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List of Abbreviations

NO.	Abbreviation	Meaning
1.	Arms-PCR	Amplification refractory mutation system PCR
2.	bp	Base pair
3.	DGGE	Denaturation gradient gel electrophoresis
4.	DNA	Deoxyribonucleic acid
5.	Hb	Hemoglobin
6.	α-hemoglobin	Hemoglobin type alpha
7.	β-hemoglobin	Hemoglobin type beta
8.	δ -hemoglobin	Hemoglobin type delta
9.	γ-hemoglobin	Hemoglobin type gamma
10.	HbA	Adult hemoglobin
11.	HbA_2	Adult hemoglobin subunit 2
12.	HbF	Fetal hemoglobin
13.	HPLC	High performance liquid chromatography
14.	MCH	Mean corpuscular hemoglobin
15.	MCHC	Mean cell hemoglobin concentration

16.	MCV	Mean corpuscular volume
17.	mRNA	Messenger ribonucleic acid
18.	PCR	Polymerase chain reaction
19.	PCV	Packed cell volume
20.	RBC	Red blood cell
21.	RFLP	Restriction fragment length polymorphism
22.	RNA	Ribonucleic acid
23.	RE	Restriction Enzyme

Abstract

In this work, seventy of β-thalassemia patients were subjected to pedigree analysis, hematological analysis of peripheral blood smears, hemoglobin electrophoresis and molecular analysis of β-globin gene in comparison with normal people.

The conventional hematological procedures were made for patients with β-thalassemia major who were attending Central Public Health Lab/Ministry of Health from different regions of Baghdad, the hematological tests were PCV, MCV, MCH and the results showed that PCV percentages were in the range 18-27% for patients and 36-43% for carriers (parents) while for normal 37-54%, MCH values were 20.7-26 pg for patients and 16.6-19.1 pg for carriers and for normal 27-31 pg, MCV values were 67.2-79.3 fl for patients and 61.5-66.6 fl for carriers in comparison with normal people 80-94 fl. On the other hand, it was found in blood samples which were collected from the patients that there were abnormalities in the morphological appearance of RBCs which showed severe degree of microcytes, hypochromia, anisocytosis and variation in shape which included target cells, tear-drop cells, in comparison for normal people the morphological appearance of RBCs are normochromic and normocytic.

HbA_2, Hb F, HbA were determined by three electrophoresis techniques which were cellulose acetate electrophoresis, agarose gel electrophoresis and hemoglobin testing system (variant), the result showed that most of the patients with β-thalassemia syndrome had low level of HbA due to the absence of either one or

both of β-globin gene, whereas, most of the patients carrier had a high ratio of HbF and HbA_2 comparison with normal people.

The results of pedigree analysis of β-thalassemia for four families showed that the syndrome is transmitted as an autosomal recessive pattern, which suggest that the condition generate from the intermarriage between relatives.

The molecular analysis of genomic DNA for Iraqi families with β-thalassemia by using specific primer for PCR amplification and Restriction analysis with *Bsu*361. The result showed the presence of point mutation (Frameshift 6 mutation) in β-globin gene in β-thalassemia major which cause a complete depression in the expression of β-globin genes.

Fragment sizes obtained from PCR amplification were 616 bp and the presence of fragment (616 bp) from Restriction analysis. The results were good indication for the type of mutation causing β-thalassemia, thus it was possible to conclude that point mutation is causing inactivation of β-globin gene.

Acknowledgment

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Still one word to say, my praise to the merciful God that he heals all patients with this disease, and all other diseases and end their suffering.

1.1 Introduction

β- thalassemia is a hereditary disease which is characterized by the deficiency in the β-globin synthesis, since human have only one set of β-globin gene, therefore, total or partial suppression of the expression of either or both β-globin gene will cause very low of hemoglobin production or none at all (Baysal and Carver, 1995) , this genetic disorder is considered as a common Mediterranean blood disorder that affecting thousands of infants each year, moreover, β - thalassemia occurs among individuals of East Indian, African and Southeast Asian (Lukens, 1999).

β-thalassemia is inherited in an autosomal recessive pattern which means that two copies of the gene must be altered for a person to be affected by the disorder, most parents of a child with homozygous beta thalassemia are not affected, but are carriers of one copy of the abnormal gene, therefore, β-thalassemia is prevalent in small countries where there are close family marriage (consanguinity) (Zlotogora, 2002).

β-thalassemia is classified depending on the clinical severity into three forms:-

Thalassemia minor: - the trait that cause mild microcytic anemia and doesn't need treatment.

Thalassemia Intermedia: - a moderate anemia which depend on the severity of the defect in β-chain production with minimal or no transfusion requirement.

Thalassemia major: - this is the most severe form and also called Cooley's anemia, severe anemia become apparent in the first two years of life when the switch from γ to β-chain should take place (Athanasios *et al*, 2005; Kan-Zhi *et al.,* 2003).

The consequence of the resulting chronic anemia includes growth retardation, extramedullar hematopoietic, splenomegaly, increased intestinal iron absorption, susceptibility to infection, hypercoagulability and bone marrow expansion which lead to the reduction of the bone mass and increase the incidence of fracture, therefore, children with β-thalassemia major will need regular blood transfusion (Anapliotou *et al*., 1995; Saka *et al*., 1995).

The consequence of chronic transfusion therapy is iron overload which adversely affect the function of the heart, liver, and other organs, therefore, iron removal with a chelating agent is required for survival (Angastiniotis and Modell, 1998).

Considering the health problems facing β-thalassemia major patients efforts have been made to control the birth of thalassemic children, this have been carried out by chorionic villi sampling (CVS) in the first trimester, and by amniocentesis or cordocentesis in the second trimester (Li *et al.,* 2005).

In this regard, the diagnosis of β-thalassemia major is important and the traditional diagnosis dependent on the measurement of the red blood cell which reveals severe hypochromic, microcytic anemia with raised in reticulocyte percentage with normobalsts, target cells and basophilic stippling in blood film (Suzan *et.al.*, 2002), other diagnostic methods have been developed; hemoglobin electrophoresis that reveals the absence of hemoglobin A with almost all circulating hemoglobin being HbF (Asadov *et al.*, 2004).

β-thalassemia major is caused by any one of more than 1000 point mutations and rarely by deletion; β-thalassemia is clinically heterozygous because of the various lesions which impair the synthesis of the β-globin chains

(Piyamongkol *et al.*, 2003), so, DNA analysis can be used to identify the defect on each allele, PCR is the most commonly used technique and may be performed by using primer pairs that only amplify in individual allele (allele specific priming) or by using consensus primers that amplify all the allele followed by restriction digestion to detect particular allele (Colosimo *et al.*, 2002).

Aims of the study:-

1- To study the molecular and genetic basis of β-thalassemia patients in Iraqi families.

2- To study the inheritance of β-thalassemia by pedigree analysis of patients and their families which is based on carrier detection and the available phenotypic and genotypic data.

3- To carry out molecular PCR analysis for detection of the mutation and to identify the molecular changes at DNA level by using specific primers for PCR amplification.

4- To start establishing gene bank for thalassemia genes for further work e.g. DNA sequencing and molecular characterization of mutations.

1.2 Hemoglobin structure

Hemoglobin is a globular protein of MW 68000 Dalton composed of four peptide chains called globins each of them is covalently linked at a specific sites of haem group consisting of iron atom which surrounded by porphyrin ring (Gwendolyn and Trefor, 2000).

The type of hemoglobin is determined by the amino acid sequence in polypeptide chains, normal human hemoglobin is composed of pair of two identical chains (two alpha & two beta), the alpha globin chain is composed of 141 amino acids (Perutz, 1978), whereas, β-globin chain is composed of 146 amino acid, both alpha and beta globin proteins are similar in secondary and tertiary structure each with 8 helical segments (Weiss *et al*., 2005).

The role of hemoglobin is to transport O_2 from lung to tissue and then return from tissue to the lung which is regulated by several factors one of them is O_2 affinity to hemoglobin in the presence of O_2 in the lung where O_2 level are high, the hemoglobin has higher affinity for O_2 and this affinity increase disproportionately with number of molecules its already has bound to it (Sears, 1999).

The O_2 binds to the iron atom tightly and if two heme molecules come together in the presence of O_2, the iron atom will oxidize and bind irreversibly with O_2, this irreversible binding would not be in use in hemoglobin molecule because O_2 need to be released in the tissue, the globin chain prevents this irreversible binding by folding the protein around heme molecule and this create a pocket to isolate the heme molecule from other heme molecule (Rachel and Regina, 2000).

1.3 Hemoglobin Types

Hemoglobin differ in their solubility, and resistance to denaturation by alkalis, these features together with electrophoretic mobility (chromatography) are used for their identification.

There are 3 types of embryonic hemoglobin:-

1- Gower 1 which is made up of 2ζ and 2ϵ.

2- Gower 2 which has 2α and 2ϵ chain.

3- Portland hemoglobin which has 2ζ and 2γ.

These hemoglobins appear in gestation and they are soon replaced by hemoglobin F which consist of 2α and 2γ chain which comprises less than 2% of hemoglobin (Harry, 1996).

After the birth, this fetal type is then replaced by adult hemoglobin (HbA) which is the major component of hemoglobin and it consists of 2α and 2β which comprises 95% of hemoglobin. Hemoglobin A_2 which is the minor component of hemoglobin and it consists of 2α and 2δ chains which comprise 2-3.5% of hemoglobin as shown in table (1-1) (Sanguansermsri *et al.*, 2001).

Hemoglobin S which is abnormal hemoglobin type which cause sickle cell anemia in African and Black America.

Hemoglobin C which is another type of hemoglobin which is also found in African and America (Janis, 2002).

Table (1-1). Summarizes various types of human hemoglobin.

Hemoglobin	structure	% of normal adult hemolysate	Increased in
Gower 1	zeta-2 epsilon-2 ($\zeta_2\varepsilon_2$)	0%	Embryo
Gower 2	alpha-2: epsilon-2 ($\alpha_2\varepsilon_2$)	0%	Embryo
Portland	zeta-2, gamma-2 ($\zeta_2\gamma_2$)	0%	Embryo
A	alpha-2, beta-2 ($\alpha_2\beta_2$)	95%	adult
A2	alpha-2, delta-2 ($\alpha_2\delta_2$)	2-3.5%	Beta thalassemia
F	alpha-2, gamma-2 ($\alpha_2\gamma_2$)	<2%	Fetal embryo

1.3.1 Cellular Heterogeneity and Fetal Hemoglobin Production

Although fetal hemoglobin synthesis persists after birth to some degree, its production is insufficient to compensate for the reduced synthesis of β-globin chains and the relative excess of α-globin chains (Arthur, 2006).

The elevated concentrations of fetal hemoglobin had been observed in patients with β-thalassemia which reflect a combination of the selection of precursors that produce relatively more fetal hemoglobin and erythroid expansion, which appears to favor the production of γ-globin chains(Atweh and Loukopoulos, 2001).

Even higher fetal hemoglobin concentrations are associated with specific β-thalassemia alleles or other genetic determinants within or linked to the β-globin complex (Thein *et al.*, 1994).

1.4 Beta globin gene locus

The five transcriptionally active β-like globin are located on chromosome 11 in the following order: 5' ϵ- Gγ -Aγ -ψß–δ -β -3', these genes in the β-globin locus are arranged sequentially 5' to 3' beginning with the gene expressed in embryonic development and ending with the adult beta globin gene as shown in the figure (1-1). The expression of the human ß-globin genes is restricted to erythroid cells (Kelly *et al*., 2003).

There are two copies of the gamma gene which is Gγ and Aγ which differ by glycine or alanine at position 136 in the polypeptide chain but the others are present in single copies, additionally; the pseudogene ψβ is located between the Aγ and δ genes (Gibson, 1994).

Human β-globin contain promoter which is found in the 5' of the gene which is either close to the initiation site or more distally, they are the site where RNA polymerase bind and catalyze gene transcription like TATA which is an initiator and required for high level of RNA polymerase II transcription in vitro(Roy *et al*., 1991; Bungert *et al.*, 1992).

Enhancers are also occure either 5'or 3' to the gene and are important in the regulation of globin gene expression and in the regulation of various globin chains during fetal and adult life in spite of being located some distance away from the gene itself (Baron, 1997).

The locus control regulatory (LCR) is a genetic regulatory element that located long away upstream of the β-globin cluster which control the genetic activity of each domain probably by physically interacting with the promoter region and opening up the chromatin to allow transcription factors to bind (Reik *et al.*,1998).

Figure (1-1). Schematic representation of the human ß-globin gene clusters (Kelly *et al*., 2003).

1.4.1 Imbalanced globin chain synthesis

The basic defect in all types of thalassemia is imbalanced globin chain synthesis; however, the consequences of accumulation of the excessive globin chains in the various types of thalassemia are different (Grosveld *et al*., 1993).

In β- thalassemia, the excessive α- chain which are unable to form Hb tetramers precipitate in the RBC precursors are insoluble, precipitate in the cell and interact with the membrane which causes significant damage and lead to excessive destruction of the RBC precursors, whereas, the surviving cells that arrive in the peripheral blood with intracellular inclusion bodies are exposed to hemolysis which lead to both hemolysis and ineffective erythropoiesis which cause anemia in the person with β- thalassemia (Perrimond, 2004).

The ability of some RBCs to maintain the production of γ- chains, which are capable of pairing with some of the excessive α- chains to produce Hb F, is advantageous to reduce the symptoms of the disease, furthermore, increased in the production of Hb F in response to severe anemia may adds another mechanism to protect the RBCs in persons with β- thalassemia.

The elevated Hb F level increases oxygen affinity which leads to hypoxia with the profound anemia which stimulates the production of erythropoietin (Weatherall *et al.*, 1995).

1.5 Thalassemia

The thalassemia syndrome are a heterogeneous group of inherited conditions characterized by defects in the production of hemoglobin and cause different forms of anemia, the severity and type of anemia depends upon the number of genes that are affected (Ta- Chin *et al.*, 1997) and as a consequence the two different kinds of protein chains (alpha and beta chains); therefore, any deficiency

in these chains causes abnormalities in the formation, size, and shape of red blood cells (Pat *et al*., 2003).

Thalassemia is classified according to the globin that is affected. The most common types of thalassemia are beta-thalassemia and alpha-thalassemia (Cohen *et al.*, 2004).

Thalassemia arises from mutations in one or more globin genes which lead to reduction or absence of the associated globin, the severity of symptoms are directly related to whether one or both genes in a pair are mutated, however, symptoms may be modified by other genetic or environmental factors (Yu *et al*., 1998).

1.6 Definition of ß-thalassemia major

β-thalassemia major is an inherited blood disorder which results from defect in the hemoglobin production, which are caused by mutations in both of beta globin chains and that lead to complete failure of beta globin production (Najmabadi *et al*, 2001).

The continued synthesis of α-chains production which bind to the red blood cell membranes and form toxic aggregates producing membrane damage and cause the excessive destruction of red blood cell and that lead to severe anemia that will appear rapidly (Schrier, 1997).

1.6.1 History of β-thalassemia major

In the past, before the discovery of the β-thalassemia worldwide distribution, it was known as a Mediterranean Anemia, It comes from the Greek word “thalasanemia” which means “anemia by the sea".

Over the centuries it was not possible to explain the death of children as a result of β-thalassemia until 1925 when the clinical picture was described by Thomas Cooley, who was able to observe severe type of anemia in children of Italian origin, he observed a nucleated RBC in the peripheral blood (Olivieri, 1999), he thought it was erythroblastic anemia, before long Cooley was realized that erythroblastemia was neither specific nor essential in this disorder, although Cooley anemia was aware of the genetic nature of the disorder but he failed to investigate the apparently healthy parents of the affected children.

On the other hand, Riette described Italian children with unexplained mild hypochromia and microcytic anemia (Hassan *et al*., 2005).

In the same year (1925) Cooley reported the severe form of anemia, later wintrobe and coworkers in the united state had reported the mild anemia in both parents of a child with Cooley anemia this anemia was similar to that describe in Italy, only then Cooley anemia was recognized as the homozygous form of the mild anemia, this severe form was then named as thalassemia major and the mild form as thalassemia minor (Linda *et al*., 2003).

1.6.2 Distribution of β-thalassemia major

β-thalassemia is considered as the most common inherited genetic disorder world wide with about 5% of the world population affected with it (Shah, 2004).

Over 300,000 infants with beta thalassemia major syndrome are born every year and the majority die undiagnosed, untreated or under treated (Elliottp, 2005).

β-thalassemia major occure in Mediterranean Sea Arab countries and other countries like Greece, Italy, turkey and North Africa. This syndrome also has been observed in Saudi Arabia, Iraq, Iran, Afghanistan and south East Asian countries like Thailand and Indonesia, but the prevalence of beta thalassemia is highest in Italy, Greece and Cyprus as shown in the figure (1-2) (Lorey *et al*., 1996).

β-thalassemia syndrome

Figure (1-2). Distribution of β-thalassemia in world (Lorey *et al*., 1996).

β –thalassemia major is also common in populations of African heritage but the highest incidences are reported in Cyprus (14%) and Sardinia (12%) and south East Asia.

The high gene frequency of β-thalassemia in these regions is most likely due to selective pressure of malaria but a lower prevalence has been reported in northern Europe (0.1%) (Flint *et al*., 1998).

The gene frequency of β-thalassemia in Iran shows a great variation from one area to another, the provinces that around the Persian gulf and the Caspian sea show gene frequency which is more than 10% while Fars province in the south of

Iran shows gene frequency between 8%-10%, whereas, the prevalence of the disease varies between 4%-8% in other parts of the country (Mahboudi *et al.,* 1996).

In India about 10,000 beta thalassemia major infants are born every year, the prevalence of beta thalassemia is very high among certain communities like Punjabi, Sindhi, Gujarati, Bengali, Parsee, lohana and certain tribes in northern, western and eastern parts while it's much less in the south of Indian (Panigrahi and Marwaha, 2006).

There are probably only 800 to 1000 patients with homozygous β-thalassemia and most of these patients live in the North America between Boston and New York, only about 15 to 20 new cases are diagnosed each year (Pearson *et al*, 1996).

1.7 Molecular basis of β-thalassemia major

The complete failure of β-chains production which characterize β-thalassemia major are caused by deletion of β-globin gene and non-deletion mutations that may arise from defects affecting transcription, RNA processing or RNA translation (Park and Cho, 2002).

The application of DNA recombinant technology had shown to molecular biologist a great insight into the molecular mechanisms to examine these mutations, to determine how these mutations influence the expression of the gene and help to develop an efficient strategy for cloning different thalassemic genes (Kim and Kay, 1984).

1.8 Types of Mutations

1.8.1 Non-deletion mutation

These defects are results from single base substitution or insertion within or immediately upstream of the β-globin gene, these mutations affect gene function by interfering with transcription of the gene into RNA, RNA processing or RNA translation into β-globin gene (Krawczak *et al.,* 1992).

1.8.1.1 Mutations that affect RNA translation

Several β-thalassemia mutations give rise to non functional RNA and that cause beta thalassemia major, these mutations are caused by either nonsense or frame shift mutation, the nonsense mutations arise from base substitution that change the amino acid codon to termination codon and that directly affect on translation (Susan *et al.*, 2001), an example is the substitution of C by T in codon 39 introducing the termination codon TAG in β-globin mRNA; this mutation is common in Sardinia which accounting for 90% of the cases there, also the codon 17 mutation is common in Chinese, also a G-to-T substitution at the first position of codon 43, which changed the glutamic acid coding triplet (GAG) to a terminator codon (TAG) (Fattoum *et al*., 2004).

These data indicated that several β-thalassemia major are result of nonsense mutation (Baserga and Benz, 1988)

The mRNA sequence is deciphered in triplets of bases (Codons) which code for individual amino acid, frame shift mutations arise from either insertion or deletion of one nucleotide or few nucleotides in the coding region which disrupts the normal reading frame and mRNA translation and cause termination further

downstream, an example is the insertion of one nucleotide between codon 8 and 9and the deletion of four nucleotide in the sequence between codon 41and 42, this cause an altered reading frame of the mRNA with an in phase termination codon (UAG), hence, nonfunctional β-globin is produced; this frame shift mutations are common in India (Laosombat *et al*., 2001).

1.8.1.2 RNA processing mutants

Mutations that cause anomalies of RNA processing represent about one third of the known beta thalassemia allele, these mutations are directly affect on the splice junction which is essential to remove the interventing sequence (IVS) and splicing of exon to produce functional mRNA (Stoilov *et al*., 2002).

It is estimated that up to 15% of all point mutations causing human genetic disease result in an mRNA splicing defect (splice).

This type of mutation is frequent among patients in southern China and Thailand which causes aberrant RNA splicing (Lewis *et al*., 1998).

These mutations alter two nucleotide at the start or end of the intron (5'-GT/AG-3') causing total absence of normal splicing and absence of β-globin gene production, as a result the individual will have β-thalassemia major (Baird *et al*., 1981).

An example is the substitution of thymine (T) with cytosine (C) at nucleotide position 654 in the second intron of the beta globin gene, this mutation is found in Chinese population, a mutation that produces a new acceptor site at position 116 in IVS – 1 results in little or no β - globin mRNA production and the phenotype of β – thalassemia major (Deidda *et al*., 1990).

1.8.1.3 Initiation codon mutation

The initation codon mutation in the β-globin gene cause beta thalassemia major, this mutation is caused when alternative triplets for ATG is non functional as initiation codon for β-globin gene, therefore, in this case the translation from the mutated allele is also impaired (Daniela *et al.,* 1998), in this regard the ATG⟶ATA mutation which was found in 7 members of 3 generation of a family living in northern Sweden, this mutation changes the initiation codon which is methionine into isoleucine and this will cause beta thalassemia major (Landin and Rudlophi, 1995).

Another initiator codon mutant (ATG to ACG) was found in Yugoslavians and the same mutation was found in a father and daughter of a family originating from Bern, Switzerland. Unlike the first reported family, of Yugoslavian origin, the Swiss patients had high Hb F levels (Beris *et al*., 1993).

1.8.2 Gene deletion

β-thalassemia major is rarely due to deletion mutations that affect the β-globin gene, the lower frequency of deletion affecting β-globin gene may be related to the less extensive sequence homology within β-globin gene (Weatherall, 1994).

The first example of deletion mutation was 619bp deletion at the 3'end of the β-globin gene found in Indian; this deletion removes 600bp involving the 3'end of β-globin gene but leaves the 5'end intact as shown in figure(1-3), whereas the other deletion mutation removes the 5'end of β-globin gene (Wen Wang *et al*., 2003).

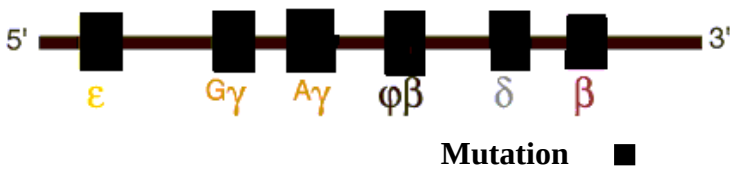

Figure (1-3) Deletion mutation of β-thalassemia in Indian (Wen Wang *et al.*, 2003).

Another β-thalassemia major deletion mutation was 105bp deletion that extends from -24 or -25 to +80 or +81 relatives β-globin gene in mRNA cap site; this deletion was due to partial deletion of the 5'end of β-globin gene that include the mRNA cap site and at of exon 1, this mutation was detected in three individual from a family originating in the area of the southern Thailand (Nopparatana *et al.*, 1999).

1.9 Inheritance of beta thalassemia major

Most individual have two normal copies of beta globin gene and the two normal copies make the normal adult hemoglobin (Bittles, 2001).

All types of thalassemia are inherited which mean that the genetic change must be inherited from both mother and father, when individual has one normal beta globin and one thalassemia gene, this state is called thalassemia trait (Alan, 2001).

If both of the parents are carrying thalassemia trait ,therefore, there is 25% chance (1/4) that any child they have will inherit thalassemia gene from each parent and have severe form of the disease ,there is 50% chance (2/4) that the child will inherit one of each kind of gene and have a trait like their parents and there is 25% (1/4) that the child will inherit normal gene from each parent and appear as a normal person as shown in the figure(1-4) (Bittles, 2005).

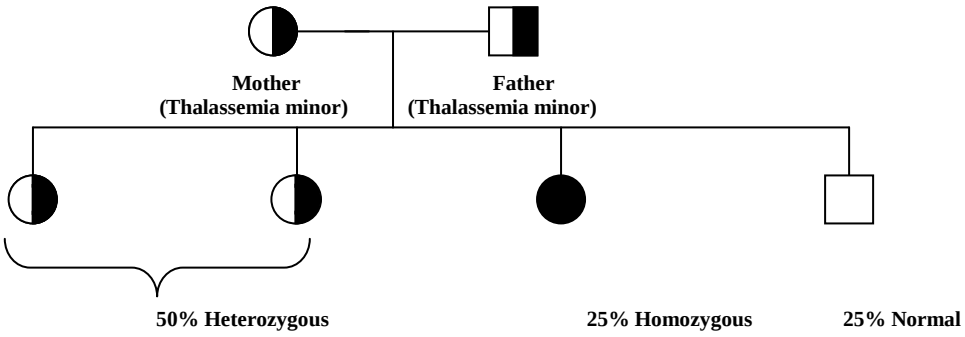

Figure (1-4) Inheritance of β-thalassemia major as an autosomal recessive trait (Bittles, 2005).

1.9.1 Genotype-Phenotype Correlations

Several genetic factors may ameliorate the severity of β-thalassemia and lead to a mild β-thalassemia phenotype (Galanello and Cao, 1998).

First, the mutations which vary in their effect on the synthesis of β-globin chains for example the compound heterozygosity of silent β^{+} - thalassemia which is produced by severe mutations give variable phenotypes that range from thalassemia intermedia to thalassemia major, therefore ,the presence of this genotype does not predict necessarily a mild phenotype (Huisman *et al*., 1997).

The clinical phenotype of homozygous β-thalassemia may be modified by the coinheritance of other genetic factors which are mapping outside the β-globin gene cluster, the best known of these modifying genes is the mutation causing Gilbert disease which when combined with thalassemia major or thalassemia intermedia lead to increased jaundice and increased risk of gallstones (Galanello *et al*., 2001).

Heterozygous β-thalassemia may lead in some instances to the phenotype of thalassemia intermedia instead of the asymptomatic carrier state as a result of mutations in HBB which lead to hyper-unstable hemoglobins that precipitate in the red cell membrane together with unassembled hemoglobin α-chains and cause ineffective erythropoiesis(Al- Qaddoumi, 2006).

1.10 Clinical features of β-thalassemia major

The clinical picture in most cases is severe, at birth the patient usually appear healthy but severe anemia usually appear during the first two years of life after birth; the patient become pale, fussy, listless, have low appetite, fatigue, they grow slowly and often which yellowish discoloration of skin (jaundice) is appear (Mohamed and Jackson, 1998).

As a result of un adequate blood transfusion, various symptoms appear e.g. enlargement of liver and spleen which are some times painful, thus, expansion of bones are caused by intense marrow hyperplasia which lead to thalassaemic facies and to thinning of the cortex of many bones with tendency to fractures and bossing of the skull, these signs are caused from increase of red blood cell destruction, therefore, most of the untransfused children die in first decade of life (Thein, 2005).

Other clinical features of β- thalassemia major which related to frequent blood transfusion begin to appear after the age 10 or 11 and including endocrine, cardiac and hepatic disturbance(Cappellini *et al*., 2000), the failure of growth, hypothyroidism, hypoparathyroidism and diabete mellitus has been mainly attributed to iron overload in the tissues (Origa *et al*., 2005).

Hepatic complications in thalassemia is also observed which affect a high proportion of patients with β-thalassemia major (De Sanctis *et al*., 2004).

The main causes that are contributing to liver injury in thalassemics patients are hepatitis C and hepatic siderosis, thus, hepatocellular carcinoma (HCC) complicates liver cirrhosis because of iron overload and viral infection therefore, patients affected by thalassemia syndromes have high risk of developing liver cancer (Angelucci *et al*., 2000).

1.11 Treatment of β-thalassemia major

Today, in the developed world the life expectancy for β-thalassemia major patients which varies between 25-55 years is dependent mainly on the medical treatment, the medical treatment involves regular and adequate red cell transfusion to counteract the anemia, iron chelation therapy with desferrioxamine and desferiprone (L1), the judicious use of splenectomy, bone marrow transplantation (Borgna *et al.,* 2004).

1.11.1 Transfusion

The most common treatment for patients who have beta thalassemia major is regular blood transfusion to maintain hemoglobin at normal or near normal level, the aim of transfusion is to suppress endogenous erythropoiesis to minimize complication such as hepatosplenomegaly, bone changes, and inhibit increased gastrointestinal absorption of iron (Ballas *et al*., 1997; Cunningham *et al*., 2004).

Hypertransfusion and supertransfusion has been used to maintain hemoglobin level above 10g/dl and the hematocrit value above 30%, as a result of treatment with repeated transfusion there is always risk of transmitting viral infection like

hepatitis B, C and HIV, so they should be carefully monitored periodically (Old *et al.,* 2001).

1.11.2 Iron chelation therapy

Iron chelation therapy by desferrioxamine is a specific treatment that help the body to prevent iron mediated injury to cells, also remove excessive iron that accumulate as a result of repeated blood transfusion which lead to heart failure and other organ damage, allow for normal growth, sexual development and increase life span for children who have β-thalassemia major (Dodd, 1992).

1.11.3 Splenectomy

Is a surgical procedure where the spleen is removed, It is beneficial for children with β-thalassemia major to reduce their blood transfusion requirement (Olivieri and Brittenham, 1997).

1.11.4 Bone marrow transplantation

A medical procedure used to treat some diseases that arise from defect in the production of red blood cell in the bone marrow, It is considered as a curative therapy for patient with beta thalassemia if there is a well HLA- matched donor for successful transplantation to cure the disease (Leandros *et al.,* 2006).

1.11.5 Gene therapy

Is a technique for correcting defective gene which is responsible for the development of the disease, the principle of gene therapy is to cure genetic disease by repairing the damaged gene which cause the disease, (Lucarelli *et al*., 1999), in early 1970s, the scientists proposed "gene surgery" for treating the inherited

diseases which caused by faulty genes, the idea was to take out the disease-causing gene and surgically implant a gene that functioned properly (Christof *et al*., 2004).

Researcher may use one of several approaches for correcting the defective gene:-

1- Normal gene is inserted into non specific location within the genome to replace non functional gene, this approach is the most common.

2- Abnormal gene could be swapped with normal gene through homologous recombination

3- Abnormal gene could be repaired by reverse mutation which returns the gene to its normal function (Geetha *et al.,* 2004; Plavec *et al.*, 1993).

In most gene therapy studies, carrier molecule called vector is used to deliver the therapeutic gene to the patient target cell, currently, the most common vector which has been used is virus which is genetically altered to carry normal human DNA (Abonour *et al*., 2000), but there is a problem which include the identification of all sequences which are required for the development of more effective and safe vectors for the transfer of gene (Malik and Arumugam, 2005).

There are two ways to deliver the therapeutic gene:-

1- *in vitro*:-

Cell such as (white blood cell from bone marrow) are removed from the body, then, the gene is incorporated into the cell DNA, and then the cell is injected back into the body.

2- *in vivo*:-

Gene containing vectors are directly injected into the body tissue (Higgs *et.al*., 1998).

The use of gene therapy is considered as curative treatment for beta thalassemia major this treatment is being developed under trade name called "thalagen"(Derek *et al.,* 2001).

1.11.6 Augmentation of Fetal-Hemoglobin Synthesis

Several trials have attempted to increase the synthesis of fetal hemoglobin to ameliorate the severity of β-thalassemia (Persons and Nienhuis, 2000).

Administration of intravenous 5-azacytidine was associated with increases in the hemoglobin concentration in a few patients ,however, the potential toxicity of the drug later shifted to less toxic alternatives which is the therapy with hydroxyurea and butyric acid compounds, these agents in combination has reduced or eliminated transfusion requirements in some patients(Olivieri, 1996).

However, other studies have reported only small increases in fetal and total hemoglobin concentrations during the administration of hydroxyurea and both intravenous and oral butyrate compounds (Arruda *et al*., 1997).

Studies in humans and animal models of β-thalassemia have suggested that there is increase in the production of γ-globin chains which influenced by the degree of erythroid marrow expansion and by sequential administration of specific combinations of agents (Collins *et al.,* 1995).

Furthermore, clinical responses had been observed in patients with mutations that delete specific sequences within the β-globin gene cluster that may have a key role in the silencing of adjacent genes which may influence the inducibility of γ-globin gene (Constantoulakis *et al.,* 1991).

1.12 Prenatal diagnosis

It's important to prevent beta thalassemia major through giving genetic counseling to married couples who are at risk of having child with homozygous beta thalassemia, when the mother is found to have hemoglobin abnormality in this case the father should be tested to determine whether he also carry hemoglobin abnormality (Kitsberg *et al*., 1993), when both of the parents show to have hemoglobin abnormality and there is a risk of serious defect in the offspring especially β-thalassemia major, in this case it is important to offer prenatal diagnosis, this method already in use in several countries (Basran *et al.,* 2005).

The prenatal diagnosis in the town of Baku is carried out during 23 week of pregnancy by means of cordocentesis and biochemical analysis of beta globin gene to determine the products of mutant gene loci, if beta thalassemia major is detected in fetus, abortion is induced on pregnancy week 25 (Mazurova *et al*., 1991).

On the other hand, prenatal diagnosis is recommended also in the case of another pregnancy, fetal blood sampling is collected by chorionic villi sample (CVS) in the first trimester and by amniocentesis or cordocentesis in the second trimester depending on the stage of pregnancy (Ayesh *et al.*, 2005).

The discovery of the presence of fetal DNA in maternal plasma is providing anew approach for prenatal diagnosis which is used to detect codon mutation in the beta globin gene (Chiu *et al*., 2002).

Prenatal diagnosis have resulted in marked reduction in the rate of birth of the affected children in Cyprus, Greece and Sardinia, therefore, there are now fewer than 600 severely affected patients with thalassemia in Cyprus and only 2-3 new cases arising each year and a similar highly effective control program was also

established by pioneering work in Sardinia (Cao *et al.*, 2002), the prenatal diagnosis technique is safe and reliable but the major disadvantage is the long period of uncertainty for the mother, therefore, the scientist need to develop quick, sensitive and accurate method (Cao *et al*., 1998).

A new approach was developed which called Preimplantation Genetic Diagnosis (PGD) which is an alternative approach to prenatal diagnosis and this approach give the couples the chance to have pregnancy without affected fetus and this approach is used to detect beta thalassemia in Thailand and south East Asia (Piyamongkol *et al*., 2006).

1.13 Polymerase chain reaction (PCR) and its application

1.13.1 Polymerase chain reaction (PCR)

PCR is a molecular biology technique for replicating DNA without using a living organism, this technique allows a small amount of DNA molecule to be amplified many times, PCR is used commonly in medical and biological research for a variety of tasks such as the detection of hereditary disease, the diagnosis of infectious disease, the cloning of genes and some of the specific advantages of PCR less contamination, minimal errors and accurate (Jinping *et al.*, 2004; Vrettou *et al*., 2003).

PCR was first discovered by kary mullis in 1983, PCR has enabled a rapid development in various field of biotechnology research (Mullis, 1990).

The PCR process is usually consist of a series of 20 to 35 cycles, each cycle consists of 3 steps:-

1- The double stranded DNA is heated at 94-96 ˚C to separate the strands and to break the hydrogen bond which connect the DNA stands but before the first

cycle, this step is called denaturing, the DNA is often denatured for an extended time to ensure that the template DNA and the primers is completely separated and become now single strand only, the required time is 1-5 minutes, also taq polymerase is activated by this step.

2- After separating the strands, the temperature is lowered so the primers can attach themselves to the single DNA strands, this step is called annealing, the temperature of this stage is dependent on the primers, therefore, wrong temperature during the annealing step can result in primers that are not binding to the template DNA at all or binding at random. The time required for this step is 1-2 minutes.

3- Finally, the DNA polymerase copying the of DNA strands which starts at the annealed primers and works along the DNA strands, this step is called extension, the extension temperature is dependent on the DNA polymerase, the time of this step is also dependent on the DNA polymerase itself and the length of DNA fragment to be amplified, the extension step is frequently used after the last cycle to ensure that the single stranded DNA which remain is completely copied, the time required is 10-15 minutes (Germer and Higuchi, 2003).

1.13.2 The application of PCR-based method

1.13.2.1 Amplification refactory mutation system (ARMS-PCR)

ARMS is a PCR-based method which uses allele specific priming, in this method an oligonucleotide primers with triple end that complement to the sequence of specific mutation which coupled with a common primer that used in one PCR reaction, in parallel, normal primer is coupled with a common primer

which used in another PCR reaction, therefore, the presence of an amplified product in the first reaction is an indication to the presence of the mutation while its absence refer to the presence of normal DNA sequence at that specific site (Old *et al*., 1990; Omar *et al*., 2005).

1.13.2.2 Restriction enzyme of amplification fragment (RFLP-PCR)

RFLP is used to diagnose many genetic disorder, single base changes are scattered along each gene cluster which vary from one individual to another these changes give rise to the site that are recognized by restriction enzyme or removes sites that are previously identified, therefore, the size of DNA fragment that produced by the restriction enzyme varies, the RFLPs which caused by these sites are inherited in mandelian manner, they are sufficiently close to the gene as the linkage markers to recognize the chromosome that carry the thalassemia or other mutations (Thakur *et al*., 2000; Kanavakis *et al*., 1997).

1.13.2.3 Denaturing gradient gel electrophoresis (DGGE-PCR)

DGGE is used to scan β-thalassemia mutation in the β-globin gene; DGGE is potentially detecting all base changes whether they are polymorphisms of disease associated mutation, when a DNA fragment is subjected to an increasingly denaturing physical environment so it partially melts when the denaturing conditions become more extreme the partially melted fragment are completely dissociates into single strands, then the discrete portions of the fragment suddenly become single-stranded.

The rate of mobility of DNA fragments in acrylamide gels changes as a consequence of the physical shape of the fragment (Losekoot *et al.,* 1990).

1.13.2.4 Gap-PCR

Gap-PCR is extremely useful in the identification of common thalassemia mutation; the primers for these deletions can be multiplexed in various combinations to capture the deletions that are most likely to be encountered in the different catchment areas, PCR primers are designed complimentary to the breakpoint sequences and amplify a deletion specific fragment which spans the deletion (Amy *et al.,* 2006).

1.14 Other forms of β-thalassemia

β-thalassemia is characterized by deficiency in β-globin chain synthesis, leading to total or partial suppression of the expression of either or both β-genes will result in low hemoglobin production.

The underlying mechanisms causing β- thalassemia are completely different from those causing α-thalassemia, α-thalassemia are the result of gene deletion , whereas, β –thalassemia are caused by deficiencies in the regulation of the β-genes (Übrahim *et al*., 2004).

1.14.1 β-thalassemia Intermedia

This condition is due to partial suppression of the β-globin gene, the most common symptoms is fatigue or shortness of breath, some children also experience heart palpitations due to the anemia, and mild jaundice, which is caused by the destruction of abnormal red blood cells that result from the disease. The liver and spleen may be enlarged (Athanassios *et al*., 2006).

Thalassemia intermedia is more severe than the usual asymptomatic thalassemia trait but milder than transfusion dependent thalassemia major, patients present with anemia later than is usual in the transfusion dependent forms of homozygous β- thalassemia and they are just able to maintain a hemoglobin level of about 6 g/dl without transfusion (Weatherall and Clegg, 2001).

1.14.2 β-thalassemia minor

β -thalassemia minor is the heterozygous state of beta thalassemia in which the individual has at least one normal functioning β-globin gene, for this reason sufficient quantities of hemoglobin A are produced and the level of hemoglobin A_2 in beta thalassemia minor is elevated from 2% to 5% or 6%, β-thalassemia minor often goes undiagnosed because kids with the condition have no real symptoms other than mild anemia. It is often suspected based on routine blood tests such as a complete blood count (CBC) and can be confirmed with a hemoglobin electrophoresis. No treatment is usually needed (Nevruz *et al*., 2006).

1.14.3 γδβ-thalassemia

The γδβ-thalasemia are group of disorders which are caused by deletion that affect the β- globin gene cluster. Over the first few months of life the clinical syndrome become progressively less severe and it will resemble heterozygous β-thalassemia (Mary *et al.,* 2003).

1.14.4 δβ-thalassemia

This is uncommon disorder which is characterized by the deletion of δ-gene and β-gene.

Homozygous δβ- thalassemia is caused thalassemia Intermedia or major which characterize by the absence of HbA_2 and the presence of 100% HbF, whereas, heterozygous δβ-thalassemia are clinically indistinguishable from heterozygous β-thalassemia. Heterozygous δβ-thalassemia is suspected when HbF is elevated but HbA_2 is normal (Fucharoen *et al*., 2000).

1.14.5 Hereditary persistence of fetal hemoglobin (HPFH)

The term hereditary persistence of fetal hemoglobin include agroup of disorders which are characterize by reduction in the expression of δ and β- globin gene, however, in HPFH the expression of γ-gene is greater than in it is in δβ-thalassemia(Qiliang *et al.,* 2001).

1.15 α-thalassemia

α- thalassemia is caused by decrease in the production of α-globin gene, the genetic basis of this disorder is a result of deletion of one or more of the four α-globin genes that are located on chromosome six.

α- thalassemia can be generally categorized as:- silent carrier, α-thalassemia trait, hemoglobin H disease, hydrops fetalis (Stanley, 2002).

1.15.1 Silent carrier

The silent carrier is characterized by deletion of one functional gene, whereas, the three remaining α- globin genes can direct the production of α-globin gene to produce normal quantities of HbF and HbA (Harteveld *et al*., 2004).

1.15.2 α-thalassemia trait

α- thalassemia trait is characterized by two functional genes that code for production of α- globin gene, the two genes can occure either on the same or on homologous chromosome and are usually not associated with anemia.

This condition is common in southest Asia, West Africa and the Mediterranean area (Stanley, 2002).

1.15.3 Hemoglobin H disease

Hemoglobin H disease is characterized by deletion of three α-globin genes which lead to under production of α-globin chains and over production of β-globin chain which lead to moderately severe microcytic, hypochromic anemia with splenomegaly(Chen *et al.,* 2000).

1.15.4 Hydrops fetalis

This condition is characterized by deletion of all α- globin genes which lead to complete failure of HbA and HbF synthesis and cause death either in uterus or very soon after birth, this phenomenon is almost found in southest asia (Chui and Waye, 1998).

2.1 Equipments

Various equipments were used in the work as shown in the table

Apparatus	Company /Country
Agarose gel electrophoresis system	Sebia / France
Autoclave	Tomy/Japan
Autoanalyzer	Gergy-pontoise / France
Cellulose acetate electrophoresis equipment	Shandon/ Germany
Electrophoresis system	Amersham / Germany
Incubator	Thermo/ Usa
Laminar flow hood	Total star/ Spain
Light microscope with camera	Carl ziess/ Germany
Magnetic stirrer Heater	Corning /USA
Micro-fuge centrifuge for capillary tubes	Beckman coulter/ England
Micro-centrifuge	Mse / England
Oven	Memmert / Germany
pH meter	Jenway / United kingdom
PCR master gradient cycler	PeQlab/ USA

Sensitive balance	Sartorius / England
Shaker	TKA / Italy
Spectrophotometer	Cecil/ England
Universal centrifuge	Universal 16A /Germany
Uv transilluminator	UVP/USA
Variant	Bio rad /USA
Vortex mixer	Steuart/ England
Water bath	Memmert / Germany

2.2 Chemicals

The following chemicals were used

Chemical	Company /Country
Boric acid	AB analitica /Italy
Bromocresol blue	
Ethylendiaminetetra acetic acid (EDTA)	
Ethidium bromide staining solution	
Glycerol	
Lysing solution	

Tris-base	AB analitica /Italy
Washing solution 1	
Washing solution 2	
Ammonium oxalate	BDH/ England
Leishman powder	
Hemoglobin A_2/F calibrator diluent set	Biorad/ USA
Hemoglobin primer set	
Sodium Azide	
Sodium phosphate buffer	
Acetic Acid	Sebia/France
Agarose	
Alkaline buffer	
Amidoblack stain	
Barbital	
Citric acid	
Destaining solution	

Ethanol	Sebia/France
Ethylene glycol	
Hemolysing solution	
Sodium Azide	
Sodium barbital	
Staining solution diluent	
Tris-barbital	
Ammonium sulphate	Shandon /Germany
Barbitone (diethyl barbiturate)	
NaOH	
Potassium cyanide	
Potassium dihydrogen phosphate	
Potassium ferricyanade	
Sodium diethyl barbiturate	
Sodium chloride	
Triton X-100	
Drabkin's reagent	Vaccine & Sera Institute / Iraq
White blood cell diluting fluid	

2.3 Blood sample collection

Seventy patients with their families who came from different parts of Iraq to Central Health Public Laboratory, blood samples were collected from the patients' family and his relatives in anticoagulant covered tube; the anticoagulant that used was EDTA.

2.4 Buffers and Solutions

2.4.1 Leishman stain (Frei *et al.*, 1995)

Leishman powder	2 g
Methanol (absolute)	1000 ml

The mixture was heated to 50°C, allowed to cool at room temperature and shacked several times during the day. After standing for 24 hour, it was filtered to obtain clear stain.

2.4.2 Leukocyte count solution

White blood cell diluting fluid	435 mmol/L

2.4.3 Hemoglobin count solution

Drabkin's reagent	435 mmol/L

2.4.4 Platelet counting solution (Rustin *et al.*, 1979)

This solution was prepared by dissolving 10 g of ammonium oxalate in 1000 deionized distilled water, and was sterilized by filtration through millipore filter (0.22 μm). This solution was stored at 4°C.

2.4.5 Hb electrophoresis by cellulose acetate paper (Dacie and Lewis, 1996)

This buffer was used in cellulose acetate paper electrophoresis.

2.4.5.1 Barbitone buffer pH 8.6

This buffer was prepared by dissolving 5.15 g of Sodium diethyl barbiturate and 0.92 g of Barbitone (diethyl barbiturate) to 1000 ml of distilled water.

2.4.6 Estimation of HbF (Betke *et al*., 1959)

The following Buffers and solution were used for estimation of HbF

2.4.6.1 Drabkin's solution pH 8.9

Potassium ferricyanade	0.2 g
Potassium cyanide	0.5 g
Potassium dihydrogen phosphate	1.4 g
Triton X-100	1 ml
Distilled water	1000 ml

2.4.6.2 NaOH (1.25 M)

NaOH	50 g
Distilled water	1000 ml

2.4.6.3 Saturated ammonium sulphate

Ammonium sulfate was added to 250 ml of distilled water until saturation.

2.4.6.4 Normal saline

Sodium chloride	8.5 g
Distilled water	1000 ml (pH 7)

2.4.7 -Agarose gel electrophoresis

The followings which were used for gel electrophoresis, were provided by Sebia Company

2.4.7.1-Agarose gel

Agarose	0.8 g/dl
Alkaline buffer ph8.5	0.1 ml

The gel was stored horizontally in the original packaging at room temperature 25°C.

2.4.7.2- Tris-barbital buffer

The vial of the stock buffer was containing

Barbital	2.45 g/dl
Sodium barbital	13.73 g/dl

The vial of the stock buffer solution was diluted with 1 liter of deionized water.

2.4.7.3 Staining solution diluent

Staining solution diluent	60 ml

The stock of staining solution diluent was diluted with 300 ml of deionized water; it is acidic solution and it was used to stain the band.

2.4.7.4 Amidoblack stain

Amidoblack stain	8 ml

The stock was diluted with 40 ml of distilled deionized water; the stock staining solution was viscous so the vial was rinsed well to collect all the staining solution, after dilution the final concentration of working staining solution was:-

Amidoblack stain	0.4 g/dl
Ethylene- glycol	0.5 g/dl

2.4.7.5 Destaining solution

Destaining solution	100 ml

One ml of the stock of destaining solution was diluted with 1 liter of deionized water, after dilution; the working destaining solution contain:-

Citric acid	0.05g/dl

The stock destaining solution was stored at room temperature; it was used to destain the slab gel and to make the background clear; 5µl/dl of proclin 300 was added to prevent microbial proliferation in the diluted destaining solution when stored for more than one week

2.4.7.6 Hemolysing solution

The vial contains 20 ml of hemolysing solution.

2.4.7.7 Saline

0.15M (0.9 g/dl) of NaCL solution was made in deionized water; it was stored at room temperature.

2.4.7.8- Fixative solution

The solution was prepared at least 15 minute before use and it is contain (v/v):-

Ethanol	60 ml
Acetic acid	10 ml
Deionized water	30 ml

2.4.8 Hemoglobin testing system (Variant)

The following buffers, solutions and reagents were provided by Biorad Company and used with HPLC method

2.4.8.1 Elution buffers

Sodium phosphate buffer	1900 ml
Sodium phosphate buffer	1800 ml

The elution buffers stored at 25°C.

2.4.8.2 Washing solution

Deionized water	1800

The washing solution stored at 25°C.

2.4.8.3 Hemolysis reagent

Deionized water	800 ml

The Hemolysis reagent was stored at 25°C.

2.4.8.4 Hemoglobin A_2/F calibrator / diluent set

The vial contain

Hemoglobin A_2/F calibrator diluent set	10 ml

Ten ml of Hb A_2/F calibrator diluent was added to reconstitute the Hb A_2/F calibrator, mixed gently to ensure complete mixing, the calibrator was allowed to stand for 10 minutes at 25°C the reconstituted Hb A_2/F calibrator stored at 8°C.

2.4.8.5 Hemoglobin primer set

10 vials of lyophilized human red blood cell hemolysate that contain gentamicin, tobramaycin and EDTA as preservative.

The vial contains

Hemoglobin primer set	1ml

Hemoglobin primer was prepared by adding 1 ml of deionized water to the vial; mixed gently to ensure the complete mixing then the hemoglobin primer was allowed to stand for 10 minute at 25°C, the reconstituted hemoglobin primer stored at 8°C.

2.4.9 Isolation of genomic DNA from whole blood

The following buffers and solution were used in the isolation of genomic DNA from whole blood which was provided by AB analitica Company

2.4.9.1 Solutions

Lysing solution
Washing solution 1
Washing solution2

2.4.10 Agarose gel electrophoresis (1%) (Maniatis *et al*., 1982)

The following solution were used for electrophoresis

2.4.10.1 Agarose (1%)

Agarose	1 g
Tris-borate buffer	100 ml

The agarose was dissolved in Tris-borate buffer using hot plate with continuous stirring and used for electrophoresis.

2.4.10.2 Tris-borate 5X

Tris-base	54 g
Boric acid	27.5 ml
EDTA (0.5M) pH 8	20 ml
Distilled water	1000 ml

2.4.10.3 Loading buffer

Glycerol	50 %
Tris-base pH(8)	0.025 M
Bromocresol purple	0.25%

2.4.10.4 Ethidium bromide staining solution

One gram of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye is dissolved.

The container was warped with aluminum foil or kept in a dark bottle and stored at room temperature.

2.4.11 PCR amplification

The following reagents were used for PCR amplification

2.4.11.1 (10X) PCR buffer

This buffer was provided by aj ROBOSCREEN Company (Germany) and is made of 10mM Tris-HCL (pH 8.3), 50mM KCL, 1.5 mM $MgCl_2$ and 0.0001% gelatin.

2.4.11.2 dNTPs mixture

It was provided by aj ROBOSCREEN Company (Germany) with concentration of 2.5 μ mol / sample.

2.4.11.3 Primers

Primers were provided by alpha DNA Company (Canada) in lyophilized form and dissolved in 500 μl of sterile distilled water to a final concentration of 15 pmol/ml. sequences of these primers were listed in table (2-1).

Table (2-1). Primer sequences used for PCR amplification of β-globin gene

	Primer	Sequence
1.	P5	5 ' CCAACTCCTAAGCCAGTCC 3'
2.	P7	5' CTTTCCCTAATCTCTTTCTTTCAGGGC 3'
3.	P10	5' CACTGACCTCCCACATTCCC 3'
4.	P12	5' CTGAGACTTCCACACTGATGC 3'

2.4.11.4 Taq polymerase

Taq polymerase was provided by aj ROBOSCREEN Company (Germany) and contain 5u/μl of enzyme.

2.4.12 Restriction Enzyme reaction

2.4.12.1 (10X) RE buffer

This buffer was provided by Promega Company (USA) and is made of 60mM Tris-HCL (pH 7.5), 1M NaCL, 60mM $MgCl_2$ and 10mM DTT.

2.4.12.2 *Bsu*361

*Bsu*361 is a restriction Enzyme that provided by Promega Company (USA) and contain 10u/µl of enzyme.

2.4.12.3 BSA

BSA was provided by Promega Company (USA) and contain 10 µg/µl.

2.5 Methods

2.5.1 Sterilization method

2.5.1.1 Autoclaving

Buffers and solutions were sterilized by pressure vessel (autoclave) at 121°C and 15 bar for 15 minutes.

2.5.1.2 Dry heat

Laboratory oven was used for glassware sterilization; the temperature of oven was fixed at 200°C for 2 hours.

2.5.2 Preparation of blood films on slides (Ingram and Minter, 1969)

1- One to two drops of patients blood sample were placed in the center of glass slide.

2- The blood was spread on the slide in a monolayer.

3- The films were stained with leishman stain and left to dry for 5-10 minutes at room temperature.

4- The film was then washed with distilled water for 10 minutes, then dried and examined by microscope.

2.5.3 Hemoglobin count

1- Five ml of Drabkin's reagent was mixed with 20 µl of blood; then left for 5 minutes at room temperature and read at 540 nm.

2- The hemoglobin count was made by the following formula:-

$$\text{Calculation} = \frac{A^{540}\ \text{sample}}{A^{540}\ \text{standard}} \times n$$

n: concentration of standard (14.3 g/dl)

2.5.4 Total leukocyte (W.B.C) count

1- 0.02 ml of blood was added to 0.4 ml of white blood cell diluting fluid and it was mixed well for 2 minutes by using the cell suspension mixer.

2- Loaded chamber was left undistributed in a wet Petri dish for at least 2 minutes for the cell to be settled down.

3- The cells were counted by using the 4 mm objective lens & 6x or 10x eye piece. The 4 corners 1 mm^2 areas were counted, the cells which touch the top and right hand margins of the ruled area were included.

4- The leucocyte count was made by the following formula:-

$$\text{White cell} / mm^3 = \frac{N \times 1}{0.4} \times 20\ (\text{diluent})$$

$$= N \times 50$$

2.5.5 Platelet count (Rustin *et al.*, 1979)

1- Blood samples were diluted 1:20 by adding 0.1 ml of blood to 1.9 ml of platelet counting solution.

2- The suspension was mixed for 10- 15 minute.

3- The neubauer counting chamber was filled with the suspension with Pasteur pipette.

4- The counting chamber was placed in moist Petri dish and left for 20 minutes to give enough time for platelet to deposit.

5- The number of platelets in one or more areas of 1mm² was counted.

Platelet counting of blood was made according to the following formula:

$$\text{Platelet count / L} = \frac{\text{No. of cell counted} \times \text{dilution} \times 10}{\text{Volume counted } \mu l}$$

2.5.6- Autoanalyzer (MS9)

The steps were performed according to the instruction of supplier Gergy-pontoise Company, the principle measuring of Autoanalyzer (MS9) is the impedance metric:-

2.5.6.1 Mechanical structure

The measuring chamber is made of two parts: - one part is to receipt the dilute in which the blood cells were analyzed and count. The other part was filled with detergent ,is called the aspiration part in which a vacuum was applied the blood cell dilution of the first part cross by aspiration a calibrated aperture (Rubis) which separates both of them.

2.5.6.2 Electrical structure

Two electrodes were in both of these two parts of measuring chamber. A constant current was established between these two electrodes across the aperture.

2.5.6.3 Phenomena

When a cell coming from the whole blood dilution part of the blood passes by a simple mechanical aspiration to the aspiration part of the measuring chamber, it crosses the aperture because of its different resistivity in front the diluent one, the cell disturbs the constant current established between the 2 electrodes and generates a pulse.

2.5.7 Hb electrophoresis by cellulose acetate paper (Dacie and Lewis, 1996)

The procedure of Hb electrophoresis by Cellulose acetate was carried out as the following

1- two ml of blood sample was centrifuged at 3500 rpm for 5 minutes to separate the plasma.

2- Lysate was washed with normal saline (0.85%) then centrifuged at 3500 rpm for 5 minutes, and then the supernatant was discarded.

3- Step (2) was repeated.

4- Step (3) was repeated but the supernatant was discarded by disposable Pasteur pipette.

5- Two volume of distilled water was added to the lysate and then the tube was shaken for 2 minutes then left it in the rack for 1 hour.

6- Five drops of chloroform were added, then shaken for four minutes and centrifuged for 15 minutes.

7- The lysate was pulled by Pasteur pipette and put in another tube, but the chlorophorm is discarded.

8- The electrophoresis champers were filled with 250 ml of barbitone buffer then the wicks were soaked and positioned in place while the power supply is still disconnected.

9- The cellulose acetate paper was soaked in a separated dish in barbitone buffer for 10 minutes; the paper was immersed slowly in the buffer solution. This is the most important step to avoid air bubbles and to obtain good results.

10- The cellulose acetate paper was dried with a filter paper and then placed between the two pieces of absorbent papers.

11- A small volume (10 µl) was taken from each sample and placed into the sample well.

12- The applicator was immersed into the sample well then the sample were applied to the cellulose acetate paper approximately three cm from one end of the paper, the tip of applicator were allowed to remain in contact with the paper for three second.

13- The cellulose acetate paper was placed across the bridge of the tank so that the surface of the paper is in contact with the buffer with the line of the cathode end.

14- The power supply was then connected and the voltage was set at 280-300 volts and the current was 4AM -5AM for 30 minutes to obtain visible separation.

15- After thirty minutes the power supply was disconnected, and then the cellulose acetate paper was removed.

2.5.8 Estimation of HbF (Betke *et al*., 1959)

1- Cyanmethaemoglobin (HiCN) solution was prepared by adding 0.2 ml of red blood cell lysate or 0.1 ml of washed packed red cells to 4 ml of Drabkin's solution.

2- A volume of 0.2 was taken from NaOH of 1.2 M and added to 2.8 ml of HiCN, mixed thoroughly and left for exactly 2 minutes at room temperature.

3- About 2 ml of saturated ammonium sulphate was added to the above mixture, mixed thoroughly and allowed to stand for 10 minutes.

4- The mixture was filtered through Watman No.42 filter paper.

5- 0.7 ml of HiCN was added to 4.3 ml of Drabkins solution to make the standard solution.

6- The absorbance was read for both test and standard tubes at 540 nm.

7- The percentage was calculated for HbF as follow:-

$$\% \text{ HbF} = \frac{A^{540} \text{ Test}}{A^{540} \text{ Standard} \times 20} \times 100$$

2.5.9- Gel electrophoresis

2.5.9.1 Sample preparation

1- One ml of the anticoagulant blood was centrifuged at 5000 rpm for five minutes.

2- The plasma was discarded.

3- The red blood cells were washed twice with ten ml of saline.

4- 10 µl of the washed packed red cells was hemolyzed with 130 µl hemolysing solution.

5- The lysate was mixed with vortex for 10 seconds and incubated for five minutes at room temperature.

2.5.9.2 procedure of gel electrophoresis

The following steps were performed according to the instruction of supplier Sebia Company

1- The hydragel k20 applicator was placed on a flat surface and the part of the applicator carrier was raised with numbered notches.

2- 120 µl distilled deionized water was pooled on the lower third of the frame printed on the hydragel k20 applicator carrier then the hydragel agarose gel electrophoresis was unpacked and uniformly one thin filter paper was rolled quickly onto the gel surface to absorb the excess of liquid. The paper was removed immediately.

3- The gel plate (the gel side up) was placed with its edge against the stop at the bottom of the printed frame.

4- The gel was bended and it was lowered down onto the water pool to ensure that no air bubbles were trapped, water was spreaded underneath the entire gel plate and the gel was lined up with the printed frame.

5- The applicator was lowered with the numbered notches down to the intermediate position with the switch in high position.

6- One applicator was placed on a flat surface with the well numbers in the right-side-up position.

7- 10 µl of the hemolyzed sample was applied into the applicator wells. The applicator was loaded within 2 minute.

8- The applicator teeth's protection frame was snapped off.

9- The sample applicator was placed into the position No.4 on the applicator carrier.

10- The applicator carrier was lowered with the switch so that the applicator contacts the gel surface.

11- After 1 minute, the switch was turned to rise up the applicator, the applicator was removed.

12- The gel was put into an appropriate electrophoresis chamber, according to the polarity indicated on the gel, the lower side of the gel on the cathodic side, when we using Sebia k20 chamber, the hydragel was placed on the bridge with the gel side facing down, the gel was dipped about 1cm into the buffer on each side.

13- The chamber was plugged to the power supply then after migration, the chamber was unplugged and the gel was removed.

Table (2-2). The details of buffer volume, voltage and migration time.

Migration condition	Sebia k20
Volume of buffer per compartment	150 ml
Total buffer volume	300 ml
Migration time	15 minute
Constant voltage	165V
Initial current (per gel)	7±2 mA

2.4.9.3- Fixation

1- The gel was placed into a gel holder (supplied with Sebia hydragel k20

accessory kit) for further processing.

2- One tank was filled with 150 ml of fixative solution.

3- The gel was immersed into the fixative solution for 15 minute.

4- The gel was removed and dried with hot 80°C air flow in incubator-dryer IS 80.

2.5.9.4 Staining – Destaining

1- The gel was immersed in the staining solution for 5 minutes.

2- Destaining was carried out in three successive baths of Destaining solution until the background was completely colorless and clear.

3- The excess liquid was soaked up on the gel surface with a tissue paper and the gel was dried with hot 80° C.

2.5.10 Hemoglobin testing system (variant)

The hemoglobin testing system utilizes the principles of cation-exchange high performance liquid chromatography (HPLC)

2.5.10.1 Sample preparation

1- Five µl whole blood from each patient sample was added into separate 1.5ml sample vial.

2 - One ml of hemalysis reagent was added to each vial (patients' hemolycate were stable for 24 hour when stored at 8° C).

3- Para film was used to cover each sample vial then mix it by inversion.

4- The sample vial was placed into the variant.

5- Variant worked as autoanlyzes and read the results automatically.

2.5.10.2 The method of variant (ß- thalassemia short program)

The following steps were performed according to the instruction of supplier Biorad Company:-

1- Hemolyzed specimens were maintained at 20°C in the automatic sampler chamber.

2- Specimens were sequentially injected into the analysis stream at 6.5 minute intervals for a throughput of 9 samples per hour.

3- 2 dual-piston pumps and a preprogrammed gradient was control the elution buffer mixture which passing through the analytical cartridge.

4- The ionic strength of the elution buffer mixture was increased by raising the percent contribution of elution buffer 2, as the ionic strength of the mixture increase ,more strongly buffer was used to retain the hemoglobin elute from the analytical cartridge.

5- A dual wave length filter photometer (415 and 690 nm) was monitoring the hemoglobin elution from the cartridge, which detect the absorbance changes at 415nm.

6- The 690 nm secondary filter was correct the base line because of the effects which caused by the mixing buffer with different ionic strength.

7- Changes in the absorbance were monitored and displayed as a chromatogram of absorbance versus time.

8- The analysis data from the detector was processed by the built-in integrator and printed on the sample report.

9- Windows (e.g. ranges) had been established for the most frequently occurring hemoglobin which based on their characteristic retention times to aid in the

interpretation.

10- Retention time was the elapsed time from the injection of the sample to the apex of a hemoglobin peak; each of the hemoglobin has a characteristic retention time.

11- The minor differences in the separation efficiency of individual analytical cartridge were corrected by using the hemoglobin A_2/F (Hb A_2/F).

12- At the end of each sample analysis, a copy of the chromatogram and report data was automatically printed, the report table includes the corrected area percent for hemoglobinsA_2 and F for all subsequent samples in the run.

2.5.11 Isolation of genomic DNA from whole blood

The following steps were performed according to the instruction of supplier AB analitica Company:-

1- 200 µL of whole blood was collected in tube which contain EDTA as preservative.

2- The bottle of solution 1 was shacked before use to resuspend the binding resin completely; 500 µl of the solution 1 was added to the sample**.**

3- The blood sample was mixed gently by inverting the tube several times, until a complete homogenous mixture was obtained.

4- The tube was incubated at 60°C for 4 minutes. At the end of the incubation, the tube was inverted several times (at least 10 times) to ensure the complete binding of DNA to the resin.

5- The filter columns were put into the 2 ml centrifuge tubes and the lysate was transferred. The cap was closed and centrifuged at 14,000 rpm for 1 minute.

The filter was retains the matrix and the DNA was bound to it, while the filtrate was contained proteins and other contaminants.

6- The filter was removed, the filtrate was poured and then replaced the filter on the test tube and 500µl of solution 2 was added, the cap was closed, vortexed for few seconds and centrifuged at 14,000 rpm for 1 minute.

7- The filter was removed, the filtrate was poured and then 500 µl of solution3 was added to the filter. The cap was closed, incubated for 2 to 4 minutes at room temperature and centrifuged at 14,000 rpm for 1 minute. (It was necessary to remove any salt traces from the sample, made an optional washing step with 500µl of Ethanol 80%).

8- The filter was removed.

9- The filter was put into a new 1.5 ml Eppendorf and 100 µl of 65-70 $^{\circ}$C preheated water was added directly to the filter. Vortexed for few seconds to resuspend the resin and the tube was allowed to stand for 5 minutes at 70 $^{\circ}$C to obtain the complete DNA releasing .Vortexed again and centrifuged at 14,000 rpm for 1 minute, to avoid DNA breaking, resuspend the resin with a pipette's tip instead of vortexing.

10- The filter was put out.

2.5.12 PCR amplification

The following reagents were mixed in a sterile 0.2 ml Eppendorf tube as given in table (2 – 3)

Table (2-3).Reagents and their addition order used in β-globin PCR amplification.

Addition order	Component	Volume	Concentration
1.	10 X PCR buffer	5 µl	1X
2.	$MgCl_2$	1.5 µl	25 mM
3.	dNTPs	0.5 µl	10 mM
4.	P5	1.5 µl	10 pmol
5.	P7	1.5 µl	10 pmol
6.	P10	1.5 µl	10 pmol
7.	P12	1.5 µl	10 pmol
8.	Taq polymerase	0.3 µl	1.5 u / R
9.	Distilled water	12.7 µl	_
10.	DNA	2 µl	_
Total		25 µl	_

Amplification was performed in Ependorff Master Cycler programmed for 35 cycles (94 °C for 5 minutes, 92 °C for 1 minute, 62 °C for 1.5 minutes and final extension step at 72 °C for 2.5 minute.

2.5.13 Restriction Enzyme reaction

The following reagents were mixed in a sterile 0.2 ml Eppendorf tube as given in table (2 – 4)

Table (2-4). Reagents and their addition order used in β-globin gene Restriction Enzyme reaction.

Addition order	Component	Volume	Concentration
1.	Distilled water	11.8 µl	_
2.	10X RE Buffer	2 µl	1X
3.	BSA	0.2 µl	10µg/µl
4.	DNA	5 µl	_
5.	*Bsu361*	1 µl	10u/ µl
Total		20 µl	_

The mixture were placed in water bath for 3 hours at 37°C.

3.1 Hematological analysis

3.1.1 Red blood cell indices in beta thalassemia

The RBC indices are the basis for classifying anaemias and they have been used to distinguish between iron deficiency and thalassaemias, therefore, it is considered as the preliminary diagnosis of β-thalassemia major (Lafferty *et al*., 1996).

The results in table (3-1) were obtained by measuring Hb, PCV, MCH, and MCV in comparison with normal values.

The results from thalassemia patients showed that MCV is less than 80, MCH and MCHC decreased in comparison with normal values.

The recognition of microcytosis has been greatly facilitated by the use of electronic cell counters that directly measure the MCV of the RBC population; this has been widely and successfully used to screen populations for β-thalassemia syndrome.

Table (3-1). Red blood cells indices in β-thalassemia patients and their parents.

Red blood cells indices	β-thalassemia minor (carrier)		β-thalassemia major (affected)	Male (normal values)	Female (normal values)
	Father	Mother	Patient		
Red blood cells count (RBCs)	6.5×10^{12}/L	6.3×10^{12}/L	3.2×10^{12}/L	4.7-6.1×10^{12}/L	4.2-5.4×10^{12}/L
Hemoglobin (Hb)	11.9 g/dl	10.5 g/dl	8.0 g/dl	14-18 g/dl	12-16 g/dl
Packed cell volume (PCV)	40%	42%	25.4%	40-54%	37-47%
Mean corpuscular hemoglobin(MCH)	18.3 pg	16.6 pg	25 pg	27-31 pg	27-31 pg
Mean corpuscular volume (MCV)	61.5 fl	66.6 fl	79.3fl	80-94 fl	80-94 fl
Mean cell hemoglobin concentration (MCHC)	29.7 g/dl	25 g/dl	31.4 g/dl	32-36 g/dl	32-36 g/dl

Thalassemia major is suspected in child younger than 2 years old when severe microcytosis, hypochromic, anisocytosis and poikilocytosis is found in peripheral blood smear.

The preliminary diagnosis of β-thalassemia major is the appearance of the large numbers of abnormal RBCs morphology in peripheral blood smear which have been taken from the patients and is a common feature in this type of disorder which lead to severe anemia and hepatosplenomegaly, however, the percentage of RBC morphologic changes differs according to the severity of β- thalassemia.

Figure (3-1),(3-2) showing more marked microcytosis and poikilocytosis in peripheral smear from a patient with β-thalassemia major, whereas, it is less severe in father and mother who have β- thalassemia minor as shown in figures (3-3)and (3-4).

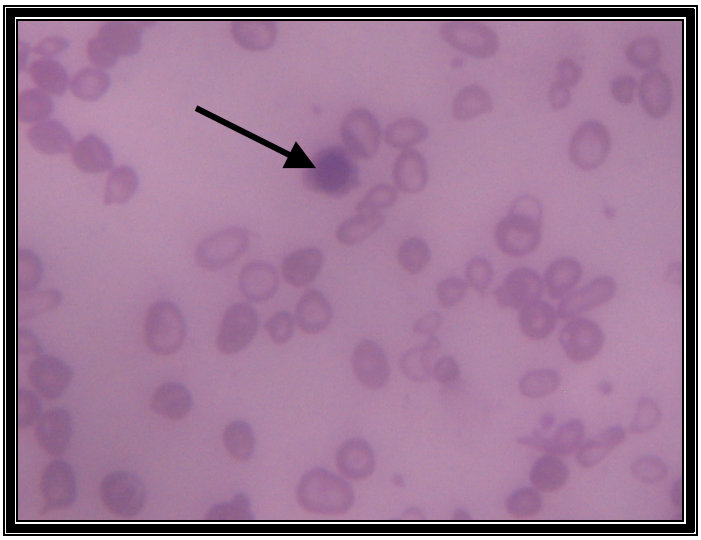

Figure (3-1) Microscopic photograph (x 400) showing blood film taken from the patient who is β-thalassemia major with normoblast which pointed by solid arrow ⟶.

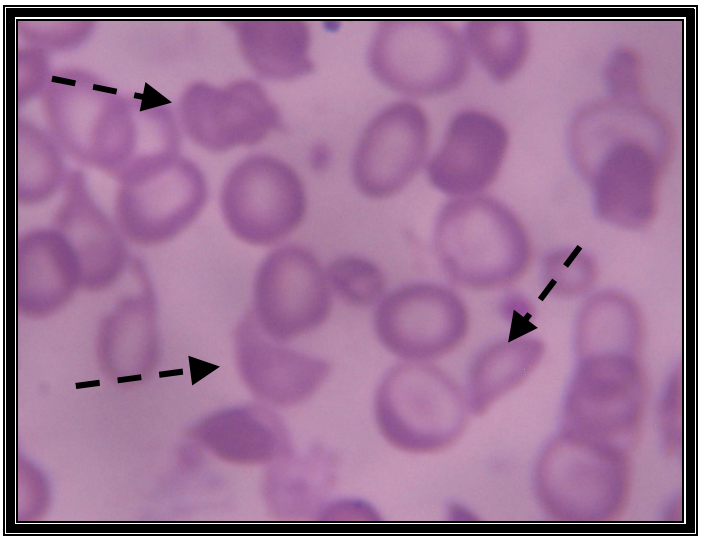

Figure (3-2).Microscopic photograph (X1000) showing blood film taken from patient who have β-thalassemia major with anisopoikilocytosis pointed by dotted arrow ⇢ .

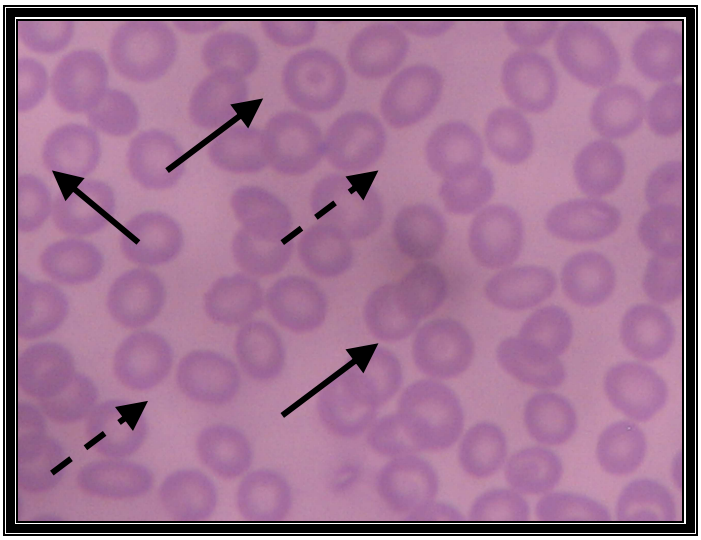

Figure (3-3).Microscopic photograph (x 1000) showing blood film taken from the mother who is β-thalassemia minor which emphasize target cells pointed by solid arrow ⟶ and Hypochromia pointed by dotted arrow - - - ▶

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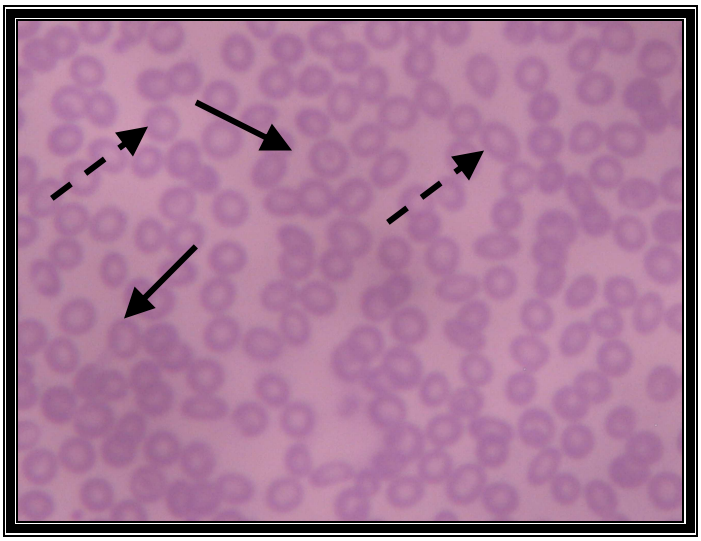


Figure (3-4). Microscopic photograph (x 400) showing blood film taken from the father who is β-thalassemia minor with target cell pointed by solid arrow ⟶ and hypochromia pointed by dotted arrow - - - ▶

Previous studies made on patients by Ahmed *et al*., (2004) were shown similar results, thus, changes in the morphology of RBC appeared as microcytosis, hypochromic, anisocytosis, poikilocytosis and cell fragment in peripheral blood smear.

Moreover, patients with β- thalassemia major had much more distinct microcytosis than those with β- thalassemia minor.

those patients who have β-thalassemia minor significantly increased red cell indices and reduced Hb levels, MCV, MCH , PCV, and MCHC which is less severe than those affected with β- thalassemia major (Padmanabhan *et al*., 2001).

3.2- Electrophoresis of Hemoglobin

Electrophoresis may be the main technique for molecular separation because electrophoresis is highly specific in the detection of certain Hb disorders such as β-thalassemia and the finding on hemoglobin electrophoresis vary from patient to patient and with the type of thalassemia.

Protein electrophoresis method is simple, easy and inexpensive; all electrophoretic separation depends up on the charge distribution of the molecular being separated (Green, 1992).

3.2.1 Hemoglobin electrophoresis by cellulose acetate paper

Blood samples were taken from the patients and their families in EDTA tubes then protein was separated and subjected to electrophoresis by cellulose acetate paper to distinguish the abnormal protein. Electrophoresis by cellulose acetate paper is rapid, simple and reliable for separating small molecules like amino acid, nucleotide as well as charged macromolecules (Badens *et al*., 2000).

The obtained results which are shown in figure (3-5) for family one demonstrate absence β-globin band in the child and this indicate that there is a complete failure of β-chain production in blood sample, whereas, β-globin band was present in father and mother which indicate that the β-chains are produced at a lower rate in comparison with normal person (control).

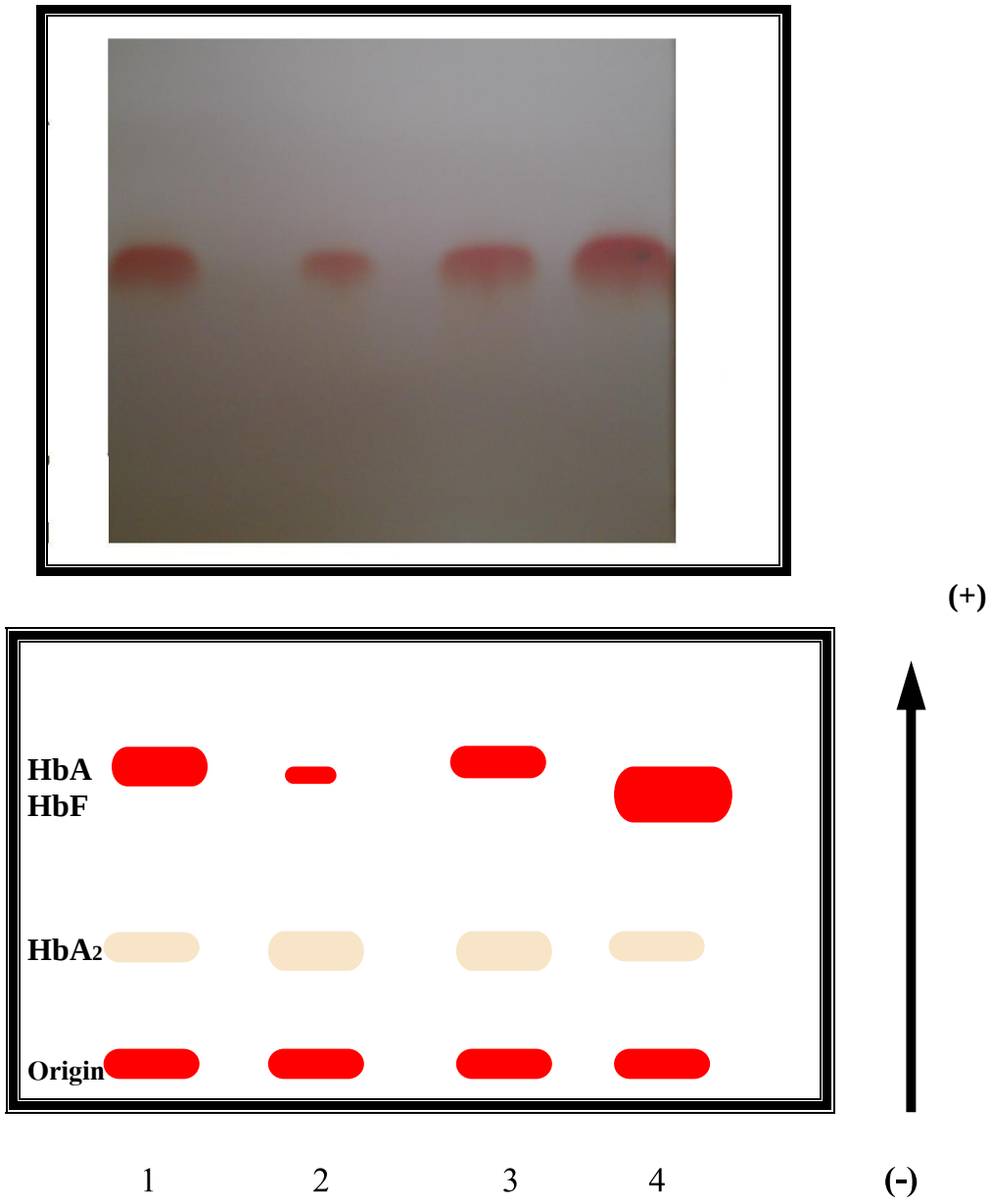
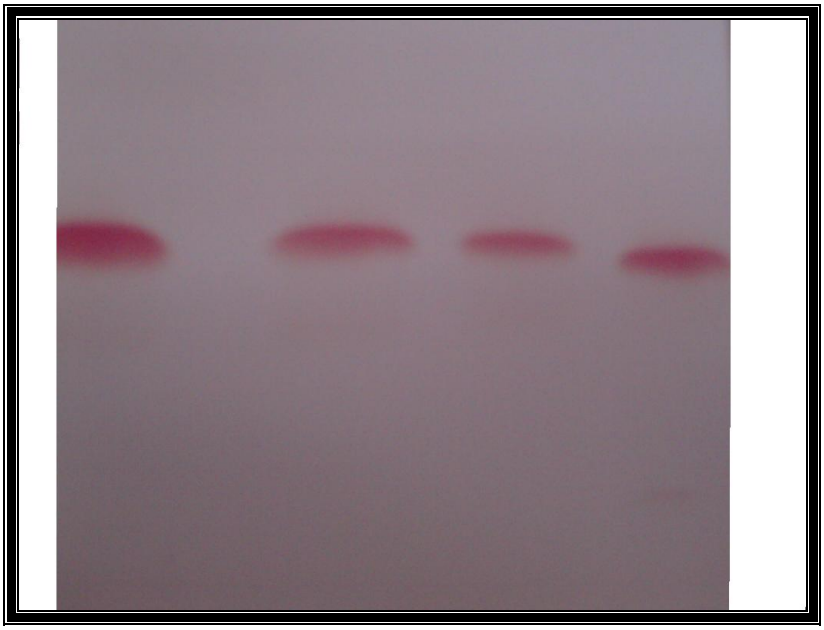


Figure (3-5). Hemoglobin electrophoresis of individuals of family one and control on cellulose acetate paper. Line (1) normal (control), line (2) father hemoglobin (β-thalassemia minor), line (3) mother hemoglobin (β-thalassemia minor) and line (4) hemoglobin from β-thalassemia major patient.

The results for family two shown in figure (3-6) indicated that the presence of HbA band in patient who have β-thalassemia major, this is as a result of continuous blood transfusion process which is necessary to prevent early death and it is usually initiated in the first year of life after biological diagnosis, therefore, in this study blood samples were taken from the patients before blood transfusion process to investigate the absence or presence of β-globin gene.

Electrophoresis by cellulose acetate paper separates proteins primarily by charge and it is satisfactory for the detection and identifies the amount and type of hemoglobin present.



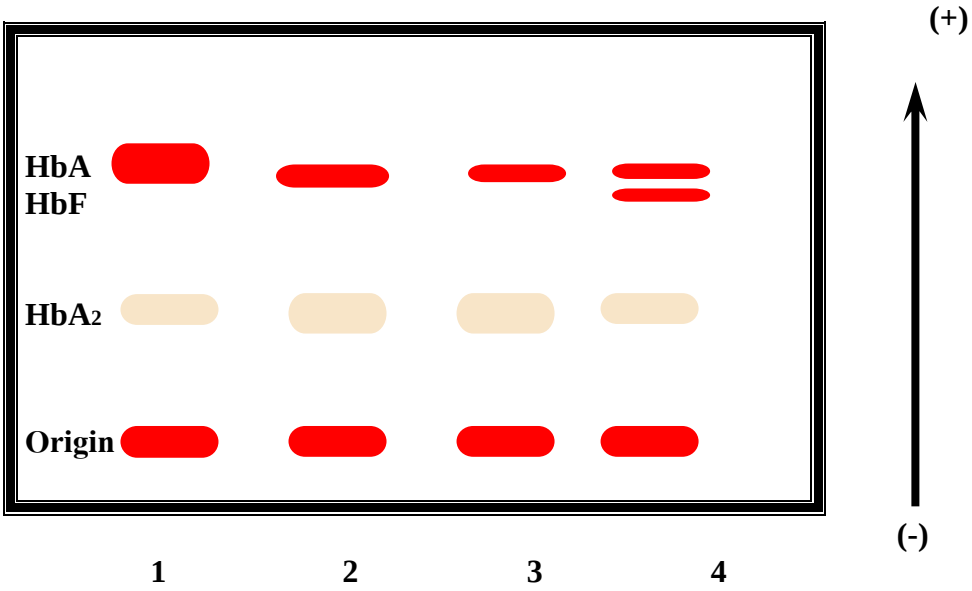


Figure (3-6).Hemoglobin electrophoresis of individuals of family two and control on cellulose acetate paper. Line (1) normal (control), line (2) father hemoglobin (β-thalassemia minor), line (3) mother hemoglobin (β-thalassemia minor) and line (4) hemoglobin from β-thalassemia major patient.

The results for family three in figure (3-7) showed that the HbF level is high in the child (son) which might indicate that the body is trying to compensate by increasing the amount of γ-globin chains and thus could eliminate the severity of anemia which is associated with the disease, whereas, the presence of high level of HbA_2 in father and mother might due to the production of δ-globin chains.

Hemoglobin electrophoresis by cellulose acetate paper were performed for all patients with their families to investigate the presence or absence of HbA and to determine HbA_2 for those who carry β-thalassemia minor and HbF for patient who carry β-thalassemia major in comparison with normal (control).

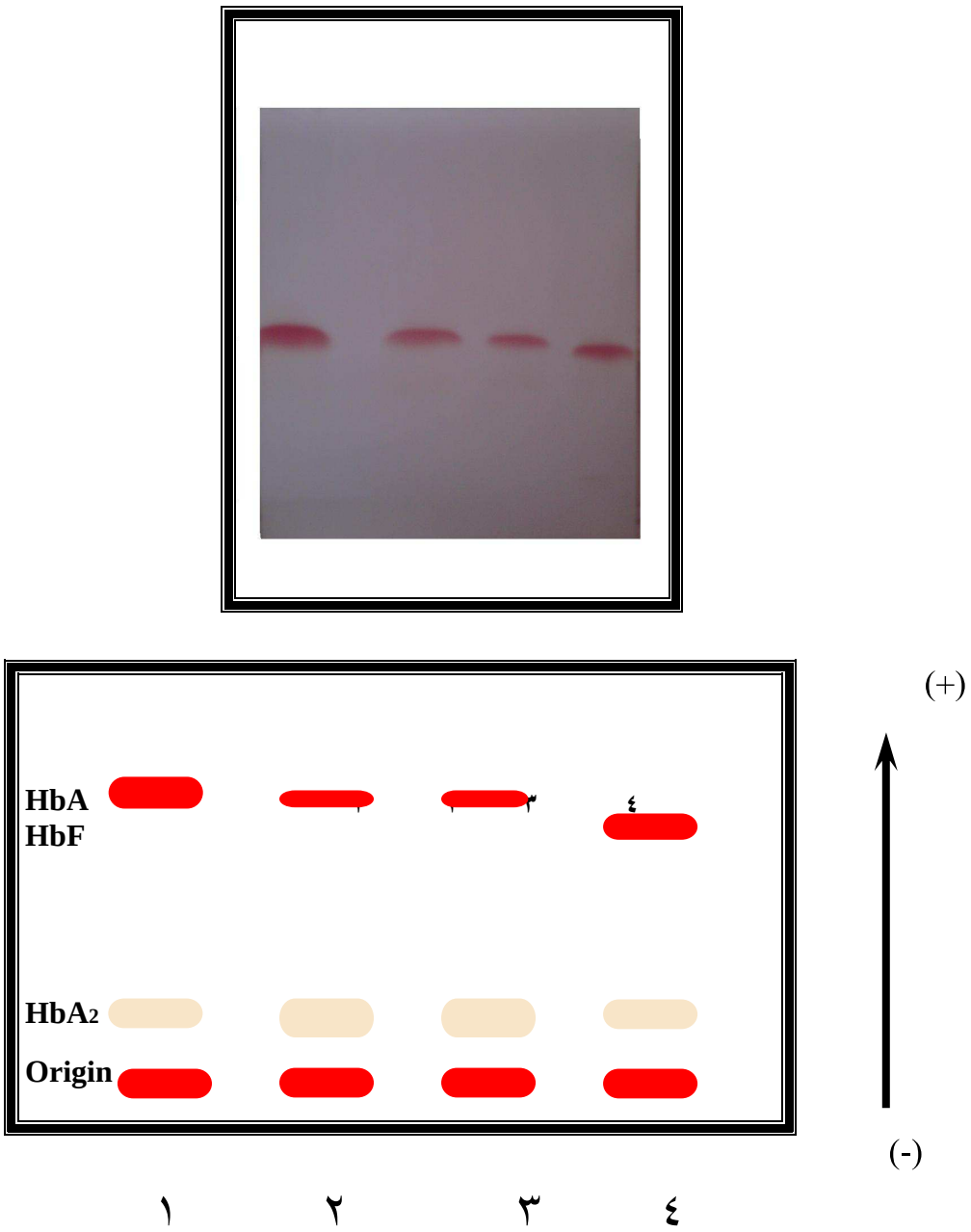


Figure (3-7).Hemoglobin electrophoresis of individuals of family three and control on cellulose acetate paper. Line (1) normal (control), line (2) father hemoglobin (β-thalassemia minor), line (3) mother hemoglobin (β-thalassemia minor) and line (4) hemoglobin from β-thalassemia major patient.

3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is method used to separate macromolecules such as proteins, nucleic acid and peptides by depending on their size and charge, this method is simple, more useful and more accurate in the detection of abnormal proteins.

The HYDRAGEL HEMOGLOBIN K20 Kit is intended for the preliminary identification of β-thalassemia which characterized by decrease in the synthesis of the β-chains (only hemoglobin A is affected).

Thus, this method was used to show that the level of HbA_2 is high in father and mother who have β-thalassemia minor, whereas, the level of HbF in patient was β-thalassemia major is high as shown in figure (3-8).

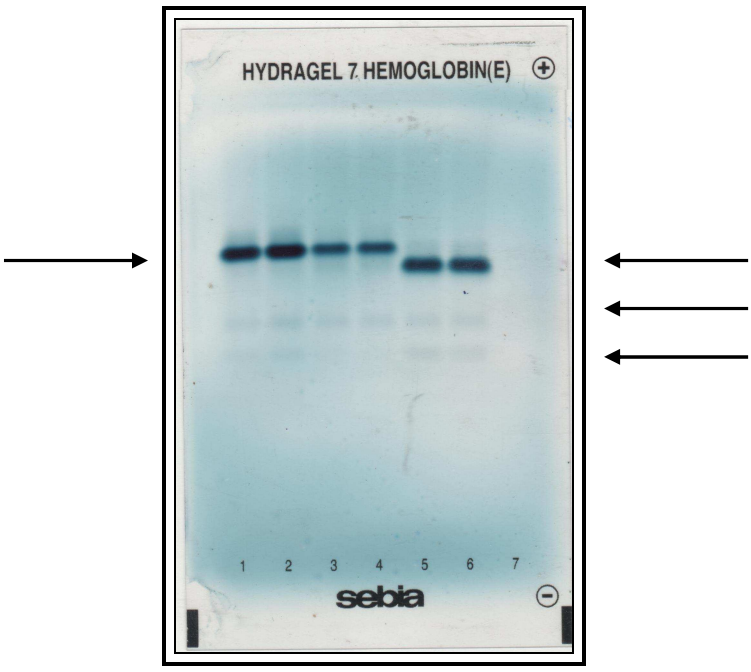


Figure (3-8). Hemoglobin electrophoresis of individual of family one on Hydragel hemoglobin K20. Line (1), (2) normal (control), Line (3), (4) patient with β-thalassemia minor and Line (5), (6) patient with β-thalassemia major.

The type of β– thalassemia can be either homozygous or heterozygous by observing the presence or absence of β – chain in blood samples as compared with standard values of these hemoglobins level in healthy people.

Previous studies by Sanguansermsri *et al.,*(2004) were show similar results, they showed elevated HbF and a decreased amount of HbA in patient with thalassemia major due to the depletion of β-globin production , whereas, HbA_2 level is high in carrier due to the reduction in β – chain formation that results in HbA malfunction.

3.2.3 Hemoglobin testing system (Variant)

The method is rapid, sensitive and precise method for detecting the abnormal hemoglobins and it has become the preferred method for thalassemia screening because of its speed and reliability (Ou-CN and Rognerud, 1993).

The results in table (3-3) showed that there is an increase in the level of Hb F in the patient who has β-thalassemia major, this finding might due to high production of $\alpha^2\gamma^2$ subunit which are the main component in the HbF architecture. Whereas, the level of HbA_2 is present in higher amount in father and mother, this might due to reduction in the β-globin formation, therefore, the continued synthesis of α-chain that stimulates the increased production of $\alpha^2\delta^2$ which lead to increased level of HbA_2. The increase in the level of HbA2 remains as a diagnostic parameter for thalassemia trait condition, the mean value for HbA_2 levels heterozygote is 5% with a range between 3.5% and 7% as shown in appendix1.

Table (3-2). Hemoglobin abnormalities for family one by hemoglobin testing system (HPLC) who have β-thalassemia syndrome.

Hemoglobin type	β-thalassemia major	β-thalassemia minor		Normal values
	Patient	Father	Mother	
HbF	95.3%	0.5%	1.7%	<1%
HbA	0.2%	82.8%	82.4%	96-98%
HbA_2	3.8%	5.7%	6.6%	2-3%

Also it is obvious from the results reported in figure (3-9) that there is severe decrease in the level of HbA in son who have β-thalassemia major because of the complete failure of β-globin genes whereas the level of HbA in father and mother who have β-thalassemia minor is appear in sufficient quantities which lead to prevent overt symptoms of anemia.

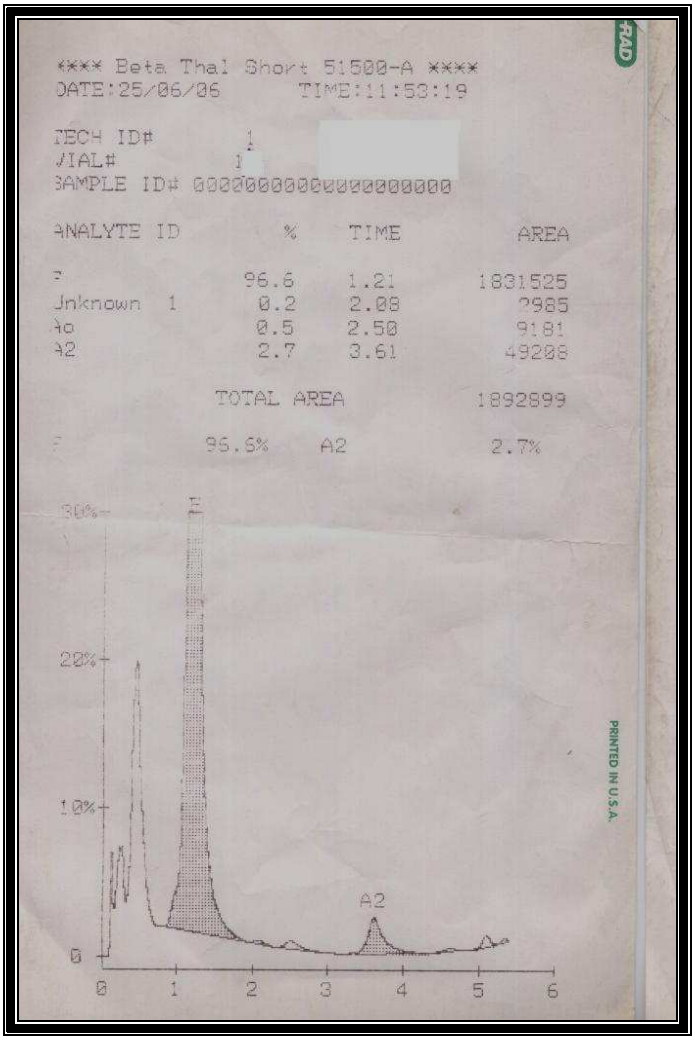


Figure (3-9). A case of patient with thalassemia major showing an elevated level of HbF and severe reduction of HbA by HPLC.

The results in figure (3-10) showed that the decrease in the level of HbF and increase in HbA in patient with β-thalassemia major is normal as a consequence of continuous blood transfusion process which are presently the only treatment to save the patients from severe mental, physical defects and death, patients who received red blood cell transfusions regularly to maintain hemoglobin level 10 to 13 g/dl every 2 to 3 weeks.

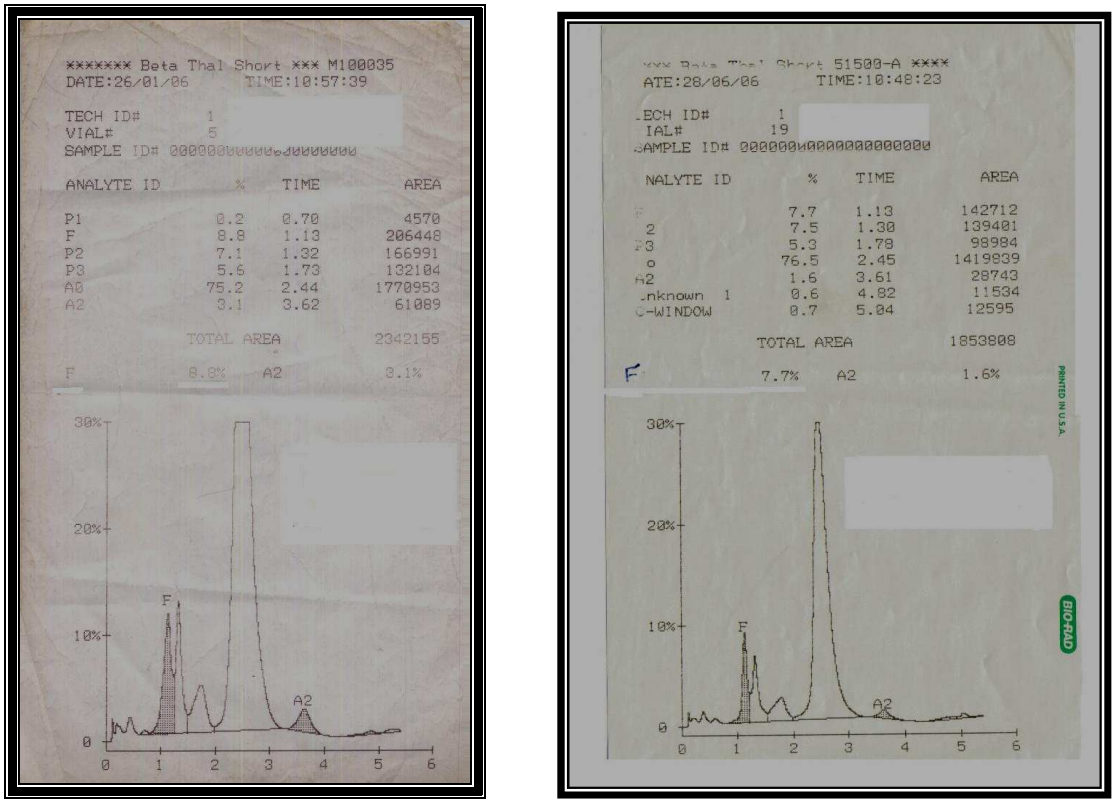


Figure (3-10). Patients with thalassemia major after blood transfusion showing decrease in the level of HbF and increase in HbA by HPLC.

The previous studies had shown that all patients with β-thalassemia major had high percentage of HbF and variable amount of HbA_2 as shown in reports for patients in Guadeloupe, China and India; since HbA_2 percentage was (1.7-4.3%) and Hb F was (69.9-90%). However, in Romania HbF percentage was (50-80.7%) and HbA_2 percentage was (2.5-2.9%), whereas, in Iraq HbF percentage (47.9%-96%) and HbA_2 percentage was (2.6%-4%).Thus, this test ascertains the diagnosis of β-thalassemia major by measure the levels of HbA_2, HbF (Jean *et.al.*, 1997).

The HbF values in patients with β-thalassemia major were significantly higher than those with β- thalassemia minor.

3.3 Pedigree analysis

Pedigree analysis was performed to study the inheritance of gene disorder in Iraqi families, males are represented by square symbols and females with circular symbols, the two lines which drawn between square and circle is indicated to consanguineous mating which mean that the two individuals are related as shown in appendix 2 (Baysal, 2005).

In this study, five families with β-thalassemia syndrome history have been studied; the information which obtained from all studied families include age, present illness, post illness, family relationships, personal history and the data obtained from Red blood indices, hemoglobin electrophoresis and hemoglobin testing system (variant).

3.3.1 Case one

In this case, the genetic analysis of family one was carried out.

This family is from Baghdad province, and composed of three persons (the father, the mother, the son).

According to the history of the family, the grandfather was carrying β-thalassemia minor, he was married to the grandmother who was also carrying β-thalassemia minor, therefore, their progeny showed typical distribution of β-thalassemia syndrome according to Mendel's law as shown in (figure 3-11).

Because of the society tradition, the father who was carrying thalassemia minor was married to his cousin who was also carrying thalassemia minor , thus , their first child showed β-thalassemia major symptom

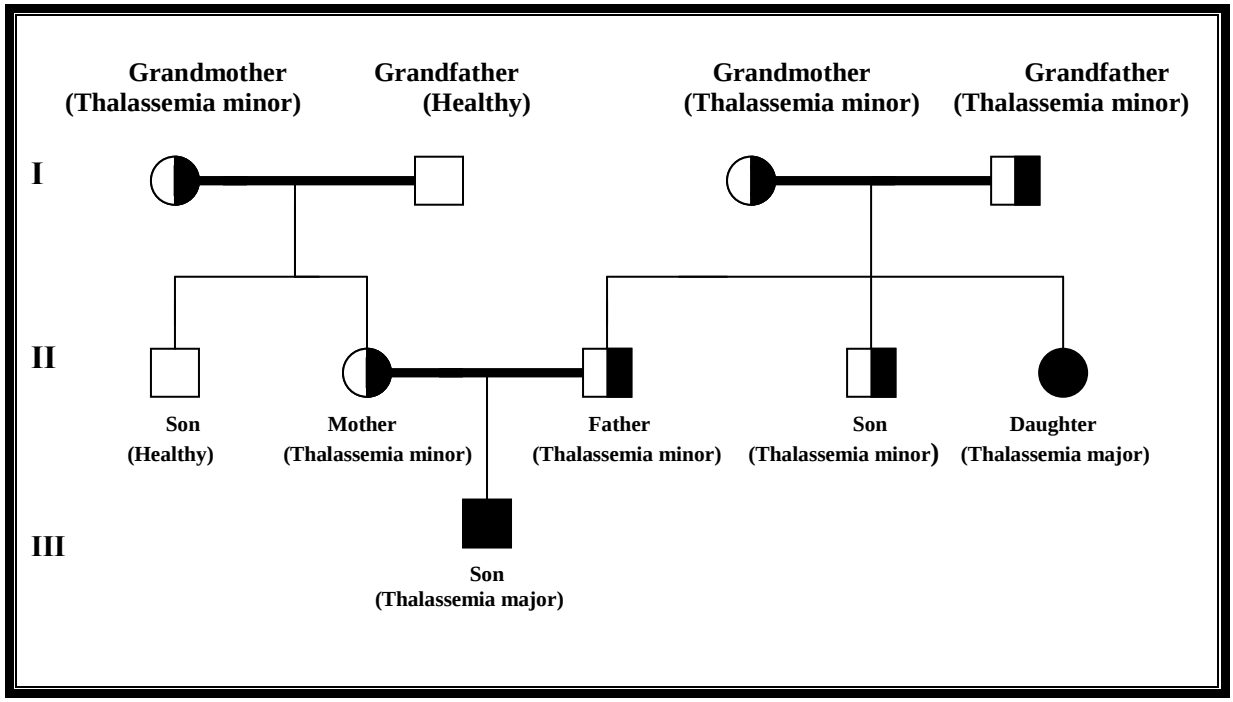


Figure (3-11).Pedigree analysis of family one showing β-thalassemia syndrome (case one).

3.3.2 Case two

The results of genetic analysis of this family is shown in the figure (3-12) which indicate that two healthy grandfather were married to two thalassemia minor grandmother , therefore, their progeny was thalassemia minor carrier when they married , their offspring showed typical distribution of beta thalassemia syndrome according to Mendel's law.

In this case, grandfather from the father and mother side were healthy while grandmother from the father and mother sides were thalassemia minor, therefore, when the father and mother were carry thalassemia trait, their offspring showed typical distribution of beta thalassemia syndrome, their progeny were: one daughter with thalassemia major (25%), two daughter with thalassemia minor (50%), and a healthy son (25%).

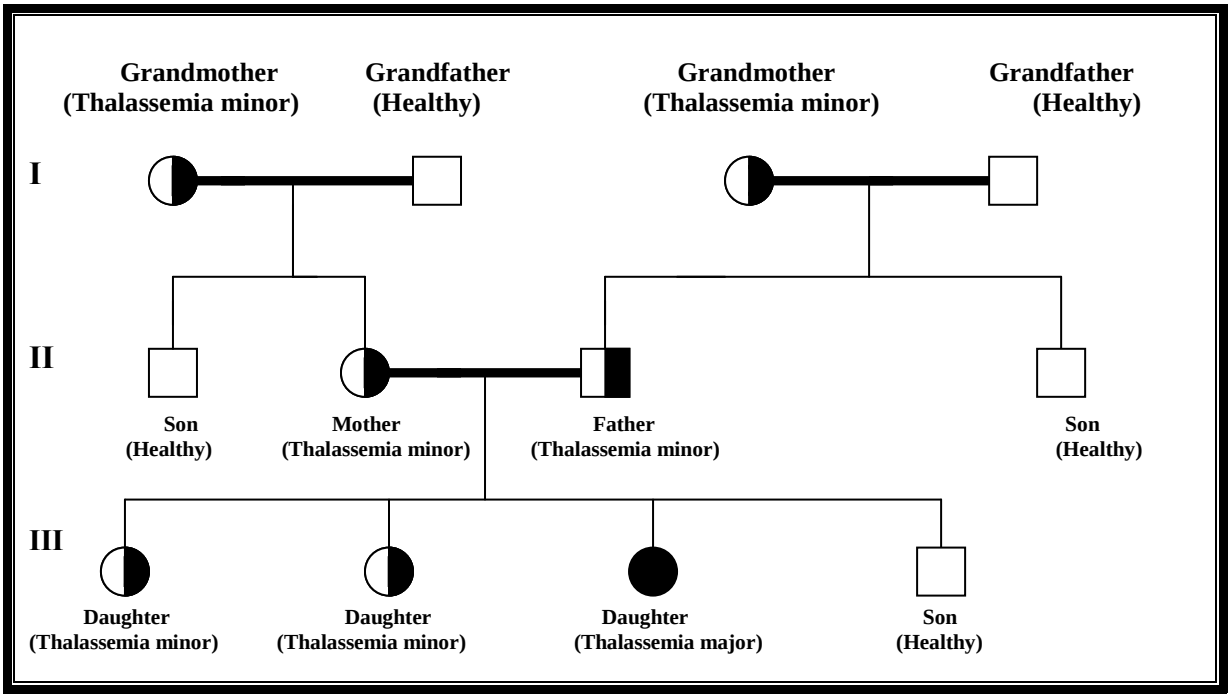


Figure (3-12).Pedigree analysis of family two showing β-thalassemia syndrome (case two).

3.3.3 Case three

The results in figure (3-13) indicated that the grandparents from the father side were carrying thalassemia trait, therefore, their offspring (father) carried thalassemia minor, while grandfather from mother side was healthy and grandmother was carrying thalassemia minor; thus, their progeny (mother) were thalassemia carrier and since when two thalassemia minor (carrier) were married, therefore, their first child show beta thalassemia major symptom.

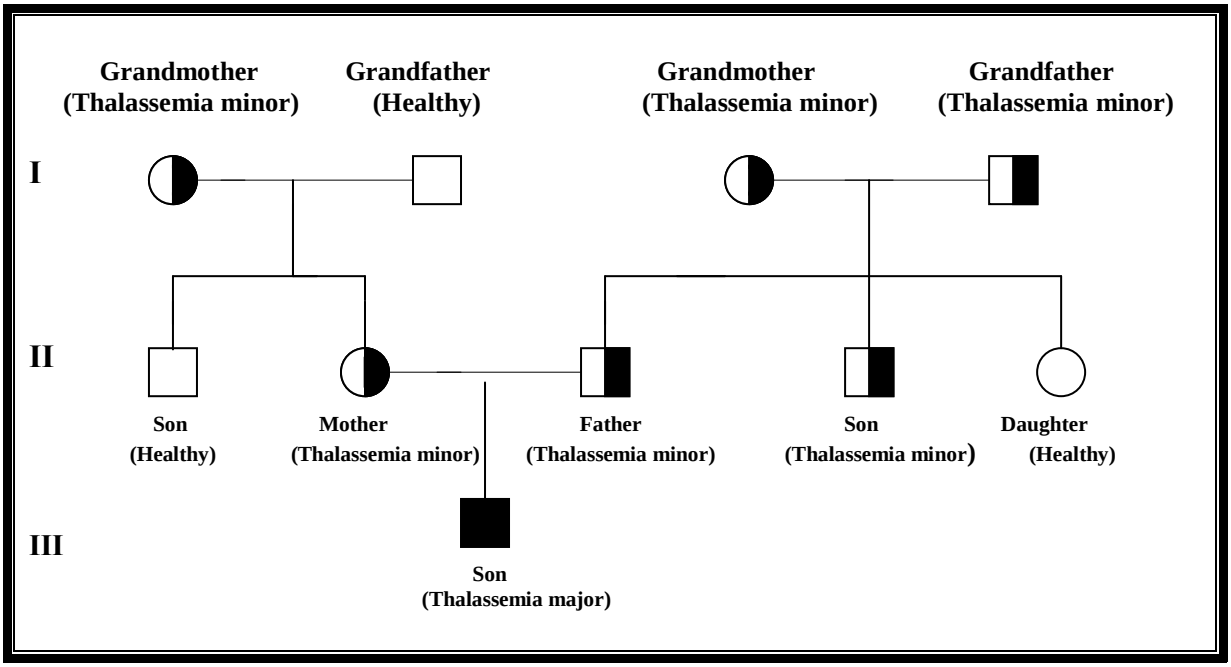


Figure (3-13).Pedigree analysis of family three showing β-thalassemia syndrome (case three).

3.3.4 Case four

The results in figure (3-14) showed that healthy grandfather was married to the grandmother who was carrying thalassemia minor from the father side, therefore, their progeny (father) was also carrying thalassemia minor; while

grandparents from the mother side were both of them carrying thalassemia minor, thus their daughter (mother) was also carry thalassemia minor .

In this case, the father was married to mother who was also carrying thalassemia minor, therefore, their offspring showed typical distribution of beta thalassemia syndrome according to Mendel's law, their progeny were daughter who affected with thalassemia major (25%) ,son (50%) with thalassemia minor and a healthy daughter (25%).

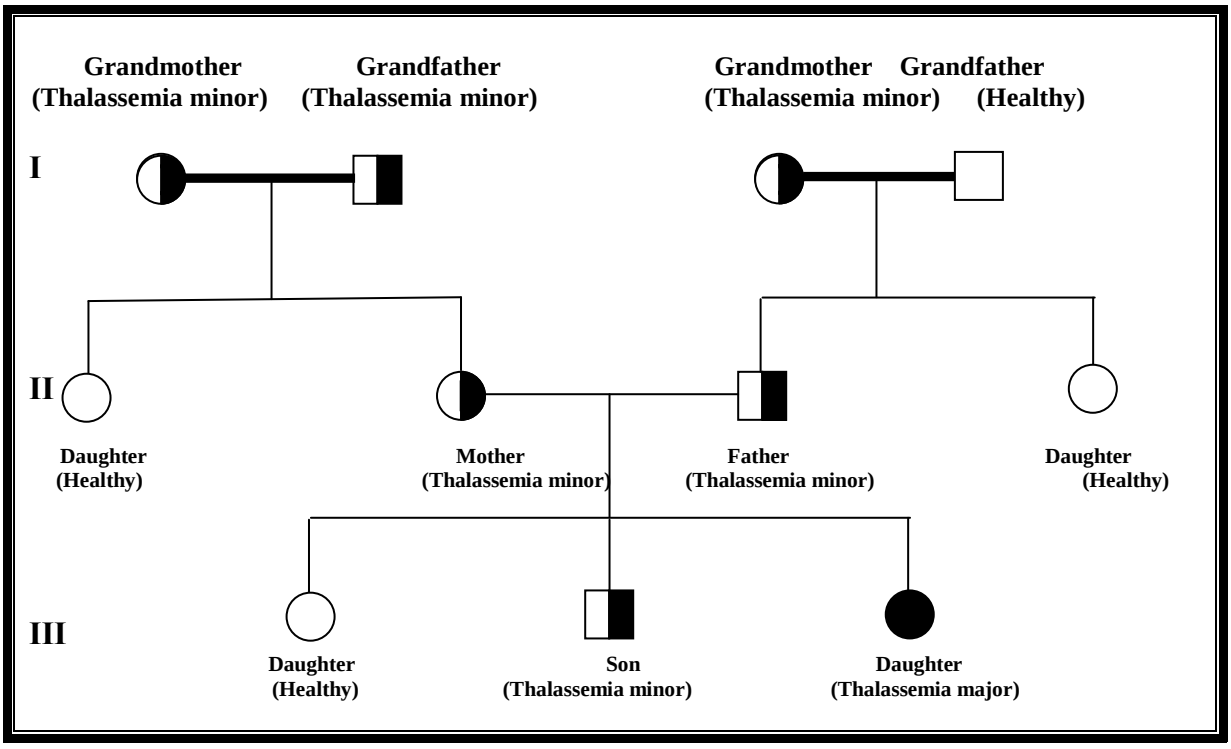


Figure (3-14).Pedigree analysis of family four showing β-thalassemia syndrome (case four).

3.3.5 Case Five

The result of genetic analysis of this family is shown in figure (3-15) different pattern.

The grand parents from the father and mother side were carrying thalassemia minor; therefore, their offspring carried thalassemia minor.

In this case, when the father and mother were carry thalassemia trait, the frequency of the syndrome of the major type increased and their progeny were daughter affected with thalassemia major, son affected with thalassemia major and a healthy daughter.

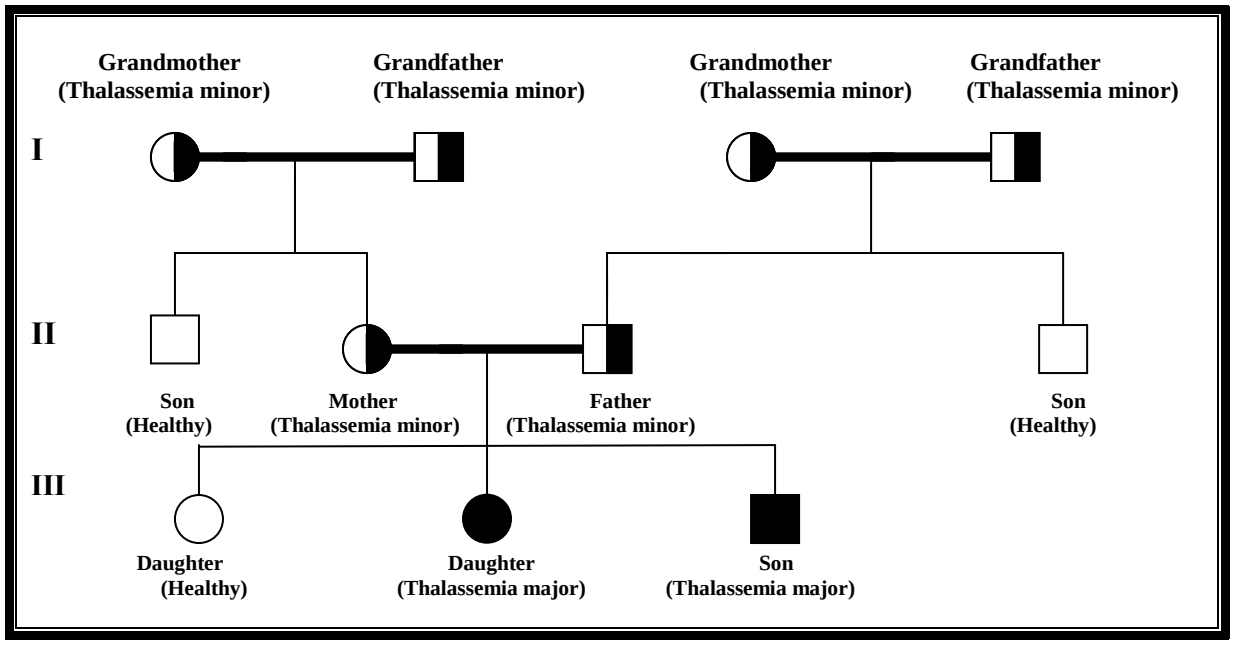


Figure (3-15).Pedigree analysis of family five showing β-thalassemia syndrome (case five).

The results obtained from the five families indicated that the β-thalassemia syndrome is transmitted through a long line of carriers before; therefore, when two carriers mate, thus, there will be a ¼ chance that any child will be affected (thalassemia major), this form of inheritance is called autosomal recessive pattern.

Consanguinity is strongly favored in iraq and many parts of Africa, the Middle East, and West, Central and South Asia, where some 20% to over 50% of all unions are between couples related as second cousins or closer, moreover, the religious differences, local and regional social regulations that can influence the prevalence and specific types of consanguineous marriage Consanguineous marriage is generally uncommon in Europe, North America and Australasia (Hussain, 1998).

3.4 Analysis of β-globin gene by polymerase chain reaction with restriction enzyme reaction

The diagnosis of β-thalassemia has been progressed from phenotype diagnosis in 1960 to genotype diagnosis in 1987. Subsequently, according to the advances in hemoglobin electrophoresis and HPLC biotecheniques, it was possible to classify β – thalassemias into heterozygous and homozygous forms. Then, molecular technologies and specifically the ability to amplify specific DNA fragments from small amounts by PCR has revolutionized many aspects of DNA analysis including genetic diseases (Costa *et al.,* 2003).

PCR is a cost-effective and non-radioactive method that used for detection of the most common mutations causing beta-thalassemia in Mediterranean people.

This approach provides an easy assay for direct detection of normal and mutant β-globin genes in homozygotes and heterozygotes (Fortina *et al*., 1992).

Three techniques which are used following PCR in the diagnosis of point mutation which is dot plot hybridization, restriction analysis and direct sequencing (Kogan *et al*., 1987).

In this study, the β- thalassemia gene was amplified with various combinations of specific primers. In the first experiment P5 and P10 specific primers were used that gave an amplification product of 1823 bp of β-globin gene. While in the second experiment following specific primers were used (P5 and P12) giving an amplification product of 799 bp of β-globin gene.

The results of the two aforementioned experiments revealed that the polymerase chain reactions DNA products were inefficient. On the other hand, when reaction was amplified with P7and P10, the amplification product of 616 bp of β-globin gene was sufficient for restriction enzyme analysis (figure 3-16).

Amplification was performed in Ependorff Master Cycler programmed for 35 cycles (94 ºC for 5 minutes, 92 °C for 1 minute, 62 °C for 1.5 minutes and final extension step at 72°C for 2.5 minutes).

The type of mutation in family two was determined with the appropriate restriction endonuclease which is *Bsu*361 which digest the following sequence (in which N in any base)

5' …CC▼TNA GG…3'.

3'…GG ANT▲CC…5'

The results for family two in figure (3-16) had showed the presence of fragment (616bp) after restriction digestion of β-globin gene with *Bsu*361 in patient with β-thalassemia major which indicate to the presence of point mutation (Frameshift 6 mutation), this point mutation eliminates the *Bsu*361 recognition site in β-globin gene, whereas, in patients who have β-thalassemia minor cleavage with *Bsu*361 had showed two fragments in the digested product.

However, cleavage with *Bsu*361 that affecting the site recognized by this enzyme in normal DNA produces two fragments.

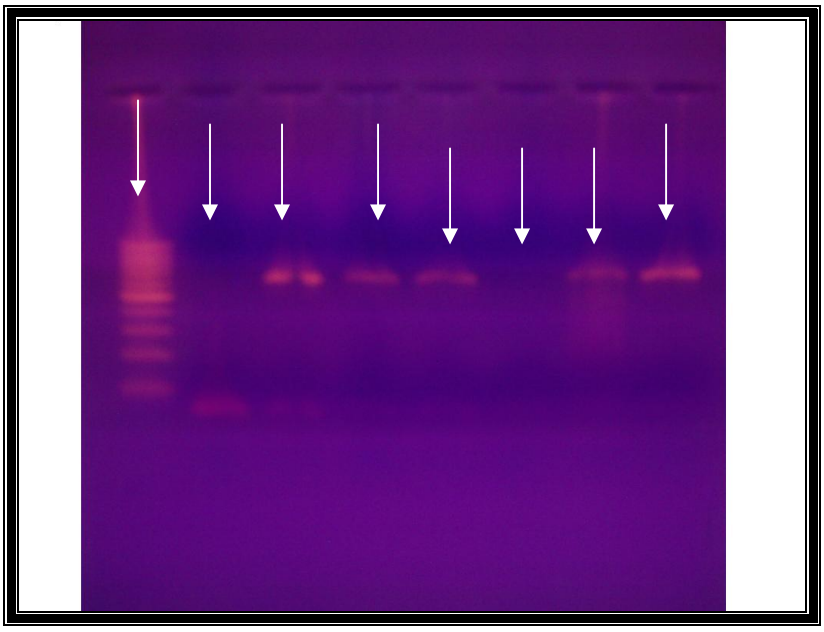


Figure (3-16). Restriction enzyme analysis of β-globin gene using *Bsu*361 for family two. Lane(1) Marker DNA(each band 100 bp) , lane(2) and lane(3) normal , lane(4) father who have β-thalassemia minor , lane(5) mother who have β-thalassemia minor, lane(6) sister who have β-thalassemia minor , lane(7) son who have β-thalassemia major, lane(8) sister who have β-thalassemia minor .

Since the main cause of β-thalassemia major can be either point mutation or base changes that interfere with the processing of the messenger RNA and deletion that produce premature chain termination codon or scrambling of the genetic code due to frame shift in β-globin gene (Clark and Thein, 2004).

Previous studies by Rund *et al.,* (1991) were show similar results, they showed the presence of one fragment after restriction digestion with *Bsu*361 in patients with thalassemia major indicates the presence of point mutation (Frameshift 6 mutation) in β – globin gene.

Conclusions

1- Complete blood picture for patient with thalassemia major had showed a severe microcytosis hypochromic anemia with nucleated red blood cells in peripheral blood smear which taken from the patient who has β-thalassemia major .

2- The analysis of β-globin chain by electrophoresis techniques had showed that most of the patients with thalassemia major have a complete absence of β-globin chain which indicates that there is defective in the production of β-globin chain.

3- The distribution of β-thalassemia major was due to the increasing homozygosity which occure in inbred populations, so the frequency of β-thalassemia disease is increased.

4- The agarose gel electrophoresis had the advantage that is more sensitive than cellulose acetate paper.

5- Hemoglobin electrophoresis and hemoglobin testing system (HPLC) will also detect the presence of other hemoglobinopathies (S, C and E) that may interact with β-thalassemia.

6- The sensitivity of hemoglobin testing system (variant) is efficient to allow the recognition of an abnormal adult Hb in a blood sample obtained from patient and is recommended for detection and identification of β-thalassemia syndrome.

7- Amplification of β-globin gene regions by polymerase chain showed an indication for the presence of point mutations in β-thalassemia major.

Recommendations

1- Prenatal diagnosis should be used to decrease the frequency of the occurrence of β-thalassemia major which based on carrier screening and counselling of couples at marriage.

2- Homogeneous allele-specific PCR amplification and detection of multiple β-globin mutations should be used as a rapid and inexpensive carrier screening tool.

3- Complete analysis of β- globin gene mutations by using :

- Multiplexing the ARMS primers to detect more than one mutation at the same time in a single reaction.
- Denaturing Gradient Gel Electrophoresis method (DGGE) in combination with (ARMS) Amplification Refractory Mutation System method allowed simple and rapid identification and characterization of the majority of β - thalassemia alleles.

4- Cloning of β-thalassemia gene in this study might be used as a start of gene bank for β-thalassemia syndrome, which could be used in future to identify the type of point mutations (base pair substitutions or frame shift) of β-thalassemia mutations in Iraqi population.

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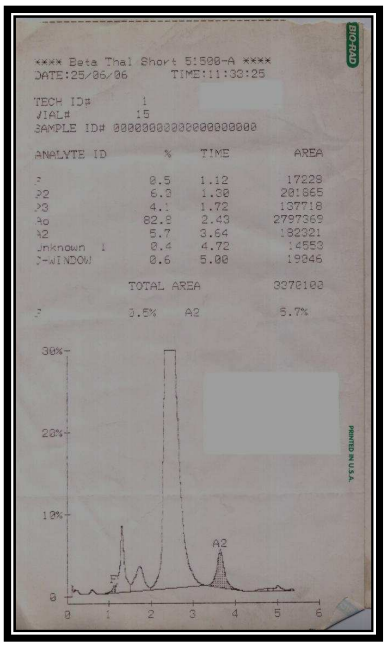
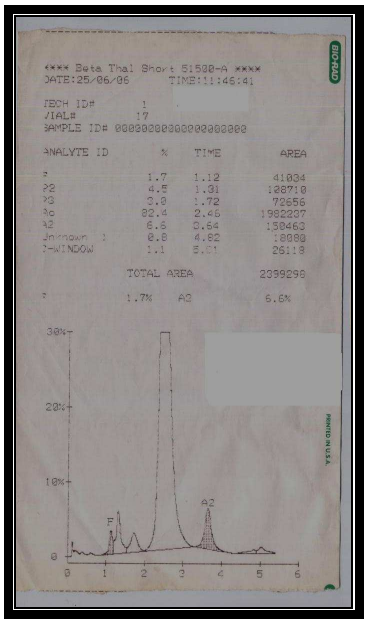
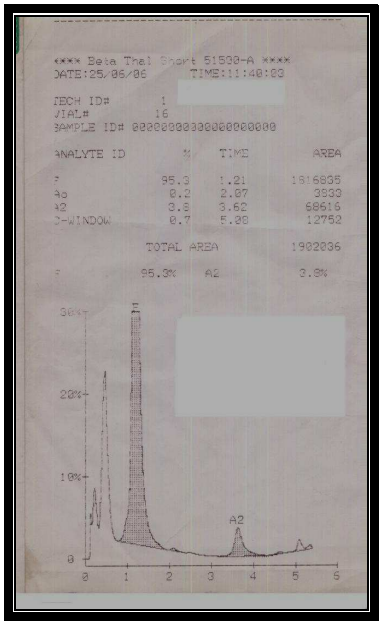
Appenedix 1



Father (β-thalassemia minor)



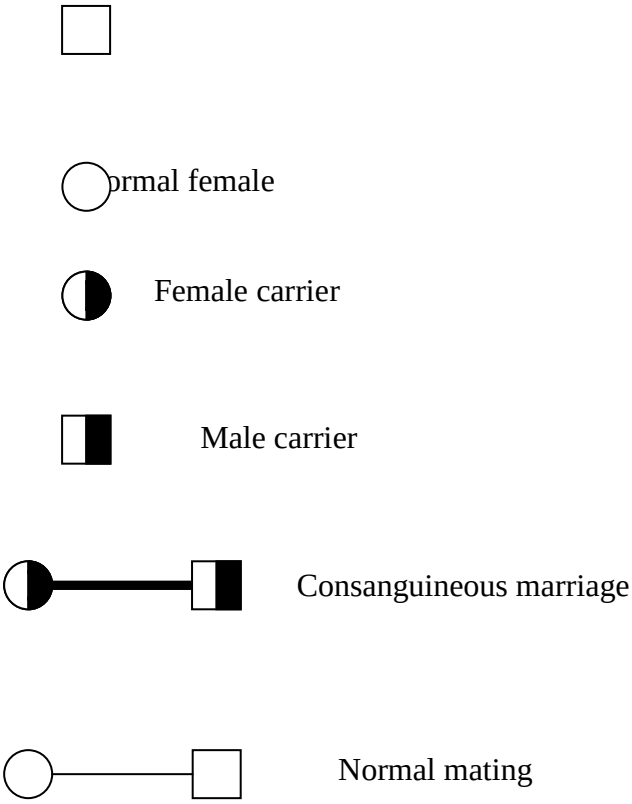
Mother (β-thalassemia minor)



Child (β-Thalassemia major)

Hemoglobin electrophoresis by Hemoglobin testing system (variant).

Appendix 2



Symbols of pedigree analysis of β-thalassemia patient

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المتواضع

بسم الله الرحمن الرحيم

قُلْ كُلٌّ يَعْمَلُ عَلَى شَاكِلَتِهِ فَرَبُّكُمْ أَعْلَمُ بِمَنْ هُوَ أَهْدَى سَبِيلاً ۞
وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً ۞

صدق الله العظيم

﴿سورة الإسراء –الآيات٨٤ و ٨٥ ﴾

الـــخلاصة

تضمنت الدراسة أجراء دراسة وراثية جزيئية على 70 مصاب بمرض فقر دم البحر الأبيض المتوسط من نوع β-major و تضمنت تحليل والكشف الأولي للدم ،والترحيل كهربائي للهيموغلوبين، وتحليل جزيئية الدنا المصابين بطريقة التفاعل التسلسلي لأنزيم البلمرة(PCR analysis) ،إضافة إلى التحليل الوراثي لشجرة العائلة لعدد من العائلات المصابة بالمقارنة مع الحالات الطبيعية.

أخذت عينات الدم من المصابين الذين يقومون بمراجعة مختبر الصحة المركزي/ وزارة الصحة ومن مناطق مختلفة من بغداد. حيث تم قياس نسبة PCV, MCH, MCV لدم هؤلاء المرضى .وأوضحت النتائج المستحصلة إن نسبة PCV للمرضى بفقر دم البحر الأبيض المتوسط كان بين 18-27% وللحاملين فقر دم البحر الأبيض المتوسط كان بين 36-43% وللأفراد الطبيعيين كان بين 37-54%، وان MCH كان بين 20.7-26 pgللمرضى بفقر دم البحر الأبيض المتوسط ولحاملي المرض كان بين 16.6- 19.1 pg وللأفراد الطبيعيين كان بين 27-31 pg ، بينما كانت نسبة MCV للمرضى بفقر دم البحر الأبيض المتوسط 67.2-79.3 fl ولحاملي المرض كان بين 61.5-66.6 fl وللأفراد الطبيعيين كان بين 80- 94 fl.

من ناحية أخرى، لقد وجد إن هناك تغير في شكل خلايا الدم الحمراء التي تظهر صغيرة الحجم، لونها شاحب، بأحجام مختلفة وتنوع في الإشكال مثل target cells، tear drops cells مقارنة بخلايا الدم للإفراد الطبيعيين التي تتصف بكونها ذات حجم طبيعي ولونها طبيعي.

كذلك تم حساب نسبة الهيموكلوبينات A_2، A، F باستخدام طرائق مختلفة للترحيل الكهربائي والتي تضمنت cellulose acetate electrophoresis، agarose gel electrophoresis، hemoglobin testing system (variant)،وأوضحت النتائج إن نسبة HbA منخفضة وذلك بسبب وجود فقدان كلي أو جزئي للموروث بيتا بينما معظم المصابين كانوا يعانون من ارتفاع نسبة HbA_2 ، HbF بالمقارنة مع الأشخاص الطبيعيين.

تضمن الجانب الأخر من الدراسة التحليل الوراثي لشجرة العائلة (pedigree analysis) لعدد من العوائل مصابة ببيتا ثلاسيميا وأظهرت النتائج انتقال المرض خلال أجيال في هذه العوائل ولقد وجد إن هذه الصفة تتناقل من الإباء إلى الأبناء كصفة متنحية ولوحظ إن معظم الحالات تتواجد خاصة في العوائل التي تفضل زواج الأقارب.

الجانب الأخر من الدراسة تضمن التحليل الجزيئي على مستوى الحمض النووي المنقوص الأوكسجين باستخدام تقنية التفاعل التسلسلي لأنزيم البلمرة الدنا المحدد والخاص بالموروث بيتا باستخدام specific primers وعملية أنزيم القطع باستخدام أنزيم القطع *Bsu*361 .وأوضحت النتائج إن هناك فقدان كلي للتعبير في الموروث بيتا نتيجة طفرة وراثية نقطية نوع

(Frame shift mutation 6) في المرضى ذوي الحالات الشديدة من مرضى (متوافقي الزيجة) وكان حجم القطعة الناتجة عن عملية التضاعف هو ٦١٦زوج قاعدي تمثل جزء الموروث بيتا وظهور حزمة واحدة ناتجة عن عملية أنزيم القطع.

Republic of Iraq
Ministry of Higher Education and
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Molecular Genetic Study of β-thalassemia major syndrome in Baghdad

A Thesis
Submitted to the College of Science at Al-Nahrain University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

By

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October 2007 Shawal 1428

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

دراسة وراثية جزيئية لتناذر الثلاسيميا β-major في بغداد

رسالة مقدمة إلى
كلية العلوم –جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم
في التقانة الاحيائية

من قبل
رؤيا هادي خزعل الحداد
بكالوريوس تقانة إحيائية، كلية العلوم، ٢٠٠٤
جامعة النهرين

تشرين الاول ٢٠٠٧ شوال ١٤٢٨