsa staining kit	Aspenbio /china	Used to blood smear
E.Z.N.A.® spin protocol Gel extraction kit spin	Omega Bio-TEK / USA	Used to nucleic acid purification

3-1-2- Instrument / Device

Table 3-2: List of devices that used in experiment.

The device	Country of origin	Manufacture
Autoclave	Japan	Hirayama
Biosafety Cabinet	Korea	Human Lab
Camera	Japan	Sony
Centrifuge	Germany	Eppendorf
Cooling centrifuge	Germany	Eppendorf
Distillator	Korea	Lab Teach
Electrophoresis	Korea	Bioneer
Gel documentation	Germany	Biometra
Hematological analyzer	China	Mindray
Laboratory shaker	China	Dragon
Magnetic stirrer	Germany	Heidolph
Master cycler personal	Germany	Eppendorf
Microcenterfuge spin and cooling centrifuge	Germany	Eppendorf
Micropipetteres	China	Dragon
Microscope	Japan	Olympus
Microwave	China	Shonic
NanoDrop spectrophotometers	USA	Thermofisher
pH meter	Romania	HANNA
Sensitive balance	Germany	Sartorius
Thermal cycler prime	UK	TECHNE
Uv- Trans illuminator	Italy	Eletrofor
Vortex	Belgium	CYAN/Belgium
Water bath	Germany	Memmert

3-1-3- Chemicals

Table 3-3: List of chemical materials that used in experiment.

Chemicals	Manufacture	Country of origin
TBE buffer (1X, 10X) (Trisbase + Boricacid + EDTA)	Promega	USA
Absolute Ethanol	Promega	USA
Agarose	Promega	USA
Distal water		
Marker or DNA ladder (100-2000 bp)	Bioneer	Korea
Ethidium Bromide	Promega	USA
Loading dye (6X)	Geneaid	Taiwan
nuclease free water (De-ionized water)	Promega	USA
Phosphate Buffer Saline (P B S)		

3-1-4- The RAPD Primers

The primers of RAPD were used for genome DNA manufactured by the company of AUGCT DNA SYN Biotechnology / China. The table below show the types of primers and sequences:

Table 3-4: Show the type of RAPD primers that used in experiment.

Primers	Length	Sequences	References
OPAA 11	10 base	ACCCGACCTG	(Allami and Dragh., 2022)
OPU15	10 base	ACGGGCCAGT	
OPAA17	10 base	GAGCCCGACT	
OPD18	10 base	GAGAGCCAAC	

3-1-5- Primers Used in Experiment

Table 3-5: Show the type of primers that used in experiment.

Gene	Sequences		Size (bp)	References
Hb	F	5'- CAATGTATCATGCCTCTTTGCACC- 3′	861	This study
	R	5'- CAATGTATCATGCCTCTTTGCACC- 3′		
GAPDH GAPDHa	5′- CCTAGTCCCAGGGCTTTGATT - 3′ 5′- CCCCACACACATGCACTTACC - 3′		57	NM_002046 (Gen Bank)
BCL11A-A (rs766432)	F	5′-TTGTTTCGCTTTAGCTTTATTAAGGTACAA- 3′	135	(Bashir *et al*., 2021)
	R	5′-GACGTGTTCTGTATCTTGATTTTGGT- 3′		
BCL11A-C (rs766432) (mutation)	F	5′-CCAAACAGTTTAAAGGTTACAGACAGACT- 3′	116	(Bashir *et al*., 2021)
	R	5′-AAAATGAATGACTTTTGTTGTATGTAGAG- 3′		

3-1-6- Design of Experiment

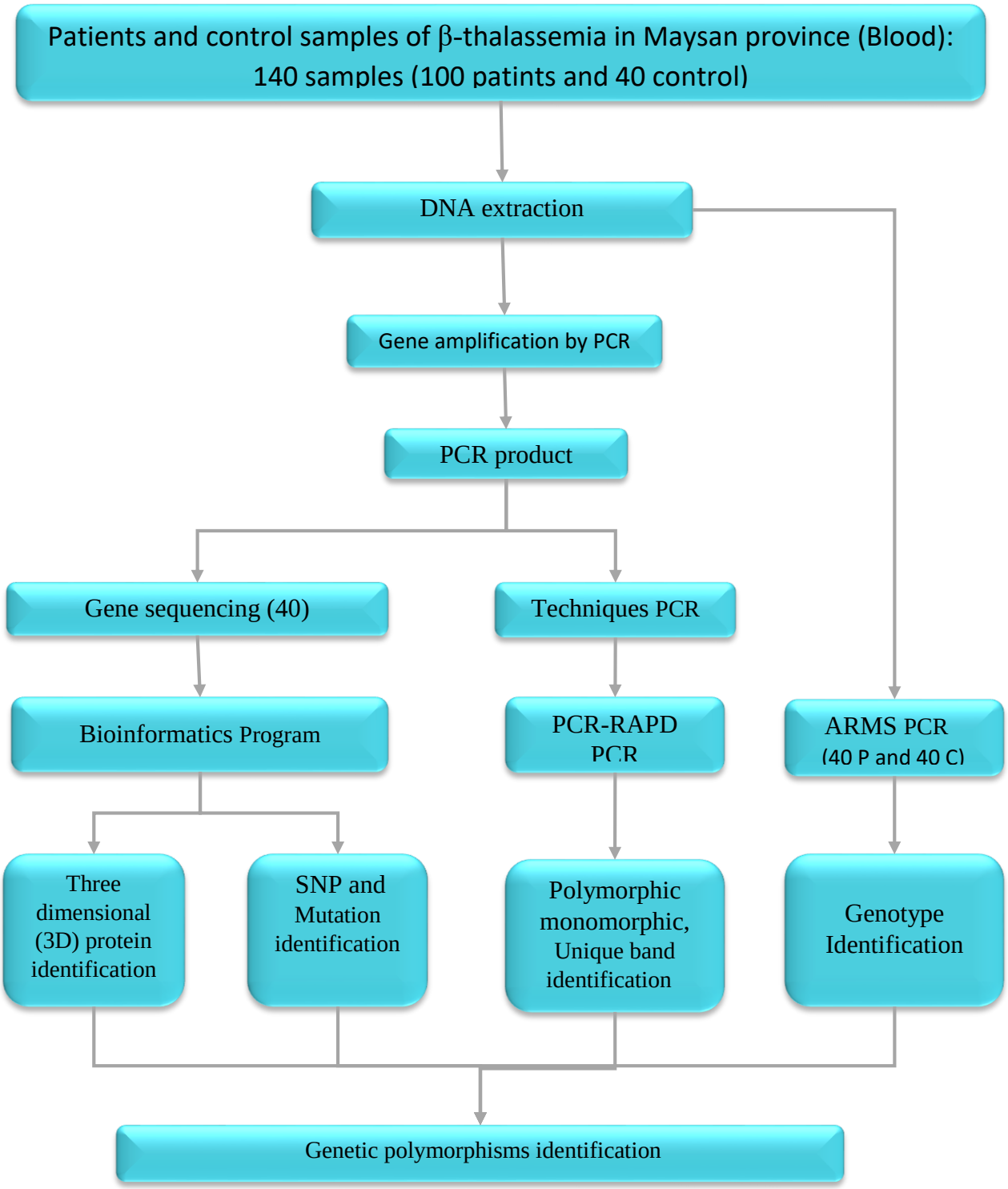


Figure 3-1: Important steps in this study.

3-2- Methods

3-2-1- Samples Collection

The study group consisted of 140 samples (100 samples with β-thalassemia major and 40 samples as a control group for healthy people without this disease). Blood samples were obtained from 100 patients with β-thalassemia during November 2021. All of them frequently Visit the Center for Genetic Blood Diseases /AL-Sader Teaching Hospital in Maysan province. These patients had a mean age of 15.3 years. In terms of their geographical distribution, 29% of them were from Alamara and 71% from the areas of Ali Al Gharbi, Ali Al Sharqi, Kumayt, Al Majar, Al Kahlaa, Al Maymouna, Al Uzair, Qal'at Saleh and Al Mushrah. In addition to the other General characteristics is listed in appendix No. 5. Blood was collected from the median cubital vein and placed in an EDTA tube that was marked and stored at - 20 ºC.

3-2-2- Sterilization Methods

Tools such as micropipette tips and RCR tubes were sterilized by an autoclave at a temperature of 121 °C and a pressure of 15 psi for 20 minutes. After that, they were exposed to dry heat by means of an oven for an hour at a temperature of 60 °C. Also, conical flasks, volumetric flask and other tools were sterilized. At 180 degrees for an hour and a half.

3-2-3- DNA Isolation

The gSYNC TM DNA Extraction Kit from Geneaid Company (Taiwan) used to extract DNA from whole blood According to the protocol procedure attached to the kit and which is included in the appendix No. 3. After blood samples were collected and stored at -20°C, the DNA was extracted from the samples and the presence of the Genomic DNA was confirmed by electrophoresis on 1% agarose

gel, and then measuring the amount of DNA by the Nanodrop device, absorbance ratio of A260/280 was obtained in the range from 1.70 to 1.96.

3-2-4- Preparation of Tris-Borate-EDTA Buffer (TBE) Buffer

TBE buffer as stock was prepared at a concentration of 10X by dissolving 108 gm of tris base, 55 gm of Boric acid and 40mL of ethylene diamine tetra acetic acid (EDTA) at a concentration of 0.5 M. All of these materials are dissolved in 800 ml of distilled water and after mixing them, the volume of the mixture is completed to 1 liter and then Heat it with constant stirring using a magnetic stirrer until the ingredients are completely melted.

3-2-5- Electrophoresis

Electrophoresis was used to detect the Genomic DNA after extraction as well as to detect the PCR products according to the following steps:

1- Preparation of agarose gel at a concentration of 1% (0.5 g agarose dissolved in 50 ml TBE buffer 1X) to detect the Genomic DNA and PCR products, and at a concentration of 2% (1 g agarose in 50 ml TBE buffer 1X), ARMS and RAPD.
2- After that, the mixture is heated in the microwave for a minute and a half.
3- Then the mixture was left to cool until it reached a temperature of 50-60 ^{o}C, then ethidium bromide was added to it (1.5 microliters per 50 ml of the mixture). Then the agarose solution was carefully poured into the gel tray after closing the gel trays like edges with rubber ruler tape and fixing the comb 1 cm away from the forward edge.
4- The agarose was allowed to harden for at least 25 minutes at room temperature.
5- DNA sample (or other samples) was transferred 4 µl from it after mixed with 2 µl of loading dye then transfer to wells of agarose gel

6- The gel tray was put into the gel tank after the fixed comb was carefully removed. Then TBE buffer (1X) was previously added to the tank until it reached approximately 3 mm above the gel's surface
7- Electric current was allowed at 90 volts to 30 minutes for Genomic DNA or 75 volts to 45 minutes for RAPD or 75 volts (5 min) than 90 volt (5min) than 120 volts (20 min) in the ARMS
8- Finally, used a UV transilumminator with a wavelength of 350 nm, the DNA bands were observed.

3-2-6- Polymerase Chain Reaction Protocol

The steps were as follows:

1. Before use, the template DNA and particular primers were dissolved.
2. For a 25 µl reaction volume, template DNA, primers, master mix and De-ionized water (nuclease free water) were added to the PCR tubes. As shown in table (3-6).

Table 3-6: Show volume of reagents used of PCR mixture.

PCR Mixture	**Volume (µl)**	
DNA template	2	2
Revers Primer	2	2
Forward primer	2	2
Taq G2 ® green master mix	12.5 Promega	5 Bioneer
Nuclease free water	6.5	14
Final volume	25	25

3. The components of PCR tubes were perfectly combined by spinning them down and up with a pipette, and then centrifuged for 10 seconds or more with a Mini spin Microcentrifuge.

4. The PCR tubes were placed in the TECHEN thermocycler, with the appropriate PCR cycling program parameter settings shown in table (3-7).

Table 3-7: Hb, GAPDH and GAPDHa genes PCR conditions.

PCR steps	HBB gene		*BCL11A* gene		Cycles
	Temperature ºC	Time	Temperature ºC	Time	
Initial denaturation	94	5 Min	94	5 Min	1
Denaturation	94	1 Min	94	30 Sec	35
Annealing	63.5	1 Min	61	45 Sec	
Extension	72	90 Sec	72	45 Sec	
Final extension	72	5 Min	72	5 Min	1
Final hold	4	10 Min	4	10 Min	-

3-2-7- Agarose Gel Extraction

The desired DNA fragment was extracted from the gel using the E. Z.N.A » Spin protocol Gel Extraction Kit Spin (OMEGA Bio-TEK/USA) companies. Agarose gel extraction was employed to identify and purify the amplified specific gene (PCR product), as well as to purify appropriate DNA fragments (PCR product band). Steps for DNA extracting the agarose gel were performed according to the steps in the procedure which are attached in the appendix No. 4.

3-2-8- Random Amplified Polymorphic DNA (RAPD)

The following table shows the components of the mixture of the RAPD PCR

Table 3-8: Show volume of reagents that used in RAPD.

Mixture	**Volume (µl)**
AccuPower® ProFi Taq PCR PreMix & Master Mix (Bioneer)	5
Nuclease free water	10
Genomic DNA	2.5
RAPD primers (OPAA 11,OPU15,OPAA17,OPD18)	2.5
Final volume	20

The mixture was inflated in a Techne prime thermocycler with program in table (3-9) and (3-10).

Table 3-9: PCR program for primers OPAA 11, OPU15 and OPAA17 of RAPD technique.

PCR steps	**Temperature (ºC)**		**Time**	**Cycles**
	OPAA11	**OPU15, OPAA17**		
Initial denaturation	95	95	1 Min	1
Denaturation	95	95	1Min	3
Annealing	36	36	45 Sec	
Extension	72	72	1 Min	
Final Extension	72	72	10 Min	1
Final hold	10	10	-	-

Table 3- 10: PCR program of RAPD for primer OPD18.

PCR steps	Temperature (ºC)	Time	Cycles
Initial denaturation	95	1 Min	1
Denaturation	95	1Min	3
Annealing	36	45 Sec	
Extension	72	1 Min	
Final Extension	72	10 Min	1
Final hold	10	-	-

RAPD-PCR product observations were carried out with Gel DOC UV Transilluminator.

3-2-9- Genotyping of *BCL11A* Polymorphism

The *BCL11A* polymorphism was genotyped using tetra-primer (ARMS).

3-2-9-1- Sample Preparation for *BCL11A* Tetra-Primer ARMS

The *BCL11A* tetra-primer ARMS was carried out using Tag 2X master mix red (Ampliqon / Denmark) and two pairs of *BCL11A* primers (*BCL11A*_A_Fwd primer, *BCL11A*_A_Rev primer and *BCL11A*/mutation_C_Fwd primer, *BCL11A/*mutation_C_Rev primer) a 25 µl PCR reaction mix. As shown in table (3-11).

Table 3-11: Volumes of reagents used for *BCL11A* reaction mix preparation.

Mixture	**Volume (µl)**
Sterile distilled water	9.5
PCR master mix	12.5
BCL11A _A_Fwd primer	0.5
BCL11A _A_Rev primer	0.5
BCL11A /mutation_C_Fwd primer	0.5
BCL11A /mutation_C_Rev primer	0.5
Genomic DNA template	1
Final volume	25

3-2-9-2- Thermal Cycling Conditions for *BCL11A* Tetra-Primer ARMS

Table 3-12: *BCL11A* & *BCL11A* -M PCR conditions (ARMS).

PCR steps	**Temperature (ºC)**	**Time**	**Cycles**
Initial denaturation	95	2 Min	1
Denaturation	95	30 Sec	30
Annealing	55	30 Sec	
Extension	72	45 Sec	
Final extension	72	10 Min	1
Final hold	4	10 Min	-

3-2-9-3- *BCL11A* Tetra-Primer ARMS Genotyping

In this tetra-primer ARMS inner primers, which are allele-specific, are utilized to create a DNA fragment containing the *BCL11A* polymorphisms special allele. While outer primers are employed to create the outer control band A-allele for homozygous, two DNA bands are synthesized; 135 bp long for the inner A-allele band and 193 bp for the outer control band.

C -allele for homozygous, two DNA bands are synthesized; 116 bp long for the inner C-allele band and 193 bp long for the outer control band.

Three DNA bands are produced for heterozygous alleles; are synthesized; the outer control band which are 193 bp, the inner A-allele band 135 bp and inner C allele band 116 bp long. The tetra-primer ARMS amplicons were visualized on a 2.5% agarose gel.

3-2-10- Molecular Size Index

The molecular size indicator (DNA marker 100 bp) was used, with 13 bands as follows (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1600, 2000) base pair, from the Promega / USA company, the next parameters were calculated using the gel images. The following parameters were calculated using the gel images as input. The numbers of total bands generated by each primer was calculated.

The primer efficiency and discriminatory power were calculated using the following law according to (Ismaeel, 2013).The percentage of primer bands was calculated using the following law according to (**Al**-Rawi, 2015) the percentages of polymorphisms (%), monomorphisms (%), and uniqueness (%) were calculated using the following laws:

Primer efficiency = $\frac{\text{The number of polymorphic bands produced by each primers}}{Total\ number\ of\ bands\ of\ all\ primers}$

Primer discriminatory power % = $\frac{The\ number\ of\ polymorphic\ bands\ produced\ from\ the\ primers}{Total\ number\ of\ poymorphic\ bands\ produced\ from\ all\ primers}$ x 100

Percentage of primer bands % = $\frac{The\ numbers\ of\ bands\ produced\ by\ the\ primer}{Total\ number\ of\ bandsfrom\ the\ primers}$ x 100

Polymorphisms % = $\frac{The\ number\ of\ polymorphic\ bands\ produced\ from\ the\ primers}{Total\ number\ of\ bands\ produced\ from\ the\ primers}$ x 100

Monomorphisms % = $\frac{The\ number\ of\ monomorphisms\ bands\ produced\ from\ the\ primers}{Total\ number\ of\ bands\ produced\ from\ the\ primers}$ x 100

Uniqueness % = $\frac{The\ number\ of\ unique\ bands\ produced\ from\ the\ primers}{Total\ number\ of\ bands\ produced\ from\ the\ primers}$ x 100

To separate the resulting bands, 40 PCR-RAPD-PCR products (20 controls and 20 patients) are transferred to a (2%) agarose gel. Monomorphic bands are those that appear in all wells at the same molecular weight, polymorphic bands are those that appear in some wells but not others at the same molecular weight, and unique bands are those that appear in only one well at a given molecular weight.

3-2-11- Determine the Sequence of Nitrogen Bases

Following confirmation of sample amplification, samples of 20 microliters of the PCR product were being sent to Macrogen Company in South Korea to acquire the real sequences of the nitrogenous bases for the required pieces of genes.

According to our request from the company, a sequencing process for single strands of DNA Forward (HBB gene) or Reverse (BCL11A gene) was performed for the purpose of identifying genetic mutations.

After the results were received, the sequence's identity was checked in the Gene Bank using algorithms and Bioinformatics techniques as a Blast search tool.

This tool assisted in determining the conformity of records sent from samples and records in the gene bank.

Sequences lined up and aligned were identified using specialized software including BioEdit, and genetic mutations were recognized using Blast, Geneious V 4.0.20 and ithaGenes.

3-2-12- Three Dimension Protein Drawing

Drawing a three dimensional protein using the programs and steps listed below:

1- Using the NCBI website's Blast program, transform DNA sequences in FASTA format to amino acid sequences in the same format (Altschul *et al*., 1990).

2- Using the Phyre 2 V.2.0 program, convert FASTA amino acid sequences to PDB format and predict the three-dimensional shape of the protein (Kelley *et al*., 2015).

3- Drawing three-dimensional protein shapes with the EzMol V.1.22 program (www.Sbg.bio.ic.ac.uk/ezmol) and determining the position of the genetic mutation on the protein (Reynolds *et al*., 2018).

4- Using Expasy and the Swiss-Model program to draw 3D proteins for some genes.

3-2-13- Statistical Analysis

Data statistical analysis was carried out by (SPSS version 26). Chi-square was used to display important statistics and significant differences with a $P < 0.05$ probability levels (Al-Rawi and Allah, 2000).

The expected genotype of Hardy - Weinberg equilibrium was examined manually and then by Michael H.Court's (2005-2008) online calculator, and the difference from HWE was accomplished using SPSS version 26 and Michael H. Court's (2005-2008) online calculator (2005-2008). MedCalc statistical software (version 20.0111) was used to calculate odds ratios (ORs) and confidence intervals (CIs) for genotypes and alleles (https://www.Medcalc.net) (Altman, 1991).

Chapter Four

Result

4- Results

4-1- Genetic Polymorphisms:

4-1-1- Detection of the Genetic Polymorphism of the *BCL11A* Gene by ARMS-PCR

The results show the presence of two alleles A and C as well as three genotypes AA, AC, and CC in β-thalassemia major. As it is shown below in Figures (4-1) and (4-2).

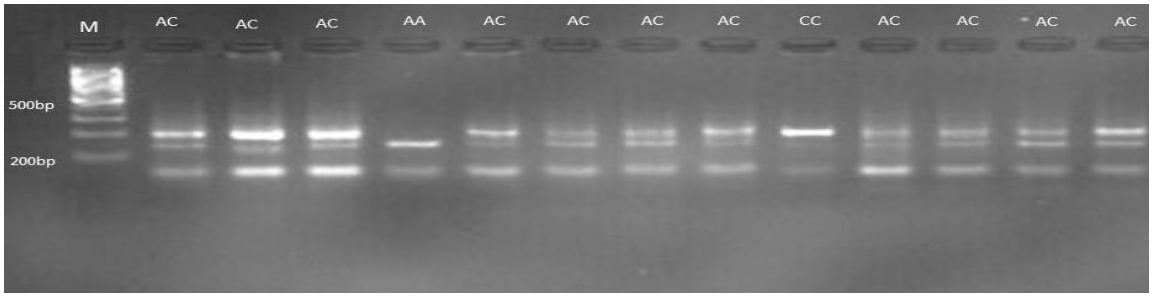


Figure 4-1: ARMS-PCR of *BCL11A* gene in control samples on 2% agarose gel electrophoresis, 75 volts (5 min) then 90 volts (5 min) then 120 volts (20 min).

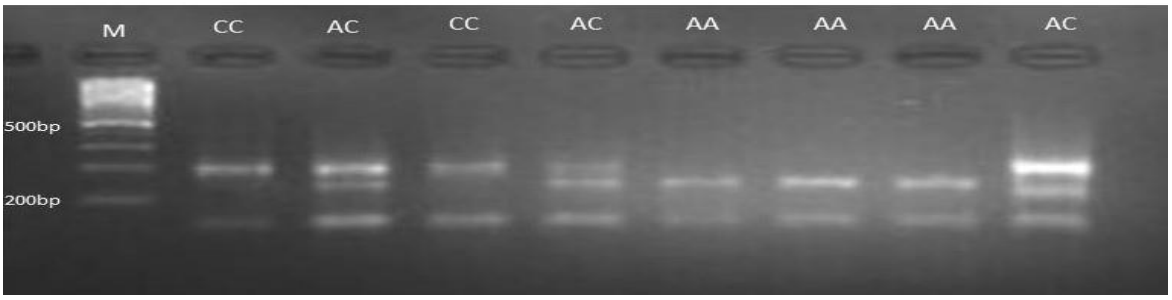


Figure 4-2: ARMS-PCR of *BCL11A* gene in β-thalassemia major patients on 2% agarose gel electrophoresis, 75 volts (5 min) then 90 volts (5 min) then 120 volts (20 min).

The results of ARMS PCR analysis of the *BCL11A* gene show that three genotypes, AA, AC, and CC, are in β-thalassemia major patients' samples with frequencies of 0.3, 0.6 and 0.1, respectively, and in control 0.1, 0.8 and 0.1, respectively. The frequency of the two alleles A and C in patients with A

(0.5625), and C (0.4375) as for the control A (0.525), C (0.475), as illustrated in table (4-1).

Table 4-1: Genotype and allele frequency in the *BCL11A* gene of β-thalassemia major patients and control.

Gene	Genotype	Genotype frequency (patient)	Genotype frequency (control)	Alleles	Alleles frequency (patient)	Alleles frequency (control)
BCL11A	AA	0.3	0.1	A	0.5625	0.525
	AC	0.6	0.8	C	0.4375	0.475
	CC	0.1	0.1	-	-	-

The results of the frequency distribution of the A and C alleles of the *BCL11A* gene showed that the percentage of their distribution was not equal among the β-thalassemia major patient and control samples, where the percentage of the A allele in patients was 56.25%, while in the control it was 52.5%, while the percentage of the C allele was in the patient at 43.75 % and in the control it was 47.5%, As illustrated in table (4-2).

Table 4-2: Genotype and alleles percentage of *BCL11A* gene polymorphism among patients and control group.

Gene	Genotype	Patients No. (%)	Control No. (%)	Alleles	Patients No. (%)	Control No. (%)
BCL11A	AA	10(25)	5(12.5)	A	45(56.25)	42(52.5)
	AC	25(62.5)	32(80)	C	35(43.75)	38(47.5)
	CC	5(12.5)	3(7.5)	-	-	-

According to the current study, the results of the frequency distribution show no significant differences $P = 0.634$ at the probability level of $P < 0.05$ between β-thalassemia major patients and control samples, as illustrated in table (4-3).

Table 4-3: Allele frequency of *BCL11A* gene among β-thalassemia major patients and control samples.

Gene	Alleles	Control (N0.)	patients (No.)	OR	CI (95%)	P-value
BCL11A	A	42	45	0.8596	0.4612 to 1.6023	0.634
	C	38	35			
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05						
X^2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)						

The statistical analysis of the results using the chi-square show that there are no significant differences between the genotype distribution of β-thalassemia major patients and controls under the P<0.05 probability level where it reached P-value 0.220. As illustrated in table (4-4).

Table 4-4: The statistical analysis of the genotype frequencies of the *BCL11A* gene among β-thalassemia major patients and control group.

Gene	Genotype	Total	Control	patients	X2	P-value
BCL11A	AA	15	5	10	3.026	0.220
	AC	57	32	25		
	CC	8	3	5		
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05						
X2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)						

Using the Hardy Weinberg equilibrium law, the results of the *BCL11A* gene showed a statistically significant difference between the expected and the observed of the control group, with a value of P 0.0001 at its P < 0.05 probability level. This means that the control group is not subject to a Hardy- Weinberg distribution. As illustrated in table (4-5).

Table 4-5: The excepted and observed frequencies under hardy Weinberg equilibrium in the *BCL11A* gene of the control group.

Gene	Genotype	Multipopulation (Observed)%	Multipopulation (Expected)%	X2 (H.W.E)	P-value
BCL11A	AA	5(12.5)	11	14.593	0.0001***
	AC	32(80)	20		
	CC	3(7.5)	9		
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05					
X2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)					

The results of genetic analysis of the ARMS-PCR technique of the *BCL11A* gene in β-thalassemia major show three genotypes, which are AA, AC, and CC. The AA genotype appeared in a percentage in β-thalassemia major patients of 25% and 12.5% of control samples, and there was no significant difference between the control sample and the patient. The P-value was 0.159 at the probability level $P < 0.05$. The odd ratio value was 2.333 and the confidence interval was between 0.717 and 7.586.

The AC genotype in the 80% control sample was compared to the 62.5% thalassemia major patient samples and there was no significant difference where the P-value was 0.08 at the $P < 0.05$ probability level. The odd ratio was 0.4167 and the confidence range was between 0.1525 and 1.138.

The Genotype CC appeared in 7.5% of β-thalassemia major patients and 12.5% of the control group. Therefore, there was no significant difference between the two samples of the patient and the control, where its value was $P = 0.46$ at the level of probability $P < 0.05$, the odd ratio was 1.7619, and the value of the confidence interval was between 0.391 and 7.929. As illustrated in table (4-6) and figure (4-3).

Table 4-6: The genotype distribution and frequency of the *BCL11A* polymorphism in patients and controls**.**

Gene	Genotype	Control No. (%)	Patients No. (%)	OR	CI (95%)	P-value
BCL11A	AA	5(12.5)	10(25)	2.333	0.717-7.586	0.159
	AC	32(80)	25(62.5)	1.2	0.201-7.182	0.08
	CC	3(7.5)	5(12.5)	1.7619	0.391-7.929	0.46
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05						
X2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)						

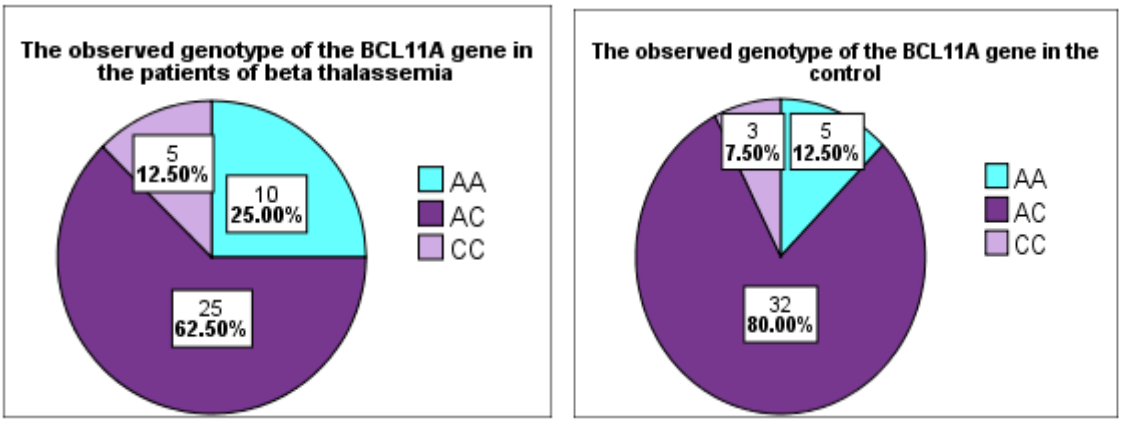


Figure (4-3): The observed genotype distribution and the frequencies of the *BCL11A* polymorphism in patients and controls.

Under the dominant model in this study, the results did not show a statistically significant difference between the β-thalassemia major patients and the control samples. Where P = 0.159 at P<0.05 Probability level. As shown in table (4-7).

Table 4-7: Distribution of the *BCL11A* genotype in the patients and controls under dominant model.

Gene	Genotype	Control (No.)	Patients (No.)	OR	CI (95%)	P-value
BCL11A	AA	5	10	2.3	0.718-7.587	0.159
	AC +CC	35	30			
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05						
X^2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)						

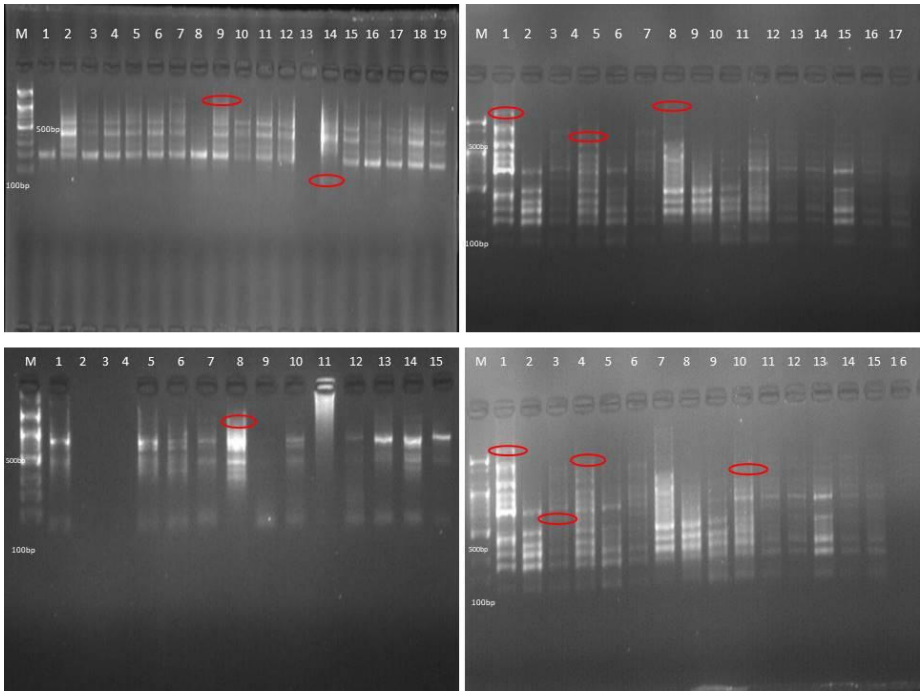
Under the recessive model of the current study, the results did not show a statistically significant difference between the β-thalassemia major patients and the control samples. Where P = 0.46 at P<0.05 Probability level. As shown in table (4-8).

Table 4-8: Distribution of the *BCL11A* genotype in the patients and control under recessive model.

Gene	Genotype	Control (No.)	Patients (No.)	OR	CI (95%)	P-value
BCL11A	AA+AC	37	35			
	CC	3	5	1.7619	0.391-7.929	0.46
Significance *P<0.05 ,**P<0.01 ,***P<0.005 , NS=No significance>0.05						
X2 Chi-square , H.W.E Hardy - Weinberg equilibrium (if P<0.05 not consistent with H.W.E)						

4-1-2- PCR-RAPD-PCR Technique for Detection of β-thalassemia Major Genetic Polymorphism

The results showed that the numbers of bands in β-thalassemia major patients are more than the control group. There is also a difference in their molecular weights according to the type of primer used. As shown in Figure (4.4).



BCL11A control *BCL11A* patients

Figure 4-4: PCR- RAPD - PCR generated by OPAA 11, OPU15, OPAA17 and OPD 18 four primer. The first lane from the right (M) is DNA ladder marker 100-2000bp in size, and the other lanes are PCR-PAPD bands on 2% agarose gel electrophoresis.

4-1-2-1- PCR-RAPD-PCR of *BCL11A* Gene to Detection of Genetic Polymorphism

The results of the PCR-RAPD-PCR for the *BCL11A* gene showed that the total bands in β-thalassemia major patients were 303 bands, while in the control group there were 97 bands. The OPAA17 primer showed the highest number of bands, whereas the total bands reached 86 28.3%, while the OPU15 primer gave the highest total of 27 bands 27.8% in the control group. As illustrated in tables (4-9), (4-10).

Table 4-9: The percentage and numbers of the total band, monomorphic, unique polymorphic bands, and primer discriminatory power and primer efficiency that were produced from the amplified four primers in the *BCL11A* gene of β-thalassemia major patients.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency (PB)	Primer discriminatory power %	Total No. of unique bands	Uniqueness, %	Total No. of monomorphic bands	Monomorphisms%
BCl11A patient	OPAA11	62	20.5	1	1.612	0.003	16.666	2	3.225	3	4.838
	OPU15	81	26.7	4	4.938	0.013	66.666	2	2.469	3	3.703
	OPAA17	86	28.3	1	1.162	0.003	16.666	2	2.325	4	4.651
	OPD18	74	24.5	0	0	0	0	4	5.405	7	9.459
Total		303	100.0	6	7.712	0.019	99.998	10	13.424	17	22.651

Table 4-10: The percentage and numbers of total band, monomorphic, unique polymorphic bands, and primer discriminatory power and primer efficiency that were produced from amplified four primers in the *BCL11A* gene of β-thalassemia major control group.

Gene	RAPD Primer	Total No. of bands	Percentage of primer bands %	Total No. of polymorphic bands	Polymorphism %	Primer efficiency (PB)	Primer discriminatory power %	Total No. of unique bands	Uniqueness, %	Total No. of monomorphic bands	Monomorphisms%
BC11A control	*OPAA11*	23	26.7	3	13.043	0.03	60	4	17.391	4	17.391
	OPU15	27	27.8	1	3.703	0.01	20	3	11.111	2	7.407
	OPAA17	26	26.8	1	3.846	0.01	20	2	7.692	4	15.384
	OPD18	21	21.6	0	0	0	0	4	19.047	3	14.285
Total		97	100	5	20.592	0.05	100	13	55.241	13	54.467

The results can be shown by PCR-RAPD-PCR for the size of the band's fragments. In patients, the largest was in the *BCL11A* gene 1900-2000 bp using OPAA11, OPU15 and OPAA17 primers, and the smallest size was 30 bp using the OPU15 primer.

For the control, the largest size fragment was 2000 bp when using the OPU15 primer, while the smallest size fragment was 30 bp when using the same primer as shown in Figure (4-5).

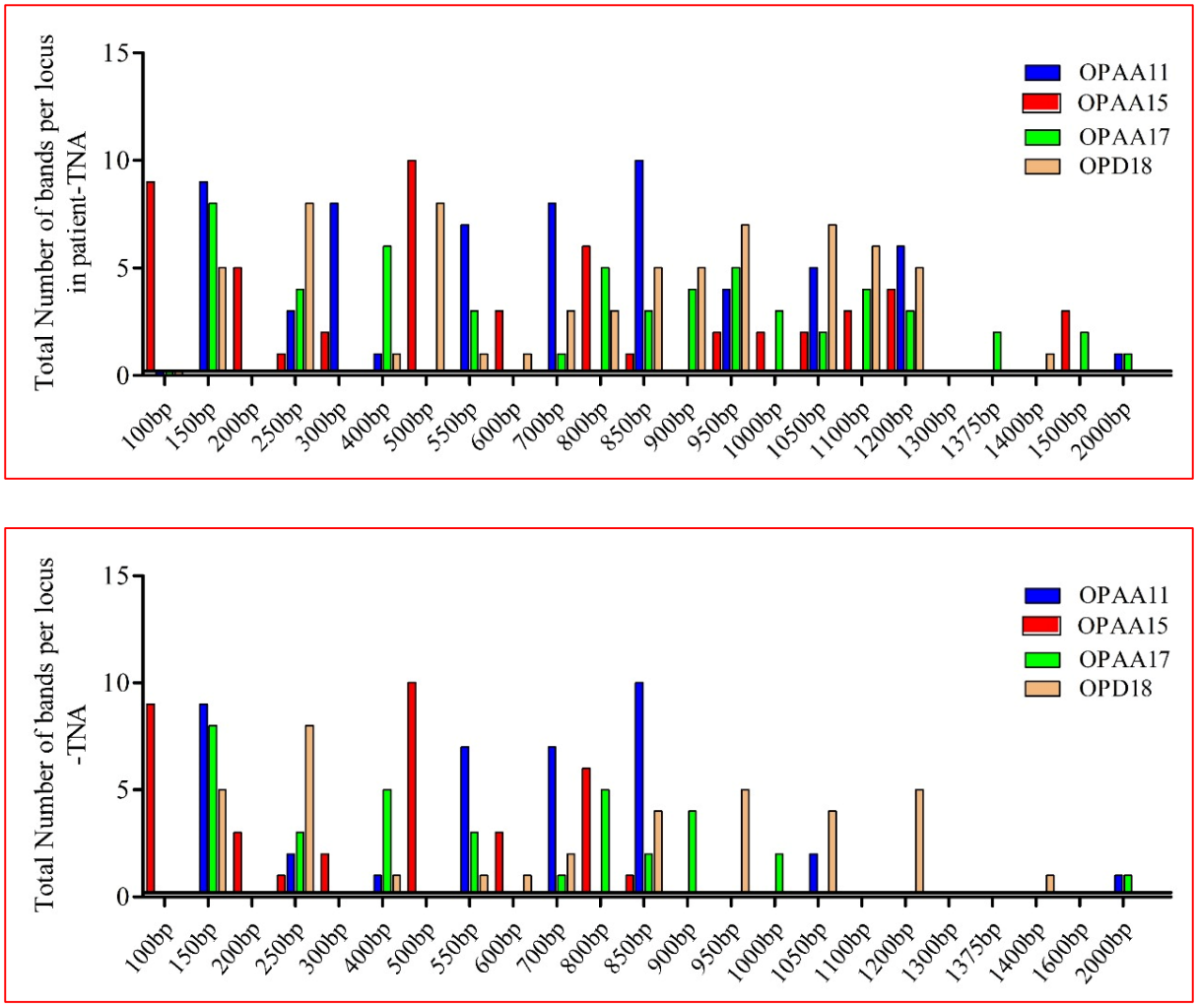


Figure 4-5: The distribution of the fragment sizes in the *BCL11A* gene β-thalassemia patients and control.

The results for polymorphic bands in the *BCL11A* gene in patient samples showed a total of 6 bands with a percentage polymorphism of 7.7%. The primer OPU15 gave the highest number of these bands were 4 with a percentage polymorphism 4.9%, and the primer efficiency was 0.013 and the primer discriminatory power was 66.66%, while the primer OPD18 did not give any polymorphic bands. As illustrated in table (4-9).

In the control samples, the total number of polymorphic bands were 5 and the percentage polymorphism was 20.5% and the OPAA 11 primer gave the highest number of these bands were 3 with percentage polymorphism 13.04 % and the primer efficiency was 0.03 and the primer discriminatory power was 60%, while

the OPD18 primer did not give any polymorphic bands. As illustrated in table (4-10).

The results for unique bands in the *BCL11A* gene in patients showed a total of 10 with a percentage uniqueness of 13.4%. The primer OPD18 gave the highest number of these bands 4 with percentage uniqueness 5.4%, while the primers OPAA 11, OPU15 and OPAA17 give number of these bands 2 with percentage uniqueness 3.2 %, 2.4 % and 2.3% respectively. As illustrated in table (4-9).

In the control, the total number of unique bands 13, and the percentage uniqueness was 55.2%, so OPAA 11 and OPD18 primers gave the highest number of these bands 4 with percentage uniqueness 17.3 % and 19.04 % respectively. The OPAA17 primer provides two band with a percentage uniqueness 7.6 %. As illustrated in table (4-10).

The results of the monomorphic bands in the *BCL11A* gene in patients showed that the total number of these bands 17, with a percentage monomorphism of 22.6%. The primer OPD18 gave the highest number of these bands 7 and the percentage of monomorphism was 9.4 %, while the primers OPAA 11 and OPU15 gave the lowest number of these bands 3 and the percentage monomorphism was 4.8% and 3.7%, respectively. As illustrated in table (4-9).

In the control, the total number of monomorphic bands 13 and the percentage monomorphism was 54.4 %, so OPAA11 and OPAA17 primers gave the highest number of these bands 4 with percentage monomorphism 17.3 % and 15.3 %, respectively. While the OPU15 primer gave only two bands with percentage monomorphism 7.4 %. As illustrated in table (4-10).

Although there is a clear difference in the total bands preparation between patients and the control group of the *BCL11A* gene, the statistical analysis showed that there were no statistical differences at the probability level of $P < 0.05$ using chi-square whereas the P-value was 0.621. As illustrated in table (4-11).

Table 4-11: Statistical analysis for monomorphic, polymorphic, unique bands of the *BCL11A* gene between patients and control group.

Gene	Type of band	Overall total band	Total No. of band (patient)	Total No. of band (control)	X^2	P-value
BCL11A	Polymorphic band	11	6	5	0.954	0.621
	Unique band	23	10	13		
	Monomorphic band	30	17	13		
Overall total		64	33	31		
Significance *P <0.05,P <0.01,***P <0.005 , NS=No significance P >0.05**						

4-1-3- Molecular Identification of β-thalassemia Mutation

The Genomic DNA was amplified by polymerase chain reaction (PCR), whereby the primer HBB was given bands of size 861 bp on an agarose gel at a concentration of 1% .As illustrated in figure (4-6)

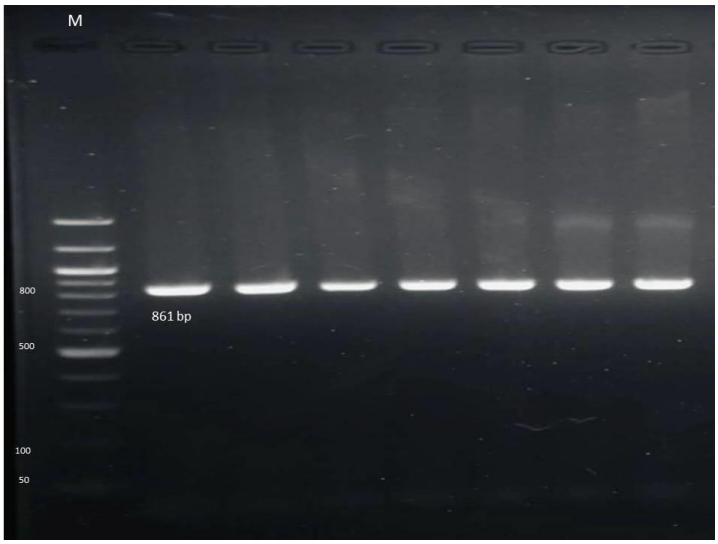


Figure 4-6: Amplification of the *HBB* gene 861 bp obtained from β-thalassemia major patient.

Since the HBB primer gave bands in patients and did not give bands in controls, the result of PCR amplification was positive in patients and negative in controls, and confirmed by using an internal control primer *GAPDH*. A result

which can be employed to be an indicator for the diagnosis of β-thalassemia disorder. As illustrated in Figure (4-7).

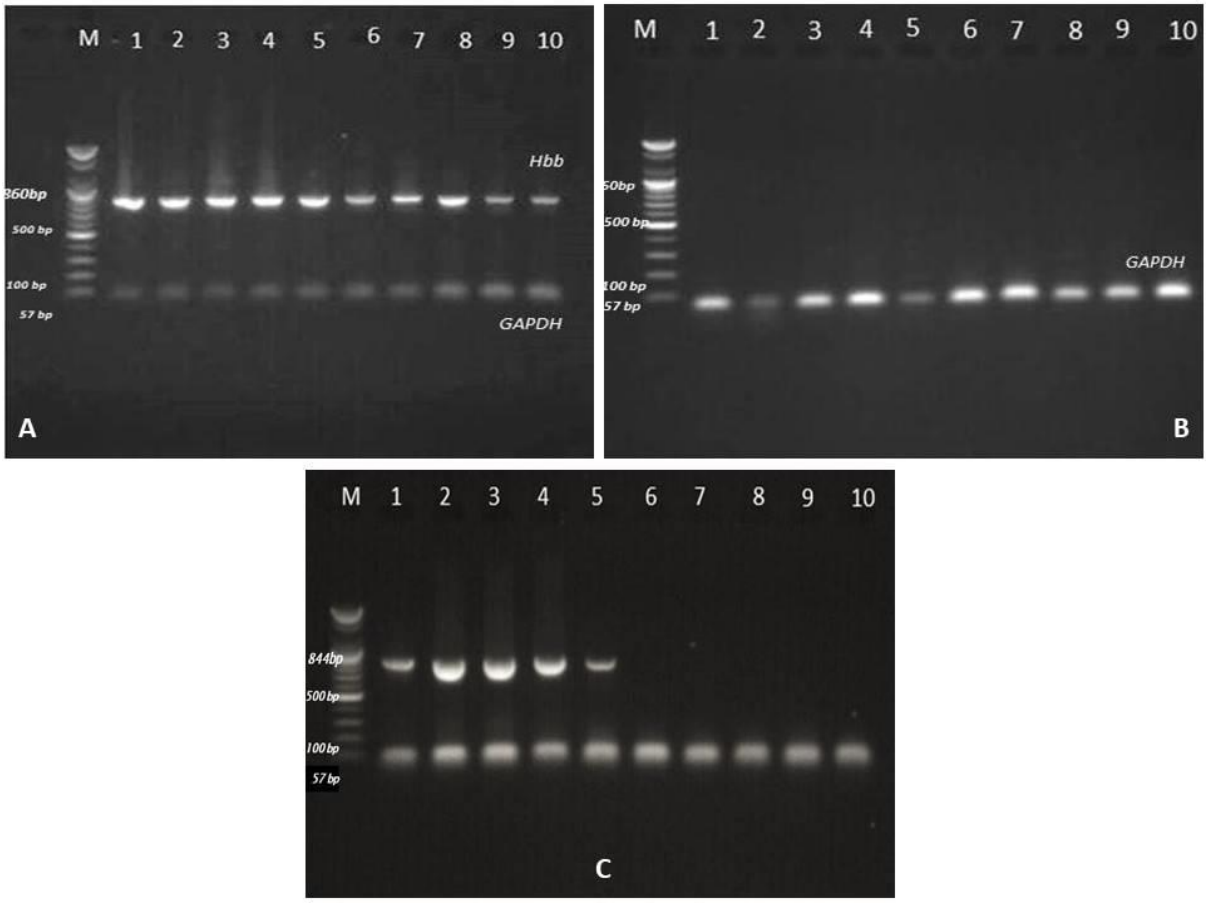


Figure 4-7: (A). Amplification of the HBB primer 861 bp with *GAPDH* primer 57 bp in the patients. **(B).** Amplification of the HBB primer 861 bp with *GAPDH* primer 57 bp control samples. **(C).** the first five samples were patients and the last five were control samples.

4-1-4- Detection Polymorphism of Beta Thalassemia Genes by Nucleotide Sequencing

4-1-4-1- *BCL11A* Gene

The results of the nucleotide base sequence analysis show the presence of alterations in the nitrogenous bases, specifically in the (65611, 65664) sites of the *BCL11A* gene as the base G changed to C (G65611C) and the base G changed to

A (G63611A), and as the base G changed to T (G65664T). As it is shown in the table (4-12), figure (4-8).

The polymorphism (G65664T) changed the genetic codes, changing the amino acids of the resulting proteins from Valine to Leucine, where the mutations were missense mutation (not synonymous), as shown in table (4-12) and figure (4-8).

Sequence results show that rs766432 A>C polymorphism of the *BCL11A* gene is detected in all patients (A 65664 C) sites of the *BCL11A* gene.

Table 4-12: Nucleotide changes and type of mutations, the resulting amino acid changes, and their impact in the translation process of *BCL11A* gene.

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation %	Silent m`utation %	Non-sense mutation %	Frameshift mutation %
BCL11A	65611 65664	G>C G>T	G>A V>L	Transversion Transversion	Missense Missense	NG-011968.1 NG-011968.1	GGC>GCC GTA>TTA	100	0	-	0
SNP:single nucleotide polymorphism ; InDel :insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G : Guanine;I:Isoleucine ; S: Serine ; A: Alanine ; T: Threonine ; D: Aspartic acid ; G: Glysine ; L:leucine											

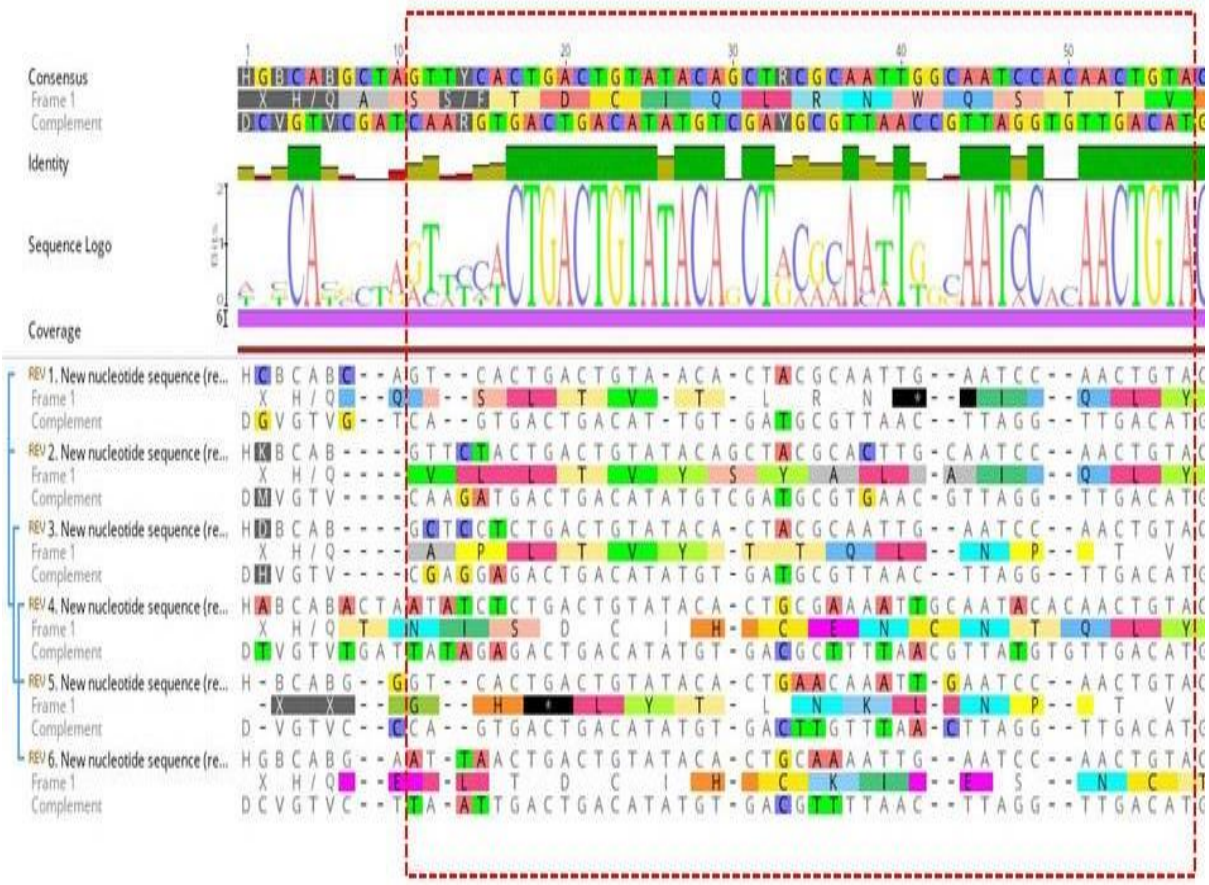


Figure 4-8: SNPs at the studied sites of the *BCL11A* gene.

4-1-4-2- *HBB* Gene

The results of the nucleotide base sequence analysis show the presence of alterations in the nitrogenous bases, specifically in the accession number (U01317.1) Of the *HBB* gene as the base T changed to A (T63279A), as the base A changed to G (A63282G) and the base T changed to C (T63297C), as the base T changed to G (T63298 G), (T64064 G) and as the base G changed to C (G63843C), (G64075C) and as the base G changed to A (G63923A), (G63962A), as the base C changed to A (C63925A). As shown in table (4-13).

As the bases C, T, A, C, C and A deletion at sites (63285, 63288, 63292, 64071, 64072 and 64074) respectively, led to a change in the genetic coding,

which changed the amino acids in the final protein. These mutations were frameshift mutations. As shown in the table (4-13).

The results showed that the polymorphisms (C63925A) despite the fact that the genetic codes had changed, the identical amino acid was provided (Alanine to Alanine). The results showed that the bases A, A, A and C insertion at sites (63296-63297, 63297-63298, 63327-63328, 63341-63342, 63915-63916) respectively, led to a change in the genetic coding, which changed the amino acids in the final protein. These mutations were frameshift mutations. As shown in figure (4-9).

Table 4-13: Nucleotide changes and type of mutations, the resulting amino acid changes, and their impact in the translation process of *HBB* gene (HBA2).

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Accession number	Triple code	Missense mutation %	Silent m`utation %	Non-sense mutation %	Frameshift mutation %
HBB	63279	T>A	K>G	Transversion	Missense	U01317.1	GGT>GGA	48	4	-	48
	63282	A>G	R>G	Transition	Missense	U01317.1	AGG>GGG				
	63285	C	-	Deletion	Frameshift	U01317.1	-				
	63288	T	-	Deletion	Frameshift	U01317.1	-				
	63292	A	-	Deletion	Frameshift	U01317.1	-				
	63296-63297	A	-	Insertion	Frameshift	U01317.1	-				
	63297	T>C	F>S	Transition	Missense	U01317.1	TTT>TCT				
	63297-63298	G	-	Insertion	Frameshift	U01317.1	-				
	63298	T>G	F>L	Transversion	Missense	U01317.1	TTT>TTG				
	63327-63328	A	-	Insertion	Frameshift	U01317.1	-				
	63341-63342	A	-	Insertion	Frameshift	U01317.1	-				
	63843	G>C	W>S	Transversion	Missense	U01317.1	TGG>TCG				
	63915-63916	C	-	Insertion	Missense	U01317.1	-				
	63923	G>A	A>T	Transition	Missense	U01317.1	GCC>ACC				
	63925	C>A	A>A	Transversion	Silent	U01317.1	GCC>GCA				
	63962	G>A	E>K	Transition	Missense	U01317.1	GAG>AAG				
	64064	T>G	C>G	Transversion	Missense	U01317.1	TGC>GGC				
	64071	C	-	Deletion	Frameshift	U01317.1	-				
	64072	C	-	Deletion	Frameshift	U01317.1	-				
	64074	A	-	Deletion	Frameshift	U01317.1	-				
	64075	G>C	Q>H	Transversion	Missense	U01317.1	CAG>CAC				

SNP:single nucleotide polymorphism ; InDel :insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G : Guanine;I:Isoleucine ; S: Serine ; A: Alanine ; T: Threonine ; D: Aspartic acid ; G: Glysine ; L:leucine

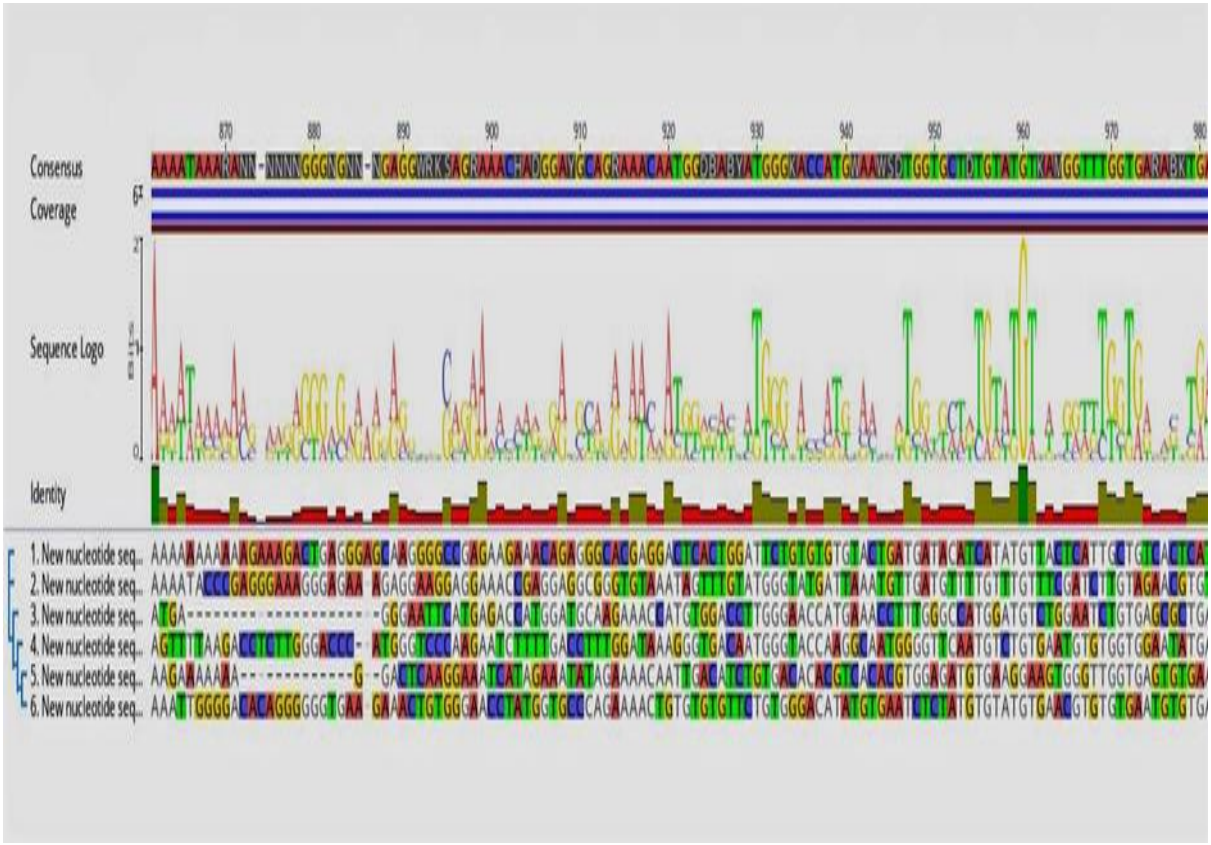


Figure 4-9: SNPs at the studied sites of the *HBB* gene.

Chapter Five
Discussion

5- Discussion

Hemoglobin is consisted of heme (iron) and globin (protein). It transports oxygen from the lungs to the tissues (Harewood and Azevedo, 2021).

Globin consists of four chains of proteins, two α- chains and two other (mostly beta) chains. Each chain is covalently linked with a heme molecule to form hemoglobin, which is bound to four oxygen molecules in the form of O2. (Ouellette and Rawn, 2018).

Thalassemia is frequent in Arab countries and in many neighboring countries, with varying frequencies. In different parts of Iraq, the prevalence of β-thalassemia ranges from 3.7 percent to 4.6 percent. The geographical distribution of thalassemia patients reflects the heterogeneity of β-thalassemia across Iraq (Saud, 2012).

The results of the current study show that 88% of the patients in the study sample are of consanguinity.

Consanguineous marriages are frequent in most Middle Eastern communities. In Iraq, consanguineous marriages account for 40 to 49 % of all marriages, with first cousin marriages accounting for about 28 percent (Alwan and Modell, 1997).

Arab countries have one of the highest rates of consanguinity in the world, ranging from 20% to 50%, with average rates of 20% to 30% across the 22 Arab countries (Tadmouri *et al*., 2009).

Consanguineous marriage is 52.8% of a total of 152 patients in Iraq (Amin *et al*., 2020).

The findings of research by Al-Ali (2016) and Al-Badran (2016), which are carried out in the provinces of Missan and Basrah, respectively, revealed differences in the distribution of β-thalassemia among residents of the same province (Al-Badran *et al*., 2016; Alali and Faraj, 2016).

The clinical presentation of the patient is impacted by numerous factors. The International Thalassemia Federation did concur that the degree of red blood cell indexes, particularly the Hb status, have a significant impact on the clinical disturbance (Cappellini MD, Farmakis D, Porter J, 2021).

The results are in agreement with (Rujito *et al*., 2016), wherein a thorough examination by the Gene Reviews team highlighted that patients with thalassemia major have very low Hb, MCV values regardless of the mutation.

Cutoff values for either MCV or MCH are typically used as the foundation for screening methods for thalassemia in large populations. As a standard for β-thalassemia screening, MCV <80 fL or MCH <27 pg is often used (Sae-ung *et al*., 2012).

Employing MCV < 80 fL as a standard threshold do result in false negative results for the screening of β-thalassemia. The MCV is below ≥ 80 fL in 26 out of 1214 (2.1%) β-thalassemia carriers. However, only 4 of 965 (0.4%) β-thalassemia carriers have MCH ≥ 27 pg. This demonstrates that MCH is a more accurate screening marker for β-thalassemia than MCV because it has fewer false negatives. Additionally, after blood specimens are stored, MCH is observed to be significantly more stable than MCV. (Singha *et al*., 2019).

The large number of patients and thalassemia carriers have lower MCV (< 80 fL) and MCH (< 27 pg), which leads to positive screening results. It is important to highlight that this screening technique do not allow for the exclusion of iron-deficient anemia with decreased MCV and MCH. Therefore, more Hb analysis needs to be done (Luo and Chui, 2016).

The fact that three-quarters of them are carriers of mild β^+-thalassemia, using MCV alone for β-thalassemia screening may result in a considerable percentage of false negatives. A superior screening marker is MCH, which is approximately five times as sensitive. It is strongly advised to use a combined MCV and MCH,

especially in areas with a high prevalence and heterogeneity of thalassemia (Singha *et al*., 2019).

Effective carrier screening and a correct thalassemia diagnosis are crucial preconditions for the prevention and management of thalassemia (Sae-ung *et al*., 2012).

The *BCL11A* locus is a Quantitative trait loci QTL that has a high persistence role for the HbF level (Akbulut-Jeradi *et al*., 2021).

Inducing Hb F in β-thalassemia is an extremely promising strategy for reducing disease severity (Musallam *et al*., 2012).

Because of their potential use in developing targeted therapeutic approaches for β-thalassemia, γ-globin and Hb switching modifier genes (J. Li *et al*., 2018).

Individuals who have severe hemoglobinopathies caused by β-globin chain disorders, such as β-thalassemia intermedia and major, typically, clinical phenotypes are inversely related to the degree of HbF expression retention (Pereira *et al*., 2015).

Only three loci with common polymorphisms account for a significant portion of the variation in HbF levels. The β-globin gene cluster, an intergenic interval among the *HBS1L* and *MYB* genes (*HMIP*), and *BCL11A* are among these loci.

The results by using ARMS-PCR show that there are two alleles A and C with three genotypes AA, AC and CC which are consistent with previous studies, such as (Bashir *et al*., 2021) and (Munkongdee *et al*., 2021) and (Genc *et al*., 2020).

According to Hardy Weinberg's equilibrium law, the results by using ARMS-PCR for *BCL11A* gene show that the control samples do not undergo Hardy Weinberg equilibrium ($P < 0.05$, $p = 0.0001$). Statistically significant differences between expected and observed frequencies caused this deviation from the HWE ($p < 0.05$).

The deviation from the hardy Weinberg equilibrium in the control sample is due to the frequency of consanguineous marriage in these societies, and this possibility is very likely in our society (Cazeneuve *et al*., 2003).

Another possibility for deviation from the hardy Weinberg equilibrium is the small size of the control sample, as the hardy Weinberg equilibrium is very sensitive to low frequencies allele in the homozygous (Chen, 2010).

While in the patient group, the results showed an agreement with the hardy Weinberg equilibrium due to the absence of statistically significant differences between the frequency of the expected and observed alleles.

Matching with Hardy-Weinberg equilibrium indicates that the alleles are inherited independently and no allele is dropped out during genotyping (James and TeralMRes, 2018).

Regarding the equilibrium between the control samples and the patient samples according to Hardy-Weinberg law. The results show a deviation from this law at the $p < 0.05$ probability level where the P-value was 0.220. This is consistent with the results of another study (James and TeralMRes, 2018).

This result can be explain as that *BCL11A* (rs766432) A>C is partly associated with β-thalassemia major and its relative effect on the phenotype of patients as well as the presence of more than one other SNP involved in the phenotypic events of the disease (Genc *et al*., 2020).

The previous studies report that polymorphism of *BCL11A* (rs 766432) A>C β-thalassemia major patients is less effective in increasing Hb F level than other SNPs present in *BCL11A* (Bashir *et al*., 2021).

The study shows that the percentage of the A allele is higher in patients 56% than it is in controls 52%, but there were no significant differences at the level of $P> 0.05$.

From the above, It is noted that the distribution of the A allele is identical to the pattern of the A allele that is observed in almost all the world's population, including the population of white Americans, whites in Europe, and Asian populations (e.g., Japan, China, and India), whereas the A allele has a frequency range of 51-88% (Rujito *et al*., 2016).

By comparing the appearance of alleles and the frequency of genotypes between control samples and patients, the results show that the AC genotype show clear differences, but without statistical significance, with a value of p = 0.08, which was higher in the control sample by 80%, compared to 62% in the β-thalassemia major patient sample. While homogeneous genotypes (AA, CC) are higher in patients, but without statistical, p>0.05 (N. Kumari and Thakur, 2014).

According to the previous studies, *BCL11A* and *HBB* genes play an important role in β-thalassemia (Bashir *et al*., 2021; Mondal and Mandal, 2016), so it is chosen to study genetic polymorphism using RAPD PCR. It is a convenient, sensitive genetic marker and is efficient for detecting genetic polymorphism (Atienzar *et al*., 2002).

There are noticeable differences in the discriminatory power of each primer between the control group and the patients in *BCL11A* gene.

The reason for this is due to the different number of polymorphic bands, because the calculation of the discriminatory power depends on the number of polymorphic bands for each primer divided by the total of polymorphic bands for all primers.

Polymorphisms are generally caused by point mutations resulting from translocations and inversions, single nucleotide substitutions, DNA section duplication, rearrangements involving insertions or deletions, and mistakes in tandemly repeated DNA replication (Selvakumari *et al*.,2017).

PCR RAPD PCR was used in a previous study (Allami and Dragh, 2022) and this technique is based on polymorphism of the amplified gene with the help of PCR. It is used to study genetic alteration and analyze genetic instability to understand molecular events in the blood of patients with β- thalassemia

In thalassemia disorder, the current study is the first that uses the amplified gene as a template instead of the Genomic DNA in the RAPD technique to study the genetic polymorphism of the *BCL11A* gene in β-thalassemia.

The results using PCR RAPD PCR technique in *BCL11A* gene show that the number of bands in each primer is higher in β-thalassemia patients than in control groups and this is due to the use of the gene amplified in the modified PCR RAPD PCR technique.

The variation in the total of bands between β-thalassemia patients and the control group can be relating to the nucleotide count of these primers, the genotype of β-thalassemia patients, and the number of identical sites of primers in β- thalassemia genes being changes by mutations and translocations. This will influence the template interaction sites of primers, and lead to an increase or loss of bands. Genetically, it shows a contrasting pool of fragments in its genotypes due to point mutations at oligonucleotide sites. Also, when there is distance between the termini sequences that changes due to deletion or insertion mutation events leads to genetic polymorphism (Amiteye, 2021).

the primer efficiency value ranges from (0 to 1) and is identified as the primer's capacity to produce polymorphisms (Yalçinkaya and Koçoglu, 1992) (Ismaeel, 2013)

The primer's efficiency is demonstrated by its ability to produce the highest proportion of polymorphic bands when compared to the sum number of amplified bands. The ability to demonstrate polymorphism between normal and patient individuals is an important feature of an efficient primer. There are noticeable

differences in the molecular size of fragments generated from primers between patients and control groups in *BCL11A* (Saleh *et al*., 2010).

The reason for using the amplifier gene is also to increase the accuracy of the results and to facilitate the comparison of the relationship between the sequencing results and the RAPD results for β-thalassemia genes in terms of the absence or presence of the genetic mutation (N. Kumari and Thakur, 2014).

The results of the nucleotide base sequence analysis show the presence of alterations in the nitrogenous bases. Sequence results show that rs766432 A>C polymorphism of the *BCL11A* gene is detected in all patients (A 65664 C) sites of the *BCL11A* gene.

The *BCL11A* rs766432 and HbF levels are nominally significantly correlated in β-thalassemia carriers, as it is previously observed in Chinese adult β-thalassemia heterozygotes (Farrell *et al*., 2011).

High Hb F levels are linked to several SNPs in the *HBG1* and *HBG2* genes. Particularly, the SNPs in the rs4671393 (G>A) and rs766432 (A>C) areas are linked to high levels of hemoglobin F (Thein *et al*., 2009).

Regardless of the mutation, the gene Reviews team's thorough assessment found that patients with thalassemia major have relatively low levels of Hb, MCV, and MCH. They noted that the average Hb level is < 7 g/dL, the MCV ranges from 50 to 70 fL, and the MCH level is between 12 and 20 pg. These data demonstrate that the HbF concentration in β-thalassemia patients ranges from 50% to 98% depending on the situation (i.e., based on the type of mutation).

According to another study, β-thalassemia individuals with homozygous or compound heterozygous mutations have HbF levels of between 70 and 90% (Rujito *et al*., 2016).

β-thalassemia patients is an adaptation to, or compensation for, the variable reductions in HbA and HbE levels brought on by cryptic mutations. Maintaining a high level of γ-globin chains and speeding up erythropoiesis are two fundamental mechanisms that contribute to this (Rees *et al*., 1999).

In a recent investigation, significant amounts of γ- globin chain were observed as a result of suppression or changes in the *BCL11A* protein-coding genes and other transcription factors, such as EKLF and GATA1 (Satta *et al*., 2012).

Regarding the second process, erythrocyte cells are more prevalent in peripheral blood circulation due to overexpression of erythropoiesis (Rahim *et al*., 2013).

Sequence results in exon 3 of the *HBB* gene show that it contains many point mutations in thalassemia patient as well as deletion or insertion type mutations. Thein in 2013 states that the range of β-thalassemia mutations in the Arab region is quite diverse and primarily share with other ethnic groups. The type of β-thalassemia allele (β^0, β^+, β^{++}) is the main factor in determining the severity of the phenotype, many Arab mutations that impact transcription, RNA processing, and translation are identified.

Numerous different mutations are identified to have an impact on mRNA processing. β^0 phenotype can emerge from point mutations that impair normal mRNA splicing, such as substitutions that affect the invariant dinucleotides GT or AG (Catterall, 1984).

The promoter elements necessary for controlling the production of genes are located in the (50-300) region where the mutation occurs. There are several reported frameshift mutations in, compound heterozygous, homozygous and heterozygous states. The majority of these mutations results in stop codons, which cause the major phenotype known as β-thalassemia major (Fattoum *et al*., 2004).

Sequence variations account for more than 90% of β-thalassemia cases compared to α-thalassemia cases. More than 280 sequence variations have been related to β-thalassemia, and some cases of the disorder are caused by gene deletions involving the *HBB* gene (Huisman *et al*., 2013).

But occasionally, individuals with a single *HBB* gene mutation in every cell experience mild anemia; these individuals are referred to as having thalassemia minor (Qurat-ul-Ain *et al*., 2011).

The *HBB* gene lead to a complete absence or decrease in β- chains. Which leads to excess of α-chains, Because α- and non-α chains pair with each other at a ratio close to 1:1 to form normal Hb, the excess unmatched α chains accumulate in the cell as an unstable product, leading to cell destruction in the bone marrow and in the extramedullary sites (Taher *et al*., 2018).

This process is referred to as ineffective erythropoiesis (IE) and is the hallmark of β-thalassemia. The excess α-chain precipitation in the red cell membrane causes structural and functional alterations with changes in deformability, stability, and red cell hydration (Hassan *et al*., 2016).

Alterations of erythroid precursors result in an enhanced rate of apoptosis, which is a programmed cell death. Apoptosis could contribute significantly to ineffective erythropoiesis and occurs primarily at the polychromatophilic erythroblast stage.

The ineffective erythropoiesis (IE) and anemia have several consequences producing the clinical picture of the disease. The first response to anemia is an increased production of erythropoietin, causing a marked erythroid hyperplasia, which may range between 25 and 30 times normal. Anemia may produce cardiac enlargement and sometimes severe cardiac failure (Schrier, 2002).

Increased erythropoietin synthesis may stimulate the formation of extramedullary erythropoietic tissue, primarily in the thorax and paraspinal

region. Marrow expansion also results in characteristic deformities of the skull and face, as well as osteopenia (Rivella, 2012).

High levels of iron, closely associated with denatured hemoglobin, have been found in the membrane of β-thalassemia red cells (Grinberg *et al*., 1995) .

Severe IE, chronic anemia, and hypoxia also cause increased gastrointestinal (GI) tract iron absorption. This is combined with increased iron from the breakdown of RBCs and the increased iron introduced into the circulation by the transfusions necessary to treat thalassemia, plus inadequate excretory pathways lead to progressive deposition of iron in tissues and hemosiderosis occurs eventual tissue damage, organ dysfunction, and death (Rivella, 2012).

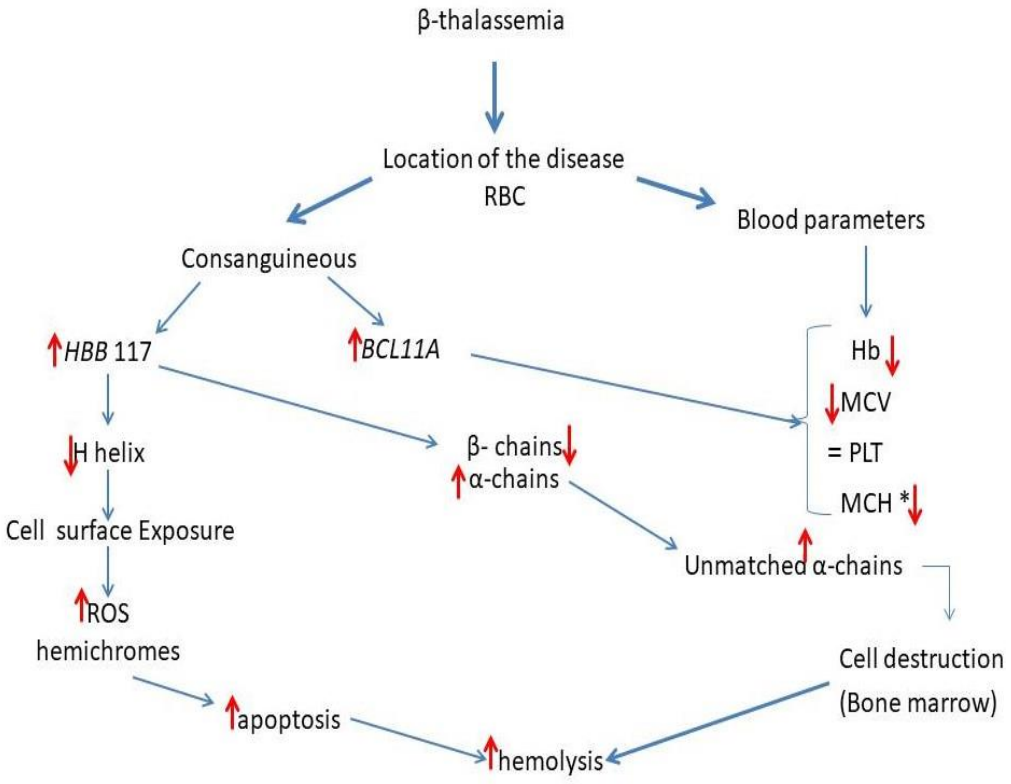


Figure 5-1: Ineffective erythropoiesis in thalassemia, either BCL11A or HBB gene mutation will lead to red blood cell distraction and apoptosis ending with hemolysis and death.

In brief, after conducting the polymerase chain reaction and electrophoresis, a positive result for the *HBB* gene (bands) appeared in the samples of patients with thalassemia major, intermedia, and minor. The result was negative; bands did not appear in the control samples. The reason for the absence of bands in the control sample is due to the *HBB* primer sequence used in the experiment, specifically reverse primer.

The reverse primer was bound to nucleotides from 64061 to 64084 in exon 3 of the *HBB* gene, and these nucleotides do non-coding protein. Sequence results show that this region contained two unregistered mutations present in all patients' samples. The first is T64064G and the second is 64073 delT. This explains the efficiency of this primer in the accurate diagnosis of thalassemia. A result which can be employed to be an indicator for the diagnosis of β-thalassemia disorder.

Chapter Six
Conclusions and Recommendations

6- Conclusion and Recommendation

6-1- Conclusion

1. The results of *BCL11A* showed three genotypes AA, AC, and CC in β-thalassemia and No significant differences between the genotype distribution of β-thalassemia patients and controls.
2. Using the Hardy Weinberg, the results of the *BCL11A* gene showed that patient group was under this equilibrium which indicate this disease genotype transfer independently among generation and not affected by location ethical group.
3. Nucleotide sequencing analysis showed 2 SNPs mutations in the *BCL11A* gene and 21 mutations in *HBB* gene.
4. The result of PCR amplification by using HBB primer was positive in patients and negative in controls, and confirmed by using an internal control primer (GAPDH). A result which can be employed to be an indicator for the diagnosis of β-thalassemia disorder.
5. The incidence of β-thalassemia was closely related to consanguinity marriage especially in first cousin marriage which it is predominant in our society.

6-2- Recommendation

1. Conducting an examination to detect thalassemia in newborns and making it one of the requirements for obtaining the child's personal identity to limit the spread of this disease, especially for those who have this characteristic. To create new data for the next generation to diagnose who is infected, who is a carrier of the disease and who is healthy. Because the examination before the marriage contract does not limit the spread of thalassemia.
2. Using the result obtained selecting the primer *HBB* gene as an indicator for diagnosing thalassemia in laboratories because it is most accurate and less expensive and timely.
3. Using the genetic data obtained in our study as a database for further studies on thalassemia.
4. Conducting future studies on α-thalassemia and other homological diseases in maysan province.

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Appendixes

Appendix 1: Sample questionnaire that was used in our experiment.

الاسم : رقم العينة : رقم الاضبارة :

نوع المرض : فصيلة الدم :

العنوان : رقم الهاتف : المواليد :

عدد افراد العائلة : ذكور : اناث :

ترتيب الشخص بالعائلة : هل لديه اخوه مصابون : عددهم :

هل لدية اقرباء مصابون : عددهم :

هل توفيه احد افراد العائلة بنفس المرض : متى :

هل اصيب بالتهاب الكبد الفايروسي : هل عولج : اين :

محل الاقامة : مركز قضاء الجنس : ذكر انثى الحالة الاجتماعية: اعزب متزوج

صلة القربى بين الوالدين : اقرباء غير اقرباء صلة القربي حسب الفئات :

التحصيل الدراسي : حالة العمل : موظف رعاية متقاعد عاطل

نوع اللقاح : التهاب كبد مكورات عنقودية

مضاعفات المرض : تضخم طحال سكري غدد درقية فوق الدرقية تأخر نمو وهن العظم وفاة

كمية العلاج : بدون علاج نقل دم نقل دم مع علاج دسيفرال اكسجيد

دسفبرال+اكسجيد 125 دسيفرال +اكسجيد 500

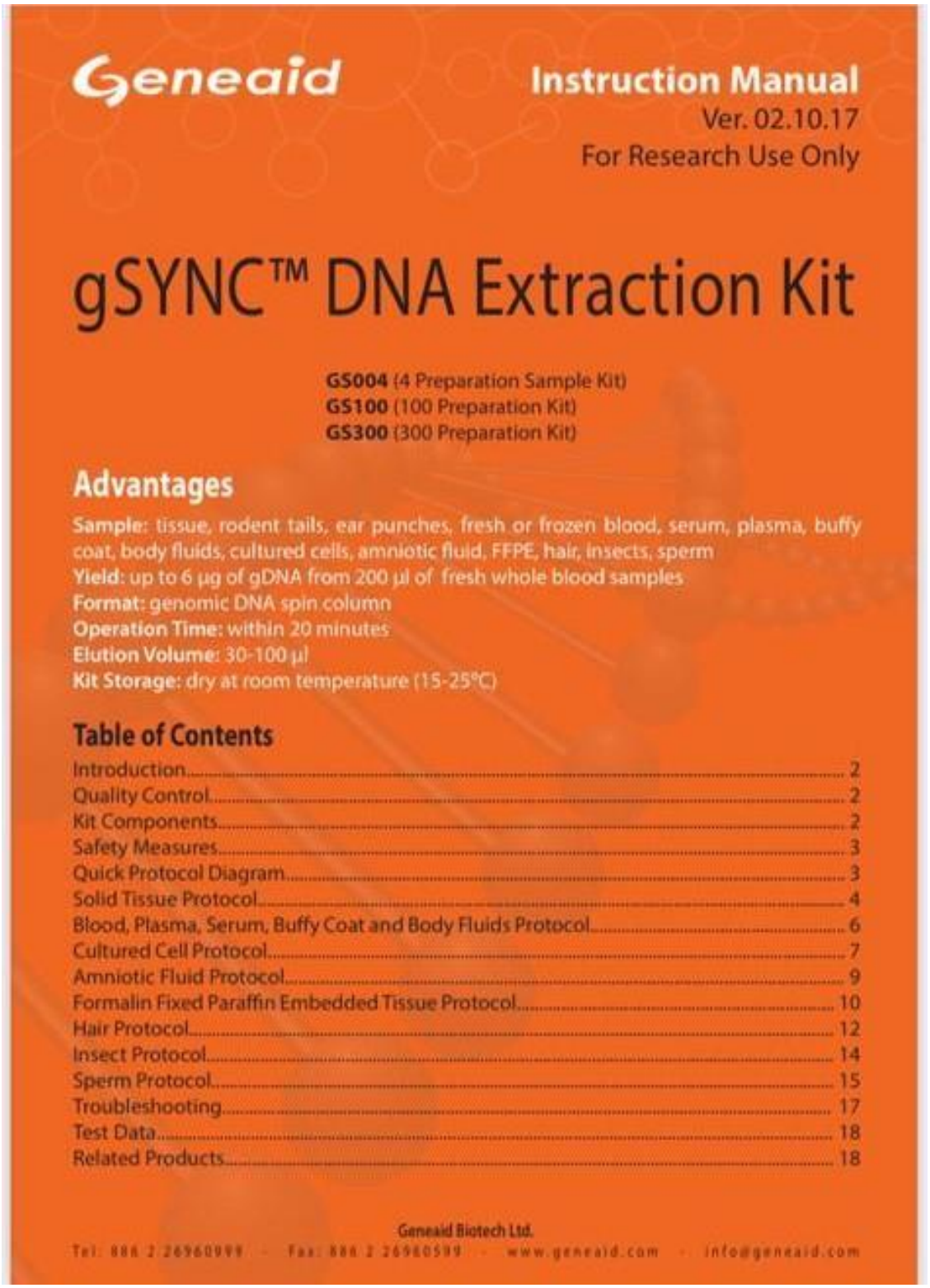
العمليات الجراحية : رفع طحال زرع نخاع عظم اخرى لاتوجد

Appendix 2: The component of Go Taq ® Green Master Mix kits that used in the present study.

The component of the Go Taq ® Green Master Mix

1	Go Taq ®DNA polymerase	2X
2	Green Go Taq ® Reaction Buffer (PH 8.5)	1X
3	µM dATP, µM dGTP, µM dCTP , µM dTTP	400µM for each
4	Mgcl2	3mM
5	Yellow and blue dyes	Trace

Appendix 3: The kit used to DNA extraction and procedure.



Geneaid

Instruction Manual
Ver. 02.10.17
For Research Use Only

gSYNC™ DNA Extraction Kit

GS004 (4 Preparation Sample Kit)
GS100 (100 Preparation Kit)
GS300 (300 Preparation Kit)

Advantages

Sample: tissue, rodent tails, ear punches, fresh or frozen blood, serum, plasma, buffy coat, body fluids, cultured cells, amniotic fluid, FFPE, hair, insects, sperm
Yield: up to 6 µg of gDNA from 200 µl of fresh whole blood samples
Format: genomic DNA spin column
Operation Time: within 20 minutes
Elution Volume: 30-100 µl
Kit Storage: dry at room temperature (15-25°C)

Table of Contents

Geneaid Biotech Ltd.
Tel: 886 2 26960999 · Fax: 886 2 26960599 · www.geneaid.com · info@geneaid.com

1. Sample Preparation

Transfer up to 200 µl of whole blood, serum, plasma, buffy coat or body fluids to a 1.5ml microcentrifuge tube. Adjust the volume to 200 µl with PBS. Add 20 µl of Proteinase K then mix by pipetting. Incubate at 60ºC for 5 minutes. NOTE: Fresh blood is recommended. However, frozen or blood treated with anticoagulants (EDTA etc.) Can also be used. Increased storage length decreases DNA yield. If using nucleated blood (e.g. bird or fish) use up to 10 µl then adjust volume to 200 µl with PBS.

2. Cell Lysis

Add 200 µl of GSB Buffer then mix by shaking vigorously. Incubate at 60ºC for 5 minutes, inverting the tube every 2 minutes.

NOTE: It is essential that the sample and GSB Buffer are mixed thoroughly to yield a homogenous solution. Inverting the sample occasionally during incubation will facilitate Proteinase K digestion and cell lysis. Using an auto shaking system is more convenient when incubating samples. During incubation, transfer the required volume of Elution Buffer (200 µl/sample) to a 1.5 ml microcentrifuge tube and heat to 60ºC (for Step 5 DNA Elution).

Optional RNA Removal Step for RNA-free gDNA, following GSB Buffer addition and 60ºC incubation, add 5 µl of RNase A (50 mg/ml) and mix by shaking vigorously. Incubate at room temperature for 5 minutes to ensure efficient RNA degradation

3. DNA Binding

Add 200 µl of absolute ethanol to the sample lysate and mix IMMEDIATELY by shaking vigorously for 10 seconds. If precipitate appears, break it up as much as possible with a pipette. Place a GS Column in a 2 ml Collection Tube. Transfer all of the mixture (including any insoluble precipitate) to the GS Column. Centrifuge at 14-16,000 x g for 1 minute.

Following centrifugation, if the mixture did not flow through the GS Column membrane, increase the centrifuge time until it passes completely. Discard the 2 ml Collection Tube containing the flow-through then transfer the GS Column to a new 2 ml Collection Tube.

NOTE: It is important that the lysate and ethanol are mixed thoroughly to yield a homogeneous solution.

4. Wash

Add 400 µl of W1 Buffer to the GS Column. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through. Place the GS Column back in the 2 ml Collection Tube. Add 600 µl of Wash Buffer (make sure absolute ethanol was added) to the GS Column. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through. Place the GS Column back in the 2 ml Collection Tube. Centrifuge for 3 minutes at 14-16,000 x g to dry the column matrix.

NOTE: Additional centrifugation at 14-16,000 x g for 5 minutes or incubation at 60ºC for 5 minutes will completely dry the GS Column to avoid any residual ethanol carryover and ensure the most effective downstream applications.

5. Elution

Standard elution volume is 100 µl. If less sample is to be used, reduce the elution volume (30-50 µl) to increase DNA concentration. If higher DNA yield is required, repeat the DNA Elution step to increase DNA recovery and the total elution volume to approximately 200 µl.

Transfer the dried GS Column to a clean 1.5 ml microcentrifuge tube. Add 100 µl of pre-heated Elution Buffer1, TE Buffer2 or water3 into the CENTER of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer or water to be completely absorbed. Centrifuge at 14-16,000 x g for 30 seconds to elute the purified DNA:

- Ensure that Elution Buffer (10 mM Tris-HCl, pH8.5 at 25ºC) is added into the center of the GS Column matrix and is completely absorbed.
- Using TE (10 mM Tris-HCl, 1 mM EDTA, pH8.0) for elution is beneficial as EDTA preserves DNA for long term storage. However, EDTA will affect PCR and other sensitive downstream applications. Ensure that TE is added into the center of the GS Column matrix and is completely absorbed.
- If using water for elution, ensure the water pH is between 7.0 and 8.5. ddH_2O should be fresh as ambient CO2 can quickly cause acidification. Ensure that water is added into the center of the GS Column matrix and is completely absorbed. DNA Eluted in water should be stored at -20ºC to avoid degradation.

Appendix 4: Extraction of PCR product from the gel agarose

Agarose gel extraction was used to isolate and purify the amplified specific gene (PCR product). Desired DNA fragment was extracted from gel by using E. Z. N. A ®

Gel Extraction kit according to company instructions (OMEGA Bio-TEK/USA), as follows with some modification in time:

1. Agarose powder (0.6 gm) was added to 50 ml of 1X TBE buffer and heated with microwave until all agarose particles were dissolved and become clearness, 2.5 µl of ethidium bromide were added to the agarose solution. The agarose was stirred in order to get mixed, the agarose solution was put into gel tray, and the agarose was allowed to solidify at room temperature for 30 minutes. The fixed comb was carefully removed and the gel tray was carefully placed in the gel tank. The tank was filled with 1X TBE buffer until the buffer reached 2 mm over the surface of the gel.
2. PCR product mixture of the specific gene (5 µl) was loaded into the wells of agarose gel. Electric current allowed at 65-70 volts for 30 min-1 hour.
3. Finally, the bands were visualized on a UV transilluminator at wave length 350 nm.
4. Carefully excised the DNA fragment of interest by using a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.
5. The volume of the gel slice was determined by weighing a clean 1.5 ml microcentrifuge tube. The volume of gel was derived as follows: The difference between the weights of the Eppendorf tube containing gel slice minus the weight of empty Eppendorf tube (weight of the Eppendorf tube containing gel slice - weight of empty Eppendorf tube). The numbers were rounded to the nearest integer number and the integer number was converted to microliter unit.
6. Added 1 volume Binding Buffer (XP2) to Eppendorf tube.
7. The tubes were incubated at 60 ºC for 7 minutes or more until the gel has

Completely melted. Shake the tube every 2-3 minutes.

8. A HiBind DNA Mini colum was placed in a 2ml collection tube.
9. Added no more than 700 µl DNA/agarose solution from step 6 to the HiBind DNA Mini column.
10. The tubes were placed in centrifuge at 10,000 rpm for 1 minute at room temperature.
11. Discard the filtrate and reuse the collection tube.
12. Repeated steps (8-10) until all of the sample has been transferred to the column.
13. Added 300 µl Binding Buffer (XP2).
14. The tubes were placed in centrifuge at 14,000 rpm for 1minute at room temperature.
15. Discard the filtrate and re use collection tube.
16. Added 700 µl SPW wash Buffer.
17. The tubes were placed in centrifuge at 14,000 rpm for 1 minute at room temperature.
18. Discard the filtrate and reuse collection tube.
19. The empty HiBind DNA Mini column was centrifuge for 2 minutes at maximum speed to dry the column matrix.
20. The HiBind DNA Mini column was transferred to the clean 1.5 ml microcentrifuge tube and was labeled.
21. Added 30 µl of Elution Buffer directly to the mid of the column.
22. We waited for at least 30 minutes or a little more to allow the Elution buffer to be completely absorbed at room temperature.
23. The column tubes were placed in centrifuge at 14,000 rpm for 1 minute. 24- Finally, the DNA (Purified gene) was stored at -20 ºC.

Appendix 5: General characteristics of the blood patients with β- thalassemia.

Characteristic	Type	Total	Percentage
Sex	Male	58	58%
	Female	42	42%
Relationship of parents	Relative	88	88%
	Not relative	12	12%
Location	City center	29	29%
	Distract	71	71%

الخلاصة

الخلاصة

أجريت الدراسة الحالية للكشف عن تعدد الأشكال الوراثية لعينات مرضى الثلاسيميا ومقارنتها مع تعدد الأشكال الوراثية للأشخاص الأصحاء والتنبؤ بشدة مرض الثلاسيميا.

تم إجراء الدراسة الحالية من أجل معرفة وتوضيح التشكلات الوراثية لبعض الجينات التي لها علاقة في الإصابة بالثلاسيميا (*HBB* and *BCL11A* genes). في تشرين الثاني من عام 2021 تم جمع عينات الدم من مركز أمراض الدم التابع لمستشفى الصدر التعليمي - مديرية صحة ميسان في محافظة ميسان جنوب العراق. حيث بلغ عدد العينات 100 عينة لاشخاص مصابين بالثلاسيميا إضافة إلى 40 عينة كانت مجموعة المقارنة لأشخاص اصحاء ليس لهم تاريخ عائلي للإصابة بالثلاسيميا. تم إجراء مسحة الدم وتعداد الدم الكامل على عينات المرضى ومجموعة المقارنة وبعد ذلك تم استخلاص الحمض النووي لدراسة تعدد الأشكال الوراثية في الاشخاص المصابين بمرض الثلاسيميا.

أظهرت نتائج ARMS-PCR وجود أليلين A و C بالإضافة إلى ثلاثة طرز وراثية AA و AC و CC في ثلاسيميا بيتا بتكرار 0.3 و 0.6 و 0.1 على التوالي وفي مجموعة المقارنة 0.1 و 0.8 و 0.1 على التوالي. تكرار الأليلين A و C في المرضى (0.56) A، C (0.437). أما في مجموعة المقارنة A(0.52) ، C (0.47). لا توجد فروق ذات دلالة إحصائية بين توزيع النمط الجيني لمرضى الثلاسيميا بيتا ومجوعة السيطره تحت مستوى احتمالية $p < 0.05$ حيث وصلت إلى قيمة p (0.220).

كما تم استخدام PCR-RAPD-PCR واستخدمت أربعة بادئات (APAA11، APU15، APAA17 ، و APD18) للكشف عن تعدد الأشكال الوراثي لجينات *BCL11A* للمقارنة بين مرضى الثلاسيميا ومجموعة التحكم.

أظهرت النتائج أن عدد الحزم (bands) في مرضى الثلاسيميا هو أكثر من المجموعة الضابطة كذلك هناك أختلاف في حجومها الجزيئية وفقًا لنوع البادئ المستخدم.

أظهرت النتائج بأن الحزم الاحادية (monomorphism) و الحزم الفريدة (unique) و الحزم المتعددة (polymorphism) في جين *BCL11A* في عينات المرضى كان مجموعها الكلي 10 و 17 و 6 على التوالي كذلك كان مجموعها 13 و 13 و 5 على التوالي في المجموعة المقارنة.

باستخدام برامج المعلوماتية الحيوية تم تحليل تتابعات النيوكليوتيدات او مايعرف ب Nucleotide sequencing حيث تم استخدام المركز الوطني للمعلومات التقنيات الإحيائيه أو ما يعرف ب NCBI لمقارنة التسلسلات لدينا مع التسلسلات القياسية المنشورة في هذا المركز.

فيما يتعلق بتسلسلات النوكليوتيدات لجين HBB ، كان عدد الطفرات 21 طفرة في هذا الجين .

اما في تسلسل النيوكلوتيدات لجين الـ BCL11A كان عدد الطفرات اثنان ، واحدة منها هي BCL11A rs766432A> C حيث تم العثور عليها في جميع المرضى في موقع (A65664C) .

يذكر بأن HBB primer أعطى حزم في المرضـــى ولم يعط حزم في مجموعة المقارنة ، بمعنى آخر ، كانت نتيجة تضـــخيم PCR إيجابية في المرضـــى وســـلبية في المجموعة المقارنة هذه النتيجة يمكن استخدامها كطريقة في تشخيص الثلاسيميا .

وزارة التعليم العالي والبحث العلمي
جامعة ميسان
كلية العلوم
قسم علوم الحياة

تعدد الاشكال الوراثية في مرضى الثلاسيميا الحاد في ميسان

دراسة مقدمة

الى مجلس كلية العلوم / جامعة ميسان

وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

زهراء قاسم موسى

بكلوريوس تربية / علوم الحياة (2011)

بأشراف

أ.م.د. ميثم عبد الكاظم دراغ

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