

**Republic of Iraq**  
**Ministry of Higher Education and Scientific Research**  
**Al Muthanna University**  
**College of Science**  
**Department of Biology**



# **Clinical Manifestation and Molecular Diagnosis in Beta-Thalassemia Patients**

A thesis submitted in partial fulfillment of requirements for the Master 's  
Degree of Science in Biology / Zoology

By

**Riham Hussein Noor Al-Mosawi**

Supervisor

Assist. Prof. Dr. **Nihad A.M. Al-Rashedi**

**2018**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

«يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ  
دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ»

صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمُ

المجادلة الآية ( ١١ )

# Supervisor's Certificate

I certify that the thesis entitled  
**(Clinical Manifestation and Molecular Diagnosis in Beta-Thalassemia Patients)**

By

Riham Hussein Noor Al-Mosawi

Has been prepared under my supervision at the College of Science, Muthanna University, as a partial fulfillment of the requirements for Degree of Master of Science in Biology / Zoology.

Assist. Prof. Dr. **Nihad A.M. Al-Rashedi**

2018 /   /

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

Assist. Prof. Dr. **Laith Abdul Hasan M.Jwaid**

Head of Department

2018 /   /

# Dedication

To ... Whom devoted his life for me and taught me patience and loyalty. He was my first teacher who brought me up and improved my education, "**my father**"

To .... Whom took me to her care and enlightened my way by her prayer .. To the river of love and flowing compassion , "**my mother**"

". Respect and Homage "

To ... whom were the beacon of the way, "presence and absence" by their thoughts and bodies, "**my brothers and sisters**"

"Pride and gratitude"

To ... **everyone** treated my hurt, exhausted my sadness and broadcast the ray of hope in my heart and supported me,

"Respect and appreciation"

**I present my modest effort,**

Riham

## **Acknowledgment**

Before anything, I would like to thank merciful **Allah** for enabling me to perform this work. Without His great assistance, the work would not have been finished. The peace and mercy upon our **messenger prophet Muhammad** and His house hold.

I would like to express my thank and gratitude to my supervisor **Assist. Prof. Dr. Nihad A.M. Al-Rashedi**, College of Science, Al-Muthanna University for his scientific guidance, encouragement, support, concern, and kind advice.

I also would like to thank **Deanship of Facility of Science**, especially, **Assist. Prof. Dr. Laith Abd-Alhassan**, for their kind cooperation and advisements.

I would like to thank the **staff members** of the blood and thalassemia unit in Women and Child Hospital for their kind cooperation and advisement and for many facilities given.

My deepest appreciation **to the patients** who expressed their assistance and made this work possible. My praise to the merciful **Allah** that he heals all patients with this disease, and all other diseases and ends their suffering

I am also grateful to **my family** for their support and help.

Finally, great thanks go **to my friends** for encouragement and moral support, and to **everyone** who supported and stood by my side.

**Riham**

## Abstract

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Beta-thalassemias are a group of hereditary hemoglobin disorders in Iraq caused by different mutations in  $\beta$ -globin gene coding for  $\beta$ -globin chains of hemoglobin. The  $\beta$ -thalassemia mutations result to insufficient or absent  $\beta$ -globin synthesis. As a result, affected individuals have different degrees of severity of anemia and hemolysis, which in turn can cause bone changes, impaired growth, and iron overload. This study was aimed to investigate molecular, serological and hematological aspects of  $\beta$ -thalassemia patients in Al-Muthanna province. Ninety-eight blood samples collected from  $\beta$ -thalassemia patients in Women and Children Hospital, thalassemia unit in Al-Muthanna province, aged 1-29 years old and the samples used 61 males and 37 females. Blood samples were taken simultaneously from 32 control individuals to be used as control. Blood samples used for serum measurement of Ferritin, Vitamin D3 and 8-hydroxydeoxyguanosine using Enzyme Linked Immunosorbent Assay technique. Genomic DNA extraction from peripheral blood for detection of  $\beta$ -globin mutations frequency by using direct DNA Sanger sequencing. Result found mean of hemoglobin was significantly decreased in patients group in comparison to control subjects ( $P<0.001$ ). The mean of ferritin concentration was significant increase in patients group in comparison to control subjects ( $P<0.001$ ). The mean 25-hydroxy vitamin D was significant decrease in patients group in comparison to control subjects ( $P<0.001$ ). The present study showed that the mean 8-OHdG was significant increase in patients group comparison to control subjects ( $P<0.001$ ). Analysis revealed the presence of nine mutations in 98  $\beta$ -thalassemia patients of which seven mutations not previously recorded in Iraq, including IVS-II-666 C> T, CD2 CAT>CAC , IVS-II-850 G>A, IVS-II-16 G>C , Hb kangsmill, Hb Saveh , and IVS-II-81 C>T, as well as two mutations previously recorded in Iraq include IVS II-1 G>A and IVS 1-5 G>C.

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## Abbreviations

| Symbols       | Meaning                                       |
|---------------|-----------------------------------------------|
| 1,25 (OH) 2D3 | 1,25-Dihydroxy vitamin D3                     |
| 25 (OH) D3    | 25-Hydroxy vitamin D3                         |
| ALP           | Alkaline phosphatase                          |
| ARMS          | Amplification-Refractory Mutation System      |
| Ca            | Calcium                                       |
| CEN           | Centromere                                    |
| DNA           | Deoxyribonucleic Acid                         |
| ECAC          | The Epithelial Calcium Channel                |
| EDTA          | Ethylenediaminetetraacetic Acid               |
| ELISA         | Enzyme Linked Immunosorbent Assay             |
| GH            | Growth Hormone                                |
| Hb            | Hemoglobin                                    |
| HbA           | Adult Hemoglobin                              |
| HbA2          | Adult Hemoglobin subunit 2                    |
| HbF           | Fetal Hemoglobin                              |
| HRP           | Horseradish Peroxidase                        |
| IGF-I         | Insulin-Like Growth Factor 1                  |
| IVSI          | Intervening Sequences Intron                  |
| MCH           | Mean Corpuscular Hemoglobin                   |
| MCHC          | Mean Corpuscular Hemoglobin Concentration     |
| MCV           | Mean Corpuscular Volume                       |
| Mg            | Mili gram                                     |
| ML            | Mili liter                                    |
| mRNA          | Messenger Ribonucleic Acid                    |
| NCBI          | National Center For Biotechnology Information |
| Ng            | Nano gram                                     |
| OD            | Optical Density                               |
| PBS tablets   | Phosphate-buffered saline tablets             |
| PCR           | Polymerase Chain Reaction                     |

|                  |                                          |
|------------------|------------------------------------------|
| Ph               | Potential of Hydrogen                    |
| PTH              | Parathormone                             |
| RBCs             | Red Blood Cells                          |
| RFLP             | Restriction Fragment Length Polymorphism |
| ROS              | Reactive Oxygen Species                  |
| SF               | Serum Ferritin                           |
| SPSS             | Statistical Packages For Social Science  |
| TB               | Tris - Borate                            |
| TBE              | Tris Base- Boric Acid- EDTA Buffer       |
| TE buffer        | Tris - EDTA buffer                       |
| TEL              | Telomere                                 |
| TMB substrate    | Tetramethylbenzidine substrate           |
| UTR              | Untranslated Region                      |
| UV               | Ultra Violet                             |
| VD               | Vitamin D                                |
| VDD              | Vitamin D Deficiency                     |
| VDR              | Vitamin D Receptor                       |
| $\alpha$ -Globin | Alpha Globin                             |
| $\beta$ -Globin  | Beta Globin                              |

# **Chapter One**

## **Introduction**

## 1. Introduction

Thalassemias are inherited blood diseases due to defects in the synthesis of hemoglobin chains. There are two types of thalassemia; alpha ( $\alpha$ ) and beta ( $\beta$ ) thalassemia.  $\alpha$ -thalassemia is caused by impaired production of the alpha globin chains, while  $\beta$ -thalassemia is a deficient synthesis of the  $\beta$  globin chains. Thus imbalances of one of these chain cause hemolysis and impair erythropoiesis. The signs of thalassemia include hemolytic pallor, poor development, and skeletal distortions. During childhood, the consequences of thalassemia disease impose the transfusions for a lifetime [1]. The clinical severity of the thalassemia depends on the type of mutations in the gene. Genetic analysis suggests that more than 200 different mutations have been reported and identified in the globin gene which is responsible for the development of thalassemia [2].  $\beta$ -thalassemia resulting from mutations in one or more of the  $\beta$ -globin gene that leads to reduced  $\beta$ -globin production and is home- and heterogeneous of gene structure at the molecular level [3]. These types of mutations have been reported to interfere with the transcription of the  $\beta$ -globin gene, splicing steps and translation of  $\beta$ -globin gene mRNA can lead to either loss or decrease in synthesis of  $\beta$ -globin chains [4].

Most types of  $\beta$ -thalassemias are due to point mutations, and large deletion mutations are found in rare cases [5].  $\beta$ -thalassemia diseases are classified based on their clinical severity into thalassemia major requires of the regular procedure of blood transfusion for a lifetime, thalassemia intermedia is described mild symptoms of the patient which require occasional blood transfusion therapy, while  $\beta$ -thalassemia minor is described as the carrier state of disease and usually do not require medical therapy [1]. Consequently, complications of diseases resulted in delayed growth, hormone-related disorders and gradual decline in liver and kidney function. The major challenge which is faced by the patient is excessive levels of free radicals can make oxidative damage [6].

Recurrent blood transfusions in  $\beta$ -thalassemia major lead to accumulation of excess iron in the body tissues. This amount of iron overload is responsible for peroxidative harm and increasing the production of reactive oxygen species inside the erythrocytes, so leading to oxidative stress [7]. Reactive oxygen species (ROS) are capable of causing oxidative damage to macromolecules, oxidation of amino acid side chains, and oxidation of polypeptide chains causing protein fragmentation, DNA damage, and DNA strand breaks [8].

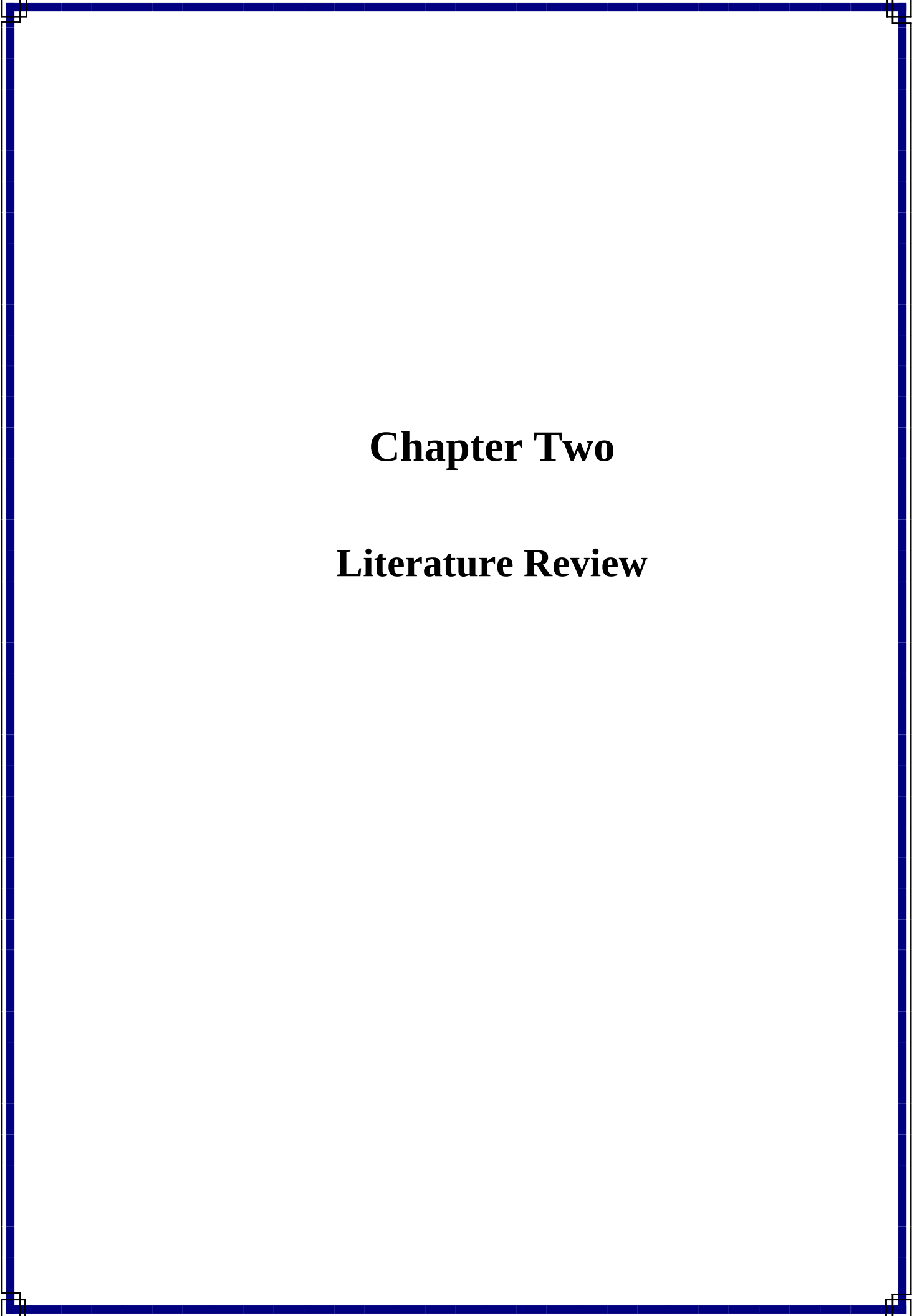
Metabolism of vitamin D has affected the  $\beta$ -thalassemia patients due to the accumulation of iron in the liver and the skin [9]. Vitamin D is essential for calcium (Ca) homeostasis and skeleton mineralization. Deficiency and insufficiency in vitamin D are reported to be in thalassemic patients. Bone disorder becomes a serious cause of morbidity and problems include fractures, scoliosis, rickets, spinal deformities and osteoporosis [10]. Impaired calcium homeostasis is thought to be a consequence of iron overload to be seen in  $\beta$ -thalassemic transfused patients. Both defective synthesis of 25 OH vitamin D (25OH D) and/ or hypoparathyroidism have been described in  $\beta$ -thalassemic patients and negatively affect their bone metabolism [11].

Severe signs management in  $\beta$ -thalassemia diseases depends on four mainstays treatment regimen: regular blood transfusion, removal of overloaded iron with chelating agents and splenectomy when enlarged spleen occur as well as compensation of 25-hydroxyvitamin D deficiency[12]. Programs based on awareness, carrier screening, genetic counseling, and prenatal diagnosis can be very effective in preventing the disease[13].

Based on the mentioned facts, and in the view of the studies published in Iraq, it found that most studies focused on screening of  $\beta$ -globin mutations were performed by using amplification refractory mutation system (ARMS) PCR assay to identify types of mutations that cause  $\beta$ -thalassemia in different regions of country. The previous studies haven't included analysis of  $\beta$ -gene sequence, nor its relationship to the impact of DNA damage and vitamin D3 to reveal the extent of this impact on  $\beta$ -thalassemia patients; therefore the present study was conducted to study the region of  $\beta$ -gene and screening a large region of  $\beta$ -gene by using direct DNA sequencing to determine the molecular basis as well as DNA damage and vitamin D3 associated with  $\beta$ -thalassemia patients in Al-Muthanna province[14]

**1.2 The aims of the study:**

- 1- Quantification of oxidative DNA damage in  $\beta$  – thalassemia patients.
- 2- Estimation of vitamin D level in the serum of patients with  $\beta$  – thalassemia.
- 3- Detection of  $\beta$ -globin mutations using direct DNA Sanger sequencing.



# **Chapter Two**

## **Literature Review**

## 2. Literature review

### 2.1 Thalassemia

The term thalassemia was firstly described by George Whipple and William Bradford in 1932 that derives from two parts 'thalassa' is the Greek word: sea and 'haema' (Latin: anemia) is the Greek word: blood. It was referred to as Mediterranean anemia; the affected people are from Mediterranean origins. It refers to a group of genetic blood disorders which characterized by anemia due to enhanced RBC destruction [15].

Thalassemia (Mediterranean anemia) is an inherited autosomal recessive blood disease. That genetic defect result in the absence or reduced rate of synthesis of the globin chains which make the hemoglobin. However, formation of abnormal hemoglobin molecules causes the anemia [16]. Thalassemia is inherited as alleles of one or more of the globin genes located on either chromosome 11 ( $\beta$ ,  $\gamma$  and  $\delta$  chains) or on chromosome 16 for  $\alpha$  chains [17]. Thalassemia patients do not produce enough of one (or occasionally both) of globin proteins, therefore their red blood cells may be abnormal and unable to carry enough oxygen throughout the body [18].

Thalassemia is a common hematological disorder worldwide, 4.8% of the world populations carry globin gene mutations include 1.6% people are heterozygous for alpha and 1.9 % for beta-thalassemia [18].  $\beta$ -thalassemia is seen most commonly in the Mediterranean populations including, (North African, and particularly Italian and Greek), Middle Eastern, Indian, African, Chinese, and Southeast Asian including, Vietnamese, Laotian, Thai, Singaporean, Filipino, Cambodian, Malaysian, Burmese, and Indonesian [19].

Thalassemia is a public hematological disease in Iraq, especially in the north region of Iraq because these regions boarded with Turkey and Iran which consider as high endemic of thalassemia and nature of their population have mixed ethnic, in addition to consanguineous marriages. In Iraq until end of 2015,  $\beta$ -thalassemia reported 8246 patients in 16 thalassemia centers throughout the country that prevalence was 27.4 per 100,000 of Iraqi population, it is consider high value may be due to lack of thalassemia prevention program and consanguine marriage[20].

## 2.2 Clinical Classification of $\beta$ -Thalassemia

### 2.2.1 $\beta$ -Thalassemia Major

Thalassemia major is the severe type of  $\beta$ -thalassemia since both  $\beta$ -globin genes are mutated, that homozygous of  $\beta$ -thalassemia cause to the disability of the  $\beta$ -globin chain production of the genotype described as  $\beta^0/\beta^0$  which are responsible for  $\beta$ -thalassemia major. The first description of clinical thalassemia major picture was Thomas Cooley in 1925 so that thalassemia major also known as Cooley's anemia. The baby affected with  $\beta$ -thalassemia major is normal at birth because fetal hemoglobin is consist of two alpha and two gamma chains, but after few months begins to progressively severe anemia because of a fail switch of  $\beta$ -globin chain production in this developmental stage [21].

Thalassemia major cases need to frequent blood transfusion for the patient survive. However, on infant fails to normally grow and also occurs problems feeding due to fever, fatigue, diarrhea, and other intestinal problems. In first and second year old of patients show clinical manifestations including pale, fatigue, fussy, poor appetite, hepatosplenomegaly, heart failure, and bone deformation and delayed puberty, laboratory abnormalities include microcytic anemia with abnormally shaped RBCs [22]. In thalassemia major, the excess unpaired alpha-globin chains aggregate to form inclusion bodies. These chain inclusion bodies damage RBC membranes, leading to intravascular hemolysis [23]. In addition, there is damage and premature destruction of RBC precursors, causing ineffective erythropoiesis. Anemia is severe and oxygen transport is compromised [24].

### 2.2.2 $\beta$ -thalassemia Intermedia

The  $\beta$ -thalassemia intermedia patients are severe symptoms of  $\beta$  thalassemia major to the mild manifestation of  $\beta$  thalassemia major, in most cases do not require regular blood transfusions [25]. This condition is milder than thalassemia major due to the inheritance of a  $\beta$ - globin gene mutation associated with reduced  $\beta$ -globin chain production, so ranging at least 6-7 g/dl of hemoglobin, the deduced genotype  $\beta$ -thalassemia intermedia is  $\beta^+/\beta^+$  or  $\beta^0/\beta^+$  [26].

The clinical phenotype of thalassemia intermedia is roughly intermediate between thalassemia major and trait (minor). Common clinical features include splenic enlargement due to entrapment of damaged RBCs, with the risk of iron overload due in part to increased intestinal absorption [27]. Although thalassemia

intermedia can be associated with poor growth and bone abnormalities. These symptoms appear later in patients posture [25]. They require regular monitoring because the clinical severity varies widely between patients and within a patient over time, with a possible change to the thalassemia major phenotype[28]. Several rare  $\beta$ -globin variants phenotypically manifest thalassemia intermedia[26]. A rare variant form called “silent  $\beta$ -thalassemia” results from a mild imbalance of globin chain synthesis due to reduced  $\beta$ -globin synthesis, leading to thalassemia intermedia, silent  $\beta$ -thalassemia mutations are found mainly in the regulatory regions,  $\beta$ -globin promoter and 5' and 3' UTRs[29].

### **2.2.3 $\beta$ - Thalassemia Minor**

Thalassemia minor is a most common form of  $\beta$ - thalassemia, known as the ‘thalassemia trait’, in which affected individuals are asymptomatic [18]. This pathogenic type is typically heterozygous for  $\beta$ -thalassemia since they carry a normal  $\beta$ -globin allele ( $\beta$ ) and another mutated allele - either  $\beta^0$  or  $\beta^+$  [30]. Asymptomatic patients are usually detected through routine hematologic testing, but in retrospect some early diagnosed patients are observed to have mild anemia and small RBCs, the primary caution for individuals with thalassemia minor is a potential risk of having children affected with more serious thalassemia if their partner is also a carrier of thalassemia minor [31].

## **2.3 Other Types of Thalassemia**

In addition to alpha and beta-thalassemia, there are other types of related disorders which occur when the gene for alpha or beta thalassemia combines to an abnormal or mutant gene [32].

### **2.3.1 $\beta$ - Thalassemia Hemoglobin (E)**

It is one of most common abnormal hemoglobin diseases. It is usually found in people of south-east Asian population, such as Cambodians, Vietnamese, Thailand and rarely in a Chinese population. Hemoglobin E has a change from lysine to glutamic acid at position 26 codon of the  $\beta$ -globin chain [32].

### 2.3.2 Sickle $\beta$ -Thalassemia

This hematological disorder is caused by a combination of beta-thalassemia and abnormal hemoglobin S, that associated with sickle cell patients. It's commonly in a population of Mediterranean ancestry. The condition varies according to the amount of normal beta globin, produced by the beta subunits of the globin gene [33].

## 2.4 Globin Gene

Hemoglobin is a heterotetramer that contains two polypeptide subunits related to the  $\alpha$ -globin gene subfamily (referred to  $\alpha$ -like globins) and two polypeptide subunits related to the  $\beta$ -globin gene subfamily ( $\beta$ -like globins). Globin polypeptides bind heme, which in turn allows the hemoglobin, in erythrocytes, to reversibly bind oxygen and transport it from the lungs to respiring tissues. Humans normally produce several kinds of the hemoglobin protein (oxygen-carrying protein, a developmental stage in individuals determines the production of primarily embryonic, fetal, or adult hemoglobin's, as shown in Figure 2-1. All types of hemoglobin proteins are consist of three components including (heme, alpha (or alpha-like) globin, and beta (or beta-like globin). All types of thalassemia are due to changes in alpha- or beta-globin gene. These changes cause less or no globin protein to be produced. Therefore, the thalassemia is considered as quantitative hemoglobin diseases [32].

All types of thalassemia are inherited. The genetic change inherited from both the father and the mother. The seriousness of the thalassemia disease is influenced by the thalassemia mutations inherited as well as environmental factors[34]

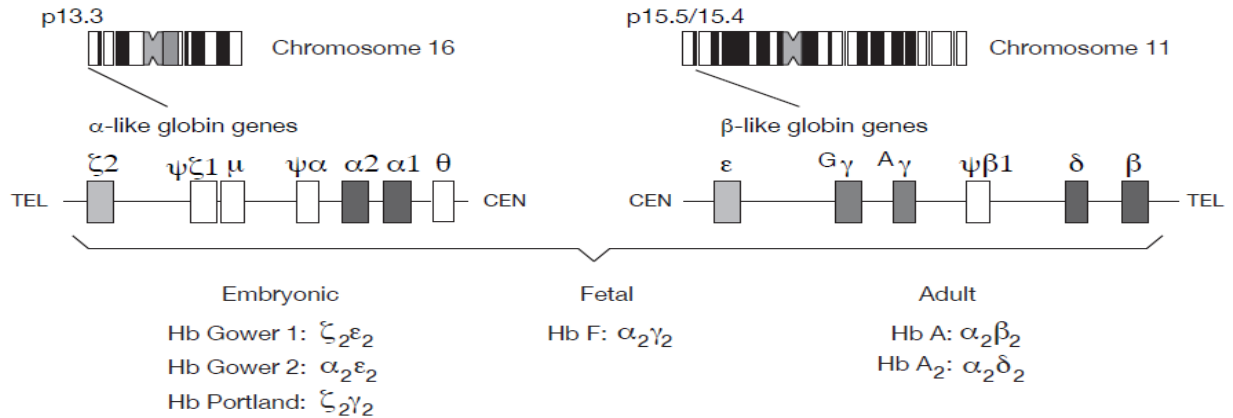


**Figure (2-1):** Basic Organization of Human Globin Gene Clusters. The locations of the alpha-globin gene cluster very close to the telomere of the short arm of chromosome 16 and the beta-globin gene cluster on the short arm of chromosome 11. The orientations with respect to the centromere (CEN) and telomere TEL) are opposite; the alpha-like globin genes are transcribed toward CEN whereas the beta-like globin genes are transcribed toward TEL. The composition of hemoglobin's produced at progressive developmental stages is showed at the bottom of the figure[35].

#### 2.4.1 $\alpha$ -Globin Gene Cluster

The  $\alpha$ -globin gene cluster is located on the short arm of chromosome 16. It is comprised of two  $\zeta$ -globin genes followed by two  $\alpha$ -globin genes. Embryonic hemoglobin is made up of two  $\zeta$ -globins that produced by the two  $\zeta$ -globin genes on the  $\alpha$ -globin gene cluster, and two  $\epsilon$ - globins produced by the two  $\beta$ -globin genes on the  $\beta$ -globin gene clusters, as shown in figure 2-2. Adult hemoglobin is made up of two  $\alpha$ -globins produced by the two  $\alpha$ -globin genes on the  $\alpha$ -globin gene cluster, and two  $\beta$ -globins produced by the two  $\beta$ -globin genes on the  $\beta$ -globin gene cluster. The genes on the  $\alpha$ -globin gene cluster are in the same order as their expression throughout development on the short arm of chromosome 16.

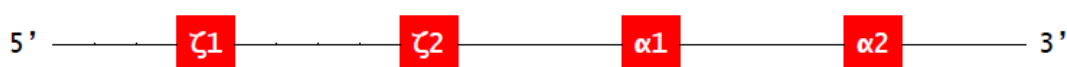


**Figure (2-2):** Structure of Human  $\alpha$ -Globin Gene Cluster on Chromosome 16.

### 2.4.2 $\beta$ -Globin Gene Cluster

Control persons have two natural copies of the beta-globin gene, located on chromosome 11, as shown in figure 2-3, and synthesis the beta-globin component of normal hemoglobin protein (hemoglobin A), there are approximately 200 genetic mutations that causing beta-Thalassemia, described as either  $\beta^0$  or  $\beta^+$  mutations, in case of  $\beta^0$  mutation where beta globin didn't produce, and only a little natural amount of beta globin is produced in case of  $\beta^+$  mutation [21, 34].

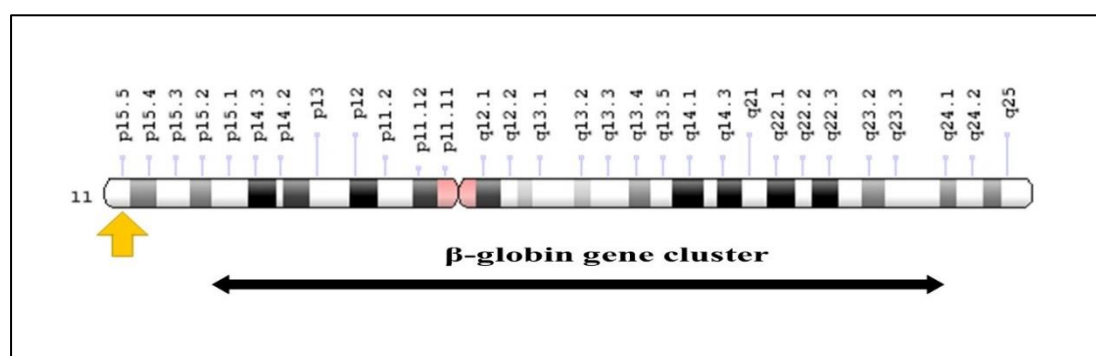


**Figure (2-3):** Schematic Represent of The  $\beta$ -Globin Locus That Resides on Chromosome 11 Cytogenetic Location: 11p15.5 at position 15.5 (Homo sapiens) (NCBI) (<https://www.ncbi.nlm.nih.gov/genome/tools/gdp>)

### 2.5 Structure and Regulation of Globin Gene

The  $\beta$ -globin gene contains three exons separated by two introns of variable length, [32], figure 2-4. Transcription begins at a site called Cap, promoter region located immediately after cap site, which consists of (100) base pairs, there are three of short sequences within promoter region, where bind RNA polymerase that regulates messenger RNA synthesis [36].

Two sequences are essential for the initiation of transcription. These sequences are called CAT box and TATA box [37] when Mutations hit these sequences reduce bind RNA polymerase and result in limit mRNA transcription. The sequence of AATAAA present downstream from the third exon, these sequences triggers the enzyme process to cuts mRNA at an appropriate point and termination of gene transcription [38, 39]. Other two promoter elements, CCAT box and CACC homology  $\alpha$  box present upstream from Cap site, these sequences required for optimal transcription [18, 40].

In the Cap site, exon one encompasses 50 bp of 5' untranslated sequences as well as codons for amino acids 1- 31 in  $\alpha$  and 1- 29 together with two bp of codon 30 in the  $\beta$ - globin gene, exons two encode amino acids 32 – 99. The site of the globin polypeptide which involved in binding of haem and amino acids 31 – 104 that is  $\alpha_1\beta_2$  and  $\alpha_2\beta_1$  [41], remaining amino acids 100 – 141 for  $\alpha$ , 105 – 146 for  $\beta$  and 3' untranslated region of (100 bp) are encoded on exon three, the intervening sequences intron (IVSI) varies in the length from one allele to another. IVSI are removed in the initial transcription phase and the sequences of the exon are joined with mRNA[42].

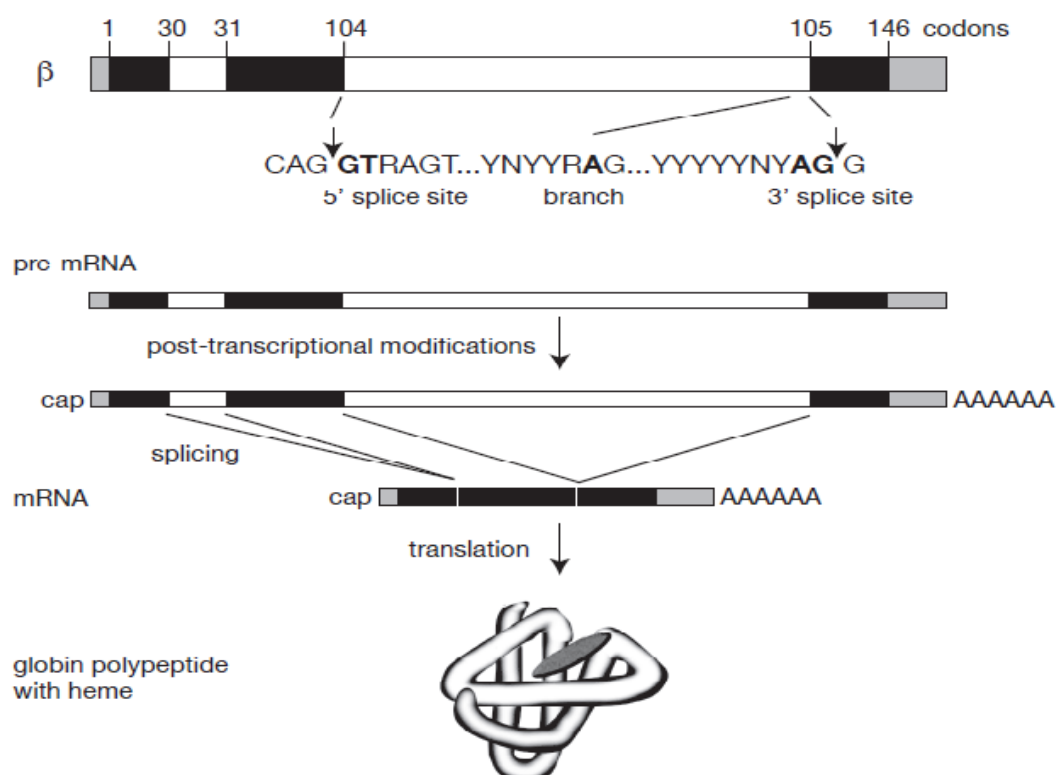


**Figure (2-4):** The Pathway for The Expression of Globin Genes. The RNA transcript is shown with short boxes corresponding to the untranslated regions (gray), coding regions (black and introns (white)) as in A, with processing and splicing steps occurring in the nucleus to form the mature mRNA. The mRNA is translated in the cytoplasm to generate a globin polypeptide to which the heme (gray disk) will bind. (Genes,2009) [43]

Synthesis of hemoglobin is heterogeneous at all developmental stages. It is confined to yolk sac in the embryo, where hemoglobin Gower 1 ( $\zeta_2\varepsilon_2$ ), Gower 2 ( $\alpha_2\varepsilon_2$ ) and Portland ( $\zeta_2\gamma_2$ ) are syntheses at 5 weeks gestation the  $\zeta/\zeta +$  ratio of synthesis  $\alpha$  chains is  $0.82 \pm 0.04$ , in the 6<sup>th</sup> week of pregnant it declines to 0.03 during

developmental stage there is turn to fetal hemoglobin (HbF,  $\alpha_2\gamma_2$ ) then to adult hemoglobin HbA<sub>1</sub> ( $\alpha_2\beta_2$ ) and HbA<sub>2</sub> ( $\alpha_2\delta_2$ ) [44]. Expression of  $\alpha$  - globin gene remains constant during life, whereas the proteins produced by  $\alpha_1$  and  $\alpha_2$  genes are identical, however, the state level of  $\alpha_2$  – mRNA predominates its steady over the  $\alpha_1$  – mRNA by approximately 3:1[45].

## 2.6 Mutations Causing $\beta$ –Thalassemia

The thalassemias are heterogeneous at the level of molecular, and there are more than 200 genetic mutations causing the disease [1], as shown in figure 2-5. Although most of the  $\beta$ -thalassemia mutations included substitutions, deletions or insertions of a single or a few nucleotides, large deletions taking away part, the entire or multiple genes [46]. Can be subdivided large deletions in the cluster of  $\beta$  globin gene into two types; the first type involving the 5' upstream sequences and the second involving the 3' downstream sequences [47]. In all types of pathological mutations, the result either absence ( $\beta^0$ -thalassemia) or reduced synthesis of  $\beta$  globin chains ( $\beta^+$ -thalassemia). Mutations located in or close to the sequences of the conserved promoter and in the 5' region (untranslated) that down-regulate of transcription, which results in mild  $\beta^+$ -thalassemia [48]. Deletions 5' involving in Cap site region of the gene also inactivate transcription and causing of  $\beta^0$  thalassemia. Mutations disrupting the mechanism of splicing and may block or reduce the precursor messenger RNA (mRNA) resulting in unstable mRNA or non-functional and then  $\beta^0$ - or  $\beta^+$ -thalassemia phenotypes [21].

Mutations involving the invariant dinucleotide at junctions of (intron/exon) are critical for splicing of exon and functional (mRNA) production, which result in  $\beta^0$  [4]. These generating “cryptic splice sites”, or those conserved nucleotide sequences may be result in both mild  $\beta^+$ - as well as severe  $\beta^0$ - thalassemia, small deletions or substitutions effect on the (Poly A) signal conserved sequences (AATAAA) in the 3' untranslated regions and cause ineffective cleavage of the mRNA transcript result in mild  $\beta^+$ -thalassemia phenotypes[49].

Stop codons that generated by mutations will interfere with initiation or termination in the production of globin-chain, which results in  $\beta^0$ -thalassemia. Approximately (50%) of all mutations in  $\beta$ -thalassemia interfere with a translation stage [50]. Mutations occur in the third exon result in the production of considerable lengths chains of globin that unsuitable to formation dimer and are too big for proteolytic disposal [4]. This, as well as a relative increase of  $\alpha$ -globin chains, lead to

precipitation in the red-cell precursors of both free  $\alpha$  and ineffective  $\beta$ -chains result in ineffective erythropoiesis, even in the heterozygous case, this case so-called dominantly inherited  $\beta$ - thalassemia or Heinz Bodies anemia [51].

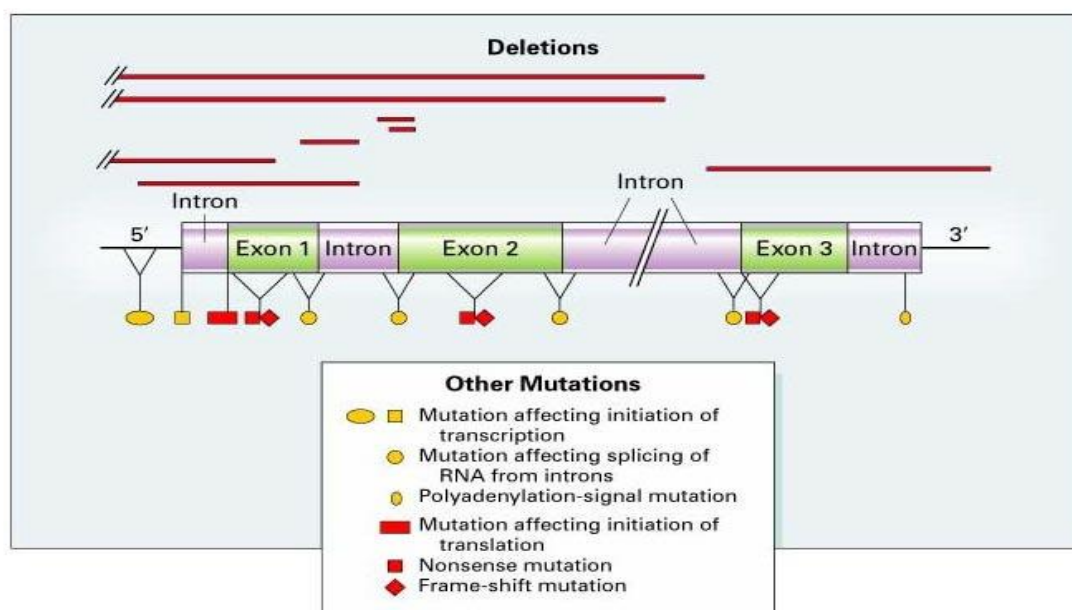


**Figure (2-5):** Location of Mutations in The  $\beta$ -Globin Gene That Cause  $\beta$ -Thalassemia(Olivier,1990) [52]

### 2.6.1 Mutations of $\beta$ -Thalassemia in Iraq.

There are two types of mutations IVS1-110 and IVS1-1 that reported in Iraqi  $\beta$ -thalassemia patients [17]. A novel beta-chain silent variant, a change of codon 10 from GCC to GTC, in association with  $\beta$ -thalassemia as described by [53]. In another study using two specific primers to detect deletion mutation in  $\beta$ -globin genes in severe forms of thalassemia syndrome  $\beta$ -thalassemia major [54].

In Dohuk city of northern Iraq, determine twelve mutations out of a total of twenty screened mutations are detected, and the most eight frequent mutations in order of frequency are: codon 39 C>T, codon.44 (-C), codon5 (-CT), IVS-I-6 T>C, IVS-II-1 G>A, IVS-I-5 G>C, codon 8/9 (+G), and IVS-I-1 G>A, the less four frequent mutations are: codon 30 G>C, codon 22 (-7 bp), IVS-I-110 G>A and codon 8 -AA. [14]

Al-Hadad study in (2007) that involved the molecular analysis of some Iraqi  $\beta$ -thalassemia families by using PCR- RFLP with *Bsu361* enzyme. The study recovery of the presence of point mutation in a  $\beta$ -globin gene in  $\beta$ -thalassemia major result in

less expression of  $\beta$ -globin genes. Thus it is maybe to conclude that point mutation leads to inactivation of the  $\beta$ -globin gene [55]. Al-Assadi in (2007) determine seven mutations types in his study by PCR -ARMS technique on the individual with  $\beta$ -thalassemia from Iraqi population are IVS2 nt.745, IVS1nt.5, IVS2 nt.1, IVS1 nt.110, codon 39, IVS1 nt.6, and IVS1 nt.1 [56]. In a previous study on three main Iraqi ethnic groups with  $\beta$ -thalassemia (Arab, Turkman, and Kurd) where four types of  $\beta$ -thalassemia mutations are determined are Codon 44, IVSInt.6, IVSInt.110 and Codon 39 by using PCR- ARMS technique [57].

In the Kurdish population of northeastern Iraq, Jalal *et al* (2010) carried out his study to detect the molecular basis of  $\beta$ -thalassemia by using multiplex PCR technique and direct sequencing, eleven different  $\beta$ -thalassemia mutations are determined in the  $\beta$ -thalassemia samples include IVS-II-1 G>A, IVS-I-1 G>A, codon 8 -AA, IVS-I-110 G>A, IVS-I-5 G>C, codon 5 -CT, IVS-I-6 T>C and codon 8/9 +G [14]. Other study determined some of the mutations in thalassemic Iraqi population by developing (multiplex ARMS technique), which can detect seven different  $\beta$ -thalassemia mutations are detected in the studied samples are IVS1 nt.6, codon 39, codon 37 IVS2 nt.1, IVSI-110 and codon 8/9, IVS1 nt.1 [58].

### 2.6.2 Mutations of $\beta$ - Thalassemia in Neighboring Regions.

In the Jordan population, 19 different mutations determined in (2001). The most nine frequent mutations is, IVS2-1 G> A, codon 37 G> A, IVS2-745 C> G, IVS1-6 T> C, IVS1-1 G>A, codon 39 C> T, IVS1-110 G>A and codon 5 -CT [59].

In another study, 13 different mutations are determined, Mediterranean alleles (7) including codon 5 -CT, IVSII-745 C>G, codon 27 G>T, IVSII-1 G>A, -87 C>G, IVSI-1 G>A and codon 6 -A, one Eastern Mediterranean IVSI-110 G>A, two the Western Mediterranean IVSI-6 T>C and codon 39 C>T, one Asian -Indian mutations IVSI-5 G>C, one Egypt, Saudi Arabia codon 37 G>A. One Egyptian IVSII-848 C>A [60].

Kyriacou *et al* in (2000) determined most common eight mutations of  $\beta$ -thalassemia in Syria are: IVS-I-1 G>A, codon 5 -CT, codon 15 TGG>TAG, -30 T>A, IVS-I-110 G>A, codon 39 C>T, IVS-II-1 G>A, and IVS-I-6 T>C [61].

The previous study by Zahid (2001) determines in his study 16 of  $\beta$ - thalassemia mutations in Syria. six of them are Mediterranean alleles codon 5 -CT, IVSII-1 G>A, IVSI-116 T>G, -87 C>G, IVSII-745 C>G and IVSI-1 G>A, four Asian -

Indian mutations codon 15 G>A, codon 8/9 +G, -88 C>T, 25 bp deletion], one Eastern Mediterranean IVSI-110 G>A, two Turkish codon 8 -AA and -30 T>A, two the Western Mediterranean IVSI-6 T>C and codon 39 C>T, and one Saudi Arabia, Egypt codon 37 G>A [60].

While Adekile *et al* (1994) detect (12) mutations of  $\beta$ - thalassemia in the Kuwaiti population. Six of them are Mediterranean alleles IVS-I-6 T>C, IVS-I-110 G->A, codon 39 C>T, codon 8 -AA, IVS-II-1 G>A and IVS-I-1 G>A, four of them are East Indian mutations IVS-I-5 G>C, , codon 8/9 +G, IVS-I-3' end -25nt deletion and 619 -bp del, two of them are Kurdish-Iranian alleles codon 36/37 -T, and codon 44 -C [62]. Other study identified  $\beta$ -thalassemia mutation in 18 patients, four of them are homozygous for IVS-I-6 T>C and four of IVS-I-II G>A mutations, while the other is found to be compound heterozygote with a combination of the subsequent mutations: -28 A>C and IVS-II-1 G>A; codon 19 A>G and IVS-I-6 T>C; -101 C<T and codon 8 -AA and IVS-I-110 G>A [47].

In Saudi Arabia, where 14 Mutations of  $\beta$ - thalassemia identified, four of them are Mediterranean alleles IVSI-1 G>A, codon 6 -A, IVSII-1 G>A and IVSI-5 G>T, four of them are Asian -Indian mutations codon 8/9 +G, IVSI-5 G>C, 25 bp deletion and Cap +1 A>C, one of them is Eastern Mediterranean IVSI-110 G>A, one Kurdish, Iran codon 36/37 -T, two Western Mediterranean IVSI-6 T>C and codon 39 C>T, one Turkish codon 8 -AA, and one Kurdish codon 44 -C [60].

Forty-three different mutations are determined in  $\beta$ - thalassemia patients from the Iranian population, the predominant mutation is IVS-II-1 G>A followed in decreasing order of frequency by the IVS-I-5 G>C, the IVS-I-110 G>A, the frameshift codon 8 -AA, the IVS-I-1 G>A, the codon 5 -CT, the[ 3' del 25 nt], the IVS-I-6 T>C, and codon 39 C>T mutations [63].

The severe  $\beta^+$  mutation IVS-I-5 G>C substitution is the most common allele in the south of Iran. This mutation, identified as (Asian/Indian) defect, is most common in regions of the Indian subcontinent, Oman, the Emirates (UAE), and the south of Iran. This reflects the last trade between the Arabic peninsula and India and the distribution of the Arabic culture. The percentage of this mutation is about (1%) in the southeast and east of Turkey, close to Iraq and Iran [64]. The deletion in dinucleotide at codon 8 -AA, described in a Turkish  $\beta$ - thalassemia patients, mostly occurs in the west part of Iran and the southwest Iran [65].

**Table (2-1):** Mutations of  $\beta$ -Thalassemia in Iraq and Neighboring Regions.

| Population          | $\beta$ -gene Mutation                                                                                                                                                                | Reference |
|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Iraq</b>         | IVS1-110 G>A, IVS1-1 G>A                                                                                                                                                              | [17]      |
|                     | Codon 10 GCC > GTC                                                                                                                                                                    | [53]      |
|                     | Codon 39 C>T, Codon.44 -C Codon5 -CT, IVS-I-6 T>C, IVS-II-1 G>A, IVS-I-5 G>C, Codon 8/9 +G Codon 30 G>C, Codon 22 (-7 bp), Codon 8 -AA                                                | [14]      |
|                     | IVS2 nt.745 C>G, IVS1nt.5 G>C, IVS2 nt.1 G>A                                                                                                                                          | [56]      |
|                     | Codon15 G>A                                                                                                                                                                           | [70]      |
|                     | Codon 30 G>C, Codon 41/42 -TCTT, - 88 C>T                                                                                                                                             | [71]      |
| <b>Jordan</b>       | IVS1-110 G>A, IVS1-1 G>A, Codon 39 C>T Codon5 -CT, IVS-I-6 T>C, IVS2 nt.745 C>G, IVS2 nt.1 G>A, Codon37 G>A                                                                           | [59]      |
|                     | Codon 27 G>T, IVSII-1 G>A, -87 C>G IVS-I-5 G>C, IVSII-848 C>A, codon 6 -A                                                                                                             | [60]      |
| <b>Syria</b>        | IVS1-110 G>A, IVS1-1 G>A, Codon 39 C>T, Codon5 -CT, IVS-I-6 T>C, IVS-II-1 G>A Codon15 G>A, -30 T>A                                                                                    | [61]      |
|                     | Codon37 G>A, -30 T>A, Codon 8 -AA, 25 bp deletion, -88 C>T, Codon 8/9 +G, IVSII-745 C>G, IVSI-116 T>G, -87 C>G                                                                        | [60]      |
| <b>Kuwait</b>       | IVS1-110 G>A, IVS1-1 G>A, Codon 39 C>T, IVS-I-6 T>C, IVS-II-1 G>A, IVS-I-5 G>C codon 8/9 +G, Codon 8 -AA, IVS-I-3' end -25nt deletion, 619 -bp del, codon 36/37 -T, codon 44 (-C)     | [62]      |
|                     | Codon 19 A>G, -28 A>C, -101 C>T                                                                                                                                                       | [47]      |
| <b>Saudi Arabia</b> | IVSI-1 G>A, codon 6 -A, IVSII-1 G>A IVSI-5 G>T, codon 8/9 +G, IVSI-5 G>C IVSI-6 T>C, 25 bp deletion, Cap +1 A>C, IVSI-110 G>A, codon 36/37 -T, codon 39 C>T, codon 8 -AA, codon 44 -C | [60]      |
| <b>Iran</b>         | IVS-II-1 G>A, IVS-I-5 G>C, IVS-I-110 G>A codon 8 -AA, IVS-I-1 G>A, codon 5 -CT 3' del 25 nt, IVS-I-6 T>C, codon 39 C>T                                                                | [63]      |
|                     | Codon 8/9 +G                                                                                                                                                                          | [66]      |
|                     | Codon 15 G>A, codon 41-42 -TTCT, codon 30 G>C,-88 C>T                                                                                                                                 | [67]      |
| <b>Turkey</b>       | Codon 8/9 +G, -30 T>A, IVS II-1 G>A, IVS I-110 G>A, IVS I-130 G>A                                                                                                                     | [68]      |
|                     | Codon 8 -AA, codon 22 (7pb del), IVS 2.1 G>A IVS 1.1 G>A, IVS 1.6 T>C, codon 39 C>T, IVS 2.745 C>G, codon 36/37 -T, codon 44 -C IVS 1.5 G>C, codon 5 -CT                              | [69]      |

Another study refers to a rare mutation in Indian Asian  $\beta^0$ codon 8/9 +G promoter, while the overall frequency is less than (1%) in Iran, two peaks are identified in two areas, one in the (Khorasan) northeast of Iran and the other in Iran center [66]. The most common mutation in studied samples in (Kerman Province) in Iran is IVSI-5 G>C; the second most mutation is codon 8/9 +G, and the fewer mutations frequently include: codon 15 G>A, codon 41/42 -TTCT, codon 8 -AA and codon 30 G>C, the -88 C>T is absent in the (Kerman province) population [67].

Most common mutations in  $\beta$ -thalassemia gene in the east of Turkey, are codon 8/9 +G, -30 T>A, IVS II-1 G>A, IVS I-110 G>A, and IVS I-130 G>A described by [68]. The most prevalent mutations of  $\beta$ -thalassemia in the city of (Gaziantep) identified by using PCR-ARMS technique, are include codon 8 -AA in (5.6%), IVS 1.110 G>A in (29.1%), codon 22 (7pb del) in (0.5%), IVS 2.1 G>A in (12.3%), IVS 1.1 G>A in (7.7%), -30 T>A in (4.6%), IVS 1.6 T>C in (4.6%), codon 39 C>T in (3.6%), IVS 2.745 C>G in (1.5%), codon 8/9 +G in (2.1%), codon 36/37 -T in (2.1%), codon 44 -C in (3.1%), IVS 1.5 G>C in (2.1%), codon 5 -CT in (0.5%) while (20.9%) are undetermined [69]

## 2.7 Iron Overload in Thalassemia

The iron deposits in thalassemics, who have received multiple blood transfusions, can exceed the storage and detoxification capacity of ferritin. Also the excess iron fully saturates transferrin. Consequently, "free" iron (or non-transferrin bound iron) begins to accumulate in tissues and blood. This "free" iron can catalyze the formation of very injurious compounds such as the hydroxyl radical (.OH) from compounds such as hydrogen peroxide, which are normal metabolic byproducts (Fenton reaction)[72]. Patients in developed countries are given routine transfusion therapy, which has lengthened survival and altered the clinical course of the disease, assessment, and treatment of iron overload patients maintained by a regular transfusion system progressively develop clinical symptoms of iron overload. Iron overload of tissue is fatal with or without transfusion if not prevented or adequately treated it is the most important complication of  $\beta$ -thalassemia and is a major focus of management [73].

### 2.7.1 Ferritin

Iron is an essential element for the living body. The human body stores iron in the form of ferritin and hemosiderin in liver, spleen, marrow, duodenum, skeletal muscle and other anatomic areas. Hemosiderin and ferritin are iron-containing proteins with magnetic susceptibility. Hemosiderin is water-insoluble and thermally denatured, but ferritin is water-soluble and heat-resistant up to 75°C. These characteristic differences were used for the fractionation of ferritin and hemosiderin [74]. In the majority of clinical centers, the standard method of evaluating the total amount of body iron is a measurement of the serum ferritin SF concentration in the blood [75].

The ferritin stores iron in a non-toxic form, to store it in a safe and stable form, and to transport iron to areas that require it. Ferritin level in serum directly relates to the amount of iron stored in the body, which is important for red blood cell production [76]. If ferritin amount is high, it leading to an excess of the iron amount, ferritin is served as a monitor for iron overload defects [77].

### 2.7.2 Effect of Iron Overload

Iron overloads in thalassemia patients cause damage to organs as well as a depression in survival so that patients are treated with iron chelators such as deferoxamine [32]. Iron overloads are harmful to biological macromolecules by the same characteristics that make it important to live. However, the forming of free radicals by its interaction with water and oxygen especially in the neutral pH, and conditions rich in oxygen and humidity which contain by mammalian, particularly nucleic acids and a intra cytoplasmic cell membranes, and iron toxicity lead to irreversible damage of tissue as well as the danger of malignant disease, when iron in form of  $\text{Fe}^{2+}$ , it will stimulate to forming free radical by generating of superoxide or hydrogen peroxide during donating a electrons to oxygen to give oxygen species with high energy interaction [78]. Hepatocytes are the particular site to storage of iron in the body, with iron excess, these cells are affected by reactive oxygen species result in death and they are replaced by other cells of type fibroblast. The collagen breakdown by fibroblasts led to forming liver fibrosis and then cirrhosis, likewise, cardiac cells are also damaged by excess iron [79]. The normal cardiac function requires the coordinated activity of all cells in the heart. Damaged, poorly functioning cells often fail in this regard. The clinical manifestations include congestive heart

failure (due to injury to myocytes) and arrhythmias due to damage to the cells of the cardiac conducting system [73].

## 2.8 Vitamin D (VD)

Vitamin D is essential for calcium (Ca) homeostasis and for mineralization of the skeleton, particularly through periods of fast growth, usually infantile and pubertal growth periods. Vitamin D Deficiency (VDD) result in rickets a mineralization defect at the epiphyseal growth plates and bone tissue and osteomalacia a mineralization defect of bone tissue [11]. VD is transported to the liver and then hydroxylated to 25-hydroxyvitamin D<sub>3</sub> (25(OH) D), regulated via parathyroid hormone (PTH), when their additional hydroxylation to form 1,25-dihydroxy vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) will occur in the kidney [80].

25 (OH)D is the good marker of vitamin D status and serves as a monitor of dietary intake and assemble in the skin [81]. When Levels 10 ng/ml(25 nmol/L) are refer to deficient ; levels 32 ng/ml(80 nmol/L) are considered insufficient [82]. 1,25 (OH)<sub>2</sub>D<sub>3</sub> considered active metabolite form of vitamin D which increases extracellular and intracellular concentrations of calcium via several mechanisms: it activates absorbs calcium in intestine, decrease excretion of calcium in kidney and, in interaction with PTH, transport calcium from bone, uptake of calcium initially is the rate-limiting step in the absorption of calcium in the intestine and highly dependent on vitamin d [83]. 1,25(OH)<sub>2</sub>D<sub>3</sub> participation in the renal treatment of calcium and phosphate because the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on serum PTH level and on intestinal calcium and phosphate absorption, which affects the filter action of both ions, 1,25(OH)<sub>2</sub>D<sub>3</sub> induce reabsorption of calcium in kidney and expression of calbindin as well as accelerates transport of calcium that dependent on PTH in the distal tubule [83].

The main determinant of the final excretion of calcium into the urine and the site with the highest Vitamin D receptor (VDR) content, the epithelial calcium channel (ECaC) is an important target in 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated calcium reabsorption, several putative VDR binding sites have been located in the human promoter of the renal ECaC, decreases in circulating levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations resulted in a marked decline in the expression of the channel at the protein and mRNA [84, 85]. In thalassemia patients bone disease becomes an important cause of morbidity, problems include osteoporosis, rickets, scoliosis and fractures [10].

Impaired calcium homeostasis is thought to be a consequence of iron overload seen in  $\beta$ -thalassemic transfused patients. Both defective synthesis of 25(OH) D and/or hypoparathyroidism have been described in these patients and negatively affect their bone metabolism [86]. Adequate calcium intake and vitamin D administration during skeletal development can increase bone mass in adolescents and decrease bone loss in adult life. In adolescents, there is an inverse relationship between serum 25 (OH) D levels and PTH levels and a positive association between serum 25(OH)D levels and bone mineral density (BMD) [10, 87].

### **2.8.1 Vitamin D and Bones Metabolism**

Without vitamin D, only 10– 15% of dietary calcium and about 60% of phosphorus is absorbed. The active form, 1,25(OH)<sub>2</sub>D<sub>3</sub> markedly increases the efficiency of intestinal calcium and phosphorus absorption[88]. Serum levels of 25(OH)D are directly related to bone mineral density with a maximum density achieved when the 25 (OH)D level reached 40 ng/ml or more [89]. Serum levels below 30 ng/ml are associated with a significant decrease in intestinal calcium absorption. In children, adolescents, and adults this is associated with increased PTH and decreased IGF-I (Insulin-like Growth Factor) [90, 91]. PTH enhances the tubular reabsorption of calcium and stimulates the kidneys to produce 1,25 (OH)<sub>2</sub> D<sub>3</sub> [88]. It also activates osteoblasts, which stimulate the transformation of preosteoclasts into mature osteoclasts. Osteoclasts dissolve the mineralized collagen matrix in bone, causing osteopenia and osteoporosis and provide enough calcium to prevent hypocalcemia [81].

Systemically IGF-I stimulates the production of 1, 25(OH)<sub>2</sub>D<sub>3</sub> by kidney cells independently of growth hormone (GH) [92]. IGF-I stimulates bone formation, even in absence of GH, through an intrinsic action on osteoblasts. It supports proliferation, differentiation, and matrix synthesis in cultures of osteoblast-like cells and bone organ cultures and potently stimulates the production of type I collagen (the main structural protein of bone) and increases pro-collagen $\alpha$ 1 (I) mRNA expression both in osteoblasts in vitro and in bone in vivo [93]. Consequently, during VDD, decreased circulating and locally produced IGF-I in children seems to be a gradual adaptive process to inhibit linear growth (in growth plate) and bone mineral accretion (diaphysis), this process conserves bone minerals and proteins to maintain normal serum Ca concentration and slows down the breakdown of the already formed bones

instead of consuming them in forming new bones. In addition, decreased circulating and locally produced IGF-I concentrations can explain in part the defective matrix calcification, the irregular maturation of chondrocytes, and the large irregular hypertrophic zone found in the growth plate of rachitic children can be explained by the prolongation of the cell cycle time. Defective maturation and impaired calcification due to the effect of high level of PTH (stimulates proliferation of chondrocytes) in the presence of low IGF-I (delays maturation and calcification of chondrocytes). In addition, low IGF-I may also allow the catabolic effect of PTH necessary for effective release of calcium and explains the osteoporotic appearance of the diaphysis in rachitic children [91, 94].

### **2.8.2 Pathophysiology of VDD**

The development of clinical manifestations of VDD is dependent on many factors including 1. the severity of the VDD (circulating concentrations of 25(OH)D. the duration of the deficiency; 3. the rate of the child's growth (which influences Ca demands); 4. the amount of Ca intake and 5, the interaction between bones (osteoblastic and osteoclastic activities) kidneys, gut and important hormones including PTH and IGF-I [95, 96]. The latter process entails an important adaptation mechanism that efficiently defends the body against the deleterious effects of hypocalcemia (PTH increases 1 alpha-hydroxylation of vitamin D in the kidney that increases intestinal calcium absorption and increases osteoclastic activity to maintain serum calcium), It is well appreciated that due to this potent adaptation process. The overt cases of rickets and osteomalacia represent only the tip of the iceberg of patients with severe VDD [85, 97]. In young children, during the early stages of VDD, decreased intestinal calcium absorption leads to moderate and/or intermittent increase of PTH stimulates the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> that increases Ca absorption from the gut, and ensures maintenance of normal serum Ca concentration important for adaptive stage, with longer and/or more severe vitamin D deficiency PTH secretion becomes higher and continuous, this high PTH maintains normal serum Ca concentration on the account of bones (significant osteoclastic activity) and with a significant phosphaturic effect that leads to hypophosphatemia. Secretion of IGF-I decreases further with the slowing of bone growth that economizes the bone use of calcium [91, 97]. Hypophosphatemia further compromises mineralization of the growth plate and bones. This stage is usually associated with significant radiological

manifestations. In the late/terminal (dysadapted ) stage, failure of these adaptive mechanisms (low IGF-I, very slow or arrested bone growth, and high PTH) and significant exhaustion of bone hydroxyapatite lead to hypocalcemia[91]. Adolescents with VDD have better adaptation to VDD compared with young children due to their larger bone mass (Ca and PO<sub>4</sub> stores) and relatively slower growth rate (a lower requirement for calcium and PO<sub>4</sub> per kg ) compared with infants and young children [97]. Thalassemic patients progressively develop iron overload, and it is possible that a deficiency in liver hydroxylation of vitamin D, or in vitamin D absorption. In thalassemic children and adolescents, many factors can compromise the adaptation process to VDD including IGF-I deficiency, hypoparathyroidism due to iron deposition in the parathyroid gland, delayed puberty and hypogonadism, decreased bone mass and decreased synthesis of 25(OH)D, due to hepatic siderosis [11, 86, 91]. Decreased outdoor activities in thalassemic patients can also compromise cutaneous synthesis of vitamin D, a blunting of PTH response to VDD and a combination of hypovitaminosis D and hypoparathyroidism has been described in many thalassemic patients [98].

Histopathology shows that in suboptimally blood-transfused thalassemics with iron overload. Osteopenia is primarily caused by focal osteomalacia as well as decreased bone formation. Densitometric and histomorphometric studies indicated impairment of both trabecular as well as cortical bones in those hemochromatosis patients [91].

# **Chapter Three**

## **Materials and Methods**

### 3. Materials and Methods

#### 3.1 Materials

##### 3.1.1 Equipments

The equipments and instruments used in this study are listed in the table (3-1).

**Table (3-1):** Instruments and Equipments.

| No. | Name of Item                       | Model                                                      | Company             | Origin  |
|-----|------------------------------------|------------------------------------------------------------|---------------------|---------|
| 1   | Analytical Balance                 | ABS/ABJ                                                    | KERN                | Germany |
| 2   | Autoclave                          | DE-23                                                      | Systec              | Germany |
| 3   | Automated DNA extraction machine   | Magtration System 12GC plus                                | PSS                 | Japan   |
| 4   | Automated Hematology Analyzer      | Sysmex XS-1000i                                            | Sysmex              | USA     |
| 5   | Automated Water distillator        | K-HWSFA08                                                  | K & K Scientific    | Korea   |
| 6   | ELISA Printer                      | LQ-300+II                                                  | EPSON               | USA     |
| 7   | ELISA Absorbance Microplate Reader | ELX800                                                     | BioTek              | USA     |
| 8   | ELISA Washer                       | ELX50                                                      | BioTek              | USA     |
| 9   | Gel Documentation system           | Mthna-Gel Doc System                                       | Muthanna University | Iraq    |
| 10  | Horizontal electrophoresis system  | Mupid-One                                                  | Mupid               | Japan   |
| 11  | Gradient Thermal Cycler            | MultiGene optiMax TC9600-G                                 | Labnet              | USA     |
| 12  | Hemoglobin Testing System          | D-10 system                                                | Bio-Rad             | USA     |
| 13  | Incubator                          | CL011                                                      | Cypress Diagnostics | Belgium |
| 14  | Microcentrifuge                    | Mikro 120                                                  | Hettich             | Germany |
| 15  | Micropipette                       | 0.1-2.5 µl, 0.5-10 µl<br>5-50 µl, 20-200 µl<br>200-1000 µl | Nanolytik           | Germany |
| 16  | Microscope                         | CM001                                                      | Cypress Diagnostics | Belgium |
| 17  | Microwave Oven                     | LMH2235ST                                                  | LG                  | Korea   |
| 18  | Mini-Vidas                         | LVD/2459                                                   | BIOMERIE UX         | France  |
| 19  | pH / Temperture bench meter        | Mi150                                                      | Martini             | Italy   |
| 20  | Refrigerator                       | GRT452XV                                                   | Concord             | Korea   |

|    |                                 |          |                |       |
|----|---------------------------------|----------|----------------|-------|
| 21 | Thermocycler PCR                | TC-3000  | Techne         | USA   |
| 22 | Upright Freezer                 | Serie 4  | Bosch          | UK    |
| 23 | UV sterilization cabinet        | GLE-UVSC | SCI-PLAS       | UK    |
| 24 | Vertical Biological Clean bench | BC-11B   | JeIO-Tech      | Korea |
| 25 | Vortex mixer                    | LVM-202  | DAIHAN LabTech | Korea |
| 26 | Water Bath                      | LWB-111D | DAIHAN LabTech | Korea |

### 3.1.2 Chemicals

The chemicals that are used in the present study are listed in the table (3-2).

**Table (3-2):** Chemical substances.

| No. | Name of Item                       | Company        | Origin       |
|-----|------------------------------------|----------------|--------------|
| 1   | Agarose                            | US biological  | USA          |
| 2   | Boric acid                         | S.d. fine-Chem | India-Mumbai |
| 3   | Decontamination nucleases solution | Bio-world      | USA          |
| 4   | EDTA                               | S.d. fine-Chem | India-Mumbai |
| 5   | Ethanol 70%                        | Scharlab S.L.  | Spain        |
| 6   | Ethanol 96% Spray                  | Amresco        | USA          |
| 7   | Ethidium bromide                   | Bio-world      | USA          |
| 8   | Nuclease free water                | Bio-world      | USA          |
| 9   | PBS tablet                         | Amresco        | USA          |
| 10  | TE buffer                          | Biovision      | USA          |
| 11  | Tris-Base                          | S.d. fine-Chem | India-Mumbai |

### 3.1.3 Kits and Reagents

The commercial kits and reagents were used in the present study that are illustrated in table (3-3).

**Table (3-3):** The commercial kits and reagents

| No. | Name of Item                                    | Kit code  | Company     | Origin  |
|-----|-------------------------------------------------|-----------|-------------|---------|
| 1   | 8-deoxyhydroxy – Guanosine ELISA Kit            | E-EL-0028 | Elabscience | China   |
| 2   | Automated MagDEA DNA 200 (GC) Kit               | E7001     | PSS         | Japan   |
| 3   | One Taq Quick-Load 2x Master Mix stander Buffer | M0486s    | BioLabs     | England |
| 4   | Universal DNA ladder with Loading dye           | E3140-01  | EURX        | Poland  |
| 5   | Vitamin D3 ELISA Kit                            | E-EL-0014 | Elabscience | China   |

### 3.1.4 Primers

The primers are specific for the amplification of beta-globin gene [51], provided by Alpha DNA company (USA), as shown in table 3-4. Each primer was diluted to a final concentration of 100 pm/μl and kept at -20 °C for the next assays.

**Table (3-4):** The primers that were used in the present study.

| Primer                                                                                    | Sequence                                                                                 |
|-------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| PCR mix I amplification<br><b>A (forward)</b><br><b>B (reverse)</b><br><b>D (reverse)</b> | 5'-ACGGCTGTCATCACTTAGAC-3'<br>5'-AAGAGGTATGAACATGATTAGC-3'<br>5'-CAGATTCCGGGTCACCTGTG-3' |
| PCR mix II amplification<br><b>C (forward)</b><br><b>D (reverse)</b>                      | 5'-GTGTACACATATTGACCAAATC-3'<br>5'-CAGATTCCGGGTCACCTGTG-3'                               |
| PCR mix I sequencing<br><b>A (forward)</b><br><b>E (reverse)</b>                          | 5'-ACGGCTGTCATCACTTAGAC-3'<br>5'-CTCCCCCTTCCTATGACATGA-3'                                |
| PCR mix II sequencing<br><b>C (forward)</b><br><b>F (reverse)</b>                         | 5'-GTGTACACATATTGACCAAATC-3'<br>5'-AATGCACTGACCTCCACAT-3'                                |

## 3.2 Methods

### 3.2.1 Preparation of Buffers and Reagents

#### 3.2.1.1 TBE (10X) Buffer:

To prepared 1 liter of 10X TBE dissolve 108 g Tris base /55 g boric acid /40 ml 0.5 M EDTA (pH 8.0) in 600 ml deionized water. Adjust the final volume to 1 liter with deionized water. The autoclave is used to avoid contamination for 15 minutes at 121 C°[99].

#### 3.2.1.2 Preparation of 1% agarose gel

One gram of agarose is added to 100 ml of 1X TBE buffer in a flask, dissolved in microwave oven until start the appearance of boiling. The flask removed and swirl agarose solution. This last step was repeated until no there were flicks. Then agarose solution left at room temperature to cooling 50 to 65 °C. Ethidium bromide added to the agarose gel (5µl of a 10mg/ml stock solution per 100 ml) of agarose gel [99].

#### 3.2.1.3 Primer Preparation

1. The primers (table 3-4) are used for the amplification of the  $\beta$ -globin gene. The lyophilized primer tubes spinned prior to open the tubes to prevent loss of pelleted oligonucleotides. The primers are prepared according to manufacturer instructions that include the amount of nuclease free water to be added to the lyophilized primers to get the stock solution with a concentration of 100 pmol /  $\mu$ l, for forward and reverse primers.
2. PCR primer mix was prepared from combination forward and reverse primer by added 10  $\mu$ l from each primer (forward or reverse) solution (100 pmol /  $\mu$ l) to a sterilized microcentrifuge tube containing 90  $\mu$ l of nuclease free water to get 10 pmol /  $\mu$ l.

## 3.2.2 Design of Experiment

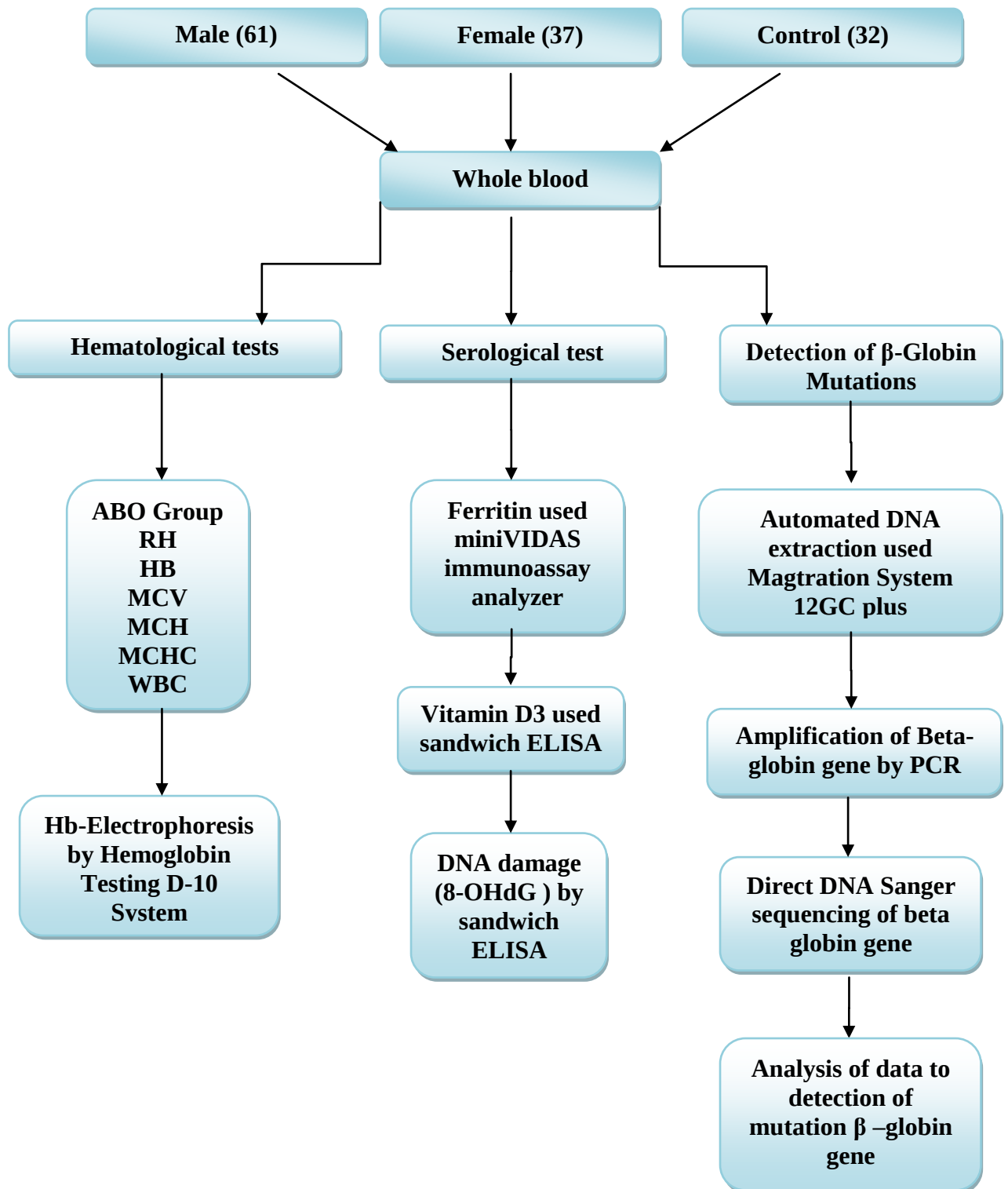


Figure (3-1): Flow Chart Illustrates The Study Design

### 3.2.3 Collection of Specimens

A total of 98 blood samples of  $\beta$ -thalassemia patients were included in this study age ranging from 1-29 years old collected during the period between December 2016 to May 2017 from the Women and Children Hospital in Al-Muthanna province. All patients have involved the frequent blood transfusion. The control group included 32 control individuals that collected from patients carrier from same age ranging they appeared without any blood disorders. The blood sample volume was 5ml collected from patient and control group[100]. The collected sample was divided into 2 sterilized tubes as follows:

A. Amount 2 ml of blood in 5 ml EDTA tube, it was used directly to hematological tests and molecular study of  $\beta$ -globin gene.

B. Amount 3ml of blood in a gel tube, it was centrifuged at 4700 RPM for 5 min to obtain blood serum then frozen at -20 °C for the screening of vitamin D3 and 8-hydroxydeoxyguanosine assays.

### 3.2.4 Hematological Parameters of Beta-Thalassemia

#### 3.2.4.1 ABO and RH typing

The blood typing is performed a blood transfusion on the same day of practice. The cause of transfusion-related morbidity is due to a patient being transfused with ABO incompatible. Reactions occur because individuals form potent, naturally occurring antibodies to ABO red cell antigens which they do not possess. When transfused with ABO, an immediate antigen/antibody reaction occurs which, if not detected in time, may be fatal [101].

#### Procedure

1. One drop was placed on a labeled slide with anti-A, one drop of anti-B reagent separately and one drop of anti-D.
2. One drop from patient blood was added to each drop of the typing antiserum.
3. The blood and reagent were mixed by a clean stick. Each mixture was spread evenly on the slide over an area of 10-15 mm diameter.
4. The slide was tilt and left for 2 minutes at room temperature (22°-24°C). Then looked for agglutination.
5. Recording the results.

#### 3.2.4.1 Complete Blood Count

Hematological parameters (WBC, HB, MCV, MCH, and MCHC) tested by an automated hematology analyzer (Sysmex XS-1000i) is designed to directly measure hematological parameters [102]. Where a minimum of 1 ml of whole blood is required for running specimen (Whole blood anticoagulated with EDTA). After running the system. The EDTA tube cap removed and analysis of a sample run by one touch.

#### 3.2.4.2 Hemoglobin Variants

**Hemoglobin Testing D-10 System** utilized the cation-exchange HPLC for automated analysis of normal and abnormal hemoglobin in adult and neonatal blood samples, four programs available of D-10 System, one of them is beta-thalassemia short program, is intended for the quantification of HbA<sub>2</sub> and HbF. It assists in detecting different types of hemoglobin variants [103].

**Assay procedure:** All operations of the system of Hemoglobin testing D-10 system with  $\beta$ -thalassemia short program for detecting HbA<sub>2</sub> and HbF quantification are controlled automatically by a built-in the microprocessor. The only manual step required was the preparation of sample hemolysates, typically by adding 5  $\mu$ l of well-mixed blood to 1 ml of hemolyzing solution. It uses the principle of cation-exchange HPLC to separate Hbs A, A<sub>2</sub> (Co-elute with E), F, S, D and C on a nonporous cation exchange resin. As the elute moves through the flow cell, a dual wavelength filter-photometer detects the absorbance of Hb at the primary wavelength (415 nm) and correct for non-specific absorbance at the secondary wavelength 690nm [104, 105]. A presumptive identification of each component is determined automatically by comparing the retention (elution) time to pre-programmed retention times for HbA, HbA<sub>2</sub> Hbs, HbD, and Hbc. HbE cannot be separated from HbA<sub>2</sub> or identified as HbE but should be suspected if the peak identified as HbA<sub>2</sub> is highly increased. A built-in integrator collects and reduces the data from each analysis, and a printer produces a report showing the identification and area percent relative to the total area of the Habs separated during the 6.5 min program. The system is calibrated at beginning of each run with the Bio-Rad variant hemoglobin A<sub>2</sub>/F calibrator, a solution labeled by the manufacturer with assigned values for the two hemoglobin components [103, 105].

### 3.2.5 Serological Methods of Beta-Thalassemia

#### 3.2.5.1 Quantitation of Ferritin

VIDAS Ferritin kit is an automated quantitative test for the determination of ferritin in human serum or plasma using by enzyme-linked fluorescent technique (miniVIDAS immunoassay analyzer). The level of serum ferritin acts as an indicator of the quantities of iron in the human body. The drop in serum ferritin levels can indicate a deficiency prior to the appearance of iron deficiency anemia, enabling anticipated treatment [106].

#### Assay Procedure

1. Amount 50  $\mu$ l of human serum sample was transferred to a tube contains (200  $\mu$ l) detection buffer.
2. The detection buffer tube lid was closed and the sample was gently mixed by shaking about 10 times.
3. Amount 75  $\mu$ l of the sample mixture was loaded into a sample well in the test strip.
4. The sample-loaded test strip left at room temperature for 10 minutes.
5. The sample-loaded strip, inserted into a strip holder of the mini-VIDAS instrument.
6. The instrument was run by press 'Select' button on the instrument to start the test.
7. The result displayed on the screen of the instrument.

#### 3.2.5.2 Vitamin D3 Quantification ELISA Assay

Vitamin D3 ELISA kit provided from Elabscience/ China, were uses sandwich ELISA method for in vitro vitamin D3 quantification in ranging 1.56-100 ng/ml. The microtiter plate provided that be pre-coated with VD3. The VD3 in the sample or standard competes with a fixed amount of VD3 on the solid phase supporter for sites on the biotinylated detection Ab specific to VD3. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm  $\pm$  2 nm. The concentration of VD3 in the samples is then determined by comparing the OD of the samples to the standard curve.

This ELISA kit is used for the determination of VD3 concentration in serum of  $\beta$ -thalassemia patients. The kit components are listed in Table (3-5).

**Table (3-5):** components of vitamin D3 ELISA kit

| No | Items                                   | Specifications             |
|----|-----------------------------------------|----------------------------|
| 1  | Biotinylated Detection Ab Diluent       | 1vial 10mL                 |
| 2  | Concentrated Biotinylated Detection Ab  | 1vial 120 $\mu$ L          |
| 3  | Concentrated HRP Conjugate              | 1vial 120 $\mu$ L          |
| 4  | Concentrated Wash Buffer (25 $\times$ ) | 1vial 30mL                 |
| 5  | HRP Conjugate Diluent                   | 1vial 10mL                 |
| 6  | Micro ELISA Plate                       | 8 wells $\times$ 12 strips |
| 7  | Plate Sealer                            | 5pieces                    |
| 8  | Reference Standard                      | 2 vials                    |
| 9  | Reference Standard and Sample Diluent   | 1vial 20mL                 |
| 10 | Stop Solution                           | 1vial 10mL                 |
| 11 | Substrate Reagent                       | 1vial 10mL                 |

### Reagent preparation

1. **Wash Buffer:** volume 30 mL of concentrated wash buffer diluted into the preparation of 750 ml of wash buffer by distilled water.
2. **Standard working solution:** The standard solution centrifuged at 10,000 $\times$ g for 1 minute and 1.0mL of reference standard & sample diluent was added, it let stand for 10 minutes and it turned upside down several times. After it dissolved fully, mixed thoroughly with a pipette. This reconstitution produces a stock solution of 100ng/mL. Then serial dilutions made as needed. The recommended dilution gradient is as follows: **100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0 ng/ml.** **Dilution method:** seven eppendorf tubes taken, a volume 500  $\mu$ L of reference standard & sample diluent added to each tube. Pipette 500 $\mu$ L of the 100ng/mL stock solution to one tube to produce a 50ng/mL stock solution. Pipette 500 $\mu$ L of the solution from former tube to the latter one in order according to this step. The illustration below is for reference. And the last tube is regarded as blank (figure 3-2).

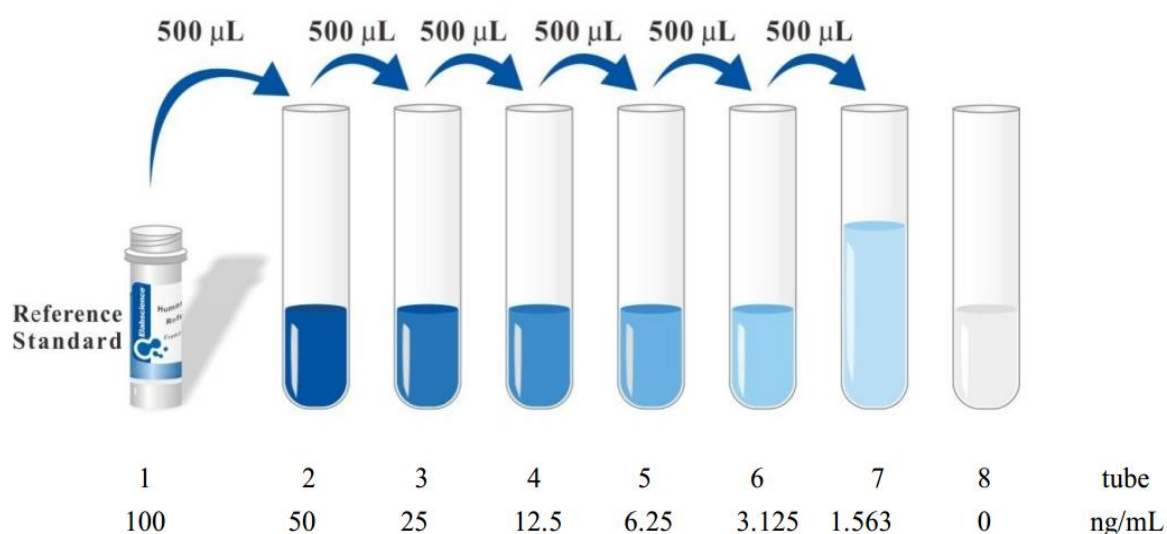


**Figure (3-2):** Dilution of The Standard of Vitamin D3 (ng/ml)

**3. Biotinylated Detection Ab working solution:** The required amount calculated before the experiment (100µL/well). In actual preparation should prepare 100-200µL more. The stock tube centrifuged before use, the 100× concentrated biotinylated detection Ab diluted to working solution by biotinylated detection Ab diluent.

**4. Concentrated HRP Conjugate working solution:** The required amount Calculated before the experiment (100µL/well). In actual preparation, should prepare 100-200µL more. The stock tube Centrifuged before use. The 100×Concentrated HRP Conjugate Dilute to working solution by Concentrated HRP Conjugate Diluent.

### Assay Procedure

1. A volume 50µL of standard or sample was added to well.
2. Biotinylated detection Ab 50 µl immediately added to each well, incubated for 45mintues at 37°.
3. Aspirate and wash 3 times by using microplate washer.
4. A volume 100µL HRP Conjugate was added and incubated for 30 minutes at 37°.
5. Aspirate and wash 5 times by using microplate washer.
6. Substrate reagent 90µL was added and incubated for 15 minutes at 37°.
7. Stop Solution 50µl was added and read at 450nm immediately.
8. The results were calculated.

### 3.2.5.3 8-OHdG (8-hydroxydeoxyguanosine) ELISA assay

This ELISA kit uses sandwich ELISA as the method were provided from Elabscience/ China. The microtiter plate provided in this kit has been pre-coated with 8-hydroxydeoxyguanosine, During the reaction, 8-hydroxydeoxyguanosine in the sample or standard competes with a fixed amount of 8-OHdG on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 8-OHdG, excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of  $450 \text{ nm} \pm 2 \text{ nm}$ . The concentration of 8-OHdG in the samples is then determined by comparing the OD of the samples to the standard curve.

This ELISA kit is used for the determination of 8-OHdG concentration in serum of  $\beta$ -thalassemia patients. The kit components are listed in Table (3-6).

**Table (3-6):** Components of 8-OHdG ELISA kit

| No | Items                                   | Specifications             |
|----|-----------------------------------------|----------------------------|
| 1  | Biotinylated Detection Ab Diluent       | 1vial 10mL                 |
| 2  | Concentrated Biotinylated Detection Ab  | 1vial 120 $\mu$ L          |
| 3  | Concentrated HRP Conjugate              | 1vial 120 $\mu$ L          |
| 4  | Concentrated Wash Buffer (25 $\times$ ) | 1vial 30mL                 |
| 5  | HRP Conjugate Diluent                   | 1vial 10mL                 |
| 6  | Micro ELISA Plate                       | 8 wells $\times$ 12 strips |
| 7  | Plate Sealer                            | 5pieces                    |
| 8  | Reference Standard                      | 2 vials                    |
| 9  | Reference Standard and Sample Diluent   | 1vial 20mL                 |
| 10 | Stop Solution                           | 1vial 10mL                 |
| 11 | Substrate Reagent                       | 1vial 10mL                 |

### Reagent preparation

1. **Wash Buffer:** volume 30 mL of concentrated wash buffer diluted into the preparation of 750 ml of wash buffer by deionized or distilled water.
2. **Standard working solution:** The standard solution centrifuged at  $10,000\times g$  for 1 minute. And 1.0mL of reference standard & sample diluent was added, it let stand for 10 minutes and it turned upside down several times. After it dissolved fully, mixed thoroughly with a pipette. This reconstitution produces a stock solution of 100ng/mL. Then serial dilutions made as needed. The recommended dilution gradient is as follows: **100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0 ng/ml.**

**Dilution method:** seven eppendorf tubes taken, a volume 500  $\mu$ l of reference standard & sample diluent added to each tube. Pipette 500uL of the 100ng/mL stock solution to one tube to produce a 50ng/mL stock solution. Pipette 500uL of the solution from former tube to the latter one in order according to this step, (figure 3-3).

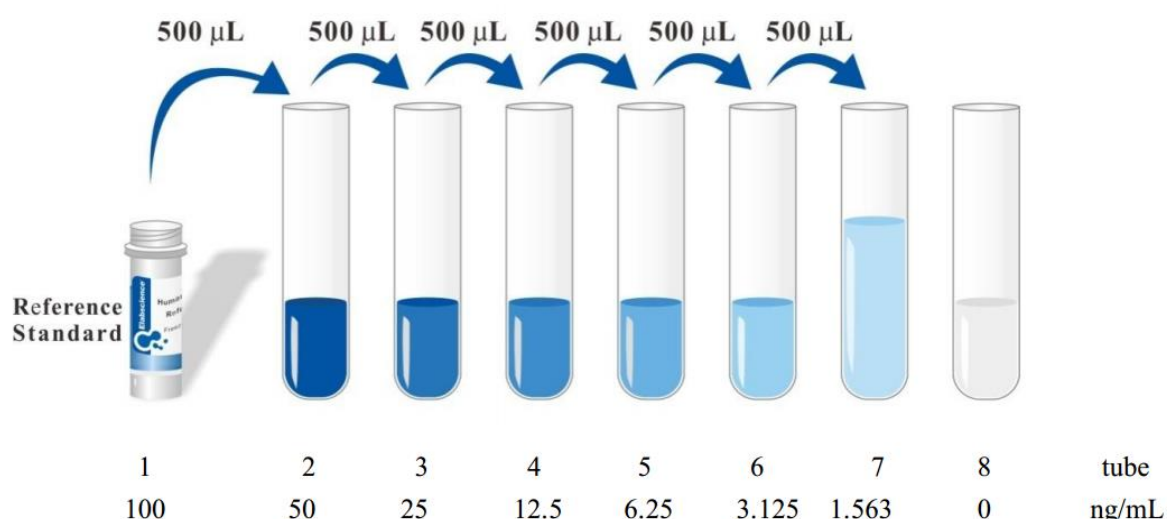


**Figure (3-3):** Dilution of Standard of 8-Hydroxydeoxyguanosine (ng/ml)

3. **Biotinylated Detection Ab working solution:** The required amount calculated before the experiment (100 $\mu$ L/well). In actual preparation should prepare 100-200 $\mu$ L more. The stock tube centrifuged before use. The  $100\times$  concentrated biotinylated detection Ab diluted to working solution by biotinylated detection Ab diluent.
4. **Concentrated HRP Conjugate working solution:** The required amount Calculated before the experiment (100 $\mu$ L/well). In actual preparation, should prepare 100-200 $\mu$ L

more. The stock tube Centrifuged before use. The 100×Concentrated HRP Conjugate Dilute to working solution by Concentrated HRP Conjugate Diluent.

### Assay Procedure

1. A volume 50μL of standard or sample was added to well.
2. Biotinylated detection Ab 50 μl immediately added to each well, incubated for 45mintues at 37°C
3. Aspirate and wash 3 times by using microplate washer.
4. A volume 100μL HRP Conjugate was added and incubated for 30 minutes at 37°C.
5. Aspirate and wash 5 times by using microplate washer.
6. Substrate reagent 90μL was added and incubated for 15 minutes at 37°C.
7. Stop Solution 50μl was added and read at 450nm immediately.
8. The results were calculated.

### 3.2.6 Detection of β-Globin Mutations

#### 3.2.6.1 Genomic DNA extraction

Genomic DNA from blood samples was extracted by using automated DNA extraction machine (Magtration System 12GC plus) be programmed the protocol I1510 IC with whole Blood MagDEA DNA 200 (GC) Kit, and done according to company instruction (Figure 3-4), as following steps:

**Principle of DNA extraction by Magtration®**

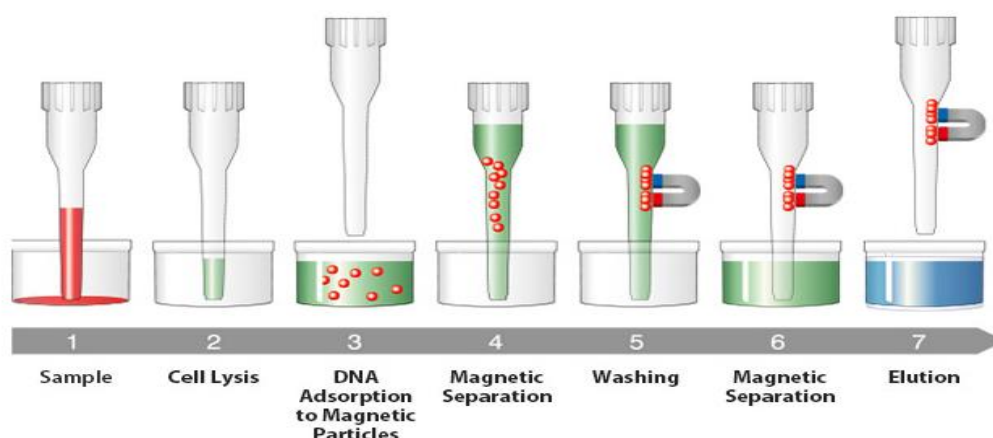


**Figure (3-4):** Principle of Extraction of DNA by Magtration System 12GC plus.

- 1- The Automated DNA extraction machine prepared by clearing their components using alcohol 70% and built-in UV sterilization lamp system to eliminating contaminations.
- 2- All microtubes and the tip holders have been set with their tips in the appropriate wells of the rack.
- 3- The rack has been set in the machine.
- 4- A volume 1300  $\mu$ l of isopropanol was added and 200  $\mu$ l of whole blood was added in the appropriate microtubes that were added in step 2.
- 5- The front door closed, preparing to begin by following the instructions on the operation panel and start the extraction after inserting IC whole blood card in their appropriate site.

#### **3.2.6.2 Detection of Genomic DNA extraction yield**

TBE (1X) buffer was added to the electrophoresis tank tray with immersed agarose that was prepared as follows (3.2.1.2) in electrophoresis tank, each well was loaded with 7 $\mu$ l of DNA samples mixed with 1 $\mu$ l loading dye, electrophoresis was run at 135 volt/cm for 15 minutes, then gel was visualized. The extracted genomic DNA from blood samples that were checked by using Gel electrophoresis to ensure the presence of DNA through visible bands on an agarose gel.

#### **3.2.6.3 Preparing Workstation Before Pre-PCR**

Sterilization must be done before all PCR reactions and primer dilutions that took place in a UV sterilization cabinet to avoid contamination of DNA samples. First, the UV cabinet for PCR was cleaned with nuclease decontamination spray before and after all works. PCR products were carefully handled to prevent contamination of the amplified DNA in the laboratory. A set of pipettes, a laboratory coat, gloves, and other equipment and consumables were designated to each working area so that they were not carried from one place to another which may cause crossover contamination.

#### **3.2.6.4 Amplification of $\beta$ -globin Gene**

The  $\beta$ -globin gene was amplified by using specific primers listed in the table (3-4). The primers designed according to (Wang et al.,2003 and Chan,2010) studies for detection of  $\beta$ -globin mutations, two PCR mix prepared needed in the amplification of

$\beta$ -globin gene assay according to [51]. PCR mix I labeled separate, new, sterile, and nuclease-free tube contain equal parts from primer A, B, and D, while similarly tube for PCR mix II was used contain equal parts from primer C and D. PCR mixtures (I and II) was provided by the manufacturing company table (3-7) and (3-8). The volume of PCR mixture was 5 $\mu$ L, including 25 $\mu$ L (1X) One Taq Quick-Load 2x master mix stander (Biolabs, England), 1 $\mu$ L of each primer (0.2 $\mu$ M), 5 $\mu$ L of genomic DNA (20 ng/ $\mu$ L) and volume were completed with Nuclease-free water, as showed in table (3-7) and (3-8).

**Table (3-7):** Component of PCR I.

| Compounds                                               | Volume     | Concentration   |
|---------------------------------------------------------|------------|-----------------|
| One Taq Quick-Load 2x Master Mix stander Buffer         | 25 $\mu$ L | 1X              |
| PCR mix I primers<br>10 $\mu$ M                         | 1 $\mu$ L  | 0.2 $\mu$ M     |
| Template DNA                                            | 5 $\mu$ L  | 100 ng/ $\mu$ L |
| <b>Add Nuclease-free water<br/>up to a final volume</b> | 50 $\mu$ L |                 |

**Table (3-8):** Component of PCR II.

| Compounds                                               | Volume     | Concentration   |
|---------------------------------------------------------|------------|-----------------|
| One Taq Quick-Load 2x Master Mix stander Buffer         | 25 $\mu$ L | 1X              |
| PCR mix I primers<br>10 $\mu$ M                         | 1 $\mu$ L  | 0.2 $\mu$ M     |
| Template DNA                                            | 5 $\mu$ L  | 100 ng/ $\mu$ L |
| <b>Add Nuclease-free water<br/>up to a final volume</b> | 50 $\mu$ L |                 |

PCR conditions are select the typical annealing temperature after optimizations that was doing for temperatures from 45 °C to 62 °C. Typical thermocycler conditions were: 95 °C for 10 minutes then 35 cycles of 95 °C for 30 seconds, 58.5 °C for 60 seconds and 72 °C for 2 minute followed by 10-minute extension at 68 °C.

Then PCR products were analyzed by electrophoresis in a (1%) agarose gel and stained with (10 mg/ml) ethidium bromide. This program was listed in the table (3-9)

**Table (3-9):** Program of PCR amplification.

| Steps                       | Temperature | Time   | No. of cycles |
|-----------------------------|-------------|--------|---------------|
| <b>Initial denaturation</b> | 95 °C       | 10min  | 1 cycle       |
| <b>Denaturation</b>         | 95 °C       | 30 sec | 35 cycle      |
| <b>Annealing</b>            | 58.5°C      | 1min   |               |
| <b>Elongation</b>           | 72 °C       | 2 min  |               |
| <b>Final elongation</b>     | 68 °C       | 10 min | 1 cycle       |

### 3.2.6.5 Gel electrophoresis after Gap-PCR

Gel electrophoresis has been used for the detection of DNA by Gel Documentation system [99]. Agarose 1% preparation as displayed in (3.1.5.2), after that sample preparation about 6µl of DNA sample was mixed with 1µl of loading dye (6x). Samples are loaded carefully into the individual wells of the agarose gel that was prepared as in (3.2.1.2) in electrophoresis tank and was run at 100 volt/cm for 20 minutes then at 50 volt/cm for 30 minutes. Finally, the gel was visualized with Gel Documentation system at 243 nm and photographed.

### 3.2. 6.6 DNA Sanger Sequencing of The $\beta$ -Globin Gene

DNA sequencing was carried out commercially at MACROGEN Inc., Korea. PCR products along with forward and reverse primers were sent to Macrogen Sequencing Service, who delivered the sequence reads. The DNA sequencing of the PCR products was done using the BigDye terminator (reaction mixture consisting of 5 µl purified products, 2 µl BigDye Terminator, 2 µl sequencing buffer and 1 µl forward primer) utilizing POP-7 polymer (Applied Biosystem, USA). The separation of the cycle sequencing products and detection were carried out by using ABI 3730 XL DNA analyzer. The analysis of quality of the input DNA sequence data by using DNA baser V4 software and sequence scanner software version 2.0 that display Quality Scores (Phred value) of output DNA data asses overall quality and individual score of each nucleotide [107].

### 3.2.6.7 Bioinformatics

The Capillary electrophoresis data produced from applied biosystem genetic analyzers (ABI genetic analyzers) can be viewed with DNA sequence editor (BioEdit). This experiment is designed to experimentally collect thalassemic DNA sequence data saved in NCBI. DNA sequencing is a method of determining the exact DNA base or series of bases at a given location in the genome. The goal of this experiment is to find the variations (mutation) in patient sequences samples that were aligned with beta globin is GenBank sequence NG\_000007.3 from NCBI by using the CLUSTAL W. NCBI blast was also used to find mutation positions by alignment query with specific RefSeq. The mutations were searched in HbVar Database to match with already reported  $\beta$ -globin mutations. HbVar is an exhaustive data bank of all the documented mutations in the globin genes either for Hb variants or thalassemia mutations (Globin Gene Server Web Site - <http://globin.cse.psu.edu/>)[108]. Any mutation which was absent from the database was considered as a new mutation (novel mutation). From ITHANET information obtained about allele frequency, databases of variations, epidemiology and clinical data of  $\beta$ - globin mutations.

### 3.2.7 Statistical Analysis

Statistical analysis was conducted using (SPSS 23). Determine the statistical differences among different groups was made using the Pearson Chi-square ( $\chi^2$ ) test, while the hierarchical log-linear models were applied to compare the observed vs. the expected frequencies. Statistical differences among different ELISA assay results determined by T- and Anova tests. The probability of ( $P \leq 0.05$ ) was considered to be statistically significant [109].

# **Chapter Four**

## **Results and Discussion**

## 4. Results and Discussion

### 4.1 Clinical Manifestation

The present study included 98  $\beta$ -thalassemic patients who are currently being blood transfusion therapy and have the clinical symptoms and manifestation of the disease at the thalassemia unit in Women and Children Hospital in Al-Muthanna Province. All patient and control individuals gave informed consent to the study.

#### 4.1.1 Classification of Samples according To Gender and Age

The sample was collected during the period December 2016 to May 2017, which included that 61 (62.2%) of patients were male, while 37 (37.8%) were female, ( $P=0.015$ ), as shown in figure 4-1.

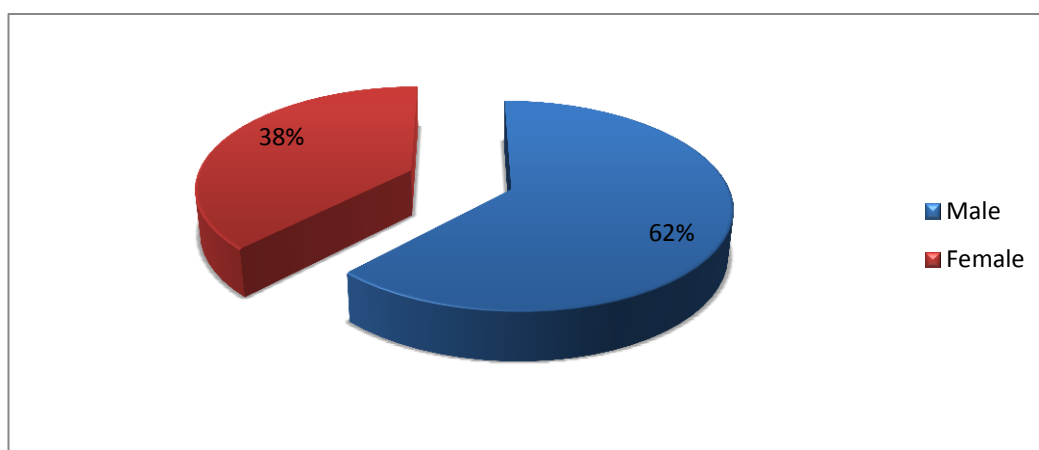


**Figure (4-1):** Distribution of  $\beta$ -Thalassemic Patients According to Gender.

The age ranging from  $\beta$ -thalassemic patients were included in this study was 1-29 years old, that divided into age categories: 51 (52 %) were 1-10 years old, 39 (39.8%) were 11-20 years old, and 8 (8.2%) were 21-29 years old ( $P<0.001$ ), figure (4-2).

In Babylon province, previous study showed that the most predominant age group is less than 20 years of  $\beta$ -thalassemic patients (93.3%) [110]. As well as another study in Tikrit city which showed (55.1%) of  $\beta$ -thalassemic patients in the age group 1-11 years, while those aged more than 12 years were (44.9%)[100]. The present study found that  $\beta$ -thalassemic patients older than 20

years were about 8 (8.2%), but the previous study reported that patients died in the 2<sup>nd</sup> trimester of life [111]

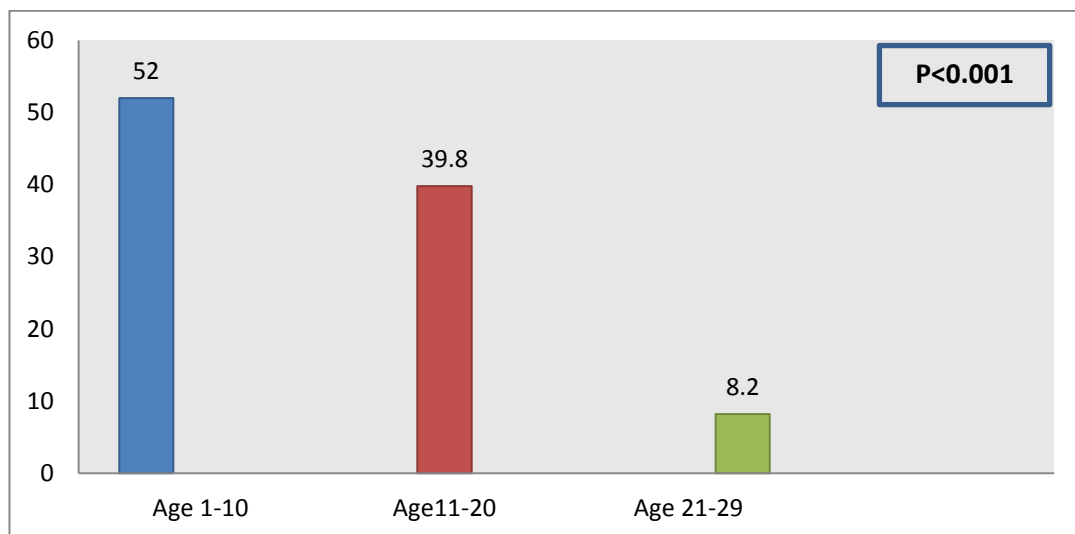


**Figure (4-2):** Percentage of  $\beta$ -Thalassemic Patients According to Age.

The current study was classified gender categories to age groups of  $\beta$ -thalassemic patients 1-10 years, males were 32 (62.7%), and the female was 19 (37.3%) from 98 patients. In patients with age 11-20 years where the male was 23(59%) and the female was 16 (41%) from 98 patients. While in the last group patients with age 21-29 years were male 6 (75%) and female 2 (25%) from 98 patients.

This study illustrated the correlation between ages and removed spleen in  $\beta$ -thalassemia patients, showed that 4 (22.2%) were ages ranging between 1-10 years old, 9 (50%) were ages ranging 11-20 years old, and 5 (27.8%) were ages ranging between 21-29 years old, the statistical analysis refer to found association between age group and removed spleen ( $P=0.001$ ), figure(4-4).

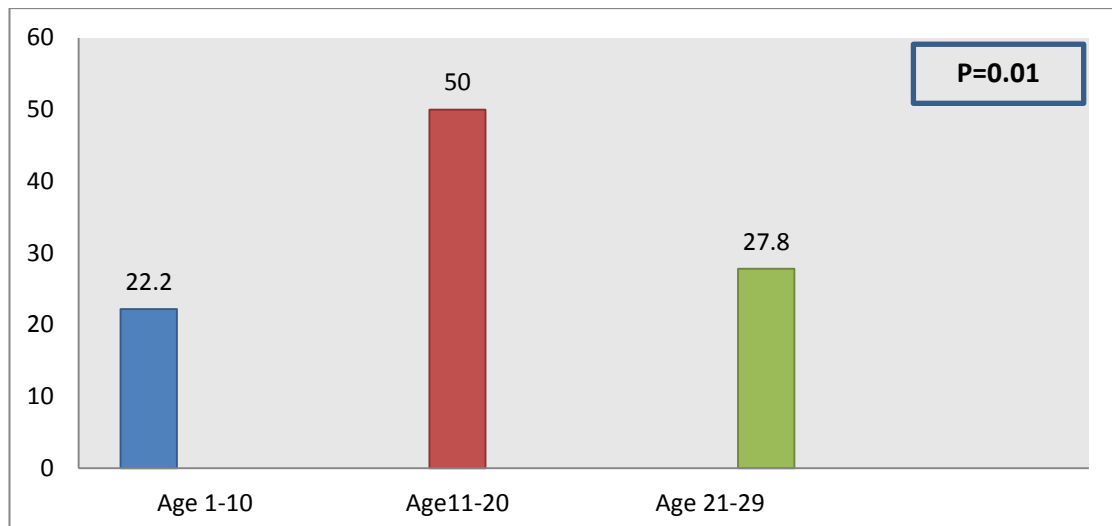


**Figure (4-3):** Correlation Between Patient's age and Removed Spleen.

Generally, the result presented in this study showed that 18(18.4%) of patients were splenectomized. While 80 (81.6%) of them were spleen presence, ( $P<0.001$ ). Regarding current data and studying distribution of patients with spleen removed among beta-thalassemia types, were found 13(19.1%) of patients were splenectomized and 55(88.9%) of them have intact spleen in  $\beta$ -thalassemia major type, ( $P<0.001$ ). In  $\beta$ -thalassemia intermediate type, 5 (17.2%) of patients were splenectomized and 24 (82.8%) of them have an intact spleen, ( $P<0.001$ ).

Red blood cells that damaged or defective are normally eliminated in the spleen. In thalassemia patients, there is a large quantity of defective red blood cells which results in an enlarged hyperfunctioning spleen (splenomegaly) lead to necessary of splenectomy [112].

#### 4.1.2 Geographical Distribution of the Samples

The total number of patients in this study was 98 collected of most cities of Al-Muthanna province which involved: Samawa city 52 (53%), Rumaitha city 20 (20.4%), Khudhur city 14 (14.3%) and Warkaa city 12 (12.3%), ( $P<0.001$ ). The geographical distribution of  $\beta$ -thalassemic patients reflects the heterogeneity of  $\beta$ -thalassemia within different regions in Al-Muthanna province. This indicates that  $\beta$ -thalassemia can be occurred in all regions. In the present study showed that the incidence of thalassemic patients in urban regions was 60 (61.2 %) more than the incidence in rural regions 38 (38.8%), ( $P=0.026$ ), figure (4-5). The difference of

geographical distribution of disease prevalence is might be due to a population density of cites, another factor should be taken into account, such as a variant in the genetic makeup of their population[113].

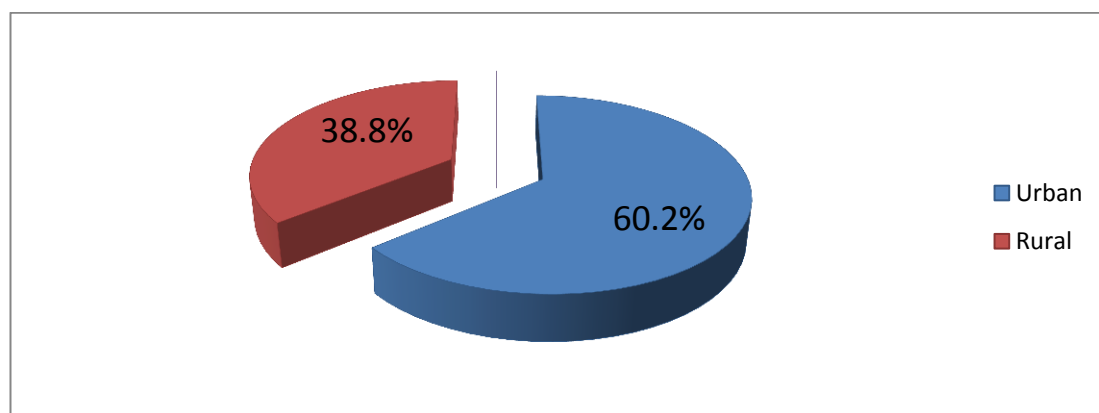


**Figure (4-4):** Distribution of  $\beta$ -Thalassemic patients According to the Geographic site.

#### 4.1.3 Classification Samples According to $\beta$ -Thalassemia Types

The distribution of  $\beta$ -thalassemia types in the present study was done by Hemoglobin Testing D-10 System, its reported in order from high to low percentage:  $\beta$ -thalassemia major 69.4%, intermediate type 29.6% and minor 1%, respectively (as shown in table 4-1). The study performed in Baghdad found the prevalence of  $\beta$ -thalassemia types 525 (51%)  $\beta$  –thalassemia major, 239 (23.2%)  $\beta$ -thalassemia intermedia and 77 (7.5%) case of  $\beta$  –thalassemia minor patients[114].

**Table (4-1):** Distribution of  $\beta$ - Thalassemia Types

| Thalassemia Type | Case | Percent% | Statistically |
|------------------|------|----------|---------------|
| Major            | 68   | 69.4%    | P<0.001*      |
| Intermediate     | 29   | 29.6%    |               |
| Minor            | 1    | 1%       |               |
| Total            | 98   | 100%     |               |

\* represent a significant difference at 0.05 levels, ( $\chi^2$ )test

The distribution  $\beta$ -thalassemia types according to gender, were in  $\beta$ -thalassemia major patients data showed that males were 46 (67.6%) more than females that were 22 (32.4%) with significantly (P=0.004), table (4-2). While no significant among patients of Intermediate type, 14 (48.3%) of them was male and 15 (51.7%) of them

was female. In Pakistan were found male more affected than female in thalassemia major, and no significant between them in Intermediate type [115], Another study in Chittagong where 47.42% were male and 52.58% were female of  $\beta$ -thalassemia patient with statically significant ( $p < 0.05$ ) [116]. While in Gaziantep, Turkey were reported no appreciable in male 55.5% and female 44.5% of  $\beta$ -thalassemia patient [117]. However, a statically non- significant ( $p = 0.07$ ) difference between a number of male and female  $\beta$ -thalassemia patient reported in Shiraz, Iran [118]. This difference in  $\beta$ -thalassemia patients (male more affected than female) might be due to the gender bias among the parents of patients who get medical care more than female.

**Table (4-2):** Distribution of  $\beta$ -thalassemia Types according to Gender

| Thalassemia Type    | Male         | Female       | Statistically |
|---------------------|--------------|--------------|---------------|
| <b>Major</b>        | 46/68(67.6%) | 22/68(32.4%) | P=0.004*      |
| <b>Intermediate</b> | 14/29(48.3%) | 15/29(51.7%) | P=0.853       |
| <b>Minor</b>        | 1/1(100%)    | 0/1(0%)      | ----          |
| Total               | 98           | 100%         |               |

\* represent a significant difference at 0.05 levels, ( $\chi^2$ )test

When the distribution of  $\beta$ -thalassemic patients studied in relation to the first diagnosis of disease, found patients diagnosed under one year old was 42 (42.9%) while 56 (57.1%) of them diagnosed above one year ( $P = 0.157$ ). Regarding present data were found that significant deference among  $\beta$ - thalassemic patients diagnosed under one year ( $P < 0.001$ ). Diagnose of patients with 6 month age was highly 17 (40.5%) compared to that observed in other age groups. Whereas the distribution of  $\beta$ -thalassemic patients diagnosis according to age (above one year), found that significant deference among  $\beta$ -thalassemic patients diagnosed above one year ( $P < 0.001$ ), and diagnose of patients with one year age was highly 24 (42.9%) compared to that observed in other age groups, table (4-3).

**Table (4-3):** Distribution of  $\beta$ -Thalassemic Patients Diagnosis according to age.

| Year           | Case     | Percent % |
|----------------|----------|-----------|
| Under one year | 42/98    | 42.86     |
| 1-5            | 49/98    | 50.00     |
| 6-10           | 6/98     | 6.12      |
| 11-27          | 1/98     | 1.02      |
|                | P<0.001* |           |

\* represent a significant difference at 0.05 levels, ( $\chi^2$ )test

## 4.2 Hematological Parameters

The study included the hematological parameters: ABO and Rhesus blood group, hemoglobin concentration, MCV, MCH, MCHC, and WBC.

### 4.2.1 Classification ABO and Rh in $\beta$ -Thalassemic Patients.

The distribution of blood group among beta-thalassemic patients. Since results in the present study showed the highest prevalence of O+ group in beta-thalassemic patients followed by A+ and B+ group, while AB+ shows only (7.1%) of patients, (P<0.001). Thus blood group O is more prevalent and 'AB' as the less common blood group in Iraqi Arab [114, 119-121], Iraqi Kurdish population [122], Kuwait [123], Saudi [124], Iranian north population [125], India [126, 127], Turkey, Hungary, Ukraine, Germany and Nigeria [128]. The high prevalence of group O individuals in nature is of great advantage because of their status as (universal donors) as this implies a viability of blood cases of emergency [129].

Present study found that highest  $\beta$  -thalassemia major of Rh-positive O blood group with least  $\beta$  -thalassemia minor and  $\beta$  -thalassemia intermedia of Rh-negative AB and A blood group, table (4-4), this result was similar to other, where they found Rh positive O blood group a high in all  $\beta$ -thalassemia types then followed by Rh negative O blood group in Iraq and Saudi Arabia [114, 127]

**Table (4-4):** Prevalence of the ABO Blood Groups among Beta-Thalassemia Patients.

| <b>Blood Groups</b><br><b>Types Beta Thalassemia</b> | <b>O+</b> | <b>O-</b> | <b>A+</b> | <b>A-</b> | <b>B+</b> | <b>B-</b> | <b>AB+</b> | <b>Statistically</b> |
|------------------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|------------|----------------------|
| <b>Major</b>                                         | 20.4%     | 2.0%      | 17.3%     | 1.0%      | 20.4%     | 2.0%      | 6.1%       | P<0.001<br>*         |
| <b>Intermediate</b>                                  | 13.3%     | 1.0%      | 6.1%      | 1.0%      | 6.1%      | 1.0%      | 1.0%       | P<0.001<br>*         |
| <b>Minor</b>                                         | -         | -         | -         | -         | 1         | -         | -          | -                    |

\* represent a significant difference at 0.05 levels, ( $\chi^2$ ) test

The frequency of Rh + was 91.8% while that of Rh - was 8.2%, as shown in figure (4-5), (P<0.001), present data consistent with reports from previous studies in Iraq which found the frequency of Rh + was highly more than Rh – [114, 120, 130-132]

#### 4.2.2 Hemoglobin Concentration

The hemoglobin concentration was significantly decreased in patients group in comparison to control subjects (7.75±0.179 g/dl) versus (12.55±0.432 g/dl) respectively (P<0.001), as shown in Figure (4-6).

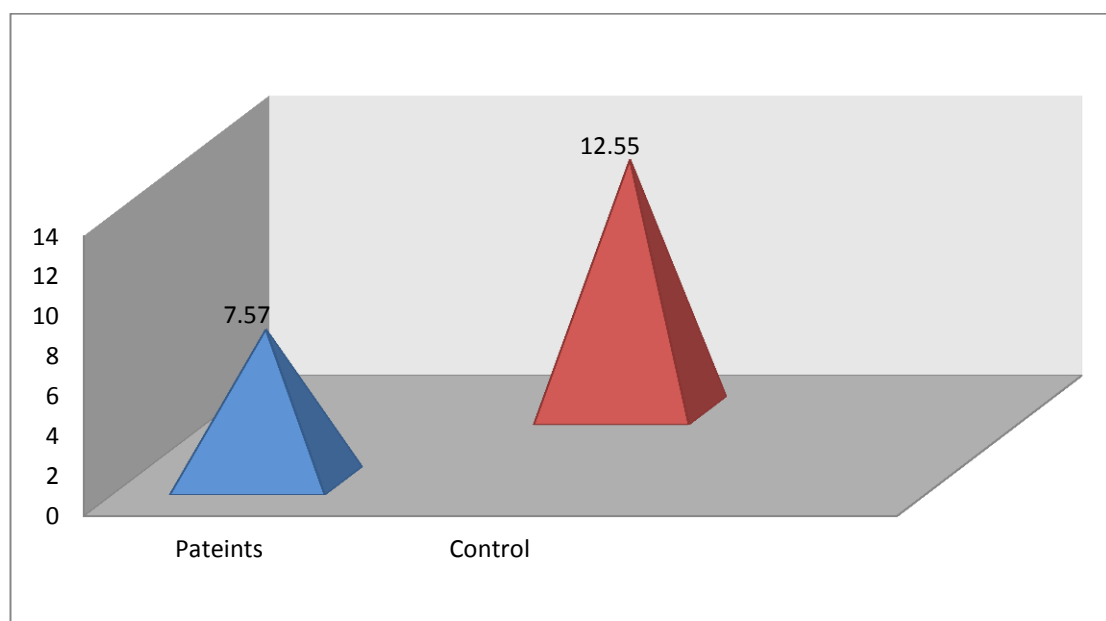


**Figure (4-5):** Comparison of Mean of Hemoglobin Concentration Between Control Group and Patient Group.

The concentration of hemoglobin in patients and control groups according variation in age, however the data showed that hemoglobin concentration in patient less than control in all age groups, since hemoglobin level in in age group 1-10 years was ( $7.782 \pm 0.313$  g/dl) in patient and ( $10.84 \pm 0.544$  g/dl) in control ( $P < 0.001$ ), in the age group 11-20 years was ( $7.875 \pm 0.193$  g/dl) in patients and ( $11.433 \pm 0.876$  g/dl) in control ( $P = 0.003$ ), and in age group 21-29 years was ( $7.214 \pm 0.579$  g/dl) in patients and ( $13.925 \pm 0.481$  g/dl) in control ( $P < 0.001$ ), figure(4-7).

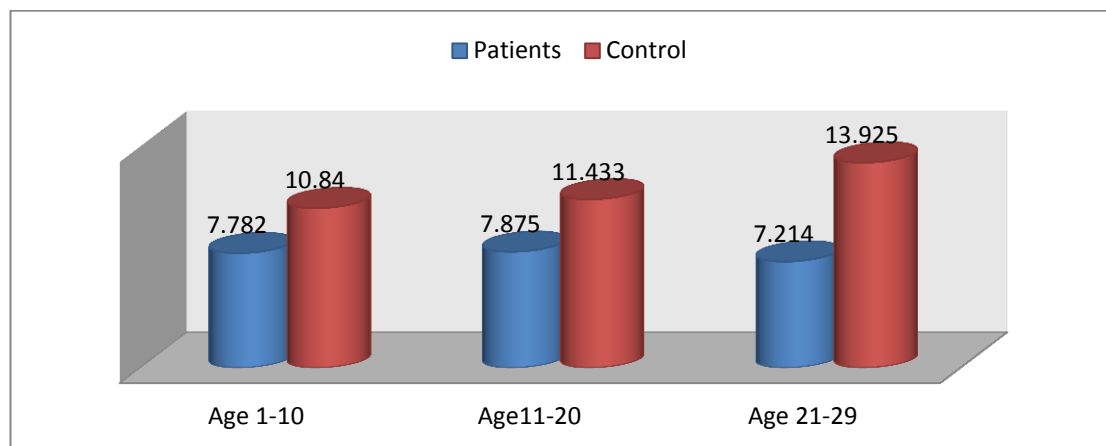


**Figure (4-7):** Correlation between Age and Hemoglobin Mean in Patients and Control.

Previous studies found a significant decrease of hemoglobin level in  $\beta$ -thalassemia patients with compared with controls, in Iraq such as AL-Jamaly in Najaf [119], Iada in Tikrit [100] and Mohssin *et al* in Baghdad Province [114], where they found expected decrease ( $p < 0.05$ ) in hemoglobin concentration in  $\beta$ -thalassemic patients as compared with the control group, as well as another study in other countries include Mansi and Aburjai in Jordon, observed there was significant decrease of hemoglobin concentration in both males and females was noticed in comparison with controls [133], Akram *et al* in Pakistan [134], Khin *et al* in Myanmar [135] and Patne *et al* in India [136], where they observed that the levels of hemoglobin in  $\beta$ -thalassemia major was lower than its levels in the control group. In  $\beta$ -thalassemia major (homozygous is caused by a mutation in the  $\beta$ -globin chain), the production of  $\beta$ -globin chains is severely impaired, because both beta globins are mutated. The severe imbalance of globin chain synthesis. The excess unpaired alpha globin chains aggregate to form precipitates that damage red cell membranes. This

leads to low production and excessive destruction of red blood cells [137]. Erythrocytes degrade reactive oxygen species (ROS) via the actions of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx). In  $\beta$ -thalassemia major, intra erythrocytic release of heme induces a glutathione (GSH)-dependent self-amplifying and self-propagating Hb oxidation pathway, resulting in injury to the thalassemic cell [138]. In addition, iron can serve as a potent catalyst of lipid peroxidation [139]. The presence of hypochromia may facilitate oxidation of the red cell membrane by reducing the amount of Hb available for buffering protection [140]. Unpaired  $\alpha$ -Hb chains may denature and bind to the cell membrane, thus giving rise to cytoskeleton alterations and lipid peroxidation [141]. The free  $\alpha$  chains in  $\beta$ -thalassemia increase autoxidation rates by about two times faster than normal Hb A [139]. It has been reported that the accumulation and autoxidation of the unpaired  $\alpha$ -globin chains in severe  $\beta$ -thalassemia would generate ROS; superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) that would cause accelerated apoptosis and ineffective erythropoiesis [138, 140].

#### 4.2.3 MCV, MCH, MCHC, and WBC Parameters

The result shows that no significant difference in (MCV, MCH, and MCHC) between patients and control, these parameters were analyzed during blood transfusion treatment, in consideration, all patients entering the study had artificially normal MCV, MCH, and MCHC due to recent blood transfusions, while WBC recorded with statically significant ( $P=0.004$ ) between patients and control, as shown in table (4-5).

**Table (4-5):** Comparison of Mean of Some Blood Parameter among Thalassemic Patients and Control Group.

| Parameter | Patients              | Control               | P value  |
|-----------|-----------------------|-----------------------|----------|
| MCV**     | 78.700 $\pm$ 0.792 fl | 78.13 $\pm$ 1.62 fl   | P=0.726  |
| MCH**     | 25.83 $\pm$ 0.479 pg  | 25.75 $\pm$ 0.626pg   | P=0.917  |
| MCHC**    | 33.24 $\pm$ 0.276 g/d | 32.91 $\pm$ 0.315 g/d | P=0.475  |
| WBC**     | 11.56 $\pm$ 1.15      | 7.74 $\pm$ 0.554      | P=0.004* |

\* represent a significant difference at 0.05 level, T-test

\*\*parameters were analyzed during blood transfusion treatment.

### 4.3 Serological Parameters

The study included the serological parameters: ferritin, 25-hydroxy vitamin D and 8-hydroxydeoxyguanosine (8-OHdG).

#### 4.3.1 Serum Ferritin Concentration

The ferritin concentration was a significant increase in patients group ( $3635.28 \pm 412$  ng /ml) in comparison to control ( $156.69 \pm 10.07$  ng /ml) with statically significant ( $P < 0.001$ ), as shown in figure (4-8), table (4-6).

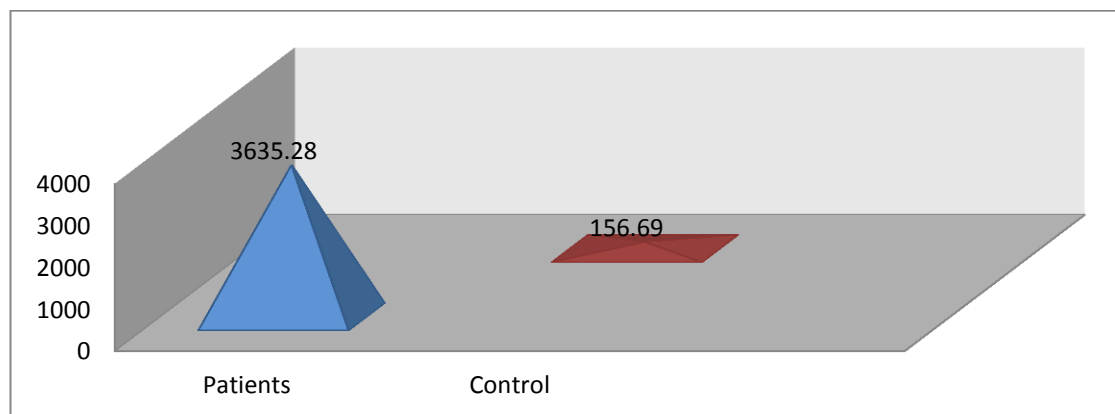


**Figure (4-7):** Comparison of Mean Serum Ferritin Between Control and Patient Group

Previous studies found significantly high concentration of ferritin in  $\beta$ -thalassemic patients In Iraq such as Iada in Tikrit [100] , AL-Jamaly in Najaf [119], and Abbas and Defer in Baghdad [142] who observed that the ferritin levels in serum were significantly higher in  $\beta$ -thalassemia major patients compared to control group , as well as other studies in other countries including Jordon such as Mansi and Aburjai, [133] and Abdalla *et al*, [143] where they observed that the ferritin concentration was significantly higher in both males and females  $\beta$ -thalassemia major patients in comparison with controls. Hashemi *et al* in Iran observed that the mean serum ferritin level was significantly higher in the patients with a final short stature than in those with a normal final height[144]. Livrea *et al* in Italy observed that the levels of ferritin in thalassemic patients was more than its levels in control subjects[145]. Nasr *et al* in Egypt showed that the levels of ferritin in thalassemic patients was more than its levels in normal subjects[106]. Arica *et al* in Turkey observed that the level of ferritin was a significant increase in beta-thalassemia patients compared to control subjects[146] Ferritin is used as a marker for iron overload disorders, such as hemochromatosis and hemosiderosis i.e., abnormally

raised ferritin levels to indicate iron overload that is due to the hemolysis and frequent transfusions to the patients [77].

The present study found that the mean ferritin level increased continuously with increasing age in patients and control groups, however this did not attain the statistical significance ( $P \geq 0.05$ ), were data showed that ferritin in patient ( $3372 \pm 553 \text{ ng/ml}$ ) in age group (1-10), ( $3908 \pm 698 \text{ ng/ml}$ ) in age group (11-20), and ( $3952 \pm 1430 \text{ ng/ml}$ ) in age group (21-29).

The concentration of ferritin level was no significant ( $P=0.568$ ) in male comparison to that observed in female in patients group, since the ferritin level in patients group in male was ( $3445 \pm 494 \text{ ng/ml}$ ) and female was ( $3931 \pm 727 \text{ ng/ml}$ ), while there are significant deference's among control group was ( $189.25 \pm 13.43 \text{ ng/ml}$ ) in male and ( $119.78 \pm 7.98 \text{ ng/ml}$ ) in female, ( $P < 0.001$ ). In same time patient male recorded height concentration ( $3445 \pm 494 \text{ ng/ml}$ ) compression to control male ( $189.25 \pm 13.43 \text{ ng/ml}$ ) with statically significant ( $P < 0.001$ ), and patients female ( $3931 \pm 727 \text{ ng/ml}$ ) more than control females ( $119.78 \pm 7.98 \text{ ng/ml}$ ), with statically significant ( $P < 0.001$ ). Mansi and Aburjai in Jordon showed that that the ferritin concentration was significantly higher in both males and females  $\beta$ -thalassemia patients in comparison with controls [133].

#### 4.3.2 Serum 25-hydroxy Vitamin D Concentration

This study reported that the mean 25(OH) D was a significant decrease in patients group ( $18.75 \pm 1.05 \text{ ng/ml}$ ) in comparison to control ( $26.27 \pm 1.39 \text{ ng/ml}$ ) with statically significant ( $P < 0.001$ ), as shown in figure (4-9), table (4-7).

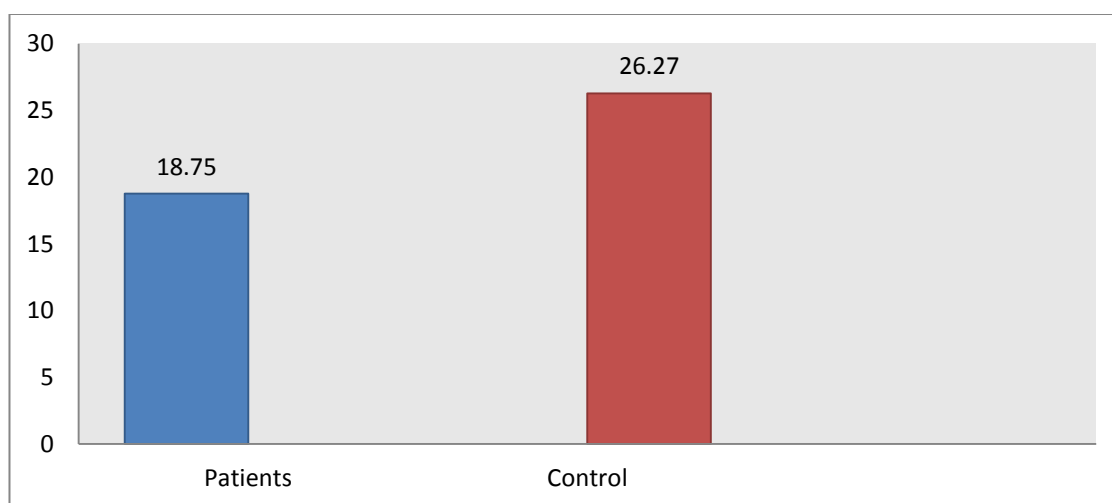


**Figure (4-8):** Comparison of Mean Serum 25-hydroxy vitamin D Between Control Group and Patient Group.

Results of previous reports such as Dandona *et al* in United Kingdom [147], Moulas *et al* in Germany [148], Polak *et al* in Israel [149] and Napoli in Italy [149], where they found that the 25(OH) D concentration was significantly lower in  $\beta$ -thalassemia patients than control subject.

Deficiency of 25(OH) D might be a correlation with iron overload. In another hand, there is a negative correlation between serum 25(OH) D and ferritin level, where a higher concentration of ferritin levels found in patients compared to the control group. Ferritin levels increased continuously with increasing age in patients and control groups, also iron overload may be increased with patient age and result in deficiency of 25(OH) D. Low vitamin D3 concentration has been found in patients with iron overload associated with thalassemia major, that found removal of iron by chelation therapy increased serum 25(OH)D levels in patients [150].

The mean concentration of 25(OH) D was significantly lower in beta-thalassemia patients than control individuals of same age categories according to variation in age, ( $P \leq 0.05$ ). the study showed that 25(OH) D mean in patient was ( $18.642 \pm 1.405$  ng/ml) in the age group 1-10 years, ( $19.251 \pm 1.777$  ng/ml) in the age group 11-20 years, and ( $16.576 \pm 3.944$  ng/ml) in age group 21-29 years, while in control group was ( $27.612 \pm 1$  ng/ml) in age group (1-10), ( $28.92 \pm 0.425$  ng/ml) in age group 11-20, and ( $22.24 \pm 1.24$  ng/ml) in age group 21-29.

The 25(OH) D level was no significant ( $P=0.821$ ) in male comparison to that observed in female in patients group, since the mean of 25-hydroxyvitamin D level in patients group in male was ( $18.936 \pm 1.349$  ng/ml) and female was ( $18.428 \pm 1.730$  ng/ml), while in the control group was significant ( $P=0.018$ ) between male ( $28.676 \pm 0.388$  ng/ml) and female was ( $24.360 \pm 2.211$  ng/ml). In the same time, patient male recorded decreased concentration ( $18.936 \pm 1.349$  ng/ml) compression to control male ( $28.676 \pm 0.388$  ng/ml) with statically significant ( $P < 0.001$ ), and patients female ( $18.428 \pm 1.730$  ng/ml) lower than control females ( $24.360 \pm 2.211$  ng/ml), with statically significant ( $P=0.014$ ).

**Table (4-6):** Comparison of Mean of Ferritin and Vitamin D according to Gender and Age Variation in Patients and Control Groups

| Age group/Tests         |          | Ferritin     | P value  | vitamin D    | P value  |
|-------------------------|----------|--------------|----------|--------------|----------|
| <b>Group1(Age1-10)</b>  | Control  | 104±8.96     | P<0.001* | 27.612±1     | P<0.001* |
|                         | Patients | 3372±553     |          | 18.642±1.405 |          |
| <b>Group2(Age11-20)</b> | Control  | 148.22±7.56  | P<0.001* | 28.92±0.425  | P<0.001* |
|                         | Patients | 3908±698     |          | 19.251±1.777 |          |
| <b>Group3(Age21-27)</b> | Control  | 185.88±15.34 | P=0.034  | 3952±1430    | P=0.012  |
|                         | Patients | 3952±1430    |          | 16.576±3.944 |          |
| <b>Gender/Test</b>      |          |              |          |              |          |
| <b>Male</b>             | Control  | 189.25±13.43 | P<0.001* | 28.676±0.388 | P<0.001* |
|                         | Patients | 3512±20      |          | 18.936±1.349 |          |
| <b>Female</b>           | Control  | 119.78±7.98  | P<0.001* | 24.360±2.211 | P=0.014* |
|                         | Patients | 3931±727     |          | 18.428±1.730 |          |

\* represent a significant difference at 0.05 level, T-test.

#### 4.3.3 Serum 8-hydroxydeoxyguanosine (8-OHdG) Concentration

The mean of 8-OHdG was a significant increase in patients group (21.176±2.13 ng/ml) comparison to control subjects (2.05 ± 2.93 ng/ml) with statically significant (P<0.001), figure (4-10).

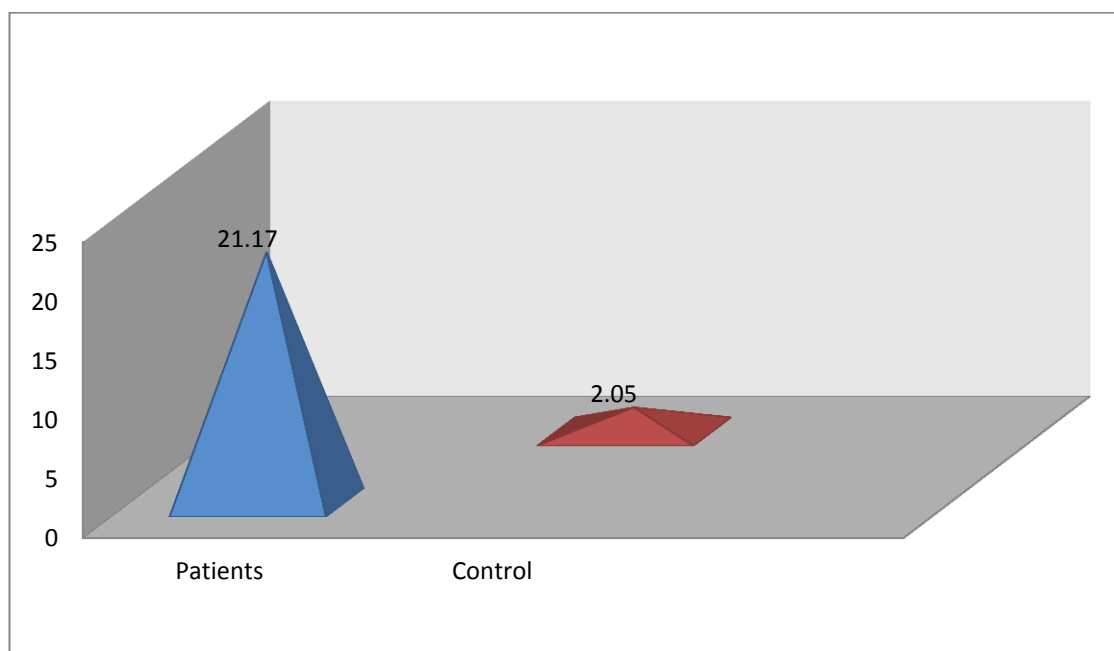


**Figure (4-9):** Comparison of Mean Serum 8-OHdG Between Control Group and Patient Group

This increase reported by other studies in as El Gindi *et al* Egypt observed 8-OHdG significantly elevated in  $\beta$ -thalassemia major ( $P < 0.001$ ) and  $\beta$ -thalassemia intermediate ( $P < 0.001$ ) compared with normal controls[151] and Ferro *et al* in Italy[152], which found 8-OHdG was significantly  $\beta$ -thalassemia patients higher than control individuals. Excess Iron is the main causative agent for increase free radical production and reactive oxygen species, and the oxidative stress[153]. The 8-hydroxydeoxy-guanosine is an indicator of oxidative stress was positively correlated with ferritin, suggesting the role of over iron in generating free radicals and the result in tissue injury and DNA damage[152]. Statically no significant comparison of the concentration of 8-OHdG in patients according to variation in age, ( $P = 0.92.1$ ). Where result showed that 8-OHdG mean was ( $21.714 \pm 3.00 \text{ ng/ml}$ ) in the age group 1-10, ( $21.375 \pm 3.791 \text{ ng/ml}$ ) in the age group 11-20, and ( $18.176 \pm 7.597 \text{ ng/ml}$ ) in the age group 21-29.

#### 4.4 Molecular Identification of $\beta$ -Thalassemia Mutations

##### 4.4.1 Genomic DNA isolation yield

Blood samples were subjected to DNA isolation in automated DNA extraction machine by using MagDEA extraction kit after hemostasis, extracting pure DNA from whole blood is required to free inhibitors of DNA polymerase which may cause, so to inhibit downstream applications. Automated DNA extraction machine Magstration 12GC System has advantages, include its high yield, quality of nucleic acid, addition to that system confer some protection from infected or contaminated blood samples. Automated DNA extraction system is an important part in workflow of molecular diagnostic laboratories and it is suitable for genetic testing of humans because eliminate cross-contamination of DNA sample, DNA genomic, integrity and high yield of genomic DNA isolation, figure (4-11).

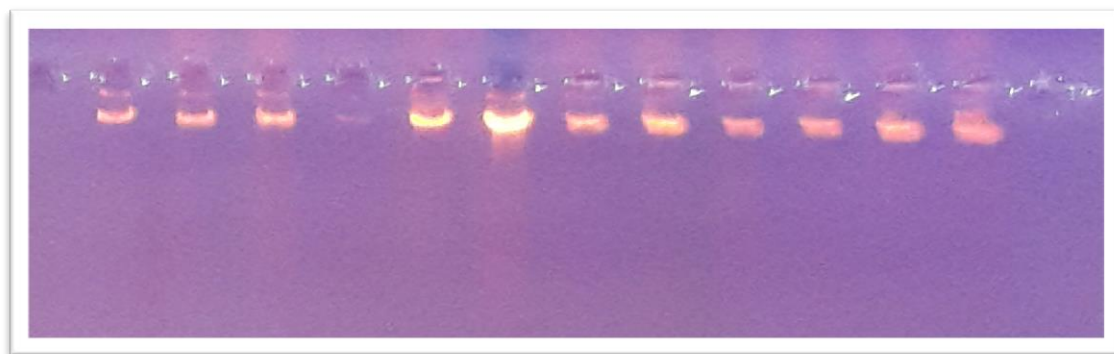


**Figure (4-10):** Genomic DNA Bands Extracted From Whole Human Blood Samples on 0.5% Agarose Gel at 135Vol/cm for 15 Minutes.

##### 4.4.2 HBB Gene Deletion and Mutations Diction

In this study, the strategy of detection of  $\beta$ -thalassemia mutation was designed in two phases. The first phase, involved detecting  $\Delta 619$ bp deletion by gap PCR technology, and the second phase involved PCR product of both mixes I and II applied to direct DNA sequencing. This enables more comprehensive detection of known and unknown  $\beta$ -globin gene mutations.

###### 4.4.2.1. Detecting $\Delta 619$ bp Deletion by Gap PCR Technology (First Phase)

The polymerase chain reaction (PCR) is the most common method to detect the deletional thalassemias, gap-PCR can detect the deleted DNA sequence using the primers flanking the deleted region[154]. The  $\Delta 619$ bp deletion of  $\beta$ -globin gene was subjected to gradient PCR amplification using primer sets which were explained in

the previous study of Chan in 2010, as shown in the table (3-4) [51]. PCR was done after the optimization step to select the typical PCR annealing temperature by gradient PCR thermocycler that was 58.5°C. The products of successful binding specific primers to target DNA template of  $\beta$ -globin gene PCR mixes I and II, as shown in figure (4-12). PCR reactions in this study, the PCR mix I product of 1457 bp was observed. PCR mix II products showed 1212 bp. Two PCR products of mix I and mix II was considered as a monitor of successful reaction that was designed to unaffected wild-type patient, as explained in figure (4-12). In an affected person with a homozygous  $\Delta 619$  bp allele, PCR I yield was a 1,671-bp product from the A and D primer pair (due to the deletion of the B primer binding site), and PCR II yield is a truncated product of 593 bp from the C and D primer pair. Heterozygous persons would have a combination of the 1,457- and 1,671-bp products from PCR I and the 1,212- and 593-bp products from PCR II. The present study showed no presence of  $\Delta 619$  bp deletion.

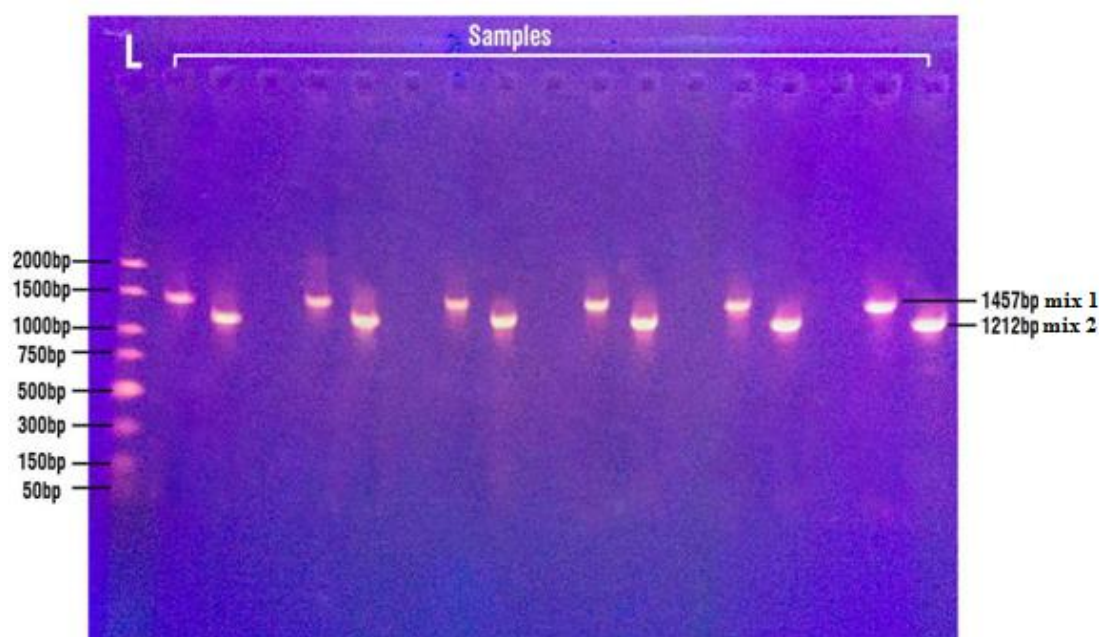


**Figure (4-11):** PCR Phase I Product of Mix I and Mix II Reactions on 1% agarose at 100 volt/cm for 20 minutes Then at 50 volt/cm for 30 Minutes.

#### 4.4.2.2 Detection of $\beta$ -globin Mutations by Direct DNA Sequencing (Second Phase)

The DNA Sequencing of the PCR products was done using the BigDye terminator V.3.1 The separation of the cycle sequencing products and detection was carried out

by using ABI 3730 xL genetic analyzer. The selected primers for PCR I was primer A + E and for PCR II was primer C + F, as previously explained in the table (3-4).

#### 4.4.2.2.1 Analysis of DNA Sequence Data

DNA sequencing was carried out commercially at MACROGEN Inc., Korea. PCR products along with forward and reverse primers were sent to MacroGen Sequencing Service, who delivered the sequence reads. The analysis of sequence data done by using DNA sequence editor (BioEdit), analysis revealed the presence of nine mutations in 98  $\beta$ -thalassemia patients, they are IVS-II-666 C>T, CD2 CAT>CAC, IVS-II-850 G>A, IVS-II-16 G>C, Hb kangsmill, Hb Saveh, IVS-II-81 C>T, Ivs-II-1 G>A and Ivs-1-5 G>C.

Positions of mutations detected by used NCBI blast by alignment query with specific RefSeq (NG\_000007.3) as well as HbVar and Ithasnet sites were used to identify local mutation In Iraq, were found seven mutations not previously recorded in Iraq, including IVS-II-666 C>T, CD2 CAT>CAC, IVS-II-850 G>A, IVS-II-16 G>C, Hb kangsmill, Hb Saveh, and IVS-II-81 C>T, as well as two mutations previously recorded in Iraq, include IVS II-1 G>A and IVS 1-5 G>C, as shown in table (4-7).

**Table (4-7):** Detection of Nine Iraqi  $\beta$ -Thalassemia Mutations Observed in The Present Study.

| Name of mutation            | Human Genome Variation Society (HGVs) | NCBI NG_000007.3 | No of total % |
|-----------------------------|---------------------------------------|------------------|---------------|
| <b>IVS-II-666 C&gt;T</b>    | HBB:C.316-185 C>T                     | 71705 C>T        | 26 (26.53)    |
| <b>CD2 CAT&gt;CAC</b>       | HBB:C.9 T>C                           | 70603 T>C        | 6 (6.12)      |
| <b>IVS-II-850 G&gt;A</b>    | HBB:C.316-1 G>A                       | 71889 G>A        | 2 (2.04)      |
| <b>IVS-II-16 G&gt;C</b>     | HBB:c.315+16 G>C                      | 71055 G>C        | 2 (2.04)      |
| <b>Hb Kings Mill G&gt;C</b> | HBB:c.79G>C                           | 70675 G>C        | 2 (2.04)      |
| <b>Hb Saveh GTT&gt;ATT</b>  | HBB:C.70 G>A                          | 70664 G>A        | 2 (2.04)      |
| <b>IVS-II-81 C&gt;T</b>     | HBB:C315+81 C>T                       | 71120 C>T        | 2 (2.04)      |
| <b>Ivs-1-5G&gt;C</b>        | HBB:c.92+G>C                          | 70691 G>C        | 24 (24.50)    |
| <b>Ivs-II-1 G&gt;A</b>      | HBB:c.315+G>A                         | 71040 G>C        | 32 (32.65)    |
| Total                       |                                       |                  | 98 (100)      |

The number frequency of mutation IVS-II-1 G>A in the present study 32(32.56%) was followed by IVS-II-666 C> T mutation which reported was 26 (26.53%), Ivs-1-5G>C was 24 (24.50%), CD2 CAT>CAC was 6( 6.12%), IVS-II-850 G>A was 2 (2.4% ), ,IVS-II-16 G>C was 2 (2.4% ), Hb Kings Mill was 2 (2.4% ), Hb Saveh 2 (2.4%) and IVS-II-81 C>T was 2 (2.4%). The present study found 61 (62.24 %) of all mutation were homozygous and 37 (37.76 %) of mutations presented heterozygous. The frequency of alleles and genotypes in the present study shown in the table (4-8).

**Table (4-8):** Frequency of Alleles and Genotypes of  $\beta$ -Globin Mutations in  $\beta$ -Thalassemia Patient.

| Name of mutation    | Percentage of total % | Genotype | Genotype frequency | Allele | Allele frequency |
|---------------------|-----------------------|----------|--------------------|--------|------------------|
| IVS-II-666 C>T      | 26.53                 | CC       | 72(73.47)          | C      | 147 (0.75)       |
|                     |                       | CT       | 3 (3.06)           | T      | 49(0.25)         |
|                     |                       | TT       | 23(23.47)          |        |                  |
| CD2 CAT>CAC         | 6.12                  | TT       | 92(93.88)          | T      | 184(0.94)        |
|                     |                       | TC       | 0(0.0)             | C      | 12(0.06)         |
|                     |                       | CC       | 6(6.12)            |        |                  |
| IVS-II-850 G>A      | 2.04                  | GG       | 96(97.96)          | G      | 192(0.98)        |
|                     |                       | GA       | 0(0.00)            | A      | 4 (0.02)         |
|                     |                       | AA       | 2(2.04)            |        |                  |
| IVS-II-16 G>C       | 2.04                  | GG       | 96(97.96)          | G      | 192 (0.98)       |
|                     |                       | GC       | 0(0.00)            | C      | 4 (0.02)         |
|                     |                       | CC       | 2(2.04)            |        |                  |
| Hb Kings Mill G > C | 2.04                  | GG       | 96(97.96)          | G      | 193 (0.98)       |
|                     |                       | GC       | 1(1.02)            | C      | 3 (0.02)         |
|                     |                       | CC       | 1(1.02)            |        |                  |
| Hb Saveh GTT>ATT    | 2.04                  | GG       | 96(97.96)          | G      | 192 (0.98)       |
|                     |                       | GA       | 0(0.00)            | A      | 4 (0.02)         |
|                     |                       | AA       | 2(2.04)            |        |                  |
| IVS-II-81 C>T       | 2.04                  | CC       | 96(97.96)          | C      | 193(98.47)       |
|                     |                       | CT       | 1(1.02)            | T      | 3 (1.53)         |
|                     |                       | TT       | 1(1.02)            |        |                  |
| IVS-1-5 G>C         | 24.50                 | GG       | 74(75.51)          | G      | 164 (0.84)       |
|                     |                       | GC       | 16(16.33)          | C      | 32 (0.16)        |
|                     |                       | CC       | 8(8.16)            |        |                  |
| IVS-II-1 G>A        | 32.65                 | GG       | 66(67.35)          | G      | 148 (0.76)       |
|                     |                       | GA       | 16(16.33)          | A      | 48 (0.24)        |
|                     |                       | AA       | 16(16.33)          |        |                  |

\* represent a significant difference at 0.05 levels, ( $\chi^2$ )test.

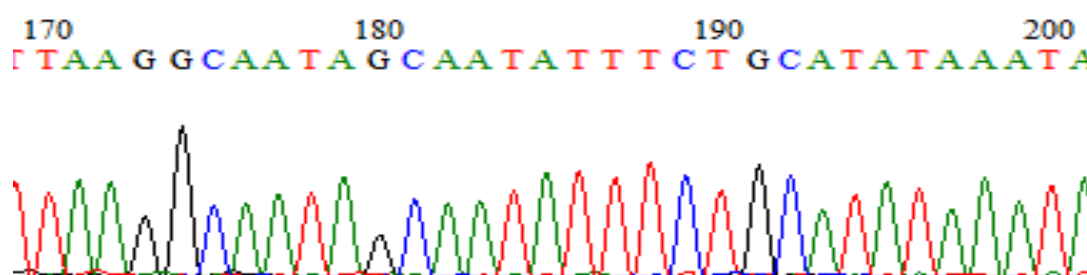


**Figure (4-12):** Homozygous IVS-II-666 C> T mutation at position 187 (according to the query sequence of this study) by using NCBI blast and BioEdit software version 7.0.5.3

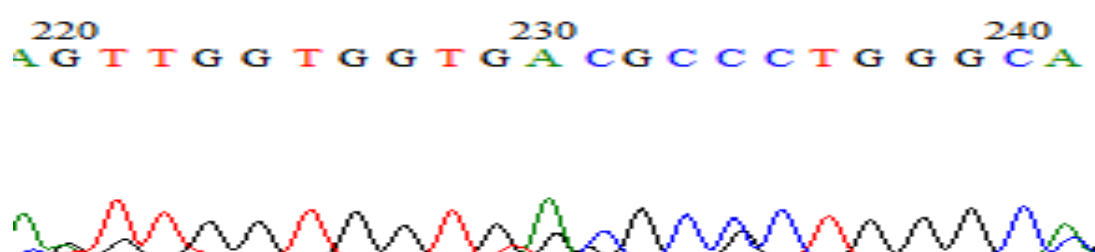


**Figure (4-13):** Heterozygous Hb Kings Mill mutation at position 231 (according to the query sequence of this study) by using NCBI blast and BioEdit software version 7.0.5.3

The mutation IVSII-666 C>T is located in the second intron of the  $\beta$ -globin gene, figure (4-16), which recorded in the present study with 26 (26.53%). Out of 26 patients studied 23 (23.47%) were homozygous and 3 (3.06%) heterozygotes. The genotypes frequency of IVS-II-666 C>T mutation were CC 73.47%, CT 3.06%, and TT 23.47%. C allele frequency of this mutation 0.75 than T allele frequency 0.25, ( $p < 0.001$ ), as shown in table 4-8. IVSII-666 C>T was identified in 15.4% of the Saudi beta-thalassemia patients[155].

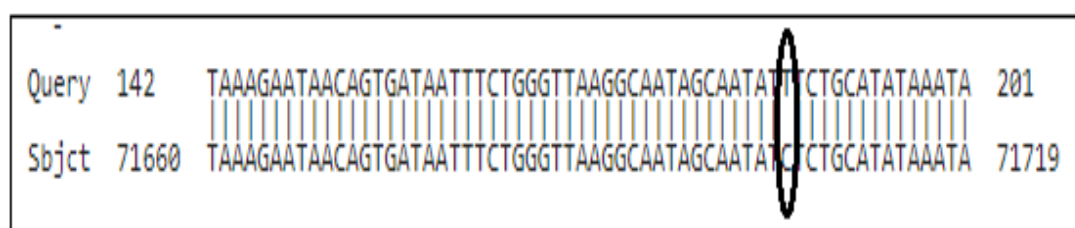


**Figure (4-14):** IVSII-666 C>T Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

The present study showed that HBB: C.9T>C (CD2 CAT>CAC) silent mutation (CAT>CAC), figure (4-17), It one of most common mutation in this study with a number frequency of 6 (6.12%). Six of patients with CD2 CAT>CAC mutation were homozygous 6.12%. In CD2 CAT>CAC mutation, the TT genotype was detected in 93.88 %, TC with percent 0% and CC 6.12% of  $\beta$ - thalassemic patients, T allele is higher frequency 0.94 than C allele 0.6, ( $p < 0.001$ ), as shown in table 4-8. This mutation is firstly described in the Portuguese population [156]. This mutation has also been reported in China and India [157]. Codon 2 T>C changes CAT to CAC but both coding for Histidine. In Saudi CD2 CAT>CAC identified as 11.1% [155]. As well as in Bangladesh 57.1% [158]. It is possible that the mutation is present throughout the population without any phenotypic manifestation of hemoglobinopathy. Alternatively, the mutation itself may be responsible for the  $\beta$ -thalassemic phenotype to some extent by changing the stability of the transcript[159, 160] or by altering translation efficiency as observed in case of other genes in mammalian systems. Both the possibilities are yet to be explored experimentally [161].



**Figure (4-15) :** HBB:C.9 T>C Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

The IVS-II-850 G>A mutation in the present study, as shown in figure (4-18), with the number frequency 2 (2.04%). Two of patients with IVS-II-850 G>A mutation were homozygous 2.04%. The current study showed that GG genotype frequency was showed in  $\beta$ - thalassemic patients IVS-II-850 G>A mutation with 97.96%, more than in GA 0% and AA 2.04%, a present result found the frequency of G allele 0.98 more than A allele 0.02, ( $p < 0.001$ ), as shown in table 4-8. This mutation in Northern Ireland population was found heterozygous IVS-II-850 G>A mutation and was associated with Haplotype [162]. It has been described previously in a North European family of Scottish/English ancestry [163], a Japanese family [164]. Thus, it is most likely that mutation has been introduced into the population by

migration. However, IVS-II-850 G>A from North European Family is described by Curuk [163]

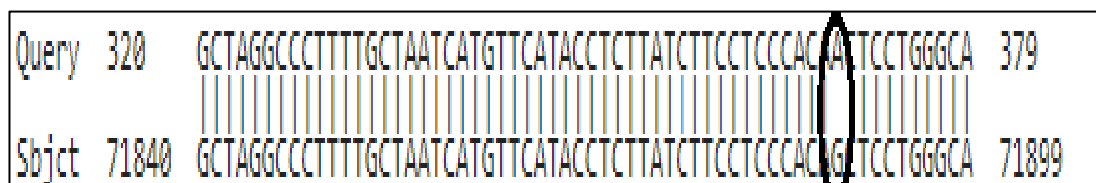


**Figure (4-16):** IVS-II-850 G>A Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

IVS-II-16: G>C mutation in the present study, as shown in figure (4-19), with a number frequency of 2 (2.04%). Two of patients with IVS-II-16: G>C mutation were homozygous 2.04%. The genotypes frequency in  $\beta$ -thalassemic patients with IVS-II-16 G-C mutation were as follow: GG 97.96%, GC 0%, and CC 2.04%. G allele is higher frequency 0.98 than C allele 0.02, ( $p < 0.001$ ), table 4-8. It's in the alternative splicing region of mRNA, which may lead to differential expression, its recorded in India with a number frequency 36% [165], This polymorphism is quite common in Indian populations, all over India fifty-five populations that were studied have revealed this C allele number frequency ranging from 0.21 in Indo-Europeans to 0.66 in Dravidian linguistic groups [166]. In Saudi Arabia recorded with a number frequency 15.4% [155]. IVS-II-16: G>C mutation identified in Bangladesh population with high number frequency 57.0%[158]. Previous study showed the occurrence of IVS-II-16 G>C mutation of thalassaemic patients in India [165], and Mexican [167].

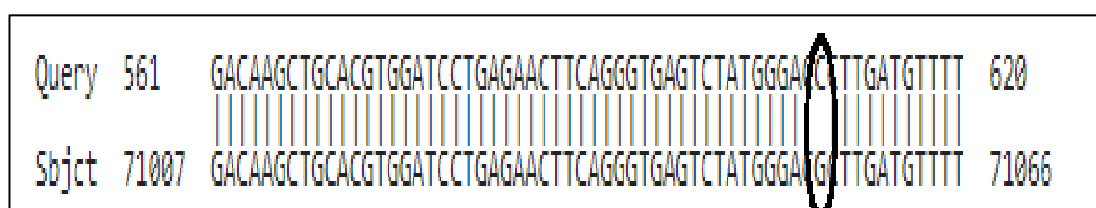


**Figure (4-17) :** Deletion IVS-II-16: G>C Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

In present study IVS-II-81 C>T mutation, as shown in figure (4-20), with a number frequency of 2 (2.04%), one of the patients with IVS-II-81 C>T were homozygous 1.02% and one other heterozygous 1.02%. In IVS-II-81 C>T mutation, where found

the CC genotype was detected in 97.96 %, CT with a percent (1.02%) and TT (1.02%) of  $\beta$ - thalassemic patients, C allele is higher frequency 0.98 than T allele 0.02, ( $p < 0.001$ ), table 4-8. This mutation is described in Bangladesh population with number frequency 1.43% [158].



**Figure (4-18) :** IVS-II-81 C>T Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

The mutation IVS-I-5 G>C was described previously in Iraq, as shown in figure (4-21). This mutation identified in the present study with a number frequency of 24 (24.50%). Sixteen of patients with IVS-I-5 G-C were heterozygous 16.33% and eight other were homozygous 8.16%. In IVS-1-5 G>C that showed GG genotype 75.71% ,GC 16.33% and CC 8.16%, of G allele frequency 0.84 more than allele C 0.16, ( $p < 0.001$ ), as shown in table 4-8. IVSI-5 leading to reduce normal splicing to very low levels, resulting in aberrant  $\beta$ -globin chain formation and causing transfusion-dependent disease, IVS1-5: G>C mutation alone can because the pathogenic condition in homozygous genotype or it can be lead to the disease combined with other mutation in both homozygous and heterozygous genotype [168].

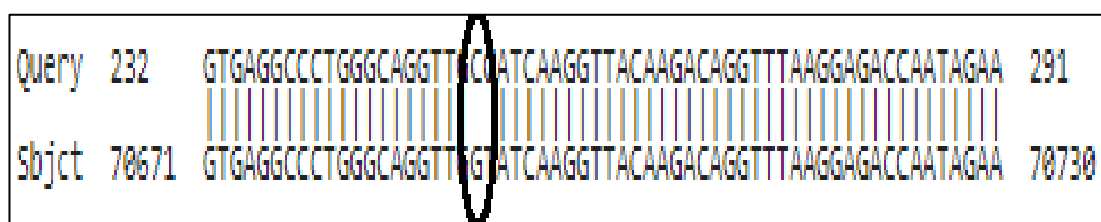


**Figure (4-19):** IVS-I-5 G>C Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

This point mutation is recorded for the 1<sup>st</sup> time in Babylon governorate with number frequency at 10%, were detected by using a PCR-ARMS technique [169], also described in Al-Muthanna province – Iraq as percent 53.8 %[70]. A previous

study of  $\beta$ -thalassemia in Dohuk city (northern Iraq) reported with number frequency of 6.7%. The disease prevalence in Iraqi Kurds was reinforced by the high rate of consanguineous marriages in the region [14].

The IVS-I-5 mutation in Iraq was recorded with number frequency 18 (21.7%) of thalassemic samples studied in Basrah city, located on the south of Iraq between Kuwait and Iran [170]. Dohuk and Basrah are border cities near from neighboring countries endemic of this mutation like Syria, Iran (Kerman Province) 66.2%[67], Kuwait 17% [47], and Turkey 2.1 % [69] and maybe there is society association between them. AlAssadi study in 2007 has been showed that the number frequency of IVS-1-5 has been 12.5% in thalassemic samples in Baghdad [56]. AL-Azzawi study in 2013 of thalassemic patient samples were 26.3% in Baghdad [71]. The comparison of the mutation frequencies in different regions of Iraq referred that the distribution of  $\beta$ -thalassemia alleles differed within each area. These findings supported by the previous observations which indicated that the number frequency of several mutations varies from one region to another,  $\beta$ -thalassemia mutations were very heterogeneous, and finally, there were no specific distribution patterns that would aid in the identification of any ethnic background [4, 56].

Distribution of IVSI-5 G>C mutation in neighboring regions as described in the study of Saleh-Gohari and Bazrafshani which referred to the most prevalent mutations in overall samples analyzed in Kerman Province in Iran was IVSI-5 G>C, with the number frequency of 66.2% [67]. The most common mutation in the United Arab of Emirates (UAE) (55%) and Oman (62%), and was quite frequent in neighboring Kuwait 17% [47] and Saudi Arabia 19% [171]. The study of Pehlivan carried out that determine of the molecular basis of  $\beta$ -thalassemia in Turkey, where found the frequent mutations of IVS 1.5 G>C was 2.1%. In Turkey, however, a broad range of mutations had been observed, probably because of Turkey's location at the three continents and of the influence of different cultures over the course of history[69]. The IVS-I-5 G>C is the most common mutation in Bangladeshi  $\beta$ -thalassemia patients recorded 81.5% [158]. Even in Indian population, this mutation is most common with a number frequency of 63.17%, [47], IVSI-5 G>C number frequency varied from 15% in the immigrant population from Pakistan and Goa on the west coast to 88.6% in the eastern state of Orissa[172], It was also found in the majority of other Arab countries, although the number frequency diminishes appreciably in countries of Northern Africa. The most frequent and widespread

Asian-Indian mutation in Arab countries is IVSI-5 G>C. As expected, its number frequency is highest in countries of the Arabian Peninsula, while its lower number frequency in Western Asian Arab countries may be explained by migration and invasion which have important roles in genetic admixtures.

In the present study was detected IVS-II-1 G>A mutation, as shown in figure (4-22), with identified with a number frequency of 32 (32.65%), sixteen patients have IVS-II-1 G>A were heterozygous 16.33% and other sixteen were homozygous 16.33%. The allele frequency of IVS-II-1 G>A mutation found high incidence in GG genotype 67.35%, GA genotype 16.33% and AA genotype 16.33%, G allele recorded in 0.76 in patients more than A allele 0.24, ( $p < 0.001$ ), table 4-8. IVS-II-1 G>A mutation recorded previously in Iraq, it's described in Sulaimaniyah city, northeastern Iraq [14] IVS-II-1, G>A transition, has serious consequences where it affects the splice site and hence causes  $\beta^0$  phenotype[173]



**Figure (4-20):** IVS-II-1 G>A Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

IVS-II- 1 G>A, was detected in high number frequency in North Jordan 20%, Saudi Arabia 15%, Jordan 19.8%, Syria 4% and Kuwait country which recorded is the most common mutation in 29%[60]. The IVS-II-1 G>A mutation is one of the most prevalent in the general population of Iran [174]. It was the major mutation reported in Iranian patients, where 61% have this mutation. All other studies from different provinces of Iran also reported this mutation at a high number frequency[175].

Other thalassemia types, HBB: c.79 G > C mutation, figure (4-23), also known as Hb Kings Mill, with a number frequency of 2 (2.04%), one of the patients with HBB: c.79 G > C were homozygous 1.02% and another one was heterozygous 1.02%. In

HBB: c.79 G > C mutation, where found the GG genotype was detected in 97.96 %, GC with percent 1.02% and CC 1.02% of  $\beta$ - thalassemic patients, G allele is higher frequency 0.98 than C allele 0.02, ( $p < 0.001$ ), table 4-8. Hb Kings Mill is a clinically mild disease with no significant symptoms. Few studies are available on clinical variability in Hb E disorders[176]. HbE, the most common Hb variant among Southeast Asian populations, results from a G to A substitution at codon 26 (GAG to AAG) in exon 1 of the HBB ( $\beta$ -globin) gene (HBB:c.79G>A) HbE is synthesized at a reduced rate and the  $\beta$ -globin gene produces symptoms resembling those of a mild form of  $\beta$ -thalassemia[177]

A wide spectrum of clinical phenotypes in  $\beta$  -thalassemia/ HbE patients has been observed. These patients show remarkable variability in clinical expression, ranging from asymptomatic or only mild clinical symptoms with normal growth development and survival without transfusions, to transfusion-dependent thalassemia major, where patients have marked anemia, growth retardation, severe bone changes, hepatosplenomegaly and heavy iron overload [178]

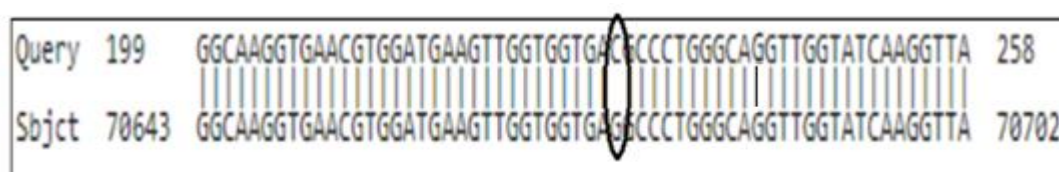


**Figure (4-21):** Hb Kings Mill Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

Hb Saveh mutation GTT>ATT at codon 23 in  $\beta$ - gene, as shown in figure (4-24), recorded in the present study with a number frequency of 2 (2.04%), Two of patients with HBB:C.70 G>A mutation were homozygous 2.04%. The genotypes frequency in  $\beta$ - thalassemic patients with HBB:C.70 G>A mutation were as follow: GG 97.96 %, GA 0%, and AA 2.04%.G allele is higher frequency 0.98 than A allele 0.02, ( $p < 0.001$ ), as shown in table table 4-8.

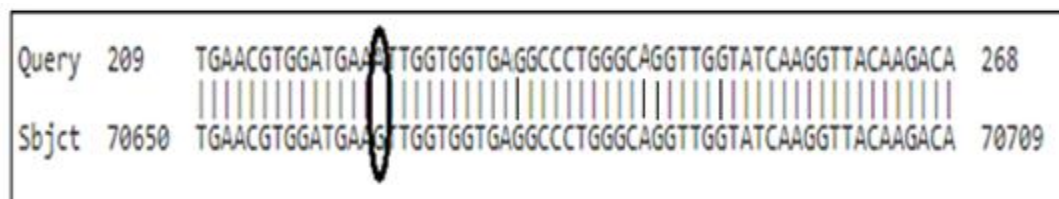


**Figure (4-22)** Hb Saveh Mutation Detected in This Study by Using NCBI Blast Nucleotide Aligned With NG\_000007.3.

#### **4.4.3 Thalassemia Cases for Possible Associations of the Hemoglobin and Three Serum Measurements with Selected $\beta$ -thalassemia Mutation.**

Differences in average values of the 3 tested serum measurements and hemoglobin between the three genotypes of each mutation were explored, as shown in the table (4-9). In IVS-II-1 G>A, were GG genotype showed the high mean concentration of hemoglobin comparison to that observed in the AA genotype, ( $p \leq 0.005$ ). The remaining mutation showed no important or statistically significant differences in mean concentration of hemoglobin between the 3 genotypes.

The differences in average values of tested serum ferritin were assessed against the presence of  $\beta$ -thalassemia mutation, as shown in the table (4-9). Serum ferritin showed an important difference in its median value between genotype in IVS-II-16 G>C mutation, was CC genotype showed the high mean concentration of ferritin comparison to that observed in GG genotype, ( $p < 0.001$ ). In IVS-1-5 G>C mutation, were values of ferritin mean highly in GG genotype more than CC genotype, ( $p < 0.001$ ). The mean serum ferritin was significantly higher among cases with AA genotype in IVS-II-1 G>A mutation compared to GG genotype, ( $p < 0.001$ ). The remaining 6 mutations showed no important or statistically significant differences in mean concentration of ferritin between the 3 genotypes.

The mean concentration of vitamin D3 and 8-hydroxydeoxyguanosine was significantly higher among cases with CC genotype compared to GG in IVS-1-5 G>C mutation. In IVS-II-1 G>A, were AA genotype showed the high mean concentration of vitamin D3 and 8-hydroxydeoxyguanosine comparison to that observed in the GG genotype. The remaining 7 mutations showed no important or statistically significant differences in the mean concentration of hemoglobin between the 3 genotypes, ( $p < 0.001$ ), as shown in the table (4-9).

**Table (4-9):** The Mean of Selected Serum Parameters and Hemoglobin by Presence of  $\beta$ -Thalassemia Mutation among Thalassemia Cases.

| Name of mutation | Severity | Age  |       |       | Gender |    | Removed spleen |    | Type of $\beta$ -thalassemia |       |       | Genotype | Hb          | Ferritin  | V.D           | 8-H           |
|------------------|----------|------|-------|-------|--------|----|----------------|----|------------------------------|-------|-------|----------|-------------|-----------|---------------|---------------|
|                  |          | 1-10 | 11-20 | 21-29 | M      | F  | Yes            | No | Major                        | Inter | Minor |          |             |           |               |               |
| IVS-II-666 C>T   | N        | 12   | 11    | 3     | 15     | 11 | 5              | 21 | 17                           | 9     | 1     | CC       | 7.940±0.195 | 2830±423  | 18.778±1.200  | 21.438±18.18  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | CT       | 7.695±0.005 | 2807±2653 | 14.00±2.039   | 13.286±3.092  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | TT       | 6.900±0.392 | 6962±922  | 18.1059±2.508 | 18.910±6.320  |
| CD2 CAT>CAC      | N        | 4    | 2     | -     | 4      | 2  | 1              | 5  | 4                            | 2     | -     | TT       | 7.807±0.188 | 3479±420  | 18.756±1.088  | 20.565±2.242  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | TC       | -           | -         | -             | -             |
|                  |          |      |       |       |        |    |                |    |                              |       |       | CC       | 7.333±0.520 | 6019±1993 | 14.630±5.090  | 13.019±10.34  |
| IVS-II-850 G>A   | P        | 1    | 1     | -     | 1      | 1  | 2              | -  | 1                            | 1     | -     | GG       | 7.750±0.184 | 3533±414  | 18.539±1.080  | 20.839±2.192  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | GA       | -           | -         | -             | -             |
|                  |          |      |       |       |        |    |                |    |                              |       |       | AA       | 8.400±0.100 | 2852±1588 | 17.354±11.64  | 0.25815±0.086 |
| IVS-II-16 G>C    | N        | 2    | -     |       | 1      | 1  | -              | 2  | 2                            | -     | -     | GG       | 7.790±0.180 | 3318±391  | 18.656±1.097  | 20.890±2.230  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | GC       | -           | -         | -             | -             |
|                  |          |      |       |       |        |    |                |    |                              |       |       | CC       | 7.00±1.40   | 12000+0*  | 13.078±2.67   | 9.609±3.768   |
| Hb Kings Mill    | P        | 2    | -     | -     | 1      | 1  | -              | 2  | 1                            | 1     | -     | GG       | 7.730±0.179 | 3590±415  | 18.959±1.076  | 21.264±2.235  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | GC       | 7.8         | 560       | -             | -             |
|                  |          |      |       |       |        |    |                |    |                              |       |       | CC       | 10.4        | 11422     | 15.31687      | 12.65045      |
| Hb Saveh         | P        | 2    | -     | -     | 1      | 1  | 1              | 1  | 2                            | -     | -     | GG       | 7.727±0.178 | 3488±421  | 18.861±1.079  | 20.964±2.244  |
|                  |          |      |       |       |        |    |                |    |                              |       |       | GA       | -           | -         | -             | -             |
|                  |          |      |       |       |        |    |                |    |                              |       |       | AA       | 9.250±1.150 | 8431±1116 | 10.511±4.805  | 7.205±5.444   |

|                             |   |    |    |   |    |    |   |    |    |   |   |    |              |            |                |               |
|-----------------------------|---|----|----|---|----|----|---|----|----|---|---|----|--------------|------------|----------------|---------------|
| <b>IVS-II-81<br/>C&gt;T</b> | N | 1  | 1  | - | -  | 2  | 2 | -  | 1  | 1 | - | CC | 7.751±0.182  | 3686±429   | 18.948±1.02    | 21.452±2.253  |
|                             |   |    |    |   |    |    |   |    |    |   |   | CT | 7.1          | 5440       | 5.705519       | 1.760736      |
|                             |   |    |    |   |    |    |   |    |    |   |   | TT | 8.7          | 264        | 29.00341       | 8.950515      |
| <b>Ivs-1-5<br/>G&gt;C</b>   | P | 12 | 10 | 2 | 16 | 8  | 3 | 21 | 17 | 7 | - | GG | 7.717±0.178  | 3552±417*  | 18.753±1.08    | 21.187±2.243  |
|                             |   |    |    |   |    |    |   |    |    |   |   | GC | 9.150±1.250  | 11422±5628 | 13.808±1.050   | 12.650±2.792  |
|                             |   |    |    |   |    |    |   |    |    |   |   | CC | 8.730±0.213  | 416±205    | 29.00341±3.65* | 44.622±9.85*  |
| <b>Ivs-II-1<br/>G&gt;A</b>  | P | 15 | 14 | 3 | 22 | 10 | 4 | 28 | 24 | 8 | - | GG | 7.790±0.181* | 3622±426   | 18.826±1.079   | 20.825±2.263  |
|                             |   |    |    |   |    |    |   |    |    |   |   | GA | 7.950±0.1500 | 3000±2444  | 5.7055±0.624   | 1.760±0.337   |
|                             |   |    |    |   |    |    |   |    |    |   |   | AA | 6.850±0.750  | 7844±3831* | 28.525±3.12*   | 43.886±8.423* |

# **Chapter Five**

## **Conclusion and Recommendation**

**Conclusions**

1. Prevalence of  $\beta$ -thalassemia was highly in age ranging (1-10) year.
2. Distribution of  $\beta$ -thalassemia in rural regions more than urban regions.
3. Incidence of disease according to cities was highly in Samawa, then by Rumaitha, Khudhur and Warkaa respectively.
4. Beta thalassemia major was high rate and followed by intermediate and by minor type.
5. The frequency of Rh + was more than Rh –and ABO blood group in the order of (O > B > A > AB) in both males and females in  $\beta$ -thalassemia patients.
6. The concentration of hemoglobin was significant decreased in  $\beta$ -thalassemia patients in comparison to control group.
7. The mean of ferritin concentration in  $\beta$ -thalassemia patients was higher than control group.
8. The mean of 25-hydroxy vitamin D was significant decreased in  $\beta$ -thalassemia patients compared with healthy persons.
9. The 8-OHdG level in patients was higher than control group.
10. The first detection of seven types of  $\beta$ -thalassemia mutations in Iraq including IVS-II-666 C>T, CD2 CAT>CAC , IVS-II-850 G>A, IVS-II-16 G>C , Hb kangsmill, Hb Saveh , and IVS-II-81 C>T and two mutations recorded previously in Iraq include IVS-II-1 G>A and IVS-1-5 G>C.
11. In IVS-II-16 G>C, were CC genotype is associated with highest levels of Ferritin comparison to that observed in GG genotype. In IVS-1-5 G>C, were CC genotype is associated with lower levels of Ferritin comparison to that observed in GG genotype, on other hand CC genotype is associated with highest levels of vitamin D and 8-hydroxydeoxyguanosine comparison to that observed in GG genotype. In IVS-II-1 G>A, were AA genotype is associated with lower levels of HB comparison to that observed in GG genotype, on other hand AA genotype is associated with highest levels of Ferritin, Vitamin D and 8-hydroxydeoxyguanosine comparison to that observed in GG genotype.

**Recommendations**

1. Build data base for  $\beta$  - thalassemia mutations types in Iraq.
2. Studying of  $\beta$  - thalassemia mutations should be extended to coverage of Iraq population to complete the profile of  $\beta$ -thalassemia mutations.
3. Upgrade the prevention program by the Ministry of Health; include examination of  $\beta$ -thalassemia mutations before married, as well as update tests of detection of mutations.
4. Monitoring program of 25 (OH) D level of  $\beta$ -thalassemic patient and take the medicine management of vit. D. deficiency in thalassemic unit.
5. Recommend to using antioxidant therapy for reduce free radicals side effects, that result in tissue injury and DNA damage.

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يعتبر مرض الثلاسيميا واحد من امراض الهيموغلوبين الشائعة في العراق . السبب الرئيسي للثلاسيميا هو حدوث طفرات في جينات ألفا وبيتا غلوبين المسؤولة عن تشفير الهيموغلوبين، هذا المرض الوراثي ينتج عنه انخفاض في انتاج احد سلاسل الهيموغلوبين. تم أعداد هذه الدراسة لغرض تحديد المفاهيم الجزيئية الوراثية والمصلية والدموية للمرضى المصابين بالثلاسيميا نوع بيتا في محافظة المثنى، تم اخذ (٩٨) مريض مصابا بالثلاسيميا خلال مراجعتهم لمستشفى النسائية والطفل التعليمي في المحافظة و(٣٢) شخص من الاصحاء كمجموعة سيطره ، كان عدد الذكور فيهم ٦١ وعدد الاناث ٣٧ اعمارهم تتراوح بين ١-٢٩ سنة . تم استخدام عينات الدم لقياس مستوى الفيريتين، فيتامين-د و 8-hydroxydeoxyguanosine في المصل باستخدام تقنية الإليزا وتم استخلاص المادة الوراثية لغرض تحديد الطفرات الوراثية في المرضى باستخدام تقنية السكونس. بينت النتائج ان مستوى تركيز الهيموكلوبين لدى المرضى منخفض مقارنة بمجموعة الاصحاء . كان تركيز الفيريتين في المرضى اعلى مما هو عليه في الاصحاء .(P<0.001). اوجدت النتائج ان نسبة تركيز فيتامين D لدى المرضى كان منخفض مقارنة بالاصحاء (P<0.001)، كانت نسبة تركيز 8-OHdG في المرضى اعلى مما تم تسجيله في مجموعه الاصحاء، (P<0.001). استخلص الحامض النووي (DNA) ، واجري التشخيص الجزيئي لجين بيتا كلوبين باستخدام تقنية التفاعل البلمره التسلسلي (PCR) وتم تحديد ٩ انواع من الطفرات الوراثية باستخدام MACROGEN Inc., Korea، سبعة من هذه الطفرات الوراثية لم تسجل في العراق سابقا تضمنت IVS-II-666 C>T, و CD2 CAT>CAC , IVS-II-850 G>A, IVS-II-16 G-C , Hb kingsmill, Hb Saveh , IVS-II-81 C>T، ونوعان منها سجلت مسبقا في العراق تضمنت. IVS-II-1G>A , IVS-1-5 G>C.



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## المظاهر السريرية و التشخيص الجزيئي في مرضى ثلاثيميا بيتا

هذه الرسالة مقدمة جزء من متطلبات نيل درجة الماجستير في  
علوم الحياة / علم الحيوان

من قبل  
ريهام حسين نور الموسوي

إشراف  
أ.م.د. نهاد عيال مطر الراشدي