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Association between Hemochromatosis *HFE*, *HAMP*, *TFR1* and *TFR2* Genes Variants and Iron Overload of Beta Thalassemia Patients

A thesis submitted to the council of College of Science / Al Muthanna
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Master of Science in Biology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿وَأَنْزَلْنَا الْحَدِيدَ فِيهِ بَأْسٌ شَدِيدٌ وَمَنْفَعٌ لِلنَّاسِ﴾

Supervisor's Certification

I certify that the thesis entitled (**Association between Hemochromatosis *HFE*, *HAMP*, *TFR1* and *TFR2* Genes Variants and Iron Overload of Beta Thalassemia Patients**)

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Has been prepared under my supervision at the College of Sciences / Al-Muthanna University as Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology.

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In view of the available recommendations, I forward this thesis for debate by the Examining Committee.

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Dedication

This thesis is dedicated to The sake of Allah, my Creator and my Master, My great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life.

To my mother and father; whom my good example to be followed, my wife and my hope in my life everything in his life for us, and to all those, who encouraged and stand beside me in all my life stages Definitely, I am not forgetting my sons, my brothers, my sisters and my friends whom I am having a good and happy life with them.

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Abstract

Thalassemia is an inherited blood disorder in which the body makes an abnormal form of hemoglobin. It is two types α and β according to hemoglobin chain defect. Treatment of β thalassemia depends on recurrent blood transfusion that leads to increased blood ferritin level. β thalassemia disease and hemochromatosis may be compatible in the same patients due to defects in the iron metabolism mechanism leading to iron overload. Many mutations may have an incidence of thalassemia and hemochromatosis. Much more iron is harmful to the body and can cause tissue damage over time. This study was aimed to determine the effect of some mutations (C282Y, H63D mutations in the *HFE* gene, G71D mutation in the *HAMP* gene, G142S mutation in the *TFR1*, and Y250X mutation in *TFR2* gene) on blood ferritin levels and iron overload in β thalassemia patients. Eighty-four samples (21 β thalassemia patients, 42 parents as carriers, and 21 healthy controls) were included in this study. The period of collected samples was from January to April 2020. The genotyping of genes mutations was conducted by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method. The mean of serum ferritin levels in β thalassemia patients were 1810.5 ng /ml and the mean of hemoglobin in β thalassemia patients was 8.2 g/dl. as well MCV, MCH was (81.5 fL, 25.9 pg) respectively. The C282Y mutation was not found in all 84 samples, for H63D mutation was nonsignificant between iron overload and β thalassemia patients and parents the P-value was ($p=0.08$, $p=0.74$) respectively. In addition, a nonsignificant association of G71D mutation between iron overload and β thalassemia patients but significant with parents, the P-value were ($p=0.44$, $p<0.001$) respectively. The G142S mutation was significant for patients and nonsignificant for parents, to the association between iron overload and β thalassemia, the P-value were ($p=0.02$, $p=0.47$) respectively and finally, the Y250X mutation was not found in all groups. The β thalassemia patient in Samawah city has a significant effect of iron overload with G142 but nonsignificant with (H63D, G71D) mutation. On the other hand (C282Y, Y250X) mutations were not found in all groups of this study.

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List of Abbreviations

Abbreviations	Whole phase
bp	Base pairs
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HAMP	Hepcidin antimicrobial peptide
HB	hemoglobin

HFE	High Fe ²
HPLC	High-performance liquid chromatography
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain Reaction
REF	Reference
RFLP	restricted fragment lengths polymorphism
SNPs	Single Nucleotide Polymorphisms
TBE	Tris base/ Boric acid / EDTA
TFR1	transferrin receptor 1
TFR2	transferrin receptor 2
UV	Ultraviolet

Chapter One

Introduction

1.1. Introduction

β Thalassemia is a group of hereditary blood disorders that results from reducing in hemoglobin chain synthesis. Two genes code for the beta-globin chain, which is caused by a lack of synthesis of the β globin chains. As a result, a defect in each of these chains leads to hemolysis and a decline in erythropoiesis. β Thalassemia can be classified into three types, major β thalassemia that requires of regular procedure of blood transfusion for a lifetime and moderate β thalassemia described mild symptoms of the patient that require partial therapeutical blood transfusion and finally minor is described as the carrier state of disease that usually does not require medical therapy. Childhood thalassemia requires transfusions for the rest of one's life (Alli *et al.*, 2021). Recurrent blood transfusions in patients with β thalassemia cause an accumulation of extra iron in the blood leading to damages body tissues that mean a great one of important complications of blood transfusion, as subject to therapy by a chelating agent to reduce the iron level in the body (Isidori *et al.*, 2020).

On the other hand, iron overload is a disorder of iron metabolism characterized by increased iron absorption. Several mutations are responsible for the majority of hereditary hemochromatosis cases. It is an autosomal recessive disease in which the body stores over the limit of iron. A genetic defect in the *HFE*, *HJV*, *HAMP*, *TFR2*, or *SLC40A1* gene causes hereditary hemochromatosis (HH). In HH, iron is continuously absorbed in the upper part of the small intestine, resulting in an increase in the overall volume of iron in the body. however the body has no pathway of removing the extra iron, it can eventually accumulate in different tissues. The accumulation of iron in tissues causes disorders such as diabetes and cirrhosis (Zhang *et al.*, 2019). While

repetitive blood transfusions cause iron overload in transfusion-dependent thalassemia patients, other factors can raise iron absorption within the body, one of the mutations can cause hemochromatosis. Association hereditary hemochromatosis and beta-thalassemia can be inherited together, the risk of iron overload is increased, which leads to a variety of endocrine disorders, heart failure, hepatic cirrhosis, diabetes mellitus, and even death (Zhang *et al.*, 2019). The most common type of hereditary hemochromatosis is caused by mutations in the HFE gene, HAMP gene, TfR1 gene, and TfR2 gene which are the subjects' objectives in this study.

In Iraq, the Ministry of Health records an increase in thalassemia cases, The prevalence of thalassemia had increased from 33.5/100,000 in 2010 to 37.1/100,000 in 2015. (Kadhim *et al.*, 2017).

1.2. Aims of the study

This study was conducted to fulfill the following objectives

1. To determine the mutation of β thalassemia patients and parents as a genetic disease in Al Muthanna province; Two points mutation (C282Y, H63D) on the *HFE* gene and G71D mutation on the *HAMP* gene.
2. To evaluate mutations on both transferrin receptors; *TFR1* (G142Y), *TFR2* (Y250X).
3. To detect the relationship between related mutation and iron overload (hemochromatosis) in β thalassemia patients.

Chapter Two

Literature Review

2. Literature Review

2.1. Thalassemia

In 1932, George Whipple and William Bradford described the word thalassemia, which is made up of two sections. The Greek word for sea is thalassa, and the Greek word for blood is haema (Latin: anemia). Mediterranean anemia was the term applied to it just because the individual who were affected were of Mediterranean ancestry. It refers to a category of genetic blood disorders that cause anemia as a result of increased RBC destruction (Ghodekar *et al.*, 2010).

Mediterranean anemia is a blood disorder that is inherited as an autosomal recessive condition. It is caused by a defect (absence or reduction) in the synthesis of globin chains, which are used to produce hemoglobin. The two most common forms of thalassemia (α and β) are caused by a mutation in the beta and alpha globin chains. The beta globin gene is coded by two genes, while the alpha globin chain is coded by four genes. Thalassemia can be caused by mutations in these genes (Wahed *et al.*, 2019). Beta thalassemia causes a genetically defective synthesis of the beta globin chain of hemoglobin. β globin is expressed by a structural gene arranged in a cluster with the other β like genes spread about 70 Kb on chromosome 11 at short arm (11p15.4). (Thein S. L. 2005). β Thalassemia is a group of diseases with declining or absent synthesis of β globin chains resulting in reduced production of adult hemoglobin (HbA), thalassemia is a form of anemia. The form of allele (β^0 , β^+) the group of inherited hemoglobin disorders characterized by a decreased synthesis (β^+ thalassemia) or absence (β^0 thalassemia) of beta globin chain formation, which induces anemia is the primary determinant of beta thalassemia severity (Thein S. L. 2005).

Table (2-1) percent of hemoglobin types in newborn and adult(Perumbeti A. 2014).

Hemoglobin	Chain phenotype	Newborn level (%)	Adult level (%)
HbF	$\alpha 2\gamma 2$	50-95	0-1.5
HbA	$\alpha 2\beta 2$	5-15	85-95
HbA2	$\alpha 2\delta 2$	0	2.3-3.3

β Thalassemia is genetic of diseases characterized by diminished or absent β globin chain synthesis, leading to decreased development of adult hemoglobin (HbA) and an abundance of α globin chains, a condition known as (α - β) globin chain imbalance (Perumbeti A. 2014) Table (2-1) show percent of hemoglobin types in newborn and adult. β globin is part of the multi gene β like globin cluster (5'- ϵ - γ - δ - β -3') on the human short arm of chromosome 11. Switching β like globin are formed at different stages of fetal erythropoietic development to result in the production of the $\alpha 2\beta 2$ heterotetramers (HbA). Deletions in β thalassemia are uncommon therefore most common of β thalassemia are caused by nondeletional point mutations in the β like globin cluster. Mutations in transcription, mRNA processing, translation, and posttranslational stability can reduce β globin incorporation into hemoglobin and result in β thalassemia. An increase in hemoglobin A2 ($\alpha 2\delta 2$) can be suggestive of some β -thalassemia phenotypes (Figure 2-2). (Perumbeti A. 2014)

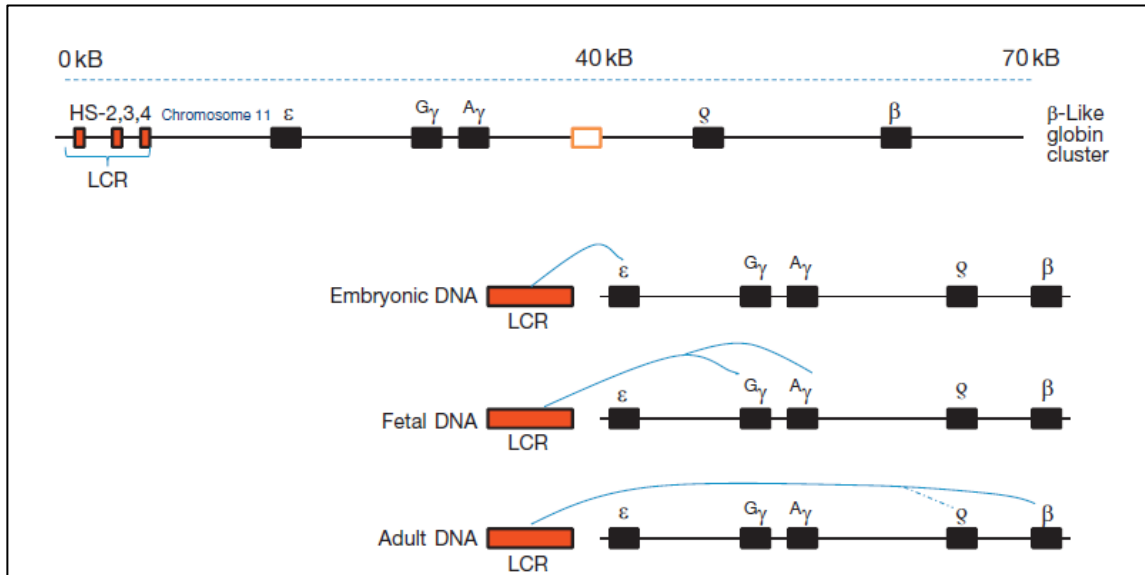


Figure (2.1) Switching β like globin are formed at different stages of fetal erythropoietic development, the top β globin locus on chromosome 11. Filled boxes indicate transcribed genes. The bottom panel indicates the locus control region (LCR) and illustrates the LCR interaction with different downstream β -like globin genes, during different stages of human development. (Perumbeti A. 2014)

Erythropoiesis starts in the yolk sac where erythroid cells contain primarily embryonic globin. At the fifth week of gestation, erythropoiesis shifts from the yolk sac to the fetal liver, and globin production switches from embryonic to fetal globin. Then, at the 20th week, erythropoiesis begins shifting from the fetal liver to the bone marrow, along with globin switching from fetal globin to adult globin (figure 2-1) (Perumbeti A. 2014)

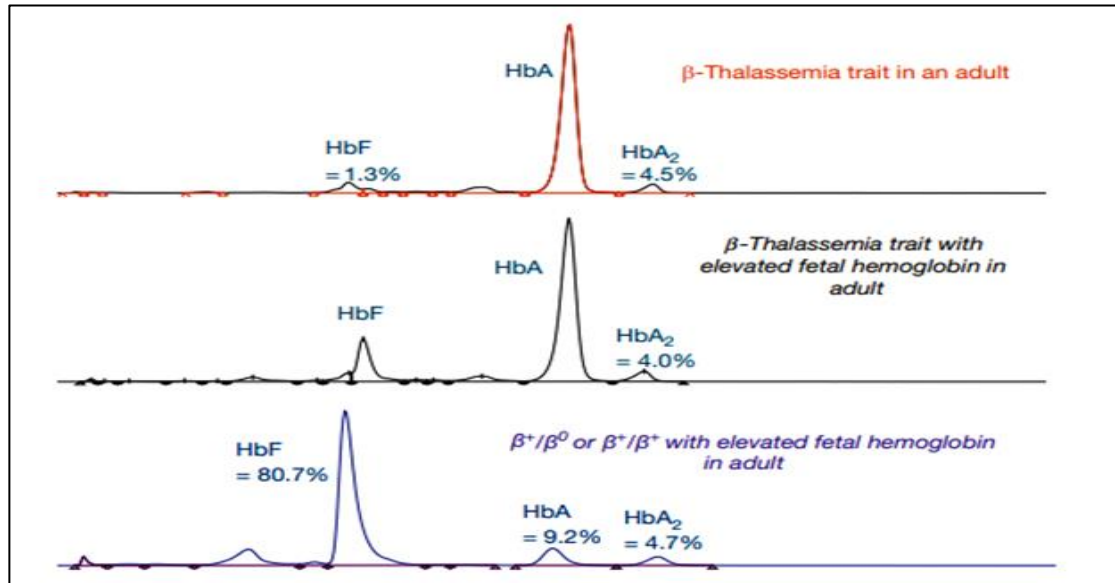


Figure (2-2) HPLC of an adult patient with β -thalassemia trait with elevated HbA₂, a parent with β thalassemia trait with a Hereditary persistence of fetal hemoglobin haplotype, and a progeny with β thalassemia major with Hereditary persistence of fetal hemoglobin. Note the presence of HbA₂ in the bottom HPLC indicates the preservation of γ -globin locus. Courtesy of Lana S. Weckbach (Perumbeti A. 2014)

2.1.1. Type of β thalassemia

Three forms of beta thalassemia can be distinguished; Firstly Thalassemia trait. Patients with this state have one abnormal gene and one normal gene, and they can have moderate anemia but are not transfusion dependent. The presence of HbA₂ in concentrations greater than 3.5 percent is the identifying characteristic. Microcytic hypochromic red cells with moderate anemia should be visible in the CBC results. The RDW (red cell distribution width) has not changed substantially. When compared to hemoglobin levels, the RBC count is disproportionately higher. The second type of β Thalassemia intermediate is characterized by two deficient genes, but these individuals also contain certain beta globin. However, certain individuals may have severe health conditions that necessitate transfusions on a routine basis. And the last one β

Thalassemia major (Cooley's anemia) caused by two mutated genes that have virtually no activity, resulting in no beta globin synthesis. These individuals have a serious type of illness that necessitates lifelong blood transfusions and may have a shorter lifespan. HbF levels are higher in these individuals. HbA2 levels may not be elevated in all cases of beta thalassemia major. (Weatherall and Clegg., 2008).

Where a deficient gene is unable to produce any β globin, it is referred to as a " β^0 " gene, resulting in a more advanced type of beta thalassemia. If the mutant gene will still operate, it is referred to as a " β^+ " gene. β thalassemia minor (trait; patients β^+/β or β^0/β) is seen in one gene mutation, and individuals are either normal or slightly anemic. HbA2 levels are higher in these patients. Furthermore, HbF levels can be increased. While MCV and MCH levels are reduced, these patients do not need blood transfusions. (Thein S. L. 2005)

The disorder is known as β thalassemia major (also known as Cooley's anemia) when both genes are a failure in significantly defective beta globin synthesis (β^0/β^0 or β^+/β^0). Illnesses of beta thalassemia major, on the other side, do not appear before 6 months of age due to the presence of fetal hemoglobin. HbA2 and HbF levels are elevated in beta thalassemia major patients (in some time individual HbA2 maybe not changed). The condition is known as β thalassemia intermediate (β^+/β^+ or β^0/β) when beta globin development is mildly disrupted. These patients have a milder version of the disorder than those of β thalassemia major. (Weatherall, D. J., & Clegg, J. B. 2008).

2.1.2. β Thalassemia diagnosis

Thalassemia character by the following clinical points

- HbA2 and/or Hb F can be higher than healthy, whereas HbA is lower.
- MCV and MCH levels are lower on a complete blood count (CBC).
- As compared to hemoglobin levels, the number of red blood cells (RBCs) in the cell will increases. blood film showed microcytic hypochromic anemia
- normal iron level (Wahed *et al.*, 2019).

RBC maturation and aging in humans are influenced by a variety of factors that occur over the course of around 120 days in a healthy individual (Lew and Tiffert., 2013; Bogdanova and Lutz., 2013). However, in the case of beta thalassemia, previous mutations in the β globin gene cause a reduction or deficiency in hemoglobin synthesis, resulting in insufficient erythrocyte development (Oikonomidou *et al.*, 2019). Blood transfusion is one of the solutions in this crucial situation for getting adequate oxygen to the tissues. Thalassemia that requires blood transfusions is also known as transfusion-dependent thalassemia (TDT). Patients with this condition have taken iron chelation therapy to avoid morbidity and mortality caused by iron overload caused by repeated blood transfusions (Oikonomidou *et al.*, 2018).

In β thalassemia major, inadequate erythropoiesis and increased iron absorption can contribute to iron overload and associated disorders as a result of repeated blood transfusions. Hemochromatosis is a common complication of beta thalassemia major (Kosaryan *et al.*, 2013).

2.1.3 Transfusion of blood

To maintain a hemoglobin level greater than 9.5 g per dL (95 g per L) and maintain normal development, individuals with beta thalassemia major need regular and lifelong blood transfusions.(Rund and Rachmilewitz., 2005), (Cazzola *et al.*, 1997). Blood transfusions may be needed as often as six months of age. The recommendation to transfuse is a more subjective health evaluation for an individual with beta thalassemia intermediate. Transfusions are only required often on a basis when individual hemoglobin is insufficient for a normal life or when anemia impairs growth and development.

2.2. Hemochromatosis

Hemochromatosis is a condition in which an individual's blood contains more normal concentration of iron. The primary *HFE* gene mutation linked to clinical symptoms was discovered in 1996. (Feder *et al.*, 1996). Hereditary hemochromatosis (HH) is an iron absorption condition caused by excessive iron intake and accumulation, which causes oxidative damage to certain organs (Hash *et al.*, 2001; Swinkels *et al.*, 2006).

Also, Iron buildups can occur unintentionally. It happens when a patient requires a repeat transfusion to treat anemia such as thalassemia. The iron in transfused RBCs overtakes around 1 mg of iron a day and should be stored. this is referred to as transfusion-related hemosiderosis. (Keohane *et al.*, 2019).

Hemochromatosis, on the other hand, maybe caused by mutations in genes that regulate iron absorption in the human body. This iron imbalance is disrupted, and the body tends to consume iron even as stocks are overloaded. This is a real case of inherited iron overload. A new technique for molecular study of the cause of an autosomal recessive disorder was identified explicitly. A mutation in the *HFE* protein was discovered at the end of the nineteenth century, and it is now thought to be one of the causes of inherited hemochromatosis (Feder *et al.*, 1996 ; Waheed *et al.*, 1997 ; Brissot *et al.*, 2010). Individuals with phenotypic disorder (excess iron accumulation in tissues) are not only *HFE* mutations identified by use adequate molecular tests. Other proteins that are now understood to play a role in iron accumulation were defined as a result of this (incessant absorption and storage). As a result, the picture of inherited hemochromatosis differed depending on the protein mutation, resulting in changes in iron regulation that manifested as various human phenotypes (Kowdley *et al.*, 2019).

2.2.1 Hemochromatosis types

2.2.1.1 *HFE* related hemochromatosis (type 1 hemochromatosis)

The *HFE* gene is composed of seven exons that extend 12 kb on chromosome 6. The protein encoded by this gene is a membrane protein that binds to β 2-microglobulin (B2M) and it is analogous to MHC class I-type proteins. It's known that this protein regulates the interaction of the transferrin receptor with transferrin to control systemic iron uptake. The production of translation is 343 amino acids coded by this *HFE* gene. A signal peptide (the protein's first part), an extracellular transferrin receptor binding region (α 1 and α 2), a fragment that resembles immunoglobulin molecules (α 3), a

transmembrane region that holds the protein in the cell membrane, and a short cytoplasmic tail are the components (Barton *et al.*, 2015) figure (2-3)

The common mutation that causes *HFE* related HH is a tyrosine to cysteine replacement at amino acid 282 in *HFE*'s $\alpha 3$ loop (C282Y), which breaks the disulfide bond in this domain figure (2-3). The depletion of this disulfide bond disrupts the association of *HFE* protein with $\beta 2$ -microglobulin (B2M), resulting in reduced appearance at the cell surface, enhanced retention in the endoplasmic reticulum, and faster breakdown in the C282Y mutant protein. The value of the *HFE* protein $\beta 2$ -microglobulin (B2M) interaction for normal *HFE* protein activity is shown by the HH like phenotype of $\beta 2$ -microglobulin (B2M) knockout mice (Barton *et al.*, 2015).

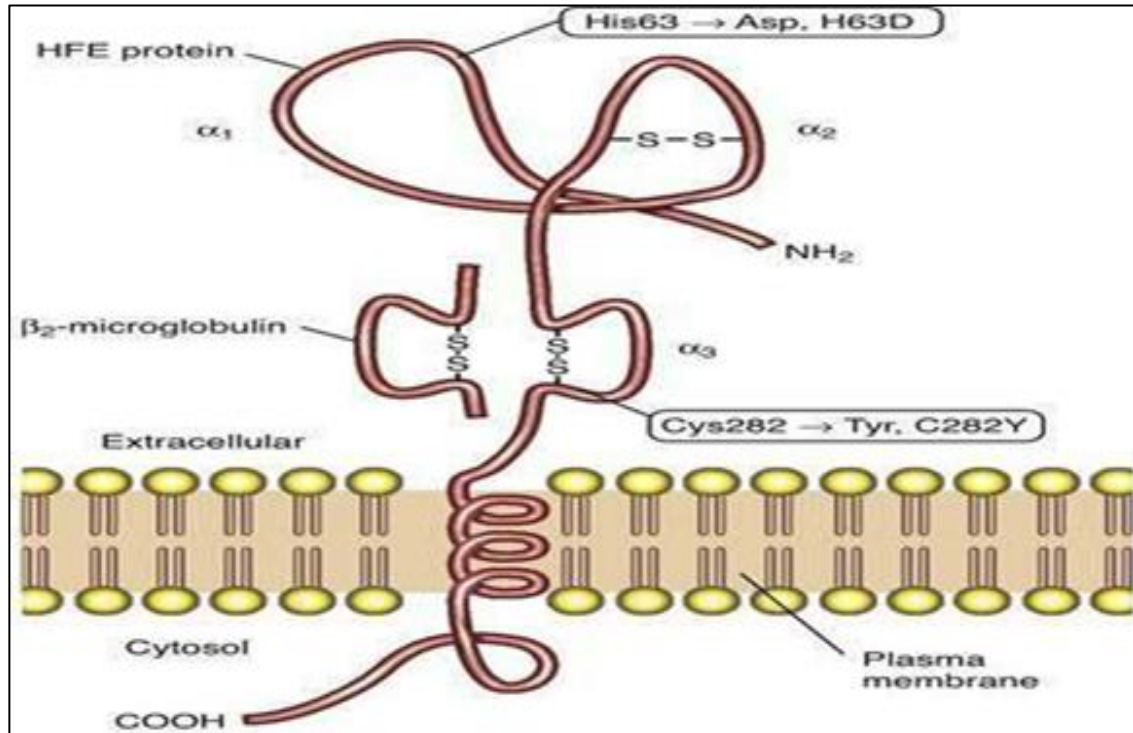


Figure 2.3 At the cell surface, *HFE* protein is seen in combination with β_2 -microglobulin. *HFE* protein has three extracellular domains, numbered α_1 , α_2 , and α_3 . The α_3 domain is chemically aligned with β_2 -microglobulin. A transmembrane domain and a short intracellular domain are also found in the *HFE* protein. The two most common *HFE* mutations, C282Y and H63D, are seen in their respective positions (Bacon *et al.*, 2011)

2.2.1.1.1 Mutations of *HFE* gene

2.2.1.1.2 H63D mutation

The most frequent *HFE* gene polymorphism is located in position 187 in exon 2, where a cytosine (C) is replaced by guanine (G) leading to histidine to aspartic acid replacement at position 63 of the *HFE* protein sequence (mutation H63D), SNP (rs1799945) the position of SNP chr6:26090951 (GRCh38.p13). This polymorphism can disrupt the iron homeostasis and cause iron accumulation, especially when it occurs simultaneously with the C282Y polymorphism (Gochee *et al.*, 2002)

The H63D mutation, which does not induce major iron overload but may serve as a cofactor for phenotypic expression of iron overload when combined with C282Y (Aguilar-Martinez *et al.*, 2001). Patients that are homozygous or heterozygous for the H63D variant may not have an elevated risk of clinical iron overload as a comparison to patients that do not have the mutation. Though they can have an improvement in transferrin saturation (TS) and serum ferritin (SF) ratios (Constantine *et al.*, 2009).

2.2.1.1.3 C282Y mutation

The C282Y mutation or SNP (rs1800562) polymorphism is the most common *HFE* gene polymorphism which results in a nonfunctional protein (Adams *et al.*, 2005). It's at position 845 in exon 4 on chromosome 6 (6p13), where guanine (G) is substituted by adenine (A), resulting in a cysteine being replaced by tyrosine at position 282 in the *HFE* protein sequence (C282Y), The position of SNP is chr6:26092913 (GRCh38.p13) (Katsarou *et al.*, 2016).

The C282Y mutation disrupts the forming of disulfide bonds in the *HFE* protein, reducing *HFE*'s ability to bind 2-microglobulin (Bacon *et al.*, 2011). The polymorphism inhibits *HFE* from touching the cell membrane, preventing it from interacting with hepcidin and *TFRs*. Despite the high prevalence of the C282Y polymorphism, some population tests have shown that hemochromatosis only affects a small percentage of carriers (Hollerer *et al.*, 2017). The clinical scan shown not all C282Y homozygotes exhibit HH clinical characteristics such as elevated iron level biomarkers. This may imply that a dynamic interplay of genetic predisposition and environmental factors could influence overall disease expression (Coppin *et al.*, 2003).

Individuals that are homozygous for the C282Y mutation are at the highest risk of iron overload. Several researchers from the United States and Europe have discovered that 60-93% of iron overload patients are homozygous for C282Y. Different causes hemochromatosis, as well as regional variations in the prevalence of *HFE* mutations, can account for the broad variety of C282Y homozygotes. Iron excess happens in a small percentage of individuals who have other *HFE* mutations. Compound heterozygotes (one copy of C282Y and one copy of H63D) or H63D homozygotes account for around 1-2% of Caucasians, but only a small percentage will experience iron overload. Furthermore, about 25% of the Caucasian population of the United States is H63D heterozygous. An increased chance of iron overload is not linked to a single copy of H63D. Almost all experiments have shown that even in the absence of *HFE* gene mutations, extreme iron overload can occur. (Johnson., 2004).

2.2.1.2 NON *HFE* related hemochromatosis (type 2 hemochromatosis)

2.2.1.2.1 *HAMP* gene

In the year 2000, the Hepcidin molecule (“hep” hepatic base, “cidin” antimicrobial activity) was discovered; it is an antimicrobial peptide that plays a role in innate immunity and iron metabolism. (Kruuse *et al.*, 2000) isolated it from human blood and urine. *HAMP* is Hepcidin antimicrobial peptide gene coding to product protein, it is located on chromosome 19 it extends 9637bp in the long arm (Park *et al.*, 2001). Hepcidin is composed of an 84 amino acid molecule called prehepcidin, which is cleaved into 64 amino acids prehepcidin by proteolytic cleavage in one portion. Prohepcidin is physiologically inert until it is cleaved in a unique NH₂ region by the

enzyme furin, resulting in pharmacologically active Hepcidin consisting of 8 cysteine residues bound by four disulfide bridges containing 25 amino acids (Krause *et al.*, 2000). (Park *et al.*, 2001). Hepcidin is a hormone consist of 25 amino acid peptide act as a prevention iron entry into the plasma compartment from the three important sources of iron: dietary absorption in the duodenum, the release of recycled iron from macrophages and the release of stored iron from liver .(Park *et al.*, 2001)

In knockout animals for the gene encoding Hepcidin, the disruption in the *HAMP* gene lead to clinical disorder similar to hemochromatosis. The association between Hepcidin and its effect on iron homeostasis was shown in many previous reports. Transgenic animals with higher Hepcidin expression had lower serum iron levels, erythropoiesis deficiency, and significant microcytic hypochromic anemia (Pigeon *et al.*, 2001; Nicolas *et al.*, 2002). Hepcidin is produced to a lesser degree outside of the liver, and it is thought that it functions as an antimicrobial peptide at these sites. Hepcidin modulates the protective barriers against urinary tract infections in the kidney. Hepcidin promotes iron homeostasis in cardiac tissue by an autocrine mechanism of ferroportin expression on cardiomyocyte surfaces (Michels *et al.*, 2015; Daher and karim 2017)

2.2.1.2.2 G71D mutation

Hepcidin activity is effected by genetic changes in the *HAMP* gene. Exon 3, the final exon of the *HAMP* gene on chromosome 19, encodes the effective peptide and is the gene's most polymorphic region (Krause, A. *et al.*, 2000; Park, C. *et al.*, 2001). Polymorphisms in the *HAMP* gene are less common than those found in the *HFE* gene. Around 16 different forms of Single Nucleotide Polymorphism (SNP) polymorphisms

have been identified so far, but only a small number are clinically significant because they are found in hemochromatosis. Patients with β thalassemia condition that utilize the *HAMP*-G71D variant in combination with the *HFE* H63D variant have more iron overload (Rahman *et al.*, 2014). A missense mutation in the *HAMP* gene that causes glycine 71 to be replaced by aspartic acid (G71D) due to a G to A substitution at nucleotide 212 in exon 3 affects the charge of amino acid 71 and is likely to affect Hepcidin production (Merryweather-Clarke, *et al.*, 2003). The transformation polymorphism G to A causes a difference in the sequence, resulting in a new irregular protein (transfer G to D in the amino acid 71). The transformation results in an incomplete protein that is destroyed shortly after the messenger RNA is translated (Lugus *et al.*, 2009). A defective or truncated Hepcidin can result from genetic changes in the *HAMP* gene. Increased ferritin levels, as well as iron stored, facilitate the progression and pathogenesis of some disorders, such as type 2 diabetes mellitus and coronary artery disease (Pechlaner *et al.*, 2016). Many reports refer to the development of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, and dyslipidemias (Crichton, R. *et al.*, 2011). Dietary iron absorption is affected by this mutation (Bomford, A. 2002). The association of mutations in genes affecting iron homeostasis with thalassemia can have a synergistic effect, increasing iron absorption and storage (Rees *et al.*, 1998; Jazayeri *et al.*, 2003). Knowing the presence of mutations in the genes that influence iron overload will allow for iron overload prevention (Palmer *et al.*, 2018).

2.2.1.3 Transferrin receptors

The transferrin receptor (*TFR*) is a transferrin carrier element. It is regulated in response to intracellular iron concentrations and is involved in iron import into the cell. The transferrin receptor 2 (*TFR2*) gene has similarities to the transferrin receptor 1 (*TFR1*) gene and codes for a transmembrane protein with a wide extracellular domain that can bind transferrin. Mutations in the transferrin receptors (*TFR2* and *TFR1*) can change the pathophysiology of the iron disorder. Changes in the genes that code for transporting receptors alter iron homeostasis, and provide a mechanism for excess iron accumulation and irregular iron distribution in iron-related disorders. (Pandey, *et al.* 2019).

2.2.1.3.1 Transferrin receptor, trafficking, and binding partners

The structural resemblance of *TFR2* to the previously characterized transferrin receptor 1 (*TFR1*) is one of its most important characteristics. They're both type II membrane proteins that form a disulfide linked dimer and have a 66 percent ectodomain homology (Kawabata *et al.*, 1999). *TFR1* and *TFR2* also bind transferrin Tf, and many of the amino acids involved in *TFR1*'s Tf binding are also constant in *TFR2* (Giannetti *et al.*, 2003). *TFR1* binds iron loaded Tf (holo-Tf) and is in control of holo-Tf endocytosis into acidic compartments. This iron is again set to release from Tf, decreased, and transferred throughout the endosomal membrane by the metal transporters: dimetal transporter (DMT1), Figure (2-5) showed transferrin cycle (Dautry-Varsat *et al.*, 1983; West *et al.*, 2000; Zhao *et al.*, 2010).

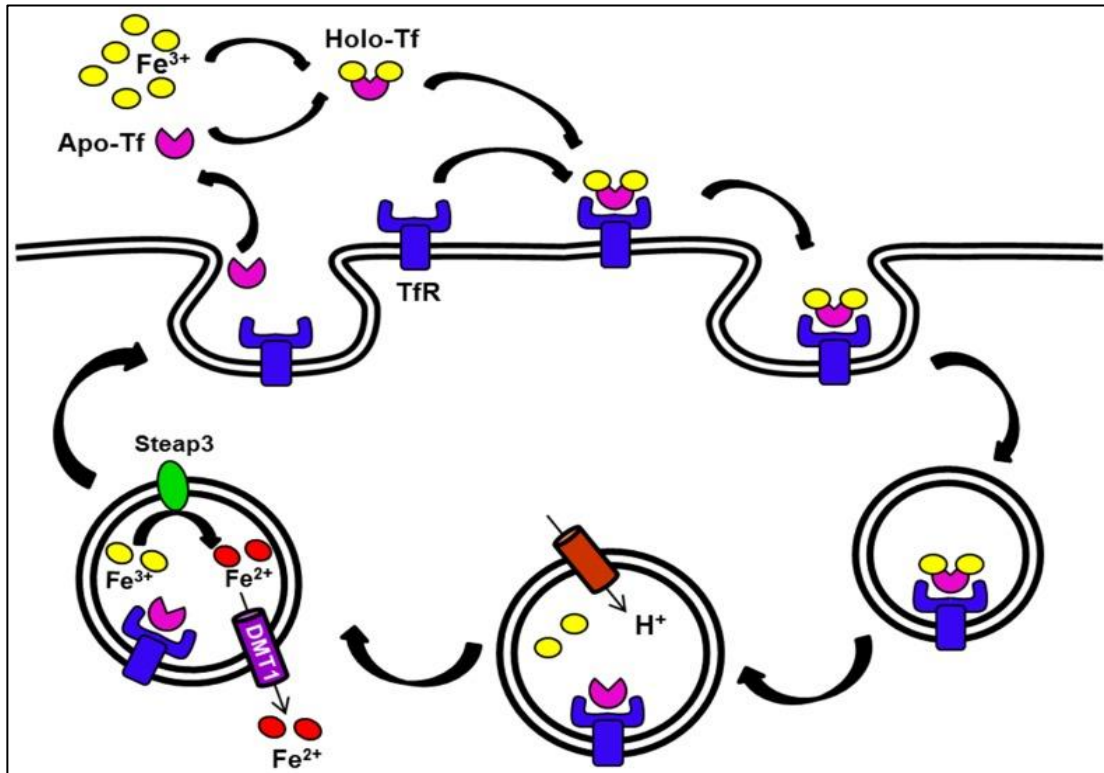


Figure (2.5) The transferrin cycle. Apo-Tf binds Fe^{3+} result Holo-Tf bind to *TfR* located on the surface of the cell. The *TfR*–Tf–Fe complex is internalized. Iron is released by endocytosis vesicles within a process that involves acidic endosomes. Apo-Tf remains bound to its receptor in the endosome and this receptor complex recycles back to the cell surface. In the endosome (Steap3) Ferric iron is reduced and then released into the cytosol by DMT1. (Veuthey and Wessling-Resnick., 2014)

TFR2 is capable to import iron, but its Tf binding affinity is 25 times lower than *TFR1* (Kawabata *et al.*, 1999; West *et al.*, 2000). *TFR2* function is an iron sensor, it can be enhanced by this variation in affinity, allowing it to detect increases in Tf saturation in the blood. *TFR1* is found in a wide variety of tissues, while *TFR2* is mainly found in the liver and erythropoietic progenitors (Sposi *et al.*, 2000)

2.2.1.3.2 Structure of *TFR1*

The *TFR1* protein in mammals is a homodimer of 760 residue disulfide bonded subunits (McClelland, A. *et al.*, 1984 ; Seligman, P. *et al.*, 1979). It can be identified and described to three regions in the primary sequence within each subunit of the protein: a globular extracellular region (residues 90–760) where Tf binds, a hydrophobic intramembraneous region (residues 62–89), and the remaining 61 residues in the cytoplasm. (Fuchs, H. *et al.*, 1998). *TFR1* (transferrin receptor 1) is a large strength affinity specific receptor that is responsible for the majority of iron absorption in most cells. *TFR1* is a single membrane spanning glycoprotein with a short cytoplasmic domain that binds iron-loaded transferrin with a high affinity at equal pH. The receptor traffics to the early acidic endosome after binding this ligand, in which it releases iron, recycles to the cell surface, and then delivers transferrin.(Fernández-Real *et al.*, 2010).

2.2.1.3.3 G142S mutation

This gene encodes a cell surface receptor necessary for cellular iron uptake by the process of receptor mediated endocytosis (sposi *et al.*, 2000)

In 1982, the *TFRC* gene was assigned to chromosome 3 it expands about 39878bp at the long arm. Transferrin receptor 1 is a unique protein that refers to the transferrin receptor like family. The *TFR1* gene consist of 19 exons in the full-length transcript, which code for 760 amino acids. Exon 4 contains one of the most significant frequencies (rs3817672), which product G142S amino acid substitution if mutatant. Differential iron uptake can be caused by gene polymorphisms that cause changes in this region's

residues. One of the few amino acid substitutions found in the open reading frame of the *TFR* gene is a nucleotide substitution (G to A) that resulted in the Gly142Ser amino acid substitution. Since the Gly142Ser coding modification is common in the general population and may be useful in the study of other diseases linked to iron metabolism. (Fernández-Real *et al.*, 2010)

2.2.1.3.4 Structure of *TFR* 2

On chromosome 7 the transferrin receptor type 2 (*TFR2*) gene is found it is 28135bp on the long arm of the human genome. The transferrin receptor 2 refers to the *TFR* family and is similar to *TFR1*. *TFR2* is an iron receptor and Hepcidin pathway activator that is used in the liver. *TFR* is found in erythroid tissue as a part of the Erythropoietin (EPO) receptor, which is needed for successful erythropoiesis (del-Castillo-Rueda *et al.*, 2012). The human *TFR2* gene has two transcripts: a 2.9 kb alpha variant (*TFR2*) and a 2.5 kb beta form (*TFR2*). *TFR2* encodes an 802 amino acid type II transmembrane protein which appears as cytoplasmic, transmembrane, and a long extracellular domain containing the C-terminus. The extracellular domain shows a high degree of homology (45% identity and 66% similarity) with *TFR1*, and contains two cysteine residues, confirming that the protein is homodimerized like *TFR1*. (Trinder, D., & Baker, E. 2003) In human *TFR2* is expressed in liver and normal erythroid precursor cells and bone marrow samples highly degree in several patients with erythroleukaemia and myeloid leukaemia. *TFR2* is found to a lesser extent in spleen, lung, muscle, prostate and peripheral mononuclear cells . The tissue distribution and the level of expression of

TFR2 are distinctly different from *TFR1*, which is expressed by all types of cells except mature erythroid cells (Trinder, D., & Baker, E. (2003))

2.2.1.3.5 Y250X mutation

. *TFR2* gene encodes a single pass type II membrane protein, which is a member of the transferrin receptor like family. This protein mediates cellular uptake of transferrin-bound iron and may be involved in iron metabolism, hepatocyte function, and erythrocyte differentiation. Mutations in this gene have been associated with hereditary hemochromatosis type III which caused by a homozygous nonsense mutation in the *TFR2* gene at the 7q22 locus (Camaschella *et al.*, 2000), (del-Castillo-Rueda, A. *et al.*, 2012). A transversion C / G at position 13451 of Homo sapiens transferrin receptor 2 (*TFR2*), RefSeqGene on chromosome 7 lead to amino acid change tyrosine (TAC) to stop sign (TAG) at residue 250 of the transcript (Y250X) (Camaschella *et al.*, 2000). TRF2 interacts with the *HFE* protein to regulate transcription in the *HAMP* gene (Mehta *et al.*, 2016). An iron overloading is caused by a defect in the formation of the *HFE* and *TFR2* proteins (McDonald *et al.*, 2014). *TFR2* is thought to be needed for phosphorylation of BMP6 in response to iron levels, with the *HFE* protein playing a role in the *HAMP* gene's signaling pathway and the *HFE* protein contributing to *HAMP* gene transcription (D'Alessio *et al.*, 2012)

Hereditary hemochromatosis is a rare recessive disorder due to mutations of the transferrin receptor 2 (*TFR2*) gene. Y250X was the first *TFR2* mutation in transferrin receptor 2 (*TFR2*) (Camaschella *et al.*, 2000). The phenotypes caused by mutations of these gene are similar to hemochromatosis conditions, manifesting increased transferrin

(Tf) saturations, periportal hepatic iron loading, and reticuloendothelial iron sparing. This observation suggests that the products of these genes are on a common pathway for the regulation of iron homeostasis. *TFR2* protein is a membrane glycoprotein that can interact with Tf (Kawabata *et al.*, 2005). Human *TFR2* has spliced transcripts α and β . *TFR2* α is the membrane bound form predominantly expressed in the liver, whereas *TFR2* β is a form that consists of only the extracellular domain of *TFR2* α . Similar to *TFR1*, *TFR2* α interacts with holo-Tf but not with apo-Tf at neutral pH (Kawabata *et al.*, 2000). Expression of *TFR2* mRNA almost exclusively occurs in the liver and erythroid precursor cells (Kawabata *et al.*, 2001). Homozygosity for one of several mutations in the *TFR2* gene, including the truncation mutation Y250X, has been associated with hereditary hemochromatosis in humans I (Camaschella *et al.*, 2000). In addition, some reports that homozygous *TFR2*(Y245X) mutant mice, which correspond to the *TFR2*(Y250X) mutation in humans, showed hepatocellular iron deposition with elevated serum Tf saturation (Fleming *et al.*, 2002).

Chapter Three

Material & Methods

3. Materials and Methods

3.1 Materials

the material includes equipment, kits and reagents, primers, chemicals, and restriction enzymes.

3.1.1 Equipment

The equipment and instruments that used in this study following in the table (3-1).

Table (3.1): Instruments and Equipment.

No.	Name of Item	Model	Company	Origin
1	Analytical Balance	ABS/ABJ	Kern	Germany
2	Autoclave	DE-23	Systec	Germany
3	Automated Water Still	Zd-1	K&K Scientific	South Korea
4	Automated Hematology analyzer	XS1000i	Sysmex	USA
5	Centrifuge	CL008	Cypress Diagnostics	Belgium
6	Gel Documentation system	Muthanna-Gel Doc System	Muthanna University	Iraq
7	Gradient Thermal Cycler	TC9600-G	Labnet	USA
8	Horizontal electrophoresis system	Mupid-One	Mupid	Japan
9	Hood	Bc-11b	Magnehelic	USA
10	Laboratory Electrical oven	LEO-080F	DAIHAN LabTech	Korea
11	Microwave Oven	LMH2235ST	LG	Korea

12	Microcenterifuge	Mikro 120	Hettich	Germany
13	Micropipette	100-1000 µl, 50-200 µl, 0.5-10 µl,	Nanolytik	Germany
14	Mini VIDAS	LDV/2459	BIOMERIEUX	F
17	Upright Freezer	Serie 4	Bosch	UK
18	PCR work station	GLE-UVSC	SCI-PLAs	UK
19	Vortex mixer	LVM-202	LabTech	Korea
20	Water Bath	LWB-111D	LabTech	Korea

3.1.2 Kits and Reagents

In this study the commercial system and reagents that are following in table(3.2).

Table (3.2) : The Commercial system and Reagents.

No.	Name of Item	Product code	Company	Origin
1	Wizard Genomic DNA Purification Kit	A1120	Promega	USA
2	GoTaq® G2 Green Master Mix	M7822	Promega	USA
3	1000bp DNA ladder	G120A	Promega	USA

3.1.3 Primers

The primers with product size shown in the table (3.3)

Table (3.3): primer set with the determination of product size

gene	Snps mutations	Primer	Nucleotide sequence	Product	REF.
HFE	rs1800562	F	5'TGGCAAGGGTAAACAGATCC'3	390bp	(Gomes <i>et al.</i> , 2009)
		R	3'CTCAGGCACTCCTCTCAACC' 5		
	rs1799945	F	5'ACATGGTTAAGGCCTGTTGC'3	208bp	(Gomes <i>et al.</i> , 2009)
		R	3' GCCACATCTGGCTTGAAATT'5		
TAMP	rs104894696	F	5'ATGCAGGGAGGTGTGTTAGGAGGCT'3	712bp	(Rahman <i>et al.</i> , 2014)
		R	CAAGGCAGGGTCAGGACAAGCTCTTAGC		
TfR1	rs3817672	F	5'CCAGGAGAGGACTTCCCTGC'3	591bp	(Pandey <i>et al.</i> , 2019)
		R	3'GATACTGACT GGCCTAGGGG' 5		
TfR2	rs80338880	F	5'TGCACTGGGTCGATGAG'3	355bp	De Gobbi <i>et al.</i> , 2001).
		R	3'CTCAAGCCCTCCCTCT' 5		

3.1.4 Chemicals

The chemical materials shown in table (3.4) according to (the material chemical datasheet) the use of this material was under safety protocol to control exposure to hazards.

Table (3.4): Chemicals used in this study.

No.	Name of Item	Company	Origin
1	Agarose	US biological	USA
3	Decontamination nucleases spray	Bio-world	USA
4	Ethanol 70% Spray	AMRESCO	USA
5	Ethanol 100%	Scharlab S.L.	Spain
6	Ethidium bromide	Bio-world	USA
8	Nuclease free water	Bio-world	USA
9	TBE buffer	Dongsheng biotech	China
10	Loading dye	Promega	USA

3.1.5 Restriction enzymes

Specific restriction enzymes show in the table (3.5)

Table (3.5): restriction enzyme used in this study.

No.	Name of restriction enzyme	Company	Origin
1	BclI 3000 units	Bio labs	USA
3	RsaI 1000 units	Promega	USA
4	Aci I 200 units	Bio labs	USA
5	NlaIV 200 units	Bio labs	USA
6	BfaI 500 units Isoschizomers for MaeI	Bio labs	USA

3.2 Design of Experiment

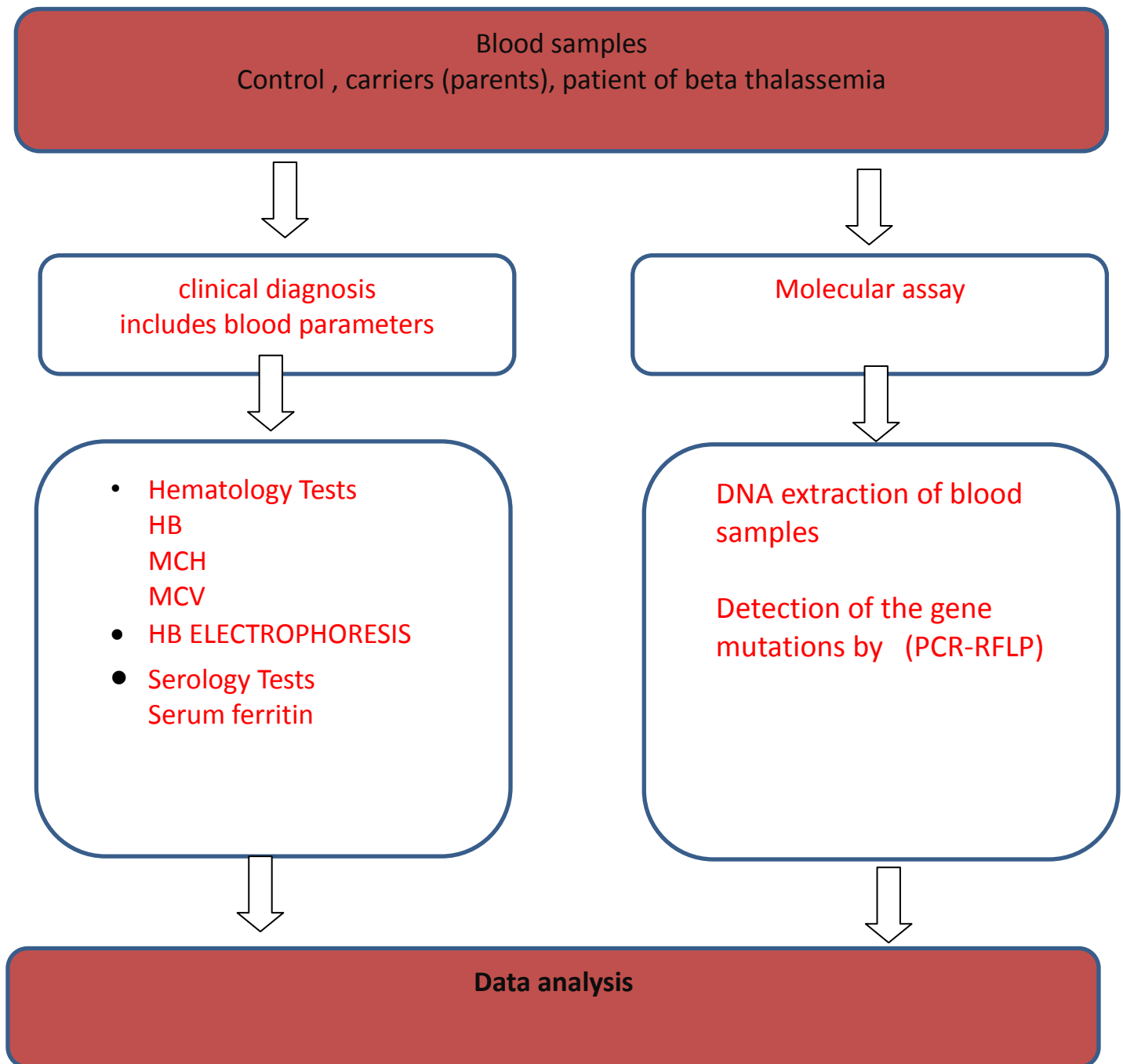


Figure (3.1): The Experimental Design of the study.

3.3 Methods

3.3.1 Preparation of Buffers and Reagent

Some components should be prepared firstly

3.3.1.1 Tris - Borate - EDTA (TBE) Buffer

TBE (10X) buffer was provided by (Dongsheng, China). This solution is reconstructed by adding 100 mL of the available 10x TBE buffer with 900 mL of distilled water and mixing to produce a homogeneous TBE (1X) buffer that can be held at room temperature until used for agarose gel electrophoresis.

3.3.1.2 Preparation of 1% Agarose Gel

In a flask, 75 ml of 1X TBE buffer was applied to 75 mg of agarose dissolved in a microwave oven until it begins to boil. Allow the agarose gel to cool to around 55°C before adding 1.6 microliters of ethidium bromide (10 mg/ml stock solution) per 75 ml of agarose gel (Sambrook and Russell., 2001).

3.3.1.3 Preparation PCR primers

1. Spin lyophilize primer tube to avoid loss by adhering oligonucleotide with tube lid.
2. Resuspension of primers by adding free nuclease water to achieve a final concentration of 100 μ M per 100 μ l of free nuclease water (according to manufacture information) to make stock primer solution for each forward and reverse PCR primer.

3. took a new 1.5ml microcentrifuge tube that is clean, sterile, and nuclease-free. To make the 10 μ M working solution should be a mix (90 μ l of nuclease-free water was mixed with 10 μ l of primer) both forward and reverse primer stock final volume of work solution 200 μ l.

4. stored at -20 C°.

3.3.2 Sterilization of DNA Extraction Station

Sterilization should be for all plastics, glassware, and components by autoclaved for 15 minutes at 2 bar. Pipette tips and microcentrifuge tubes were autoclaved for 15 minutes at 1.5 bar. Before starting work, clean the cabinet with 70% ethanol and then a nucleases decontamination spray apply to the working surface to remove and clear any nuclease condition, and then 20 minutes of the ultraviolet (UV) germicidal lamp is applied to the work surface of the cabinet. To prevent infection, all products and containers in the cabinet should be disinfected before use..

3.4 Sampling

3.4.1 Sample Collection

Whole blood samples were collected from 84 individuals (63 samples collected 21 samples from patients fathers, and mothers) from Al-Samawah city, Woman and children hospital. In addition 21 samples from healthy children as a control. The period of collected samples was from January to April 2020. The sample (2ml) was placed in a blood collection tube containing EDTA.

3.4.2 Extraction of DNA from Blood Sample

1. Added to a sterile 1.5ml microcentrifuge tube 750µl cell lysis Solution for a 250µl sample volume.
2. Gently motion the blood tube until it is fully mixed, then transport the blood to the Cell Lysis Solution tube. invert the tube 5–6 times.
3. To lyse the red blood cells, incubate the mixture for 10 minutes at room temperature (invert 2–3 times during the incubation). centrifuge at 13,000–16,000 xg for 20 seconds at room temperature.

4. As much supernatant as possible should be removed and discarded without damaging the visible white pellet. In the 1.5ml tube, about 10–20 µl of residual liquid will remain.

Repeat Steps 1–4 until the pellet becomes white if the blood sample has been frozen. It's possible the DNA from frozen samples would be lost.

Along with the white blood cells, certain red blood cells or cell debris can be visible. If the pellet continues to contain only red blood cells, after separating the supernatant above the cell pellet, add another amount of Cell Lysis Solution and repeat Steps 3–4.

5. Vortex the tube strongly for 10–15 seconds. To achieve effective cell lysis, fully resuspend the white blood cells.

6. Fill the tube containing the resuspend cells with Nuclei Lysis Solution (250µl with a 250 µl sample volume). To lyse the white blood cells, pipette the solution 5–6 times. The solution can thicken to a viscous consistency. If you see clumps of cells after mixing, keep the solution at 37°C until the clumps are disrupted if the clumps remain visible after 1 hour, add more Nuclei Lysis Solution (100µl for a 250 µl sample volume) and incubate again.

7. Add RNase Solution (1.5 μ l per 250 μ l sample volume) to the nuclear lysate and invert the tube 2–5 times to blend the sample. Cool the mixture to room temperature after incubation at 37°C for 15 min.

8. Vortex the nuclear lysate strongly for 10–20 seconds with Protein Precipitation Solution (100 μ l for 250 μ l sample volume). After the vortex, small protein clumps can appear.

Note: If you used more Nuclei Lysis Solution in Step 6, you'll need 130 μ l Protein Precipitation Solution for a total of 250 μ l sample volume.

9. For a 250 μ l sample volume, centrifuge at 13,000–16,000 $\times$ g for 3 minutes at room temperature. There should be a dark brown protein pellet visible.

10. Transfer the supernatant to a sterile 1.5ml microcentrifuge tube containing 250 μ l of room-temperature isopropanol for a 250 μ l sample amount.

NOTE: A small amount of supernatant can remain in the protein pellet channel. To prevent contaminating the DNA solution with the precipitated protein, leave this remaining liquid in the container.

11. Gently invert the solution to create a transparent mass of white thread-like strands of DNA.

12. For a 250 μ l sample, centrifuge at 13,000–16,000 $\times$ g for 1.5minute at room temperature. As a tiny white pellet, the DNA would be clear.

13. Decant the supernatant and transfer it to one sample volume of 70 percent ethanol to the DNA at room temperature. to pure the DNA pellet and wash the sides of the microcentrifuge tube by gently inverting the tube several times. Centrifuge at 13,000–16,000 $\times$ g for 1.5minute at room temperature.

14. carefully aspirate the ethanol. At this stage, the DNA pellet is very loose. Invert the tube onto absorbent paper and let the pellet air dry for 10–15 minutes.

15. Filled the tube with DNA Rehydration Solution (70 μ l for a 250 μ l sample volume) and rehydrate the DNA at 65°C for 1 hour.

Alternative rehydrate the DNA by incubating the solution at room temperature or at 4°C for an overnight period.

16. Keep the DNA between 2 and 8 degrees Celsius.

3.5 Optimizing of PCR conditions

In this technic, specific and typical resolution of PCR products can obtain. We tested different ranges of annealing temperatures. one DNA sample was amplified in twelve different PCR tubes with identical PCR components. The thermal cyclic conditions of all the twelve amplification reactions were kept the same program but the annealing temperature was changed. Different temperatures were tested from (55 - 61°C) in order to determine optimal temperatures that would give good quality PCR products and hence the shortest possible run time of the PCR. The results were showed that 56 temperatures for the (H63D, C282Y, Y250X) mutations and 60 temperatures for the (G71D, G142S) mutations were appropriate to obtain an adequate amplification product without smearing or dimer .

3.6 PCR Amplification

To make successful DNA amplification, mix all components (DNA template, primers, and PCR master mix) in PCR tube and centrifuge briefly to bring the contents to the bottom of the tubes. Assemble all reaction components on the ice during the work. PCR mixture was set up in a total volume of 25 μL included 12.5 μL of PCR premix, 1 μL of each primer and 2.5 μL of template DNA have been used. The volume is completed with nuclease-free water up to 25 μL . As shown in table (3.6).

Table (3.6): The components of PCR reaction.

Components	Reaction Volume	Final Concentration
GoTaq® G2 Green Master Mix	12.5 μL	1X
10 μM Forward Primer	1 μL	0.5 μM
10 μM Reverse Primer	1 μL	0.5 μM
Template DNA	2.5 μL or variable	< 1,000 ng
Nuclease-Free Water added up to	to 25 μL	—

The tubes were placed in a programmed thermal cycler. Close the lid and select run the amplification program follows table (3.7) that shows the PCR conditions.

Table (3.7): PCR program reaction .

PCR stage		Temperature	Time	Cycle number
Initial Denaturation		95°C	120 second	
PCR step	Denaturation	95°C	60 second	35
	Annealing *	Accordin tog specific region study °C	60 second	
	Extension	72°C	60 second	
Final Extension		72°C	120 second	
Hold		4-10°C	—	—

* 56 C° for the (H63D, C282Y, Y250X) mutations and 60 C° the (G71D, G142S)

mutations

3.6.1 Conventional PCR product analysis

The conventional PCR product was analyzed by agarose gel electrophoresis according to the following steps:

1. Agarose gel 1 percent was prepared by dissolving 750 mg of agarose powder in 1X TBE (75 ml) and moving the flask to blend the contents, then inserting it in the microwave for 2 minutes. Then set aside to cool at room temperature.
2. Add a 1.6 µl Ethidium bromide stain to the agarose solution.
3. Pour agarose solution in the tray after the comb was fixed in the correct spot and left to solidify for 15 minutes at room temperature, then the comb was gently withdrawn from the tray and 5µl of PCR product and 1.8 µl of (100bp Ladder) were applied to each comb well.

4. The gel tray was placed in the electrophoresis chamber and 1X TBE buffer was added to it.
5. The voltage was in two steps firstly 100 volts for 15 minutes and then reduce to 50 volts for 40 minutes.
6. Using the Muthna-Gel Documentation system's UV transilluminator, a conventional PCR product was visualized the size of the PCR product depends on the region amplification.

3.7 Digestion of PCR product with restriction enzyme

After amplification of the specific region of DNA, the PCR product digest with a specific restriction enzyme to cleavage the PCR product and determination the mutations. A mix of retraction enzyme with PCR product and other components incubate in specific digestion temperature for sufficient period according to company information of restriction enzyme digestion. The most important point (critical point) is that the balance between the quantity of PCR product and restriction enzyme any extra amount will not give typical digestion.

The following table of a typical RE digest.:

Table (3.8): reaction of restriction enzyme digestion by (Bcl1,Aci1,NlaIV,Bfal)used in this study from Biolabs / USA.

material reaction	volume	Typical reaction from neb
Sterile, deionized water	39 μ l	complete to a final volume
RE 1X Buffer	5 μ l	5
DNA, 1 μ g/ μ l	5 μ l	1 μ g
Mix by pipetting then add.		
Restriction enzyme, 10 μ / μ l	1 μ l	10 units generally about 1 μ l
Final volume	to 50 μ l	to 50 μ l

Table (3.9): reaction of restriction enzyme Real used in this study from Promega / USA

material reaction	volume
Sterile, deionized water	16.3 μ l
RE 10X Buffer	2 μ l
Acetylated BSA,10 μ g/ μ l	0.2 μ l
DNA, 1 μ g/ μ l	5 μ l
Mix by pipetting then add.	
Restriction enzyme, 10 μ / μ l	0.5 μ l
Final volume	20 μ l

In a sterile tube, add all component tables (3.8) (3.9) mixed gently by pipetting. Close the tube and centrifuge for a few seconds in a microcentrifuge. Incubated at the optimum temperature according to manufacture information table (3.10)

Note Unit definition: one unit is defined as the amount of enzyme required to completely digest 1µg of lambda DNA in one hour at a specific temperature in 50µl.

Table (3.10): specific condition of restriction enzyme used in this study.

.restriction enzyme	recognition sequence	incubation period	temperature of incubation
BclI * 3000 units	5'... T▼GATCA ...'3 3'... ACTAG▲T...'5	1-4 hour may be overnight	50°
RsaI 1000 units	5'... GT▼AC...'3 3'... CA▲TG...'5	1-4 hour	37°
AccI * 200 units	5'... GGC▼G...'3 3'... C▲CGC...'5	1-4 hour may be overnight	37°
NlaIV 200 units	5'... CCN▼NGG...'3 3'... GGN▲NCC...'5	60 minute	37°
Bfal * 500 units Isoschizomers for MaeI	5'... C▼TAG...'3 3'... GAT▲C...'5	60 minute	37°

*no star activity *star activity

3.8 Gel electrophoresis for PCR product digested by restriction enzyme

After PCR products were digested with a specific restriction enzyme analyzed by agarose gel electrophoresis. Small volumes (5-10 μ l) of the PCR products that were digested with restriction enzyme were separated using 1.5% agarose gels containing 1.125gm agarose 75ml TBE buffer. Digestion products should be mixed with 6x loading dye. A 100bp DNA ladder (1.8 μ l ladder was used as a molecular marker to monitor the migration of samples and determine the size of PCR products).

The voltage was in two steps firstly 100 volts for 15 minutes and then reduce to 50 volts for 40 minutes. Using the Muthna-Gel Documentation system's UV transilluminator, digestion of PCR product was visualized the size of fragment band depend on the presence of the recognition site for restriction activity.

3.9 Statistical Analysis

Differences in blood parameters were expressed as mean $\pm$ SD. The Chi-square (χ^2) analysis was performed to evaluate the Hardy–Weinberg equilibrium test was used for the single nucleotide polymorphism variables as needed. Statistical significance was set at $P < 0.05$

Chapter Four

Results & Discussion

4.1 The Molecular Study of DNA

4.1.1 DNA isolation from Blood Sample

Extraction of blood samples done by wizard genomic DNA purification kit. The DNA isolated shown in the figure (4.1) by migration on agarose gel electrophoresis., where 84 DNA was isolated from the blood samples collected from all groups for the study

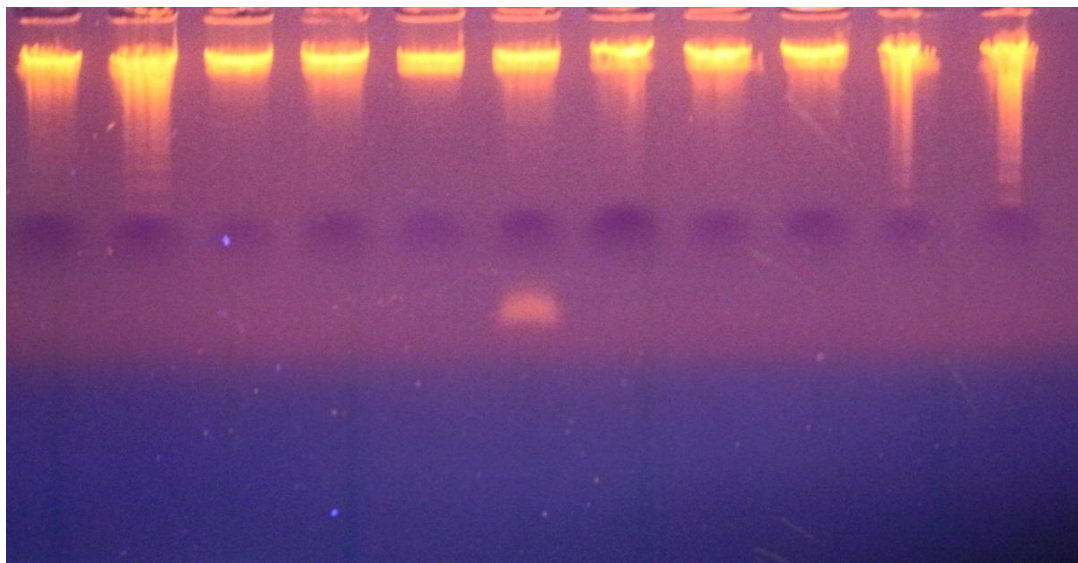


Figure (4.1): DNA isolation of blood samples was electrophoresis on 1% agarose gel in 100 volt for 15 min.

4.1.2 Optimizing the annealing Temperatures

The results of optimized annealing temperatures were SNP mutation (H63D:208bp at temp. 56 c°, C282Y:390bp at temp. 56 c°, Y250X:355bp at temp. 56 c°, G71D:712bp at temp. 60 c°, G142S:591bp at temp. 60 c°) respectively figure (4.2) showed optimal annealing temperature according to PCR products on agarose gel electrophoresis. A 100 bp DNA ladder was used as a molecular marker letter M

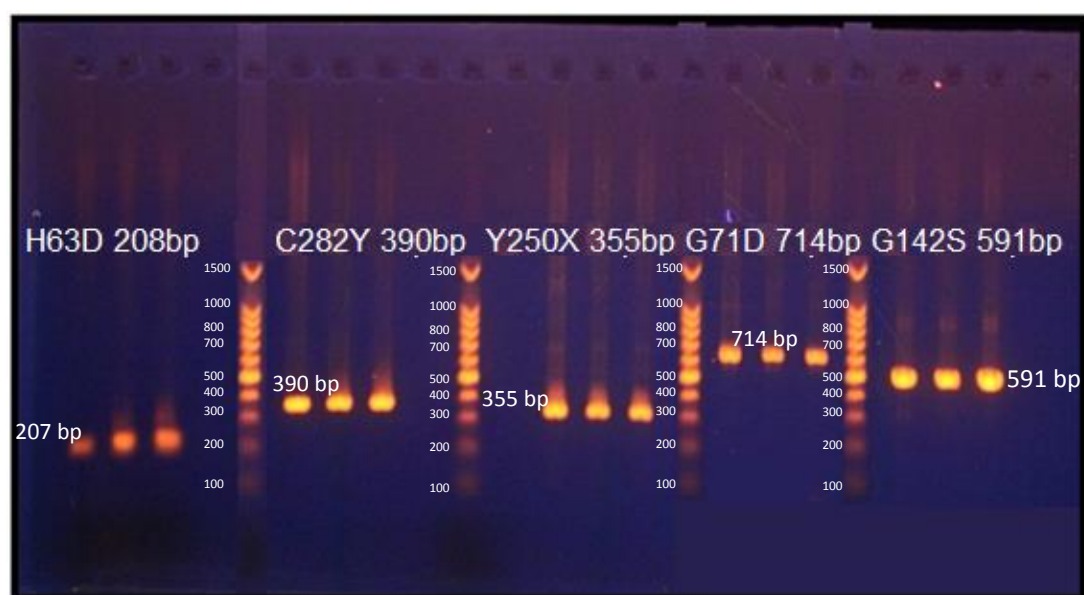


Figure (4.2): Optimization of annealing temperature 52-72 °C. PCR products of mutations (H63D:208bp at temp. 56 c°, C282Y:390bp at temp. 56 c°, Y250X:355bp at temp. 56 c°, G71D:714bp at temp. 60 c°, G142S:591bp at temp. 60 c°) were separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3 PCR Amplification

PCR product according to SNP target sequence was amplified by using specific forward and reverse primers

4.1.3.1.1 SNP rs1800562 : (C282Y)

The product size of this SNP was 390 bp at annealing temperature 56 c° , which is shown at the following (4.3)figure

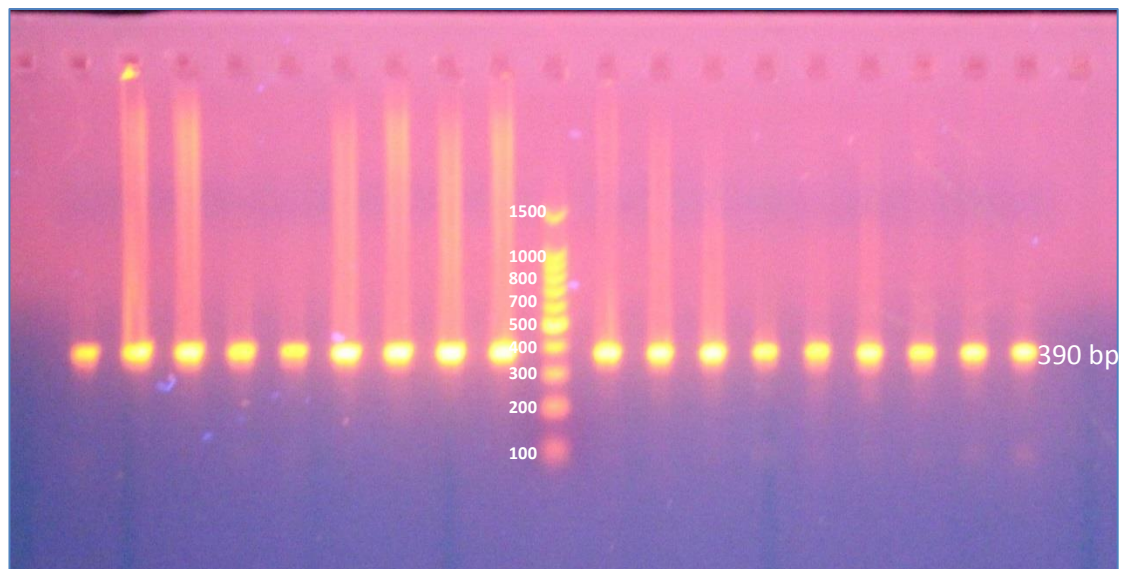


Figure (4-3) PCR product of SNP rs1800562 : (C282Y) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3.1.2 Digestion of C282Y mutation by (RsaI) restriction enzyme

RsaI digestion of a 390 bp PCR product. In the *HFE* wild-type, the digestion produced sizes are 247 and 143 bp. The mutation form generates a new restriction site, the digestion will give three fragments. Homozygous mutant genotypes are given three fragments of (247,111,32) bp while heterozygous are given four fragments (247,140,111,32bp) figure (4-4)

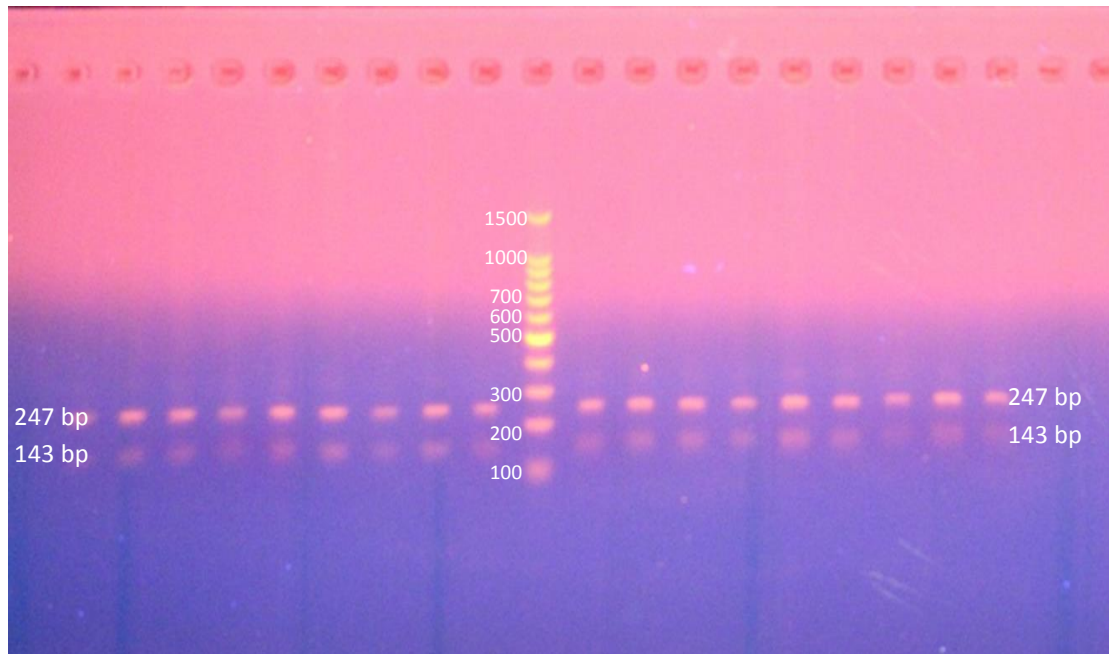


figure (4.4) RsaI digestion of a 390 bp PCR product. In the *HFE* wild-type digestion product sizes are 247 and 143 bp. The mutation generates a new restriction site, the digestion will give three fragments. Homozygous mutant genotypes are three fragments of (247,111,32) bp while heterozygous are four fragments (247,140,111,32bp).

The results of digestion of all PCR production given wild type. Sanger sequencing was made to conformation the result. The C282Y mutation was not found in β thalassemia patients, parents as carriers, and healthy control. These results showed the absence of this SNP in all cases. The FASTA format (results of sanger sequence) was aligned with *homo sapiens* homeostatic iron regulator (*HFE*) reference sequence gene (NG_008720.2) and appeared a wild type, shown in figure (4-5). Also, the FASTA format viewed by using FinchTV software showed wild type single-nucleotide under nucleotide number 10633 on *HFE* gene as a genotyping in these study figures (4-6)

Score	Expect	Identities	Gaps	Strand
531 bits(287)	2e-146	287/287(100%)	0/287(0%)	Plus/Plus
Query 1	TACGGTGTCTGGGCTTGAACCTACTACCCCAAGAACATCACCATGAAGTGGCTGAAGGATA	60		
Sbjct 10456	TACGGTGTCTGGGCTTGAACCTACTACCCCAAGAACATCACCATGAAGTGGCTGAAGGATA	10515		
Query 61	AGCAGCCAATGGATGCCAAGGAGTTCGAACCTAAAGACGTATTGCCCAATGGGGATGGGA	120		
Sbjct 10516	AGCAGCCAATGGATGCCAAGGAGTTCGAACCTAAAGACGTATTGCCCAATGGGGATGGGA	10575		
Query 121	CCTACCAGGGCTGGATAACCTTGCGTGTACCCCTGGGGAAGAGCAGAGATATACGTGCC	180		
Sbjct 10576	CCTACCAGGGCTGGATAACCTTGCGTGTACCCCTGGGGAAGAGCAGAGATATACGTGCC	10635		
Query 181	AGGTGGAGCACCCAGGCTGGATCAGCCCTCATTGTGATCTGGGGTATGTGACTGATGA	240		
Sbjct 10636	AGGTGGAGCACCCAGGCTGGATCAGCCCTCATTGTGATCTGGGGTATGTGACTGATGA	10695		
Query 241	GAGCCAGGAGCTGAGAAAATCTATTGGGGGTGAGAGGAGTGCCTGA	287		
Sbjct 10696	GAGCCAGGAGCTGAGAAAATCTATTGGGGGTGAGAGGAGTGCCTGA	10742		

Figure (4-5) alignment of the sample sequencing with *homo sapiens* homeostatic iron regulator (*HFE*) reference sequence gene (NG_008720.2)

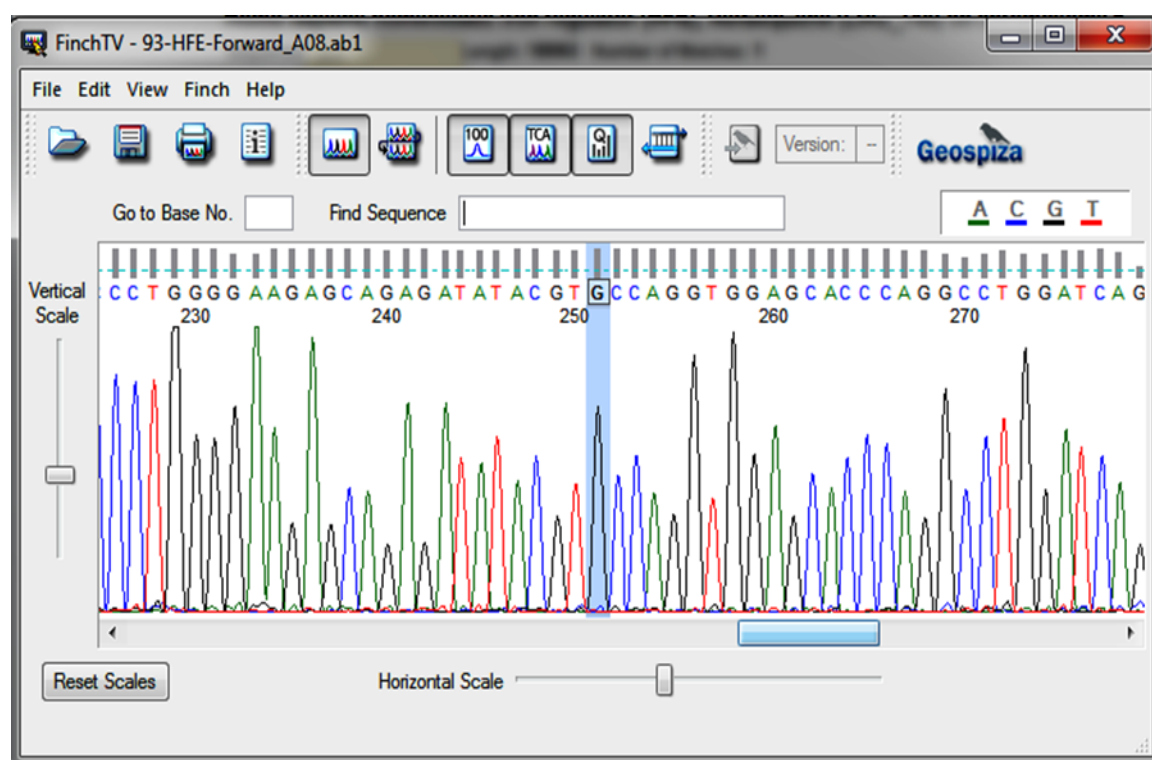


Figure (4-6) showed wild type single nucleotide under nucleotide number 10633 on *HFE* gene as a genotyping by Finch TV software

Many previous studies reported that neither homozygous nor heterozygous mutation was found in patients or in controls (Yasir et al., 2019; Mokhtar ET AL., 2018; Nadkarni *et al.*, 2017; Enein *et al.*, 2016). This study confirms the absence of the C282Y mutation in all groups with the Samawah population. Dasgupta et al. study illustrated that the population was not at risk for iron deposition from the C282Y mutation(Dasgupta et al., 2017). On the other side, some studies explain the C282Y allele frequencies were significant with iron overload. The allele frequency of C282Y in 870 West African blacks was $P < 0.001$. The result of another group in this study illuminate allele frequencies of C282Y in European Americans were $p=0.0600$ and allele frequencies in African Americans were 0.0102. The C282Y are consistent with those using other autosomal markers, differ across regions, and reflect paternal and maternal contributions of European American ancestry in African Americans (Acton et al., 2020). The incidence of SNP mutation in this study was not found in all target groups and was not associated with iron overload.

4.1.3.2.1 SNP rs1799945 : (H63D)

The product size of this SNP was 208 bp at annealing temperature 56 c°, which is shown at the following (4.7)figure

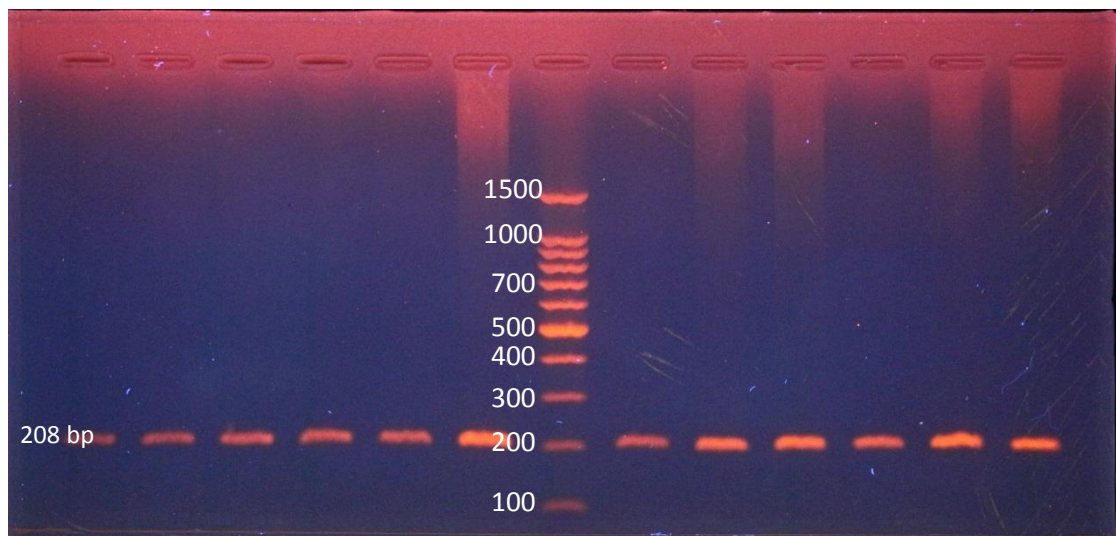


Figure (4-7) PCR product of SNP rs1799945 : (H63D) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3.2.2 Digestion of SNP rs1799945 (H63D) with BclI restriction enzyme

The BclI restriction enzyme cleaves the PCR product (208bp) to 138bp and 70bp in wild type but in homozygosis mutant this restriction enzyme same PCR product 208bp due to the mutant nucleotide effect on the restriction site and abolish it. figure (4-8)

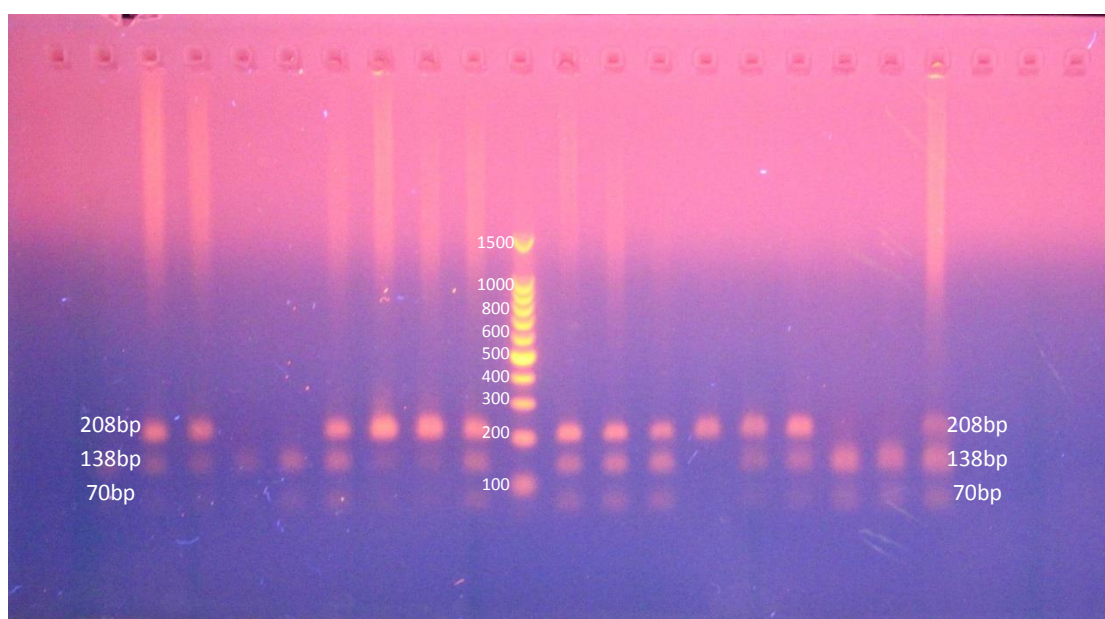


Figure (4-8) The BclI restriction enzyme cleaves the PCR product (208bp) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

PCR-RFLP showed nonsignificant association has obtained between serum ferritin level with β thalassemia patients, parents as carriers, and control ($p=0.08$, $p=0.74$, $p=0.82$) respectively. The allelic frequency of (H, D) alleles in β thalassemia patients was (0.738, 0.262) respectively and for parents was (0.607, 0.393) respectively table (4-1). Although of P-value was nonsignificant but the value was nearest of a significant level

Table (4-1) allele frequency of H63D mutation (rs1799945) for β thalassemia patients and parents.

allele frequency	β thalassemia patient	parents as carriers
H	0.738	0.607
D	0.262	0.393

In the H63D mutation, the homozygous variant was found 3(14.28%), 7(16.66%), 0(0%) for patients, parents, and control respectively, and heterozygous was found 5(23.8%), 19(45.23%), 2(9.52%) of patients, parents, and control respectively. The study of (Fekri *et al.*, 2019) was reported that the noninfluence of the presence of the H63D mutation of *HFE* polymorphisms in blood ferritin level of β thalassemia. (Sun *et al.*, 2019) the study reported that one hundred thirteen iron overload patients showed only one homozygous H63D mutation, and showed one heterozygosis H63D mutation. Other documents showed that H63D mutation of 210 cases (186, 16, and 8 cases) was a wild type, heterozygote, and homozygote mutant respectively in this study and recorded 92 % of cases showed wild type alleles for the H63D mutation with a significantly higher amount of iron deposition (Dasgupta *et al.*, 2017).

On the other side was observed H63d mutation can be associated with iron overload in β thalassemia, one of these documents described the collection of blood samples from 204 β thalassemia patients same numbers of normal controls. The H63D mutation was observed in 23 (11.3%) cases, out of which 19 cases were heterozygous for H63D and 4 cases were homozygous. In control samples, 4(2%) cases were found heterozygous for the H63D, and no homozygous mutation was found. Significantly high serum ferritin levels were found in β thalassemia patients with the H63D mutation ($p < 0.001$) (Yasir *et al.*, 2019). (Enein *et al.*, 2016) showed the H63D heterozygous state was detected in 5/50 (10%) thalassemia patients and in 1/30 (3.3%) controls. Significantly higher levels of serum ferritin and serum iron in patients with C282Y mutation ($p = 0.001$). The result of SNP mutation was nonsignificant but the value was nearest of a significant level and for conformation, this result suggested to retest with a large target sample.

4.1.3.3.1 SNP rs104894696 : (G71D)

The product size of this SNP was 714 bp at annealing temperature 60 c°, which is shown at the following (4.9)figure

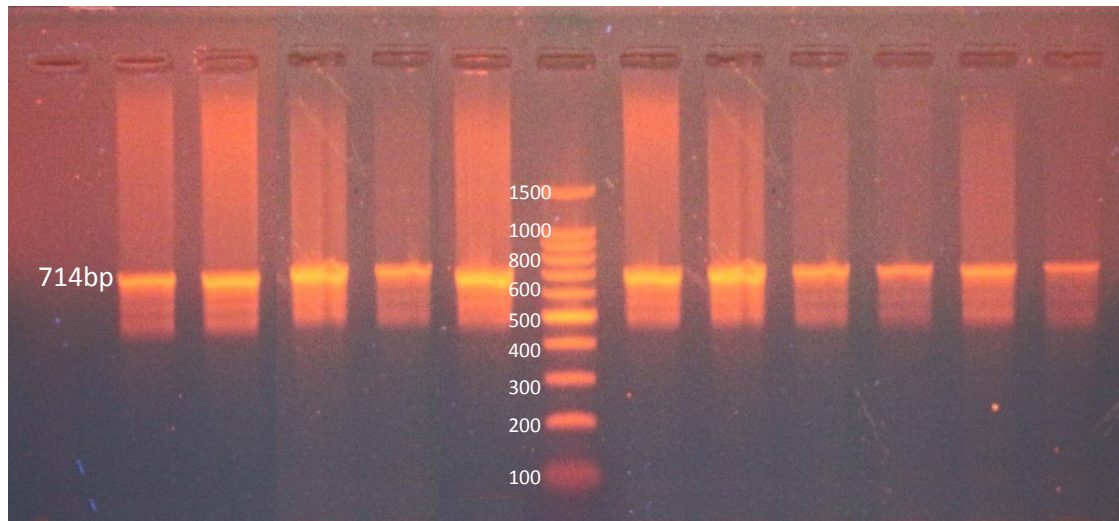


Figure (4-9) PCR product of SNP rs104894696 : (G71D) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3.3.2 Digestion of SNP rs104894696 (G71D) with AclI restriction enzyme

The AclI restriction enzyme cleaves the PCR product (714bp) to 370bp, 217bp, 94bp, and 33bp in wild type but in homozygosis mutant this restriction enzyme same PCR product 587bp, 94bp, and 33bp due to the mutant nucleotide effect on one restriction site and abolish it figure (4-10)

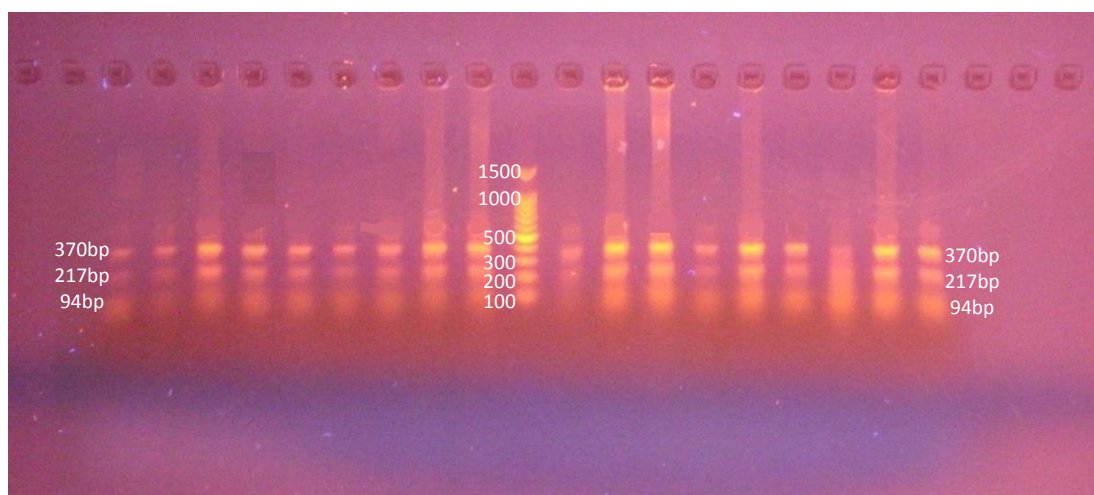


Figure (4-10) The *Acil* restriction enzyme cleaves the PCR product (714bp) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

The G71D mutation of the *HAMP* gene was non-significant with β thalassemia patients and healthy control but significant for parents as carriers individuals ($p = 0.44$, $P = 0.82$, $P < 0.001$) respectively. The significant P value of parents was heterozygous with not homozygous mutant.

The allelic frequency of (G, D) alleles of β thalassemia patients was (0.83, 0.17) respectively and for parents was (0.62, 0.38) respectively table (4-2).

Table (4-2) showed the allele frequency of patients and parents with G71D mutation rs104894696

allele frequency	β thalassemia patients	parents as carriers
G	0.83	0.62
D	0.17	0.38

The G71D mutation homozygous variant was not found in β thalassemia patients, parents, and control respectively. the G71D mutation heterozygous was found 5(33.3%), 23(76.6%), 2(9.52%) of patients, parents, and control respectively.

The previous study explained the *HAMP* gene G71D mutation is not a major determinant of iron overload in β thalassemia patients (Abou-Elew et al., 2012). (Rahman et al., 2014) the study was described the *HAMP* gene (G71D) mutation as nonsignificant with the major determinant of total body iron status. Another study was the *HAMP* G71D mutations are rare among Spanish hemochromatosis patients, and their impact on this population is not significant (Altès et al., 2009). But on the other side (Shah et al., 2020) study showed *HAMP* gene G71D mutations show high frequency in β thalassemia major patients and mean significant association between mutations and high serum ferritin level with β thalassemia major patients. Other documents suggest that the association of heterozygous mutations in *HAMP* genes (G71D) mutation could lead to an adult form of primary iron overload. (Jacolot et al., 2004). The results included unaffected this SNP mutation on iron overload also most parents hold G71D mutations without effect on iron overload.

4.1.3.4.1 SNP 3817672 : (G142S)

The product size of this SNP was 591 bp at annealing temperature 60°C which shown at the following (4.11)figure

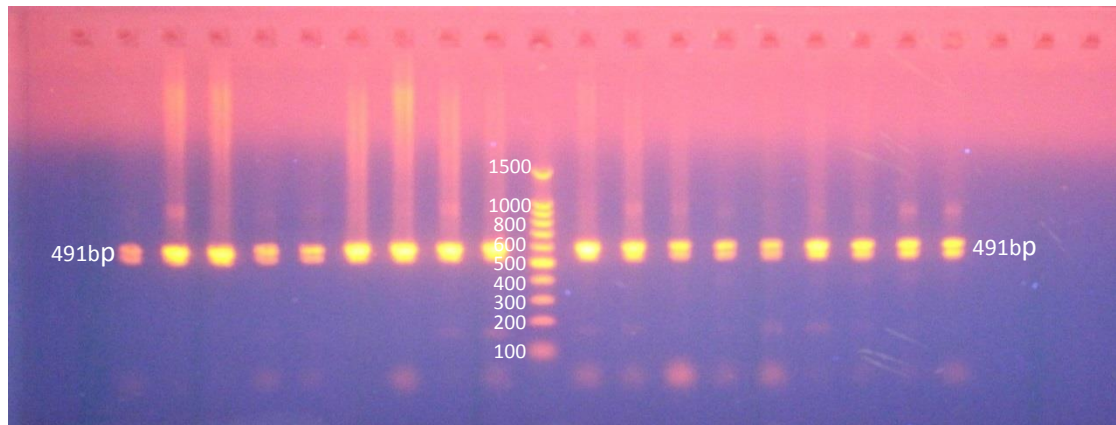


Figure (4-11) PCR product of SNP 3817672 : (G142S) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3.4.2 Digestion of SNP rs3817672 (G142D) with NlaIV restriction enzyme

The NlaIV restriction enzyme cleave the PCR product (591bp) to 347bp and 244bp in wild type but in homozygosis mutant these restriction enzyme cleave PCR product to 347bp, 151bp and 93bp. Figure (4-12)

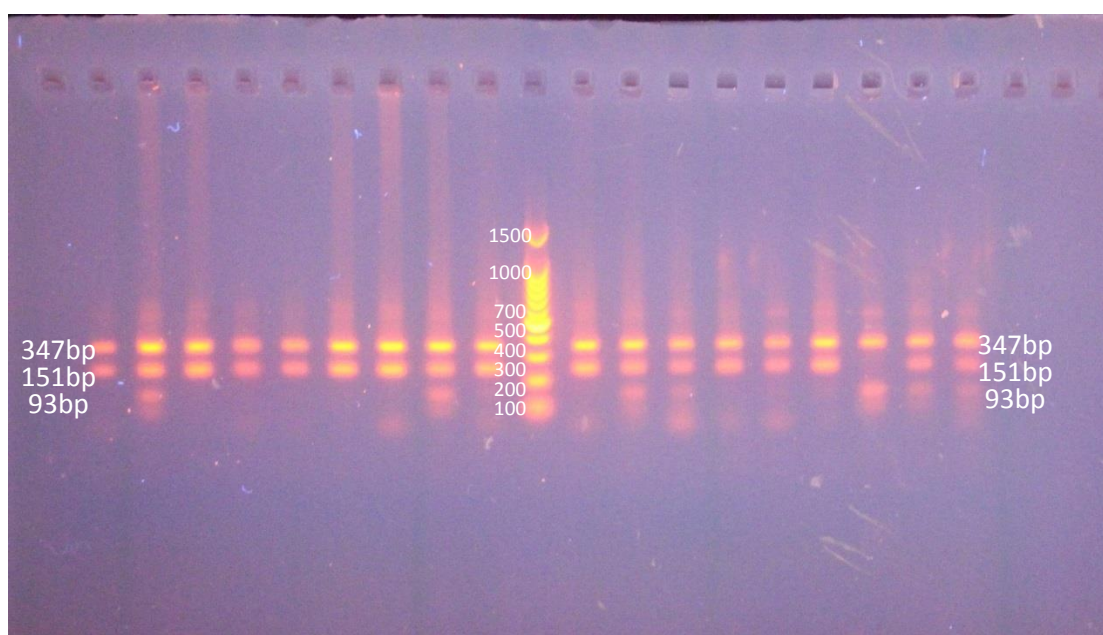


Figure (4-12) The NlaIV restriction enzyme cleaves the PCR product (591bp) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

The G142S mutation was significant with β thalassemia patients, but nonsignificant with parents as carriers, control ($p=0.02$, $p=0.47$, $p=0.74$) respectively. The homozygote variant was 6(28.57%), 5(11.9%), 1(4.76%) in β thalassemia patients, parents as carriers, and control respectively. The heterozygote were 5(23.80%), 22(52.38%), 6(28.57%) in β thalassemia patients, parents as carriers, and control respectively.

The allelic frequency of (G, D) alleles of β thalassemia patients was (0.60, 0.40) respectively and for parents was (0.62, 0.38) respectively table (4-3).

Table (4-3) The allelic frequency of β thalassemia patients and parents as carriers for SNP rs3817672 : (G142S)

allele frequency	β thalassemia patients	parent as carrier
G	0.6	0.62
S	0.4	0.38

A previous report that showed our result was (Fernández-Real et al., 2010) The frequency of the G allele at the position 13222 of the *TFRC* gene was significantly higher in T2D patients. The increased prevalence of the G allele run in parallel to increased sex-adjusted log serum ferritin and slightly increased soluble transferrin receptor among patients with T2D. On the other side (Fujihara et al., 2019) showed that no effects of (rs3817672) on iron concentrations in the blood of Japanese subjects also (Sukiennicki et al., 2019) study showed the polymorphisms rs3817672 in iron-related *TFR1* genes did not reveal a significant difference between lung cancer patients who have significant iron overload. Moreover, There were no statistically significant differences in the distribution of genotype and allele frequencies *TFRC* genes between analyzed groups of patients (Sijmons, R. 2018). The results were affected and associated with this SNP mutation with iron overload. To conformation, the results may be retested with large β thalassemia cases.

4.1.3.5.1 SNP rs80338880 : (Y250X)

The product size of this SNP was 355 bp at an annealing temperature 56 c°, which is shown in the following figure (4-13)

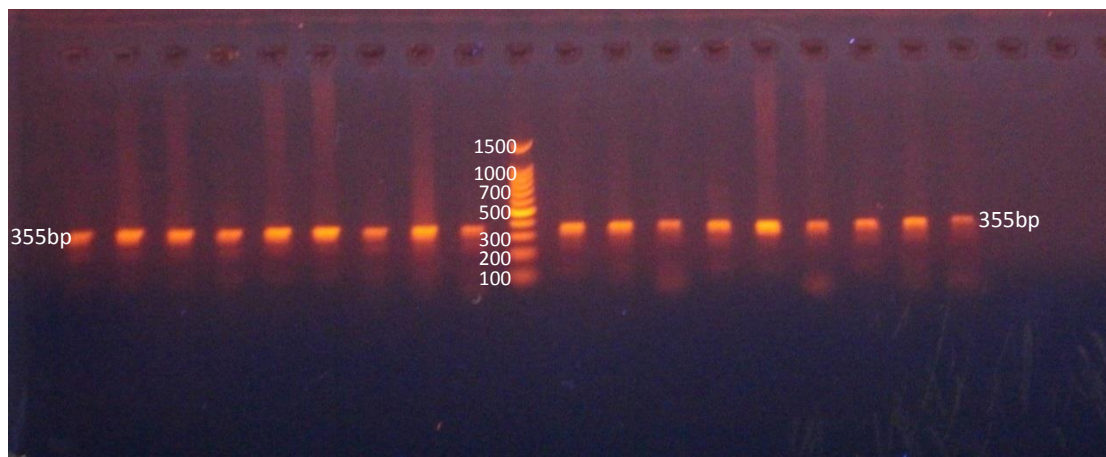


Figure (4-13) PCR product of SNP rs80338880 : (Y250X) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

4.1.3.5.2 Digestion of SNP rs80338880 (Y250X) with BfaI Isoschizomers for MaeI restriction enzyme

The BfaI restriction enzyme cleaves the PCR product (355bp) to 238bp and 117bp in wild type but in homozygosis mutant, these restriction enzymes cleave the PCR product to 134bp, 104bp, and 117bp. Figure (4-14)

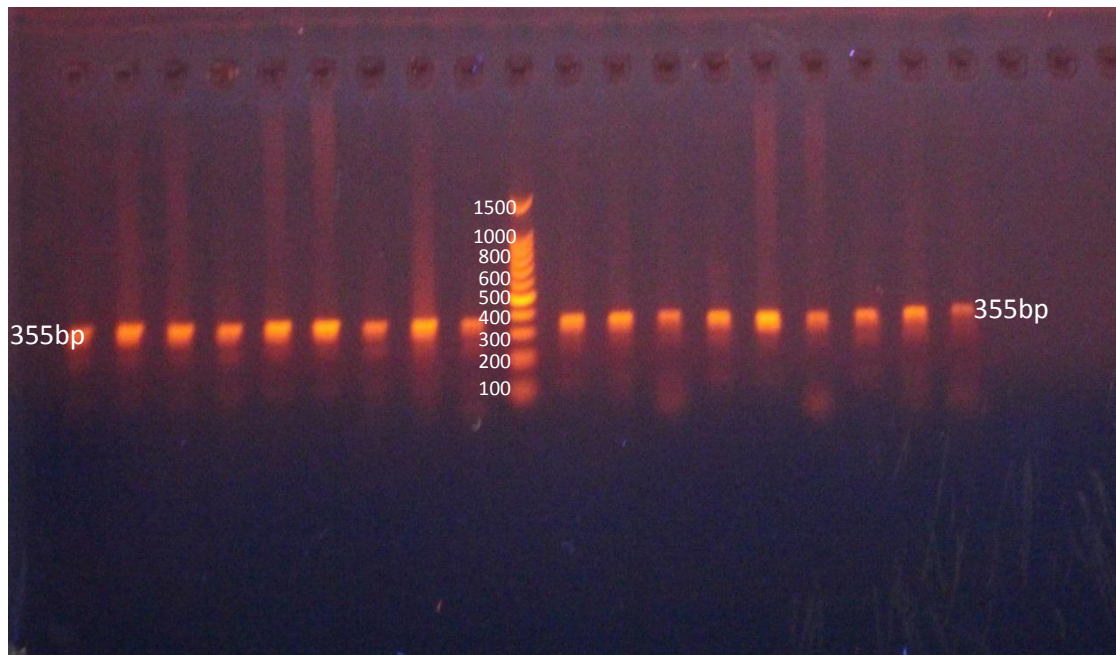


Figure (4-14) The BfaI restriction enzyme cleaves the PCR product (355bp) separated by electrophoresis on 1.5% agarose gel in 100 volts for 15 min then decrease to 50 volts for 40 min.

The results were wild-type for all samples target. Sanger sequencing was made to conformation the result. The Y250X mutation was a mutation not found in β thalassemia parents, and healthy control. Normal reference nucleotide in all samples of this study showed by FinchTV software figure (4-15). The FASTA format which result of the sequence showed by alignment with the sequence of homo sapiens transferrin receptor 2 (*TFR2*), accession number (NG_007989.1) shown in figure (4-16)

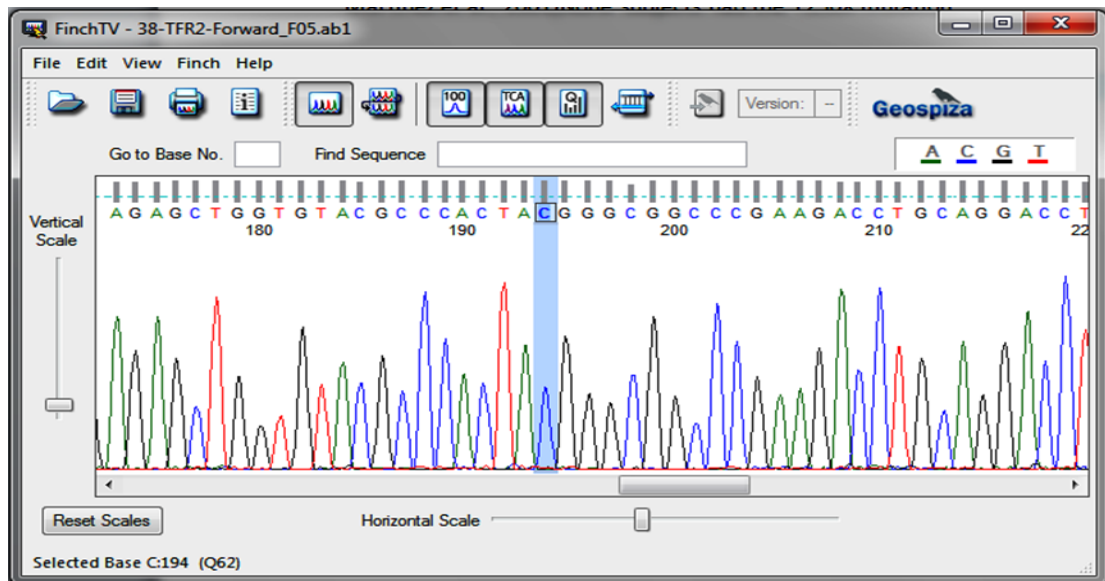


Figure (4-15) showed wild type nucleotide reference nucleotide in G142S: rs80338880 by FinchTV software

Score	Expect	Identities	Gaps	Strand
438 bits(237)	8e-119	237/237(100%)	0/237(0%)	Plus/Plus
Query 1	CCCCTGCCCGCTCCTAGGATGGGGCGGGCAGAGCCCTTTCTGGGTCTCGAAGGAGGG	60		
Sbjct 13331	CCCCTGCCCGCTCCTAGGATGGGGCGGGCAGAGCCCTTTCTGGGTCTCGAAGGAGGG	13390		
Query 61	ACGCGGGGAGCGGCCTACCGTCCTCGTGTCCCAGGGAGAGCTGGTGTACGCCCACTA	120		
Sbjct 13391	ACGCGGGGAGCGGCCTACCGTCCTCGTGTCCCAGGGAGAGCTGGTGTACGCCCACTA	13450		
Query 121	CGGGCGGGCCGAAGACCTGCAGGACCTGCGGGCCAGGGGCGTGGATCCAGTGGGCCGCCT	180		
Sbjct 13451	CGGGCGGGCCGAAGACCTGCAGGACCTGCGGGCCAGGGGCGTGGATCCAGTGGGCCGCCT	13510		
Query 181	GCTGCTGGTGC GCGTGGGGGTGATCAGCTTCGCCCAGAAGGTAAGTGTCCCTGGACA	237		
Sbjct 13511	GCTGCTGGTGC GCGTGGGGGTGATCAGCTTCGCCCAGAAGGTAAGTGTCCCTGGACA	13567		

Figure (4-16) alignment of the sample with specific reference sequence *homo sapiens* transferrin receptor 2 (*TFR2*), accession number (NG_007989.1)

Iron overload is caused by a defect in the iron absorption pathway. Several studies reported the mutations in *TFR2* gene to induce increased intestinal iron absorption by increased iron uptake during diet and macrophage iron release leading

to tissue iron overload (Drake *et al.*, 2007; Piperno *et al.*, 2020). Some studies refer to the effect of Y250X mutation on the *TFR2* gene may elevate iron levels in the blood (Schmidt *et al.*, 2021)). In major β thalassemia patients, the blood transfusion dilutes some complications of thalassemia syndrome while iron level raised (Cappellini, M. D., 2007). This mutation has an important role in iron hemostasis in the body (Camaschella *et al.*, 2000).

In this study, the Y250X mutation was absent in all samples. This result was in agreement with the study of Sun *et al.* (2019) that reported Y250X mutation in the *TFR2* gene does not occur in Tibetan patients with iron overload (Sun *et al.*, 2019). Santos *et al.* (2010) were described Y250X mutation not detected of iron overload Brazilian blood donors (Santos *et al.*, 2010). Moreover, Y250X mutation effect on clinical manifestations of iron overload. This document searched for the Y250X mutation in 63 individuals who have hereditary hemochromatosis. The results were most of the patients had C282Y and H63D mutations but the Y250X mutation was not found in any of the 63 individuals that were tested (Aguilar-Martinez *et al.*, 2001).

On the other side, several studies reported Y250X mutation of *TFR2* gene has a role in the homeostasis of iron and the abnormal iron absorption, study by Kawabata *et al.* (2005) found that homozygous *TFR2* (Y245X) mutant mice ((mirror to the *TFR2*(Y250X) mutation in humans)) showed a hereditary hemochromatosis phenotype (Kawabata *et al.*, 2005). (Fleming *et al.*, 2002) affirms the essential role for Y250X mutation in iron homeostasis and may consider a prognostic marker of abnormal iron absorption (Fleming *et al.*, 2002). The study of Piperno *et al.*, (2004) was Two Italian patients who carried homozygous Y250X mutation, both cases have

increased iron concentration in hepatic cells and elevated serum ferritin (Piperno *et al.*, 2004). The results were unaffected by the iron overload with Y250X mutation also the frequency of this SNP was rare.

4.2. Blood Parameters

The Serum Ferritin level of 21 beta-thalassemia patients was measured by mini VIDIS close system. The mean and stander deviation of serum ferritin levels in patients were (1810.5 g/dl $\pm$ 1158.2). The most number of patients (76.20 %) had a Serum ferritin level of more than 1000 g/dl. The P-value ($P < 0.001$) was significant for serum ferritin in β thalassemia patients. While nonsignificant of HB, MCV, and MCH with β thalassemia patients. The P-value were ($P = 0.07$, $P = 0.99$, $P = 0.23$) respectively table (4-4).

Table (4-4) P value of blood parameters and serum ferritin of β thalassemia patients

blood parameter of patients	mean $\pm$ stander deviation	p value
age in year	6 $\pm$ 2.4	0.23
HB g/dL	8.2 $\pm$ 1.4	0.07
MCV fL	81.5 $\pm$ 5.1	0.99
MCH pg	25.9 $\pm$ 2.2	0.23
Serum Ferritin ng/mL	1810.5 $\pm$ 1158.2	<0.001

One of the blood parameters; the result of HB was nonsignificant the mean $\pm$ sd. (8.2 $\pm$ 1.4) P-value was 0.07. Our result disagreement with (Jameel *et al.*, 2017) which describe low Hb and low MCV (Hb < 9 gram/dl & MCV <80fl) (Jameel *et al.*, 2017). Another study document The hemoglobin level in patients is decreased of the level recorded in controls, (7.05 $\pm$ 1.43; 13.19 $\pm$ 0.95) g/dL. (Wilson *et al.*, 2015 ; Mokhtar *et al.*, 2013) illuminated lower mean hemoglobin levels compared to thalassemia carriers (7.9 $\pm$ 1.6 g/dl vs. 9.9 $\pm$ 2.3 g/dl. Also, there was a significant decrease in Hb (P< 0.001), HCT (P< 0.001) values in beta-thalassemia patients compared to controls (Karim *et al.*,2016)

The result of MCV was nonsignificant the mean $\pm$ sd. (81.5 $\pm$ 5.1) P-value was 0.99. Also, MCH was nonsignificant in the mean $\pm$ sd. (25.9 $\pm$ 2.2) P-value was 0.23. Our result disagreement with (Jameel *et al.*, 2017) which describes low MCV (MCV <80fl) (Jameel *et al.*, 2017). Another study document there was a significant decrease in MCV (P< 0.05) and MCH (P< 0.05) values in beta-thalassemia patients compared to controls(Karim *et al.*,2016). Our results of HB, MCV, and MCH were nonsignificant may be due to repeated blood transfusion with a short period.

My studies report significant. In our study, the Serum ferritin values were significant with β thalassemia patients the P-value was less than 0.001. The results were in agreement with Yasir *et al.* in 2019 which described serum ferritin levels for all patients were >1000ng/mL (Yasir *et al.*, 2019). Other documents showed High serum ferritin levels and serum iron are detected in β thalassemia patients as compared to controls (Al-Abedy *et al.*, 2019). (Shah *et al.*, 2020) reported The serum ferritin level of all the cases was in the range of 2000–4000 g/dl with a mean value of 3,080.9 g/dl. The highest number of patients (26%) had Serum ferritin levels between 3500 and 4000 g/dl. While 21% had 2500–2900 g/dl, 19% had 3000–3400 g/dl, and

5% had between 2000 and 2400 g/dl. Finally, in a study by Soltanpour et al, it was shown that mean serum ferritin levels were significantly higher in both β thalassemia major and β -thalassemia (Soltanpour et al., 2017).

Recurrent blood transfusion in the case of β thalassemia that targets samples of our study refers to increase serum ferritin this may be due to a short period with an increase of the number of blood transferred.

Chapter Five

Conclusions & Recommendations

5.1 Conclusion

1. elevate serum ferritin level in β thalassemia patients.
2. blood parameter value was normal in β thalassemia patients (hemoglobin, MCV, and MCH).
3. The H63D on HFE gene and G71D on mutations were not associated with an iron overload of β thalassemia patients.
4. The mutations C282Y and Y250X were not found in all target groups.
5. The G142S mutation was associated with iron overload in β thalassemia patients.

5.2 Recommendation

1. Identifying the effect of other mutations that predicted to the interaction of iron overload in β thalassemia patients.
2. Genetic tests conducted by the Ministry of Health before marriage to investigations for thalassemia and hemochromatosis must include.
3. Retested the H63D mutation with a wide sample as an iron overload in thalassemia patients due to the P-value being nearest to borderline result.
3. In patients with a liver disorder or iron abnormal tested diagnosis of hemochromatosis should be done.
4. Serum ferritin levels should be monitored in thalassemia patients and other liver disorders.

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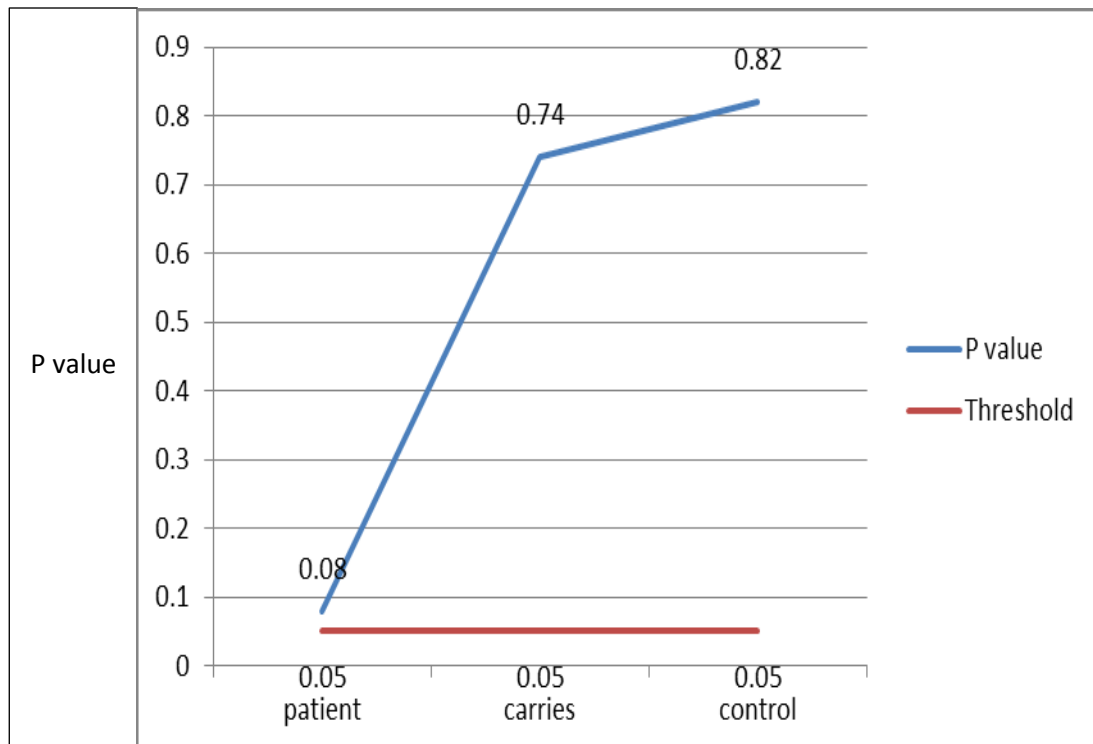
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Appendixes

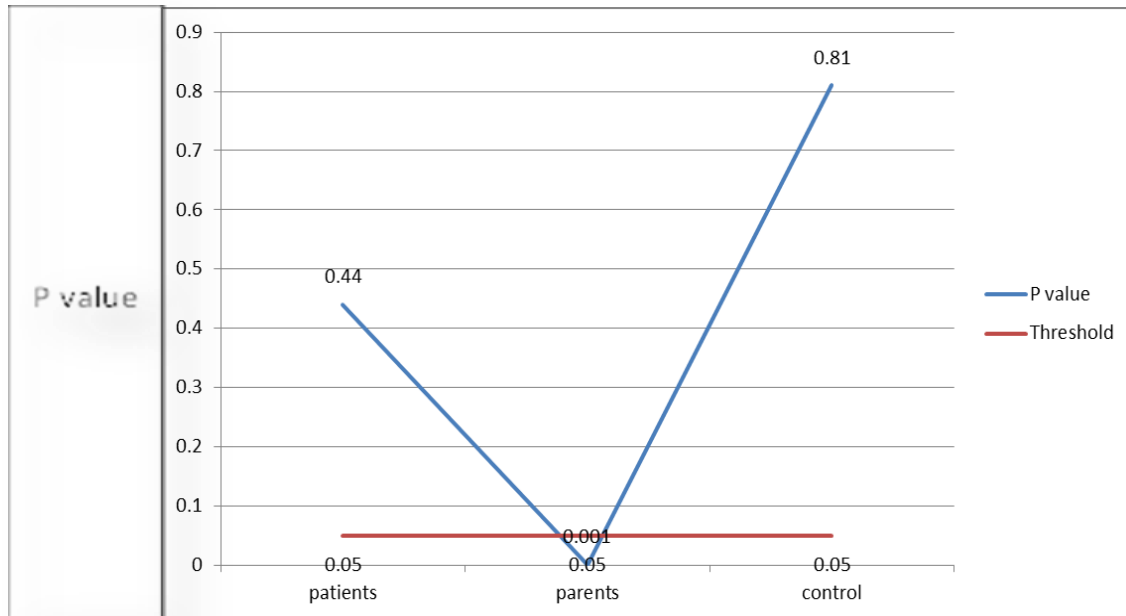
Appendix 1:

distribution the P-value with all samples (patients, parents, and control) of rs1799945: H63D mutation



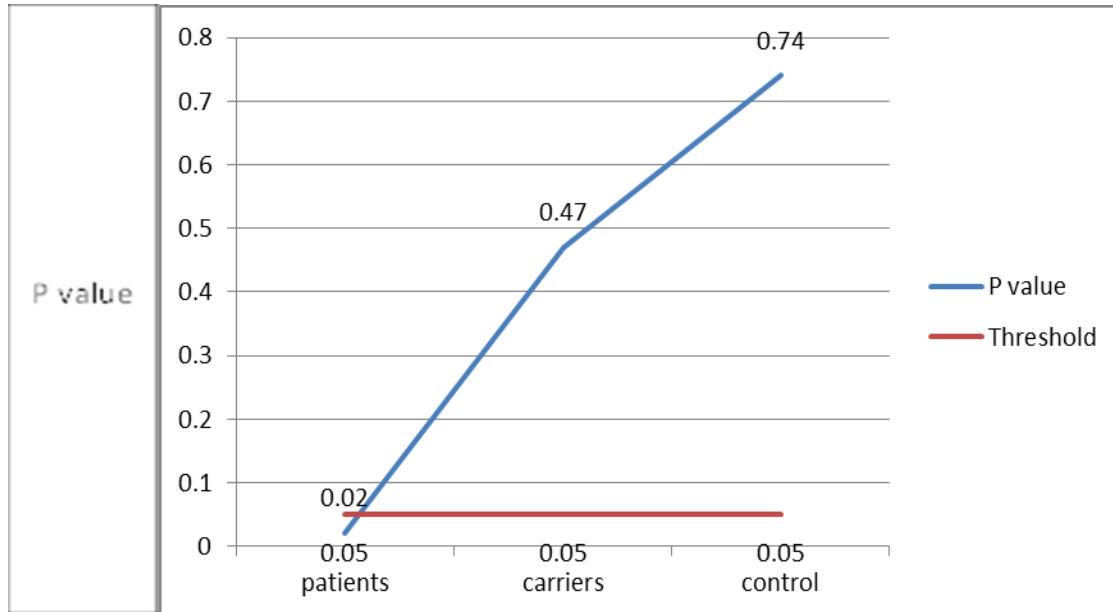
Appendix 2:

distribution the P-value with all samples (patients, parents, and control) of rs104894696: G71D mutation



Appendix 3:

distribution the P-value with all samples (patients, parents, and control) of rs3817672 : (G142S)



الثلاسيميا هو مرض دم وراثي يصنع فيه الجسم شكلاً غير طبيعي من الهيموجلوبين. وهو نوعان α و β وفقاً لعبق في سلسلة الهيموجلوبين. يعتمد علاج الثلاسيميا على تكرار نقل الدم الذي يؤدي إلى زيادة نسبة الفيريتين في الدم. قد يتوافق مرضى الثلاسيميا مع داء ترسب الصبغات الدموية عند نفس المريض نتيجة خلل في آلية استقلاب الحديد مما يؤدي إلى زيادة الحديد. قد يكون للعديد من الطفرات الإصابة بالثلاسيميا وداء ترسب الصبغات الدموية الكثير من الحديد الضار بالجسم ويمكن أن يسبب تلف الأنسجة بمرور الوقت. هدفت هذه الدراسة إلى تحديد تأثير بعض الطفرات (طفرة C282Y و H63D في جين *HFE* و طفرة G71D في جين *HAMP* و طفرة G142S في طفرة *TFR1* و Y250X في جين *TFR2*) على مستويات الفيريتين في الدم والحمل الزائد للحديد في مرضى الثلاسيميا. تم تضمين أربعة وثمانين عينة (21 مريض ثلاسيميا ، 42 ابوين كحامل و 21 عينة سيطره). كانت فترة العينات التي تم جمعها من كانون الثاني الى نيسان 2020. تم إجراء التنميط الجيني للطفرات الجينية بواسطة طريقة تعدد الأشكال لطول تفاعل البوليميراز المتسلسل (PCR-RFLP). كان متوسط مستويات الفيريتين في الدم في مرضى الثلاسيميا 1810.5 نانوغرام / مل ومتوسط الهيموجلوبين في مرضى الثلاسيميا 8.2 جم / ديسيلتر. و MCV ، MCH كانت (81.5 فيمتولتر ، 25.9 بيكوغرام) على التوالي. لم تكن طفرة C282Y موجودة في جميع الحالات الـ 84 ، بالنسبة لطفرة H63D كانت غير معنوية بين الحمل الزائد للحديد عند مرضى الثلاسيميا والابوين ، كانت قيمة P (0.08, $p=0.74$) على التوالي. بالإضافة إلى ذلك كان الارتباط غير المعنوي لطفرة G71D بين الحمل الزائد للحديد ومرضى الثلاسيميا ولكن معنوية مع الوالدين ، كانت قيمة P (0.44, $P=0.001$) على التوالي. كانت طفرة G142S معنوية بالنسبة للمرضى الثلاسيميا وغير معنوية للابوين للارتباط بين الحمل الزائد للحديد ومرض الثلاسيميا ، وكانت قيمة P (0.02, $p=0.47$) على التوالي وأخيراً لم يتم العثور على طفرة Y250X في جميع الحالات. مرضى β الثلاسيميا في مدينة السماوة لهم تأثير معنوي في زيادة الحديد مع G142 ولكن غير معنوي مع طفرة (H63D, G71D) ، من ناحية أخرى ، لم يتم العثور على طفرات (C282Y, Y250X) في كل المجاميع هذه الدراسة.



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